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ORIGINAL ARTICLE

Improving the topical delivery of zinc phthalocyanine using oleic acid as a penetration enhancer: *in vitro* permeation and retention

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Abstract

Topical photodynamic therapy with zinc phthalocyanine (ZnPc), second-generation photosensitizer, can be an alternative method for the treatment of skin cancer. However, ZnPc has poor penetration in the skin. This study was aimed at investigating whether the presence of oleic acid (chemical enhancer) in propylene glycol can improve the topical delivery of ZnPc. The topical (to the skin) and transdermal (across the skin) delivery of ZnPc were evaluated *in vitro* using suine ear skin mounted in Franz diffusion cell. Photosensitizer was quantified by fluorescence emission, which is a sensitive and selective method. At 5 and 10%, oleic acid increased the topical and transdermal delivery significantly. When the concentration of oleic acid was further increased (20–60% w/w), the topical delivery of ZnPc was still elevated, but its transdermal delivery was substantially reduced. It was concluded that oleic acid (in propylene glycol formulations) can promote the topical delivery of ZnPc, with reduced transdermal delivery. This approach can be effective for the treatment of skin cancer by topical photodynamic therapy.

Keywords: Photosensitizer, photodynamic therapy, cutaneous delivery, skin cancer

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Introduction

Photodynamic therapy (PDT) has been established as an alternative treatment for skin cancer (Roberts & Cairnduff, 1995; Ibbotson, 2010) with minimal side and systemic adverse effects (Hopper, 2000; MacCormack, 2006). This therapy is based on local administration of a photosensitizer, which involves drug accumulation in the target tissue and then exposure to visible light of 600–800 nm (therapeutic region). After absorption of light, the photosensitizer moves from a ground singlet to a triplet-excited state. This excited state can produce singlet oxygen ($^1O_2$) via an energy transfer reaction between triplet-excited state and molecular oxygen ($^3O_2$). Frequently, it has been proposed that singlet oxygen ($^1O_2$) is the main element involved in the PDT effect, inducing an irreversible destruction of target tissues (Davies, 2003; Stief, 2003, Robertson et al., 2009). PDT has been actively exploited in many clinical applications, such as cutaneous infections (Dai et al., 2009), age-related macular degeneration (Schmidt-Erfurth & Hasan, 2000), and cancer treatment (Sharman et al., 2004; Castano et al., 2005; Boiy et al., 2007; Yslas et al., 2009; Ibbotson, 2010).

Systemic intravenous administration of photosensitizers, such as hematoporphyrins (Photofrin®), cause a generalized photosensitivity in skin that remains for up to 8 weeks after treatment since it is slowly removed (Szeimies et al., 1996, Steluti et al. 2005).

Serious side effects have encouraged dermatologic research on topical application of photosensitizers in the last 10 years. Some promising results were obtained

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by using topical application of 5-aminolaevulinic acid (ALA), which was metabolically converted *in situ* into the photodynamically active protoporphyrin IX (De Rosa et al., 2000; De Rosa et al., 2003; Lopes et al., 2004; Steluti et al., 2005; Pierre et al., 2006). ALA topical application has been successfully used for the treatment of cutaneous diseases, such as the squamous cell carcinoma, the basal cell carcinoma, and the Bowen's disease (Kassab et al., 2000; De Rosa et al., 2003, Bagnato et al., 2005; Ibbotson, 2006; Ibbotson, 2010). Topical PDT has been used for other photosensitizers, such as zinc-tetrakis-(4-oxy-*N*-methylpiperidinyl) phthalocyanine (Kassab et al., 2000), Hypericin (natural photosensitizer) (Boiy et al., 2007), and chlorin (Primo et al., 2007). Kassab et al. (2000) and Boiy et al. (2007) used chemical permeation enhancers to improve cutaneous retention of the photosensitizers. Moreover, Primo et al. (2007) used nanoemulsion to promote topical delivery of Foscan® (chlorin).

Zinc phthalocyanine (ZnPc) has been clinically used as a promising photosensitizer in cancer phototherapy in Russia (Brown et al., 2004), and chosen as photosensitizer due to its intense absorbance in the red spectral region of clinical application (therapeutic region). It has shown high photosensitizing activity besides the low cost. In this context, our group started researches on the potential use of ZnPc for PDT.

