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RESEARCH PAPER

Microneedle Pre-treatment of Human Skin Improves 5-Aminolevulininc Acid (ALA)- and 5-Aminolevulinic Acid Methyl Ester (MAL)-Induced PpIX Production for Topical Photodynamic Therapy Without Increase in Pain or Erythema

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ABSTRACT

Purpose To determine the impact of skin pretreatment with microneedles (MNs) on ALA- and MAL-induced protoporphyrin IX (PpIX) production, as well as MN impact on pain sensations during light exposure and erythema after PDT.

Methods The skin of 14 healthy volunteers was preteated with MNs. Equal amounts of creams containing 2%, 8% and 16% (w/w) ALA and MAL were applied on 1 cm^2 areas for 4 h. Additionally, 16% ALA and MAL creams were applied for 24 h. Afterwards, PpIX fluorescence spectra were measured. Sixteen percent ALA and MAL spots were exposed to red light (632 nm, 77 mW/cm^2). Time for pain to occur was measured in seconds, and erythemal response was monitored up to 6 h after the end of the light exposure.

Results Use of MNs increased the PpIX fluorescence after 4 h incubation time with 2% and 8% ALA or MAL, but not with 16% ALA or MAL. Pretreatment with MNs did not increase the pain sensations during light exposure, nor did it influence erythema occurrence.

Conclusions MNs are a promising tool for improving the efficiency of topical PDT by improving the cutaneous delivery of ALA and MAL, without increase in side effects.

KEY WORDS aminolevulinic acid (ALA) · aminolevulinic acid methyl ester (MAL) · erythema · microneedles (MNs) · photodynamic therapy (PDT)

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INTRODUCTION

Topical photodynamic therapy (PDT) with 5-aminolevulinic acid (ALA) and methyl aminolevulinate (MAL) is considered highly effective for superficial basal cell carcinoma, Bowen's disease, premalignant lesions called actinic keratoses and other dermatoses (1, 2). Lately, several reports show the positive effect of PDT against bacteria, viruses, fungi and protozoa (3–6). The major drawbacks of topical PDT are shallow penetration depth (<2 mm) of ALA and MAL and pain experienced during light exposure (2, 7–9). The distribution of a topically applied drug/prodrug in skin is dependent upon many parameters, such as its permeability through *stratum corneum*, diffusion through epidermis and dermis, application time and drug delivery vehicle (10). The hydrophilic nature of ALA may limit its permeation through the *stratum corneum*, the principal barrier for effective penetration (11). In order to increase the penetration depth of ALA into tissue, several methods have been proposed, such as removal of the *stratum corneum*, different formulations containing penetration enhancers, physical methods (curettage, ultrasound, iontophoresis, electroporation and electrophoresis), and chemical derivatization of ALA (12). MAL, as a more lipophilic ALA derivative, should penetrate more efficiently through skin and has been shown to induce less

pain during light exposure than ALA (13, 14). Despite that, ALA-PDT and MAL-PDT seem equally efficient in the treatment of acne vulgaris (15) and nodular basal cell carcinoma (16).

Recently, silicon microneedles (MNs) were employed in animal model to improve skin penetration and facilitate accurate targeting of ALA (17) and meso-tetra (N-methyl-4-pyridyl) porphine tetra tosylate (TMP) (18). MNs provide a safe and efficient method for improvement of skin permeability in a minimally invasive manner in comparison with, e.g. tape stripping, needle puncturing or painful curettage (12, 19–21). MNs create microscopic perforations in the *stratum corneum,* causing small or no erythemal reactions (22). This is not surprising, as skin repairs itself without infections or scarring on a regular basis from everyday microscopic scrapes, scratches, shaving, and other mechanical traumas. In comparison, skin permeation improvement with tape stripping causes barrier disruption, which does not repair significantly within the following 24 h (23). Furthermore, MNs are designed to penetrate *stratum corneum* without stimulating the pain receptors found in deeper tissue. Thus, they are able to penetrate the delivery barrier without causing pain or bleeding (19). Skin puncturing with hypodermic needles, on the other hand, leads to inhomogeneous and unrepeatable skin perforations, causing skin trauma and bleedings (21, 24). Moreover, we have recently shown that the risk of microbial infections is lower with MNs than with hypodermic needles (20).

