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Protective effects of sunscreen (50+) and octatrienoic acid 0.1% in actinic keratosis and UV damages

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ABSTRACT

Actinic keratosis is a form of dysplastic epidermal lesion resulting from chronic and excessive UV exposure with a certain risk of becoming cancerous. Current guidelines advocated the use of sunscreens to prevent photodamage. An efficient photoprotection must involve both primary protective factors such as UV filters and secondary factors (eg, antioxidants) able to disrupt the photochemical and genetic cascade triggered by UVs. An in vitro model of human skin (Phenion FT) was used to assess the photoprotective potential of a sunscreen containing inorganic sun-filters (50+ SPF) and 0.1% octatrienoic acid (KERA'+) after UVA (10 J/cm^2) and UVB (25 mJ/cm^2) by means of evaluation of the number of sunburn cells (SBCs) and apoptotic keratinocytes. Also resulting alterations in the gene expression of markers involved in apoptosis (Tumor protein 53), inflammation/immunosuppression (IL-6 and IL-8), oxidative stress (oxidative stress response enzyme heme oxygenase 1), remodeling (metalloproteinase 1) and cell-cell adhesion (E-cadherin) were investigated. Gene expression was investigated using quantitative real-time PCR. This work demonstrated that the sunscreen preparations under study (with and without 0.1% octatrienoic acid, respectively) can be distinguished about their ability to prevent UVs-induced damage. Synergism between the inorganic filters and 0.1% octatrienoic acid was found (KERA'+) on all end points analyzed and this effect was found to be statistically significant ($p<0.05$). Our data revealed that topical application of a sunscreen containing inorganic filters (50+SPF) and 0.1% octatrienoic acid can protect from SBC formation, reduce the number of apoptotic keratinocytes and protect from the main molecular alterations caused by UV radiations.

Significance of this study

What is already known about this subject?

- Actinic keratosis (AK) results from chronic and excessive UV exposure.
- AK may become cancerous.
- AK progression cannot be predicted, consequentially, current guidelines advocated the use of sunscreens to prevent photodamage.

What are the new findings?

- An efficient photoprotection must involve both primary protective factors such as UV filters and secondary factors (active substances) able to disrupt the photochemical and genetic cascade triggered by UVs.
- The topical application of a sunscreen containing inorganic filters (50+ SPF) and 0.1% octatrienoic acid can protect from SBC formation and reduce the number of apoptotic keratinocytes and protect from the main molecular alterations caused by UV radiations.
- The topical application of a sunscreen containing inorganic filters (50+ SPF) and 0.1% octatrienoic acid also protects from the main molecular alterations caused by UV radiations.
- By decreasing p53 and increasing E-cadherin expression, topical application of a sunscreen containing inorganic filters (50+ SPF) and 0.1% octatrienoic acid could act on AK persistence, progression, and regression.

How might these results change the focus of research or clinical practice?

- A sunscreen containing inorganic filters (50+ SPF) and 0.1% octatrienoic acid can be useful not only for the prevention of UV damages but also to AKs progression in malignant form, specifically acting on p53 and E-cadherin expression.

INTRODUCTION

Actinic keratosis (AK) is a form of dysplastic epidermal lesion resulting from chronic and excessive UV exposure.[1 2] It is characterized by skin lesions with keratinocytic atypia in the lower epidermis part.[1 3–6] From a clinical perspective, AK lesions extend from small, erythematous papules to large plaques with brown or yellow scales and crusts appearing on the sun-exposed areas of the face, scalp and extremities.[7 8] About 10% of AK will eventually become cancerous and majority of squamous cell carcinomas (SCCs) begin as AKs.[9]

Fargnoli *et al*[10] reported a high AK prevalence in patients attending Italian dermatology clinics even though data on the prevalence of AK remain limited.

