Tocotrienol-rich fraction attenuates UV-induced inflammaging: A bench to bedside study

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Summary

Background: UV radiation from the sun is the most common environmental stressor to damage the skin. It is now well established that photodamaged skin manifests signs of mild but chronic inflammation, termed as "inflammaging." Thus, there is an urgent need for anti-inflammatory regimes that can limit the damage caused by inflammation.

Objectives: This study aimed to evaluate the possible palliative effects of a new topical nanoemulsion formulation containing tocotrienol-rich fraction (TRF) on UV-induced inflammation (erythema) of human skin.

Methods: An in vitro model was used to demonstrate the ability of TRF to alleviate photodamage via attenuation of UV-induced oxidative stress and inflammation. Two ex vivo models (skin antioxidative potential and radical sun protection factor) were used to determine the efficacy of different formulations of TRF on the skin. A UV-induced erythema protection test in 20 subjects was conducted.

Results: In vitro studies involving HaCaT keratinocytes revealed that TRF possesses marked anti-inflammatory properties, as indicated by the attenuation of UV-induced upregulation of pro-inflammatory cytokines. A 1% TRF formulation was found to be more effective in enhancing the endogenous antioxidative protection of skin compared to 1% TRF in medium chain triglycerides because of its higher penetration kinetic profile. The clinical study showed that formulated TRF was effective in reducing skin redness after UV irradiation as early as after 6 hours of application. A significant depigmentation was also observed in TRF treatment subjects.

Conclusion: TRF may serve as an anti-inflammatory compound that is safe to be applied daily to protect the skin from UV-induced inflammaging.

KEYWORDS
diclofenac, erythema, inflammation, sunlight, tocotrienol

1 INTRODUCTION

There is increasing evidence that aging involves low grade, systemic, and chronic inflammation, collectively termed as inflammaging. First coined in 2000 to describe a person’s aging process, this mild inflammation manifests as elevated levels of C-reactive protein (CRP) and Interleukin-6 (IL-6), which are associated with age-related pathologies. While acute and transient inflammation serves as an...
immediate defense against physical injury and pathogen infection, this inflammation leads to detrimental damage of internal tissues and organs for a prolonged period.

Our skin is the largest external organ and serves as an important dynamic barrier (physical, chemical, and immunological) to external pathogens and harmful radiation. There are two types of skin aging: intrinsic (chronological) and extrinsic (accelerated). The former depends on the passage of time. As one ages, skin changes are accompanied by flattening of epidermal-dermal junction, epidermal thinning, and changes of connective tissues structure. In addition, the inflammatory response becomes exaggerated and thus aged skin become mildly inflamed. Extrinsic aging is known as photoaging/accelerated aging due to constant environmental insults such as UV radiation. This process accounts for a wide range of biological effects including physiological processes and alterations to the skin. UV radiation, even at insufficient levels to cause a sunburn, induces small amounts of damage to the skin. These damages initiate a sequence of events that can lead to inflammation and free radical generation (ie reactive oxygen species [ROS] and reactive nitrogen species [RNS]), which stimulate the restoration of the skin’s barrier function. It is now well established that photodamaged skin also shows signs of mild but chronic inflammation. Photodamaged skin shows signs of inflamming including coarse and deep wrinkles, mottled pigmentation, sallowness, and telangiectasia.

Many strategies have been employed to prevent the damage caused by the complex cascade of these biochemical reactions. Examples include blocking UV penetration using sunscreen, reducing inflammation using anti-inflammatory compounds, and quenching of free radicals by antioxidants. Sunscreens with different absorption wavelength exist to filter or scatter UV radiation. They are effective but remain insufficient, in preventing photoaging and skin cancers. The major public health concern is the potential risk of vitamin D deficiency by sunscreen use. Furthermore, some UV filters have been reported to cause photo-allergy and endocrine disruption. Therefore, natural botanical products with photo-protection properties have been gaining attentions nowadays for the prevention of sunburn and UV-induced skin disorders.

Vitamin E—a ubiquitous yet indispensable liposoluble antioxidant—has been used for more than 60 years in experimental and clinical dermatology. The hydroxyl group on the chromanol ring acts to scavenge chain-carrying lipid peroxyl radicals on skin. Vitamin E consists of two members: tocopherol (TP) with saturated phytyl chain and tocotrienol (T3) with unsaturated farnesyl isoprenoid chain. Each member has four homologs: alpha (α), beta (β), gamma (γ), and delta (δ) depending on the number and location of methyl groups on the chromanol ring. TPs are mainly found in olive, sunflower, soybean, and corn oils, while T3s are present in edible oils from plants such as the palm tree, rice bran, and annatto.

