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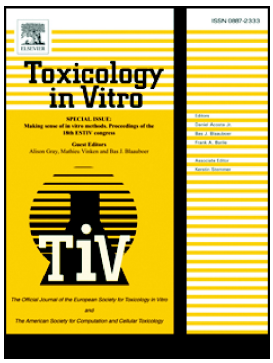

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UVA and UVB Formulation Phototoxicity in a Three-dimensional Human Skin Model: Photodegradation Effect

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ABSTRACT

In vitro three-dimensional human skin models are an innovative alternative to evaluate cytotoxicity and phototoxicity in the cosmetic industry. The aim of this study was to use a skin model to evaluate the potential toxicity of sunscreen formulations with or without exposure to UV radiation. In addition, the toxicity of these formulations was evaluated after exposure to photodegradation. The results showed toxicity with all formulations/conditions tested, including the control formulation, compared to PBS. Cell viability of photodegraded formulations - prior to the phototoxicity radiation process - was higher, indicating that some formulation components were degraded into products with reduced toxicity. The results also indicated that avobenzone was more unstable/toxic than octyl p-methoxycinnamate under the same test conditions. The sunscreens and their formulations were shown to be toxic to skin model cells to some extent, even when not exposed to UV irradiation; however the biological role of this toxicity is unclear. This result shows the importance of testing sunscreen formulations in real in-use conditions. Finally, since we used an *in vitro* assay based on a human cell model, this non-invasive technique represents a suitable alternative to animal models for phototoxicity tests in general and could have application in screening new sunscreen products.

Keywords:
Three-dimensional human skin model, toxicity, phototoxicity, photoprotectors, UV-induced cell damage, Neutral Red.

Abbreviations:
Octyl p-methoxycinnamate (OMC), avobenzone (AVB), Neutral Red (NR), solubilizing solution (SS), hematoxylin-eosin (HE).

1. Introduction

In the twenty-first century, it is becoming increasingly important to develop *in vitro* alternatives to animal testing. Three-dimensional human skin models or tissue-engineered constructs are adaptable and powerful research tools with numerous applications, such as the study of cancer, pigmentation and toxicity. In particular, many different techniques have been proposed to assess the photo- and cytotoxicity of potentially irritant chemicals such as sunscreens in a parallel development with the reduction in the number of animals used for marketing approval of these substances (Fernandez et al., 2012; Ponec, 2002).

In this context, i*n vitro* phototoxicity tests that are based on possible toxic reactions shown by cells or skin models after direct contact with specific substances, followed by exposure to UV sources, are extensively used. These allow the effects of different factors that may determine the levels of toxicity, such as the application volume, contact time with the model system, and the time of exposure to UV light to be discerned (OECD, 2004).

The burden on public health systems due to excessive exposure to sunlight represents the negative side of actively promoted lifestyles that keep individuals exposed to UV radiation. At the same time, however, the increasingly wide dissemination of information about the dangers of excessive exposure to solar radiation and the value of sunscreen use has led to a significant increase in the consumption of sunscreens. This increased sunscreen use, along with the need to evaluate new substances or products, makes it essential to develop robust *in vitro* toxicity tests.

The active ingredients in an effective sunscreen formulation may be inorganic particles, organic molecules, or a combination of both. The particles absorb, reflect or scatter UV radiation. The organic compounds contain conjugated aromatic carboxylic groups and generally have an electron donating group such as an amine or methoxyl in the *ortho* position of the aromatic ring which absorbs radiation in different spectral regions (UVA or UVB) (Giokas et al., 2007).

The sunscreen agent octyl p-methoxycinnamate (OMC) is currently the most frequently used sunscreen worldwide for protection from short wavelength UVB radiation. Avobenzone (AVB) is one of the agents that protects specifically against longer wavelength UVA radiation. AVB is very unstable and changes according to/depending on the formulation, which can lead to degradation by sunlight, placing its

phototoxicity protection in question (Gaspar et al., 2013). However, despite the extensively evaluated and well-known phototoxic effects of AVB and OMC, the increase or decrease of their toxicity when photodegraded and applied to human skin models has never been tested.

In addition, regulatory and commercial agencies have stressed the importance of evaluating sunscreen formulation stability under in-use conditions, to ensure the efficacy and the safety of the final product (Klein and Palefsky, 2001). The aim of this study was to investigate the application of a human keratinocyte and fibroblast-based skin model using a de-epidermized dermal culture substrate as a tool to evaluate phototoxicity of photodegraded sunscreen formulations. The skin model was used to characterize the epidermal photoresponse to AVB and OMC sunscreen formulations after prior exposure to UV radiation that led to photodegradation.

2. Materials and methods

2.1. Human cell culture

Human keratinocytes were obtained from skin fragments devoid of subcutaneous tissue by serial enzymatic cell separation using a 0.05% trypsin/ 0.02% EDTA solution (GIBCO-BRL Life Technologies, Rockville, MD, U.S.A.). The cells were plated on an irradiated 3T3 feeder layer in 75 cm^2 culture flasks and cell culture was performed according to the methodology proposed by Rheinwald and Green (1975). The culture bottles were stored in a 5% CO_2 incubator at 37°C.

Human fibroblast suspensions were obtained by a similar procedure. Keratinocytes and fibroblasts were both enzymatically released and seeded onto prepared, de-epidermized, glycerol-preserved allodermis (Herson et al., 2001).

