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Research Paper

Oleic Acid as Optimizer of the Skin Delivery of 5-Aminolevulinic Acid in Photodynamic Therapy

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Purpose. In photodynamic therapy (PDT), topically applied aminolevulinic acid (5-ALA) is converted to protoporphyrin IX (PpIX), which upon light excitation induces tumor destruction. To optimize 5-ALA-PDT via improving the highly hydrophilic 5-ALA limited penetration into the skin, we propose the use of the known skin penetration enhancer, oleic acid (OA).

Methods. *In vitro* skin penetration and retention of 5-ALA (1% w/w) were measured in the presence or absence of OA (2.5, 5.0, and 10.0% w/w) in propylene glycol (PG) using porcine ear skin as the membrane. *In vivo* accumulation of PpIX, 4 h after application, was determined fluorometrically in healthy mice skin by chemical extracton of skin samples. *In vivo* PpIX fluorescence kinetics was also investigated by noninvasive techniques using an optical fiber probe, for 30 min up to 24 h after topical application of 1.0% 5-ALA + 10.0% OA in PG on hairless mice skins.

Results. The flux and *in vitro* retention of 5-ALA in viable epidermis increased in the presence of 10.0% (w/w) OA. The amounts of PpIX, evaluated both by chemical tissue extractions and *in vivo* measurements by an optical fiber probe, increased after applying 5-ALA formulations containing 5.0 or 10.0% OA. Moreover, *in vivo* kinetic studies showed an increase in skin PpIX accumulation when formulations containing 10% OA were used; PpIX accumulation was also maintained longer compared to controls.

Conclusions. Both *in vitro* and *in vivo* results show the OA potential as an optimizer of 5-ALA skin delivery.

KEY WORDS: 5-aminolevulinic acid; *in vitro* skin permeation; oleic acid; photodynamic therapy; protoporphyrin IX.

INTRODUCTION

Photodynamic therapy (PDT) has progressed in the past 25 years to become an important new clinical treatment modality for skin cancer (1). It involves the use of a class of molecules accumulated in certain abnormal tissues (photosensitizers) and activated by light at specific wavelengths. At these conditions, several ensuing photochemical reactions cause cell damage and death in the cancerous tissue (2).

PDT with topical applications of 5-aminolevulinic acid (5-ALA), a protoporphyrin IX (PpIX) precursor, is also a potential treatment for cutaneous nonmalignant tumors (3,4). The transformation of PpIX into heme induces a negative feedback on 5-ALA biosynthesis and, under normal circumstances, concentrations of porphyrins low enough to avoid induced photodynamic tissue damage. Exogenous administration of 5-ALA bypasses this feedback control, leading to intracellular accumulation of porphyrins, usually PpIX, which can be activated by visible light (5,6).

PDT in superficial basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) using 5-ALA has shown promising clinical results. The selective accumulation of PpIX in BCC and SCC could be explained by several factors. One of them relates to the permeability through overlying abnormal stratum corneum, responsible for accumulation of the exogenous ALA in the lesion (7,8). Another explanation may be based on differences in the porphyrin and heme synthesis rates. By adding exogenous ALA, the naturally occurring PpIX transiently accumulates, probably as a result of the limited capacity of ferrochelatase to complete the transformation of PpIX into the heme group. Furthermore, the activity of porphobilinogen deaminase, another enzyme of the heme synthesis pathway (responsible for the formation of uroporphyrinogen from porphobilinogen), is higher in some skin tumors whereas the ferrochelatase level is lower, leading to the accumulation of PpIX (9,10). The combined enzymatic effects, together with the changes in permeability, lead to the accumulation of PpXI with a high selectivity degree after the administration of exogenous photosensitizers.

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However, for deeper lesions or when the skin barrier (SC) is thicker (hyperkeratolytic areas, for example, in nodular tumors), the efficacy of 5-ALA-PDT is dramatically decreased by a limited diffusion of hydrophilic 5-ALA into the stratum corneum (SC), the lipophilic barrier of the skin (11), as well as by poor penetration of red light ($\lambda \sim 635$ nm) (12,13). Consequently, it leads to a limited 5-ALA bioavailability or its unfavorable distribution in the skin, an essential factor for the success of 5-ALA-PDT. In superficial tumors and lesions, a damaged SC can contribute to the 5-ALA increased permeability (14,15). Various strategies have been developed to improve 5-ALA delivery and tissue penetration such as iontophoresis (16,17), or the use of liposomes (18), cubic phase gels (19), 5-ALA ester derivatives (20–22), and permeation enhancers such as DMSO (23,24) among others.