Unlike TP, T3 is considered to be a lesser known form of vitamin E. T3 has, for decades, been overshadowed by TP, in particular αTP, which is a more relevant Vitamin E form for human physiology. Nonetheless, over the years, T3 has been shown to exert biological properties that are distinct from those performed by αTP because of T3’s three double bonds in the side chain. This has prompted a surge of interest to study the feasibility of using T3 as a potential active ingredient in sun protection products, particularly on those related to T3’s improved anti-oxidation, anti-inflammatory, and anticancer properties compared to αTP. These activities are believed to play a vital role in ameliorating UV-induced inflamming.

To create an effective protective defense system for the skin against UV-induced accelerated aging, a topically applied therapy should be (i) photostable; (ii) exhibit high cutaneous absorption and penetration; (iii) exhibit resistance against cutaneous endogenous oxidation; (iv) reduce inflammation; and (v) exit in an effective carrier system (either formulation or emulsion). Thus, in this study, several experiments were conducted under realistic experiment conditions to understand the mechanism on how TRF (a commercially available mixture of T3 and αTP) acts as an anti-inflammatory ingredient and antioxidant to prevent UV-induced inflamming. Firstly, electron spin resonance spectroscopy (ERS) was used to determine the skin antioxidative potential (SAP) to measure the ability of different formulations of TRF to penetrate the skin and scavenge-free radicals. Secondly, radical sun protection factor (RSF) test was performed to test the ability of TRF to reduce free radical formation in skin following UV exposure. Lastly, a UV-induced erythema protection test in 20 subjects was carried out to indicate degree of anti-inflammation by a 1% TRF formulation. In addition, an in vitro model using HaCaT keratinocytes was used to demonstrate at the cellular level the ability of TRF to alleviate photodamage via the prevention of oxidative stress and inflammation. To the best of our knowledge, this is the first study to demonstrate the efficacy of TRF for treating UV-induced inflammation from cellular and ex vivo models to human application.

## Methods

### Methodology for in vitro study

#### Cell lines and reagents

The immortalized human keratinocyte cell line (HaCaT) was supplied as A/P Gautam Sethi (National University of Singapore, Singapore). HaCaT cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 1% Pen/Strep in a humidified incubator in equillibration with 5% CO₂ at 37°C. Malondialdehyde (MDA) and protein carbonyl enzyme-linked immunosorbent assay (ELISA) kits were obtained from Cell Biolabs, Inc (San Diego, CA, USA). 8-hydroxy-2'-deoxyguanosine (8-OHdG) was obtained from Cayman (Ann Arbor, MI, USA). Cyclooxygenase-2 (COX-2), IL-6, and Interleukin-8 (IL-8) were purchased from R&D systems (Minneapolis, MN, USA).

#### Tocotrienol-rich fraction (TRF)

TRF with purity ≥95% (DavosLife E3) was supplied by Davos Life Science Pte Ltd (Singapore). This TRF was extracted using state of the art technology from non-GMO palm fruits sourced directly from Roundtable on Sustainable Palm Oil (RSPO)-certified plantations of...
Kuala Lumpur Kepong (KLK). TRF was dissolved in dimethylsulfoxide (DMSO) as a 100 mM stock solution and stored at -20°C. Working solution was prepared by further dilution in cell culture medium as required.

### 2.1.3 Cell viability measurement

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) proliferation assay was used to determine the cell viability of HaCaT cells after TRF treatments or UVB irradiation. Two hours before the end of treatment, 20 μL of 5 mg/mL of MTT solution was added into each well containing cells. The cells were then incubated at 37°C with 5% CO2. Two hours after the incubation, the medium was aspirated and 200 μL of DMSO was added to each well to solubilize the crystals. The number of metabolically active cells is directly proportional to the level of formazan product formed. The quantity of formazan present was determined by measuring its absorbance at 595 nm using the EnSpire® Multimode Plate Reader (Perkin Elmer, Waltham, MA, USA).

### 2.1.4 UV irradiation and Enzyme-linked immunosorbent assay (ELISA)

An 8-watt model UVB lamp with 302 nm wavelength (Cole Parmer, Vernon Hills, IL, USA) was used for the UVB exposure. UVB intensity was measured using photodetector probes (Scitec Instrument, UK). The emitted irradiation consisted of 100% UVB with a total output of 2000 μW/cm² at a distance of 15 cm from the source and had a maximum irradiating area of 356 cm². HaCaT cells were pretreated with 30 μM TRF for 24 hours prior to 20 mJ/cm² UVB exposure (1 MED). HaCaT cells were collected at 24 hours for the quantification of the following ELISA assay according to the manufacturer’s protocols: oxidative markers (8-OhdG, MDA, and protein carbonyl), and inflammatory markers (IL-8, IL-6, and COX-2).

