A topical antioxidant solution containing vitamins C and E stabilized by ferulic acid provides protection for human skin against damage caused by ultraviolet irradiation

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Background

Skin cancer and photoaging changes result from ultraviolet (UV)-induced oxidative stress. Topical antioxidants may protect skin from these effects.

Objective

We sought to determine whether a stable topical formulation of 15% L-ascorbic acid, 1% alpha-tocopherol, and 0.5% ferulic acid (CEFer) could protect human skin in vivo from substantial amounts of solar-simulated UV radiation.

Methods

CEFer and its vehicle were applied to separate patches of normal-appearing human skin for 4 days. Each patch was irradiated with solar-simulated UV, 2 to 10 minimal erythema doses, at 2-minimal erythema dose intervals. One day later, skin was evaluated for erythema and sunburn cells, and immunohistochemically for thymine dimers and p53. UV-induced cytokine formation, including interleukin (IL)-1α, IL-6, IL-8, and IL-10, and tumor necrosis factor-α, were evaluated by real-time polymerase chain reaction.

Results

CEFer provided significant and meaningful photoprotection for skin by all
methods of evaluation.

Limitations

The number of patients evaluated was relatively small.

Conclusion

CEFer provided substantial UV photoprotection for skin. It is particularly effective for reducing thymine dimer mutations known to be associated with skin cancer. Its mechanism of action is different from sunscreens and would be expected to supplement the sun protection provided by sunscreens.

Ultraviolet (UV) radiation in sunlight generates oxidative stress in skin that can result in skin cancer and photoaging changes. The skin protects itself using low molecular–weight antioxidants that neutralize the oxidative stress before it can cause damage. Most antioxidant protection depends on dietary intake and subsequent delivery to skin. Because the antioxidant is destroyed or altered by oxidation during neutralization, protection is often limited by the concentration of antioxidants remaining in skin. In an attempt to augment this protective strategy, our laboratory has been interested in developing the topical use of antioxidants for photoprotection. To accomplish this goal, it has been necessary to develop formulations that allow for stability of these inherently unstable substances. Of even more importance, it has been necessary to find formulations that allow these antioxidants to enter into skin, so that they are in the right place to provide protection.

Using porcine skin as a model for human skin, we reported that 15% L-ascorbic acid, protonated to remove charge, was capable of entering skin and protecting it from UV irradiation. After maximizing the concentration of the solution for effectiveness, we demonstrated that skin concentration, once saturated, was somehow stabilized with a half-life disappearance of about 4 days. This first-generation topical product provided protection against UVB and UVA. Because the solution had no absorption against these wavelengths, it was concluded that the protection was not caused by a sunscreen effect. In addition, the solution protected UV-induced immunosuppression. The topical L-ascorbic acid added to the skin’s own concentration and most probably augmented its antioxidant effect.

We next formulated 15% L-ascorbic acid together with 1% α-tocopherol. These antioxidants form an interactive pair in tissues. When α-tocopherol
neutralizes oxidative stress in lipids, its oxidation product can be regenerated by L-ascorbic acid.\textsuperscript{5} \textsuperscript{6} \textsuperscript{7} This interaction helps to renew antioxidant protection in the tissues. After maximizing the solution for concentration and stability, this second-generation product provided doubled UV photoprotection for skin, in comparison with L-ascorbic acid alone.

More recently, we have undertaken a search for antioxidants that could be shown to increase the stability of the solution of L-ascorbic acid and α-tocopherol. We have demonstrated that the addition of 0.5% ferulic acid, a common plant antioxidant, both improved stability and again doubled UV photoprotection for skin.\textsuperscript{8} Ferulic acid, a hydroxycinnamic acid, probably protects L-ascorbic acid and α-tocopherol in solution by serving as a sacrificial substrate. We demonstrated that the antioxidant combination provided superior protection when compared with the individual ingredients. This effect is apparently unrelated to a sunscreen effect. In this article we describe the extension of these observations to human skin. We demonstrate that this third-generation product provides effective UV photoprotection, inhibits thymine dimer formation, and inhibits p53 activation. In addition UV activation of interleukin (IL)-1α, IL-6, IL-8, IL-10, and tumor necrosis factor (TNF)-α is blunted.

