Skin phototoxicity of cosmetic formulations containing photounstable and photostable UV-filters and vitamin A palmitate

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ABSTRACT

The aim of this study was to evaluate the in vitro skin phototoxicity of cosmetic formulations containing photounstable and photostable UV-filters and vitamin A palmitate, assessed by two in vitro techniques: 3T3 Neutral Red Uptake Phototoxicity Test and Human 3-D Skin Model In Vitro Phototoxicity Test. For this, four different formulations containing vitamin A palmitate and different UV-filters combinations, two of them considered photostable and two of them considered photounstable, were prepared. Solutions of each UV-filter and vitamin under study and solutions of four different combinations under study were also prepared. The phototoxicity was assessed in vitro by the 3T3 NRU phototoxicity test (3T3-NRU-PT) and subsequently in a phototoxicity test on reconstructed human skin model (H3D-PT). Avobenzene presented a pronounced phototoxicity and vitamin A presented a tendency to a weak phototoxic potential. A synergistic effect of vitamin A palmitate on the phototoxicity of combinations containing avobenzene was observed. H3D-PT results did not confirm the positive 3T3-NRU-PT results. However, despite the four formulations studied did not present any acute phototoxicity potential, the combination 2 containing octyl methoxycinnamate (OMC), avobenzene (AVB) and 4-methylbenzilidene camphor (MBC) presented an indication of phototoxicity that should be better investigated in terms of the frequency of photoallergic or chronic phototoxicity in humans, once these tests are scientifically validated only to detect phototoxic potential with the aim of preventing phototoxic reactions in the general population, and positive results cannot predict the exact incidence of phototoxic reactions in humans.

1. Introduction

The level and quality of UV protection provided by sunscreen products have improved considerably over the past three decades. Modern sunscreen products should provide broad-spectrum UV protection, offering uniform UVB/UVA protection, because this assures that the natural spectrum of sunlight is attenuated without altering its quality. Modern sunscreens may contain at least two UV filters, one with optimal performance in the UVA region and the other one in the UVB region. However, the presence of different UV filters, which usually leads to synergistic effects regarding both the final performance and photostabilization of the sunscreen, can also accelerate their decomposition if a photoreaction occurs between the single components (Osterwalder and Herzog, 2010; Gonzalez et al., 2007; Chatelain and Gabard, 2001; Lhiaubet-Vallet et al., 2010).

Despite the wide range of UVB filters, appropriate UVA filters are rare; among them avobenzone is probably the most important representative. This active ingredient is present in numerous commercial sunscreen and cosmetic formulations. Avobenzene strongly absorbs UVA, but presents significant degradation under UV exposure reducing its UVA protecting effect (Paris et al., 2009; Bouillon, 2000).

The reactive intermediates of photounstable filter substances come into direct contact with the skin, where they may behave as photo-oxidants or may also promote phototoxic or photoallergic contact dermatitis. The interaction of photodegradation products with sunscreen excipients or skin components like sebum may lead to the formation of newmolecules with unknown toxicological properties (Cambon et al., 2001; Deleo et al., 1992; Rieger, 1997; Schrader et al., 1994; Nohynek and Schaefer, 2001). Consequently, there is an increasing concern about the phototoxicity and photoallergy of UV filters.

Phototoxicity is defined as a toxic response from a substance applied to the body which is either elicited or increased (apparent at lower dose levels) after subsequent exposure to light, or that is induced by skin irradiation after systemic administration of a substance (OECD, 2004). It is as a non-immunological light-induced...
skin response (dermatitis) to a photoactive chemical, and the skin response is characterized by erythema and sometimes edema, vesiculation, and pigmentation. Phototoxic reactions are comparable with primary irritation reactions in that they may be elicited after a single exposure, thus no induction period is required (Marzulli and Maibach, 1985).

Photoallergic contact dermatitis is thought to arise when UV radiation interacts with a chemical to form a hapten or antigen, which in turn triggers a type IV hypersensitivity reaction (Bryden et al., 2006).

As organic UV filters are used in increasing amounts, there is gradual emergence of reports of allergic and photoallergic reactions to UV filters on human skin. Epidemiological studies performed using human photopatch test, showed that avobenzone and many other UV-filters were the causal agents of these allergic and photoallergic reactions (Schauder and Ippen, 1997; Lodén et al., 2011). Among the organic UV absorbers, octocrylene, benzophenone-3 and avobenzone most frequently elicited photoallergic contact dermatitis. On the other hand, despite cinnamates and salicylates are used in large quantities, reports of allergic reactions are relatively low (Kerr et al., 2012; Kerr and Ferguson, 2010).