### 2.1.5 ROS measurement by flow cytometry

HaCaT cells (2 × 10⁵ cells/well) were seeded in 6-well plate and incubated with 30 μM TRF for 24 hours, followed by 1 MED UVB exposure. 5 μM Dichloro-dihydro-fluorescein diacetate (DCFH-DA) solution was added, and 2′,7′-dichlorofluorescein (DCF) fluorescence (Excitation 488 nm; Emission 520 nm) was detected after 15 minutes using FACS LSR LL (Becton Dickson, Franklin Lakes, NJ, USA).

### 2.1.6 ROS measurement by fluorescence plate reader

5 × 10⁴ HaCaT cells were seeded in each well of a 24-well plate and pretreated with 30 μM TRF for 24 hours. The next day, the cells were washed with KRH buffer (125 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 2.6 mM MgSO₄, 5 mM Hepes, pH 7.2) and then incubated with 100 μM DCFH-DA at 37°C for 30 minutes before 1 MED UVB irradiation. Fluorescence intensity per each well was detected at an excitation wavelength of 485 nm and at an emission wavelength of 530 nm using a EnSpire multimode reader (Perkin Elmer, Waltham, MA, USA).

### 2.2 Methodology for ex vitro studies

#### 2.2.1 Test products

A proprietary aqueous nanoemulsion skin care formulation containing 1% TRF (DavosLife E3) was developed by Vesifact AG (Switzerland). This formulation and other products (1% TRF in medium chain triglycerides [MCT], and a commercial sun repair product containing 0.1% diclofenac sodium) that used for comparison were coded and sent to Gematria Test lab (Berlin, Germany) for SAP and RSF testing.

#### 2.2.2 Skin Antioxidative Potential (SAP)

This method has previously been described by T.Herrling et al. to measure the ability of topically applied antioxidants to penetrate the skin and quench free radicals. Briefly, skin biopsies from the external lobe of fresh pig ears were washed, and subcutaneous fat was removed. The skin was cut into 1 × 1 cm pieces and placed on a filter paper presoaked with 100 μL Epilife media (Gibco, MA, USA) containing 0.1 mM Ca²⁺. Test samples were then applied on the epi-dermal layer at a concentration of 2 mg/cm². The skin samples were kept at room temperature protected from light for different time point to allow the test products to penetrate the skin. After the predetermined application time, the dermis side of the skin biopsies was placed onto a filter paper saturated with 100 μL Epilife media (Gibco, MA, USA) containing 0.1 mM Ca²⁺. The skin biopsies were then placed in electron spin resonance (ESR) tissue cell, and the ESR spectra of the TEMPO were monitored over time to measure the quenching of radicals. The amplitudes of the ESR spectra were plotted against time, and the obtained kinetics were fitted using monoexponential phase decay algorithm. The SAP value is the kinetics parameter of treated skin samples as expressed relative to untreated skin.

#### 2.2.3 Radical sun protection factor (RSF) test

RSF is the ratio of the number of free radicals generated in unprotected skin to the number of free radicals generated in protected skin, all following UV radiation. This method has been previously described by Wang SQ et al. to measure the amount of free radicals generated in the skin by ESR spectroscopy following UV irradiation. Briefly, skin from the outer lobe of fresh pig ears was washed, and subcutaneous fat was removed. The skin was cut into 1 × 1 cm pieces. Skin samples were soaked in 1 mM solution of the nitroxyl spin trap, 2, 2, 5, 5-tetramethylpyrrolidine-N-oxyl (TEMPO, Sigma-Aldrich, MO, USA) for 5 minutes. The skin biopsies were then placed in electron spin resonance (ESR) tissue cell, and the ESR spectra of the TEMPO were monitored over time to measure the quenching of radicals. The amplitudes of the ESR spectra were plotted against time, and the obtained kinetics were fitted using monoexponential phase decay algorithm. The SAP value is the kinetics parameter of treated skin samples as expressed relative to untreated skin.
skin. A 6 mm punch biopsy specimen of the pig skin was taken after 10 minutes. The specimen was then exposed to 1.2 MED with a solar simulator (SOL F2, Höntle AG, Germany). This MED is equivalent to a UV output of UVA 22 mW/cm² and UVB 1.2 mW/cm² for an irradiation dose of 8.1 J/cm². The RSF was calculated from means of a calibration curve that was constructed using untreated skin samples covered with optical density filters of different transmission rates.

