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Research paper

Assessment of physical and antioxidant activity stability, in vitro release and in vivo efficacy of formulations added with superoxide dismutase alone or in association with α-tocopherol

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Abstract

A topical formulation was added with different concentrations of superoxide dismutase (SOD) alone or in association with α-tocopherol (α-TOC). The physical stability was evaluated by rheological behavior of formulations stored at 4 °C, 30 °C/60% RH and 40 °C/70% RH for 6 months. SOD alone and formulations containing SOD 0.2%, 0.4% or 0.6% or SOD and α-TOC were stored in the same conditions and the enzymatic activity was evaluated by the superoxide anion scavenging using chemiluminescence measurement. In vitro release study was carried out using modified Franz diffusion cell and SOD formulations photoprotection against skin erythema was observed for 72 h. SOD and α-TOC formulation proved to be instable, since the interaction between the antioxidants led to both physical and enzymatic activity instability. SOD formulations showed to be physically stable and maintained the enzymatic activity for 6 months when stored at 4 and 30 °C/60% RH. Despite the fact of low SOD release from the formulation, it was effective in inhibiting the UVB-induced skin erythema for 48 h after a single application. Topical administration of antioxidants provides an efficient way to enrich the endogenous cutaneous protection system, and SOD formulations could be used for improving photoprotection of skin.

Keywords: Superoxide dismutase; Antioxidant; Stability; Formulation; Chemiluminescence; Release; Erythema

1. Introduction

Ultraviolet-radiation-induced skin damage includes acute reactions such as erythema and edema, as well as premature skin aging (photoaging) largely determined by chronic exposure. Acute and chronic exposure to ultraviolet irradiation can result in reactive oxygen species (ROS) generation such as superoxide anion ($O_2^{-\cdot}$), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) [1]. It has been also reported that ultraviolet B (UVB) irradiation is capable of inducing lipid peroxidation and an impairment in the antioxidant defense system like SOD [2].

SOD is an essential enzyme, which protects cells from oxidative damage by catalyzing the reduction of the $O_2^{-\cdot}$ to H_2O_2 and molecular oxygen (O_2) [3]. This enzyme already has been shown to influence aging, cancer, and some very important diseases, such as arteriosclerosis and age-dependent immune deficiency [4]. Inal et al. [5] and Andersen et al. [6] reported that there were age related decreases in SOD activity. In addition, the enzyme activity was found to be decreased immediately after solar-simulated UV-irradiation, and UVB can be considered to be mainly responsible [7].

Treatment with exogenous SOD can reduce the activity loss and prevent the UV-induced formation of sunburn cells in skin [8]. Mizushima et al. [9] and Niwa [10] showed that a topically applied SOD-cream was effective against several skin diseases associated with increased ROS levels in humans. Nevertheless, the potential of SOD as an active pharmaceutical ingredient, fragility of

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enzymes has been a problem. Most enzymes lose biological activity at room temperature in a month or longer. Topical formulations consist of various oils and surfactants that may disturb enzyme structure and consequently deactivate the enzyme [11]. The present study was designed to evaluate both physical and antioxidant activity stability of formulations added with different concentrations of SOD as well as in vitro release and in vivo photoprotection effect of these formulations. Since the association of antioxidants could be more efficient in protecting skin against UV-irradiation, a formulation containing SOD and α-tocopherol was also evaluated concerning the physical and enzymatic activity stability.

2. Materials and methods

2.1. Test formulation

Dismutin-BT® (SOD) was purchased from Pentapharm (Basel, Switzerland), DL-α-Tocopherol (α-TOC) was a gift from Roche (São Paulo, Brazil), and the remaining raw materials for the formulations were purchased from Galena (Campinas, Brazil). An emulsion stabilized by an anionic hydrophilic colloid (carboxypolymethylene – Carbopol® 940) was developed, based on a commercially available self-emulsifying wax; macadamia nut oil was added as an emollient, and propylene glycol as a moisturizer. The preservative used was a mixture of phenoxyethanol and parabene (Table 1). This formulation was added or not with SOD 0.2%, 0.4% and 0.6%. The formulation containing the antioxidant association was added with SOD 0.4% and α-TOC 2%. Preservative, enzyme and α-TOC were incorporated at room temperature. All formulations were allowed to equilibrate for 24 h prior to use in studies.

2.2. Physical stability evaluation

Physical stability was evaluated by submitting the formulations to storage at 4 °C, 30 °C and 60% RH (relative humidity) and 40 °C and 70% RH for a period of 6 months. At initial time and after 1, 7, 15, 30, 60, 90 and 180 days, samples were collected for evaluation of rheological behavior, viscosity, pH and microscopy analysis.