2.2. Reconstruction of human skin containing a de-epidermized allodermis

The reconstruction of the three-dimensional human skin model started by placing the de-epidermized allodermis, previously sterilized by 50 kGy, as described in Herson et al. (2001), into metal grids. Metal rings were fixed to limit the cell seed area. Onto that system ~500,000 cells/cm^2 of each cell type (keratinocyte and fibroblast) were seeded and cultured for 21 days. After 24 h following inoculation, the metal rings were

carefully removed and more medium was added so that the culture was submerged. The first total medium change was performed after 48 h and then every third day for the next 2 weeks. From the fourteenth to the twenty-first day of the experiments, culture medium was changed daily. The dermal fragments with proliferating keratinocytes were kept in submerged conditions for 7 days, allowing the cells to proliferate and for the last 14 days the system were maintained under the air-liquid interface, required in order to obtain a fully differentiated epidermis with a *stratum corneum* layer (Herson et al., 2001). The culture medium change was performed in order to keep the dermis in contact with the medium and the cells exposed to air, mimicking the *in vivo* skin.

2.3 Sunscreen formulation

The OMC (UVB) and AVB (UVA) filters were chosen to be tested in this skin model as they are the most commonly used cosmetic ingredients in commercial sunscreen products (Table 1).

Table 1. Composition of the formulations used for the *in vitro* cytotoxicity and phototoxicity assays

Ingredients	BF (%)	OMC (%)	AVB (%)
BHT	0.05	0.05	0.05
Solution of Methylchoroisothiazolinone/ Methylisothiazolinone	0.1	0.1	0.1
EDTA disodium	2.0	2.0	2.0
Glyceryl stearate	2.0	2.0	2.0
Stearyl alcohol	2.5	2.5	2.5
Cetearyl alcohol	3.0	3.0	3.0
Peg 100 stearate/glyceryl/stearate	3.0	3.0	3.0
Caprylyl methicone	4.0	4.0	4.0
Glycerin	10	10	10
C12–C15 alkyl benzoate	0.05	0.05	0.05
Octyl Methoxycinnamate (OMC)	-	10	-
Butyl Methoxydibenzoylmethane (AVB)	-	-	4.0

Acqua	73.3	63.3	69.3

A basic oil-in-water formulation (BF) was prepared with ingredients commonly used in the composition of commercial sunscreens. Formulation BFOMC and Formulation BFAVB were prepared by adding either the UVB filter OMC or the UVA filter AVB respectively to BF.

Cosmotec (Brazil) supplied all formulation components, including the active photoprotective ingredients. All formulations cited above, with or without pre-irradiation (i.e. degraded and non-degraded, respectively), were tested.

The experimental design and protocol under which the different formulations were tested - in the presence and absence of radiation exposure - is shown in Figure 1.

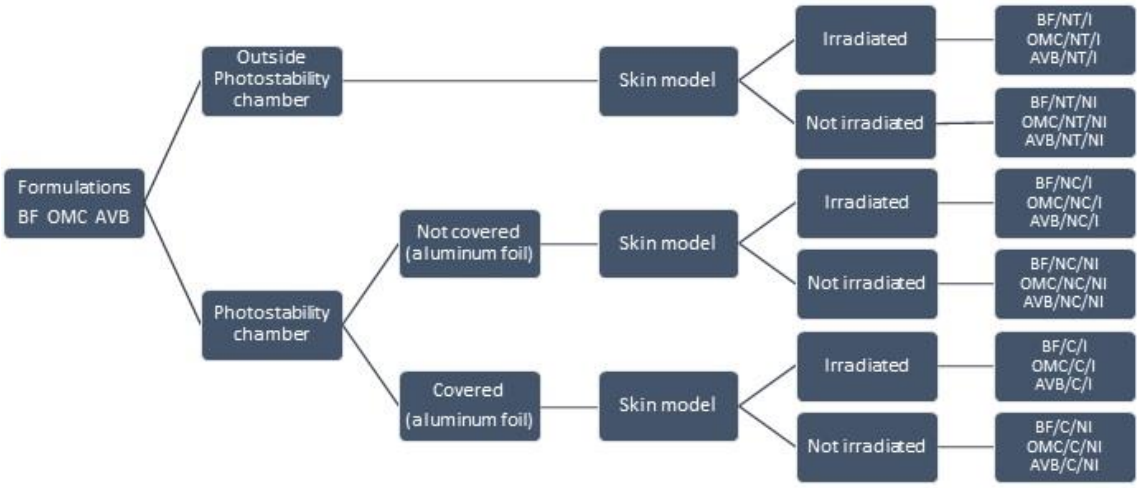

Fig. 1. Experimental design for the photodegradation test (UVA exposure) and phototoxicity tests (UVA exposure) of BF (basic formulation); OMC (basic formulation with OMC); AVB (basic formulation with AVB) in the conditions NT: no-treatment (not submitted to photostability chamber); I: irradiated; NI: not irradiated; C: covered with aluminum foil when submitted to photostability chamber; NC: not covered with aluminum foil and submitted to photostability chamber.

2.4. Photostability chamber

The degradation process of all formulations tested, using 2 g of each sample spread as a thin film in Petri dishes (90 mm diameter), was conducted in a photostability chamber with UVA (with distributed spectrum between 320 nm and 400 nm, UV energy > 200w.h/m²) (Fotoestabilidade Farma 424 CF - Nova Ética) at 24.5 ºC for 60 hours (exposure time obtained by actinometric procedure using a 2% w/v solution of quinine monohydrochloride dihydrate to achieve 200 watt hours/square meter as described in ICH Q1B guideline). The chamber used was in accordance with Q1B guide, published by the International Conference on Harmonization, and the light source corresponding to option 2 (ICH, 1996). UVA radiation was chosen to degrade the formulas (BF/NC, AVB/NC, OMC/NC), due to this type of radiation being normally present throughout the day. The control formulation samples that were not degraded (BF/C, AVB/C, OMC/C) were covered with aluminum foil and maintained in the photostability chamber for the same period under the same conditions as the degraded samples in order to provide the same environment.