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AK is more frequently reported in individuals over the age of 45 years due to escalating photodamage derived from cumulative sun exposure.[11 12]

The progression of AK cannot be predicted, and, for this reason, current guidelines suggest treating all AKs, regardless of the grade, suggesting also the use of sunscreen as effective in the prevention of AK.[13 14]

From a molecular point of view, AK development has been associated with the generation of DNA photolesions, mostly the cyclobutane pyrimidine dimer (CPD),[15] and alteration in some gene expression profiles, even though the exact mechanism of AK pathogenesis development remains unknown.[16] The most altered gene in AK is tumor protein 53 (TP53) (p53.[17–20] Other common alterations include the oxidative stress response enzyme heme oxygenase 1 (HMOX1), inflammatory cytokines such as interleukin 8 (IL-8) and IL-6, and the matrix-remodeling enzyme marker of photoaging such as metalloproteinases (eg, MMP-1 and MMP-3).[21] Therefore, in a recent work, Bakshi *et al* reported that the loss of E-cadherin and the contextual increase of p53 can be linked to the dynamic interplay between the persistence, progression, and regression of AKs.[22] An efficient photoprotection must involve both primary protective factors such as UV filters and secondary active ingredients able to disrupt the photochemical and genetic cascade triggered by UV.

Previous experimental evidence[23 24] suggests that 2,4,6-octatrienoic acid, a parrodiene derivative, possesses all the necessary characteristics for use in sunscreen to protect from photodamage and the deriving molecular alterations. Indeed, it is reported to act as an antioxidant and anti-inflammatory agent by activating peroxisome proliferator-activated receptor gamma (PPAR-γ).[23 24]

In the present study, we studied the ability of a sunscreen containing inorganic sun-filters (50+ SPF) and 0.1% octatrienoic acid (KERA'+) to protect against molecular photodamage in an in vitro model of human skin.

MATERIALS AND METHODS

Chemicals

The sunscreen emulsion was formulated using VP/eicosene copolymer was purchased from Ashland Industries Italia S.r.l (Italy). Silica dimethyl silylate was purchased from Evonik Operations AG (GmbH). Glyceryl behenate was purchased from Gattefossé Italia S.r.l. (Italy). Sorbitan isostearate, diisopropyl adipate, zinc oxide, c12-15 alkyl benzoate, polyhydroxystearic acid, isostearic acid, titanium dioxide, c12-, aluminum stearate, alumina, and di-ppg-3 myristyl ether adipate were purchased from Croda Italiana S.p.A. (Italy). Caprylyl methicone was purchased from Clariant SE (Italy). Caprylic/capric triglyceride was purchased from LEHVOSS Italia S.r.l. (Italy) and 2,4,6-octatrienoic acid was purchased from Dr. Alberto Milanese Prodotti Chimici (Italy).

The emulsion was in the form of an anhydrous solution for better incorporation of the inorganic mineral filters, titanium oxide, and zinc oxide. The emulsion also contains a blend of modern oils, ultra-light and inert towards the epidermis, able to perfectly disperse mineral filters creating a homogeneous mixture. The above sunscreen is in the market under the commercial name of 'Kerà Sole 50+' (Giuliani SpA, Italy) (KERA'+). A similar sunscreen without octatrienoic acid was also tested (KERA'−).

Human full-thickness aged skin model

The sunscreens were tested in vitro on a Phenion full-thickness aged skin model purchased from Henkel (Phenion FT; Düsseldorf, Germany). This model includes epidermal keratinocytes and dermal fibroblasts organized in a multilayered skin equivalent that resembles human aged skin multilayered structure and tissue functionality. The Phenion AGED Skin Model is characterized by a connective tissue with senescent fibroblasts, reduced synthesis of extracellular matrix (ECM) proteins such as collagen and elastin, and elevated MMP secretion.[25] This experimental model allows testing the products under examination at the same concentration and mode of application as in vivo.

On arrival, the FT models were transferred in small Petri dishes (3.5 cm in diameter) filled with 4 mL prewarmed air-liquid-interface (ALI) medium, refreshed one time after an initial overnight equilibration period. Tissues were subjected to 24 hours treatment with the above-cited compounds after overnight equilibration at 37°C and 5% CO_2.

