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Research paper

Effect of ultraviolet filters on skin superoxide dismutase activity in hairless mice after a single dose of ultraviolet radiation

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ABSTRACT

Organic sunscreens may decrease their protective capability and also behave as photo-oxidants upon ultraviolet radiation (UVR) exposure. The present study investigated the effect of a cream gel formulation containing the UV filters benzophenone-3, octyl methoxycinnamate, and octyl salicylate on skin superoxide dismutase (SOD) after a single dose of UVR (2.87 J/cm^2). The retention of these UV filters was first evaluated *in vivo* using hairless mice to guarantee the presence of the filters in the skin layers at the moment of irradiation. The *in vivo* effect of the UV filters on skin SOD was then assayed spectrophotometrically via the reduction of cytochrome c. The cream gel formulation promoted the penetration of the three UV filters into the epidermis and the dermis at one hour post-application. A significant decrease in SOD activity was observed in irradiated animals treated with sunscreen formulation. However, no effect on SOD activity in skin was observed by the isolated presence of the sunscreens, the formulation components, or the exposure to UVR. The sunscreens may have formed degradation products under UVR that may have either inhibited the enzyme or generated reactive species in the skin.

1. Introduction

Oxidative stress and inflammatory responses induced by ultraviolet radiation (UVR) can cause a variety of harmful effects in skin, including the induction of premature photoaging, immunosuppression, and skin carcinogenesis [1,2]. Cellular chromophores of the skin absorb the energy of UVR and convert it into chemical energy. These energized chromophores can react with molecular oxygen to generate reactive oxygen species (ROS) such as hydroxyl ($HO^{\cdot}$) and superoxide (O_2^-) radicals, singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2) [3].

Among the antioxidant enzymes present in organisms, superoxide dismutase (SOD) plays an important role as a scavenger of superoxide radicals and has been implicated as a major player in the cellular defense system against cytotoxicity and cell lethality [4]. There are three isoforms of SOD in mammals: cytoplasmic and nuclear Cu–Zn-SOD, mitochondrial Mn-SOD, and extracellular SOD (EC-SOD). The latter is located within the extracellular space and binds to sulfated glycosaminoglycans. These enzymes catalyze the conversion of the superoxide anion to hydrogen peroxide (H_2O_2). Cu–Zn-SOD and Mn-SOD are generally thought to act as bulk scavengers of the superoxide anion in the cell cytosol and mitochondria. EC-SOD is thought to be more involved in signal transduction, particularly in the regulation of nitric oxide–mediated signaling across extracellular spaces [5].

To limit sun exposure, the government advises us to cover up with loose-fitting, tightly woven clothing, stay in the shade between 11 a.m. and 3 p.m., and use a sunscreen with a sun protection factor (SPF) of 15 or higher [6]. Ideally, sunscreens should protect against both skin cancer and the sun's effects on the immune system and photoaging of the skin. The increasing use of sunscreens has also increased interest in the photostability of these products. In this regard, it has been shown that organic sunscreens may decrease their protective capability and also behave as photo-oxidants upon UV exposure [7].

Studies have shown that the use of sunscreen promotes longer UVR exposure time because people feel more protected against erythema [8,9]. However, only considering erythema as the *in vivo* response to UVR may not reflect the protective action of sunscreens against other responses, such as immunosuppression, the depletion of the skin's antioxidant system, photoaging, and carcinogenesis. In this context, it is important to consider that sunscreen formulations are applied over a large area of the body and for a long period of time, producing a constant and high input of the UV filters into the viable skin layer and to the systemic circulation [10].

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For the above-mentioned reason, it is important to know the behavior of sunscreens on the skin, including the following: their penetration, the possible depletion of the antioxidant system when treated skin is exposed to UVR and whether UV filters can cause alterations in the skin's natural antioxidant protection without being exposed to radiation. The present study first evaluated the penetration of the UV filters benzophenone-3 (BP3), octyl methoxycinnamate (OMC), and octyl salicylate (OS) in the skin of hairless mice after the topical application of a cream gel formulation. The activity of the SOD antioxidant enzyme in the skin of mice treated with a cream gel formulation containing these UV filters was then investigated in both the presence and absence of UVR.