Reports in the literature have shown that penetration enhancers may alter the composition and/or the organization of SC intercellular lipids, thus decreasing the barrier function and allowing an appropriate diffusion of drugs through the skin (25,26).

Oleic acid (OA), a *cis*-unsaturated free fatty acid abundantly found in nature, including human skin, has been generally recognized in various *in vitro* studies as a skin penetration enhancer (27,28). The effects of OA involve disturbances in the inherent SC lipid structure as a separate fluid phase (29–31), resulting in a decrease in lipid glass transition temperatures (32) as well as an increase in the absorbance frequency of infrared stretching vibrations of the SC lipids (33). It has been shown that the effect of OA is more pronounced with hydrophilic than lipophilic compounds, that is, a higher enhancement ratio (ratio between drug flux across the skin in the presence of penetration enhancers and drug flux alone) can be observed with very hydrophilic compounds ($\log P_{\text{octanol/water}} < 0$), decreasing toward a minimum effect with increasing $\log P$ toward zero value and slightly increasing to reach a plateau when $\log P > 0$ (lipophilic compounds) (34).

Because 5-ALA is a highly hydrophilic and polar substance, we suggest the use of the OA adjuvant as a new approach to topical 5-ALA PDT. The *in vitro* 5-ALA porcine ear skin penetration and the accumulation of PpIX in healthy hairless mouse skin *in vivo* were evaluated. Moreover, the 5-ALA-induced accumulation of PpIX in the skin was considered an important tool for understanding the optimization effect of this penetration enhancer.

MATERIALS AND METHODS

Chemicals

5-ALA hydrochloride, PpIX, and OA were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany).

In Vitro Retention and Permeation Studies

Full thickness porcine ear dorsal skins were excised and immediately mounted in a modified Franz diffusion cell (Microette-Hanson Research, Chatsworth, CA, USA), with the dermal side facing downwards into the receptor compartment, which was filled with 7.0 mL of isotonic phosphate buffer (pH 5.0) (35). To achieve higher reproducibility, the skin samples were then allowed to prehydrate with receptor fluid for 2 h before the formulation was applied. The donor compartment was then filled with 120 μL of the formulations containing 5-ALA (1% w/w) and one of the concentrations of OA (2.5, 5.0, or 10.0% w/w) in propylene glycol (PG). The total available diffusion area of the cell was 1.7 cm^2. The system was maintained at 37°C and the receptor medium stirred at 300 rpm. At regular intervals for up to 12 h, 1 mL of the receptor phase was removed for the determination of total drug content by fluorometric assay and replaced by an equal volume of fresh receptor solution. The amount of 5-ALA permeated through the skin was expressed in $\mu g/cm^2$ as a function of time.

At the end of the *in vitro* permeation study, the diffusion cells were dismantled; the skin surfaces were carefully washed with distilled water, gently dried by pressing between two tissue papers, and then submitted to the tape stripping method to remove SC from the remaining skin, i.e., epidermis (E) without SC + dermis (D) (36). The skins were fixed on a flat surface and the diffusion area of SC was removed by 10 pieces of adhesive tape (Scotch Book Tape, n. 845; 3M, St. Paul, MN, USA). According to our previous report (22), to extract 5-ALA from SC in the adhesive tapes and remaining skin, 10 mL methanol was added to the adhesive tapes, left overnight, and shaken for 1 min before filtering. The remaining skin [E without SC + D] was homogenized with 5 mL methanol for 1 min, sonicated in an ultrasound bath (40 kHz, continuous mode) for 30 min at 25°C, and filtered through a 0.45-μm polytetrafluoroethylene (PTFE) membrane (Corning Incorporated, Corning, NY, USA).

5-ALA extracted from these samples was assayed fluorometrically after derivatization, at $\lambda_{\text{excitation/emission}}$ = 378/464 nm. The extraction procedure presented a recovery of 97.9% and the 5-ALA retained in the skin was expressed in $\mu g/cm^2$.