2.5. UV-vis spectrophotometry evaluation of formulations – degradation process

The sunscreen formulations containing AVB 4% w/w or OMC 10% w/w were analyzed by UV-vis spectrophotometry. Samples of formulations that were taken from previously exposed or protected Petri dishes in the photostability chamber were weighed and dissolved in absolute ethanol at 2 mg/mL w/v (80 µg/mL of AVB or 200 µg/mL of OMC). The solutions were diluted with absolute ethanol up to concentration of 0.04 µg/mL and analyzed by Thermo spectrophotometer (Evolution 201 - Thermo Scientific) at the range of 200 to 500 nm, using a quartz cuvette of 1.0 cm optical path. Formulation samples without sunscreens were submitted to the same analyses for comparison. Absolute ethanol was used as a blank.

2.6. Phototoxicity chamber

The simulation of sunlight by irradiation with UVA lamps (340 nm) was conducted in a phototoxicity testing chamber constructed in stainless steel frame and fitted with two 15 Watts Xenon lamps and a filter system, designed and built in accordance with the protocol ©ECVAM DB-ALM: INVITTOX protocol. This chamber provides UVA radiation at 1.7 mW/cm^2 faithfully following the protocol OECD

Toxicity Guide 432, which proposes a radiation dose of 5 J/cm^2 for testing the phototoxicity performance of chemicals in direct contact with the studied cells (ECVAM, 2008; OECD, 2004). Two reference substances - Bergamot oil (*Citrus bergamia* - Givaudan-Roche*)*, as a positive control and Sodium Lauryl Sulfate as a negative control, both at a concentration range of 0.005 to 0.100 mg/mL, were used to validate the phototoxicity-testing chamber (Sufi, 2013).

The multiwell plates containing the reconstructed skins (item 2.2) on grids were irradiated after topical application of ~ 2 mg/cm^2 of formulations BF, OMC and AVB (uncovered: NC - photodegraded; or covered: C – non- photodegraded) as shown in Fig. 1. These multiwell plates were divided in two groups; one that was subjected to UVA exposure and irradiated with 5 J/cm^2 (BF/NC/I, BF/C/I, AVB/NC/I, AVB/C/I, OMC/NC/I, OMC/C/I), and a second group that was placed in the dark area of the chamber (BF/NC/NI, BF/C/NI, AVB/NC/NI, AVB/C/NI, OMC/NC/NI, OMC/C/NI) under the same conditions of temperature and time (75 minutes). Samples were rinsed after irradiation using PBS with Ca^{2+} Mg^{2+}, following the addition of fresh medium, maintained at 37°C and 5% CO_2 overnight.

The choice of this light source is a crucial factor in phototoxicity testing. Radiation in the UVA and visible regions is usually associated with phototoxic reactions *in vivo* (Lambert et al., 1996; Spielmann et al., 1994), whereas UVB is generally of less relevance but is highly cytotoxic (Tyrrell and Pidoux, 1987).

2.7. Assessment of UV-induced cytotoxicity

After 24 hours, Neutral Red (NR) solution was added to the human skin model, which was maintained at 37 °C and 5% CO_2 for a further 3 hours. After that period, the NR solution was removed, the model washed and a solubilizing solution (SS) added to the wells. The multiwell plates were protected from light and agitated for 10 minutes on a plate shaker. After this time, the supernatant comprising the SS and the NR released by the cells was placed in 96-well multiwell plates to be read in a multiplate reader (Multiskan EX 355, Thermo Electron Corporation) at 540 nm.

2.8. Statistical analysis

The data obtained from the phototoxicity test were processed by statistical analysis using one-way analysis of variance (ANOVA one-way, Duncan test, Tukey test and t- test) at a confidence range of 95%, so the changes were considered statistically significant for $p < 0.05$.

2.9. Histological analysis

Immediately after the phototoxicity experiments, the skin models were fixed in a formaldehyde solution 10%, buffered, dehydrated and embedded in histological paraffin. Histological sections (4 µm thickness) for each of the test groups were stained with hematoxylin-eosin (HE) to assess and compare the conditions of the structure of epidermal equivalents.

The present study is part of a research project approved by the Research Ethics Committee of Faculdade de Saúde Pública of the Universidade de São Paulo – FSPUSP (CAAE 00583812.6.0000.5421).

3. Results and Discussion

Human skin models – a reliable way to assess toxicity UV skin damage

Several *in vitro* cytotoxicity assays have been developed and performed by significant private and government agencies around the world. The Multicenter Evaluation of *in vitro* Cytotoxicity (MEIC) and the European Centre for the Validation of Alternative Methods (ECVAM) both recommend the Neutral Red uptake (NRU) assays, performed with mouse 3T3 fibroblast cells (3T3 NRU assay) or normal human keratinocytes cells (NHK NRU assay). These assays have been used to test a range of chemicals/pharmaceuticals already marketed and therefore tested *in vivo*, with mainly assays in monolayer culture, obtaining similar and reproducible laboratory results and making the assessment of *in vitro* cytotoxicity a feasible technique and also a source of highly reliable results (Clothier et al., 2013; Paris et al., 2005; Strickland et al., 2005). The 3T3 NRU phototoxicity assay is best suited for individual chemicals, and thus final formulations must be tested with 3D skin models, since solubility is not a limiting

factor. Further, chemicals are tested topically, and therefore testing conditions should resemble end user applications (Institute for *in vitro* Sciences, 2013).