The object of this work originates from the fact that most studies on the permeability of ALA and ALA derivatives across the *stratum corneum* and porphyrin production have been done in rodent models. The thickness and the structure of the *stratum corneum* and the hair follicles in mouse skin differ from those in human skin (25). These differences may influence the drug penetration rate, the ability to penetrate the *stratum corneum* and the production of endogenous porphyrins. Thus, to gain clinically relevant data, the present study was performed on healthy volunteers. Lately, a few articles concerning the use of MNs on human skin were published. However, the first study concerns not as much the use of MNs as about a new condition, Recalcitrant Malassezia Folliculitis (6). The second study, although about the use of MNs in PDT, has a few drawbacks. MNs have been applied to improve MAL penetration through stratum corneum and increase PpIX amount, yet fluorescence of PpIX was not measured. Alopecia areata (hair-loss condition of the scalp and body) does not respond generally to ALA/MAL-PDT (26). Moreover, some dermatological clinics use ALA-PDT for hair removal (27). Accordingly, we think that our study concerning MN use in topical PDT is the first clinical study.

The aims of the project were two-fold: first, to determine the impact of MNs on the production of PpIX induced by ALA and MAL; second, to determine the impact of the MNs on pain sensations during PDT with ALA and MAL delivered in a topically applied cream vehicle.

MATERIALS AND METHODS

Chemicals

A copolymer of methylvinylether and maleic anhydride (PMVE/MA) was provided by ISP Co. Ltd. (Gantrez® AN-139, Guildford, UK). Silicone elastomer was obtained from Dow Corning (Wiesbaden, Germany). 5-aminolevulinic acid hydrochloride (ALA) and 5-aminolevulininc acid methyl ester (MAL) were obtained from Sigma-Aldrich Norway AS (Oslo, Norway). All chemicals were of the highest purity commercially available. The active compounds were diluted in cream base (Unguentum®, Almirall Hermal GmbH, Reinbek, Germany) to reach 2%, 8% or 16% (w/w) of ALA or MAL concentration.

Volunteers

The influence of MNs on ALA and MAL penetration and PpIX production was investigated on 14 healthy volunteers (six women and eight men) with an average age of 40 (Fitzpatrick skin types II-IV). All volunteers were informed about the procedure of the study, possible risks, protection of sensitive data and the right to withdraw any time they wanted. The project did not involve any health risk for the volunteers.

The study was approved by the local ethical committee, Regional komite for medisinsk forskningsetikk Sør-Norge (Ref.nr. S-07434b).

Microneedles (MNs)

Silicone elastomer micromoulds were prepared using custom-made aluminium containers with fitted aluminium stubs in the centre. The silicone elastomer was carefully poured into the aluminium container so that the level of the silicone was approximately 5.0 mm above the surface of the aluminium stubs. To eliminate entrapped air, the containers were centrifuged for 15.0 min at 3500.0 rpm before curing overnight at 40.0°C. The silicone elastomer mould was then removed from the aluminium container by gently pressing a metal rod against an opening in the base of the device. Laser-engineered micromould templates were prepared as described previously (28), with microneedle geometries of 600 μm in height, 300 μm in width, an interspacing of 300 μm and 121 microneedles in the 1 cm^2

of the array. These templates were cut in dimensions to exactly fit the silicone mould. The templates were then glued into the mould using silicone elastomer, which was cured at 40°C for 40 minutes. In order to clean the prepared moulds, they were sonicated in warm water for 30 min. A 30% w/w aqueous solution of PMVE/MA was prepared by adding the required mass of PMVE/MA to ice-cold deionised water, followed by vigorous stirring and heating at 95.0°C until a clear gel was obtained, due to hydrolysis of the anhydride form of the copolymer to the corresponding acid. Upon cooling, the blend was then readjusted to the final concentration of 30% w/w by addition of an appropriate amount of deionised water. In each case, the resultant solutions (0.5 g) were then poured into the silicone micromoulds, centrifuged for 15.0 min at 3,500 rpm, and allowed to dry under ambient conditions for 24 h. Formed microneedle arrays were then eased gently out of the flexible moulds (Fig. 1a and b).

