Original Contribution

Protective effects of a topical antioxidant mixture containing vitamin C, ferulic acid, and phloretin against ultraviolet-induced photodamage in human skin

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Summary

Background Ultraviolet (UV) irradiation of the skin leads to acute inflammatory reactions, such as erythema, sunburn, and chronic reactions, including premature skin aging and skin cancer.
Aim In this study, the effects of a topical antioxidant mixture consisting of vitamin C, ferulic acid, and phloretin on attenuating the harmful effects of UV irradiation on normal healthy volunteers were studied using biomarkers of skin damage.
Subjects/methods Ten subjects (age, 18–60 years; Fitzpatrick skin types II and III) were randomized and treated with antioxidant product or vehicle control on the lower back for four consecutive days. On day 3, the minimal erythema dose (MED) was determined for each subject at a different site on the back. On day 4, the two test sites received solar-simulated UV irradiation 1–5× MED at 1× MED intervals. On day 5, digital images were taken, and 4-mm punch biopsies were collected from the two 5× MED test sites and a control site from each subject for morphology and immunohistochemical studies.
Results UV irradiation significantly increased the erythema of human skin in a linear manner from 1× to 5× MED. As early as 24 h after exposure to 5× MEDs of UV irradiation, there were significant increases in sunburn cell formation, thymine dimer formation, matrix metalloproteinase-9 expression, and p53 protein expression. All these changes were attenuated by the antioxidant composition. UV irradiation also suppressed the amount of CD1a-expressing Langerhans cells, indicating immunosuppressive effects of a single 5× MED dose of UV irradiation. Pretreatment of skin with the antioxidant composition blocked this effect.
Conclusion This study confirms the protective role of a unique mixture of antioxidants containing vitamin C, ferulic acid, and phloretin on human skin from the harmful effects of UV irradiation. Phloretin, in addition to being a potent antioxidant, may stabilize and increase the skin availability of topically applied vitamin C and ferulic acid. We propose that antioxidant mixture will complement and synergize with sunscreens in providing photoprotection for human skin.

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Conflict of Interest. Dr. Sheldon Pinnell is a consultant for SkinCeuticals/L'Oreal. Drs. Thomas Stephens, Peter Hino, and Robert Law are independent contractors for the study. The other authors are employees of L'Oreal USA, who is the sponsor of the study.

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Introduction

Ultraviolet (UV) irradiation of the skin leads to acute inflammatory reactions such as erythema, sunburn, and chronic reactions, including premature skin aging and skin tumors. UV irradiation is a potent generator of oxidative stress in the skin. Exposure of mammalian skin to UV increases the cellular levels of reactive oxygen species, which damages lipids, proteins, and nucleic acids in both epidermal and dermal cells and contributes to the sunburn reaction as well as photocarcinogenesis and photoaging.[1]

As the outermost organ exposed directly to the pro-oxidative environment, the skin is equipped with an elaborate system of antioxidant substances and enzymes, including a network of redox-active antioxidants. The endogenous antioxidant capacity of the skin is a major determinant in its response to oxidative stress-mediated damage.[1] Normal aging process, as well as environmental stress, can deplete the epidermis of protective antioxidants. Thus, antioxidants potentially constitute an important group of pharmacological agents capable of preventing the occurrence and reducing the severity of UV-induced skin damage and skin aging.

In recent years, we have investigated the protective effects of antioxidants applied topically to skin to prevent UV-induced oxidative damage. Although there are many known low molecular weight antioxidant substances, their efficacy is limited if they fail to penetrate skin. A systematic study of endogenous antioxidants resulted first in a topical formulation of L-ascorbic acid which was maximized for chemical stability, concentration, availability, and subsequent photoprotection for skin.[2] Addition of α-tocopherol improved stability and photoprotection, demonstrating the interacting balance achieved by combination antioxidants.[3] Investigation of plant phenolic antioxidants revealed that ferulic acid improved the stability of the antioxidant formulation of L-ascorbic acid and α-tocopherol (CEFer) as well as its photoprotective properties.[4–6] Subsequent studies have identified phloretin, another plant antioxidant, as a useful antioxidant that is capable of penetrating the skin and interacting with other antioxidants to provide effective photoprotection. Phloretin found in both the flesh and peel of apples is a potent antioxidant. Unlike other major plant-derived phenolic compounds, phloretin has not been used for UV protection of skin. In preliminary studies measuring photoprotection to UVA-induced erythema and pigmentation, we found that a solution containing vitamin C, ferulic acid, and phloretin (CFerPhlor) provided protection. In this study, we compared the role of UV irradiation on skin damage, and its protection by CFerPhlor, in human subjects using erythema, sunburn cell formation, thymine dimer formation, as well as other biochemical markers as end points.