2. Materials and methods

2.1. Materials

3-benzophenone (99.9%, Eusolex® 4360), octyl methoxycinnamate (99.9%, Eusolex® 2292), and octyl salicylate (99.8%, Eusolex® OS) were purchased from Merck (Darmstadt, Germany). Acetic acid (chromatographic grade) was supplied by Merck (Darmstadt, Germany), and methyl alcohol (MeOH) for high-performance liquid chromatography (HPLC) was purchased from J. T. Baker (Phillipsburg, NJ, USA). The water used to prepare the solutions and the mobile phase was purified by a Milli-Q-plus System (Millipore®, Bedford, MA, USA). Ethyl alcohol was supplied from Synth (Sao Paulo, Brazil). All of the raw materials used for the formulations were purchased from Galena (Campinas, SP, Brazil) or Clariant (Sao Paulo, SP, Brazil). Superoxide dismutase, xanthine, xanthine oxidase, and cytochrome c were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of reagent grade and used without further purification.

2.2. Formulation

Considering that commercial products usually not only contain the chemical filters, but also physical filters such as zinc oxide and titanium dioxide, vitamin E, plant extracts, and other substances, which may interfere in the *in vivo* data, in this study, the formulations were prepared in order to eliminate these interferences, since their study were not the aim of this work.

The present study was performed using a cream gel formulation that is an emulsion that contains high percentage of aqueous phase and low oily content stabilized by hydrophilic colloids [11]. The gel cream was added by a mixture of the three organic UV filters at concentrations of 4.0% (w/w) BP3, 7.5% (w/w) OMC, and 5.0% (w/w) OS. The selection of UV filters and their concentrations were determined based on the concentrations usually employed in commercial formulations. A placebo formulation was prepared containing all of the components without the UV filters. The percent composition of the formulation is described in Table 1.

Table 1
Percent composition (w/w) of the cream gel formulation.

Components	Cream gel
Polawax®[a]	1.5
Carbopol® 940 [b]	0.5
Cetyl alcohol	1.75
Stearic acid	2.0
Gliceryl monostearate	2.75
Propylparaben	0.025
Methylparaben	0.175
EDTA	0.075
Propylene glycol	10.0
Distilled water	81.23

[a] Self-emulsifying wax (cetostearyl alcohol and polyoxyethylene derived from fatty acid ester sorbitan 2OE).
[b] Anionic hydrophilic colloid (carboxypolymethylene).

2.3. Study of photostability

Samples of the cream gel formulation both with and without UV filters were irradiated and evaluated by HPLC. Forty milligrams of each formulation was spread onto a 20 cm^2 (approximately 2 mg/cm^2) area of a glass plate and left to dry for 30 min before exposure to UVR [12]. The UVR source was a Philips TL/12RS 40 W lamp (Medical-Holand). This source emits in the range of 270–400 nm with an output peak at 313 nm, resulting in an irradiation of 0.27 mW/cm^2 at a distance of 20 cm as measured by an IL 1700 radiometer (Newburyport, MA, USA) equipped with UVB and UV detectors. UVB output accounts 78% of the total UVR. The cream gels were irradiated for 3 h, which corresponds to a dose of 2.87 J/cm^2, and the exposed samples were then diluted in ethanol. This radiant exposure corresponds approximately to the maximum dose of UVR that reaches the surface of the earth during one full day close to the equator. This protocol is similar to the one used by Tarras-Wahlberg et al. [13].

The ultraviolet filters, BP3, OMC, and OS, were quantified on a Shimadzu (Kyoto, Japan) HPLC system equipped with an LC-10 AT VP solvent pump unit and an SPD-10A VP UV–Visible detector operating at 305 nm. Separation was performed on a Supelcosil™ LC-18 column (25 cm × 4.6 mm, 5 μm) equipped with a C-18 precolumn (4 × 4 mm, 5 μm, Merck). This method was adapted from methods developed by Gaspar and Maia Campos [12] and Santoro et al. [14]. The mobile phase consisted of methanol–water (84:16, v/v) containing 0.1% (v/v) acetic acid at a flow rate of 1 mL min^{-1}. The HPLC was run at room temperature. Data were collected using a Chromatopac C-R6A integrator (Shimadzu, Kyoto, Japan). All solutions and solvents were filtered through a 0.45-μm Millipore® filter membrane and vacuum degassed by sonication before use.

2.4. In vivo skin retention

In vivo experiments were performed on 3-month-old, sex-matched hairless mice. The animals, weighing 20–30 g, were housed in a temperature-controlled room with access to water and food *ad libitum* until use. They were housed in cages with a 12-h light and 12-h dark cycle. All experiments were conducted in accordance with the National Institutes of Health guidelines for the welfare of experimental animals and with the approval of the Ethics Committee of the Faculty of Pharmaceutical Science of Ribeirao Preto (University of Sao Paulo, Ribeirao Preto, SP, Brazil – Process n 08.1.1399.53.4).