Another tendency in photoprotection in the topical application and systemic administration of antioxidants acting as photoprotectives, which could maintain or restore a healthy skin barrier (Pinnell, 2003). Among the frequently used antioxidants in anti-aging products we can point out vitamin A, C and E derivatives. Vitamin A palmitate acts on epithelization in dry and rough skin, as well as on keratinization considered being abnormal (Maia Campos et al., 1999). In addition, it also absorbs UV radiation between 300 and 350 nm, with a maximum at 325 nm (Antille et al., 2003), which can suggest that it may have a biologically relevant filter activity as well. However some studies have shown that vitamin A and its ester undergo photo-oxidation to give a variety of photodecomposition products and reactive oxygen species (Xia et al., 2006). Therefore, since some studies show that vitamin A generates toxic photoproducts or allergens when exposed to UV radiation, the US FDA selected vitamin A palmitate by the National Toxicology Program (NTP) as a high priority compound for phototoxicity and photocarcinogenicity studies (Xia et al., 2006; Tolleson et al., 2005).

In Europe, since the year 2000, in vivo testing in animals for acute phototoxic potential is no longer permitted, since a successfully validated in vitro alternative method has been accepted for regulatory purposes. Due to its high sensitivity and specificity, the validated 3T3 Neutral Red Uptake Phototoxicity Test (3T3-NRU-PT) is the core test, which is usually the only phototoxicity test required when the substance is not considered phototoxic (Liebsch et al., 2005). Reconstructed human skin models closely resemble the native human epidermis due to the presence of a barrier function similar to the barrier function of human epidermis.

Thus, the reconstructed human skin models are proposed as an additional tool for verification of positive results of the 3T3 NRU-PT, with respect to bioavailability in human skin, and/or for testing of substances incompatible with the 3T3 NRU-PT (Liebsch et al., 2005; Kejlová et al., 2007). Human in vivo photopatch method can also be performed, but they must be carried out only after prior risk assessment in vitro studies and in compliance with the ethical principles avoiding unnecessary risks to human subjects. Some studies report a good correlation among 3T3-NRU-PT, Human 3-D Skin Model and human in vivo photopatch tests (Kejlová et al., 2007; Spielmann et al., 1998). However, despite the proposed tests are scientifically validated to detect phototoxic potential with the aim of preventing phototoxic reactions in the general population, the extrapolation of in vitro results to the human situation may be performed only to a limited extent. These limitations are in part due to the higher permeability of the skin tissues compared to human skin in vivo (Kand’árová, 2006).

There are also some other concerns involving the predictability of phototoxicity testing in animals and humans (Maibach and Marzulli, 2004). For example, Marzulli and Maibach (1970) discussed the correlation between skin permeability and bergapten phototoxicity performed in animals and humans. They found that animals with more permeable skin (rabbits and hairless mice) were more reactive to bergapten than monkey and swine that have less permeable skin. In addition, they found that stripped skin had more pronounced biological effects than intact skin or less permeable forearm skin.

Nevertheless, even human photopatch tests need to be standardized in order to investigate photoallergic reactions and obtain consistent results. Such points are related to experimental design, irradiation sources, specify exposure time and distance of source to the skin, as well as UV dose (Maibach and Marzulli, 2004). In 2004 a group of interested European Contact Dermatologists/Photobiologists met to produce a consensus statement on methodology, test materials and interpretation of photopatch testing (Bruynzeel et al., 2004). In 2012, this group provided current information on the relative frequency of photo-allergic contact dermatitis to common photoallergenic organic UV-filters and they also stated the relevance of such investigations as well as of some cross-reactions between some UV-filters combinations (EMCPPTS, 2012). This way, it is of great importance to investigate the phototoxic potential of new combinations of UV-filters and antioxidant substances like vitamin A. However, for ethical reasons before in vivo testing on human volunteers and to avoid confirmatory testing in animals, 3T3 NRU-PT and H3D-PT are offering an attractive in vitro alternative approach, since H3D-PT is characterized by skin barrier function.

