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Vitamin E Delivery to Human Skin by a Rinse-Off Product: Penetration of α-Tocopherol versus Wash-Out Effects of Skin Surface Lipids

S. Ekanayake-Mudiyanselage[a,c] A. Tavakkol[b] T.G. Polefka[b] Z. Nabi[b]
P. Elsner[a] J.J. Thiele[c]

[a]Department of Dermatology, Friedrich Schiller University, Jena, Germany; [b]Colgate-Palmolive Company, Piscataway, N.J., and [c]Department of Dermatology, Northwestern University Medical School, Chicago, Ill., USA

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Vitamin E · Tocopherol · Sebum · Skin surface lipids · Photoprotection · Squalene

Abstract
α-Tocopherol, the major biologically active form of vitamin E, represents a frequently added lipophilic compound of skin care products. Despite its emerging use in rinse-off formulations, little is known on its efficacy with respect to its deposition or its antioxidant potential in human skin. The objective of this study was to investigate whether the single use of an α-tocopherol-enriched rinse-off product provides effective deposition of α-tocopherol on human stratum corneum. To test this, forearm skin of 13 volunteers was washed either with an α-tocopherol-enriched rinse-off product (test product, TP) or with an α-tocopherol-free vehicle control (control product, CP) (contralateral arm) using a standardized wash protocol. Thereafter, skin surface lipids were extracted with pure ethanol after the wash procedure as well as after 24 h. Additionally, one group of volunteers was subjected to irradiation of their forearms with low-dose UVA (8 J/cm^2) prior to lipid extraction. Skin lipid extracts were analyzed by high performance liquid chromatography using electrochemical detection for vitamin E and UV detection for squalene (SQ) and squalene monohydroperoxide. The results of this in vivo study demonstrated that (1) while CP treatment lowers, TP treatment strongly increases α-tocopherol levels of skin barrier lipids; (2) increased vitamin E deposition levels were maintained for a period of at least 24 h, and (3) TP treatment significantly inhibited photooxidation of SQ. In conclusion, the use of α-tocopherol-enriched rinse-off products may help to maintain the integrity of the skin barrier by providing protection against photooxidative stress at the level of skin surface lipids.

Introduction

Located at the interface between body and environment, the outermost layers of the skin are frequently and directly exposed to a prooxidative environment, including air pollutants, ultraviolet solar light, chemical oxidants and microorganisms [1, 2]. Oxidative stress has been associated with cutaneous carcinogenesis [3] and photoaging [4]. To counteract oxidative injury, human skin is equipped with a network of enzymatic and non-enzymatic antioxidant systems [5]. The outermost epidermal layer, the stratum corneum (SC), is comprised of a

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Jens Thiele, MD
Department of Dermatology, Northwestern University
303 East Chicago Avenue
Chicago, IL 60611 (USA)
Tel. +1 312 503 4856, Fax +1 312 503 4843, E-Mail j-thiele@northwestern.edu

unique, highly lipophilic two-compartment system of structural, enucleated cells (corneocytes) embedded in a lipid-enriched intercellular matrix. This matrix contains stacks of bilayers that are rich in ceramides, cholesterol and free fatty acids [6]. The SC lipid composition and structure plays a key role in determining barrier integrity, which is essential for skin moisturization, normal desquamation and a healthy skin [7]. α-Tocopherol, the major biologically active vitamin E homologue, is generally regarded as the most important lipid-soluble antioxidant in human tissues [8]. Previously, we have demonstrated the presence of a vitamin E gradient in the SC of untreated, healthy human skin, with the lowest α-tocopherol concentrations at the surface and the highest concentrations in the deepest SC layers [9]. Besides its protection against lipid peroxidation, vitamin E is suggested to stabilize lipid bilayers, which may also be of relevance for SC lipid bilayers. Based on the good correlation found between physiological levels of α-tocopherol and squalene (SQ) in human sebum, it was suggested that the role of vitamin E is to maintain low levels of SQ oxidation products in skin surface lipids (SSL) [10]. Thus, adverse effects known to result from SQ peroxide exposure to skin [11, 12] may be prevented by physiological antioxidants such as vitamin E.

While a large body of evidence points to protective effects of topically applied vitamin E against immunosuppression, DNA damage and carcinogenesis [5, 13], little is known on the penetration characteristics and efficacy of vitamin E in rinse-off products. In general, washing of the skin with a surfactant leads to delipidation and dehydration of the SC, a pH shift of the skin towards the alkaline region and a deterioration of barrier function [14–18].