Materials and methods

Formulations

The preparation used in this study was a solution of 10% L-ascorbic acid in a hydroglycolic base (water, butylene glcycol, dipropylene glycol, and ethanol) containing 0.5% ferulic acid and 2% phloretin. The solution was adjusted to pH 2.5 to achieve maximum topical absorption. A vehicle-only solution (without the active ingredients) served as a control. These mixtures had no appreciable SPF value as measured by *in vitro* UV absorption profiles.

UV source

The UV radiation was supplied by single port solar simulator (Model 16S, Solar UV Simulator, Solar Light Co., Philadelphia, PA) with a 150-W xenon arc lamp with a spectral output in the UV range similar to that of the natural solar spectrum (UVR – UVB: 290–320 nm and UVA: 320–400 nm). The filters used were UG-11/1 mm and WG-320 filters (Schott Glass Technologies, Scranton, PA) to obtain a 290- to 400-nm solar spectrum. An adjustable patient stop was used to keep the distance from the solar simulator to the radiated surface constant. At a distance of approximately 6.5 cm from the lamp housing, the radiated surface was exposed to a 1.0-cm diameter spot UVR. Exposures were performed by varying the time of exposure (in seconds) while keeping the energy level constant. Opening and closing of the light shutter was performed manually. The radiation output of the xenon bulb was measured using the 3D-600 meter (Solar Light Co.). The relative UVB and UVA output of the solar simulator under the conditions of the experiment was determined to be approximately 4.1 mW/cm^2, and the relative output of UVA and UVB was approximately 88.9% of UVA and 11.1% UVB.

Experimental protocol

Ten subjects (male and female; age, 18–60 years; Fitzpatrick skin types II and III) were recruited for this study. Written informed consent was obtained from each subject prior to enrollment in the study. The protocol, informed consent agreement, and any protocol amendments were reviewed and approved by an institutional review board. The subjects were randomized for product application. Two areas (7.5 cm × 5 cm) were marked on the hairless lower back skin of each subject. Both active and vehicle-only formulations were applied (2 mg/cm^2) daily for four consecutive days.

On day 3, the minimal erythema dose (MED) was determined for each subject. Six separate sites near the treatment area were irradiated with a range of intensities (20–70 mJ/cm^2 at 10 mJ/cm^2 intervals). On day 4, the MED was determined as the spot receiving the lowest dose with erythema extending to the borders. On day 4, the two test sites received solar-simulated UV irradiation 1–5× MED at 1× MED intervals. On day 5, digital images were taken of the entire treatment area under standardized conditions.

Four-millimeter punch biopsies were collected from the two 5× MED test sites and a control site from each subject and fixed in 10% buffered formalin. Biopsy specimens were processed for morphology and immunohistochemical studies.

Erythema

Digital images were analyzed using colorimetry to determine a* values (degree of redness). The difference between the unexposed sites from the exposed site was calculated and plotted for each MED.

Skin biopsy sections were fixed in formalin and processed for routine hematoxylin–eosin staining to detect "sunburn cells," which exhibit pyknotic nuclei and dense, eosinophilic cytoplasm. Total number of cells was counted over the linear length of epidermal surface, and results are expressed as mean number of cells/mm ± SD.

Immunohistochemistry

All antibodies used were mouse monoclonal or rabbit polyclonal antibodies made against human antigens.