Irradiation procedure

For UV irradiation, FT models were incubated in ALI medium and irradiated at a dose of 10 J/cm^2 for UVA and 25 mJ/cm^2 for UVB using an Opsytec irradiation chamber BS-03 (Opsytec Dr. Groebel, Germany). Control models were treated identically but without UV exposure. The UVA lamps in the illuminator emit ultraviolet rays between 355 nm and 375 nm, with peak luminosity at 365 nm; UVB lamps emit ultraviolet rays between 280 nm and 320 nm, with peak luminosity at 312 nm. Neither UVA nor UVB lamps have UVC emission. UVA and UVB were supplied by a closely spaced array of three UVA lamps or three UVB lamps that delivered uniform irradiation at a distance of 10 cm. Based on a programmable microprocessor, the Opsytec system constantly monitors the UV light emission stopping the irradiation automatically when the energy received matches the programmed energy (range of measure: 0 to 9999 J/cm^2). FT models were irradiated after topical application of 2 mg/cm^2 (checked by weighing) of both sunscreens applied using sterilized brushes. Control FT models were incubated at room temperature for the same time. At the end of irradiation, samples were rinsed thrice with phosphate-buffered saline (Gibco, Italy) before adding fresh medium. FT models were further incubated for 24 hours in a 5% CO_2 atmosphere at 37°C and then taken for histological and immunofluorescence analysis and real-time PCR. Each experiment was conducted in duplicate and has been reproduced at least three times.

SBC counting

After 24 hours postirradiation, FT models were transferred from the ALI culture vessel onto the lid of a polystyrene Petri dish (Ø 10 cm) and carefully cut into nearly equal stripes using a scalpel with a curved blade. Each obtained section was fixed in tissue freezing medium (Cryomatrix, Thermo Fisher Scientific, Italy) and frozen in liquid nitrogen. FT models were then sectioned (7 µm) in a cryomicrotome Leica CM1510 (Leica Biosystems, Italy) and mounted onto

glass slides. The sections were stained with H&E and the number of SBCs was evaluated on six different sections per sample All values are expressed as mean±SEM

Detection of apoptotic keratinocytes

Tissue sections of irradiated FT models were analyzed for the presence of apoptotic keratinocytes using in situ terminal deoxynucleotidyl transferase 2′-deoxyuridine, 5′-triphosphate (dUTP) nick-end labeling (TUNEL) assay (Roche Applied Science) according to the manufacturer's protocol. Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) solution (Sigma Aldrich S.r.l., Italy). Images from stained sections were acquired using fluorescence microscopy and the number of apoptotic keratinocytes was evaluated on six sections per sample. All values are expressed as mean±SEM

RNA extraction and real-time PCR

RNA was extracted from FT models 24 hours after irradiation . Tri Reagent (Sigma Aldrich, Italy) methods as described by Chomczynski and Mackey[26] were used. A 2 µg RNA template was used for cDNA synthesis in a 20 µl reaction volume, using the PrimeScript real-time PCR (RT-PCR) Kit (Takara, Japan). The cDNA was amplified and detected by the Stratagene Mx3000P RT-PCR System (Agilent Technologies Italia S.p.A., Milan, Italy). Following Taqman gene expression assays were used for quantitative RT-PCR: Hs01034249_m1 (Tumor Protein P53, TP53), Hs00985639_m1 (IL-6), Hs00174103_m1 (IL-8), Hs01110250_m1 (HMOX1), Hs00899658_m1 (MMP1) and Hs99999905_m1 (human glyceraldehyde-3-phosphate dehydrogenase, GAPDH). GAPDH was used as the housekeeping gene. PCR amplifications were performed in a total volume of 20 µl. The mixture of reaction contained 10 µl of 2× Premix Ex Taq (Takara, Japan), 1 µl of 20×TaqMan gene expression assay, 0.4 µl of RoX Reference Dye II (Takara, Japan), 4.6 µl of water, and 4 µl of DNA. PCR conditions were the following: 95°C for 30 s followed by 40 cycles of 95°C for 5 s, 60°C for 20 s. PCR reactions were performed in duplicate using an MX3000p PCR machine (Stratagene, La Jolla, California, USA). For the calculation of the relative abundance in the expression of each gene, Δ cycle threshold[27] was used.