The goal of the present study was to investigate (1) the changes of the SSL SQ after single use of a rinse-off product; (2) an α-tocopherol-containing rinse-off product with respect to its capability of delivering α-tocopherol to the SC, (3) the antioxidative efficacy of this strategy with respect to preventing photooxidation of SQ in vivo.

Material and Methods

Chemicals

All chemicals and solvents were of the highest analytical or high performance liquid chromatography (HPLC) grade, unless specified otherwise. Rothisolv® HPLC grade ethanol and methanol were from Carl Roth GmbH (Karlsruhe, Germany). Lithium perchlorate was from ABCR Company (Karlsruhe, Germany), pure SQ from Sigma-Aldrich Chemie (Steinheim, Germany) and α-tocopherol from Merck GmbH (Darmstadt, Germany).

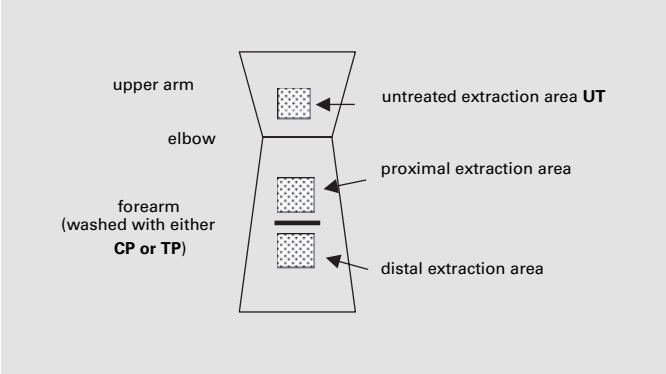

Fig. 1. Scheme of the test area locations. The distal upper arm served as site for UT. Forearms were washed with either CP or TP (contralateral sites). Skin lipids extracted at t = 0 h and t = 24 h from proximal and distal forearm location, respectively. For SC stripping experiments, only one time point (t = 0 h) was investigated. Use of smaller extraction areas allowed placement of all 3 treatment modalities (UT, CP and TP) on forearm sites.

Volunteers

α-Tocopherol and SSL were collected from 13 volunteers: 8 females and 5 males, all between 21 and 33 years old (average age 27.7 years), including Fitzpatrick skin types I–IV (I/II: 1, II/III: 10, III/IV: 3). Exclusion criteria for subject recruitment were a history of dermatological disorders, current medical problems or systemic medication, and tanned skin on the volar forearms. During the entire study period, volunteers were instructed not to use any oral vitamin E, cosmetics, oils, sunscreens or moisturizers and to avoid sun/UV exposure. Permission of the human subject study was granted by the ethical commission of the Friedrich Schiller University of Jena.

Clinical Protocol

During a 7-day preconditioning phase, all volunteers were instructed to exclusively use a shower gel (Baby Magic® Laugh & Splash™ Baby Bath, Playtex Products Inc., Dover, Del., USA) and shampoo (No More Tears®, Johnson & Johnson, Skillman, N.J., USA) free of vitamin E. After the preconditioning phase, the following protocol was applied: squared areas of 3 × 3 cm were delineated on both upper arms. Skin lipid extractions were performed in these areas at t = 0 h and t = 24 h (untreated areas; UT) (fig. 1). For an evaluation of the test product (TP) enriched with vitamin E and its vehicle control product without vitamin E (CP), one forearm was washed with TP (Palmolive Vitamins® Shower Crème, containing 0.15% α-tocopherol; Colgate-Palmolive, Piscataway, N.J., USA) and the other arm with CP as stated below. After the wash procedure, the proximal forearm area close to the elbow served as test area for skin extractions at t = 0, and the distal area for skin extractions at t = 24 h, respectively (fig. 1). The wash procedure was performed as described below on both forearms. Test subjects were not informed as to which arm received CP or TP treatment (blinded application).