Therefore, the aim of this study was to evaluate the in vitro skin phototoxicity of cosmetic formulations containing photostable and photostable UV-filters and vitamin A palmitate, assessed by two in vitro techniques: 3T3 Neutral Red Uptake Phototoxicity Test and Human 3-D Skin Model In Vitro Phototoxicity Test.

2. Materials and methods

2.1. Chemicals

UV-filters samples were supplied by Symrise (Germany): benzophenone-3, butyl methoxydibenzoylmethane (avobenzone), ethylhexyl methoxycinnamate, Octocrylene, methyldibenzilidene camphor, ethylhexyl salicylate. Vitamin A palmitate (retinylpalmitate) was supplied by DSM (Switzerland). Positive controls Chlorpromazinehydrochloride and Bergamot oil were purchased from SIGMA AG (Germany).

2.2. Formulations

Four UV filter combinations often used in SPF 15 sunscreen products were chosen for this study. The combined UV filters were added to a formulation containing 0.5% of hydroxyethyl cellulose, 3% of glycerin, 0.05% of BHT, 3.5% of phosphate-based self-emulsifying wax (ceteryl alcohol, dicetyl phosphate, ceteth-10 phosphate), 6% of C12–C15 alkyl benzote, 3% of propylene glycol, 4% of a blend of polyglyceryl-10 myristate, diphenylmethicone, trietixadecano, 2.0% of cyclopentasiloxane, 0.8% of phenoxyethanol and parabens and distilled water. The combinations were: 7% of octyl methoxycinnamate (OMC), 2% of benzophenone-3 (BP-3) and 1.5% of octyl salicylate (OS) [formulation 1]; 10% of OMC, 2% of avobenzone (AVB) and 2% of 4-methyldibenzilide camphor (MBC) [formulation 2]; 7% of OMC, 4% of BP-3 and 5% of octocrylene (OC) [formulation 3]; 5% of OMC, 2% of AVB and 7% of OC [formulation 4] (Gaspar and Maia Campos, 2006).
2.3. Combinations under study

For the 3T3 Neutral Red Uptake Phototoxicity Test, a stock solution was prepared in DMSO for each UV-filter and the vitamin under study. This stock solution was diluted in eight different concentrations in EBSS ranging from 0.1 to 316 μg/mL in a geometric progression (constant factor of 3.16).

Four different combinations under study were also analyzed, these combinations contained the UV-filters under study in the same proportion (1:1:1) (Comb 1, Comb 2, Comb 3, Comb 4) or the same proportion used in the formulations under study (Comb 1+, Comb 2+, Comb 3+, Comb 4+). The different combinations of UV-filters in the presence of vitamin A, in different proportions were also analyzed. The stock solutions of the combinations in DMSO were diluted in 8 different concentrations in EBSS ranging from 3.16 to 178 μg/mL in a geometric progression (constant factor of 1.78).

For the EpiDerm Skin Phototoxicity test, all combinations were diluted in C12–C15 alkyl benzoate.

2.4. Source of irradiation

The UV light source used in phototoxicity tests in cell culture (3T3 NRU) and in human 3-D skin model (H3D-PT) was a doped mercury metal halide lamp (SOL 500, Dr. Hönle, Germany) which simulates the spectral distribution of natural sunlight. Asperctrum almost devoid of UVA (<320 nm) was achieved by filtering with 50% transmission at a wavelength of 335 nm (Filter H1, Dr. Hönle, Germany). The emitted energy was measured before each experiment with a calibrated UVA meter (Type No. 37, Dr. Hönle, Germany) [OECD, 2004; Kejlová et al., 2007].

2.5. Phototoxicity test in cell culture (3T3 NRU)

The 3T3 Neutral Red Uptake Phototoxicity Test was performed according to INVITTOX Protocol No. 78 [Liebsch and Spielmann, 1998], using 3T3 Balb/c fibroblasts (L1, ECACC No. 86052701). For this purpose, after the evaluation of the fibroblasts sensibility of radiation), for that, firstly 100 μL of a cell suspension of 3T3 fibroblasts in Dulbecco’s Modification of Eagle’s Medium (DMEM) containing New Born Calf Serum and antibiotics (1 x 10^5 cells/mL, 1 x 10^6 cells/well) was dispensed in two 96-well plates. After a 24 h period of incubation (7.5% CO2, 37 °C), plates were washed with 150 μL of Earle’s Balanced Salt Solution (EBSS) and different concentrations of the test chemicals or combination were applied in sextuplicate in the 96-well plates. After 1 h incubation, the +UVA plate was irradiated for 50 min with 1.7 mW/cm² (=5 J/cm²) of UVA radiation from UV-sun simulator, type SOL-500 (Dr. Hönle, Germany). The –UVA plate was kept in a dark box for 50 min. The test solutions were replaced by culture medium and plates were incubated overnight. Neutral Red medium was added in each well and after an incubation period, cells were washed with EBSS and a desorb (ethanol/acetic acid) solution was added. Then, neutral red extracted from viable cells formed a homogeneous solution and the +UVA and –UVA plates were analyzed in a micro-liter plate reader at 540 nm.