Topical PDT can be an option for the treatment of skin cancer because it reduces the systemic toxicity of the photosensitizers. Another advantage of this therapy is disease removal without surgery. Topical delivery of ZnPc is of great interest for the treatment of skin cancer, however, photosensitizer penetration in the skin is not easily achieved due to its large molecular weight (MW = 577.91) and cyclic structure. Thus, formulations for topical administration of photosensitizers need to be developed and studied to determine the feasibility of topical PDT with ZnPc. Techniques that reduce the diffusional resistance of the stratum corneum (SC) allow to optimize topical delivery of drugs (Barry, 2001). Many studies have used chemical techniques (chemical enhancer) that disrupt the cutaneous barrier, represented by the corneum stratum. Theoretically, a suitable vehicle containing a chemical penetration enhancer, which increases the topical delivery without transdermal delivery, is interesting for topical PDT.

Oleic acid (OA), a lipid used as a penetration enhancer, is biodegradable, nontoxic, biocompatible, and safe; it has been the object of interest in dermatological researches. Furthermore, OA causes temporary and reversible disorder of the corneum stratum, increases fluidization of the intercellular lipid medium, and reduces skin barrier function (Barry, 1987; Naik et al., 1995; Larrucea et al., 2001; Gwak & Chun, 2002). In addition, it has been used as a penetration enhancer for nifedipine (Squillante et al., 1998), piroxicam (Santoyo & Ygartua, 2000), tenoxicam (Larrucea et al., 2001), hypericin (Boiy et al., 2007), meloxicam (Jantharaprapap & Stagni, 2007), and indapamide (Ren et al., 2008).

In this study, the influence of the OA as cutaneous penetration enhancer to ZnPc was investigated. Time and concentration of penetration enhancer were evaluated to determine the optimum condition for topical delivery of the photosensitizer.

Methods

Chemicals

OA was purchased from Tedia (São Paulo, Brazil). Zinc (II) phthalocyanine (MW = 577.91) was purchased from Aldrich (Milwaukee, USA). Sodium dodecyl sulphate (SDS), ethanol, propylene glycol (PG), NaH_2PO_4, and Na_2HPO_4 all of analytical grade were purchased from Tedia (Brazil).

Formulations

Different quantities of OA were mixed with PG, having solutions at different concentrations of OA (0, 5, 10, 20, or 60% w/w). ZnPc was added to the previous solutions to achieve a final concentration of 1 mg/ml.

Characterization

The photosensitizer in the formulation was analyzed using a Leica microscope DML30 at bright field and polarized light microscopy at room temperature (28°C) to determine the system structure and possible crystallization of the drug. The formulation viscosity was measured at 37°C using a rheometer (SR-200, Rheometric).

The formulations and control were placed in glass vials and stocked at room temperature (28°C) ($n = 3$ determinations). Samples were collected at 1, 2, 5, 10, and 30 days and diluted with phosphate-buffered saline (PBS) (0.1 M, pH 7.4, containing 1% SDS) before analysis. Photosensitizer concentration was determined by fluorescence emission method as described below.

Analytical methodology for ZnPc

ZnPc was dissolved in ethanol at 100 μg/ml. The solution was diluted with PBS (0.1 M, pH 7.4, containing 1% SDS) for the analytical curve preparation. The curve was done in triplicates ($n = 3$ determinations). The standard solutions were excited at 608 nm and the fluorescence emission spectra recorded between 650 and 800 nm using a Jasco FP 750 fluorimeter. The fluorescence emission intensity of ZnPc in PBS was correlated with the concentration in a range of 10–200 ng/ml. The determination coefficient (R^2) exceeded 0.99997, with excellent linearity. Intra- and interdays precision and accuracy of the method showed a variation coefficient (CV%) and a relative error (E%) not greater than 2.3 and 3.1%, respectively. Selectivity was measured by a fluorometric analysis of homogenized skin in PBS containing 1% SDS. Samples were excited at 610 nm and the fluorescence emission spectra recorded between 650 and 800 nm. No band of fluorescence emission was observed, indicating that the fluorometric method was suited to the photosensitizer determination in aqueous medium. Analytical method was used for the

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detection of photosensitizer in the *in vitro* permeation studies. Therefore, the method was sensitive, reproducible, and selective.