Fluorometric Assay of 5-ALA

In *in vitro* permeation and retention studies, the amounts of 5-ALA were determined after conversion to the fluorescent derivative by reaction with acetylacetone and formaldehyde as previously described (24,37). Briefly, the assay consisted in the spectrofluorometric quantification of the derivative using a Spectrofluorometer Fluorolog-3 (Jobin Yvon-SPEX, Edison, NJ, USA) with excitation at 378 nm and emission at 464 nm (bandwidth 1.5/2.5 nm), by reference to a 5-ALA standard curve. This method was linear in a concentration range of 0.5–100.0 μg/mL, presenting a correlation coefficient of 0.999. The lower detection sensibility of the assay was 0.2 μg/mL, with less than 7.69% intraday variation, and less than 5.34% interday variation.

In Vivo Studies of 5-ALA-Induced PpIX Accumulation

Protocol for Application of Formulations on Animal Skins

For *in vivo* PpIX production studies, the formulations containing 5-ALA (1% w/w) and different concentrations of

OA (0, 2.5, 5.0, and 10.0% w/w) in PG were applied on the dorsal skin of male hairless mice, 6–8 weeks old (strain HRS/J Jackson Laboratories, Bar Harbor, ME, USA) showing injury-free healthy skins. The animals were housed at 24–26°C, exposed to a daily 12:12 h light/dark cycle (lights on at 6:00 A.M.), and had free access to standard mice chow and tap water. To reduce the stress associated with the experimental procedure, mice were handled daily for 1 week before experimentation. Mice (n = 4 for each group) were immobilized on a holder, and after nonocclusive application of the experimental formulations (200 mg) on the dorsal region (in an area of 2 cm^2, delimited by a template) kept in darkness for 4 h. They were euthanized by carbon dioxide vapor and the treated skin areas removed and submitted to PpIX extraction. The protocols were in accordance with the guidelines of the University of Sao Paulo Animal Care and Use Committee (authorization number: 04.1.995.53.9).

The controls were hairless mice skin without any treatment, mice skins treated only and respectively with OA, with PG, with 10.0% OA in PG, and with 5-ALA at 20% w/w in PG, which is the usual concentration applied in human clinical studies (38,39).

PpIX Chemical Extraction

PpIX extraction from skin samples was based on a previously described methodology (40). Briefly, treated skin areas weighing approximately 200 mg (n = 4 for each group) were homogenized in 25 mL methanol/water (9:1), sonicated for 15 min in an ultrasound bath (40 kHz, continuous mode), centrifuged at 450 g for 10 min, and filtered first on filter paper and then through a 0.45-μm PTFE membrane (Corning Incorporated, Corning, NY, USA). The extraction procedure was repeated with the filtered tissue residue material, as described above. The fluorescence of the two supernatants was measured spectrofluorometrically at $\lambda_{excitation}$ = 400 nm and $\lambda_{emission}$ = 632 nm (bandwidth excitation/emission = 5/5). PpIX concentrations were estimated from a standard curve prepared with solutions containing PpIX in 9:1 methanol/water (concentration range, 2.3–30 ng/mL) (r^2 = 0.999) The PpIX assay presented a variability of less than 2.3% for intraday measurements and less than 3.1% for interday measurements. The error was less than 2.9% and the minimum detectable amount was 1 ng/mL. The PpIX concentrations in both filtrates were summed up and expressed as ng PpIX/g skin weight. The extraction procedure showed a recovery of 99.8%. Intrinsic fluorescence present in untreated skins (controls) from the same animals was considered as background and subtracted from experimental readings. All procedures were performed under subdued light.

In Vivo PpIX Fluorescence Measurements on the Skin

A Spectrofluorometer Fluorolog-3 (Jobin Yvon-SPEX) equipped with an optical fiber probe (1 cm diameter) was used for measuring PpIX fluorescence directly on the skin. Four hours after the formulation was applied, the optical fiber probe was placed on the skin surface and the fluorescence intensity measured at $\lambda_{excitation}$ = 400 nm and $\lambda_{emission}$ = 637.5 nm (slit excitation/emission = 5/5), which is similar to the maximum of the PpIX emission spectrum in human skin affected by psoriasis (41). The fluorescence of skin controls were also measured in hairless mouse skins without any treatment and skins treated only with 10% OA or PG. All procedures were performed under subdued light.