Skin Washing Procedure

Forearms were rinsed under running tap water of 35 °C for 5 s. To reflect consumer usage conditions of the body wash, 0.2 ml (equiva-

lent to 0.76 mg/cm^2) of the product (CP or TP) was delivered to the forearm test sites using a syringe. The product was applied by gentle massage and equal pressure for 15 s by the investigator's hands covered by wetted gloves (No Powder EXAM Gloves, Sensi Clean Ansell Medical, Munich, Germany) on the entire volar forearm. Thereafter, the product was left on the skin for another 30 s, then rinsed off thoroughly under running tap water at 35 °C for 2 min, and then allowed to air dry for 10 min. Contamination between CP and TP application sites was strictly avoided by routinely changing gloves.

Tape-Stripping Procedure and Vitamin E Penetration Experiments

Tape-stripping experiments were carried out after the wash procedure in a separate set of volunteers to investigate the depth of vitamin E penetration after a 10-min allowance for air drying (n = 10). In order to remove the upper part of the SC that contains the SSL, sequential tape stripping was used as described [19]. Briefly, round adhesive tapes (D-Squame®; Cuderm, Dallas, Tex., USA), 2.2 cm in diameter, were applied onto the skin in defined extraction areas. After a total of 4 sequential tape strippings at the same site, ethanol extractions were performed using a glass tube of 2.2 cm inner diameter which was placed exactly onto the stripped skin area to avoid contamination with nonstripped areas. Previous publications by our own group have documented that this procedure of using 4 consecutive D-squame tape strippings removes the largest portion of SSL, such as SQ [10]. Since the goal of this experiment was to investigate vitamin E penetration after the use of TC at one time point only, it was possible to include UT in the volar forearm area. Prior to the wash procedure, the proximal volar sites of both forearms served as areas for UT measurements (n = 2, per subject). On both forearms, a 3 × 3 cm square area was marked close to the flex. In the center of this area, a tape-stripping procedure was performed prior to the ethanol extraction steps. After collection of the UT samples, each forearm was subjected to CP or (contralateral) TP treatment. One forearm served for duplicate test sites with identical treatments in different areas. Lipid extractions were carried out on both forearms immediately after the tape-stripping procedure.

Irradiation Protocol

To evaluate possible photoprotective effects of TP treatment, the forearms of another group of volunteers were washed with TP and CP, respectively (n = 6). Half an hour after the washing procedure, the forearms of subjects were exposed to a mild, suberythemal UVA dose of 8 J/cm^2 to induce lipid photooxidation at the skin surface as described [20]. Briefly, UVA radiation was performed using a Sellamed 24000 UVA lamp (Sellas Sunlight, System Sellmeier, Gevelsberg, Germany), spectrum 340–440 nm, UVA irradiance 80 mW cm^{-2}, at a distance of 50 cm. Directly after irradiation, skin lipids were extracted and analyzed for SQ photooxidation products by HPLC.

Skin Lipid Extraction

The classical method for extracting human SC lipids in vitro/ex vivo as described by Bligh and Dyer [21] is based on the use of a chloroform/methanol mixture. However, since toxic reactions, as evidenced by epidermal necrosis, may occur [22], this approach may not be suitable for direct use on human skin in vivo. In the present study, ethanol was used for skin surface lipid extraction, since it is known to induce only little to none skin toxicity and has much lower skin irritant properties than stronger organic solvents, as detected by measurement of skin blood flow, erythema or edema induction [23, 24]. Ethanol can be used to extract SSL [25] and, moreover, represents an excellent solvent for α-tocopherol [10, 19]. In brief, round glass cups of 3 cm diameter were placed on each skin test site and filled with 1 ml ethanol. The ethanol-covered skin was gently rubbed for 1 min using a glass rod and a standardized pattern of movement. Ethanol extracts were transferred into a 1.5-ml Eppendorf tube and centrifuged at 4,000 rpm and 4 °C for 10 min. Two hundred and fifty microliters of supernatant were directly subjected to HPLC analysis.

HPLC Analysis

HPLC was performed using a Gynkotek HPLC system (Dionex Softron GmbH, Germering, Germany). This system included an autosampler (Gina 50), pump (M480G), degasser, UV/Vis detector (UVD 340; all from Gynkotek/Dionex Softron), an electrochemical detector (ECD) from Antec (Antec Leyden, Zoeterwoude, Netherlands) and a Luna 5 μ C18 column (250 × 4.6 mm; Phenomenex®, Hösbach, Germany). The mobile phase consisted of HPLC grade ethanol and methanol (1:1, v/v) including 20 m*M* lithium perchlorate and was filtered before use. The flow rate was 1.2 ml min^{-1}. Control standards were prepared from commercially available pure α-tocopherol. Control standards of SQ were prepared from commercially available pure SQ and detected by in-line UV detection at 210 nm. Squalene monohydroperoxide (SqmOOH) standards were prepared and detected by in-line UV detection at 210 nm as described [20]. ECD detection of α-tocopherol was performed at an oxidation potential of 0.500 V, at 0.02 × 100 nA/V. Peak integration and quantization was performed by Gynkotek Software 5.6 (Dionex Softron GmbH).