**Methods**

*Experimental protocol*

Studies were conducted at with the approval of our institutional review board. Nine adults with Fitzpatrick skin type II or III (white skin with the ability to tan slightly to moderately in response to UV irradiation) were entered into the protocol. Two solutions were applied (2 mg/cm\textsuperscript{2}) daily for 4 days to separate patches of back skin. Subjects were instructed not to wash the area for at least 2 hours. One solution was an aqueous solution containing 15% L-ascorbic acid, 1% dl-α tocopherol, and 0.5% trans ferulic acid (CEFer). The other solution was a vehicle only control. Solutions (CEFerlic and its vehicle) were supplied by SkinCeuticals (Garland, Tex). On day 3, each subject received solar-simulated irradiation to an untreated patch of skin, to determine the minimal erythema dose (MED). Irradiations were from 20 to 60 mJ/cm\textsuperscript{2} at 10-mJ/cm\textsuperscript{2} intervals of UVB as determined by a radiometer (IL1700, International Light, Newburyport, Miss). MED was determined 24 hours later as the lowest dose resulting in perceptible borders of erythema (about 40 mJ/cm\textsuperscript{2}). Details of irradiation have been published.\textsuperscript{4} A 1000-W xenon solar simulator (Lightning Cure 200, Hamamatsu, Hamamatsu City, Japan) fitted with a dichroic mirror to filter
infrared and visible light and a Schott 295 bandpass filter (Mainz, Germany) to remove wavelengths less than 295 nm fitted with a liquid light guide to create a 1-cm spot of light on skin was used at an output of about 5 mW/cm$^2$ of UVB and about 40 mW/cm$^2$ of UVA. On day 4, the vehicle-treated patch received 2 to 6× MED and the CEFer-treated patch received 2 to 10× MED, each at 2×-MED intervals. One day later, skin was evaluated by colorimetry for erythema, and 4-mm punch biopsy specimens of skin receiving 6× MED of irradiation were evaluated for sunburn cells.

**Measurement of erythema and sunburn cells**

Erythema was measured by computerized colorimetry in the “a” mode of digital color skin photographs. Each spot and unirradiated adjacent skin was measured in triplicate. The color difference between irradiated and unirradiated skin determined the erythema. Sunburn cells were determined in formalin-fixed skin sections stained with hematoxylin and eosin. Results are expressed as mean ± SD. The P values were determined by two-tailed Student t test using unequal variance.

**Immunohistochemistry for thymine dimers and p53**

**Thymine dimer**

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized with xylene and rehydrated through a graded alcohol-water series. Sections underwent heat-induced epitope retrieval by incubating them in a solution of citrate buffer (pH 6.0) (Zymed, San Francisco, Calif) for 20 minutes at 95°C, followed by equilibration in phosphate-buffered saline (PBS). Nonspecific peroxidase was blocked by 3% hydrogen peroxide, and nonspecific binding was blocked with 1.5% horse serum in PBS. Mouse monoclonal antithymine dimer antibody clone KTM-53 (Kamiya Biomedical, Seattle, Wash) was diluted in 1.5% horse serum-PBS to 1/1000 and incubated on sections for 1 hour at 37°C. Detection was accomplished with a horse antimouse secondary antibody using an avidin-biotin link (Vector Elite ABC peroxidase kit, Burlingame, Calif) as described by the supplier. Slides were counterstained with hematoxylin.

**p53**

The procedure was identical to that described above except that antigen retrieval was with heat-induced epitope retrieval in pH8 Tris-EDTA buffer (Zymed). Detection was achieved with mouse monoclonal antihuman p53
clone DO-7 (Dako, Glostrup, Denmark) diluted in 1.5% horse serum-PBS to 1/250.