In Vivo PpIX Fluorescence Kinetics in the Skin

For *in vivo* PpIX accumulation kinetics, formulations tested contained 5-ALA (1% w/w) and one of the two different concentrations of OA in PG, i.e., 5 and 10% w/w. They were applied, as described in "Protocol for Application of Formulations on Animal Skins," on the dorsal skin of hairless mice (n = 7 for each group). A control formulation (5-ALA 1.0% w/w in PG, only) was also assayed.

The fluorescence of PpIX induced by topical application of 5-ALA was assayed noninvasively, as described in "*In Vivo* PpIX Fluorescence Measurements on the Skin," and monitored from 30 min up to 24 h after treatments. The fluorescence background (autofluorescence) of the skin was recorded for each animal before the application, and the values subtracted from each measurement.

Statistical Analysis

Results are reported as means ± SEM. Data were statistically analyzed by Kruskal–Wallis test or one-way ANOVA (Tukey's multiple comparison test). The level of significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Figure 1 shows *in vitro* 5-ALA skin permeation results for up to 12 h, by using formulations containing 1% w/w 5-ALA and OA at different concentrations (2.5, 5.0, and 10.0% w/w) in PG. Permeation profiles with different slopes can be observed, whereas OA concentrations increase from 2.5 to 10.0%. After 12 h, the amount of 5-ALA permeated (μg/cm^2)

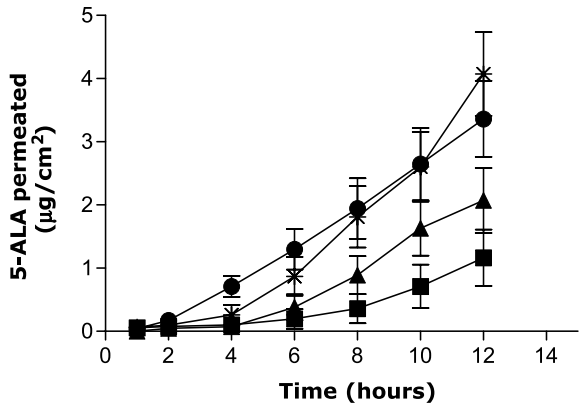

Fig. 1. 5-ALA *in vitro* permeation through full-thickness porcine ear skin from formulations containing 5-ALA (1% w/w) and OA at 0% (■), 2.5% (▲), 5.0% (●), and 10.0% (∗) w/w in propylene glycol (PG). Results presented are averages ± SEM of six experiments. Statistical analysis: one-way ANOVA (multiple comparisons Tukey's test). Values considered significant for formulations containing 5.0% OA (**$p < 0.01$) and 10.0% OA (***$p < 0.001$) compared to the control formulation.

through full thickness porcine skin was significantly higher for formulations containing 5.0% ($p < 0.01$**) or 10.0% ($p < 0.001$***) OA, compared to the control formulations.

However, there was no significant difference between formulations containing 5.0 and 10.0% OA ($p > 0.05$). 5-ALA flux values (J) were calculated from the slopes in the linear portion (steady state) of *in vitro* permeation curves. Table I shows that the fluxes J increased as the OA concentration increased, but the difference was significant ($p < 0.001$) only for the formulation containing 10.0% OA (w/w) in relation to the control. In these conditions, the 5-ALA flux through the skin was approximately 2.4 times higher. The fluxes J for formulations containing 2.5 or 5.0% OA showed statistically significant differences when compared to the formulation with 10.0% OA. Table I also reveals that there is a linear correlation (r) relating the amount of permeated 5-ALA ($\mu g/cm^2$) to time, which characterizes a diffusion model. This observation seems to be compatible with the main mechanism suggested for the action of OA as a penetration enhancer, i.e., by increasing SC permeability; the compound diffusion through the membrane is made easier (30). Another possible mechanism for the action of OA is the lamellar solid fluid phase separation (31), which leads to an increased permeability of SC to several drugs. It has been shown that OA is a more efficient enhancer to highly hydrophilic than lipophilic compounds ($P_{octanol/water} < 0$), and this supports our proposal for its use to enhance skin delivery of the highly hydrophilic drug, 5-ALA.