Statistical Analysis

Statistical analysis was carried out by repeated-measures paired ANOVA (Graph Pad Instat®, Graph Pad Software Inc., San Diego, Calif., USA). Vitamin E, SQ and SqmOOH values are given as means ± standard errors.

Results

Washing with CP Free of Vitamin E Decreases Human Skin α-Tocopherol at t = 0

The amount of α-tocopherol in the CP-treated skin was significantly reduced immediately after the washing procedure (2.9-fold lower) compared with UT (t = 0 h; $p < 0.05$; fig. 2a). After 24 h, vitamin E levels were restored almost back to normal levels; differences between UT and CP treatment were no longer significant (fig. 2b).

Vitamin E Is Significantly Increased in TP-Treated Skin at t = 0 h, and This Vitamin E Enrichment Is Still Significant at t = 24 h

Immediately after the rinse-off and air-drying procedure (t = 0 h), vitamin E was strongly elevated in skin extracts from all TP-treated skin sites (fig. 2a). α-Tocopherol levels were significantly higher in TP-treated than

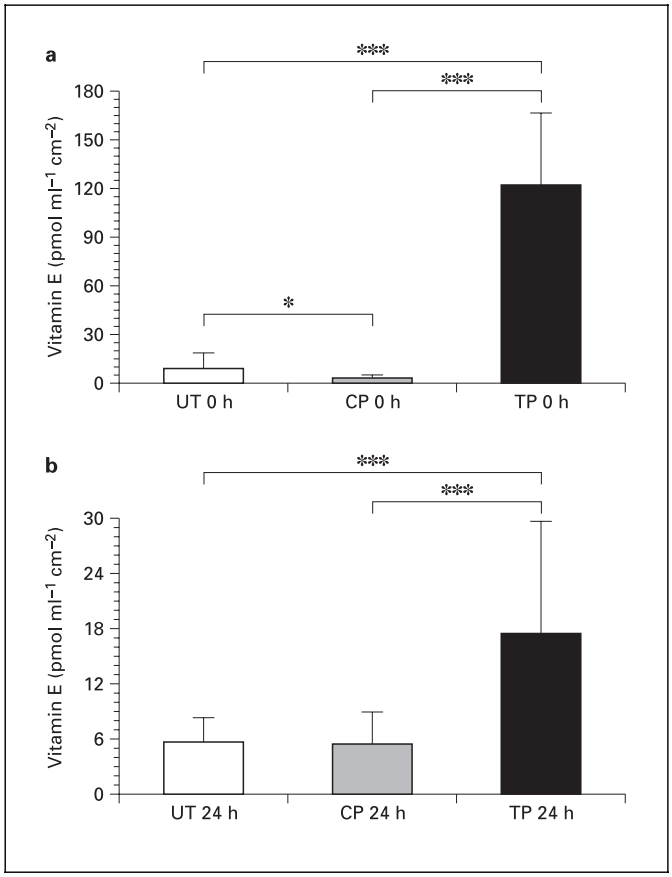

Fig. 2. Vitamin E is strongly increased in TP- versus UT- or CP-treated skin at t = 0 h (**a**) and remains significantly elevated after 24 h (**b**). Vitamin E was detected in ethanolic skin extracts by HPLC using electrochemical detection (ECD). Means and standard errors of UT (□). CP-treated (▨) and TP-treated (■) skin areas are given (n = 13; * $p < 0.05$; *** $p < 0.001$).