Real-time polymerase chain reaction studies

An identical study was conducted in an additional 10 subjects with the exception that subjects received irradiation at 2× MED. The 6-mm biopsy specimens of CEFer and vehicle-treated skin were bisected, half for real-time (RT) polymerase chain reaction (PCR) studies and half for immunohistochemistry.

Messenger RNA isolation from skin biopsy specimens

Biopsy specimens of untreated, UV + vehicle, and UV + CEFer skin of each subject were immediately placed in RNALater (Qiagen, Valencia, Calif) and stored at –80°C until processing could occur. Total RNA was extracted using fibrous tissue mini kits (RNeasy, Qiagen). Tissue was homogenized in RLT buffer using a tissue homogenizer (Omni International, Bethesda, Md) and RNA isolated according to kit protocol (Qiagen). To avoid contaminating genomic DNA, Rnase-Free Dnase (Qiagen) was used. The concentration of RNA was measured by a spectrophotometer (ND1000, Nanodrop Inc, Wilmington, Del) and stored at –82°C in water. For complementary DNA (cDNA) synthesis, approximately 2 μg of total RNA was transcribed with cDNA transcription reagents (iScript, BioRad, Hercules, Calif) using random hexamers. Thermal cycler parameters included 5 minutes at 25°C for incubation, 30 minutes at 42°C for RT reaction, and 5 minutes at 85°C for enzyme inactivation. cDNA was purified with PCR purification kits (QiaQuick, Qiagen) and then stored in elution buffer at –82°C until quantitative RT PCR was performed.\textsuperscript{10}

RT quantitative PCR

Measurement of gene expression was performed using RT PCR system (iCycler, BioRad) as previously described.\textsuperscript{11} TaqMan probes were labeled at the 5'-end with the reporter dye molecule FAM (6-carboxyfluorescein; emission λmax = 518 nm) and at the 3'-end with the quencher dye molecule 3' Black Hole Quencher (Integrated DNA Technologies, Coralville, Iowa) (absorbance λmax = 534 nm). RT PCR reactions of cDNA specimens and cDNA standards were conducted in a total volume of 25 μL with 2× iQ SuperMix (BioRad) and primers and probes at optimized concentrations. Parameters for the thermal cycler were 3 minutes at 95°C, and 40 cycles involving denaturation at 95°C for 30 seconds, annealing/extension at 59°C for 30 seconds. RT monitoring of fluorescent
emission from cleavage of sequence-specific probes by the nuclease activity of taq polymerase allowed definition of the threshold cycle during the exponential phase of amplification.\textsuperscript{11, 12}

For analysis of control 18S RNA, IL-1\(\alpha\), IL-6, IL-8, IL-10, and TNF-\(\alpha\) messenger RNA (mRNA) expression, a standard curve was constructed with serial dilutions of cDNA obtained from a single sample of human peripheral blood mononuclear cells that had been stimulated for 4 hours by phytohemagglutinin (5 \(\mu\)g/mL).\textsuperscript{10, 13} Cells were removed, mRNA isolated, and cDNA produced as described above. Relative quantitation of cytokine mRNA expression was determined using the relative standard curve method (User Bulletin No. 2, Applied Biosystems, Foster City, Calif). Individual results were normalized using 18S ribosomal RNA as an internal control with 18S-specific primers and probe. All samples, analyzed by RT PCR, were performed in triplicate. Cytokine mRNA expression was expressed as arbitrary units. Ratios of UV-CEFer–treated skin mRNA expression to UV-vehicle–treated skin \(\times\) 100 was determined to evaluate the percent suppression of cytokine mRNA expression for each cytokine tested.