Image analysis

Digital image analysis of stained sections was performed using ImageJ software (public domain software downloaded from http://rsb.info.nih.gov/ij/).[28]

Statistical analysis

One-way analysis of variance with Tukey's post hoc tests (group comparisons) was applied to analyze the data. Statistical significance was accepted when $p<0.05$.

RESULTS

Evaluation of sunscreen protection against UV-induced alterations

Many SBCs were formed within the suprabasal compartment of the epidermis 24 hours after irradiation because of the irradiation of FT models with UVB (25 mJ/cm^2) (positive control, figure 1A). Macroscopic histological examination revealed that the morphology in FT models was severely altered by UVB exposure. This effect was most evident in the FT models used as the positive control (figure 1A). The application of both sunscreens before UVB exposure protected against epidermal disorganization and especially the formation of SBCs (figure 1B,C). The effect was most evident in the FT models treated with KERA'+ (sunscreen containing octatrienoic acid) (figure 1C). Negative control (no UVB exposure) tissue lacked SBCs (figure 1D). The number of SBCs was evaluated on six histological sections per sample. The number of counted SBCs was higher in the positive control and decreased significantly in the FT models treated with sunscreen (figure 2). A higher significant effect was found following treatment with KERA'+ (figure 2).

UVB irradiation leads to irreparable DNA damage on keratinocytes. They, consequently, undergo apoptosis aiming at preventing the propagation of photolesions. DNA fragmentation, a marker of apoptosis was detected in UVB-irradiated FT models using the TUNEL assay (figure 3). The presence of apoptotic keratinocytes was detected 24 hours after irradiation with 25mJ/cm^2 UVB among suprabasal keratinocytes populations. The highest number of TUNEL-positive cells was found in FT models pretreated with none of the sunscreens (positive control) (figure 3A). Pretreatment with both sunscreens, especially one containing octatrienoic acid (KERA'+), significantly reduces the number of apoptotic keratinocytes (figure 3E).

The relative level of crucial genes involved in UV alteration was quantified in FT models after UV (UVA and UVB) exposure. The sunscreen containing octatrienoic acid (KERA'+) inhibited UV-induced expression of genes encoding p53 (figure 4A), inflammatory cytokines (IL-8 and IL-6) (figure 4B,C), oxidative stress response enzyme HMOX1 (figure 4), and the matrix-remodeling enzyme marker of photoaging (MMP-1) (figure 4E) 24 hours post-irradiation. This inhibition was significant ($p<0.05$) for both UVA (10 J/cm^2) and UVB (25 mJ/cm^2) irradiation compared with positive control. A significant ($p<0.05$) but lower inhibition of the above genes was also reported in FT models pretreated with the sunscreen without octatrienoic acid (KERA'−) (figure 4) specifying the activity exerted by octatrienoic itself. A significant ($p<0.05$) increase in the level of E-cadherin was contextually reported only in FT models pretreated with KERA'+ compared with the positive control (figure 4F).

DISCUSSION

AKs are defined as preneoplastic lesions, which may eventually evolve to SCC.[29–33] AK can also be referred to as the 'field of cancerization', referring to the histologically abnormal epithelium adjacent to tumor tissue.[34 35] The field of cancerization is accompanied by molecular changes caused by UV damage, DNA damage, and resulting alterations in important pathways involved in cellular proliferation, cell survival, inflammation, immunosuppression, differentiation, remodeling, and apoptosis.[36 37]

In this view, recurrent use of sunscreen is advisable for protection from photo-induced damages, preventing AK development, and remission of existing AK lesions.[13 14 38–40] Indeed, many studies have reported a significant decrease in AK lesions when sunscreens are used regularly.[38]