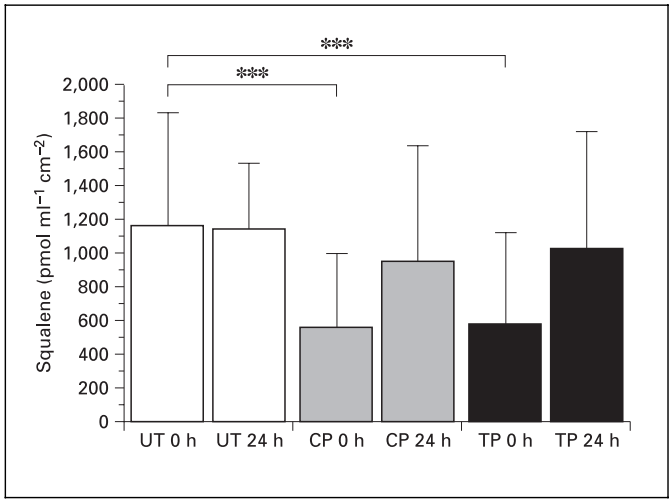

Fig. 3. SQ levels are reduced by a single use of the rinse-off products and recovered after 24 h. SQ was detected in ethanolic skin extracts by HPLC using UV detection. SQ levels are shown at t = 0 h and t = 24 h in UT-, CP- and TP-treated areas (n = 13; *** $p < 0.001$).

in untreated skin or CP-treated forearms (both $p < 0.001$; fig. 2a). Mean α-tocopherol levels of the TP-treated areas were 39.2-fold higher than the CP-treated areas (UT: 9.0 ± 9.4 pmol ml^{-1} cm^{-2}; CP: 3.1 ± 1.7 pmol ml^{-1} cm^{-2}; TP: 121.7 ± 44.2 pmol ml^{-1} cm^{-2}; fig. 2a). One day after the washing procedure, α-tocopherol levels remained significantly elevated when compared with either UT- or CP-treated skin (both $p < 0.001$; fig. 2b). The vitamin E levels in the TP-treated areas were 3.2-fold higher compared with the CP-treated areas and 3.1-fold higher than the UT, respectively. (UT: 5.7 ± 2.7 pmol ml^{-1} cm^{-2}; CP: 5.5 ± 3.5 pmol ml^{-1} cm^{-2} ; TP: 17.5 ± 12.1 pmol ml^{-1} cm^{-2}; fig. 2b).

SQ Levels Are Reduced by the Use of the Rinse-Off Products (Both CP and TP) and Recovered after 24 h

Immediately after the wash procedure, the amounts of SQ in CP- and TP-treated skin areas were significantly reduced by 51 and 50%, respectively, when compared with the UT (both $p < 0.001$; UT at 0 h: 1156.7 ± 676.7 nmol ml^{-1} cm^{-2}; CP at 0 h: 565.0 ± 395.2 nmol ml^{-1} cm^{-2}; TP at 0 h: 578.6 ± 435.5 nmol ml^{-1} cm^{-2}; fig. 3). Twenty-four hours after the wash procedure, SQ levels were recovered in the CP- and TP-treated forearms, and no significant differences were detected compared with the UT (at 24 h: 1,136.6 ± 680.1 nmol ml^{-1} cm^{-2}; CP at 24 h: 952.9 ± 547.2 nmol ml^{-1} cm^{-2}; TP at 24 h: 1,026.9 ± 694.1 nmol ml^{-1} cm^{-2}; fig. 3).

Vitamin E in TP-Treated Skin Penetrates into Deeper SC Layers as Assessed by Lipid Extractions after Removal of SSL and Upper SC Layers

Vitamin E levels obtained from skin sites that were first treated with either CP or TP, then tape stripped, and finally subjected to lipid extraction were significantly increased in TP-treated skin ($p < 0.001$). Levels in TP-treated skin were 10.8-fold higher than in UT-treated skin, and 18.2-fold higher than CP-treated areas. Remarkably, as was observed in nonstripped skin, vitamin E levels were significantly lower (1.70-fold; $p < 0.05$) in CP-washed skin than in untreated skin (UT: 7.6 ±