2.3 Methodology for the human study

The clinical trial of the UV-induced erythema protection test (Study code: DCC07W084) was carried out by a contracted research organization, Derma Consult GmbH (Germany). A proprietary skin care formulation containing 1% TRF was developed by Vesifact AG (Switzerland). The composition of the TRF used in this study consisted of 92% total T3 (29.5% αT3, 3.3% βT3, 43.0% γT3, and 16.3% δT3) with low content of αT2P (8%). This formulation and a commercial product containing diclofenac sodium were coded and compared. The trial protocol was performed in accordance with the ethical principles derived from the Declaration of Helsinki and in accordance with the International Conference on Harmonisation (ICH) Good Clinical Practice. Two phases were carried out: preconditioning phase (MED determination) and clinical evaluation phase (product testing).

2.3.1 Preconditioning phase

The UV protection of the 1% TRF formulation was evaluated in 20 healthy subjects and compared to a commercial product that contained diclofenac sodium (Novartis, Switzerland). All participating subjects gave their written informed consent prior to entry into the trial. A physical examination of the back was performed. The subjects were between 31 and 56 years of age (average 42.9), clinically healthy and of skin prototype I to III. Exclusion criteria were those with skin disease, uneven skin tone, sunburn, tan, scar, or other lesions in the test area. After enrollment into the trial, the subjects were instructed not to use any topical preparations on the test area starting from 7 days prior to the clinical evaluation phase until the final measurement.

The UV source was a set of 4 Ultra-Vitalux 300 W (Osram GmbH, Germany) lamps mounted as a square with 30 cm between the midpoints of the lamp holders. The spectrum of Ultra-Vitalux lamps is designed to resemble natural sunlight. The distance between test area and bulb crown was fixed at 50 cm to achieve a uniform intensity across the test area. Lamp intensity was checked prior to irradiation by a Solar Light PMA 2100 Photometer equipped with an erythema UV detector PMA2103 (Solar Light Company, PA, USA). Areas to be irradiated were delineated using a skin marker (Edding International GmbH, Germany) with the general test area restricted to a 3 cm square in the center of each test area.

Skin color was measured with a Minolta Chromameter CR400 using the Commission International de l’Eclairage (CIE) L*a*b* color space. This device uses independent and standardized methodology to express colors adjusted to nonlinear color sensitivity of human eye. In the L*a*b* color space, a color is expressed in a three-dimensional coordinate system with green-red (a*)—negative values are green, positive values are red), yellow-blue (b*), and L* axes (lightness). The range of a* and b* axes was from −128 to +127, and the range L* axes was from 0 (black) to 100 (white). The individual typology angle (ITA<sup>a</sup>) is a vector representation in the plane of the L* vs b* values<sup>13</sup> and defined as:

\[
ITA^a = \left( \frac{L^* - 50}{b^*} \right) \times 180/\pi
\]

Smaller ITA<sup>a</sup> values correspond to a darker toned skin and it can be categorized as:

- Brown — 10° ≥ ITA<sup>a</sup>
- Tan — 28° ≥ ITA<sup>a</sup> > 10°
- Intermediate — 41° ≥ ITA<sup>a</sup> > 28°
- Light — 55° ≥ ITA<sup>a</sup> > 41°
- Very light — ITA<sup>a</sup> > 55°

During measurement, the skin surface was illuminated by a Xenon flashlight, and reflected light was registered and analyzed by a photoreceiver. The Chromameter CR400 is sensitive and accurate for the characterization of skin color and measurement of a spot of 8 mm diameter. Before each measuring series, the instrument was calibrated against a standard white tile. Each color measurement reported was the average of three measurements.

To determine the MED for each volunteer, a series of six UV exposures (1 × 1 cm) was given to the test area during the preconditioning phase. The time intervals selected comprised a geometric series, where each exposure was 25% greater than the previous exposure time. The visual MED on unprotected skin was defined as the quantity of radiant energy required to produce the first perceptible unambiguous redness reaction with clearly defined borders, when assessed 16–24 hours after irradiation. The irradiation dose was measured in time.

2.3.2 Clinical evaluation phase

Subjects were instructed not to wash the test area for 8 hours before the commencement of the trial. Test areas of 5 × 5 cm for the products and untreated controls were delineated using a skin marker (Edding International GmbH, Germany) with the general test area between the waistline and the shoulder blades. The minimum distance between the test areas was 1 cm, and their location was selected to arrive at a maximum uniformity of skin color. The test products were randomly applied to the test areas. To avoid distortions and inhomogeneity of product application at the edges, color measurements were restricted to a 3 × 3 cm square in the center of each test area.

Baseline measurements were obtained before the test areas were irradiated with 1.5 MED. Products were applied 10 minutes after the completion of irradiation. Products were applied at a dosage of 4 mg/cm² and spread using a gloved finger. Upon completion of application, the subject remained in the prone position for 20 minutes to allow absorption of the test materials.