MN Pretreatment and Creams Application

Ten spots (1 cm^2 each) were selected and marked on the arms of each volunteer using a hydrokolloid dressing (DuoDERM®, ConvaTec, Deeside,UK). Five of them were pretreated with MNs. A 1 cm^2 MN array was pressed against the skin of volunteer for 10 seconds using gentle finger pressure and then removed. Approximately 0.1 g of cream containing 0%, 2%, 8% or 16% (w/w) ALA or MAL was applied on each spot for 4 h or 24 h. Afterwards, creams were removed. Due to a large number of tested spots, the study was performed in two separate rounds. The first round involved MN pretreated and non-pretreated spots with 4 h cream application time of 0%, 2% and 8% (w/w) ALA or MAL. The second round involved MN pretreated and non-pretreated spots with 0% and 16% (w/w) ALA or MAL applied on the spots for 4 h and 24 h. Every round of the study involved a total of ten volunteers.

Fluorescence Measurements

The fluorescence of porphyrins produced in the skin was measured in all spots in all volunteers after cream removal using a fiber-optic probe coupled to a spectrofluorimeter (LS50B, PerkinElmer, Norwalk, CT). Excitation light was set on 407 nm, while the emission spectra were in the range of 560–700 nm. With this set-up, the major part of the recorded fluorescence is related to PpIX molecules accumulated in the skin (29). Typical fluorescence emission spectra of PpIX were observed in human skin after topical application of the creams containing ALA and MAL with and without MNs pre-treatment (data not shown).

Light Exposure

All spots containing 16% ALA or MAL were exposed to red light. A CureLight® lamp (Photocure ASA, Oslo, Norway) with peak wavelength at 631 nm was used for light exposure. The lamp was placed 5 cm above the skin surface. Light exposure was interrupted at the onset of pain, and the time for this to occur was exactly measured. If no pain sensation occurred, the light exposure continued until the full treatment dose was delivered (8 min, 77 mW/cm^2).

Erythemal Response Measurements

Erythema index was measured using a narrowband reflectance spectrometer DermaSpectrometer® (Cortex Technology, Hadsund, Denmark). This instrument applies light from diodes emitting at 655 nm (red). An erythema index is computed from the intensity of the reflected light (30). Changes in erythemal response were measured before light exposure, immediately after light exposure and every second hour up to 6 h following light exposure. The measurements were performed only on spots where 16% ALA or MAL was applied.

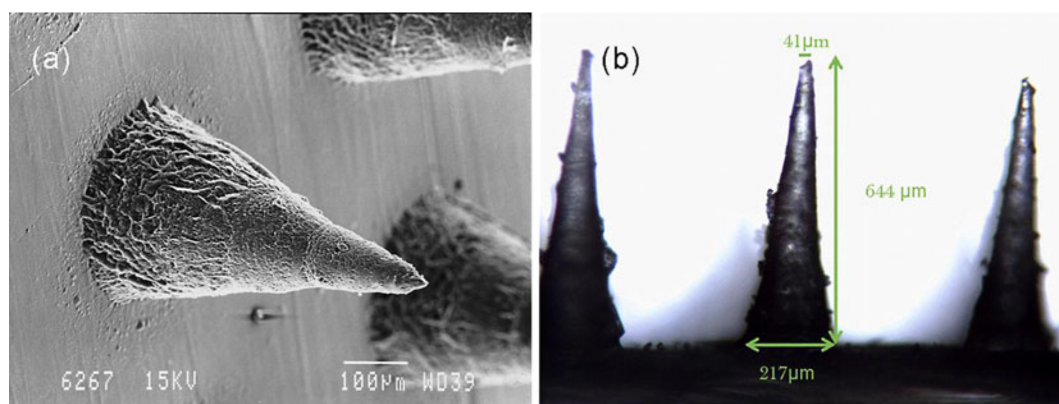

Fig. 1 Polymeric microneedle cones used in this study (**a**). The measures of the microneedles (**b**).