The minimum apparent viscosity and rheological behavior were determined using a rotational rheometer with a cone-plate configuration (Brookfield DV-III) with a CP52 spindle, and 0.5 g of the sample. A Brookfield software program, Rheocalc 1.01, was also used. Measurements were made at progressively higher rotation speeds and shear rates (10–100 rpm and 20–200 s^{-1}, respectively) to obtain the ascending curve and the procedure was repeated in reverse with progressively slower rates (100–10 rpm and 200–20 s^{-1}) to obtain the descending curve. The rate was kept constant for 10 s at each shear rate before a measurement was made [12].

Table 1
Composition of the emulsion added or not with different concentrations of SOD alone or SOD and α-TOC

Component	Emulsion (g)
Self-emulsifying wax	1.00
Macadamia nut oil	2.00
Propylene glycol	6.00
Phenoxyethanol and parabene	0.40
Carbomer	0.18
Triethanolamine	0.15
Deionized water	90.27

The pH of formulations diluted 1:10 in distilled water was measured using a Digmed pH meter. All measurements were made at room temperature in triplicate for each analyzed sample. The microscopy analysis was made using a Leica DMLB microscope with a Leica DC 300 photograph.

2.3. Antioxidant activity stability evaluation

The enzymatic activity stability was evaluated by the superoxide anion scavenging activity, measured by the inhibition of chemiluminescence intensity of the xanthine–xanthine oxidase-luminol system. Xanthine and xanthine oxidase were purchased from Sigma (St. Louis, MO, USA), Luminol was purchased from Acros. All other chemicals were of reagent grade and were used without further purification. Formulations were submitted to storage at 4 °C, 30 °C and 60% RH and 40 °C and 70% RH for a period of 6 months. At initial time and after 1, 7, 15, 30, 60, 90 and 180 days the enzymatic activity was evaluated. Formulations were diluted in different volumes of phosphate buffer (pH 7.4; 0.1 M) and 50 μL of these samples was added in the reaction mixture in order to obtain SOD in a final volume of 0.04 μL.

Chemiluminescent mixture was prepared immediately before analysis by mixing: 400 μL glycine buffer (0.1 M, pH 9.4, 1 mM EDTA), 150 μL xanthine (6 mM in glycine buffer), 50 μL sample and 10 μL luminol (0.6 mM in DMSO). Adding 100 μL xanthine-oxidase solution (20 mU/mL) started the reaction [13]. The buffer and the xanthine solution were stable for 4 and 2 weeks, respectively, when kept at 4 °C, while the xanthine-oxidase and luminol solutions were freshly prepared each time.

In order to verify the stability of the enzyme alone, SOD (raw material) was also submitted to storage at 4 °C, 30 °C and 60% RH and 40 °C and 70% RH for a period of 6 months. The samples were diluted in phosphate buffer and added in the reaction mixture in order to obtain SOD in a final volume of 0.04 μL. Two controls were used for this test: the SOD-free formulation and phosphate buffer for SOD formulations and SOD alone, respectively. Chemiluminescence was measured for 5 min at 25 °C with an EG&G Berthold Autolumat LB953 apparatus. Experiments were conducted in triplicate.

2.4. In vitro release studies

The release rate of the formulation added with SOD 0.6% was studied using cellulose Millipore® 100,000 as

membrane model and modified Franz diffusion cell with a diffusion area of 1.77 cm^2. One gram of the formulation containing or not SOD was placed on the membrane surface in the donor compartment while the receptor was filled with 10 mL of 0.1 M phosphate buffer solution (pH 7.4) containing 0.9% (w/v) NaCl. During the experiment, the receptor solution was stirred at 100 rpm, kept at 37 °C and protected from the light. Samples were collected after 3, 6, 8, 10 and 12 h for the release evaluation. The IU of SOD present in the receptor phase was analyzed by the inhibition of the xanthine–xanthine oxidase-luminol chemiluminescence reaction. The SOD-free formulation was used as a control. The mean of 10 determinations was used to calculate the SOD release from the formulation.

2.5. In vivo evaluation of the photoprotective effect

In vivo experiments were performed on 30 hairless male mice of the HRS/J strain (30 g) at 3 months of age. Skin erythema was induced by UVB irradiation using an ultraviolet lamp model TL/12RS 40 W Philips. This source emits in the range of 270–400 nm with an output peak at 313 nm resulting in an irradiation of $1.713 \times 10\text{-}4$ W/cm^2 at a distance of 20 cm as measured by an IL 1700 radiometer (Newburyport, MA, USA) equipped with UVB and UV detectors. The minimal erythemal dose (MED) was preliminarily determined (61.56 mJ/cm^2 for these mice) and irradiation doses corresponding to 2.5 and 5 MED were used.

The back of each animal was divided in two sides. The formulations containing or not SOD were applied on the upper side 15 min prior exposure, while the lower side was protected from the irradiation. The animals were irradiated within their cages. Visual inspection of the erythema was done 24, 48 and 72 h after the exposure. The animals were fed with a standard diet and allowed to drink water *ad libitum*. They were housed within cages with a 12-h light and 12-h dark cycle. The three mice per cage were allowed to move freely.