In vitro permeation studies

The experiments were performed using a fragment (4 × 4 cm) of the suine ear skin. The ear skin was obtained less than 1 h after killing of the animals and used at once or stored in freezer for 7 days before use. The *in vitro* permeation studies were assessed using a Franz diffusion cell (diffusion area of 1.77 cm^2; Hanson Research, Chatsworth, CA, USA), with the SC facing the donor compartment (where the formulation was applied) and the dermis facing the receptor compartment, which was filled with 7.0 ml of receptor medium (PBS, 0.1 M, pH 7.4, containing 1% SDS). The solubility of ZnPc in the receptor medium was 100 μg/ml. The system was kept to 37 ± 0.5°C for a bath of circulating water which surrounded the cell, and a magnetic bar continuously stirred the acceptor medium at 900 rpm to avoid diffusion layer effects. This temperature was selected based on the facts that the receptor phase is in contact with the deepest skin layers and that the deep body temperature of humans is maintained between 36.2 and 37.2°C during the circadian cycle (Duffy et al., 1998; Lopes et al., 2005). To achieve higher reproducibility, the skin samples were prehydrated with acceptor medium for 1 h before the formulation application. The donor compartment was filled with 1 ml of formulation (see item 2.2). At the end of the experiment (2, 4, 6, 8, 10, and 12 h), the amount of photosensitizer, which permeated the skin fragment, was analyzed by the fluorometric method as described above (see Analytical methodology for ZnPc section).

At the end of the *in vitro* permeation studies, skin fragment was removed and carefully washed with 10 ml of PBS (0.1 M, pH 7.4) to recover the formulation. Excess water was absorbed with filter paper. To separate the SC from the remaining epidermis (E) plus dermis (D), skin sections were subjected to tape stripping. Eleven adhesive tapes (Scotch Book Tape no. 845, 3 M) were applied with uniform pressure on the skin surface and removed for 11 times; the first tape was discarded. The adhesive tapes were placed in glass tubes containing 5 ml of PBS (0.1 M, pH 7.4, containing 1% SDS), sonicated for 10 min, stored overnight, and stirred for 1 min before filtration. The samples were filtered through a filter (0.45 μm) to remove the insoluble particles. Photosensitizer extracted from SC was analyzed by the fluorometric method as described in Analytical methodology for ZnPc section.

The remained skin, that is, the rest of the epidermis plus dermis, was cut into small pieces and added in tubes containing 5 ml of PBS (0.1 M, pH 7.4, containing 1% SDS). Small skin fragments in suspension were homogenized using a tissue homogenizer (Ultraturrax T25, Ika, Germany) at 12,000 rpm, sonicated for 10 min in ultrasonicator (UP100H, 50W, 30 kHz, Hielscher, Germany), stored overnight, and stirred for 1 min before filtration. The samples were filtered through a filter (0.45 μm) to remove the insoluble particles and analyzed by the fluorometric method as described above. The photosensitizer was quantified in SC, the remained E plus D, and acceptor solution for a complete investigation. The concentrations of photosensitizer in SC and E plus D were indexes of topical delivery, whereas the concentration in the receptor phase was an index of transdermal delivery.

Removal evaluation of the SC

Skin fragments were manipulated as *in vitro* permeation studies. Skin samples were prehydrated with acceptor medium (PBS 0.1 M at pH 7.4, containing 1% SDS) for 1 h before the formulation application. The donor compartment was then filled with 1 ml of formulation containing ZnPc in OA-PG 10:90 (w/w, %). The study was performed during 4 h. Afterward, skin fragments were carefully removed and washed with 10 ml of PBS 0.1 M at pH 7.4. Excess of water was dried using filter paper and SC of diffusion surface was removed by application with uniform pressure of 11 adhesive tapes (Scotch Book Tape no. 845, 3 M).

For light microscopic analysis, skin fragments were fixed in 10% buffered formaldehyde for 48 h and/or in Bouin's liquid during 18 h and processed according to the standard histological techniques for paraffin embedding. Five-micrometer serial slices were stained with hematoxylin-eosin (HE) (Kiernan, 1990). Skin slices were analyzed using a light microscope Leica DML30 to characterize the skin morphology and evaluate the SC removal.

