Thermo-Mechanical Fractional Injury Enhances Skin Surface- and Epidermis- Protoporphyrin IX Fluorescence: Comparison of 5-Aminolevulinic Acid in Cream and Gel Vehicles

Camilla Foged,[1] Merete Haedersdal,[1] Liora Bik,[1,2] Christine Dierickx,[3] Peter A. Phillipsen,[1] and Katrine Togsverd-Bo[1]*

[1]*Department of Dermatology, Copenhagen University Hospital Bispebjerg and Frederiksberg, Nielsine Nielsens Vej 17, entrance 9, 2. floor, Copenhagen, Nordvest DK-2400, Denmark*

[2]*Department of Dermatology, Erasmus MC University Medical Center Rotterdam, Dr. Molewaterplein 40, Rotterdam, 3015, The Netherlands*

[3]*Skinperium, Private Dermatology Clinic, Rue Charles Martel 52, Luxembourg, 2134, Luxembourg*

Background and Objectives: Thermo-mechanical fractional injury (TMFI) impacts the skin barrier and may increase cutaneous drug uptake. This study investigated the potential of TMFI in combination with 5-aminolevulinic acid (ALA) cream and gel formulations to enhance Protoporphyrin IX (PpIX) fluorescence at the skin surface and in the skin.

Study Design/Materials and Methods: In healthy volunteers ($n = 12$) a total of 144 test areas were demarcated on the upper back. Test areas were randomized to (i) TMFI (6 milliseconds, 400 μm at a single pass) or no pretreatment and (ii) 20% ALA in cream or gel formulations. Skin surface PpIX fluorescence was quantified by PpIX fluorescence photography and photometry in 30-minute intervals until 3 hours. PpIX fluorescence microscopy quantified separate PpIX fluorescence in the epidermis, and in superficial-, mid-, and deep- dermis from punch biopsies sampled after 3 hours of ALA incubation. Local skin reactions (LSR) and pain intensities (numerical rating scale 0–10) were evaluated immediately, at 3 hours and 14 days after the intervention.

Results: TMFI exposure before photosensitizer application significantly increased *skin surface* PpIX fluorescence, both for ALA cream (TMFI-ALA-cream 7848 arbitrary units [AU] vs. ALA-cream 5441 AU, 3 hours, $P < 0.001$) and ALA gel (TMFI + ALA-gel 4591 AU vs. ALA-gel 3723 AU, 3 hours, $P < 0.001$). The TMFI-mediated increase in PpIX fluorescence was similar for ALA-cream and -gel formulations ($P = 0.470$) at the skin surface. In the *epidermis*, PpIX fluorescence intensities increased from combination treatment with TMFI and ALA-cream (TMFI + ALA-cream 421 AU vs. ALA-cream 293 AU, $P = 0.034$) but not from combination with TMFI and ALA-gel (TMI + ALA-gel 264 AU vs. ALA-gel 261 AU, $P = 0.791$). *Dermal* fluorescence intensities (superficial-, mid-, or deep dermis) were unaffected by TMFI pretreatment in both ALA-cream and ALA-gel exposed skin ($P = 0.339$). ALA-cream generally induced higher PpIX fluorescence intensities than ALA-gel (skin surface $P < 0.001$ and epidermis $P < 0.03$). TMFI induced low pain intensities (median 3) and mild LSR that were resolved at 14 days follow-up.

Conclusion: Given the present study design, TMFI, in combination with the standardized application of 20% ALA cream and gel formulations, significantly enhanced skin surface PpIX fluorescence compared to no pretreatment. Additionally, TMFI increased epidermal PpIX fluorescence combined with 20% ALA cream vehicle. Thus, TMFI pretreatment and formulation characteristics exert influence on PpIX fluorescence intensities in normal skin. Lasers Surg. Med. © 2020 Wiley Periodicals LLC

Key words: 5-aminolevulinic acid; actinic keratoses; fluorescence microscopy; Protoporphyrin IX; photodynamic therapy; stratum corneum; thermo-mechanical fractional injury; thermo-mechanical system; vehicle viscosity

INTRODUCTION

Topical photodynamic therapy (PDT) in dermatology is based on light activation of the endogenous photosensitizer

Conflict of Interest Disclosures: All authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest and have disclosed the following: TMFI device was loaned by Novoxel (Novoxel LTD., Israel) for this particular study, accompanied by a research grant to Bispebjerg Hospital, represented by Merete Haedersdal, Novoxel Ltd. had no influence on data collection or interpretation of results. CD, CF, KTB, LB, and PAP have no conflicts of interest.