Skin color was measured and the product re-applied 6 hours after irradiation. Subsequent skin color measurements were made
every morning at 24, 48, 72, 96, and 120 hours after UV irradiation. The test products were also re-applied. In the evening, approximately 8 hours after the application in the morning, the product was re-applied. Subjects were requested not to wash the test area for 2 hours after each application. On each visit, the delineation of the test area was re-assessed and marked again if necessary.

2.4 | Statistical analysis

All statistical analyzes were performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). For in vitro and ex vivo studies, data are presented as the means ± standard deviation (SD). The one-way analysis of variance (ANOVA) with Bonferroni’s post hoc test was used to compare the differences between the experimental groups (TRF treatment after UV irradiation) and the control group (vehicle treatment after UV irradiation). For the human study, data are presented as mean ± standard error of the mean (SEM). The repeated measures ANOVA with Bonferroni’s post hoc test was used for area under curve (AUC) analysis. Significant differences in reducing of skin redness or tan between treated vs untreated were tested in a pairwise manner using Wilcoxon Signed-Rank test. \( P < .05 \) (*) was considered to be statistically significant. Other values were indicated as follows: \( P < .01 \) (**), \( P < .001 \) (***) , and \( P < .0001 \) (****).

3 | RESULTS

3.1 | Determination of doses of TRF and UV

We first confirmed the cytotoxicity of the TRF dose use because the compound’s photo-protective effect studied here is independent of the compound’s antiproliferation effect. According to Figure 1A, TRF did not affect the proliferation rate of HaCaT cells during a 24-h incubation period, even at a high concentration (100 \( \mu \)M). In the subsequent cell line experiment, 30 \( \mu \)M TRF treatment dose was used as it mimics to the physiological dose of vitamin E (\( \alpha \)TP) in human epidermis (31.0 ± 3.8 nmol/g of skin).\(^{14}\) Secondly, we determined the effect of UVB irradiation on the proliferation of keratinocytes by irradiating HaCaT cells with increasing doses of UVB (0, 20, 40, 80, 100, or 200 mJ/cm\(^2\)) for 24 hours. Figure 1B shows that the exposure to UVB significantly decreased cell viability in a dose-dependent manner. A dose of 20 mJ/cm\(^2\) UVB was selected for the subsequent in vitro experiments on the basis of these criteria: (i) This dose is equivalent to 1 MED under skin testing conditions for Fitzpatrick skin types I to III;\(^{15}\) (ii) this dose is within the physiological range of exposure of UV light that reaches the basal layer of the epidermis during midday;\(^{16}\) and (iii) this dose decreases viability of keratinocytes approximately by 45%-50% at 24 hours.

3.2 | TRF prevents UV-induced oxidative stress and inflammation in human keratinocytes

Vitamin E is a well-known antioxidant that quenches free radicals during oxidative stress. ROS scavenging of TRF after UV irradiation was measured by analyzing the 2',7'-dichlorofluorescein fluorescence (DCF) amount. As shown in Figure 2A, 30 \( \mu \)M TRF was effective at reducing intracellular ROS concentration after being treated with different doses of TRF (0, 10, 25, 50, or 100 \( \mu \)M) for 24 h. No statistically significant differences for the TRF-treated cells compared to untreated cells were present. (B) HaCaT cells were irradiated with increasing doses of UVB (0, 20, 40, 80, 100, or 200 mJ/cm\(^2\)) for 24 h. 20 mJ/cm\(^2\) is equivalent to 1 MED for skin testing conditions for Fitzpatrick skin type I to III. All data represent the means ± SD of at least 3 independent experiments. \( P \) values were assessed by one-way ANOVA, followed by Bonferroni’s post hoc test.
malondialdehyde (MDA), and protein carbonyls, which are hallmarks of oxidation in DNA, lipid, and protein, respectively. In addition, ROS has been reported to affect secretion of pro-inflammatory cytokines and chemokines by activating numerous cell signaling pathways. Figure 2C shows that 30 μM TRF has significant anti-inflammatory effects by suppressing UV-induced upregulation of IL-6, IL-8, and COX-2. Taken together, these data indicate that TRF inhibits UV-induced oxidative damage and downregulates pro-inflammatory markers through suppression of ROS formation.

3.3 Formulated TRF enhances skin penetration ex vivo

The SAP test measures the increase in free radical scavenging potential of a formulation after allowing the formula to penetrate the skin for a predetermined period of time. A SAP value of the vehicle-treated skin was set to 100%. The SAP value of 150% indicates that application of a product increases the antioxidant potential of the skin by 50% after application. In our study, three different formulations were tested as follows: 1% TRF formulation as an aqueous nanoemulsion, 1% TRF in MCT, and 1% αTP in an oil/water emulsion. As shown in Figure 3A, skin treated with 1% αTP showed an increase in antioxidant potential of 19 ± 5% after 10 minutes and remained increased to 47 ± 7% for 20 minutes application. Meanwhile, compared with αTP, 1% TRF formulation had a drastic increase after 10 minutes (46 ± 4%), and this increase was sustained for up to 20 minutes (55 ± 7%). However, no increase in SAP was observed when TRF was applied to skin as a solution in MCT.