The animals were divided into two groups: a group treated with a cream gel formulation containing the three ultraviolet filters and a group treated with a cream gel formulation without sunscreens. Ten milligrams of the formulations was applied to the back of the animals in a delimited area of 1.77 cm^2. One hour after application, the animals were sacrificed by inhalation of carbon dioxide. The treated area of skin was removed and subjected to tape stripping, in which the skin was stripped with 15 pieces of adhesive tape to remove the stratum corneum. The remaining epidermis and dermis were cut into small pieces, sonicated for 30 min in 2.5 mL of methanol, vortex mixed for 1 min, and centrifuged for 15 min at 15,000 rpm. The supernatant was transferred to a 5-mL volumetric flask. The remaining precipitate was added to 2.5 mL of methanol, and the extraction procedure was repeated. The supernatant of the second extraction was added to that of the first in a volumetric flask, and the volume was adjusted with methanol. The resulting mixture was filtered through a 0.45-μm membrane, and BP-3, OMC, and OS were assayed by HPLC as described previously.

2.5. Irradiation of animals

Experiments included three or four animals per group and were repeated three times. The animals were divided in six groups: Group NIC = non-irradiated control, Group IC = irradiated control, Group NIP = treated with a placebo formulation and non-irradiated, Group IP = treated with a placebo formulation and irradiated, Group NIF = treated with a formulation containing the filters and non-irradiated, and Group IF = treated with a formulation containing the filters and irradiated.

The treatment protocol consisted of applying 30 mg of the formulations topically to the back of the animals one hour before irradiation. The groups exposed to UVR were placed inside a wooden enclosure containing the lamp and were irradiated for 3 h, which corresponds to a total dose of 2.87 J/cm^2. The mice were sacrificed by inhalation of carbon dioxide 6 h following UVR exposure, and full dorsal skins were removed and stored at −80 °C until analysis [15,16].

2.6. In vivo evaluation of SOD activity

SOD activity was quantified in epidermal extracts according to the reduction of cytochrome c method of McCord and Fridovich [17]. The total skin of hairless mice (1:2, w/w dilution) was homogenized for 2 min in 0.1 M phosphate buffer (pH 7.5) using a Turrax TE-102 (Turratec, Sao Paulo, Brazil). Whole homogenates were centrifuged at 1900*g* for 6 min. The supernatants were transferred to Eppendorf tubes and centrifuged again at 10,621*g* for 10 min at 4 °C, yielding the supernatant fraction. This fraction was diluted 10 times with phosphate buffer before starting the reaction.

The SOD assay mixture contained 2.9 mL of a solution composed of 0.76 mg (5 μmol) xanthine, 10 mL of 1 mM NaOH, 24.8 mg (2 μmol) cytochrome c, and 100 mL of 50 mM phosphate buffer (pH 7.8 with 0.1 mM EDTA). To this solution, 50 μL of epidermal extract was added. The reaction was initiated by adding 50 μL of a xanthine oxidase solution at approximately 0.2 U/mL, obtained by dilution in 0.1 mM EDTA. The increase or decrease in absorbance was followed at 550 nm for 10 min. One unit of SOD activity was defined as the amount of SOD sufficient to inhibit the rate of reduction of cytochrome c by 50%, and specific activity was expressed as units/mg protein.

3. Results and discussion

3.1. Study of photostability

The HPLC technique employed for the determination of UV filters in the cream gel formulation had been previously validated by evaluating the specificity, linearity, accuracy, and precision parameters. In the presence of the different formulation components, the chromatographic profiles of BP3, OMC, and OS were not affected. The technique was linear over the concentration ranges used (correlation coefficients above 0.999), and the relative standard deviation of the repeatability and intermediate precision for the three sunscreens were all less than 5%. The percent recoveries for the UV filters from the formulations were 94%, 100%, and 87% for BP3, OMC, and OS, respectively [18].

The chromatographic profile of the UVR-exposed formulation was compared with the non-exposed one. The chromatographic profiles of these formulations are shown in Fig 1. A reduction of approximately 14 ± 7% of BP3, 15 ± 8% of OS, and 45 ± 6% in the level of OMC was observed in the irradiated formulation when compared with the non-irradiated one. The photostability results of the present study corroborate to earlier studies developed by Gaspar and Maia Campos [12].