2.6. Statistical analysis

The initial values of pH, minimum apparent viscosity and the rheological parameters (flow index and hysteresis loop), as well as the changes in viscosity in terms of period of study, storage temperature and presence of SOD or SOD and α-TOC, were analyzed statistically by ANOVA following the Tukey test. The level of significance was set at $p < 0.05$.

3. Results

3.1. Physical stability evaluation

Table 2 shows the initial values of pH, flow index, minimum apparent viscosity and hysteresis loop found for formulations containing or not SOD in different concentrations or SOD and α-TOC. There was no significant difference for these values after addition of the enzyme in different concentrations, but after the addition of SOD in association with α-TOC, the minimum apparent viscosity and hysteresis loop values showed a significant decrease ($p < 0.05$). During the 6 months of study the formulations showed a pH range of 7.55–6.95. The lowest values were found after 6 months at 40 °C/70% RH. The flow index of all formulations was less than 1, indicating a pseudoplastic behavior. The flow index range was 0.24–0.16 for base and SOD formulations. Formulation containing SOD and α-TOC showed a flow index range of 0.37–0.19. The highest value was found after 3 months at 40 °C/70% RH.

In addition to the statistical analyses of the initial values, the minimum apparent viscosity was analyzed in terms of storage time and temperature. Although the apparent viscosity showed a small decrease after 6 months at 40 C/70% RH, no significant changes were observed regarding storage time and temperature or presence of SOD. The range for the SOD-free formulation was 618–742.5 cP. Formulations added with SOD 0.2% or 0.4% showed a range of 615–737.3 and 597.7–741.2 cP, respectively. Formulation added with SOD 0.6% showed a little decrease, with a range of 567.8–729.7 cP. These results show that the increase in SOD concentration does not change the formulation viscosity. Even when 0.6% of SOD was added, no significant change in viscosity was observed. On the other hand, formulation added with SOD and α-TOC showed a great reduction in viscosity values, the range was 640.3–187.8. The lowest values were found for the higher temperature ($p < 0.01$) and after 3 months of storage ($p < 0.001$). The viscosity found for formulations containing or not SOD in different concentrations or SOD and α-TOC concerning the period of study at 4 °C, 30 °C/60% RH and 40 °C/70% RH is shown in Fig. 1.

Table 2
Initial values of pH, flow index, minimum apparent viscosity and hysteresis loop found for formulations added or not with different concentrations of SOD

Formulation	pH	Flow index	Minimum apparent viscosity (cP)	Hysteresis loop (Dinas/cm^2 s)
Base	7.55	0.20	679.6	8597
SOD 0.2%	7.40	0.19	701.9	8691
SOD 0.4%	7.39	0.20	649.3	7635
SOD 0.6%	7.28	0.19	633.1	7558
SOD and α-TOC	7.51	0.18	553.8	4934

Although the presence of SOD and α-TOC caused a decrease in the formulation viscosity, no changes in the formulation droplets were observed in the microscopy analysis during the study period. Fig. 2 includes the microphotographs of formulations at initial time.

All formulations were found to be thixotropic. The hysteresis loop was calculated by the area under the curve for the rheograms obtained for each formulation. There were no significant differences in the initial values of the hysteresis loop between the formulations containing or not the enzyme in different concentrations. Again, formulation added with SOD and α-TOC showed a significant reduction (Table 2).

3.2. Antioxidant activity stability evaluation

Based on the measurements of the areas under the time-course curves of chemiluminescence in the presence of SOD alone (raw material), SOD formulations or controls, we estimated the relative inhibitory activity of each sample during the study period. The percent inhibition caused by each sample was calculated using the following equation:

$$\text{Inhibition } (\%) = 100 - \frac{100 \times \text{AUC}_1}{\text{AUC}_0} \quad (1)$$