Thymine dimers

Formalin-fixed, paraffin-embedded tissues were sectioned at 5-μm, mounted on adhesive slides, and immunostained manually using standard methods. After de-paraffinization of the slides, endogenous peroxidase was quenched, and the slides were subjected to heat-induced epitope retrieval in 1 mM EDTA for 30 min using a vegetable steamer. The slides were then immersed in previously optimized dilutions of mouse monoclonal antithymine dimer (clone H3, Affitech) and incubated for 20 min at 25 °C. Following this, the slides were immersed in the appropriate peroxidase-conjugated polymer reagent (Vision BioSystems, Norwell, MA) for 45 min at 25 °C. The reaction product was developed by immersing the slides in prepared diaminobenzidine solution (Invitrogen, Carlsbad, CA) at 25 °C for 5 min, followed by enhancement in 0.5% copper sulfate, dehydration, and counterstaining with hematoxylin. Following immunostaining, total number of thymine dimers (positive nuclear staining) was quantified in epidermal cells and results expressed as mean number of cells/mm ± SD.

p53

Procedure was identical as described above using mouse monoclonal anti-p53 antibody (clone DO7, Immunovision, Burlingame, CA). Following immunostaining, the total number of thymine dimers (positive nuclear staining) was quantified in epidermal cells and results expressed as mean number of cells/mm ± SD.

Matrix metalloproteinase-9

Procedure was identical as described above using rabbit polyclonal anti-matrix metalloproteinase-9 (MMP-9) antibody (NeoMarkers, Fremont, CA). Following immunostaining, the level of cytoplasmic MMP-9 expression in both dermal and epidermal cells was graded on a scale from 0–4.

Langerhans cells CD1a

The procedure was identical as described above using mouse monoclonal anti-CD1a antibody (clone O10, Immunotech, Westbrook, ME). Following immunostaining, the total number of CD1a-positive cells within the epidermis was quantified, and results were expressed as mean number of cells/mm ± SD.

Statistics

Digital images of the control site and antioxidant-treated sites were analyzed using a computer-aided colorimetry algorithm to determine the a* value (degree of redness). Since each subject serves as his or her own control, *P*-values were determined by two-tailed Student's *t*-test. For the immunohistochemistry slides, the average ± SD was

calculated for the vehicle only and antioxidant (CFerPhlor)–treated sites, and the *P*-values were determined by two-tailed Student's *t*- test. The non-UV-exposed, untreated site (control) was used as negative control.

Results

UV absorption profile of CFerPhlor

Figure 1 shows the UV profile of CFerPhlor mixture used in this study: The UV peak at 240 nm is typical of the vitamin C component of the mixture. In general, the UV absorption in the UVA/UVB area is negligible, suggesting that the mixture by itself does not act as a sunscreen.

Erythema

UV irradiation significantly increased the erythema response of human skin essentially in a linear manner from 1–5× MEDs (Fig. 2a,b). Colorimetry revealed that CFerPhlor provided significant protection ($P < 0.01$) against UV-induced erythema at all MEDs tested (Fig. 2b).

Sunburn cells

Sunburn cells are epidermal apoptotic cells resulting from extensive UV damage. CFerPhlor provided significant protection ($P < 0.01$) in comparison to vehicle against UV generation of sunburn cells at 5× MEDs (vehicle 8.2 ± 3.8 cells/mm skin vs. CFerPhlor 0.8 ± 0.8 cells/mm skin; Fig. 3). The non-UV-exposed site showed no sunburn cells as expected.

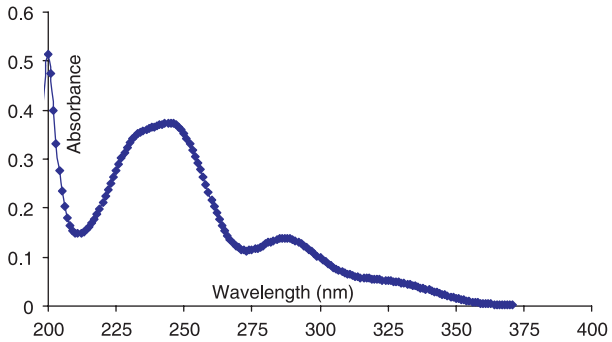

Figure 1 Ultraviolet (UV) absorption profile of vitamin C, ferulic acid, and phloretin (CFerPhlor). CFerPhlor was diluted to 100 p.p.m. and the UV profile was measured using a spectrophotometer in the range of 200 nm to 400 nm. The UV absorbance (*Y*-axis) was plotted against the wavelength (*X*-axis).