Confocal laser scanning microscopy (CLSM)

Skin fragments were manipulated as *in vitro* permeation studies. They were prehydrated with acceptor medium for 1 h before the formulation application. The donor compartment was then filled with 1 ml of formulation containing ZnPc in OA-PG 10:90 (%, w/w). The *in vitro* permeation study was performed during 4 h. Afterward, skin fragments were carefully removed and washed with 10 ml of PBS (0.1 M at pH 7.4). Excess of water was dried using filter paper. The skin pieces were embedded in a matrix, frozen at –17°C and sliced in sections of 7 μm thickness with the help of cryomicrotome. Helium-Neon laser at 633 nm was used for excitation, and fluorescence emission was detected at longer wavelengths using filter that captures emissions of 650 until 750 nm.

Data analysis

The results are reported as mean ± SD. Data were statistically analyzed using nonparametric tests. The Mann–Whitney test was used to compare two experimental groups. The Kruskal–Wallis test (followed by Dunn posthoc test) was used to compare more than two experimental groups. The level of significance was set at $P < 0.05$.

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Results and discussion

The polarized light microscopy showed that all formulations were isotropic liquids at room temperature (28°C) (data not shown). No photosensitizer crystal was observed in the formulations even when using the higher magnification (100×) (data not shown). Table 1 shows the formulation viscosity. The addition of OA in the formulation slightly increased the system viscosity.

The addition of OA was essential to maintain the stability of the formulation. The photosensitizer concentration in the control was maintained at 0.996 ± 0.011 mg/ml by 1 day. After this period, the control showed instability characterized by precipitation of drug. The photosensitizer concentration in the formulations containing OA at 10 and 20% (w/w) is showed in Table 2. The formulations were stable during the stability study and no precipitate was observed during the test. Photosensitizer concentration was reduced to 0.977 ± 0.010 and 0.981 ± 0.006 mg/ml on the 13th day of study for the formulations containing OA at 10 and 20% (w/w), respectively. The reduction in the concentration was very small and we can consider that the formulations composed of OA and PG are suitable as vehicles for photosensitizer.

The penetration of a drug through skin layers can be predicted by its diffusivity and solubility properties (Barry, 2001). Lipophilic drugs such as ZnPc generally present high partition in the SC. However, their penetration in viable epidermis (E) and dermis (D) is low, presumably due to their accumulation in the SC. Different strategies can be used to increase the topical or transdermal delivery of lipophilic drugs (Barry, 2001).

In this study, we verified whether the OA (penetration enhancer) (5–60%, w/w) promotes topical and/or transdermal delivery of ZnPc. Initially, we studied the effect of OA at 10% (w/w) in PG on the topical delivery of ZnPc as a function of time. Compared to the control, the preparation containing OA enhanced the topical delivery of ZnPc within few hours: the penetration of ZnPc in SC and E plus D was significantly ($P < 0.05$) improved at 4 h after application (Figure 1). The same preparation also enhanced the transdermal delivery of

Table 1. Formulations viscosity at 32°C. All samples containing 1 mg/ml of photosensitizer.

Formulations	Viscosity (mPa.s)
PG	19.1 ± 0.2
OA	22.7 ± 0.3
5% OA-PG	20.2 ± 0.1
10% OA-PG	20.5 ± 0.4
20% OA-PG	20.3 ± 0.3
60% OA-PG	21.2 ± 0.2

Data shown are mean ± SD $n = 6$ determinations.
OA, oleic acid; PG, propylene glycol.

Table 2. Photosensitizer concentration in the formulations during the stability study.

Sample	Time (days)	ZnPc concentration (mg/ml)[a]
10% OA in PG	0	1.002 ± 0.011
	1	1.009 ± 0.007
	2	1.005 ± 0.010
	5	0.997 ± 0.008
	10	0.991 ± 0.004
	30	0.977 ± 0.010
20% OA in PG	0	1.005 ± 0.007
	1	1.002 ± 0.005
	2	0.997 ± 0.011
	5	0.995 ± 0.004
	10	0.991 ± 0.007
	30	0.981 ± 0.006

OA, oleic acid; PG, propylene glycol.
[a]Mean ± SD $n = 3$ determinations.