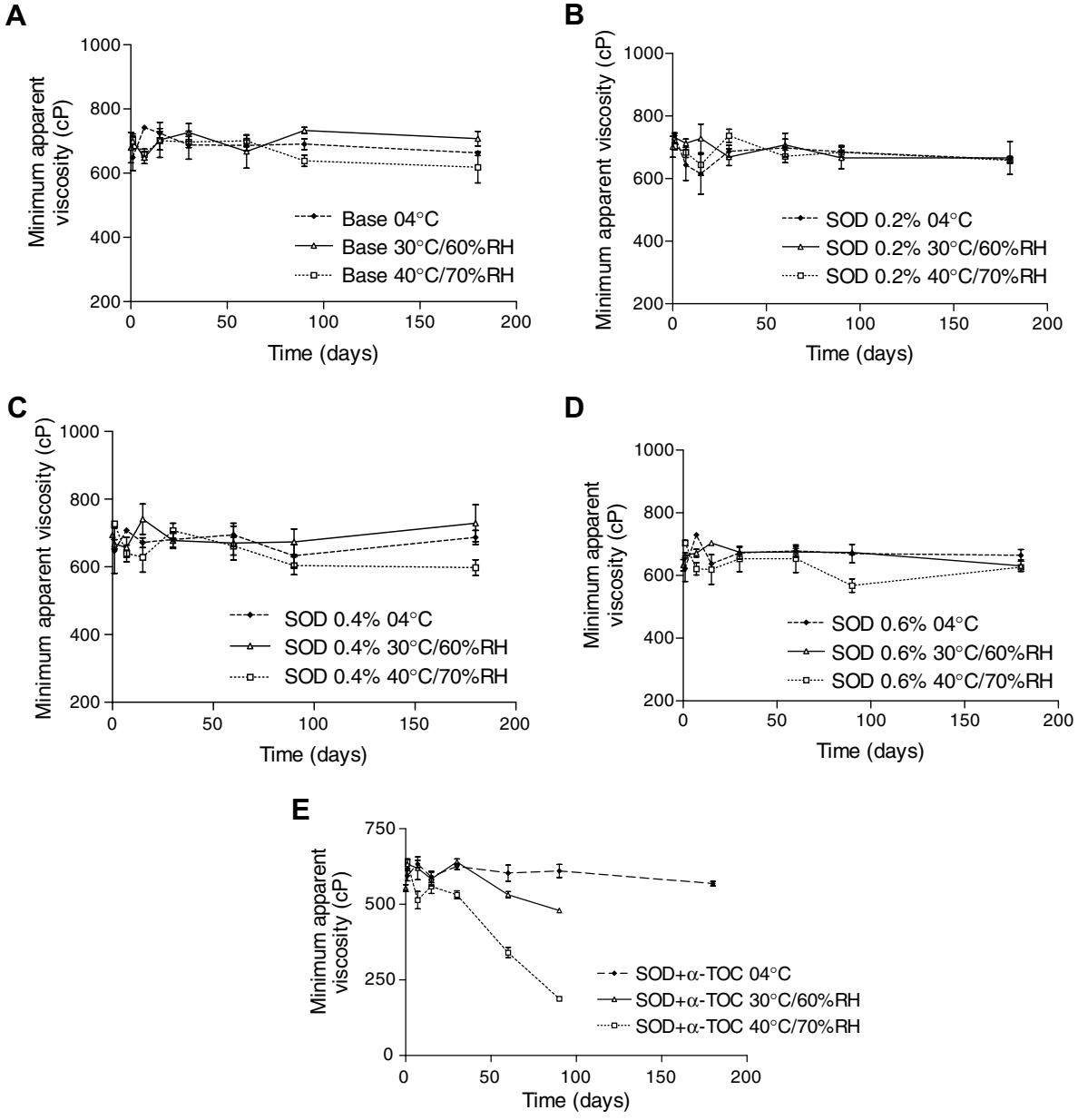

Fig. 1. Minimum apparent viscosity of formulations when stored at 4 °C, 30 °C/60% RH and 40 °C/70% RH during 6 months. (A) Base, (B) formulation added with 0.2% SOD, (C) formulation added with 0.4% SOD, (D) formulation added with 0.6% SOD and (E) formulation added with 0.4% SOD and 2% α-TOC. Results are means ± SD of three measurements.

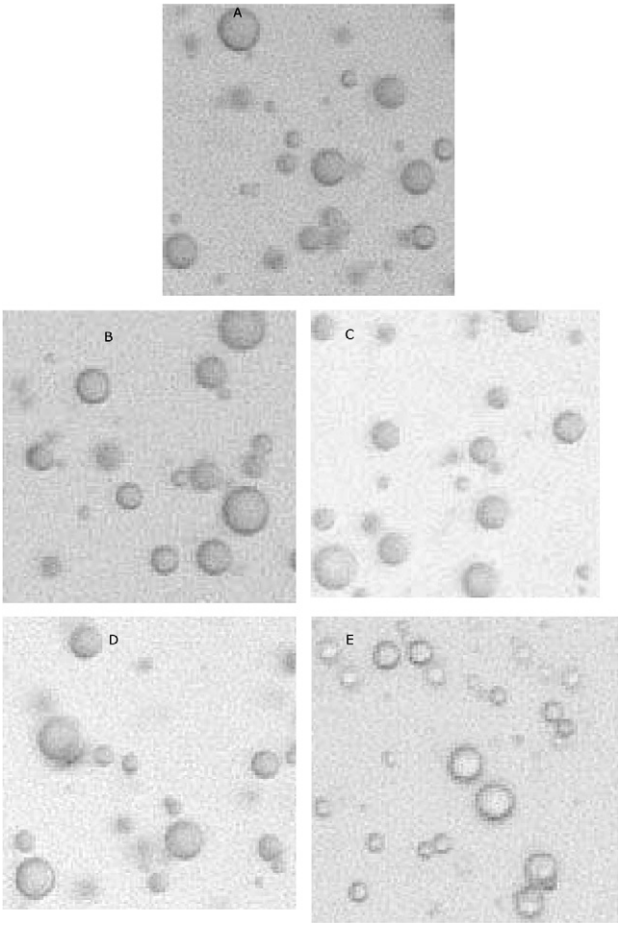

Fig. 2. Photomicrographs of formulations at initial time at 200× magnification. (A) base, (B) formulation added with 0.2% SOD, (C) formulation added with 0.4% SOD, (D) formulation added with 0.6% SOD and (E) formulation added with 0.4% SOD and 2% α-TOC.