The establishment of three-dimensional skin models has addressed many previous limitations of toxicity tests. These skin models overcome important problems related to the use of animal models, such as strict ethical regulations, substantial costs and structural and biological inconsistencies (Adler et al., 2011; Ponec, 2002).

In order to promote keratinocyte differentiation and epidermogenesis, construction of the skin models ends with an air-liquid interface, resulting in the formation of an epidermal layer that is histologically similar to native human skin, including the presence of a relatively normal stratum corneum (Fernandez et al., 2014; Kairuz et al., 2007; Topping et al., 2006). Due to the value of skin models for *in vitro* testing, we established in our laboratory a three-dimensional skin model as a tool to study wound healing and toxicity of extracts or formulations (Herson et al., 2001; Kamamoto et al., 2003).

Here, we describe an additional application of our in-house skin model, to characterize the epidermal toxicity and photoresponse to a photodegraded sunscreen formulation. Since topical application of sunscreen formulations on these models mimics real human *in vivo* conditions, they are suitable for evaluating sunscreens in response to UV radiation. In addition, the use of UVA and UVB specific filters allowed the assessment of damage caused by each of these substances as well as the stability of these formulations when exposed to UV radiation.

It is important to note that this approach can also be used to reveal whether a UV-absorbing ingredient is non-phototoxic or has the potential to transfer energy to other molecules in a formulation to induce toxic effects (Institute for *in vitro* Sciences, 2013).

Although the 3D skin models are generally designed to simulate the epidermis or the full human skin, none is currently approved for testing of skin absorption, an important point when we evaluated toxic effects (Abd et al, 2016). Nevertheless, these models are important tools for performing screening tests and thereby reducing animal experiments.

Evaluation of formulations – degradation process

UV-vis spectrophotometry analyses revealed that the formulation samples without sunscreen (basic formulation - Formulation 1) underwent a change in their absorption

spectra when subjected to irradiation (without aluminum foil protection) in the photostability chamber. This is easily seen in the formulation absorption peak due to the alteration of the peak intensity at $\lambda = 227$ nm, with absorbance of 0.9939 and 0.7984 for irradiated and protected formulations, respectively (Figure 2A).

It is also clear that this change observed in the emulsified system does not interfere with the main absorption peaks of the sunscreens. In fact, for both formulations – AVB (Formulation 3) whose absorption peak is at 357 nm and for OMC (Formulation 2) with a peak at 307 nm – there is no influence of the basic formulation absorption spectrum.

The comparison of the absorption spectra of the irradiated and protected AVB formulations showed a clear change in the sunscreen due to the exposure to light, which is indicative of photodegradation. This fact can be verified by the significant reduction in absorbance at 357 nm (0.4060 to 0.1573) and increased absorbance at a secondary wavelength peak of 271 nm (0.1469 to 0.2276) (Figure 2B).

On the other hand, after irradiation, OMC showed no significant change in its absorption spectrum for the basic formulation compared to the protected formulation. For this sunscreen, the characteristic peak at 307 nm had absorbance values of 0.8300 and 0.8092 for the protected and irradiated formulations, respectively. Further, it is possible to attest that the sunscreen absorption spectra substantially overlap, being indicative of the OMC absorptivity maintenance in the analysis media, suggesting the maintenance of sunscreen structure (Figure 2C).

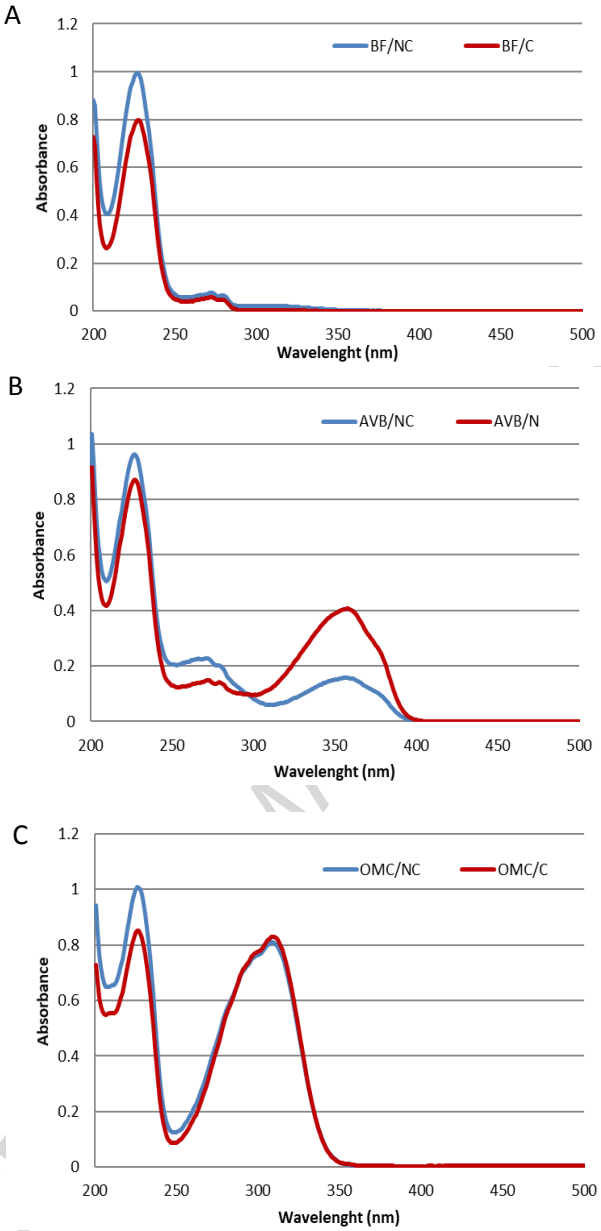

Fig. 2. UV-vis absorption spectra after UVA exposed in photostability chamber for the three formulations covered (C) and not covered (NC). A- Basic formulation (BF); B- AVB formulation (AVB), C- OMC formulation (OMC); The concentration of all formulations in ethanol for UV-vis assay was 0.04 mg/mL.