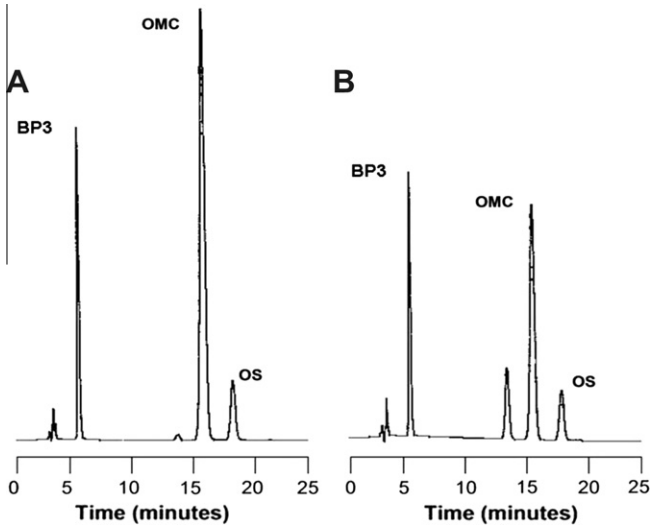

Fig. 1. Chromatogram of the cream gel formulation with added UV filters: (A) non-irradiated and (B) irradiated. Chromatographic conditions: reverse-phase C_{18} column with a mobile phase of methanol–water (84:16, v/v) containing 0.1% (v/v) acetic acid at a flow rate of 1 mL min^{-1} and UV detection at 305 nm.

Moreover, an increase in the chromatographic peak of a non-identified substance, eluted at 13 min, was also observed. Individual chromatographic analysis of the UV filters before exposure to UVR suggests that this peak might be a degradation product of OMC. This proposal is based on the fact that this peak was already present in lower concentrations when the OMC was analyzed individually and it increased after UV exposure. Our photostability study showed that OMC was the most photounstable filter compared with BP3 and OS. Butt and Christensen [19] demonstrated that when subjected to UV light, OMC produces successive breakdown products that have been found to be more toxic than the non-irradiated OMC.

The photoinstability of organic UV filters is an important problem because, after exposure to UVR, they undergo photolysis processes that promote the dissociation of these agents in reactive fragments, and the sunscreens undergo photoisomerization reactions that may yield species that absorb less UV light than the parent species [20].

3.2. In vivo skin retention

The same HPLC technique employed for photostability studies was used to evaluate the *in vivo* penetration of UV filters. The components of mice skin did not interfere chromatographically with the measurements of BP3 and OMC. However, a small amount of components of mouse skin co-eluted with OS. Due to the fact that these interfering peaks areas have been reproducible in all samples, it was possible to calculate the skin retention amounts of OS. For that, we subtracted the average value of the interfering peak areas from the peak areas which correspond to the OS plus the interfering peak.

The results of the *in vivo* skin retention study showed that BP3, OMC and OS were able to penetrate the skin 1 h post-application of formulation (Fig. 2).

Table 2 shows that BP3 was retained more in the skin than the other two filters, when retentions were expressed as a percentage of the applied dose. This result is likely due to the different physical characteristics and chemical properties of each molecule [21,22]. BP3 physical properties, such as relatively low molecular mass (228.25) and a log *P* of 3.58 confer to this filter greater ability to penetrate the stratum corneum and reach the deeper layers of the skin, when compared with OMC and OS [21,22]. However, regarding the quantities of filters added to the formulation, OMC

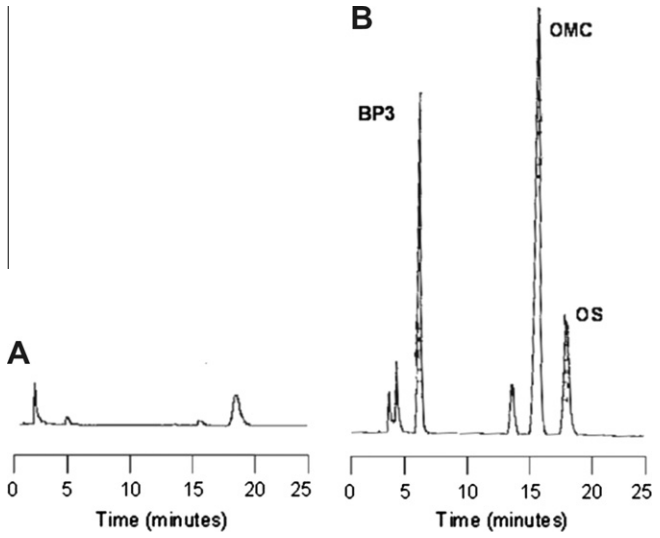

Fig. 2. Chromatogram showing the skin retention of the cream gel formulation: (A) without UV filters and (B) with UV filters. Chromatographic conditions: reverse-phase C_{18} column with a mobile phase of methanol–water (84:16, v/v) containing 0.1% (v/v) acetic acid at a flow rate of 1 mL min^{-1} and UV detection at 305 nm.