Transepidermal Water Loss Measurements

Transepidermal Water Loss (TEWL) was measured to determine the level of disruption to skin barrier function following application of the MN arrays in human volunteers. Six non-smoking healthy volunteers (three men and three women) aged between 23 and 31 years, with no pre-existing skin conditions, participated in the study. They were asked not to apply any cosmetic formulations on the ventral forearm during the study period. The study was approved by the School of Pharmacy's Ethical Committee at Queen's University Belfast, United Kingdom. Before the study, the volunteers were given volunteer's information sheet, and were asked to sign the consent forms.

A VapoMeter® (DelfinTechnologies Ltd. Kuopio, FINLAND) was used to measure TEWL at a non-skin-treated control site and a MN-treated skin site. TEWL was measured using a closed chamber attached to the skin with a moisture probe reading the humidity in the chamber. The VapoMeter® consists of sensors which measure the % relative humidity (RH) and convert it to a value representative of TEWL. Before TEWL measurement, each volunteer was rested for 15 min to acclimatise to the ambient room temperature and relative humidity, which were maintained at 20°C and 45 ± 5%, respectively. TEWL measurements were taken by carefully resting the TEWL probe horizontally on the intended skin site, with the probe head vertical and perpendicular to the skin. Once the participant was comfortable, a reading was taken, and the values presented on the digital display unit of the VapoMeter® were recorded. TEWL readings were taken at 0, 2, 15, 30, 45, 60, 90, 120, 180 and 240 min from both MN and non-treated skin sites.

Statistics

Data are presented as means +/− S.E. (standard error). The Student's paired *t*-test was used to compare the significance between data points. Values of $p < 0.05$ were considered as indicating significant differences. Statistical analyses of TEWL results were performed using a one-way analysis of variance (ANOVA), where $p < 0.05$ was taken to represent a statistically significant difference. When there was a statistically significant difference, post-hoc Tukey's HSD multiple comparison tests were performed.

RESULTS

Pretreatment with MNs significantly increased PpIX production in the case of 2% and 8% ALA creams, as compared with spots non-pretreated with MNs (Fig. 2a). The PpIX fluorescence level after 4 h incubation with 8% ALA pretreated with MNs was similar to the PpIX fluorescence level obtained after 4 h incubation time with 16% ALA alone (Fig. 2a). MN pretreatment did not increase PpIX production in spots where 16% ALA was applied (Fig. 3a).