where AUC_0 and AUC_1 represent the area under the curve observed for the controls (phosphate buffer, pH 7.4, or SOD-free formulation) and experimental samples, respectively.

SOD alone showed to be stable at different temperatures for 90 days, but after 180 days of storage, the enzyme showed an activity reduction of 14.3% and 26.2% at 30 °C/60% RH and 40 °C/70% RH, respectively, showing that increased temperatures for a long time may cause the enzymatic activity loss. At 4 °C, no reduction in the enzymatic activity was observed. The percent inhibition of chemiluminescence as a function of the time for SOD alone is shown in Fig. 3.

Formulations added or not with SOD in different concentrations or SOD and α-TOC were diluted in phosphate buffer in order to obtain the same final volume of SOD (0.04 μL), so the enzymatic activity was evaluated in the same way for all formulations. All SOD formulations showed the same inhibition percentage of the enzyme alone at the initial time, thus the formulation components did not interfere with the analytical method used. Although the temperature caused the reduction in the enzymatic activity only after 6 months for the enzyme alone, SOD formulations showed activity loss after 60 days of storage at 40 °C/70% RH. The activity reduction, concerning the initial was, 26%, 29.3% and 35.4% for formulations added with 0.2%, 0.4% and 0.6%, respectively. After 6 months, the loss was 66% of the initial activity found for formulation added with SOD 0.2% and about 85% for formulations with SOD 0.4% and 0.6% (Fig. 4).

Formulation containing SOD and α-TOC showed a great reduction in the enzymatic activity (Fig. 4). After 15 days at 30 °C/60% RH, the loss concerning the initial activity was 68.3% and after 7 days at 40 °C/70% RH, it was 81.2%. After 7 days, there was an increase of light emission in the chemiluminescence reaction. These results show that formulations containing SOD and α-TOC may have a prooxidant action rather than an antioxidant effect. The higher temperatures favor the enzymatic activity loss, since at 4 °C the reduction in activity occurred only after 6 months (66.7%).

3.3. In vitro release studies

Since SOD is an enzyme, the evaluation of the released amount of SOD from the formulation containing SOD 0.6% was made changing the chemiluminescence inhibition percentage found for the samples of receptor solution in SOD units. One SOD unit is defined as the amount that reduces the light emission by 50%. The amount of SOD released in the receptor solution is shown in Fig. 5. SOD showed a low release. After 12 h, only 5.3% of the amount present in 1 g of the formulation was released.

3.4. In vivo evaluation of the photoprotective effect

The animals from the control group (no treatment) and the ones treated with the SOD-free formulation showed evident erythema 24 h after exposure to both 2.5 and 5 MED, followed by desquamation. All SOD formulations

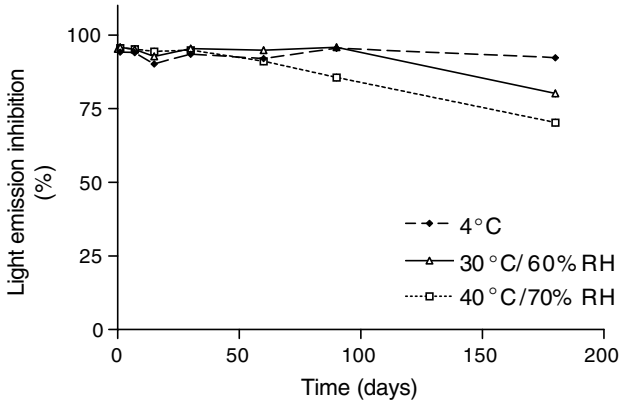

Fig. 3. Percent inhibition of light emission from the xanthine/xanthine oxidase-catalyzed luminescent reactions with luminol found for SOD when stored at 4 °C, 30 °C/60% RH and 40 °C/70% RH for 6 months. Results are means ± SD of three measurements run in parallel.