Table 2
Amount of UV filters retained in the epidermis and dermis from cream gel one hour post-application.

	Cream gel	
	Retention (μg/cm^2)	Retention (% of applied dose)
BP3	17.91 ± 4.03	2.6 ± 0.6
OMC	29.04 ± 8.01	2.3 ± 0.6
OS	18.63 ± 5.59	1.5 ± 0.4

Results are represented by means ± S.D (n = 6).

reached the viable epidermis in a level approximately 60% higher than BP3 and OS.

The results of *in vivo* skin retention study showed that one hour post-application was sufficient to retain the sunscreens in mice skin. Therefore, to assess the *in vivo* SOD activity, the formulation was applied one hour before UVR exposure to ensure that the UV filters were present in the epidermis and dermis at the beginning of the animal's irradiation process.

3.3. In vivo evaluation of SOD activity

To investigate the effect of sunscreens on SOD activity in both exposed and non-exposed skin, we used a UVR dose of 2.87 J/cm^2.

As demonstrated by Fig 3, the quantity of SOD activity units by mg of skin protein observed for the non-irradiated groups NIC, NIP, and NIF were statistically similar with no significant difference between them. We also observed a slight decrease in SOD activity on the irradiated group IP in comparison with the non-irradiated groups, but this difference was not statistically significant. These results demonstrate that neither the formulation components, even when irradiated, nor the non-irradiated UV filters interfered in the determination of SOD activity.

In addition, these results showed that both irradiated and non-irradiated formulation components and the non-irradiated UV filters do not interfere with any of the reactions involved in the spectrophotometric assay used to quantify SOD activity: generation of the superoxide radical by xanthine/xanthine oxidase, superoxide radical dismutation by SOD, and the reduction of cytochrome c by the superoxide anion. The effect of the BP3, OMC, and OS filters on the SOD and xanthine oxidase activities was evaluated *in vitro*

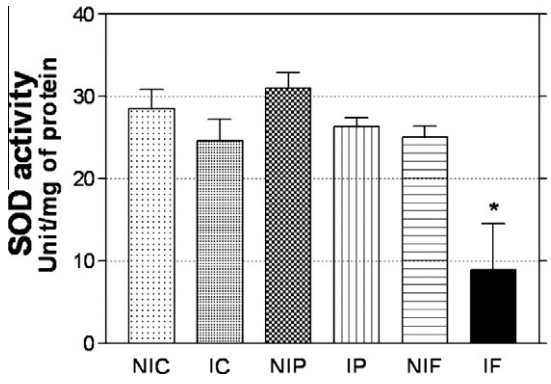

Fig. 3. Evaluation of SOD activity in the skin of hairless mice. Group NIC = non-irradiated control, Group IC = irradiated control, Group NIP = treated with a placebo formulation and non-irradiated, Group IP = treated with a placebo formulation and irradiated, Group NIF = treated with a formulation containing the filters and non-irradiated, and Group IF = treated with a formulation containing the filters and irradiated. Bars represent means ± SEM of three separate experiments (n = 3–4 animals per group). Statistical analysis was performed by one-way ANOVA followed by Tukey's test of multiple comparisons. $^*p < 0.05$ significant difference compared with NIC, IC, NIP, IP, and NIF groups.

using purified enzymes, and the results showed no interference to these enzymes activities (data not shown). These data show that the analysis method employed in the present work is selective and suitable for quantifying SOD activity in skin samples.

The obtained data also showed that after 6 h post UVR exposure, the SOD activity in the IC group was slightly decreased in relation to the non-irradiated control group (NIC), but it was not statistically confirmed. This result corroborates with other studies that demonstrated significant decreases in SOD activity induced by UVB radiation only between 12 and 72 h after exposure [23,24].