Figure 3B illustrated that all the vehicles—MCT, base formulation, and base oil/water emulsion—have shown no appreciable SAP value after 10 minutes of skin penetration. Of the three products, 1% TRF formulation was found to be effective in enhancing endogenous antioxidative protection level (P < .0001). In addition, 1% TRF formulation showed a more robust SAP enhancement compared to 1% αTP (P < .05).

3.4 Formulated TRF reduces free radicals after UV exposure ex vivo

The SAP test indicated that 10 minutes were sufficient for significant increase in antioxidant level; therefore, comparisons further tests were conducted using 10 minutes as the permeation time. The RSF is similar to the SPF test. While the SPF test measures the effectiveness of a sunscreen at preventing UV-induced erythema compared to unprotected skin, the RSF represents how much more effective a formulation is at protecting against UV-induced free radical formation. The RSF is defined as a number of free radicals in untreated skin over the amount of free radicals in treated skin. An RSF of 1 indicates no protection effect is observed.

As shown in Figure 4, neither MCT vehicle nor formulation vehicle influenced the RSF. TRF had an RSF of 3 when dissolved in MCT, and TRF had an RSF of 4 when it dissolved into a nanoemulsion formulation. These data indicate that application of TRF in an optimally formulated product can reduce radical formation after UV exposure by 75%. A high RSF for 1% TRF in nanoemulsion formulation confirmed effective antioxidant activity, as shown in Figure 3B. Nonetheless, TRF in MCT showed good enhancement of RSF despite having an unmeasurable SAP value.

3.5 TRF mitigates UV-induced erythema and tanning in human subjects

A human clinical study to determine the anti-inflammatory properties of TRF after UV exposure was conducted by a qualified dermatologist from Derma Consult GmbH. To rule out the possibility that any effects of TRF were secondary to shielding skin from UV rays, the formulation was applied after UV irradiation. Skins of subjects were measured using a chromameter, and the changes in redness were calculated by taking the a* value and subtracting the baseline a* value from it. Likewise, changes in skin color (tan) were measured by subtracting the ITA® from its baseline. A gel that contained 0.1% diclofenac sodium (Feniderma sun repair gel, Novartis, Switzerland) was used as the positive control. As shown in Figure 5A, 0.1% diclofenac sodium significantly reduced skin redness at 6, 24, 48, and 72 hours after UV irradiation (P < .05). A nanoemulsion formulation of 1% TRF also reduced skin redness and the effects were statistically significant at 6 and 72 hours after UV exposure (P < .05). An integrated approach was adopted to compare the changes in redness over the whole test period of 96 hours. The AUC is a powerful statistical method that represents the effects of the test products over the whole trial period of 120 hours. The AUC of data in Figure 5A was calculated and is shown in Figure 5B. In this analysis, the smaller the AUC, the faster the skin returned to normalcy. Both diclofenac sodium and TRF were effective in reducing skin redness after UV irradiation (P < .05).

Skin color was also measured by chromameter and expressed as ITA®. UV exposure caused a decrease in the ITA® value due to tanning. Figure 6A shows that the reduction in ITA® values for TRF was statistically significant at 72 and 96 hours, and the reduction in ITA® values for diclofenac sodium was statistically significant at 24 hours (P < .05). These results further visualized by computing the AUC values as an efficacy time profile, illustrated in Figure 6B. This analysis showed that 1% TRF in formulation appeared to decrease the UV-induced tanning significantly over the whole test period of 120 hours (P < .05). No significant differences were observed for 0.1% diclofenac sodium-treated skin compared with untreated skin.

4 DISCUSSION

Naturally occurring αTP and its synthetic derivative (αTP acetate) are commonly used antioxidants, accounting for 58% of all personal care or cosmetic products in the consumer market, according to Mintel (Personal Care Consumer, Nov 2015). Their photo-protection efficacy has been evaluated in numerous clinical studies, either as oral supplementation or topical application. The results of these studies
are contradictory. One human study demonstrated topically applied αTP acetate had no effect in 11 subjects with actinic keratosis, as this synthetic vitamin E was not metabolized to free form of αTP.\(^\text{18}\) Another study on 16 healthy volunteers revealed that dietary supplementation with 400 IU/d αTP has no effect on UV-induced oxidative stress in skin even though the MDA concentration is reduced.\(^\text{19}\)
This finding is in agreement with the results of another study that involved 6 months of dietary supplementation with αTP at 400 IU/d, showing an insignificant reduction in MED and sunburned cell numbers. Nevertheless, the combination of 1000 IU/d of αTP and 2 mg of ascorbic acid given orally reduced sunburn effects in 10 healthy volunteers. While most of these studies have focused on αTP alone, one recent study described a topical vitamin E mixture (10% αTP and 0.3% T3) that showed protective effects against UV-induced erythema, edema, itching, and vesiculation in 30 subjects.