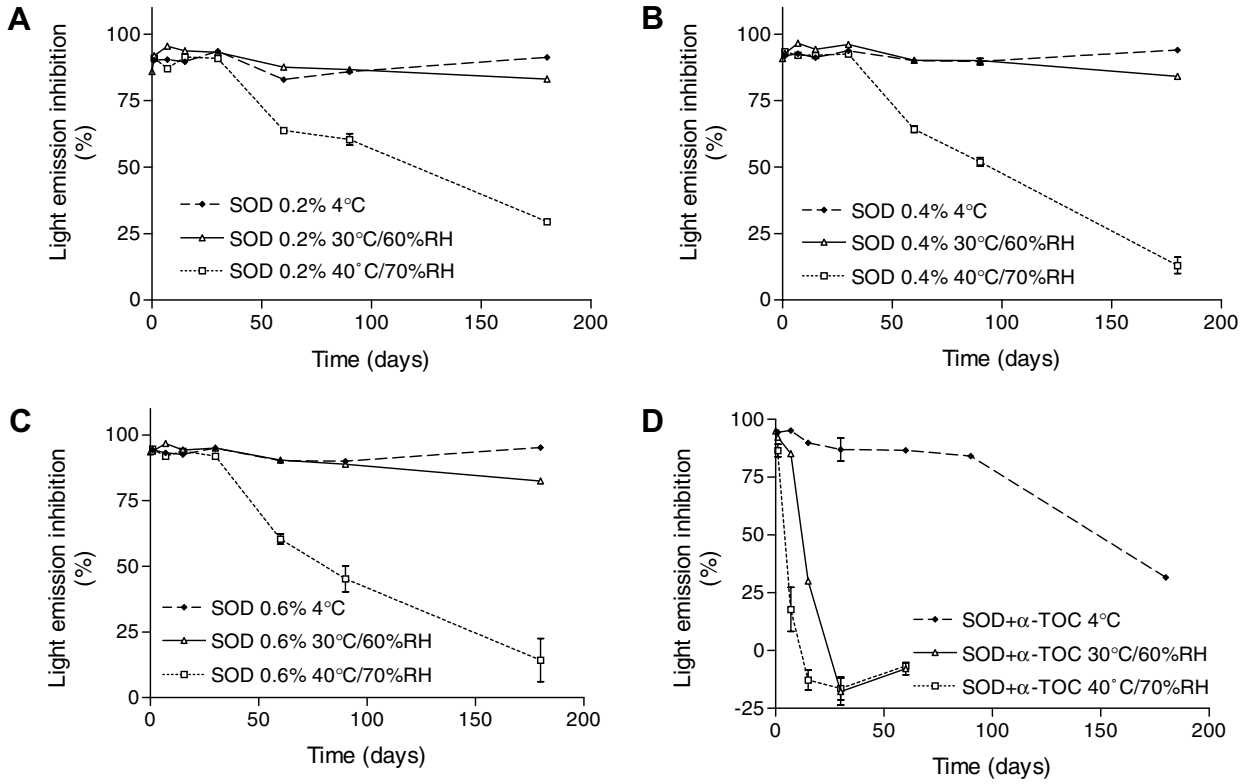

Fig. 4. Percent inhibition of light emission from the xanthine/xanthine oxidase–catalyzed luminescent reactions with luminol found for diluted SOD formulations when stored at 4 °C, 30 °C/60% RH and 40 °C/70% RH for 6 months. Results are means ± SD of three measurements run in parallel. (A) Formulation added with 0.2% SOD, (B) formulation added with 0.4%, (C) formulation added with 0.6% and (D) formulation added with 0.4% SOD and 2% α-TOC.

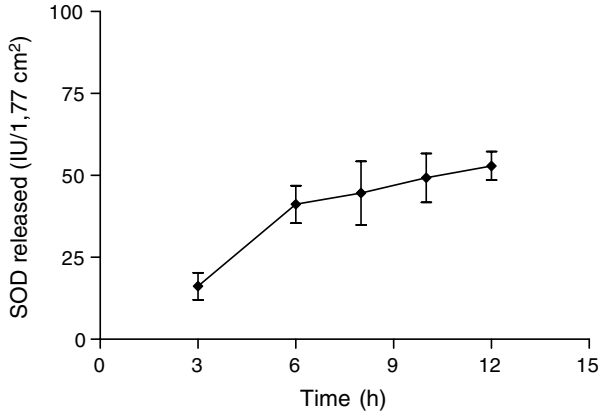

Fig. 5. In vitro release profile of SOD from the formulation containing 0.6% through the cellulose membrane. All the data were measured in duplicate for each diffusion cell and are expressed as means ± SD ($n = 10$).

were effective in inhibiting the erythema of the groups exposed to irradiation corresponding to 2.5 MED. Nevertheless, 72 h after the irradiation, the group treated with SOD 0.2% showed a slight desquamation.

SOD formulations were also efficient in protecting the animals from the irradiation corresponding to 5 MED. Twenty four hours after the UVB exposure, the animals showed no visible skin alterations; however, after 48 h it was possible to observe a slight erythema. The animals from the control and SOD-free formulation groups showed great desquamation and visible thickened skin 72 h after irradiation, animals treated with SOD 0.2% and 0.4% also showed a little desquamation and thickened skin, but it was less serious than control and SOD-free formulation groups. Formulation containing SOD 0.6% was the one that offered the best protection against irradiation corresponding to 5 MED during the visual inspection period.