Thymine dimers

Thymine dimers are signature DNA damages resulting from UV irradiation that appear to be important in the generation of nonmelanoma skin cancer. Enumeration of thymine dimers in epidermal keratinocytes from 5× MED UV-irradiated skin revealed significant protection ($P < 0.01$) by CFerPhlor when compared to vehicle (vehicle 92.9 ± 36.9 cells/mm skin vs. CFerPhlor 6.8 ± 5.3 cells/mm skin; Fig. 4). The non-UV-exposed site showed no thymine dimers.

p53

p53 is a cellular protein induced by UV irradiation as a response to DNA damage. It slows the cell cycle to allow for DNA repair and if the damage is great induces cellular apoptosis. Enumeration of epidermal cells showing p53 induction revealed significant protection ($P < 0.01$) by CFerPhlor when compared to vehicle control (vehicle 69 ± 22.8 vs. CFerPhlor 45 ± 29.8; Fig. 5). The non-UV-exposed site showed minimal amount of p53-positive cells, much lower than that of either vehicle-treated or antioxidant-treated sites.

MMP-9

MMP-9 or type IV collagenase is induced by UV irradiation. It degrades basement membranes and is thought to be important in tissue remodeling. Skin stained for histochemistry was evaluated on a scale of 1 to 4 based on the numbers of positive cells. CFerPhlor protected against the induction of MMP-9 by UV when compared to vehicle (vehicle 2.7 ± 1.2 vs. CFerPhlor 1.2 ± 0.9; Fig. 6). The levels of MMP-9 in the CFerPhlor-treated site were as low as the non-UV-irradiated site.

Langerhans cells CD1a

Langerhans cells are epidermal antigen-presenting cells of the skin essential for initiating the immune response. They are reduced in number, and their morphology is altered by UV irradiation. CFerPhlor essentially completely prevented ($P < 0.01$) UV-induced reduction of Langerhans cells (vehicle 7.8 ± 5.1 vs. CFerPhlor 18.1 ± 4.5 vs. control skin 15.8 ± 3.8; Fig. 7) to the same levels as the non-UV-irradiated site.

Discussion

These studies document that substantial photoprotection can be achieved by an antioxidant solution containing