Photodegradation and toxicity using skin models – cellular viability assessment

A testing strategy was developed to minimize the number of cultures required, being the toxicity assessed in the dark (NI: not irradiated) and the phototoxicity assessed in the light (I: irradiated).

Based on the results achieved with the permanence of skin models (n=4) only in PBS (NT: no-treatment, not submitted to photodegradation in a photostability chamber) for each situation (NI and I), we concluded that the skin model had suitable viability and sensitivity for this test (Table 4). The skin models that were treated with PBS and left in the dark (NI) had absorbance values greater than 1.0, while the absorbance values became slightly lower in the presence of UV light (0.9). This indicates that the skin model was capable of detecting the influence of UV radiation on cell viability - a finding already pointed by Chatelain and Gabard (2001).

Using the t-test it was possible to assess that there is no statistical difference between the replicates of all the conditions evaluated, showing that the test is reproducible. We also showed a statistically significant difference ($p = 2.61 \times 10^{-5}$) between the irradiated samples and those without irradiation (NI) in the phototoxicity control test with PBS only, demonstrating the appropriate functioning of the phototoxicity chamber.

Since the skin model incorporates a well-formed epidermis (after 21 days of cultivation in an air-liquid interface), it has the necessary feasibility to test complete formulations, such as oil-in-water based creams for phototoxic potential. Also, due to the 3-D structure, the formulations could be adequately spread on the culture without damaging the surface. The formulations were made in the same way as commercial sunscreens and tested without dilution to mimic usage concentrations. All formulations were divided in two groups for testing: with or without prior radiation in the photostability chamber.

One-way analysis of variance (ANOVA one-way) of cell viability results of formulations covered with aluminum foil (Table 2) showed significant difference ($p = 6.4 \times 10^{-28}$). Duncan's post-hoc test (Table 2), was then applied to these data to evaluate which cell viability means (%) were statistically different from each other. Formulations covered with aluminum foil containing OMC or AVB (OMC/C/I, OMC/C/NI, AVB/C/I and AVB/C/NI) showed lower cell viability than control formulations (BF/C/I, BF/C/NI) and PBS (PBS/I and PBS/NI) independently of UVA exposure (I or NI). Cell viability from formulations subjected to UVA photodegradation without aluminum foil (Table 3) also showed significant difference ($p = 8.2 \times 10^{-24}$) and Duncan's test was also applied to the data. The values of cell viability from formulations containing OMC or AVB (OMC/NC/I, OMC/NC/NI, AVB/NC/I and AVB/NC/NI) were lower than those from control formulations (BF/C/I, BF/C/NI) and PBS (PBS/I and PBS/NI) (Table 3).

The toxicity (low cell viability values) found for the three formulations (BF, OMC and AVB) can be related to their own formula components, especially AVB and OMC filters that exhibited lower cell viability values, when compared to PBS control (100% cell viability).

Table 2. Clusters of the formulations covered with aluminum foil (C) and PBS according to Duncan's test in six groups of cell viability means (%). BF: basic formulation; AVB: basic formulation with AVB; OMC: basic formulation with OMC; C: covered with aluminum foil when submitted to photostability chamber; I: irradiated; NI: not irradiated; PBS: phosphate buffer solution.

Formulation	**Cell viability mean (%)**	**1**	**2**	**3**	**4**	**5**	**6**
AVB/C/NI	62.11	****					
OMC/C/I	65.03		****				
AVB/C/I	65.47		****				
OMC/C/NI	71.57			****			
BF/C/I	73.74			****			
BF/C/NI	79.08				****		
PBS/I	84.95					****	
PBS/NI	100.05						****

The groups represent clusters of cell viability means (%) grouped according to their similarities. Considering $p < 0.05$, there are significant differences between means in different groups and there are no significant differences between means in the same group.

Table 3. Clusters of the formulations without aluminum foil (NC) and PBS according to Duncan's test in five groups of cell viability means (%). BF: basic formulation; AVB: basic formulation with AVB; OMC: basic formulation with OMC; NC: not covered with aluminum foil when submitted to photostability chamber; I: irradiated; NI: not irradiated; PBS: phosphate buffer solution.

Formulation	Cell viability mean (%)	1	2	3	4	5
AVB/NC/I	63.17	****				
AVB/NC/NI	72.74		****			
OMC/NC/I	80.39			****		
OMC/NC/NI	81.14			****		
BF/NC/I	82.44			****	****	
BF/NC/NI	84.44				****	
PBS/I	84.95				****	
PBS/NI	100.05					****

The groups represent clusters of cell viability means (%) grouped according to their similarities. Considering $p < 0.05$, there are significant differences between means in different groups and there are no significant differences between means in the same group.