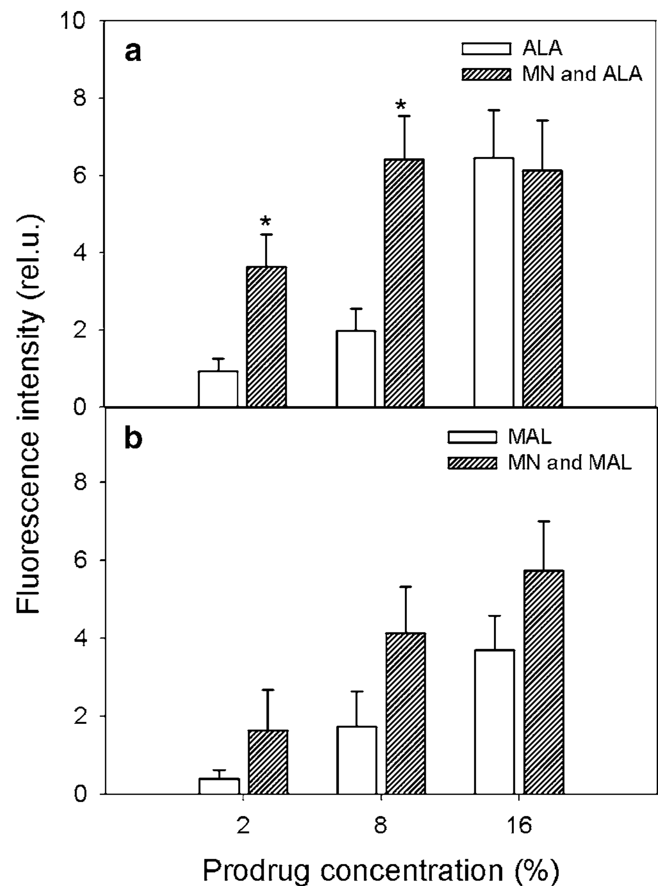

Fig. 2 Porphyrins (mainly protoporphyrin IX (PpIX)) fluorescence recorded by means of fluorescence spectroscopy (excitation at 407 nm, emission at 636 nm) from the surface of 1 cm^2 spots of the skin of healthy volunteers after 4 h incubation with creams containing different concentrations of (**a**) aminolevulinic acid (ALA) or (**b**) methyl aminolevulnate (MAL). Statistically significant differences between MN-pretreated and non-pretreated spots indicated for values of * $p < 0.05$.

Pretreatment with MNs increased PpIX fluorescence induced by 2%, 8% and 16% MAL (Fig. 2b). As with ALA, 4 h incubation of 8% MAL pretreated with MNs induced PpIX production similar to that obtained by 4 h incubation of 16% MAL alone (Fig. 2b).

The use of MNs before cream application was painless and did not influence the PpIX fluorescence as compared with spots non-pretreated with MNs (Fig. 3a). After 4 h incubation time there was no difference in time needed for pain to occur between spots where MNs were used and spots where MNs were not used, neither for 16% ALA nor for 16% MAL (Fig. 3b). After 24 h, although the differences did not reach statistical significance, ALA-PDT alone