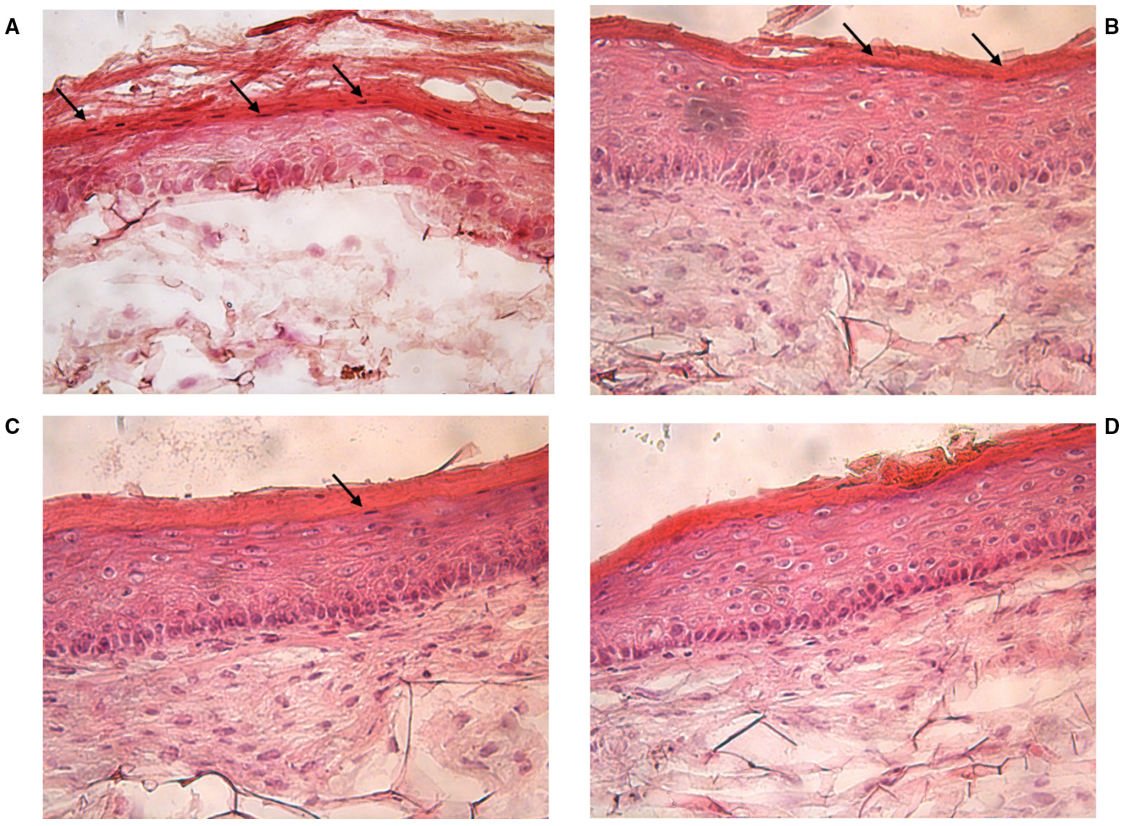

Figure 1 H&E stain of: (A) Positive control (24 hours after 25 mJ/cm^2 UVB); (B) KERA'– treated tissue (24 hours after 25 mJ/cm^2 UVB); (C) KERA'+ treated tissue (24 hours after 25 mJ/cm^2 UVB); (D) Negative control (no UVB irradiation). Sunburn cells (SBCs) are marked with black arrows.

In general, most sunscreens reduce erythema induced by UV radiation, and this reduction represents the basis of the SPF efficacy rating.[41]

Sunlight-protected human skin expresses a unique distribution pattern for various antioxidant, anti-inflammatory, and apoptosis-preventing molecules.[36] On UV radiation a highly complex program of auto-orchestrated cell death (p53-dependent apoptosis) starts aimed at protecting the skin from damaged cells. Keratinocytes undergo individual apoptosis and are histologically known as SBC. UV penetration into the skin also produces reactive oxygen species causing oxidative damage, which disrupts normal signal transduction pathways, including the production of cytokines associated with inflammation, immunoregulation, and photoaging.[42 43]