As described in the literature, the limited 5-ALA tissue penetration in the SCC and nodular BCC carcinoma treatments results in the production of inadequate amounts of PpIX, with insufficient depth for the efficiency of topical PDT (42). The present *in vitro* study was carried out in healthy skin samples, in which 5-ALA penetration may be different from human skin with BCC or SCC lesions. However, for the screening of potential formulation, this *in vitro* skin model is useful. Thus, skin retention studies were carried out to verify the extent of 5-ALA retention and its localization in the main skin layers, SC, and [E without SC + D]. The amount of 5-ALA retained in the SC (Fig. 2) was significantly smaller for formulations containing OA at 2.5 or 5.0% w/w ($p < 0.001$**) when compared to controls. On the other hand, the amounts of 5-ALA retained in [E without SC + D] was only significantly increased for formulations with 5.0% ($p < 0.01$*) and 10.0% ($p < 0.001$**) OA compared to the values for the controls. This may indicate that 5.0 or 10.0% OA facilitates 5-ALA skin penetration, quantitatively increasing its presence in the tissue, as well as its release to the deeper layers [E without SC + D]. This behavior is probably a result of the penetration enhancing effect of OA, a skin barrier disturber, which can easily interact with lipids or cellular membranes, particularly in the intercellular domains of SC. These *in vitro* permeation and retention data corroborate our recent study, in which a chemically similar polar lipid (glycerol monooleate) increased both flux and retention of 5-ALA in hairless mouse skin (43).

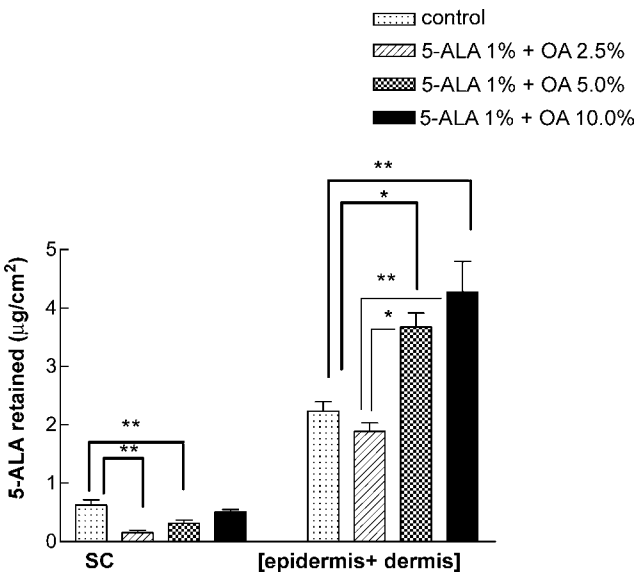

Fig. 2. 5-ALA *in vitro* retention in the SC and (epidermis without SC + dermis) porcine ear skin after 12 h of an *in vitro* permeation study. Statistical analysis: one-way ANOVA (multiple comparisons Tukey's test). Values considered significant at $p < 0.05$. Results presented are averages ± SEM obtained from six animals in each group.

Table I. 5-ALA (1% w/w) *in Vitro* Skin Permeation Through Full Thickness Porcine Ear Skin in the Presence of Different Concentrations of OA

Formulations	Flux, J ($\mu g\ cm^{-2}\ h^{-1}$)[a]	Correlation Coefficient, r^2
Control	0.2601 ± 0.128	0.994
OA 2.5 % (w/w)	0.2444 ± 0.133[b]	0.995
OA 5.0 % (w/w)	0.372 ± 0.122[c]	0.999
OA 10.0 % (w/w)	0.626 ± 0.226[d]	0.988

[a] Means ± SEM of the results in six experiments are shown (Kruskal–Wallis test).
[b] Statistically significant ($p < 0.001$) compared only with formulation containing 10% (w/w) OA.
[c] Statistically significant ($p < 0.01$) compared only with formulation containing 10% (w/w) OA.
[d] Statistically significant ($p < 0.001$) in comparison to control.