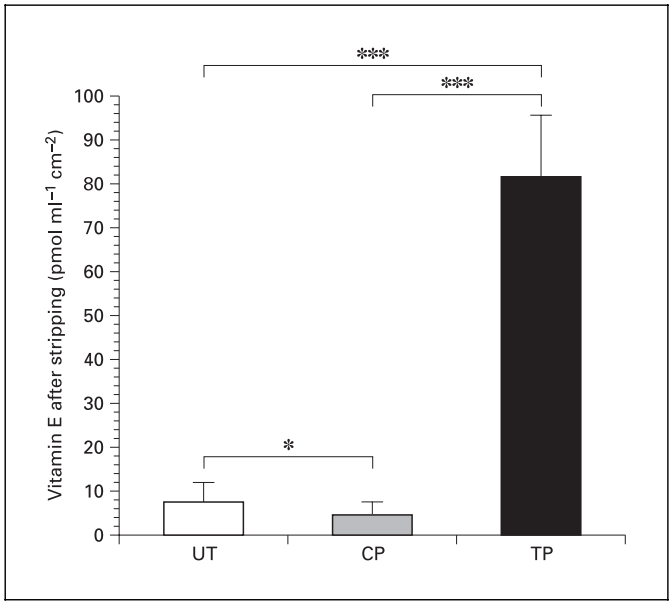

Fig. 4. Vitamin E in TP-treated skin penetrates into deeper SC layers as assessed by lipid extraction after removal of SSL and upper SC layers. Vitamin E was detected in ethanolic skin extracts by HPLC using ECD. Samples were obtained from skin sites that were first treated with either CP or TP, then tape stripped, and finally subjected to lipid extraction. TP treatment (■) led to a significant increase in vitamin E compared with the UT (□) and to CP-treated areas (▨), respectively (n = 8; * ■■■■; *** $p < 0.001$).

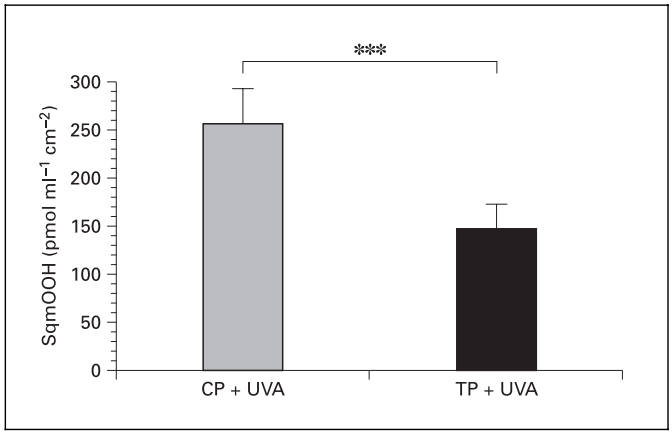

Fig. 5. TP treatment protects against UVA-induced photooxidation of SQ. SqmOOH levels were detected by HPLC using UV detection. SqmHHO was analyzed in ethanolic SSL extracts obtained from test sites after washing with either vehicle CP (□) or TP (■) enriched with vitamin E and subsequent UVA irradiation with 8 J/cm^{-2} (n = 6; *** $p < 0.001$).

4.4 pmol ml^{-1} cm^{-2}; CP: 4.5 ± 3.1 pmol ml^{-1} cm^{-2}; TP: 81.9 ± 14.0 pmol ml^{-1} cm^{-2}; fig. 4).

TP Treatment Protects against UVA-Induced Photooxidation of SQ

In untreated skin areas, no detectable SqmOOH levels were found. Irradiation with 8 J/cm^{-2} UVA in control-treated skin (without vitamin E) induced 1.7-fold higher levels of the toxic photooxidation product SqmOOH than skin that was supplemented with vitamin E by TP treatment ($p < 0.001$; CP: 254.9 ± 38.3 pmol ml^{-1} cm^{-2}; TP 147.8 ± 24.2 nmol ml^{-1} cm^{-2}; fig. 5).

Discussion

The present study demonstrates that (1) single use of a rinse-off product free of vitamin E (CP) lowers the levels of the SSL SQ as well as of α-tocopherol; (2) both are recovered physiologically after 24 h; (3) single use of an α-tocopherol-containing rinse-off product (TP) enriches skin barrier lipids with α-tocopherol for a period of at least 24 h, (4) skin that is enriched with vitamin E by washing with TP is more resistant to UVA-induced photooxidation of SQ than skin washed with CP.