For concentration–response analysis Phototox Version 2.0 software (obtained from ZEBET, Germany) was employed. A test substance is predicted as having a potential phototoxic hazard if the photoirritation factor (PIF), calculated as the ratio of toxicity for each substance with and without UV light, is higher than 5 [Spielmann et al., 1998]. Using the Phototox software, a second predictor of phototoxicity, the mean photoeffect (MPE) was also calculated. The MPE is a statistical comparison of the dose–response curves obtained with and without UV and a test substance is predicted as phototoxic if MPE is higher than 0.1 (Holzhütter, 1997). According to the Organisation for Economic Cooperation and Development (OECD) Test Guideline 432, a test substance with a PIF >2 and <5 or an MPE >0.1 and <0.15 is predicted as “probably phototoxic” (OECD, 2004; Kejlová et al., 2007). Results are the mean of at least two independent experiments ± SEM.

Chlorpromazine was used as positive control for phototoxicity test in cell culture. According to the validation procedures, the test meets acceptance criteria, if for chlorpromazine EC50 (+UVA), i.e. the concentration inhibiting cell viability by 50% of untreated controls, is within the range of 0.1–2.0 μg/mL, and the chlorpromazine EC50 (–UVA) is within the range of 7.0–90.0 μg/mL (OECD, 2004).

2.6. Phototoxicity test in human 3-D skin model (H3D-PT)

The EpiDerm Skin Phototoxicity Test was conducted according to Liebsch et al. (1999) and Kejlová et al. (2007). 3D skin models, Epi-Derm EPI-200 (0.63 cm²), were supplied by MatTek, USA. Before dosing, the tissues were preincubated in fresh medium for 1 h to release transport stress related compounds and debris. After that, the medium was replaced by fresh medium and the tissue was incubated over night (18–24 h) (37 °C, 5% CO₂). The test formulations and substances were applied overnight (16–20 h) in a volume of 15 μL of each formulation per tissue or 25 μL of each combination diluted in C12–C15 alkyl benzoate per tissue. One set of tissues was irradiated with a nontoxic dose of 6 J/cm² (as measured in the UVA range). One day after the treatment and UVA exposure the cytotoxicity was detected as reduction of mitochondrial conversion of MTT to formazan. The optical density of the formazan extract was determined at 540 nm by means of Spectrophotometer Infinite 200 (Tecan Trading AG, Switzerland). The results of mean tissue values in the presence and absence of UV light were compared and a test substance was considered to be phototoxic, if one or more test concentrations of the (+UVA) part of the experiment revealed a decrease in viability exceeding 30% when compared with identical concentrations of the (–UVA) part of the experiment (Liebsch et al., 1997). Bergamot oil was used as positive control (Kejlová et al., 2007).

3. Results

3.1. Phototoxicity test in cell culture (3T3 NRU)

The results obtained in the 3T3 Neutral Red Uptake Phototoxicity test showed that only avobenzone was considered phototoxic, since it presented mean MPE of 0.327 and mean PIF of 11.478 (Table 1). Despite vitamin A palmitate presented a borderline mean MPE (0.106), some obtained values were classified as phototoxic or probably phototoxic, thus, the basis of these borderline results, this vitamin was submitted to a UV dose/response study to confirm its phototoxic potential.

The results obtained when avobenzone and vitamin A palmitate were submitted to 3T3 NRU Phototoxicity test under various intensities of UVA (2, 4 and 8 J/cm²) showed that avobenzone presented a pronounced phototoxicity enhancement (increased MPE) with higher UVA doses, showing that its phototoxicity was UVA dose dependent (Table 2). However when vitamin A was analyzed, no dose response effect was observed. Thus, the obtained results showed that vitamin A presented a tendency to a weak phototoxic potential that was not confirmed in the dose response study (Table 2).