4. Discussion

Enzymes are one of the potential active ingredients in pharmaceutical and cosmetic products. SOD has a high capacity of removing free radicals that are one of the main causes for skin aging. Topical administration of antioxidants is capable of diminishing oxidative injury, but it is necessary to develop a stable formulation that maintains the enzymatic activity. The prepared formulation was added with SOD in different concentrations in order to verify the influence of the enzyme concentration on physical and antioxidant activity stability, as well as the photoprotective effect.

Proteins can influence physical stability of a formulation because they adsorb to oil–water interfaces. Adsorption then results in the reduction of the interfacial tension and

a formation of a large interface area in emulsion. Upon adsorption, globular proteins unfold to an extension depending on their intrinsic structural stability, and progressively constitute an interfacial film exhibiting viscoelastic properties [14].

The addition of SOD, even in higher concentration, did not change the physical parameters of the formulations. The rheograms obtained showed no instability signals for formulations added with SOD (data not shown), and indicated a shear-thinning behavior with hysteresis loop and non-Newtonian behavior, with flow index less than 1, this reflects their pseudoplastic tendency, a desirable rheological behavior in these preparations. Formulations with a pseudoplastic flow produce a coherent film covering the skin surface, which is important for a better antioxidant protection of the skin surface. Also, the addition of this macromolecule does not affect the formulation viscosity, once it was constant concerning time, temperature and SOD concentration, even when SOD was added in higher concentration.

The opposite occurred when SOD was added in association with α-TOC. The initial viscosity significantly decreased and, after 2 months at 40 °C/70% RH, the reduction of viscosity was about 66% after 3 months concerning the initial value. This may be due to interactions between SOD and α-TOC, which cause disarrangement in the formulation structure. This interaction could be favored by the temperature since no changes in viscosity were observed for formulation stored at 4 °C.

Thixotropy (hysteresis loop) was observed for formulations and the presence of SOD in different concentrations did not affect the thixotropy values. Again, the presence of both SOD and α-TOC caused a reduction in thixotropy value. Thixotropy is desirable in topical formulations because it helps to maintain the suspending components stable, moreover it can influence the active substances releasing to the skin due to the structural disarrangement of the system, where the active substances diffusion is facilitated. The results show that formulations containing SOD, but not SOD and α-TOC, could be considered stable since the rheological parameters were constant during the period of study.

The enzymatic activity stability of these formulations was measured by an indirect method using inhibition of the chemiluminescent reaction produced in the system xanthine–xanthine oxidase-luminol, in which SOD, in competition with luminol for superoxide anions generated by the xanthine oxidase, decreases light emission. Under these conditions, 50% of light emission inhibition was considered as corresponding to 1 U of SOD activity. The measurement of SOD activity at pH 9.4 was chosen since a good sensitive signal is obtained in this pH while it avoids the problems of enzyme inactivation and other inconveniences due to a higher pH [15].

This method was validated by the observation of 0.6% within-assay precision and 1.3% between-day repeatability when 0.016 or 0.04 μL of SOD was added in the reaction mixture. The different dilutions of SOD-free formulation caused no effect on the observed chemiluminescence intensity (data not shown), also SOD formulations inhibited the light emission in the same way of the enzyme solution demonstrating, in the conditions employed, that the formulation components do not interfere with the antioxidant activity measurements by chemiluminescence [16]. These results together suggest that chemiluminescence was a suitable method for evaluating SOD activity stability after addition in a emulsion.

With this analytical method, we observed that SOD alone and SOD formulations maintained the activity when stored at 4 °C during all the period of study. After 6 months at 30 °C/60% RH, the enzyme alone showed a reduction in its activity of 14.3%, while SOD formulations showed less activity reduction. The activity loss was 11.8% for formulation added with SOD 0.6%, while formulations added with 0.2% or 0.4% showed no reduction in the enzyme activity, showing that SOD is a stable enzyme and it maintains its activity after addition in emulsions, even when stored at 30 °C. The emulsion also seems to protect the enzyme activity in this temperature. Nevertheless, the opposite occurred when the samples were stored at 40 °C/70% RH. Although SOD alone was stable for 90 days at 40°/75% RH, SOD formulations maintained their activity only for 30 days. After 6 months, the activity loss was 66% for formulation with SOD 0.2% and about 85% for formulations with SOD 0.4% and 0.6%.

Storage time and temperature can affect enzyme stability by changes in the secondary, tertiary, and quaternary structure leading to protein unfolding and/or aggregation [17]. Several investigations of the secondary structure of protein when exposed to the oil–water interface have been published lately. The prevailing tendency is that the secondary structure considerably changes [18].