*Correspondence to: Katrine Togsverd-Bo, MD, Department of Dermatology, Copenhagen University Hospital, Bispebjerg and Frederiksberg, Nielsine Nielsens Vej 17, entrance 9, 2. Floor, DK-2400 Copenhagen Nordvest, Denmark. E-mail: katrine.togsverd-bo@regionh.dk

Accepted 13 September 2020
Published online in Wiley Online Library (wileyonlinelibrary.com).
DOI 10.1002/lsm.23326

Protoporphyrin IX (PpIX). PDT is an attractive treatment concept for keratinocyte dysplasia due to high efficacy, excellent cosmetic results, and the possibility to treat large skin areas in the same session [1,2]. Following topical application of PpIX precursors 5-aminolevulinic acid (ALA) or methyl aminolevulinate (MAL), the accumulated amount of PpIX can be quantified by fluorescence detection on the skin surface and in the skin [3–5].

The viable epidermis is an effective barrier for exogenous substances, including cutaneous drug delivery. The skins barrier function is due in large part to the stratum corneum (SC) that allows minimal skin penetration except for lipophilic ($\log P > 1$) and small molecules (molecular weight [MW] < 500 Da) [6,7]. Targeting SC, physical pretreatment techniques facilitate cutaneous uptake of ALA or MAL and is recommended in European PDT protocols for the treatment of actinic keratoses and basal cell carcinomas [1]. The most common method is curettage, but newer interventions, such as microneedling, microdermabrasion, ablative fractional laser (AFL), and non-AFL, are increasingly applied to enhance PpIX accumulation and PDT efficacy [8,9].

Thermo-mechanical fractional injury (TMFI) is a relatively new technique that by the protrusion of pyramid-shaped, titanium covered micro-tips, conducts heat to the skin, generating an array of fractional microscopic vertical columns of thermal injury [10,11]. TMFI has previously been explored for cutaneous uptake of hydrophilic drugs, such as verapamil, diclofenac sodium, 5-fluorouracil, and magnesium ascorbyl phosphate, after TMFI exposure [12–14]. In a recent study, TMFI pretreatment enhanced PpIX fluorescence at the skin surface following incubation with 20% ALA gel [14]. However, the formation of PpIX fluorescence in normal skin remains to be clarified, given different ALA formulations. Thus, in a randomized controlled trial applying standardized interventions, fluorescence photography, and fluorescence microscopy, we aimed to assess PpIX fluorescence intensities at the skin surface and in the skin after exposure to TMFI and ALA formulated in cream and gel vehicles.

MATERIALS AND METHODS

The study was conducted from April to July 2019 and approved by the Danish Medicines Agency (2018-004397-96), Ethics Committee of Capital Region (H-1900394), the Data Protection Agency (2019-3247), as well as registered with Clinical-Trials.gov (NCT04221126). The GCP Unit at the University of Copenhagen monitored the study according to Good Clinical Practice. Written informed consent was obtained from all volunteers prior to enrollment, and the study was performed in accordance with the Declaration of Helsinki.

Participants

The study was a randomized, controlled, intra-individual clinical trial. Healthy volunteers were assessed for eligibility and subsequent treatment at the Department of Dermatology, Bispebjerg Hospital, Copenhagen, Denmark. Inclusion criteria were age above 18 years, Fitzpatrick skin type I–III, and normal skin on the upper back. Exclusion criteria were lactating or pregnant women, allergy to ALA and lidocaine, conditions associated with risk of poor compliance, and PDT or laser treatment of the test areas within the past 6 months.

Interventions

Photosensitizing agents. 5-ALA powder (Gliolan, Medac, Roskilde, Denmark) was prepared at 20% weight/weight (w/w) concentrations in a cream vehicle (viscosity value 116,000 counts per second [cps]) and a gel vehicle (viscosity value 971 cps). Both vehicles were manufactured by the Capital Region Hospital Pharmacy (Herlev, Denmark) under good manufacturing practice (GMP) and the viscosity determined by an external laboratory (AminoLab, Ness Ziona, Israel). The excipients per 100 g vehicles included for the cream vehicle: 700 mg cetrimide, 5 g glycerol 85%, 8 g cestosterayl alcohol, 40 g paraffin liquid in purified water; and for the gel vehicle: 5.9 g glycerol, 0.6 g benzalkonium chloride, 0.3 g disodium edetate, and 1.8 g carmellose sodium in purified water.

