Photoprotective complementary effects of sun filters and a combination of active molecules on UV-exposed human volunteers

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Figures: 2

Supplementary information: Table S1

Abstract

Background: Chronic exposure to ultraviolet (UV) irradiation causes immunosuppression, photoaging, and carcinogenesis by induction of a cascade of skin damage. Although sunscreens with a very high sun protection factor (SPF) absorb most of the sun's UVB rays, no sunscreen is effective in reducing total UV effects, particularly those induced by UVA. In the context of an ecobiological approach where skin natural resources and mechanisms must be preserved, and thus to increase UVA protection, UV filters and antioxidants have been combined to enhance their photoprotective effect, but studies in humans are lacking.

Methods: Therefore, we studied an association of ectoine and mannitol to characterize its photoprotection properties *in vitro* and in humans, combined with UV filters.

Results: Using *in vitro* irradiated skin cell model, we demonstrated that this association has a global ecobiological effect on skin, by preserving intracellular ROS levels. Non-invasive skin samplings in ten subjects on irradiated areas with and without pre-treatment with the active association and/or with SPF30 UV filters showed that use of UV filters with this active compound association presented significantly higher protection of a natural defence system altered by UV compared to UV filters alone: squalene oxidation.

Conclusion: This study demonstrates the ecobiological potential of combining UV filters with biological protection to increase skin photoprotection provided by specific active ingredients with antioxidative properties.

Keywords : sunscreen; antioxidant; squalene oxidation; biomarkers

1 Introduction

Chronic exposure to ultraviolet (UV) irradiation causes immunosuppression, photoaging, and carcinogenesis[1]. A cascade of reactions occurs upon UV exposure of human skin, including the generation of reactive oxygen species (ROS), oxidation of lipids and proteins, DNA damages, p53 mutations, sunburn cell formation, release of cytokines and matrix metalloproteinases (MMP), isomerization of trans-urocanic acid (UCA), and reduction of the antigen presentation function of Langerhans cells (LC), key cells in the cutaneous immune response[1]. UVB rays (280–315 nm) are predominantly absorbed by the skin's epidermis, while longer wavelength UVA rays (315–400 nm) penetrate the dermis more deeply, which makes them the primary driver of photoaging[2]. Moreover, UVA rays have been shown to induce oxidative stress through ROS, including singlet oxygen inducing oxidative stress leading to oxidative DNA lesions, which cause mutations[3]. So photoprotection against UVA rays, in addition to UVB rays, is essential.

No sunscreen is currently capable of significantly reducing the total UV exposure effect. Common commercialized sunscreens can provide a higher degree of UVB protection, evaluating erythema measured by the Sun Protection Factor (SPF), than UVA protection for two reasons. First, the currently available UVA filters do not block the corresponding wavelengths as much as UVB filters do. Second, the International Organization Standardization 24443 guidelines use for all sunscreens marketed in Europe recommends a minimal UVA protection factor to SPF ratio of 1:3[4]. Moreover, for many decades the SPF has assessed sun protection using UV-induced erythema as an endpoint, but it does not accurately reflect all photoprotection benefits. An ecobiological approach would consider not only the immediate consequences of sun exposure but aim to better understand the causes to prevent on long term consequences.

To increase the protection derived from filters against UVA, which strongly induces oxidative stress, many studies have investigated the effect of additional molecules with physical and/or biological actions when applied topically. These molecules can act as UV blockers, SPF enhancers, or through biological effects [5–7]. Some studies suggest that complementing sunscreens with natural ingredients can increase their photoprotective effect, particularly in case of natural antioxidants (vitamins C and E) or botanical extracts[6–9]. Indeed, the body may not be able to completely neutralize ROS generated by excessive sun exposure, which can lead to significant skin damage. Thus, topical application of photoprotective antioxidants could maintain or restore skin functions[10]. Cosmetic formulations combining UV filters with vitamin derivatives and/or botanical extracts have been shown to enhance antioxidant activity and increase protection against UV radiation[8, 9, 11–13], but few were clinical studies[13]. The present study aimed to characterize the photoprotection properties of an ecobiological association of ectoine and mannitol and to investigate its additional photoprotection effect in humans in combination with UV filters based on the assessment of a UV-induced biomarker, the squalene.