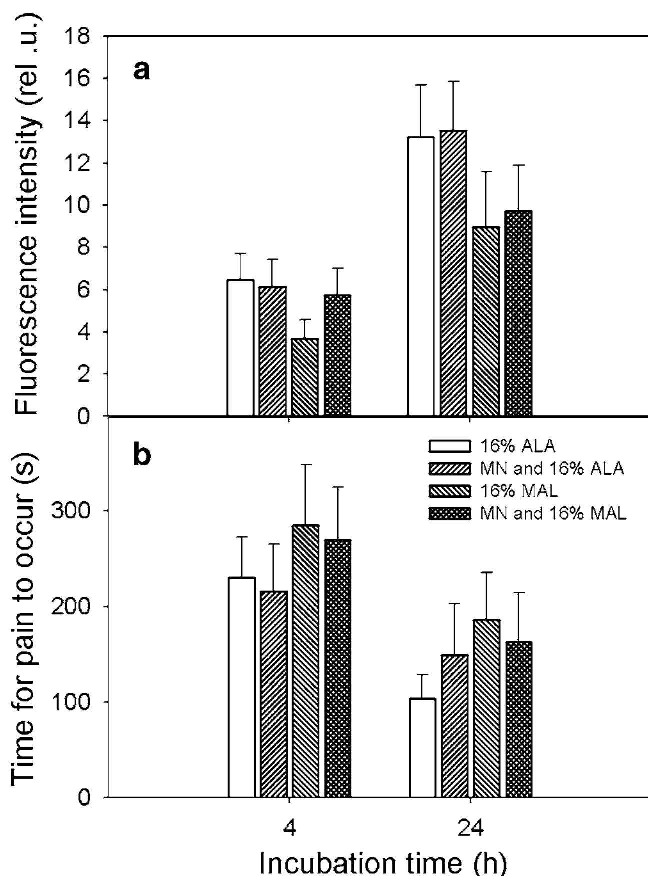

Fig. 3 Creams containing 16% aminolevulnic acid (ALA) and 16% methyl aminolevulinate (MAL) were applied on the skin of healthy volunteers. On half of the spots, the skin was pretreated with microneedles (MNs). After 4 h and 24 h, incubation creams were removed, and fluorescence intensity at 636 nm was measured (**a**). Afterwards, spots were exposed to red light (CureLight, peak wavelength at 631 nm, 90 mW/cm^2) until onset of pain. Time for pain to occur was measured in seconds from the beginning of light exposure (**b**).

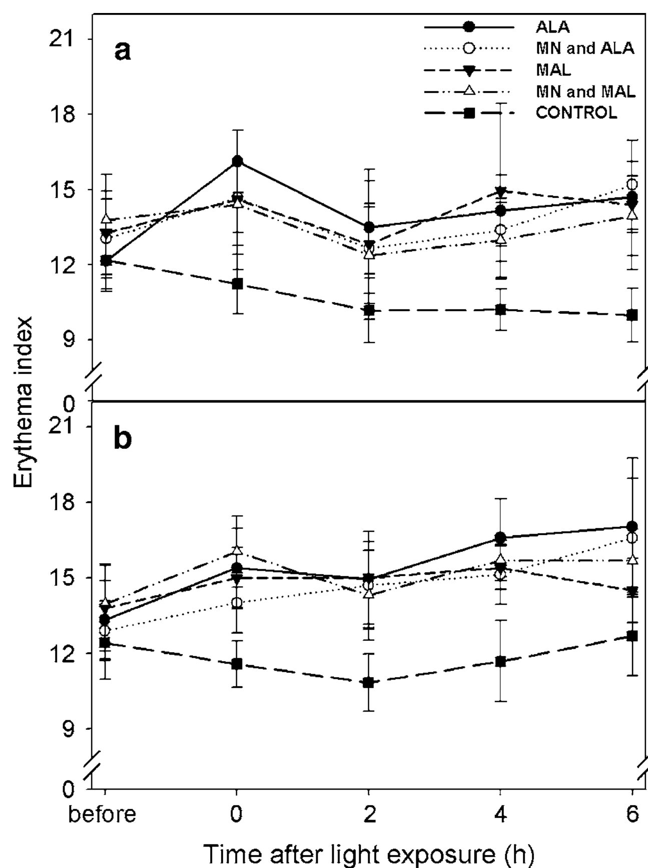

Fig. 4 Erythema index was computed from the intensity of the reflected light at 655 nm. Measurements were taken from each spot before light exposure, immediately after light exposure (time-point "zero") and every second hour within 6 h following light exposure both for 4 h incubation time regimen (**a**) and 24 h incubation time regimen (**b**). The active compounds concentration used was 16% (w/w) ALA and 16% (w/w) MAL. Control represents spots where cream with no active compound was applied.

induced pain ~ 15 s earlier than ALA-PDT in combination with MNs. MAL-PDT, on the other hand, induced pain ~20 s later than MAL-PDT in combination with MNs.

Changes in erythema index were followed up to 6 h after light exposure (Fig. 4). In all spots an increase of erythema was observed immediately after light exposure. In the spots incubated with creams for 4 h the increase in erythemal response was strongest right after light exposure and then leveled off. After 24 h application the increase was less profound in the beginning and was steadily increasing. No increase in erythema in the spots where MNs were used was observed as compared with spots where MNs were not used.