\textit{Design of cytokine primers and probes}

Oligonucleotide primers and TaqMan probes for 18S, IL-1\(\alpha\), IL-8, and IL-10 were designed using PrimerQuest (Integrated DNA Technologies). We conducted BLASTN searches to confirm the total gene specificity of the nucleotide sequences chosen for primers and probes and the absence of DNA polymorphism. To avoid amplification of contaminating genomic DNA, one of the two primers for cDNA was placed either at the junction between two exons or within different exons. Cytokine primers were designed to result in amplicons less than 150 base pair to enhance efficiency of PCR amplification. Gene expression sets for IL-6 and TNF-\(\alpha\) were purchased from Applied Biosystems.

\textit{Statistical analysis}

Results were analyzed using Mann-Whitney test and the Wilcoxon signed rank test using software (Analyse-it, Analyse-It Software Ltd, Leeds, United Kingdom). Statistical significance was assumed for a \(P\) value less than .05.

\textbf{Results}

\textit{Erythema}
CEFer provided substantial protection against UV-induced erythema when compared with vehicle-treated skin (Fig 1, Fig 2). At 2 to 6× MED irradiance, colorimeter readings for vehicle versus CEFer revealed significant ($P < .01$) protection by CEFer at all irradiance levels (Fig 2). Moreover, CEFer provided significant protection ($P < .01$) at 8× MED and 10× MED irradiances when compared with 6× MED-irradiated vehicle-treated skin.

Fig 1. CEFer inhibits ultraviolet (UV)-induced erythema. CEFer and vehicle were applied to back skin (2 mg/cm$^2$). Skin was irradiated with solar-simulated UV radiation, 2× to 10× minimal erythema doses (MED) to ferulic-treated skin and 2×- to 6×-MED intervals to vehicle-treated skin. Erythema was determined 1 day later. Digital photographs. Mean ± SD (n = 9). $P < .02$ versus vehicle. $P < .01$ versus vehicle at 6× MED.

Fig 2. CEFer inhibits ultraviolet (UV)-induced erythema. CEFer and vehicle were applied to back skin (2 mg/cm$^2$). Skin was irradiated with solar-simulated UV radiation, 2× to 10× minimal erythema doses (MED) to ferulic-treated skin and 2×- to 6×-MED intervals to vehicle-treated skin. Erythema was measured with digital photographs. Mean ± SD (n = 9). $P < .02$ versus vehicle. $P < .01$ versus vehicle at 6× MED.

Sunburn cells

Sunburn cell enumeration (Fig 3) of 6× MED-irradiated skin when compared with similarly irradiated vehicle-treated skin revealed significant protection ($P < .01$) by CEFer, (vehicle 31.5 ± 14.3 vs CEFer 8.4 ± 7).

Fig 3. CEFer inhibits ultraviolet (UV)-induced sunburn cell (SBC) formation. CEFer and vehicle (2 mg/cm$^2$) were applied daily for 4 days. Skin was irradiated with solar-simulated UV radiation, 2× to 10× minimal erythema doses (MED) at 2× MED intervals to CE ferulic–treated skin and 2×- to 6×-MED intervals to vehicle-treated skin. Skin biopsy specimens of 6× MED-treated skin were taken 1 day later. SBCs were counted and are expressed as cells/mm of epidermis. Mean ± SD (n = 9). $P < .01$ vs vehicle.

Thymine dimers and p53

Immunohistochemistry of skin receiving 2× MED irradiation revealed
virtually complete protection by CEFer, when compared with vehicle, against generation of thymine dimers in DNA (Fig 4) and against induction of p53 (Fig 5).

Fig 4. CEFer inhibits ultraviolet (UV)-induced thymine dimer formation. CEFer and vehicle were applied to back skin (2 mg/cm² daily for 4 days. Skin was irradiated with solar-simulated UV radiation, 1× to 5× minimal erythema dose (MED) at 1× intervals. Skin biopsy specimens of 2× MED-treated skin and unirradiated, untreated skin were taken 1 day later and formalin fixed tissue was stained for immunohistochemistry using mouse monoclonal antibody to thymine dimer (clone KTM53 Kamiya Biomedical, Seattle, Wash). UV generation of cellular thymine dimers was almost completely protected by CEFer application. (Original magnification: ×40.)