However, after 6 h of UVR exposure, it was observed that in the IF group a decrease in SOD activity of 69% compared with the NIC group. Even when SOD activity in the IF group was compared with the IC group, there was an activity decrease of 64%. The SOD activity decrease in the IF group was higher than the one obtained by Shindo et al. [25] who used a UV dose approximately 5-fold higher (25 J/cm^2) than the dose used in the present study.

Moreover, it can be suggested that the decrease in SOD activity was not due to the presence of UV filters on the viable epidermis once the enzyme activity in NIF group were maintained close to the NIC group. Thus, the enzymatic activity decrease observed in the IF group cannot be attributed solely to the UV filters penetration in the skin.

Given these results, we hypothesized that the presence of UV filters in the skin associated with UVR exposure might be involved in SOD activity decrease in viable epidermis.

Studies have shown that SOD enzyme can be inhibited more due to indirect mechanisms that involve free radicals and their oxidation products than by direct effect of UVR alone [26]. Recent experiments have demonstrated that the UV filters octocrylene and OMC produce ROS such as singlet oxygen ($^1O_2$). Studies have also shown that when the filters BP3, OMC, and octocrylene penetrate into the nucleated layers, the level of reactive oxygen species increases above what is produced naturally by epidermal chromophores under UV illumination [27]. The mechanism by which these UV filters generate $^1O_2$ is not fully understood, but one explanation is that they, mainly OMC, has an available triplet state capable of energy transfer to O_2, and thus $^1O_2$ may be generated. Singlet oxygen can lead to the formation of superoxide radical anion [27,28]. The generation of singlet oxygen by UV filters and consequent generation of superoxide radical in the skin might lead to an increase in SOD consumption that might explain the SOD activity decrease observed in the IF group.

Another possible explanation to this decrease in SOD activity could be the enzyme inactivation at the active Fe or Cu centers via the Fenton reaction [23,29]. Additionally, this inactivation may also be a result of the formation of cross-links in enzyme molecules by generation of reactive species [30,31].

Moreover, SOD inactivation might also be due to the reaction between the enzyme and the UV filters degradation products, as demonstrated for BP3 in other studies. Studies have shown that BP3 is rapidly photo-oxidized, yielding BP3 semiquinone, a potent electrophile that reacts with thiol groups in important antioxidant enzymes and substrates [32].

Another indirect mechanism that may be involved in the observed decrease in SOD activity is the one proposed by Dhar et al. [33]. This work demonstrated that low levels of stress induced by a low dose of UVB increase MnSOD expression. The expression of MnSOD was significantly suppressed with high-dose UVB exposure and was correlated with higher levels of the tumor suppressor p53 induction also caused by high-dose UVB exposure. In the present study, the association of the UV filters with UVR exposure (2.87 J/cm^2) might have generated high levels of oxidative stress that mediated increases in p53 and consequently suppressed MnSOD expression. This would involve a cellular defense mechanism that causes cells to die under high levels of stress induced by the association of the UV filters in the skin and UVR.

Our photostability and *in vivo* skin penetration studies have shown that the UV filters were degraded, with OMC being the one that most degraded and penetrated in higher quantities into the epidermis. These data suggest that OMC degradation may be highly responsible for the significant decrease in SOD activity on UVR-exposed skin treated with the cream gel formulation containing BP3, OMC, and OS.

These results create the need for future studies to evaluate the concomitant *in vivo* effect of sunscreens and UVR, as most of the data on the photostability of sunscreens come from *in vitro* work and thus cannot be associated with the effects in biological structures. In addition, the results of this work show the importance of skin penetration studies during sunscreen formulation development because filter molecules can penetrate through the epidermis, causing highly reactive intermediates of the photounstable filter to directly contact epidermal and dermal structures. Topical sunscreen formulations should be selected to decrease absorption and improve the safety and effectiveness.

4. Conclusion

The present data have demonstrated that neither the presence of UV filters, formulation components in the skin nor exposure to UVR alone is able to decrease SOD activity in the skin. However, when the presence of sunscreen on the skin was combined with ultraviolet exposure, there was a significant decrease in SOD activity. The sunscreens may have degraded due to the radiation, and these degradation products may have inhibited the enzyme or generated reactive species in the skin.

Based on the results of this study, we emphasize that photoprotection should consist of several lines of defense. In addition to the use of clothing and sunscreens that block the penetration of sunlight, the incorporation of antioxidant agents in sunscreen formulations might reduce photodamage and enhance the photostability of chemical UV filters.

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