It is important to highlight that the toxicity exhibited in dark conditions (UVA^-) by the three formulations (BF, OMC and AVB) when photodegraded could indicate that the sun filters and their formulations are inherently toxic to the skin model cells in the dark, although the actual mechanism of this toxicity remains unclear. The results showed higher cell viability in skin models exposed to UVA radiation (BF/NC/I, BF/NC/NI, OMC/NC/I, OMC/NC/NI, AVB/NC/I and AVB/NC/NI) compared to those that were protected from radiation in photodegradation chamber (BF/C/I, BF/C/NI, OMC/C/I, OMC/C/NI, AVB/C/I and AVB/C/NI) as shown in Table 4.

High cell viability presented by all photodegraded formulations (i.e. those that underwent prior radiation – BF/NC/I, BF/NC/NI, OMC/NC/I, OMC/NC/NI, AVB/NC/I and AVB/NC/NI, shown in Table 4) and formulations which underwent two radiation exposures indicates that some formulation ingredients were inactivated by exposure to UV radiation. Formulations that received two radiation processes (I) presented cell viability of 82.44% (BF/NC/I), 80.39% (OMC/NC/I) and 63.17% (AVB/NC/I), suggesting that AVB is more unstable and toxic than OMC. This reinforces the idea that a long period of photodegradation in sunscreens by UV radiation can result in less toxicity and even phototoxicity when compared to formulations/sunscreens that did not receive any UV radiation or remained in the dark.

It is already known that chemical sunscreens have the capability to absorb UV light and this ability can generate adverse effects. Once a sunscreen molecule absorbs a UV photon, it is raised to an excited state and can eventually release the absorbed energy in the form of lower energy photons that are free to interact with other molecules. Such effects are called secondary effects, and can lead to the creation of free radicals that may cause irritation and damage to skin (Benson, 2000; Herrling et al., 2007).

Our results indicate that the existence of photoproducts of AVB could lead to an increase in cell death in the skin models. However, there is an increase in cell death when the formulations/sunscreens are exposed to UV radiation only once (BF/C/I, BF/C/NI, OMC/C/I, OMC/C/NI, AVB/C/I and AVB/C/NI), when compared to formulations/sunscreens exposed twice (BF/NC/I, BF/NC/NI, OMC/NC/I, OMC/NC/NI, AVB/NC/I and AVB/NC/NI), as shown in Table 4. After the degradation process and UVA exposure of the formulations/sunscreens applied on the skin models, this effect appears to be reduced, which possibly indicates the breakdown of primary reactive photoproducts to less reactive photoproducts.

However, if the testing of a formulation like a sunscreen as-supplied is needed, it could be interesting and even required to test through a reduced pre-incubation time, the photodegradation as in this study, or investigate their component substances on a case-by-case basis, and its impact in toxicity tests. Nevertheless, the three formulations were not found to be overtly toxic, although all show toxicity to a greater or lesser extent, which might be expected when a chemical is directly applied and maintained for many hours on the skin. All the statistics comparation were plotted at Table 4.

Table 4. The statistic comparison of all formulations and treatments.

Formulation	BF						OMC						AVB					
Photodegradation			Photodegraded						Photodegraded						Photodegraded			
Treatment	NT		C		NC		NT		C		NC		NT		C		NC	
Irradiation	I	NI	I	NI	I	NI	I	NI	I	NI	I	NI	I	NI	I	NI	I	NI
Formul	B	B	B	B	B	B	O	O	O	O	O	O	A	A	A	A	A	A

ations/ Treatment/ Irradiation	F/ NT/ I	F/ NT/ NI	F/ C/ I	F/ C/ NI	F/ NC/ I	F/ NC/ NI	MC/ NT/ I	MC/ NT/ NI	MC/ C/ I	MC/ C/ NI	MC/ NC/ I	MC/ NC/ NI	VB/ NT/ I	VB/ NT/ NI	VB/ C/ I	VB/ C/ NI	VB/ NC/ I	VB/ NC/ NI
% Cell viability	66.06	67.21	73.75	79.09	82.44	84.44	78.09	76.52	65.04	71.57	80.39	81.15	58.83	82.91	65.47	62.11	63.17	72.75
SD	3.23	2.19	3.14	1.80	2.23	3.19	2.18	1.66	1.52	1.52	1.25	1.59	0.93	0.70	1.86	1.39	2.56	1.95
t-test	=		≠		=		=		≠		=		≠		≠		≠	
p-value	p = 0.244		p = 0.002		p = 0.118		p = 0.095		p = 1.09×10^{-5}		p = 0.189		p = 1.04×10^{-13}		p = 0.002		p = 1.33×10^{-5}	

BF: basic formulation; OMC: basic formulation with OMC; AVB: basic formulation with AVB; Photodegraded: submitted to photodegradation in a photostability chamber; NT: no-treatment (not submitted to photodegradation in a photostability chamber); C: covered with aluminum foil; NC: not covered with aluminum foil; I: irradiated (submitted to UVA radiation in a phototoxicity chamber); NI: not irradiated (not submitted to UVA radiation in a phototoxicity chamber); = without significant difference ($p > 0.05$); ≠ with significant difference ($p < 0.05$)

A t-test was used to assess the statistical differences between the average cell viabilities with samples exposed to UVA radiation in the phototoxicity chamber. In the presence of AVB, with (AVB/NC) or without (AVB/C) photodegradation, there was no statistically significant difference in cell viability between irradiated (AVB/NC/I and AVB/C/I) and non-irradiated (AVB/NC/NI and AVB/C/NI) samples (Table 4).

In the base formulation and the formulation with OMC there were significant differences between irradiated and non-irradiated forms for those subjected to photodegradation protected with aluminum foil (Table 4).

Cell viability evaluation described in Table 4 were obtained using one-way ANOVA, and the statistic difference found ($p = 1.18 \times 10^{-49}$), was taken considering significant $p < 0.05$. Tukey test was used to show the differences among the data analyzed (Table 5).