Fig 5. CEFer inhibits ultraviolet (UV)-induced p53 formation. CEFer and vehicle were applied to back skin (2 mg/cm² daily for 4 days. Skin was irradiated with solar-simulated UV radiation, 1× to 5× minimal erythema dose (MED) at 1× intervals. Skin biopsy specimens of 2× MED-treated skin and unirradiated, untreated skin were taken 1 day later and formalin fixed tissue was stained for immunohistochemistry using mouse monoclonal antibody to human p53 (clone DO). UV generation of p53 was almost completely protected by CEFer application. (Original magnification: ×40.)

Cytokine mRNA expression

Cytokine mRNA expression was increased in UV-irradiated vehicle-treated skin when compared with unirradiated normal-appearing skin for all cytokines tested. This was significant for all cytokines (P < .05, Wilcoxon signed rank test, 2-tailed). UV-irradiated vehicle-treated skin showed an increase in mRNA expression, mean ratio of UV vehicle/no UV of IL-1α (7.2), IL-6 (245), IL-8 (275), IL-10 (5.6), and TNF-α (2.5). Treatment by CEFer resulted in a statistically significant (P < .05) suppression of cytokine mRNA expression for all cytokines except TNF-α (P = .0654, Wilcoxon signed rank test, 1-tailed). Treatment with CEFer resulted in suppression of mean ratio of cytokine mRNA expression of IL-1α (2.2), IL-6 (25.8), IL-8 (42.1), IL-10 (5.6), and TNF-α (0.8). Suppression ranged from 16.4% for TNF-α to 89.6% for IL-8 (Fig 6).

Fig 6. CEFer inhibits ultraviolet (UV)-induced increases in cytokine messenger RNA (mRNA)
back skin (2 mg/cm²) daily for 4 days. Skin was irradiated with solar-simulated UV radiation and treated with the minimal erythema dose–treated skin treated with CEFer, vehicle, and of unirradiated, untreated skin. Cytokine mRNA measured by real time polymerase chain reaction. Mean suppression of mRNA CEFer for each cytokine. IL, Interleukin; TNF, tumor necrosis factor. $P < .05$.

**Discussion**

This study demonstrates that a combination of physiologic antioxidant vitamins C and E stabilized by a powerful plant antioxidant, ferulic acid, can be applied topically to skin and supplement the skin's own antioxidant pool to protect against UV-induced oxidative damage. In addition to protecting the skin against erythema and apoptosis associated with cellular damage, protection was also provided against UV-induced DNA mutations that have been demonstrated to be associated with skin cancer.

UV radiation generates reactive oxygen species in skin that can oxidize nucleic acids, proteins, and lipids, reactions that depend on proximity to the oxidizing species. Over time these changes can result in photoaging and cancer. More than 90% of UV radiation in sunlight is UVA, and UVA more than UVB is responsible for oxidative stress in skin. UVA generates singlet oxygen in skin that in turn generates other reactive oxygen species including free radicals. Sunscreens offer very effective protection for skin against UVB and sunburn, but protection against UVA and its effects is incomplete. No sunscreen chemical provides protection against long-wave UVA above about 390 nm. Sunscreen protection depends on careful application and can be removed by sweating, rubbing, and degradation in the presence of sunlight. Even the best sunscreens block no more than about half of UV-induced free radical generation in skin. Antioxidants, in contrast, work inside the skin. They cannot be removed by washing and rubbing. Once inside skin, they are stabilized and can offer protection for days.