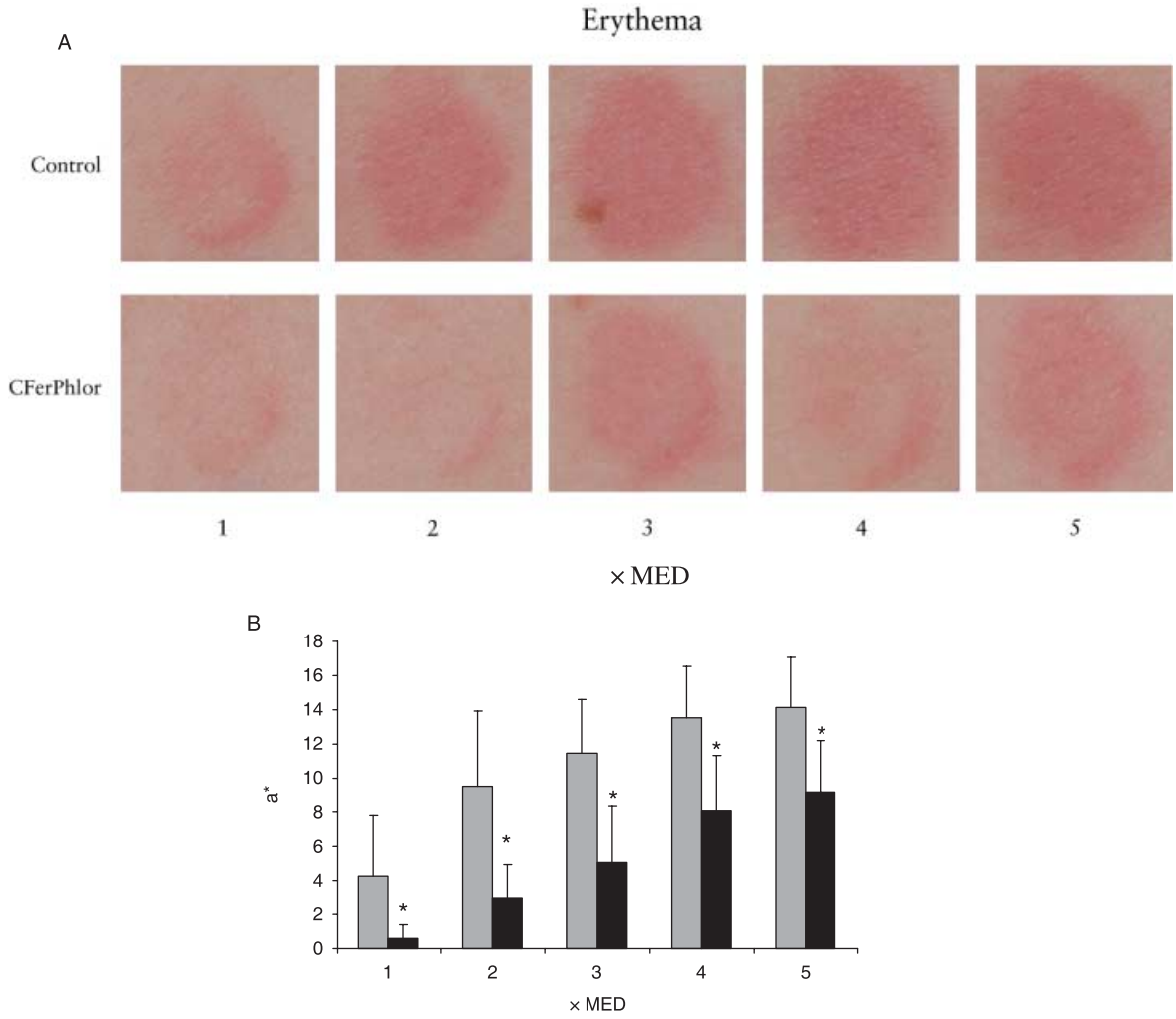

Figure 2 Effect of vitamin C, ferulic acid, and phloretin (CFerPhlor) protection against ultraviolet (UV)-induced erythema. CFerPhlor or vehicle alone (control) were applied to back skin (2 mg/cm^2) daily for 4 days. Skin was irradiated with solar-simulated UV 1×–5× minimal erythema dose (MED). Erythema was determined 1 day later (Fig. 2a). Figure 2b shows the colorimetry of digital photographs. Gray bar represents the erythema values for the vehicle-only control and the black bar represents erythema at the CferPhlor-treated site. Mean ± SD (n = 10). CferPhlor-treated site showed statistically significant reduction of erythema compared to the vehicle-treated control site (*P < 0.01) at all MEDs tested.

vitamin C, ferulic acid, and phloretin. We have previously reported the usefulness of vitamin C and ferulic acid. The addition of phloretin resulted from a systematic testing of plant polyphenols for their ability to get into skin following topical application and to provide subsequent photoprotection. Phloretin, a dihydrochalcone polyphenol, together with its glucoside phloridzin are found relatively uniquely in apples and are thought to be important in the health-promoting effects of this fruit.[7,8] Phloretin has strong antioxidant effects when tested against experimental stable free radicals,[9–11] hydroxyl radical,[9] and prevention of lipid peroxidation.[12,13] Phloretin inhibited MMP-1 and elastase,[14] enzymes that degrade connective tissue and are considered important in photoaging. Phloretin has been demonstrated to inhibit tyrosinase activity in human melanocytes and as such may be a useful ingredient as a topical lightening agent for pigment disorders of skin.[15] Phloretin has also been studied as a penetration enhancer for topical application to skin.[9,15–17] It is possible that phloretin enhanced photoprotection of CFerPhlor partly by increasing skin uptake of vitamin C and ferulic acid.