Figure 5 shows the results of TEWL values determined for MN-treated and non-treated (control) skin sites. Prior to MN application, mean TEWL values were 10.39 ± 1.63 g h^{-1} m^{-2}. Following microporation of the skin, TEWL values increased significantly to 16.67 ± 2.57 g h^{-1}m^{-2} within the

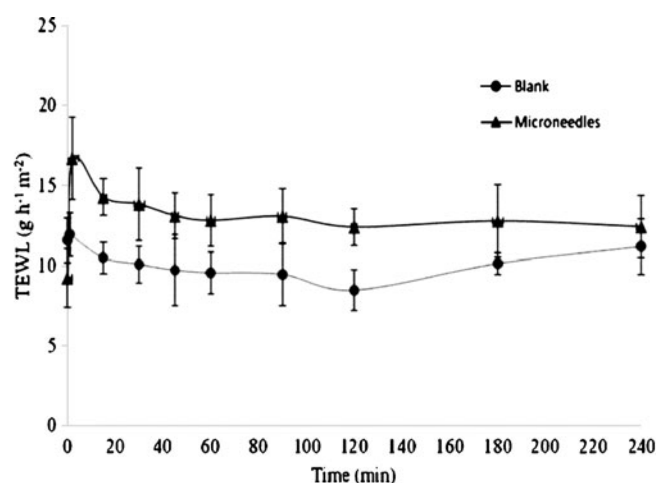

Fig. 5 Transepidermal water loss measurements to study the integrity of barrier function in microneedle-treated and non-treated skin (Mean ± SD, $n=6$).

first 2 min compared to baseline values ($P<0.001$). The TEWL values then remained significantly elevated over a 90 min period, before returning to baseline values at 120 min. In contrast, no significant changes in TEWL values were observed in the non-MN-treated control group over the time period studied. Measurement of TEWL after cream removal proved difficult, due to non-uniform removal of cream from volunteer to volunteer. Washing with detergent solution in water and excessive scraping of skin only compounded this problem. Consequently, reliable data could not be obtained.

DISCUSSION

Microneedles (MNs) can be regarded as a third-generation transdermal delivery system (31). For over a decade, MNs have been extensively investigated and improved to efficiently overcome the barrier function of the skin, which resides almost entirely in the outermost layer, the *stratum corneum* (31). In the present study, application of low concentrations of the protoporphyrin IX (PpIX) prodrugs ALA and MAL for short times (4 h) combined with the use of MNs resulted in increase of PpIX fluorescence as compared with the PpIX fluorescence from non-pretreated spots (Fig. 2). Our results are in agreement with the previously published results showing a significant increase in the ALA-PpIX production in mouse skin pretreated with MNs (17).The mechanism by which MNs increase PpIX production may involve one or more prodrug penetration paths. There are two different paths by which a prodrug can penetrate through skin: through the *stratum corneum* itself or through aqueous pores (hair follicles and sweat glands) (32, 33). The first path mainly depends on the ability of a prodrug to pass through the intercellular lipid matrix (34). Perforations created by MNs can disorder the *stratum corneum* intercellular lipids, thus increasing the prodrug diffusion coefficient. It has been shown by Moser *et al.* that a two-fold increase in the drug diffusion coefficient significantly enhances the amount of drug permeated (34). Simultaneously, by creating microperforations in the *stratum corneum*, MNs increase the total surface area of the aqueous pores in the skin. The observed lack of increase of PpIX fluorescence in 16% ALA spots pretreated with MN compared to non-pretreated 16% ALA spots (Fig. 2a) can be explained by the possibility of saturation of the hem biosynthesis pathway, which leads to PpIX accumulation in cells. These results are in agreement with a study where a bioadhesive patch containing 19 mg/cm^2 ALA applied on MN-pretreated murine skin did not increased significantly the PpIX fluorescence recorded (17).

As mentioned above, MN treatment before application of 16% ALA and MAL did not increase the PpIX fluorescence as compared to non-MN-pretreated spots (Fig. 3). Therefore, 16% ALA and MAL were chosen for investigation of MN influence on PDT-induced pain and erythema. Given that the PpIX levels were comparable for MN-pretreated and non-pretreated spots, a significant increase in pain or erythema would point towards MN-induced changes.