When the combinations under study were analyzed, the phototoxicity test showed that only the combinations containing
When combinations 2 and 4 (containing the different UV-filters in the proportion 1:1:1) were evaluated, there was an enhancement of MPE values, which were closer to borderline phototoxicity values. When vitamin A palmitate was added to these combinations, comb 2A and comb 4A had their MPE enhanced to 0.310 and 0.229, respectively, indicating a synergistic effect of vitamin A palmitate on phototoxicity of these combinations containing avobenzone. When a lower concentration of vitamin A was added to these UV-filters combinations, comb 2A and 4A (containing a proportion of UV-filters/vitamin A 1:0.1), a reduction of MPE values was observed (0.169 and 0.181, respectively), however these combinations were still considered phototoxic (Table 4).

3.2. Phototoxicity test in human 3-D skin model (H3D-PT)

In order to evaluate the relevance of positive results obtained in the 3T3-NRU-PT with respect to bioavailability in human skin, the four formulations under study, containing or not vitamin A palmitate, as well as the combinations 2 and 4, containing avobenzone were submitted to the H3D-PT test.

The results of the phototoxicity assay using the human skin model are given in Figs. 1 to 3 as the mean% solvent control MTT conversion (n = 2) in the presence and absence of UV light. Untreated control tissues gave a mean OD value in the MTT assay of 1.983 without UV and there was no significant effect of solvent treatment (C12–15 alkyl benzene (mean OD value 1.854)) on MTT conversion. In addition, the UV exposure did not have any effect on MTT conversion indicating that the cultures were of satisfactory viability (85%).

Bergamot oil was phototoxic only in the highest concentration tested (10% in C12–15 alkyl benzene) as expected (Kejlová et al., 2007), with a reduction in MTT conversion in the presence of UV to approximately 40% of that of control tissues.

Fig. 2 shows that no phototoxicity was detected with the application of the formulations 1, 2, 3 and 4, since none of the (+UVA) tissues revealed a decrease in viability exceeding 30% when compared with the (−UVA) tissues. The presence of vitamin A palmitate did not alter tissue viability.

Fig. 3 shows that no phototoxicity was detected with the application of the combinations studied, since none of the (+UVA) tissues revealed a decrease in viability exceeding 30% when compared with the (−UVA) tissues, except combination 2 in the highest concentration tested (10% in C12–15 alkyl benzene), with a reduction in MTT conversion in the presence of UV to approximately 53% of the −UVA tissues (Fig. 3A). There was a slight dose-related reduction in MTT conversion with the enhancement of concentrations of combination 2 tested. The enhancement of vitamin A palmitate concentration did not reduce tissue viability (Fig. 3D) or protected the tissues from UVA-induced damage.

Previous studies showed that bergamot oil from different companies was classified as phototoxic in the 3T3 NRU PT and presented borderline results in H3D PT, which was also dependent on the solvent used (Kandˇárová, 2006; Kejlová et al., 2007).

Despite the higher permeability of Human 3-D Skin Model compared to human skin in vivo, these authors found a good correlation of the photopotency of bergamot oils diluted in sesame oil, when Human 3-D Skin Model and human in vivo photopatch tests result were compared; however they stated that the extrapolation of in vitro results to the human situation may be performed only to a limited extent.

However other studies showed that phototoxicity prediction of avobenzone did not have a good correlation when the monolayer (3T3 NRU) and the reconstructed human skin (H3D PT) were compared, which could be due to its low skin penetration, which reduces its viable epidermis availability (Kandˇárová et al., 2005, 2006; Trauer et al., 2006).