2 Materials and Methods

2.1 Chemicals

The active association comprised ectoine and mannitol. The combined UV filters comprised diethylhexyl butamido triazone, bis-ethylhexyloxyphenol methoxyphenyl triazine, butyl methoxydibenzoylmethane, and ethylhexyl triazone. In the *in vivo* study, the active association and the UV filters were formulated in a vehicle cream (see Table S1).

2.2 H_2DCF-DA assay

2.2.1 Cell culture and treatment

NHEK were pre-incubated for 24 hours with the active association (0.01% ectoine and 0.01% mannitol). After incubation with the fluorescent probe 2,7-DCDHF-DA, the culture plates where the SPF30 filters were applied (1 mg/cm^2) were left as is or covered with a quartz plate. The cells were left untreated or irradiated with UVB at 100 mJ/cm² and UVA at 0.7 J/cm² using a SOL500 Sun Simulator equipped with an H2 filter (Dr. Hönle, AG). After irradiation, the cells were incubated for 30 minutes in PBS with and without the active association (0.01% ectoine and 0.01% mannitol). All experimental conditions were performed in triplicate. A viability assay was performed on cell layers using a standard MTT reduction assay.

2.2.2 Quantification of ROS production

The fluorescence emitted by the 2,7-DCDHF-DA probe was measured (λex = 485 nm, λem = 538 nm) using a microplate reader (Perkin Elmer). The fluorescence intensity of the metabolized probe (DCF) is proportional to the quantity of ROS. The production of ROS was expressed as relative fluorescence units.

2.2.3 MTT assay

The cells were incubated with MTT (tetrazolium salt), which is reduced to blue formazan crystals by succinate dehydrogenase (a mitochondrial enzyme). After cell dissociation and formazan crystal solubilization using DMSO, the optical density of the extracts at 540 nm, proportional to the number of living cells and their metabolic activity, was determined with a microplate reader (VERSAmax, Molecular Devices).

2.3 In vivo study

2.3.1 Subjects and study design

This open intra-individual study was approved by the Internal Ethics Committee at the Clinical Research Center Eurofins Dermscan Poland in February 2021 (study n# 20E0976). Ten men between 18 to 60 years of age with phototype II to III according to the Fitzpatrick scale were included with oily skin type on the back (according to the supplier, with a sebum rate ≥ 27 µg/cm² verified using a Sebumeter®). The subjects received the requisite written and verbal information and provided their written informed consent. The exclusion criteria were as follows: suntan, tattoos, beauty marks and/or excessive pilosity on the back, cutaneous pathology on the back, topical or systemic treatment during the previous week, professional beauty treatment, exfoliation cosmetics and/or antioxidant cosmetic on the back in the month before and during the study, sun allergy, participated in an SPF test in at least the past two months before the study, having undergone surgery under general anesthesia within the previous month, and excessive exposure to sunlight or UV-rays within the three previous months. Subjects were not allowed to sunbathe for three months before the study or to wash their back for the entire duration of the study but they were allowed to wash their hair with the head tilted forward between day 0 (D0) and day 4 (D4).