The formulation of CEFer used in this study has been maximized for percutaneous absorption and subsequent photoprotection for skin. Antioxidants in the body work in balance. Large increases in a single antioxidant usually result in only a modest effect. The balanced concentration of antioxidants in CEFer provides interactive support that results in synergistic protection. There are many antioxidants that have been demonstrated to provide photoprotection for in vitro and cellular systems but fail in vivo mostly because they are unable to enter the skin. Because antioxidants are inherently chemically unstable, derivatives have been synthesized to provide stability. Thus, L-ascorbic acid has been
stabilized to ascorbyl-6-palmitate and magnesium ascorbyl phosphate, and α-tocopherol has been stabilized to tocopherol acetate or succinate. These derivatives are widely used in topical skin products. The derivatives provide stability for the antioxidant and are useful for vitamin pills where conversion to the native antioxidant occurs in the stomach. On the skin, however, conversion is inefficient and little antioxidant is available to skin.\textsuperscript{2, 22, 23}

It is unclear why antioxidants should affect UV-induced thymine dimer formation. A recent laser-capture study of squamous cell carcinoma of skin and actinic keratoses revealed abundant thymine dimer mutations throughout the tumors.\textsuperscript{24, 25} Although generation of thymine dimer mutations has been considered to be caused by direct UVB absorption and unrelated to oxidation, recent observations have revealed abundant thymine dimer mutations in skin generated by UVA irradiation\textsuperscript{26, 27} even though UVA is only minimally absorbed by DNA.\textsuperscript{28} Moreover, skin structure efficiently prevents UVB penetration and protects UVB-induced DNA lesions, but only weakly protects UVA-induced lesions.\textsuperscript{27} UVA preferentially generates oxidative stress in skin in comparison with UVB.\textsuperscript{14} Antioxidants have recently been demonstrated to effectively inhibit UVB-induced cyclopyrimidine dimers in human HaCaT cells but not in naked DNA.\textsuperscript{29} The data suggest a cellular photosensitization reaction involving a triplet energy transfer mechanism, resulting in cyclopyrimidine dimer formation.\textsuperscript{26, 27, 29, 30} In addition, UVA may inhibit DNA repair enzymes, allowing thymine dimers to persist unrepaired and enter into DNA replication.\textsuperscript{27, 30}

P53 is induced by UV irradiation in response to DNA damage\textsuperscript{31} and oxidative stress.\textsuperscript{32} P53 causes the cell to slow DNA replication and subsequent cell division, allowing the cell more time to repair DNA damage. Reduction of p53 induction by CEFer in this study may relate to protection of DNA damage and reduction of oxidative stress afforded by the antioxidants.

UV irradiation has been shown to increase cytokine production in the epidermis, and this cytokine production is thought to play an important role in both the inflammatory response associated with UV damage and in subsequent local immunosuppression. UV irradiation has been shown to increase proinflammatory cytokines such as IL-1, IL-6, IL-8, and TNF-α and to increase the immunosuppressive cytokine IL-10.\textsuperscript{33, 34, 35} Animal studies have demonstrated that local production of IL-10 is important in the control of UV-induced carcinogenesis in mice.\textsuperscript{36} UV irradiation has been shown to result in nonligand-dependent activation of epidermal growth
factor receptor, TNF-α receptor, and IL-1 receptor with downstream responses including expression of NF-κB–regulated genes, including numerous cytokines.\(^3^7\), \(^3^8\) The mechanism for this UV-induced activation is not known but it has been suggested that reactive oxygen species may play a role in this process.\(^3^7\), \(^3^8\) We have demonstrated that CEFer, a topical antioxidant, is able to significantly decrease the expression of proinflammatory and immunosuppressive cytokines that occurs with UV damage. The suppression of these cytokines may play a role in decreasing the inflammation and immunosuppression associated with UV exposure. Further studies are needed to determine whether UV-induced activation of epidermal growth factor receptor, TNF-α receptor, and IL-1 receptor and the subsequent downstream signaling are inhibited by CEFer.

CEFer has been developed stepwise to provide maximal antioxidant photoprotection for skin. Antioxidants protect skin by neutralizing reactive oxygen species before they can damage skin. Although this study provides information only about protection against acute UV injury, it would be expected that sustained protection might prevent chronic UV injury over time. Topical antioxidants work by a mechanism that is different from that provided by sunscreens. Their use would be expected to be supplemental, and the combination should provide maximal photoprotection for skin.

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