This beneficial effect may be secondary to αTP due to its higher concentration in the mixture. To the best of our knowledge, our current study is the first to elucidate the beneficial roles of TRF in human cutaneous erythema when a nanoemulsion formulation is applied topically after UV exposure.

Our SAP data demonstrated that the skin penetration of TRF improved with nanoemulsion formulation compared to using MCT as a carrier. Skin penetration of αTP in oil/water emulsion showed slower increase in the kinetic profile compared to the 1% TRF formulation. (B) Comparison of SAP for test products and their own vehicle. For (A) and (B), results shown are mean ± SD of at least 3 independent experiments.

FIGURE 3 Effects of different test products on skin antioxidative potential (SAP) as a function of penetration time. Three products: 1% TRF in formulation of aqueous nanoemulsion, 1% TRF in MCT, and 1% αTP in oil/water emulsion were allowed to penetrate skin biopsies for different amounts of time before being exposed on the dermis layer to the stabilized-free radical TEMPO. The decay of the radical was monitored by ESR and fitted onto an exponential decay curve. The SAP value is the kinetics parameter of treated skin exposed as a percentage of untreated skin. (A) 1% TRF nanoemulsion formulation (but not for 1% TRF in MCT) increased the SAP of the skin with longer application times. Approximately 1% αTP in oil/water emulsion showed slower increase in the kinetic profile compared to the 1% TRF formulation. (B) Comparison of SAP for test products and their own vehicle. For (A) and (B), results shown are mean ± SD of at least 3 independent experiments.

*P < .05, and ****P < .0001, as assessed by one-way ANOVA, followed by Bonferroni’s post hoc test.

FIGURE 4 Radical sun protection factor (RSF) values of TRF in different vehicles. Samples were allowed to penetrate the skin for 10 min before the skin samples were exposed to UV irradiation. The amount of free radicals produced was then monitored by ESR using 2, 2, 5, 5 TEMPO as the spin trap. The amount of radicals produced in untreated samples was compared to treated samples and expressed as a ratio. The results are shown as the means ± SD of at least 3 independent experiments.

***P < .0001, as assessed by one-way ANOVA, followed by Bonferroni’s post hoc test.

The skin penetration profiles of antioxidants are measured by SAP and will normally correlate positively with the efficacy of quenching free radicals under UV stress. We found that topical application of TRF in a nanoemulsion formulation with the highest SAP value reduced UV-induced free radicals as much as 75%. Nonetheless, it was surprising that TRF in MCT with a low SAP value also showed a significant protection against UV-induced free radicals after 10 minutes of application. As an antioxidant, Vitamin E has been known to be an efficient quencher of photogenerated 1O2 and other ROS, such as superoxide anion (O2·−), perhydroxyl radical (HOO·), hydrogen peroxide (H2O2), and hydroxyl radical (OH). Vitamin E quenches these compounds by converting them to less reactive and harmful molecules. In addition, Vitamin E can stop the
chain reaction of lipid peroxidation. Within the family of Vitamin E compounds, T3 has been found to be a more potent antioxidant (40–60 times) than αTP when skin cells were treated with identical concentrations of the two. The higher antioxidant potency of T3 in the membrane may be secondary by a more uniform distribution of T3 within membrane bilayers, stronger interaction of chromanols with lipid radicals, and higher recycling efficiency for chromanoxyl radicals.

The SAP test and RSF help to describe the efficacy of the TRF formulation inside the skin by taking into account the compound’s penetration kinetics as well as free radical scavenging performance.

FIGURE 5 Effects of 1% TRF in formulation on skin redness after exposure to UV irradiation. Twenty subjects were exposed to 1.5 MED of UV irradiation. The test areas were left untreated, treated with 1% TRF in formulation, or treated with 0.1% diclofenac sodium gel. Changes in skin redness were measured as $a^*$ using a chromameter. (A) The reduction in redness for TRF was statistically significant at 6 and 72 h while diclofenac sodium was statistically significant at 6, 24, 48, and 72 h ($P < .05$). Statistical analysis was performed using Wilcoxon Signed-Rank Test. (B) Comparison of the AUC for the change in skin redness over time is shown. AUC was calculated using the trapezium rule. The smaller the AUC value, the faster the skin returned to normalcy. The results were analyzed using repeated measures ANOVA. The data are shown as the means ± SEM.