Non-invasive samplings were performed with a swab sampling kit supplied by QIMA Synelvia. A swab previously wetted in a cocktail solution was applied to the selected area for 45 s using a template and then cut and placed in an Eppendorf tube. The sampling was repeated twice per area. The personal minimal erythema dose (MED) of each subject was previously determined. At D0, a skin surface sampling was performed to determine the basal value of the squalene oxidation for each subject. From D0 to D2 (three days), a technician at the clinical centre applied the vehicle cream twice daily alone or containing the active association (0.1% ectoine and 0.1% mannitol) and/or UV filters (2 mg/cm²) according to the

four studied areas defined on the backs of the subjects (vehicle, UV filters, active association [0.1% ectoine and 0.1% mannitol], UV filters with the active association). On the morning of D3, after a standardized cleansing and a final application (30 minutes before irradiation), the four selected UV sub-areas were irradiated by 2 MED (UVA and UVB spectrum) with a Monoport 300W xenon lamp (Solar Light). On D4, skin surface samplings were performed on the eight sub-areas of each subject (four irradiated and four non-irradiated) and stored at -20 °C until the biochemical analyses were performed.

2.3.2 Oxidized squalene quantification by LC/MS

The swab homogenates were centrifuged at 10,000 x g for 5 min. Samples were extracted by a double liquid/liquid extraction method, evaporated under nitrogen at 60 °C, and the residue was dissolved in 50 µL of ethanol (Honeywell). Squalene monohydroperoxide and squalene were detected with an UltiMate 3000 liquid chromatography system (Dionex) coupled to an ISQ Plus detector (Fisher Scientific, Waltman). For mass spectroscopy (MS) detection, atmospheric pressure chemical ionization was used as the ion source. Positive ion spectra were recorded in the 50–450 m/z range. The ratio of the concentration of monohydroperoxide squalene and squalene in each sample was calculated after being normalized to the total protein content.

2.3.3 Total protein

The total protein content of the swab samples was determined using the Bicinchoninic Acid (BCA) method with alkaline medium (Protein Assay Kit, Bio Basic Inc.), where proteins reduced Cu^{2+} into Cu^{+}. Cu^{+} reacts with BCA to produce a coloured complex that was measured at 562 nm with a Tecan Spark™ plate reader after 45 min of incubation at room temperature while protected from light. Total protein content in each sample was expressed as µg/ml.

2.4 Statistical analysis

For the *in vitro* experiments, the p-value was calculated with the non-paired equal variance Student's test. For the *in vivo* study, if normality was proven by the Shapiro-Wilk test, a Student's test was used, otherwise the non-parametric Wilcoxon's signed-rank test was used. For all statistical analyses, a significance threshold level of 5% was chosen.

3 Results

3.1 In vitro efficacy of the active association with and without UV filters on UV-induced oxidative stress

UV irradiation significantly increased the fluorescence (2.5-fold; $p<0.001$) in NHEK, corresponding to intracellular ROS production, compared to non-irradiated cells (Figure 1). ROS formation was significantly reduced in irradiated cells protected by UV filters compared to non-protected irradiated cells ($p<0.05$) but was still significantly higher compared to non-irradiated non-treated cells (1.75-fold; $p<0.05$). Interestingly, the formation of ROS was not significantly increased in NHEK protected with UV filters and pre-treated with the active association compared to non-irradiated non-treated cells. Although the formation of ROS was decreased by 35% in cells protected by UV filters and pre-treated with the active association compared to UV filters alone, the difference was not significant ($p=0.08$). The combination of UV filters and the active association promoted a level of antioxidant protection of 86% compared to the irradiated non-treated cells.

Similarly, higher genomic protection was also observed by comet assay after UVA irradiation and treatment with the active association combined with UV filters compared to UV filters alone (data not shown).

3.2 In vivo efficacy of the active association with the UV filters after UV-irradiation on oxidative stress

This study was conducted on 10 men aged 20 to 44 years (mean age of 27.6 years). Compared to non-irradiated vehicle-treated areas, UV irradiation induced a significant 2-fold ($p<0.01$) increase in the oxidized squalene/non-oxidized squalene ratio (Figure 2). Compared to the irradiated vehicle-treated areas, the active association and UV filters alone protected squalene oxidation by 58.4% ($p<0.01$) and 50.6% ($p<0.01$), respectively. Combination of the active association with UV filters provided the best level of protection in terms of squalene oxidation (76.8%; $p<0.01$) compared to the irradiated vehicle-treated areas. Compared to UV filters alone, the active association combined with UV filters provided significant additional protection of squalene by 26% ($p<0.05$).