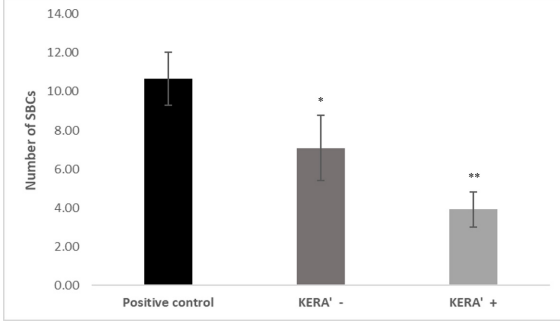

Figure 2 Sunburn cell (SBC) counting. The number of SBCs was evaluated on histological sections as described in the Materials and methods section. Statistical differences between mean values were determined with one-way analysis of variance with Tukey's tests. Asterisks indicate a significant difference to the control (*p<0.05; **p<0.01).

The work presented here investigated relevant molecular biomarkers associated with photo-damage to human skin in FT aged models following exposure to simulated UVA (10 J/cm2) and UVB (25mJ/cm^2) radiation, and effective protection of SBC formation by a sunscreen containing inorganic sun-filters (50+ SPF) and 0.1% octatrienoic acid (KERA'+).

Previous work in this area reported the utility of in vitro skin equivalents to determine specific biological effects induced by UVB and UVA irradiation taking advantage of the ability to apply products topically on the skin surface just like in real-life conditions. UV damage was evaluated after irradiation, using SBC or CPD formation as primary markers of DNA damage[9 44] and evaluating changes in gene expression.[42] The sunscreen understudy inhibited the expression of genes associated with apoptosis, antioxidant activity (HMOX-1), inflammation and immunoregulation (IL-6 and IL-8), and photoaging caused by UVs-induction of cutaneous matrix MMPs (eg, MMP3).

Therefore, in line with previous works, our data demonstrate a significant increase in p53, IL-6, and IL-8 levels in reconstructed skin models after UV irradiation.[45 46] Also, UV induction of oxidative stress in the skin has been well documented.[47]