CFerPhlor provided virtually complete inhibition of UV-induced thymine dimers. These DNA changes, if unrepaired, may lead to mutations ubiquitous in non-melanoma skin cancers.[18,19] The mutations are signature for UV induction and until recently have been considered

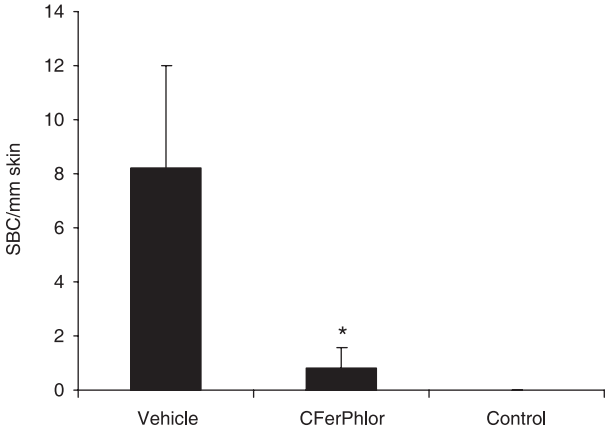

Figure 3 Effect of vitamin C, ferulic acid, and phloretin (CFerPhlor) on sunburn cell formation. CFerPhlor or vehicle was applied to back skin (2 mg/cm^2) daily for 4 days. Skin was irradiated with solar-simulated UV 1×–5× MEDs. Skin biopsies of 5× MED-treated skin were taken 1 day later. Sunburn cells were counted and are expressed as positive cells per millimeter of epidermis. Unirradiated control skin contained no sunburn cells. CferPhlor-treated site showed statistically significant reduction in sunburn cell formation (*$P < 0.01$) compared to vehicle-treated site. Mean ± SD ($n = 10$).

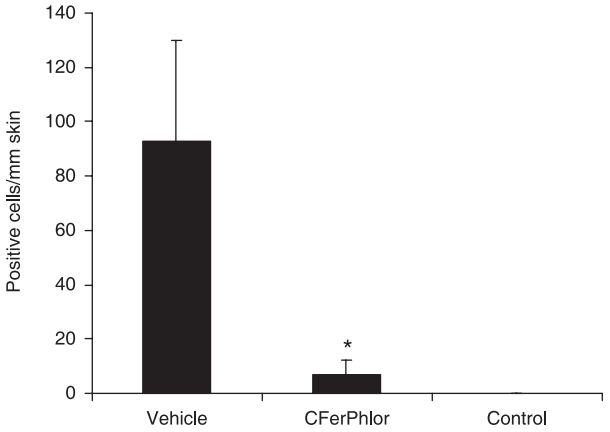

Figure 4 Effect of vitamin C, ferulic acid, and phloretin (CFerPhlor) on thymine dimer formation. CFerPhlor or vehicle was applied to back skin (2 mg/cm^2) daily for 4 days. Skin was irradiated with solar-simulated UV 1×–5× MEDs. Skin biopsies of 5× MED-treated skin were taken 1 day later, and formalin-fixed tissue was stained for immunohistochemistry using a mouse monoclonal antibody to thymine dimers. Positive cells were counted and are expressed as cells per millimeter of epidermis. Unirradiated control skin contained no cells positive for thymine dimers. CFerPhlor-treated site showed statistically significant reduction in thymine dimer formation (*$P < 0.01$) compared to vehicle-treated site. Mean ± SD ($n = 10$).

to result from DNA absorption of UVB energy. Recent observations have revealed that these DNA changes can result from UVA irradiation[20,21] even in the absence of appreciable absorption of UVA by DNA.[22] The potential