However, as there is a relationship between 5-ALA cutaneous release and PpIX accumulation in optimized PDT, it is important to determine the production/accumulation of the endogenous photosensitizer besides the *in vitro* evaluation of the skin permeation and retention of 5-ALA.

In Fig. 3, it is possible to observe a significant increase in PpIX accumulation, *in vivo*, after the application of formulations containing 5-ALA associated to 5.0 or 10.0% OA in PG when compared to the control formulation (1.0% 5-ALA in PG). The highest value for PpIX accumulation in the skin (ng/g) was obtained with OA at 10.0% w/w. These results indicate that the *in vivo* production and accumulation of PpIX in healthy hairless mice skin increased approximately 1.8 and 2.16 times by the association of 5-ALA (1% w/w) to 5.0 and 10.0% AO, respectively. There was a significant difference between formulations containing 2.5 and 10.0% OA, but not between the ones with 5.0 and 10.0% OA. Moreover, the concentration of PpIX in untreated hairless mice skin and treated with control formulation without 5-ALA were smaller than in the ones receiving 5-ALA

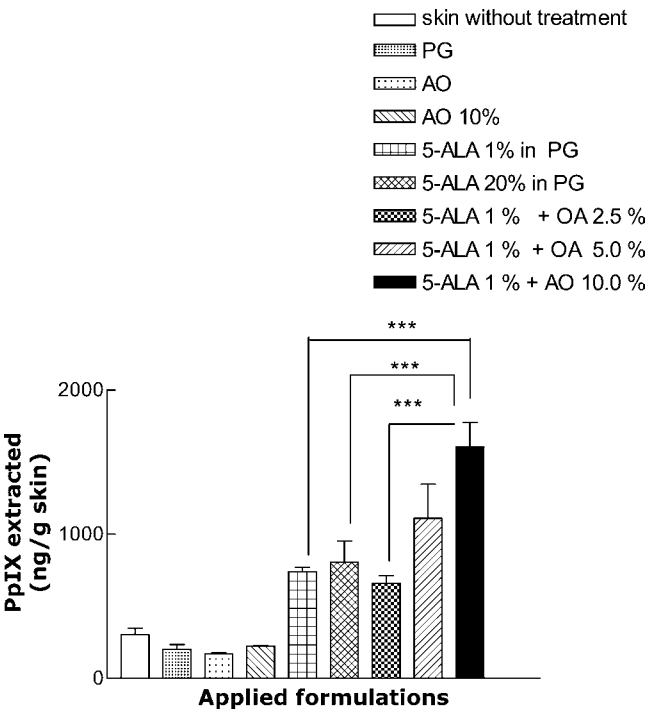

Fig. 3. PpIX extracted from hairless mice skin 4 h after *in vivo* topical application of formulations. Statistical analysis: one-way ANOVA (multiple comparisons Tukey's test). Values considered significant at $p < 0.05$. Results presented are averages ± SEM obtained from four animals in each group. PG: propylene glycol.

formulations. Today, formulations containing 20% 5-ALA are commonly used in clinical PDT, ensuring an appropriate PpIX accumulation in the skin (38,39). However, it is interesting to point out that a 5-ALA concentration 20 times lower, 1.0% w/w, associated to 10% OA was able to produce in the target tissue a PpIX accumulation 2 times higher than the control with only 5-ALA at 20%, probably as a result of the reduced penetration of 5-ALA in the absence of the penetration enhancer. The use of effective lower 5-ALA concentrations suggests a therapy with lower costs.

The 5-ALA-induced skin PpIX accumulation was also determined by direct evaluation on mice skin by fluorescence spectroscopy through an optical fiber probe. The optical fiber probe technique is useful for *in vivo* 5-ALA-induced PpIX kinetic studies (44–48) to evaluate, noninvasively, parameters such as best periods of time for laser light radiation after formulation applications and PpIX skin accumulation. These parameters play important roles in determining the conditions for topical 5-ALA-PDT and characterizing the influence of formulations on the treatment, but it should be remembered that for human use these conditions need to be reevaluated.