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### Table 1

Phototoxicity of isolated UV-filters and vitamin under study.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Run</th>
<th>PIF</th>
<th>MPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octyl methoxycinnamate (OMC)</td>
<td>1</td>
<td>1.303</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.196</td>
<td>−0.067</td>
</tr>
<tr>
<td>Octocrylene (OC)</td>
<td>1</td>
<td>1.664</td>
<td>−0.003</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.818</td>
<td>−0.040</td>
</tr>
<tr>
<td>Octyl salicylate (OS)</td>
<td>1</td>
<td>1.756</td>
<td>0.109</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.043</td>
<td>0.061</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.748</td>
<td>0.016</td>
</tr>
<tr>
<td>4-Methylbenzilidene camphor (MBC)</td>
<td>1</td>
<td>1.057</td>
<td>−0.197</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.514</td>
<td>0.109</td>
</tr>
<tr>
<td>Benzophenone-3 (BP-3)</td>
<td>1</td>
<td>2.514</td>
<td>0.109</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.415</td>
<td>0.025</td>
</tr>
<tr>
<td>Avobenzone (AVB)</td>
<td>1</td>
<td>3.428</td>
<td>0.177</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>21.034</td>
<td>0.358*</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>9.973</td>
<td>0.435*</td>
</tr>
<tr>
<td>Vitamin A palmitate (Vit A)</td>
<td>1</td>
<td>6.076</td>
<td>−0.050</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.076</td>
<td>0.075</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.023</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.133</td>
<td>0.017</td>
</tr>
<tr>
<td>Avobenzone</td>
<td>1</td>
<td>4.327</td>
<td>0.101*</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.240</td>
<td>−0.034</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.282</td>
<td>0.282*</td>
</tr>
</tbody>
</table>

---

### Table 2

Phototoxicity of avobenzone and vitamin A palmitate under various intensities of UVA radiation (2, 4 and 8 [J/cm²], (n = 1).

<table>
<thead>
<tr>
<th>Substance</th>
<th>UVA dose [J/cm²]</th>
<th>PIF</th>
<th>MPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A palmitate</td>
<td>2</td>
<td>−</td>
<td>−0.050</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>−</td>
<td>0.075</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>−</td>
<td>0.023</td>
</tr>
<tr>
<td>Avobenzone</td>
<td>2</td>
<td>0.826</td>
<td>0.017</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3.027</td>
<td>0.101*</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>12.140</td>
<td>0.294*</td>
</tr>
</tbody>
</table>

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### Table 3

Phototoxicity of UV-filters and vitamin under study in combination: combinations 1 and 3.

<table>
<thead>
<tr>
<th>Combination 1 (OMC, BP-3 and OS)</th>
<th>PIF</th>
<th>MPE</th>
<th>Combination 3 (OMC, BP-3 and OC)</th>
<th>PIF</th>
<th>MPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Comb 1</td>
<td>1.25</td>
<td>0.016</td>
<td>Comb 3</td>
<td>1.240</td>
<td>0.022</td>
</tr>
<tr>
<td>Comb 1A</td>
<td>1.033</td>
<td>0.003</td>
<td>Comb 3A</td>
<td>1.145</td>
<td>0.031</td>
</tr>
<tr>
<td>Comb 1A+</td>
<td>1.315</td>
<td>0.027</td>
<td>Comb 3A</td>
<td>2.011</td>
<td>0.032</td>
</tr>
<tr>
<td>Comb 1a</td>
<td>1.157</td>
<td>0.007</td>
<td>Comb 3a</td>
<td>1.383</td>
<td>−0.004</td>
</tr>
<tr>
<td>Comb 1a+</td>
<td>1.452</td>
<td>0.057</td>
<td>Comb 3a+</td>
<td>2.12</td>
<td>0.073</td>
</tr>
</tbody>
</table>

Comb 1 and 3: containing the different UV-filters in the proportion (1:1:1). Comb 1A and 3A: containing a proportion of UV-filters/vitamin A 1:1. Comb 1A+ and 3A+: containing the different UV-filters in the same proportion used in the formulations under study.

Comb 1A and 3A: containing a proportion of UV-filters/vitamin A 1:0.1. Comb 1A+ and 3A+: containing the different UV-filters and vitamin A in the same proportion used in the formulations under study.

vavobenzone, comb 2 (OMC, AVB, MBC) and comb 4 (OMC, AVB, OC), presented phototoxic potential (Table 4). The other combinations, comb 1 (OMC, BP-3 and OS) and comb 3 (OMC, BP-3 and OC), did not present any phototoxic potential, even when combined with vitamin A (Table 3).

Both combination 2 and 4 (containing the different UV-filters in the same proportion used in the formulations under study) were not considered phototoxic (MPE lower than 0.15). There was an enhancement of MPE values, when vitamin A palmitate was added to these combinations (comb 2A and comb 4A), however these combinations where still considered not phototoxic (Table 4).
Hayden et al. (2005) observed that avobenzone had the highest toxicity in culture human keratinocytes, however it did not penetrate the skin and thus the concentration of the UV-filter detected on viable epidermis after topical application was at least 5-fold lower than the toxic concentration on keratinocytes monolayer. Other authors observed that 1 h after application of a sunscreen formulation containing avobenzone (Parsol™ 1789), this UV-filter were located in the upper 30% of the horny layer and did not reach the living cells (Lademann et al., 2009).