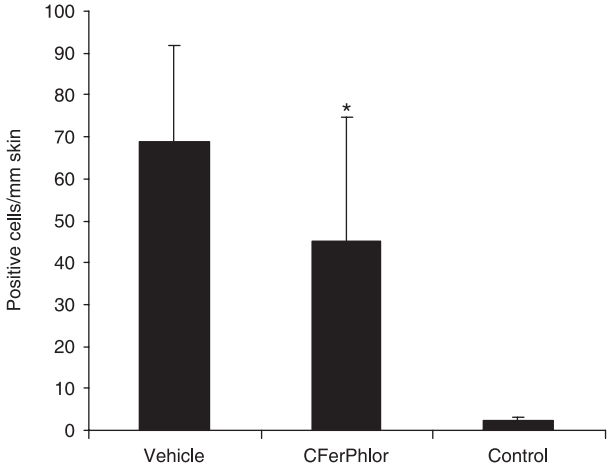

Figure 5 Effect of vitamin C, ferulic acid, and phloretin (CFerPhlor) on p53. CFerPhlor or vehicle was applied to back skin (2 mg/cm^2) daily for 4 days. Skin was irradiated with solar-simulated UVR 1×–5× MEDs. Skin biopsies of 5× MED-treated skin were taken 1 day later, and formalin-fixed tissue was stained for immunohistochemistry using a mouse monoclonal antibody to p53. p53-positive cells were counted and are expressed as positive cells per millimeter of epidermis. Unirradiated, untreated skin served as control. CFerPhlor-treated site showed statistically significant reduction in p53-positive cells (*$P < 0.01$) compared to vehicle-treated site. Mean ± SD ($n = 10$). Unirradiated site (control) showed minimal p53-expressing cells.

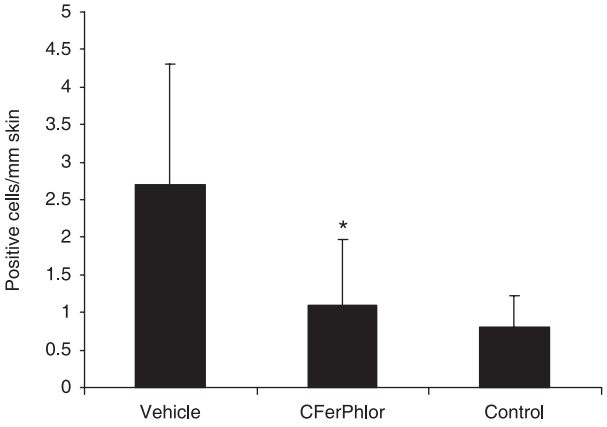

Figure 6 Effect of CFerPhlor on MMP-9 levels. CFerPhlor or vehicle was applied to back skin (2 mg/cm^2) daily for 4 days. Skin was irradiated with solar-simulated UV 1×–5× MEDs. Skin biopsies of 5× MED-treated skin were taken 1 day later, and formalin-fixed tissue was stained for immunohistochemistry using a rabbit polyclonal antibody to MMP-9. Levels of cytoplasmic MMP-9 expression were graded on a scale from 1–4. Unirradiated, untreated skin served as control. CFerPhlor-treated site showed statistically significant reduction in MMP-9-expressing cells (*$P < 0.01$) compared to vehicle-treated site. There was no statistically significant difference between CFerPhlor-treated site and the control site. Mean ± SD ($n = 10$).