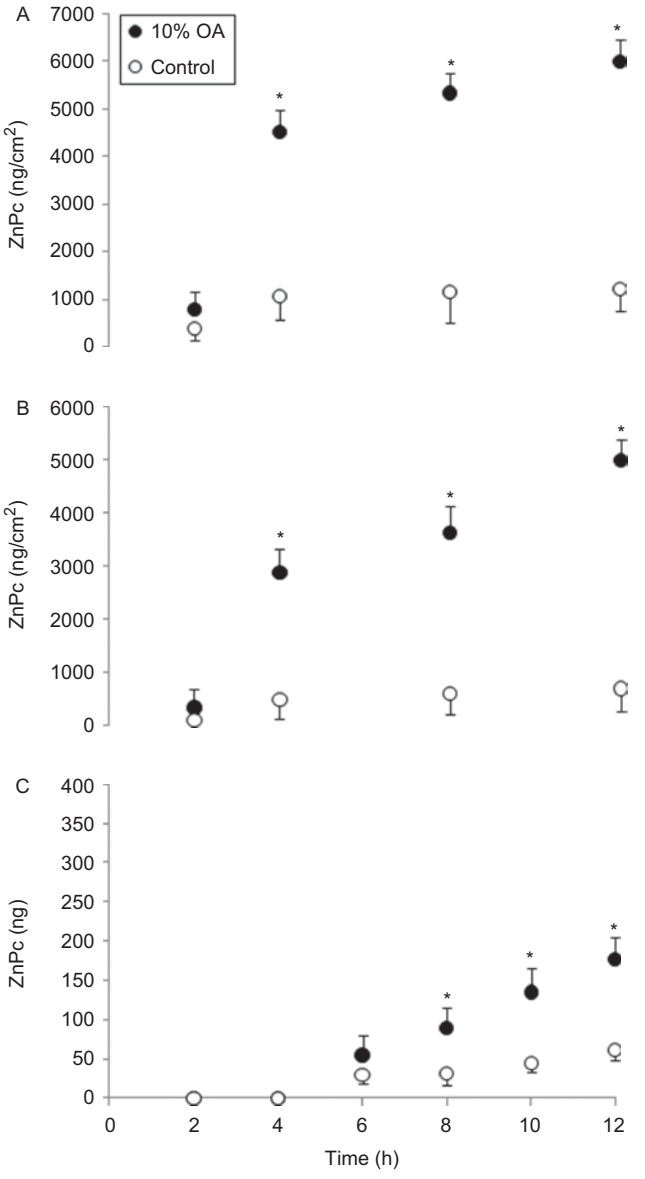

Figure 1. Time-dependent effect of the formulation containing OA at 10% (w/w) in propylene glycol (PG) or control (only PG) on the cutaneous penetration and transdermal delivery of ZnPc. The photosensitizer concentration in the SC is shown in A; the concentration in the epidermis plus dermis in B; and the concentration in the receptor phase in C. $n = 6$ determinations.

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ZnPc: the presence of the photosensitizer in the receptor phase was detected in 6 h and significantly ($P < 0.05$) enhanced 8 h postapplication (Figure 1). The relatively long lag time for the permeation of ZnPc across the skin is probably due to its high molecular weight. Because both the topical and the transdermal delivery of ZnPc were enhanced 8 h postapplication, this time point was chosen to evaluate the influence of OA concentration on these parameters.

Figure 2 shows the influence of OA concentration on the topical delivery of ZnPc with 8 h postapplication. At 5 and 10% (w/w), OA significantly ($P < 0.05$) increased the transdermal and topical delivery and of ZnPc compared to control formulation. When the concentration of OA was further increased to 20 and 60% (w/w), an interesting phenomenon was observed: significant increases in the concentrations of ZnPc in SC ($P < 0.05$ for 20 and 60%) and E plus D ($P < 0.05$ for 20 and 60%) were seen, whereas the concentration of ZnPc in the receptor phase did not differ from that obtained with the control formulation ($P > 0.05$ for 20 and 60%).