Organic UV-filters are among the most common agent groups currently responsible for photo-allergic contact dermatitis. A multicenter photopatch test study conducted with 1031 patients in 30 European centers found that the UV-filters octocrylene, benzophenone-3 and avobenzone most frequently elicited photo-allergic contact dermatitis and they also reported some cross-reactions between some UV-filters combinations (EMCPPTS, 2012).

This way, although the extrapolation of the positive results obtained in the present study to the human situation may be performed only to a limited extent, they are valid to investigate the phototoxic potential of new combinations of UV-filters and antioxidant substances like vitamin A.

The results obtained in the present study showed that despite the four formulations studied did not present any acute phototoxicity (human skin model) and negative control (indicator of tissue viability). Results are the mean of two independent experiments ± SEM.

Fig. 1. Bergamot oil (positive control) phototoxicity (human skin model) and negative control (indicator of tissue viability). Results are the mean of two independent experiments ± SEM.

Fig. 2. Phototoxicity (human skin model) of the formulations containing (form 1A, form 2A, form 3A and form 4A) or not (form 1, form 2, form 3 and form 4) vitamin A palmitate. Results are the mean of two independent experiments ± SEM.
phototoxicity potential due to their reduced penetration, the combination 2 containing octyl methoxycinnamate (OMC), avobenzone (AVB) and 4-methylbenzilidene camphor (MBC) presented an indication of phototoxicity that should be better investigated. On the other hand, some previous studies of our group performed in humans showed that some formulations containing vitamins could induce allergic responses after a 2-week period of application, mainly when applied on the face (Gaspar et al., 2008). Thus, although no acute phototoxicity was detected in the H3D PT model, the formulations may have photoallergic or chronic phototoxicity and nowadays the development of additional models and endpoints is a challenge among researchers to avoid underpredictions and to increase the sensitivity of the these in vitro assays to replace animal use.

**Fig. 3.** Phototoxicity (human skin model) of the combinations of UV-filters and vitamin A palmitate. (A) Combination 2: containing the three different UV-filters in the same concentration (1:1:1). (B) Combination 2A: containing UV-filters/vitamin A palmitate in the same proportion (1:1). (C) Combination 2a: containing UV-filters/vitamin A palmitate in a different proportion (1:0.1); combination 2a=: containing UV-filters and vitamin A palmitate in the same proportion used in formulation 2 (1:1); combination 2a=: containing UV-filters and vitamin A palmitate in the same proportion used in formulation 2A. (D) Vitamin A palmitate. (E) Combination 4: containing the three different UV-filters in the same concentration (1:1:1). (F) Combination 4A: containing UV-filters/vitamin A palmitate in the same proportion (1:1). Results are the mean of two independent experiments ± SEM.
4. Conclusions

The results obtained when avobenzone and vitamin A palmitate were submitted to 3T3 NRU Phototoxicity test showed that avobenzone presented a pronounced phototoxicity enhancement that was UVA dose dependent. However when vitamin A was analyzed, the obtained results showed that vitamin A presented a tendency to a weak phototoxic potential that was not confirmed in the UVA dose response study.

When combinations 2 and 4 containing avobenzone were evaluated, there was an enhancement of MPE values, which were closer to borderline phototoxicity values. A synergistic effect of vitamin A palmitate on the phototoxicity of combinations containing avobenzone was observed.

The results of the phototoxicity assay using the human skin model (3T3-DPT) did not confirm the positive results obtained in the 3T3-NRU-PT; however despite the four formulations studied did not present any acute phototoxic potential, the combination 2 containing octyl methoxycinnamate (OMC), avobenzone (AVB) and 4-methylbenzilidene camphor (MBC) presented an indication of phototoxicity that should be better investigated. Thus, although no acute phototoxicity was detected in the 3T3-DPT model, the formulations may have photoallergic or chronic phototoxicity and thus additional studies must be performed in terms of the frequency of photoallergic or chronic phototoxicity in humans, since the proposed tests cannot predict the exact incidence of phototoxic reactions in humans.

Conflict of interest

The authors do not recognize any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations that could inappropriately influence the work.

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