FIGURE 6 Comparison of skin color (tan) with 1% TRF in formulation and diclofenac sodium after exposure to UV irradiation. Twenty subjects were exposed to 1.5 MED of UV irradiation. The test areas were left untreated or treated with 1% TRF in formulation or 0.1% diclofenac sodium gel. Changes in skin color were monitored using the ITA° measured with a chromameter. (A) The reduction in tan for TRF was statistically significant at 72 and 96 h while diclofenac sodium was statistically significant at 24 h ($P < .05$). Statistical analysis was performed using the Wilcoxon Signed-Rank Test. (B) Comparison of the AUC for the change in skin tan over time. Smaller AUC values correspond to darker skin tones. Significant differences between TRF-treated and untreated skin over the whole test period of 120 h is indicated as $P < .05$. The results were analyzed using repeated measures ANOVA. The data are shown as the means ± SEM.

Our results suggest that TRF in an enhanced cutaneous delivery formulation preserves the skin’s antioxidative potential after UV exposure. We then further tested if this product has beneficial functions on human skin. According to our clinical findings, application of the formulated TRF after UV exposure significantly reduces sun burn, indicating that TRF has anti-inflammatory properties. The inflammatory response initiated by UV exposure is characterized by the involvement of ROS or by the production of prostaglandin and...
other cytokines, such as tumor necrosis factor-α (TNF-α), interleukin 1 (IL-1), IL-6, IL-8, and interleukin-10 (IL-10). Among these cytokines, IL-6 and IL-8 were studied in the present work because they are well-characterized keratinocyte-derived pro-inflammatory cytokines that are induced by various stimuli including UV exposure. IL-6 is a known inflammaging biomarker and is responsible for keratinocyte proliferation associated with epidermal hyperplasia. IL-8 is a neutrophil attractant and is activated by myeloperoxidase activity. These cytokines stimulate the production of COX-2, which catalyzes the synthesis of prostaglandin E2 in response to an inflammatory event. We assessed the elevated production of IL-6, IL-8, and COX-2 in UV-irradiated keratinocytes and determined how TRF modulates these cytokines at the cellular level. The cell line model showed that the levels of IL-6, IL-8, and COX-2 increased markedly in response to UV, and TRF reduced these elevated levels significantly. Moreover, the anti-inflammatory responses that are mediated by TRF observed in keratinocytes seem to alleviate the erythema associated with UV exposure in human skin, according to the analysis of skin redness (*a*). This finding is in agreement with another study that reported the mechanism of UV-induced erythema is attributed to the inflammatory response mediated by mast cell activation leading to histamine-mediated vasodilation. Our results indicate that TRF has anti-inflammatory effects on the skin, possibly by affecting on mast cell degranulation as suggested previously.

Notably, TRF but not diclofenac sodium, restored skin pigmentation after UV radiation, as evidenced by a higher value of *ITA* (∗a*). ITA measurement has been commonly used to separate skin types into different physiological groups: very light, light, intermediate, tan, brown, and dark. This observation may be explained by our previous work, suggesting that T3 suppressed of tyrosinase, a melanogenic enzyme that catalyze the rate-limiting and two-part reaction in melanin biosynthesis. Additionally, the data suggested this antimelanogenesis effect is unrelated to the antioxidant properties of T3 but could be associated with T3’s unsaturated isoprenoid side chain.

Another area of our study focuses on the toxicology and safety of TRF in inhibiting UV-induced inflammation compared to a commercially available sunscreen product that contains 0.1% diclofenac sodium. Our data show that TRF possesses comparable anti-inflammatory effects as diclofenac sodium by treating the sunburn on human skin. While diclofenac is commonly used to treat a variety of acute and chronic inflammation skin conditions (via suppression of prostaglandin synthesis through inhibiting COX-1 and COX-2), there are a few studies showing significant adverse effects of this NSAID after topical application, including severe photosensitivity reactions, pruritus, and paresthesia. TRF received the Generally Recognized as Safe (GRAS) status from the US FDA in 2010, which stated that TRF is well tolerated on the skin at the 1% dose used in our current study. Therefore, this naturally occurring TRF is a safe compound for the prevention and treatment of inflammaging caused by sunlight.

Taken together, our work clearly shows that the biological activities of TRF in photo-protection are different than other sunscreens. Sunscreens physically block or absorb UV rays, but they do not ameliorating UV-induced inflammation. TRF scavenging-free radicals formed by UV rays and have anti-inflammatory properties comparable to those of over-the-counter drugs but are very well tolerated by the skin. Thus, complementing TRF with conventional sunscreens or after-sun products may serve as a good strategy for protection from inflammaging.

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