4 Discussion

Our work shows that the production of ROS in irradiated NHEK was not significantly increased in cells pre-treated with the active association in combination with UV filters compared to non-irradiated cells, unlike UV filter protection alone. Furthermore, *in vivo* application of the active association combined with UV filters significantly protected a natural defense system, namely squalene oxidation, so more than the UV filter application alone. This suggests that the active association provides an ecobiological composition which provide significant additional skin photoprotection to UV filters with an SPF of 30.

To our knowledge, to date, only one study in humans has investigated skin UV protection by active ingredients (caffeine, vitamin E, vitamin C, *Echinacea pallida* extract, gorgonian extract, and chamomile essential oil) in combination to UV filters, and this was with an invasive method[13]. In this preliminary study on biopsies from five subjects, Matsui *et al.* showed no significant difference in the LC number but significant collagen protection based on measurement of MMP-1 levels on the two MED-irradiated areas treated with an

SPF25 sunscreen containing the antioxidants compared to the area treated with the sunscreen alone[13]. In our current studies, the active association, comprising ectoine and mannitol, also exhibited antioxidative properties. Indeed, mannitol is a polyol that has been reported to be a hydroxyl radical scavenger[14, 15] that can limit damage induced by oxidative stress, including oxidative DNA base damage, lipid peroxidation, and protein denaturation[16–19]. Ectoine, on the other hand, is a cyclic amino acid produced by extremophile microorganisms that exhibits many interesting dermatological properties. It was initially described as a cell membrane protector with outstanding water-binding and stabilizing properties that could reduce transepidermal water loss, chemical and physical penetration, and subsequent inflammation (for review[20]). At concentrations of 2% and higher, ectoine improves dryness and impaired skin barrier function, and it has been described as being effective for treatment of atopic skin and prevention of skin aging[20, 21]. Interestingly, other studies have shown that ectoine prevents UV-induced migration of LC[22] and pro-inflammatory cytokines release[23], protects DNA against visible light [19], mtDNA against UV rays[24], and prevents sunburn cell formation[22]. The antioxidant potential of ectoine remained controversial until the study by Brands *et al.*, demonstrating that ectoine is a good hydroxyl radical scavenger, similar to mannitol[14], thus explaining its irradiation protective effect[25]. Moreover, in daphnids, an aquatic invertebrate, the glutathione (GSH) to glutathione disulfide (GSSG) ratio, catalase activity, and NOx levels returned to near control values when H_2O_2 was applied in combination with ectoine[26].

Our current studies confirmed the antioxidative properties of ectoine and mannitol association by H_2DCF-DA assay. These antioxidant properties of the active association can explain the higher protection against squalene oxidation induced by UVA irradiation than with UV filters. Following the principle of ecobiology, which is an original approach considering the skin as an ever-evolving ecosystem whose natural resources and mechanisms

must be preserved[27, 28], the association of ectoine and mannitol combined the UV filters protects skin components (lipids, proteins, DNA) and preserves the skin antioxidant system.

The main limitation of this work is the small sample size of subjects in the *in vivo* study. A study including more subjects and investigating other sun protection biomarkers, such as sunburn cells formation, p53, caspase-3, and DNA repair enzymes, could be interesting but would require the use of invasive technique to obtain biopsies.

5 Conclusion

In conclusion, our study performed *in vitro* as well as *in vivo* with non-invasive analysis of biomarkers showed that this active association provides significant additional skin photoprotection to UV filters in terms of reduction of oxidative stress induced by UV rays. Sunscreens containing active ingredients, especially antioxidants, appear to provide better photoprotection at a cellular level than UV filters alone, and this ecobiological approach should be taken into consideration by dermatologists and users of such products.