SSL are derived from epidermal lipids as well as from sebaceous gland lipids secreting sebum [6, 26, 27]. One of the main components of human sebum is SQ, an unsaturated lipid, which is generated in sebaceous glands [28, 29]. Thus, SQ is considered a good marker for sebum secretion and SSL. We have previously demonstrated that human sebum contains high levels of α-tocopherol and thus contributes to high levels of vitamin E in SSL and the upper SC [10]. There is a large body of evidence that topically applied vitamin E is photoprotective [13, 30–33]. Furthermore, animal studies suggest that vitamin E protects skin from photocarcinogenesis when administered topically or systemically [34, 35]. However, there is controversy whether or not an oily compound such as vitamin E can be administered to human skin by use of rinse-off products. This appears contradictory, since rinse-off products are commonly used to clean the skin by removing skin surface oils. The results obtained from the present study indicate that the delipidation of the human skin barrier occurring during skin cleansing procedures includes a depletion of vitamin E (fig. 2, 4). Specifically, washing the skin with both CP and TP decreased SQ levels by approximately 50% as compared with untreated skin (fig. 3), while vitamin E levels were depleted by 66%

after the washing procedure with CP. Similarly, vitamin E levels obtained from skin sites that were first washed with CP, then subjected to removal of the upper SC by consecutive tape stripping, and finally subjected to lipid extractions were significantly lower than in untreated skin (fig. 4). Therefore, the wash-out effect of natural vitamin E is not restricted to loss of SSL vitamin E, but also to loss of skin barrier vitamin E. Barrier abnormalities are found in patients suffering from atopic dermatitis, a skin condition known to deteriorate upon frequent or intense skin wash procedures. Since recent evidence suggests that oral vitamin E supplementation improves clinical manifestations of atopic dermatitis [36], topical vitamin E supplementation in leave-on and rinse-off products may help to improve the skin care protocols for patients suffering from atopic dermatitis or other skin conditions exhibiting a disturbed skin barrier.

Our data further confirm that a physiological mechanism restores skin barrier vitamin E (fig. 2) along with the SSL SQ deriving from sebum secretion (fig. 3) within 24 h. However, one may conclude that these mechanisms to reconstitute the physiological antioxidant protection of the SC would be overwhelmed by repetitive skin washing procedures. In contrast, identical wash procedures with an α-tocopherol-containing TP increased superficial SC vitamin E levels almost 40-fold. Even 1 day after washing the skin with TP, α-tocopherol levels were still more than 3-fold higher than in untreated or CP-treated skin. These results indicate that, overall, the skin penetration of applied α-tocopherol was more relevant than the loss of inherent α-tocopherol caused by delipidation and resulted in a net uptake of vitamin E. In order to reflect a typical shower behavior more realistically, the skin washing procedure employed in the present study included a 5-fold smaller amount of cleanser applied per square centimeter skin surface, a shorter skin contact time (45 s versus over 1 min) and a longer rinse-off time (2 min versus 15–20 s) than in a previously reported protocol [37]. To investigate whether the deposited vitamin E remains on the skin surface or penetrates deeper into the SC, further skin extractions were carried out in tape-stripped skin. Remarkably, even after removal of the superficial SC layers by sequential tape stripping, a significant vitamin E deposition of 18.2-fold the levels found in CP-treated areas and 10.8-fold higher than in untreated skin was detected. These results demonstrate that TP wash treatment not only results in a net uptake of vitamin E into SSL, but penetrates into deeper SC layers. There is conflicting evidence as to what extent the conversion of vitamin E esters occurs in the SC [38–41]. Most studies suggest that in human SC the bioconversion to active vitamin E is far weaker than in nucleated epidermal layers. Therefore, α-tocopherol should provide a more efficient antioxidant protection of SSL and skin barrier constituents than vitamin E esters.

In order to evaluate the actual antioxidant protection of skin lipids provided by the additional α-tocopherol supplied by a single TP wash treatment, a recently described method for the quantitation of UVA-induced SqmOOH was used [20]. SQ peroxides have been reported to induce skin roughness, comedogenicity and skin wrinkling [11, 42]. Due to its antioxidant protection against lipid peroxidation, vitamin E dose dependently protects again UVA-induced peroxidation of SQ in vitro [43]. A single, suberythemogenic UVA dose of 8 J/cm^{-2} was chosen, since humans are frequently exposed to equivalent solar UVA doses in that range. Remarkably, UVA-induced generation of SqmOOH was significantly reduced in TP- versus CP-washed skin (fig. 5).

The results presented herein indicate that daily use of α-tocopherol-enriched rinse-off products may help to maintain the integrity of the environmentally exposed skin barrier by providing protection against photooxidative stress to SSL. Further studies are needed to investigate the deposition characteristics provided by repetitive, daily use of such products and evaluate possible clinical benefits for skin conditions exhibiting barrier abnormalities.

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