Table 5. Clusters of formulations in nine groups according to average cell viability using Tukey test. There is no significant difference ($p < 0.05$) between means in the same group. BF: basic formulation; AVB: basic formulation with AVB; OMC: basic

formulation with OMC; NC: not covered with aluminum foil when submitted to photostability chamber; I: irradiated; NI: not irradiated; PBS: phosphate buffer solution.

Formulations	**Cell viability mean (%)**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
AVB/NT/I	58.83	****								
AVB/C/NI	62.11	****	****							
AVB/NC/I	63.17		****	****						
OMC/C/I	65.04		****	****						
AVB/C/I	65.47		****	****						
BF/NT/I	66.06		****	****						
BF/NT/NI	67.21			****						
OMC/C/NI	71.57				****					
AVB/NC/NI	72.75				****	****				
BF/C/I	73.75				****	****				
OMC/NT/NI	76.52					****	****			
OMC/NT/I	78.09						****	****		
BF/C/NI	79.09						****	****		
OMC/NC/I	80.39						****	****	****	****
OMC/NC/NI	81.15							****	****	****
BF/NC/I	82.44								****	****
AVB/NT/NI	82.91								****	****
BF/NC/NI	84.44									****

The groups represent clusters of cell viability means (%) grouped according to their similarities. Considering $p < 0.05$, there are significant differences between means in different groups and there are no significant differences between means in the same group.

Tukey test (Table 5) showed the mean cell viability of the formulations was distributed into nine groups for the different samples and their treatments. BF without photodegradation (no-treatment) – formulations BF/NT/I and BF/NT/NI (group 3) – presented a high level of cytotoxicity, however, this effect remains equal for irradiated and non-irradiated formulations. Also, it is important to highlight that the cytotoxicity of the BF without photodegradation (no-treatment) was higher than the OMC-containing formulations (OMC/NT/I and OMC/NT/NI) in groups 5, 6 and 7. This finding suggests that the sunscreen may at least be exerting a protective action against the toxicity exerted by the components of the base. In addition, the OMC did not suffer degradation as shown in the spectra (Figure 2C), probably due to its photoprotector characteristic whose spectrum of action is due to UVB radiation, therefore out of photodegradation range applied in this study.

In general, when formulations containing AVB (AVB/NT/I, AVB/C/I, AVB/C/NI and AVB/NC/I) were subjected to different treatments, lower cell viability was shown (Groups 1, 2 and 3 in Table 5). However, the same formulations containing AVB with no-treatment (AVB/NT/NI) and not exposed to UVA (Group 8, Table 5) showed lower levels of cytotoxicity. This result shows the importance of testing formulations containing AVB in real conditions of use, i.e., how the consumer will in fact be exposed. The test formulations without that simulation can generate false-negative results in safety tests. This can be extended to other topical formulations.

These finding were also corroborated by Briasco et al. (2017) in a study of the stability of a sunscreen product packed in a LDPE/HDPE mixture, simulating the possible stress conditions that solar products could meet during their "real in-use" life. Their results confirm the importance of studying all aspects related to the final product, as they stress the conditions that sunscreen products are exposed to could affect both quality and safety of the product.

Histological analysis of the reconstructed skin model

After the cytotoxicity and phototoxicity tests, the skin models were sectioned, and HE stained, in order to assess their structural conditions after application and removal of formulations with OMC (Fig. 3, B1 - B2) and AVB (Fig. 3, C1 - C2) when compared to PBS (Fig. 3, A1 - A2).

Figure 3 shows no evidence of abrasive or deconstructive effects on the skin model epidermis, regardless of whether they were treated with the formulations or immersed in PBS. The same result can be seen in the presence (I) and absence of radiation (NI).

Some cellular disorganization was observed in a couple of analyzed skin models, in spite of the presence of a confluent and stratified epithelium. This may be explained by the loss of the undifferentiated basal keratinocytes that were not retained on the dermal scaffolds due to loosening of collagen fibers that formed their base. This was most likely caused by the prior 50 kGy irradiation used for sterilization of the acellular dermis used as a scaffold to keratinocytes and fibroblasts growth. In future experiments, sterilization of these dermis scaffold will be performed at 25 kGy to prevent the loss of some of these binding cells for an improved composition of the skin model.

Ponec et al. (2001) studied the barrier function in a reconstructed epidermis model very similar to the one used here. They reported that the formation of the stratum corneum (SC) barrier *in vitro* proceeds similarly as *in vivo*, as judged from the extensive production of lamellar bodies, and the formation of multiple lamellar structures in the intercorneocyte space. In fact, all the construction process to obtain the skin model, mimics the *in vivo* process, with the exposure of the keratinocytes to air/liquid environment, to guarantee the differential process of keratinocytes to corneocytes and consequentially the stratum corneum formation.

The stratum corneum formation in our skin model is illustrated in the histological photomicrography in Figure 3. In addition, in our study the AVB and OMC formulations remained in contact with the skin model for only two hours, during which time significant permeation is unlikely to occur. As described by Yang et al. (2008) a formulation containing AVB showed very low permeation, as required of UV-absorbing agents such as both AVB and OMC that must remain in the outermost layer of the skin to be effective.