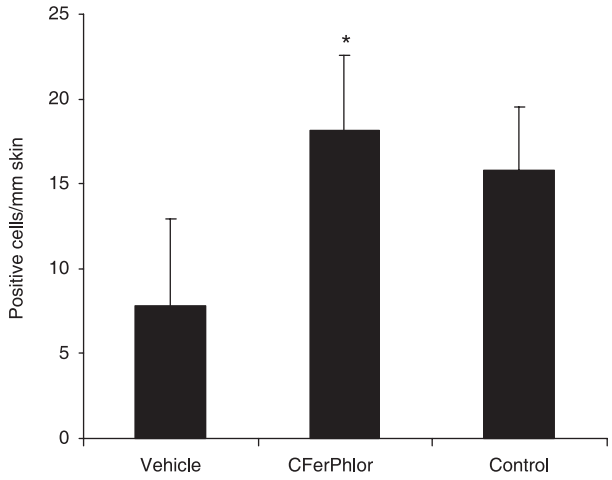

Figure 7 Effect of CFerPhlor on Langerhans cells. CFerPhlor and vehicle were applied to back skin (2 mg/cm^2) daily for 4 days. Skin was irradiated with solar-simulated UV 1×–5× MEDs. Skin biopsies of 5× MED-treated skin were taken 1 day later, and formalin-fixed tissue was stained for immunohistochemistry using a mouse monoclonal antibody to CD1a. Positive cells were counted and are expressed as cells per millimeter of epidermis. Unirradiated, untreated skin served as control. UV irradiation suppressed CD1a-positive cells in vehicle-treated site. CFerPhlor treatment significantly protected skin and increased the levels of CD1a-positive cells to the same level as the unirradiated control site (*$P < 0.01$). Mean ± SD ($n = 10$).

importance of UVA in the generation of thymine dimer is magnified when one considers that skin structure may essentially prevent most UVB radiation from getting to the epidermal stem cell layer that would presumably be necessary for an oncogenic event.[21] Since UVA generates more oxidative stress than UVB,[23] and since antioxidants prevent UVB-induced cyclopyrimidine dimer formation in human HaCaT cells but not in naked DNA,[24] it has been hypothesized that there is a cellular photosensitiztion process that is capable of transferring energy that results in cyclopyrimidine dimer formation.[20,21,24,25] In addition, if DNA repair enzymes are altered by oxidative stress, timely repair of these DNA mutations may not happen, further allowing mutant DNA to enter the cellular replicating pool.[21,25] p53 monitors this DNA damage slowing the cell cycle to allow optimal repair. p53 is induced in response to DNA damage[26] as well as oxidative stress.[27] The absence of both of these triggers protected by CFerPhlor presumably relates to the lack of induction of this substance.

MMPs are important enzymes involved in turnover of the extracellular matrix in skin. Several MMPs including MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, and MMP-12 are up-regulated following UV irradiation of skin leading to increased breakdown of skin collagen and elastins.[28,29] Destruction of the extracellular matrix following UV irradiation is thought to be responsible for the photoaging response and induction of MMP-9, which destroys basement membranes. Inhibition by CFerPhlor would be expected to be protective.

UV irradiation exposure is recognized to suppress cell-mediated immunity in human subjects. This effect is mediated in part, by alterations of immune functions of Langerhans cells and dermal dendritic cells.[30] Langerhans cells are active in the capture, uptake, and processing of microbes (antigens) that infect skin. Activated Langerhans cells travel to the lymph tissue and present the processed microbial antigens to lymphocytes as a first step to initiate immunity. A single exposure of human skin *in vivo* to UV irradiation results in alterations of density and/or morphology of Langerhans cells including reduction of dendricity.[31] CFerPhlor was quite efficient for preventing UV reduction of Langerhans cells.

Use of sunscreens for skin photoprotection directly by preventing the penetration of harmful UV rays into skin is well established. However, sunscreens often do not provide 100% protection against UV irradiation due to nonuniform application to skin surface.[32] Studies have shown significant free radical generation by UV irradiation in skin even when protected by sunscreens.[33] Antioxidants exert protective effects from inside skin and, because they stimulate cellular defense mechanisms, remain active for several days.[34] Since sunscreens and antioxidants work by different mechanisms, they would be expected to be complimentary. We previously have reported the lack of a dose–response effect of an antioxidant mixture containing vitamins C and E stabilized by ferulic acid demonstrating no apparent sunscreen effect.[5] In addition, the UV profile of CFerPhlor shows no UV absorption in the UVA/UVB range (320–400 nm), suggesting no SPF value. Therefore, the contribution to the photoprotective effect of CFerPhlor by UV absorption would be expected to be minimal. In conclusion, this study demonstrates the beneficial effects of a unique mixture of antioxidants for photoprotection, and this antioxidant mixture is expected to work in synergy with sunscreens in photoprotection of human skin.

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