Study procedures. In each participant, 12 test areas of each 3×3 cm were mapped on the upper back. Test areas were randomization to (i) TMFI or no pretreatment and (ii) 20% ALA gel or cream vehicle formulation, Table 1 illustrating an overview of test areas. Randomization was performed by a computer-generated random sequence (MatLab®; MathWorks, Natick, MA, USA) and treatment allocations selected from opaque, sequentially numbered, sealed envelopes. Treatment areas were documented at baseline and during all study procedures by clinical photography under standard lighting conditions (Canon EOS 60D, Tokyo, Japan). All study procedures were performed under dimmed light conditions after ALA application.

TMFI (Tixel; Novoxel Ltd., Hamelacha St 43, 420573 Netanya, Israel) exposure was delivered at 6 milliseconds pulse duration and 400 μm protrusion in a single pass to allocated test areas. The TMFI settings were based on previously published results, showing higher PpIX fluorescence at this setting compared to longer pulse durations with similar histologically evaluated tissue response as in the current study [14]. Immediately after pretreatment, 125 μg ALA-cream, ALA-gel, and vehicle controls were applied to relevant test areas and left without occlusion up to 3 hours (Table 1). The test areas were shielded from ambient light with non-occluding aluminum foil. Skin surface PpIX fluorescence was quantified every 30 minutes using a PpIX fluorescence photometer and PpIX fluorescence camera. After 3 hours, five punch biopsies (3 mm diameter) were sampled from each participant for PpIX fluorescence microscopy. The time point for biopsy sampling was determined in a pilot study ($n = 4$ participants) that documented higher PpIX fluorescence intensities at the skin surface and in the epidermis at 3 hours incubation with ALA gel formulation

TABLE 1. Study Setup and Standardization

Test area	Pretreatment	Vehicle	Incubation (min)[a]	PpIX fluorescence median (IQR), AU[b]	PpIX Photometry median (IQR) AU[b]
1	TMFI	ALA-cream	180	7848 (4285–12,836)	52 (41–63)
2	None	ALA-cream	180	5441 (2612–8235)	43.5 (28–52)
3	TMFI	ALA-gel	180	4591 (3821–7398)	36 (24–49)
4	None	ALA-gel	180	3723 (1722–5449)	20.5 (13–35)
5	TMFI	ALA-cream	120	6693 (3189–13,366)	47 (36–60)
6	None	ALA-cream	120	5992 (1414–6572)	35 (22–43)
7	TMFI	ALA-gel	120	5541 (3090–9807)	42.5 (28–56)
8	None	ALA-gel	120	3729 (20–7125)	18 (15–28)
9	TMFI	None	–	–	–
10	Untreated control	None	–	–	–
11	None	Cream vehicle	180	–	–
12	None	Gel vehicle	180	–	–

ALA, 5-aminolevulinic acid; AU, arbitrary units; PpIX, protoporphyrin IX; TMFI, thermo-mechanical fractional injury.
[a]Incubation: time when vehicle formulation was wiped off the skin.
[b]PpIX fluorescence intensities measured at 3 hours.

compared with 1 or 2 hours incubation. A total of 12 biopsies were assessed in the pilot study.

Outcome Assessment

Local skin response (LSR) and patient-evaluated pain. LSRs were evaluated as erythema, edema, scaling, pustules, and crusting. Each parameter was graded on a standardized 5-point severity scale representing none, mild, moderate, prominent, and severe [15]. LSR was supported by noninvasive reflectance measurement of skin redness and pigmentation percentages (UV Optimize Scientific Model, Chromo-light, Espergaerde, Denmark). LSR and reflectance measurements were conducted at baseline, immediately and 3 hours after interventions, and at 14 days follow-up. Finally, patient-assessed pain during TMFI was performed on a 0–10 numerical rating scale in which 0 is no pain and 10 worst imaginable pain.

Skin surface PpIX fluorescence. Skin surface PpIX fluorescence was quantified using two noninvasive techniques, a handheld fluorescence photometer, and PpIX fluorescence photographs. PpIX photometer (FluoDerm, Dia-Medico, Gentofte, Denmark) illuminates a circle at 4 cm diameter with blue light (400–420 nm) and detect emitted fluorescence between 610 and 700 nm [16]. The photometer was calibrated prior to the study start, and measurements adjusted for autofluorescence by subtracting the baseline value from corresponding PpIX fluorescence measurements.