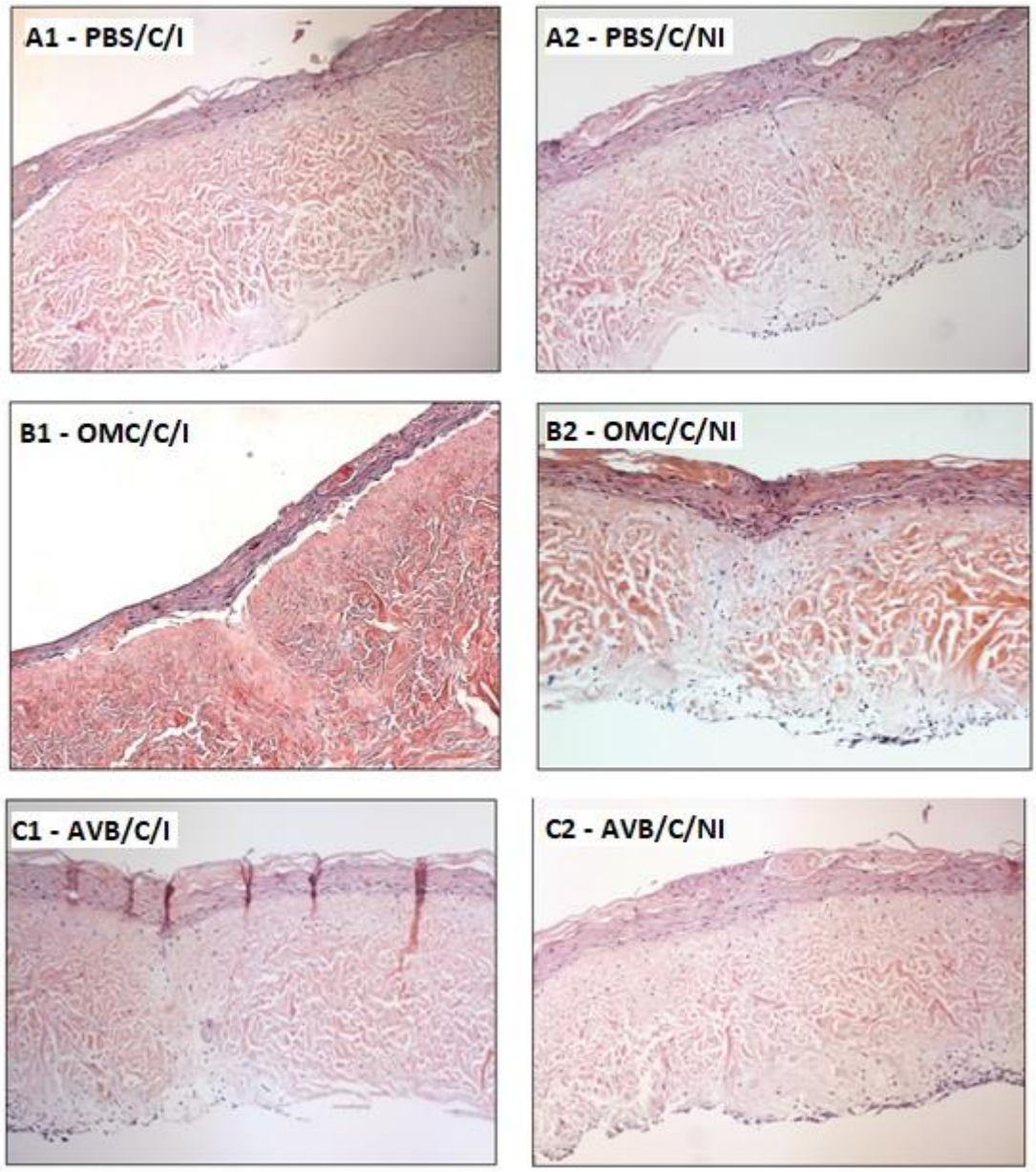

Fig. 3. Evaluation of the integrity of the skin models employed in the tests. Control group PBS (A1 – PBS/C/I and A2 – PBS/C/NI) and groups tested using formulations with OMC (B1 – OMC/C/I and B2 – OMC/C/NI) and AVB (C1 – AVB/C/I and C2 – AVB/C/NI). Optical microscopy (HE). Original magnification 100X.

4. Conclusions

An *in vitro* three-dimensional human skin model was developed and used to assess the phototoxicity resulting from application of sunscreens and their oil-in-water formulations. This skin model could overcome the need for *in vivo* animal testing, at least in the early stages of testing of currently marketed sunscreens, but also for new

formulations and other innovations in photoprotection, as a partial replacement test, within a tiered testing strategy. The development of photoprotectors containing organic products is a considerable challenge to the formulator due to the inherent instability of certain filters. Most substances used as UV filters are photoreactive.

The permanence of skin models only in PBS in dark and light conditions shows the skin model's ability to accurately assess the influence of UV radiation on cell viability. When considering the topical application of sunscreens, the results showed a lower toxicity in all formulations/conditions tested, even in the control formulation (BF) when compared to PBS.

This reinforces the concept that a long period of photodegradation by UV radiation can result in less toxicity and even phototoxicity when compared to formulations/sunscreens that were not exposed to UV radiation or remained in the dark. This indicates that the sun filters and their formulations are inherently toxic to the skin model cells in the dark; however, the mechanism of this toxicity remains unclear.

The combination of results presented in this work indicates that the in house *in vitro* skin model can be used as a reference for future testing of toxicity in different kinds of cosmetic and drug formulations.

Conflict of interest

The authors declare that there are no conflicts of interest.

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HIGHLIGHTS

- Skin models can evaluate the potential phototoxicity of sunscreens;
- The importance of testing sunscreen formulations in real consumer use conditions;
- Suitable alternative to animal models for phototoxicity tests;
- Photodegradation and phototoxicity radiation could eliminated intermediate products;
- Most substances used as UV filters are photoreactive.