The enzyme may interact with formulation components, and in higher temperature, more interactions occur. Proteins spontaneously adsorb to oil–water interface, essentially due to the hydrophobic properties of these interfaces [14]. Surfactant–protein interactions are complex, especially in emulsion, because adsorption leads to conformational changes and to a reduction in both protein flexibility and exposed surface area. Partial unfolding of adsorbed molecules results in higher exposure of hydrophobic regions of protein molecules. An adsorbed protein tends to orientate itself so that the hydrophobic segments protrude into the oil phase, whilst the hydrophilic segments protrude into the aqueous phase, because it minimizes the unfavorable contact between polar and non-polar regions [19]. When SOD was added in topical emulsions only 18% of the amount added could be recovered in the aqueous phase after extraction process, and therefore 82% of the protein could be interacting with the formulation components present in the fatty phase [20].

The surfactants that are normally used in formulations for their stabilizing properties are non-ionic. Non-ionic surfactants have also been shown to exhibit hydrophobic

protein–surfactant interactions [21]. Once there was no activity loss in SOD formulations stored at 4 °C and 30 °C/60% RH, the enzyme-formulation interaction could be due to the enzyme conformational change caused by the higher temperature, which may expose the hydrophobic groups of the molecule. With formulation aging, there is a greater exposure of these groups leading to conformational changes that may cause the loss of the enzymatic activity [20].

Formulation containing both SOD and α-TOC showed a great reduction of enzymatic activity after 7 days of storage at 40 °C/70% RH. In addition to the activity loss, these formulations showed a prooxidant effect, since the light emission increased rather than inhibited. This result confirms that SOD may interact with α-TOC leading to conformational change resulting in reduction of viscosity formulation and enzymatic activity. α-TOC contains several groups or regions (the aromatic ring, carbon-hydrogen skeleton) which provide a multiplicity of sites of a potentially hydrophobic nature to participate in such interaction. In addition, the hydrophobic interaction between SOD and α-TOC can be enhanced by hydrogen bonding of the phenolic group to points on the protein, in particular, the carbonyl group.

Lo et al. [22] reported that there was no interaction between SOD and a lipid such as dipalmitoylphosphatidylcholine, but an interaction did occur when the lipid was dipalmitoylphosphatidylglycerol, and this interaction resulted in considerable reduction in the thermal stability of the enzyme. From several studies, it was concluded that a combination of antioxidants was much more effective than the sum of the separate compounds. Nevertheless, the combination of SOD and α-TOC in the studied formulation proved not to be suitable since the interaction between the antioxidants led to both physical and enzymatic activity instability.

The enzyme-formulation interaction may explain the low percentage of SOD released found in the in vitro release studies. The overall effectiveness of a topical formulation depends on the releasing characteristics of the vehicle. Vehicle–protein interactions determine the protein releasing profile [23]. Emulsions are flexible systems, to which releasing properties can be adjusted [18]. The release of SOD could be improved by adjusting the self-emulsifying wax or polymer concentration.

Although SOD showed a low release from the formulation, it was effective in inhibiting the erythema. UVB erythema is regarded as one of the most suitable models for studying in vivo skin damage after acute UV exposure and provides a useful tool to evaluate radical scavenger activity of topically applied compounds [24–26].

With the visual inspection, it was possible to notice that SOD-formulations were effective in inhibiting the erythema caused by the exposure to an irradiation corresponding to 2.5 MED. When the animals were exposed to 5 MED the most efficient formulation was the one containing SOD 0.6%. These results suggest that SOD can protect skin against UVB-induced erythema in a concentration-dependent manner. Nevertheless, the results here have to be interpreted very carefully and other assays need to be done in order to have a complete understanding of the protective effect of SOD formulations. Since formulations were applied in animals only once before the irradiation, a repeated application should increase the efficacy.

Youssefi et al. [27] reported that a gel containing SOD 0.1% was effective in protecting hairless mice when given as a prolonged pretreatment. Shorter pretreatments were less protective, and posttreatment was practically non-protective. SOD showed ability in inhibiting UVB skin erythema in human volunteers exposed to 2 MED from both formulations gel and emulsion added with 0.1% [28]. Pathak et al. [29] studying the effects of UVB and UVA radiation on skin SOD content found that SOD activity was significantly affected by skin exposure to UV irradiation, and they suggested that topical application of antioxidants or enzymes, including SOD, could prevent its inactivation. Duval et al. [30] reported that topical application on human volunteers of gels containing SOD was effective in inhibiting methyl nicotinate (MN) skin erythema and they concluded that since MN-induced erythema is based on a mechanism similar to the one involved in UVB erythema, topical free scavengers, like SOD, can be used to reduce skin damage induced by UVB irradiation.

Topical administration of antioxidants provides an efficient way to enrich the endogenous cutaneous protection system, and SOD formulations could be used for improving photoprotection of skin.

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