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Conflict of Interest Statement

Arnaud Fontbonne, Baba Teme, Elise Abric, Sylvie Callejon, Benoît Cadars, Félix Giraud, Marlène Chavagnac-Bonneville, Nathalie Ardiet, Aurélie Guyoux, and Sandra Trompezinski are employees of NAOS Group (Aix-en-Provence, France).

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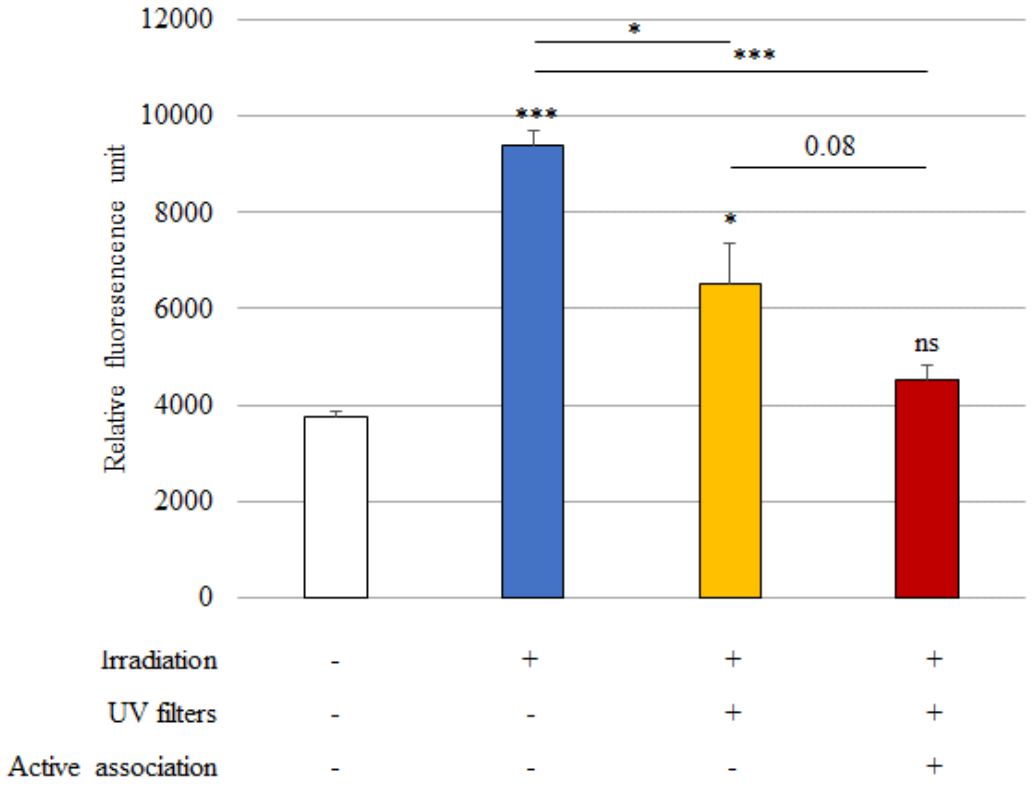
Figures legends

Figure 1: *In vitro* assessment of intracellular ROS production quantified by H_2DCFDA fluorescence in NHEK pre-treated with active association with and without UV filters before UVA/B irradiation. The mean values of the relative fluorescence units, with standard deviations, are presented. *p<0.05, ***p<0.001; ns=not significant

Figure 2: *In vivo* quantification of the oxidized squalene/squalene ratio induced by UV irradiation after application of the active association with and without UV filters. The results are presented as box plots, with whiskers representing the maximum values or 1.5 times the interquartile range of the data, whichever was smaller. Wilcoxon's signed-rank test for squalene. *p<0.05, **p<0.01, and ***p < 0.001; ns=not significant