Table II shows that 5.0 or 10.0% (w/w) AO associated to 1.0% (w/w) 5-ALA increased the PpIX concentration (3.5 and 5.6 times, respectively), compared to control, a 1% 5-ALA solution in PG. It confirms that using a penetration enhancer (OA), which increases 5-ALA skin uptake (Fig. 2), may produce a higher PpIX accumulation. PpIX quantitative assays using solvent extraction procedures (Fig. 3) further corroborate this finding.

Table II. *In Vivo* PpIX Fluorescence Intensity Values Measured by an Optical Fiber Probe, ($\lambda_{excitation}$ = 400 nm/$\lambda_{emission}$ = 637.5 nm) 4 h After Topical Application of Formulations

Formulation	PpIX Fluorescence Intensity × $(10)^{5a}$
PG	4.534 (± 0.369)
OA 10.0% in PG	3.535 (± 0.491)
5-ALA 1.0% in PG	6.857 (± 0.756)
5-ALA 1.0% + OA 5.0% in PG	24.328 (± 6.765)[b]
5-ALA 1.0% + OA 10.0% in PG	38.789 (± 5.824)[c]

Statistical test: one-way ANOVA (Tukey's multiple comparisons test).

[a] Means ± SEM of the results in four experiments are shown. The intrinsic skin autofluorescence was about 3.018 (±0.606) × 10^5.

[b] Statistically significant ($p < 0.001$) in comparison to control (5-ALA 1.0% in PG).

[c] Statistically significant ($p < 0.001$) compared with formulation containing OA 5.0% in PG.

The skin *in vivo* fluorescence was also measured just after the application of formulation and no significant change was found compared to the autofluorescence in the hairless mouse skin [about 3.018 (±0.606) × 10^5] 30 min earlier. Thus, this time was chosen for the first measurement of skin fluorescence in the PpIX fluorescence kinetic studies. *In vivo* fluorescence of PpIX was monitored from 30 min to 24 h after the application of formulations, and both control and the results showed that the peak of maximum PpIX accumulation was at 2 h after application in both cases (Fig. 4). However, the highest OA-induced PpIX accumulation was maintained for up to 4 h compared to that in the control, where the smaller values decreased rapidly after 2 h. PpIX skin accumulation in the presence of AO-containing formulations showed a desirable kinetic profile for use in PDT, i.e., a short time interval between formulation administration and photosensitizer maximal accumulation in tumor tissue during a period that enables light application, followed by rapid tissue clearance (49).

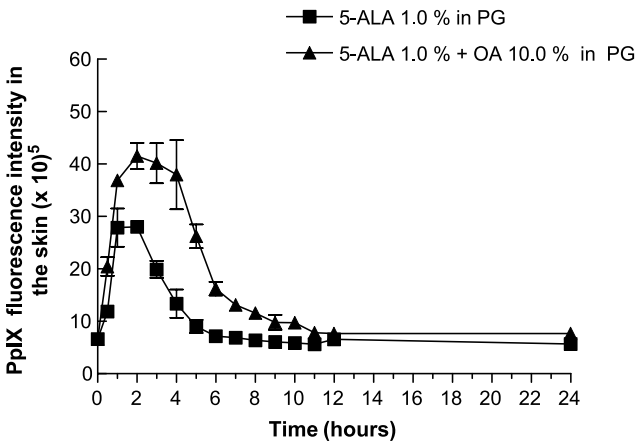

Fig. 4. Kinetic study of skin PpIX increased bioavailability induced by 5-ALA formulations in propylene glycol (PG). Values of fluorescence intensity, measured by an optical fiber probe ($\lambda_{excitation}$ = 400 nm/$\lambda_{emission}$ = 637.5 nm), from 30 min to 24 h after application on healthy mice skin. Bars represent the SEM of seven experiments.

CONCLUSIONS

Both *in vitro* and *in vivo* results showed that formulations containing OA can optimize the 5-ALA cutaneous delivery and *in vivo* PpIX accumulation in the skin. Formulations containing 10% OA increase PpIX accumulation in the animal skin by optimizing 5-ALA skin delivery. In addition, the presence of the penetration enhancer, OA, also extended the time period of maximum PpIX accumulation in the target tissue, a crucial condition for the success of topical 5-ALA-PDT.

In conclusion, this study emphasizes the potential of simple manipulation and composition of formulations containing 5-ALA and a penetration enhancer, OA, for the treatment of skin cancers by topical PDT.

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