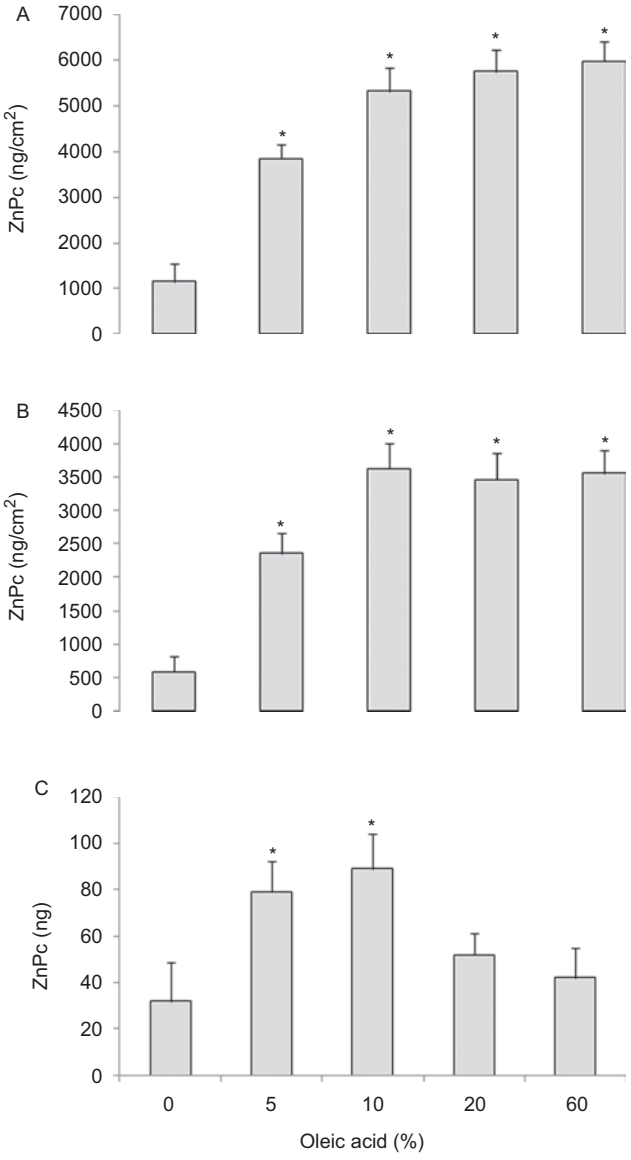

Figure 2. Concentration-dependent effect of oleic acid on the cutaneous penetration and transdermal delivery of ZnPc with 8 h postapplication. The photosensitizer concentration in the SC is shown in A; the concentration in the epidermis plus dermis in B; and the concentration in the receptor phase in C. $n = 6$ determinations.

Formulations containing OA have been shown to increase the topical and transdermal delivery of timolol maleate (Soni et al., 1992), piroxicam (Hsu et al., 1994), dideoxynucleoside-type anti-HIV drugs (Kim & Chien, 1996), ketoprofen (Singh et al., 1996), tenoxicam (Larrucea et al., 2001; Gwak & Chun, 2002), ondansetron hydrochloride (Gwak et al., 2004), meloxicam (Jantharaprapap & Stagni, 2007), methylxanthines (Thakur et al., 2007), indapamide (Ren et al., 2008), alendronate (Choi et al., 2008), curcumin (Patel et al., 2009), and ubidecarenone (Jung et al., 2009). It has been reported that the enhancing effect of OA is dependent on its concentration in the vehicle. Permeation of drugs increases with the amount of enhancer until a maximum, after which the penetration decreases (Squillante et al., 1998; Gwak & Chun, 2002; Jantharaprapap & Stagni, 2007). The present observation that OA at concentration of 20% (w/w) or higher markedly increases topical delivery without promoting transdermal delivery is interesting for topical PDT. Formulations containing high concentrations of OA may thus be a simple, relatively inexpensive method to obtain an optimal topical delivery of ZnPc, associated with minimal systemic side effects.

The fact that high concentrations of OA do not increase the permeation of photosensitizer while low concentrations are effective is intriguing. The answer to this question may lie on the fact that the activity of OA as a penetration enhancer depends not only on its actions on the skin, such as extraction of ceramides and enhancement of skin fluidity (Ogiso et al., 1995), but also on its physicochemical interaction with the drug of interest (Lopes et al., 2005). Due to their lipophilic nature, OA and ZnPc have a high affinity to each other. Interactions between OA and photosensitizer may result in photosensitizer retention in the skin where the fatty acid is better partitioned.

Figure 3 shows the images of the CLSM experiment. The red fluorescence emission on the skin fragment corroborates the *in vitro* permeation studies showing the topical delivery of the photosensitizer (Figure 3A). The CLSM image presents some distinctions between the skin layers as SC and E with high fluorescence emission and D low emission. Red fluorescence emission was not observed in image without excitation (Figure 3B). We can see the SC on top of the image, followed by E (circular cells), and D with its collagen fibers.