Figure 1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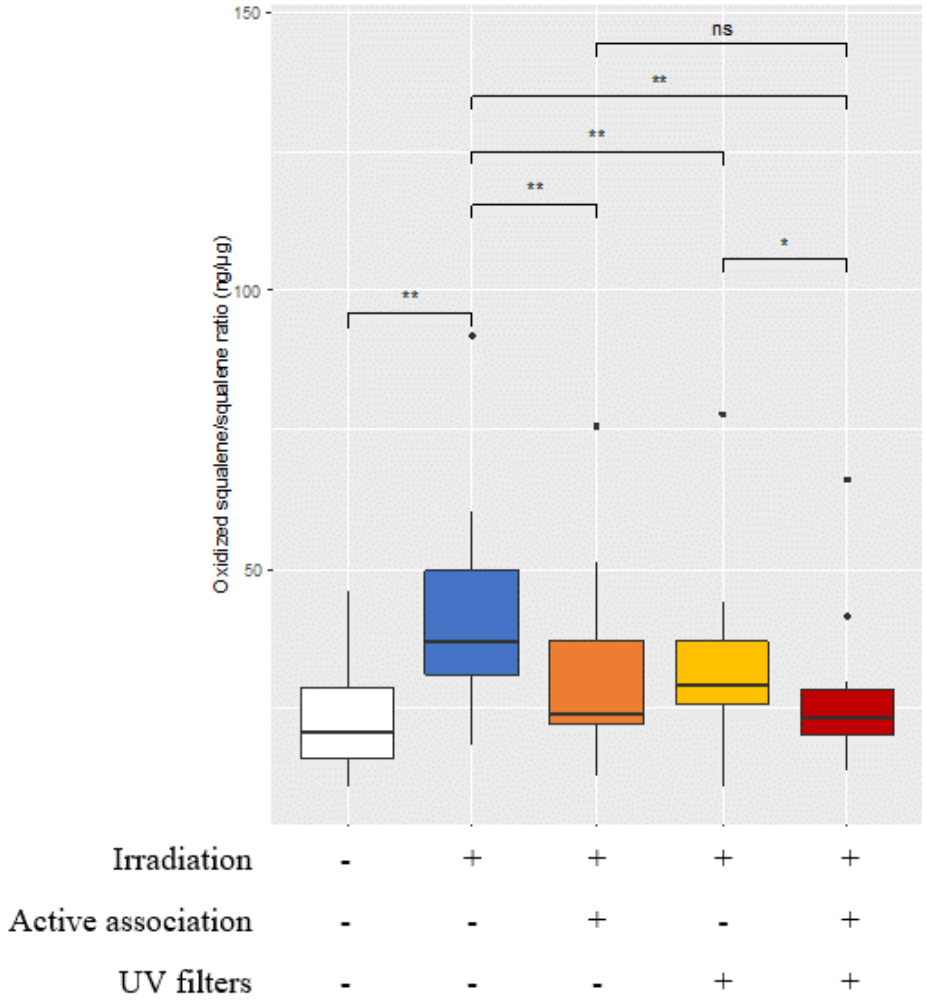
Irradiation - + + +
UV filters - - + +
Active association - - - +

Figure 2


150
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Irradiation - + + + +
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UV filters - - - + +

Table S1: Ingredients (INCI names) in the tested associations and in the vehicle cream

Active association	UV filters	Vehicle cream
ectoine mannitol	diethylhexyl butamido triazone bis-ethylhexyloxyphenol methoxyphenyl triazine butyl methoxydibenzoylmethane ethylhexyl triazone	aqua/water, dibutyl adipate, dicaprylyl carbonate, propanediol, polyglyceryl-6 stearate, glycerin, microcrystalline cellulose, 1,2-hexanediol, C20–22 alkyl phosphate, C20–22 alcohols, caprylyl glycol, polyglyceryl-6 behenate, sodium citrate, cellulose gum, citric acid, o-cymen-5-ol, sodium hydroxide, xhanthan gum, tocopherol