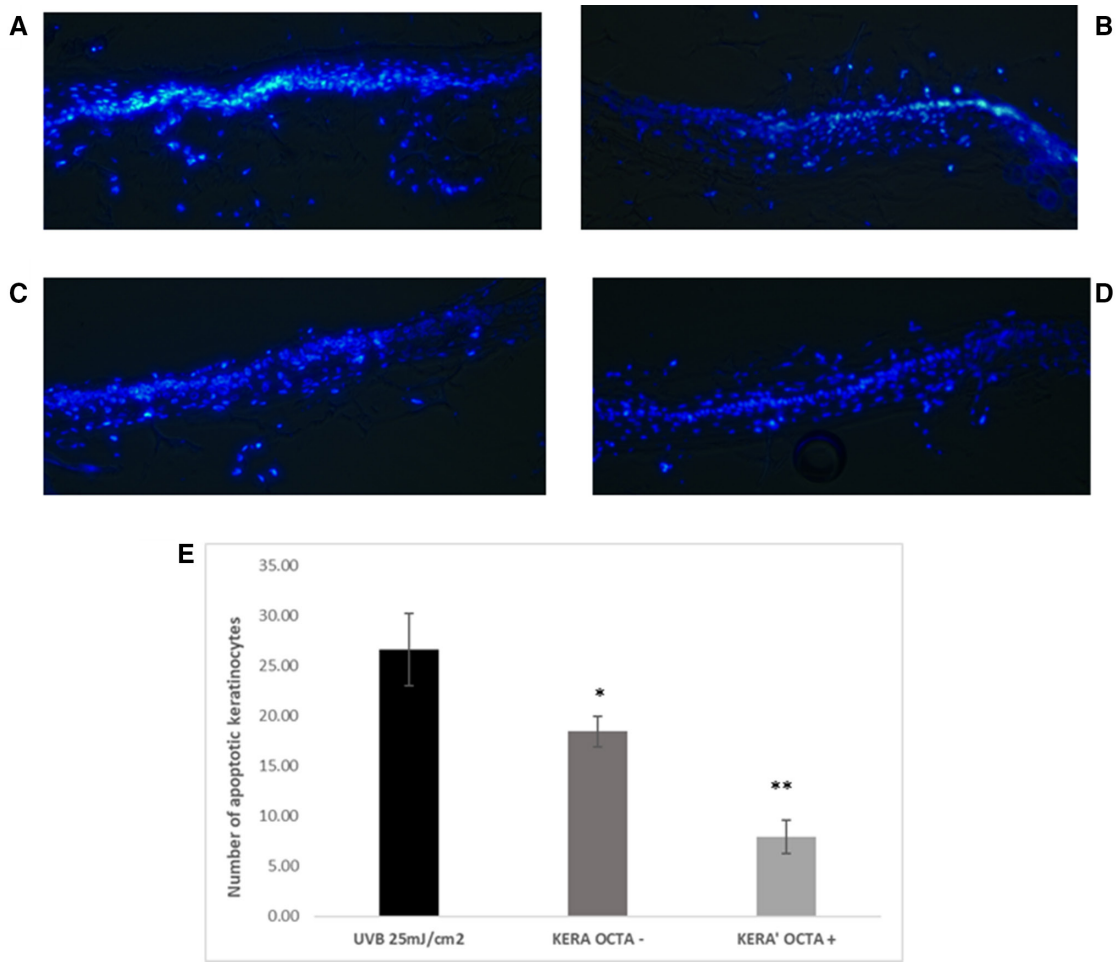

Figure 3 Apoptotic keratinocytes in UVB-irradiated FT models. Merged images of FT sections stained with transferase 2′-deoxyuridine, 5′-triphosphate (dUTP) nick-end labeling (TUNEL) assay; TUNEL-positive nuclei (apoptotic nuclei, green) and DAPI-stained nuclei (total nuclei, blue) on sections of FT models. (A) Positive control (24 hours after 25 mJ/cm^2 UVB); (B) KERA'– treated tissue (24 hours after 25 mJ/cm^2 UVB); (C) KERA'+ treated tissue (24 hours after 25 mJ/cm^2 UVB); (D) Negative control (no UVB irradiation); (E) Number of TUNEL-positive cells was evaluated on histological sections as described in the Materials and methods section. Statistical differences between mean values were determined with one-way analysis of variance with Tukey's tests. Asterisks indicate a significant difference to the control (*p<0.05; **p<0.01; ***p<0.005).

Major alterations characterize photoaged skin such as alterations in the expression of members of the MMP family (MMP-1, MMP-3, and MMP-9); it results in the degradation of collagen and other ECM proteins in the connective tissue.[48] Finally, the expression of *E-cadherin* was evaluated. The role of p53 and E-cadherin in the dynamics of AK was previously reported.[22] A significant increase in *E-cadherin* gene expression was also reported. Taken together with the contextual decrease of *p53* gene expression, these results highlight the potential role of the sunscreen containing 0.1% octatrienoic acid in AK persistence, progression, and regression.

The in vitro photoprotective efficacy evaluation demonstrated that there was a protective effect from UV damage attributable to SPF of inorganic filters (KERA'−) and this was expected.

Many advantages are derived from the use of sunscreen filters. Physical/inorganic filters possess many advantages compared instead with chemical ones. Physical sunscreen filters such as zinc oxide and titanium oxide are safer for use than chemical sunscreens since they can reflect UV radiation away from the skin and are not systemically absorbed. Consequently, physical filters result as less irritating and more suitable for sensitive or irritated skin.

This work demonstrated that the two sunscreen preparations under study (with and without 0.1% octatrienoic acid, respectively) can be distinguished about their ability to prevent UVs-induced damage. Indeed, synergism between inorganic filters and 0.1% octatrienoic acid was found (KERA'+) on the biomarkers analyzed and this effect was found to be statistically significant ($p<0.05$).