Erythema is one of the main markers of skin inflammation that may reflect vasculature changes in the skin (vasoconstriction or vasodilatation) (22, 35). Changes in vasculature can potentially influence the tissue oxygenation, and, thus, the PDT effectiveness (36, 37). Longer application times (24 h) resulted in longer lasting erythema (Fig. 4). Observed slight hypopigmentation after 24 h cream application (results not shown) might be caused by a temporal vasoconstriction of small blood vessels and has been reported before as a common side effect after light exposure in topical PDT (36). Bal *et al.* (22) showed that MNs did induce minimal and short-lasting skin irritation as compared with non-treated skin. In the present study, no differences between the spots pretreated with MNs and non-pretreated with MNs were observed as far as erythema is concerned. The recorded erythema was a result of the topical PDT and not of the use of MNs.

The present study shows that MNs do not increase the sensation of pain during light exposure in topical PDT, regardless of the applied prodrug or the application times (Fig. 3b). Moreover, the applications of MNs were perceived by the volunteers as painless. These observations are in agreement with a study on a number of solid and assembled MN arrays up to 550 μm in length, where pain and skin irritation was assessed (22), as well as with a study using 150 μm silicon MNs that also showed no influence of MN treatment on pain sensations (38). Furthermore, it was found that the application of MNs in this study resulted in a reversible disruption of skin barrier function, with the skin regaining its normal barrier function after a period of 2 h. The choice of MN is of great importance, as it has been reported that a roller with 1 mm microneedles induced bleeding that might have actually prevented the absorption of the photosensitizer (26). Safety concerns have been expressed also over the possibility of metal or silicon MN fragments being retained in the skin following MN removal (39). However, the polymeric system employed in the current study is made from a biocompatible pharmaceutical polymer which is biodegradable and negates these safety matters. Moreover, it has been shown *in vivo* that hand application of the MNs on human skin resulted in reproducible penetration depth of approximately 460 μm.

In conclusion, skin pretreatment with MNs is not only safe to use on human skin, but also allows reduction of

prodrug concentration and application time of the treatment. Results of the present study point to the fact that MNs used prior to application of ALA-containing cream, apart from displaying a tendency of reducing the experienced discomfort, caused a bigger increase in the amount of PpIX produced than when used prior to application of MAL-containing cream. Reduction of concentrations of prodrugs improves stability of the formulations (40). It also reduces costs per treatment and, therefore, makes the treatment available for more patients. Shorter application time increases patients' convenience as well as the total number of patients that can be treated per day.

This study has been performed on the healthy skin of volunteers. Carrying out the study on skin lesions would undoubtfully provide additional valuable information about the microneedle-enhanced penetration of ALA and MAL. However, for the proof of concept, it would have been difficult to assure similar conditions for spot-to-spot comparisons of PpIX production. In the further studies, it is nevertheless desirable to obtain data from lesions on human patients.

Fluorescence spectroscopy was chosen for measuring the PpIX content in the upper parts of the skin, as it is a non-invasive and relatively fast method that proved to be sufficient for the scope of this work. Yet we do not negate that acquiring additional insight from biopsy samples would be advantageous.

Knowing the great potential of MNs, the next step is to ensure that every clinician applies the MNs in exactly the same way to every patient every time. As such, an in-depth study into the characteristics of pore formation and pore closure following MN application and how these affect the PDT outcome on a patient-to-patient basis is now needed. Currently, the possibility of incorporating ALA and preformed photosensitizers directly into polymeric microneedles is being investigated. This approach will simplify the two-step method: microneedle puncture followed by the application of a cream/patch into a one-step method and application of a microneedle patch. Development of an easy-to-apply, prodrug-containing microneedle device will have a great impact on the PDT field. Not only will it ensure reproducible results when applied by clinicians, but also will open the possibility for patients to apply it themselves before visit in the clinics for light exposure.

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CONFLICT OF INTEREST

The manuscript contains original data which are not published or intended to publish elsewhere. We state no conflicts of interests or of financial nature or others.

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