As described in other mammals, the suine skin has an epidermis and a typical dermis (Figure 4). The epidermis is subdivided into the germ layer, the spinosum layer, the granulosum layer, and the corneum layer (or horny layer) (Figure 4A and 4B). The dermis is typically

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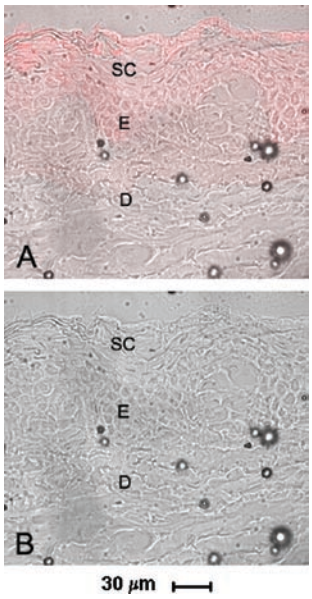

Figure 3. CLSM images of a suine skin fragment treated with ZnPc in oleic acid-propylene glycol (10:90, w/w): (A) samples irradiated with laser and (B) without excitation. Stratum corneum (SC), epidermis (E), and dermis (D). Fluorescence emission indicate the sites of topical delivery of photosensitizer in the skin. Magnification: 400×.

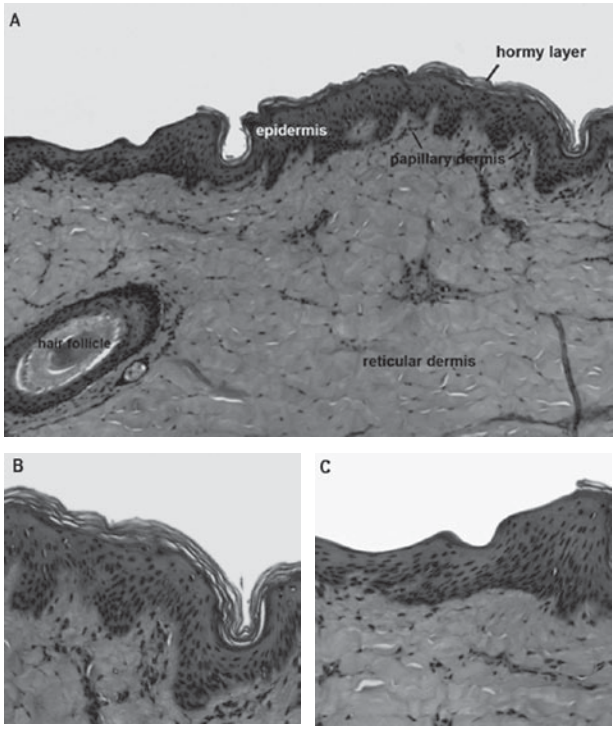

Figure 4. Light micrograph of the suine skin after tape stripping: (A) the entire skin (the epidermis and the dermis, including the horny layer) can be observed on the right side of the photograph and the corneum stratum was fully removed by the adhesive tapes on the left side (Magnification: 200×; Staining: HE—hematoxylin and eosin); (B) and (C) are magnifications (400×; Staining: HE) showing the skin section with or without the horny layer, respectively (HE staining).

subdivided into a papillary dermis and a reticular dermis. The papillary dermis is formed by loose connective tissue, which differs from the reticular dermis that is made up of dense irregular connective tissue. The reticular layer of the dermis contains appendages, mainly hair follicles. The suine skin submitted to tape stripping showed that the corneum stratum was totally removed (Figure 4A, panoramic, and C, magnified) indicating that this method maintained the integrity of other skin layers.

Conclusions

We found that OA in PG formulations affect both the topical and the transdermal delivery of ZnPc of a time- and concentration-dependent manner. At 5 and 10% (w/w), OA enhances both the topical and the transdermal delivery. However, higher concentrations (20% w/w or more) increase the topical delivery, but have no effect on the transdermal delivery. Formulations containing high concentration of OA may thus be a simple, relatively inexpensive method to achieve a topical delivery of ZnPc, associated with minimal systemic side effects. This initial *in vitro* study allowed the selection of a suitable formulation (20% OA in PG) and time (8 h) to optimize the topical delivery of the photosensitizer. Future tests of *in vivo* permeation and photobiological activity using hairless mice can demonstrate the effectiveness of the formulation. This approach can be effective for the treatment of skin cancer by topical PDT.

Declaration of interest

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