Previous works highlighted the efficacy of octatrienoic acid on photodamages.[23 24] Indeed, acting as a parrodiene-derivative it possesses antioxidant, anti-inflammatory activity, and the ability to inhibit cell membrane lipoperoxidation,[49 50] acting via PPAR-γ activation. Through these mechanisms octatrienoic acid is able to reduce oxidative damage, inflammation and DNA damages following UV exposure. Its activity as an antioxidant agent is particularly important since it allows tamping

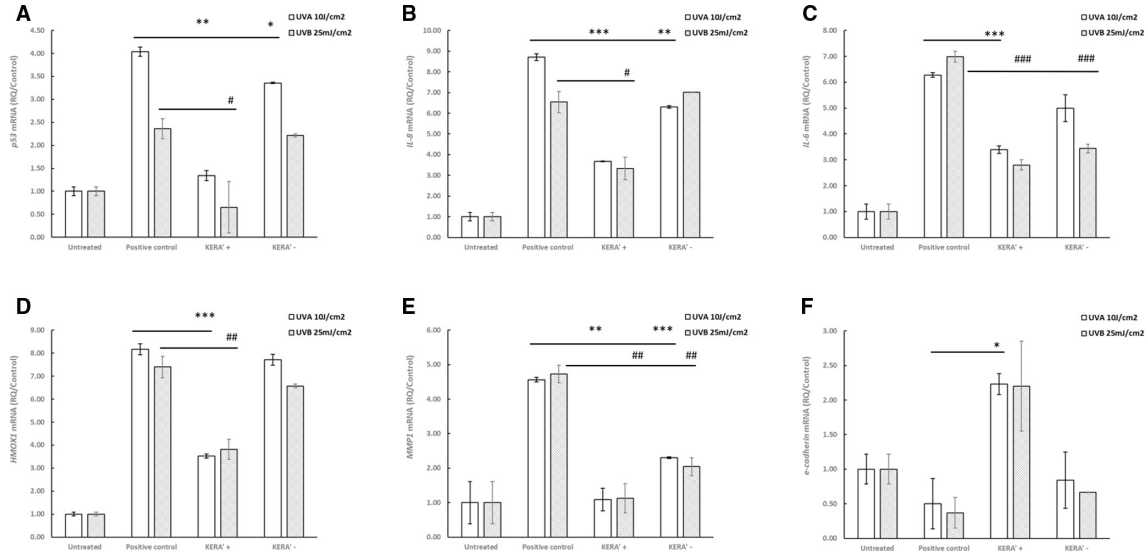

Figure 4 Relative quantification of (A) p53, (B) the interleukin 6 (IL-6), (C) the interleukin 8 (IL-8), (D) oxidative stress response enzyme heme oxygenase 1 (HMOX1), (E) metalloproteinase 3 (MMP3) and (F) E-cadherin in FT models.Gene expression has been determined by real-time PCR. FT models were treated with UVA 10 J/cm^2 or UVB 25 mJ/cm^2 (positive control); KERA'+ (inorganic filters and 0.1% octatrienoic acid); KERA'– (inorganic filters) and medium alone (untreated). Analyses were carried out after incubation at 37°C for 24 hours postirradiation, under 5% CO_2. Data are means±SD of three separate experiments performed in triplicate. Statistical differences between mean values were determined with Tukey's HSD test. The asterisk indicates a significant difference ($p<0.05$) to the control. Statistical differences between mean values were determined with one-way analysis of variance with Tukey's tests. Symbols indicate a significant difference to the control (*/# $p<0.05$; **/## $p<0.01$; ***/### $p<0.005$).

down inflammatory cytokines and damages derived from oxidative stress.

In conclusion, the above study demonstrated the utility of the used in vitro skin model for determining the protection afforded by sunscreens against photo damages. Our data revealed that topical application of a sunscreen containing inorganic filters (50+ SPF) and 0.1% octatrienoic acid can protect from SBC formation, reduce the number of apoptotic keratinocytes and protect from the main molecular alterations caused by UV radiations. The contextual decrease of *p53* and increase in *E-cadherin* gene expression also suggest a role of the sunscreen containing 0.1% octatrienoic acid in AK persistence, progression, and regression.

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