PpIX fluorescence photographs were obtained by a fluorescence imaging system (Medeikonos AB, Gothenburg, Sweden) [4,17]. PpIX excitation was delivered with UVA2 and blue light at 365–405 nm for 2 seconds with the red fluorescent light captured by a CDD camera equipped with a long pass filter (610–715 nm). Subsequently, PpIX fluorescence intensities in each study area were calculated from the fluorescence photograph using an imaging analysis program (MatLab®, Natick, MA, USA), with each fluorescence photograph calibrated to a fluorescence standard (Bio-Science, Gilleleje, Denmark). ALA-induced PpIX fluorescence was defined as pixels with a value 500 higher than background fluorescence pixel values and adjusted for autofluorescence by subtracting baseline vehicle fluorescence values in each photograph. PpIX fluorescence values measured in arbitrary units (AU) can be relatively but not directly compared since different methods were used to quantify PpIX fluorescence.

PpIX fluorescence microscopy. Biopsies were immediately frozen (−20°C) and cut into 10 μm vertical sections for fluorescence microscopy [4]. Digital fluorescence microscopy was performed using a fluorescence microscope (Olympus IX70, Fluorescence Microscope, Olympus Germany, Düsseldorf, Germany) in a room with dim light to minimize photobleaching. PpIX was excited by a xenon lamp equipped with a 400–440 nm excitation filter (AT420/40x; Chroma, VT, USA) and fluorescence emission captured with a 510 nm long-pass filter (ET510lp; Chroma, VT, USA). Images were captured using a CCD camera (ORCA-R2 Digital CCD camera; Hamamatsu Photonics, Shizuoka, Japan) with associated software (HCImage Live; Hamamatsu Photonics). The stability of the excitation light was monitored prior to each microscopy session with a Grimson Blue fluorescent standard (Bio-Science, Gilleleje, Denmark) and varied less than 10%.

From each biopsy, three cryosections were selected for fluorescence microscopy. PpIX fluorescence quantification was obtained from four regions of interest (ROI), defined at epidermis (0–100 μm skin dept), superficial dermis (125–300 μm skin dept), mid dermis (800–1200 μm skin dept), and deep dermis (1600–2000 μm skin dept). From each section, three images were captured, representing epidermis, superficial dermis, mid dermis, and deep dermis, and median fluorescence intensities calculated from three measurement areas within each ROI. Image

analysis was performed by an investigator (CF), blinded to treatment intervention using an image analysis program (ImageJ, National Institutes of Health, Bethesda, Maryland, USA). Correction for autofluorescence was made by subtracting the untreated control value from the corresponding ROI-structure. A total of 540 images were reviewed for PpIX fluorescence intensity analysis. Subsequently, 90 sections were stained with hematoxylin and eosin (HE) and reviewed for histologic analysis of TMFI-tissue interactions.

Outcome Measures

The primary outcome measure was PpIX fluorescence intensities at the skin surface and in the skin, whereas ALA concentrations were not determined. Secondary outcome measures were histological assessment of TMFI impact on the skin, LSRs supported by reflectance measurements of skin redness and pigmentation, as well as participant-assessed pain.

Statistics

A study sample size of 10 patients was calculated, aiming at a 40% minimally relevant difference (MIREDIF) of PpIX skin surface fluorescence, a standard deviation of 20%, a 5% significance level, and power of 80%. To statistically allow for premature withdrawals, 12 participants were included in the study. Descriptive data were presented as medians, interquartile ranges (IQR), and minimum and maximum values. Nonparametric Friedman and Wilcoxon signed-rank tests were used to compare PpIX fluorescence intensities as data were non-normally distributed. $P < 0.05$ were considered significant. All statistical tests were performed using SPSS Statistics version 25 (IBM, Armonk, NY, USA) and visualized using Prism 6 (GraphPad Software, San Diego, CA, USA).

RESULTS

Twelve healthy participants with a median age of 22.5 years (range 18–25), male to female ratio of 1:1, and Fitzpatrick's skin types I ($n = 1$), II ($n = 5$), and III ($n = 6$) were enrolled in the study. All participants completed the study protocol and were included for outcome analysis.

Histology-Assessed TMFI-Tissue Interaction

HE sections of TMFI-exposed skin visualized a localized dermo-epidermal coagulation zone with a median maximum depth of 251 μm (range 154–498 μm) and a median maximum width of 463 μm (range 206–1054 μm) (Fig. 1G). Tissue interactions presented with intra-epidermal vacuolization, subepidermal clefting, and intense eosin staining corresponding to the photothermal treatment zone, as shown in Figure 1G.

Skin Surface PpIX Fluorescence

Skin surface PpIX fluorescence accumulated continuously from baseline to 3 hours, as visualized in Figure 2. By gross evaluation, TMFI induced homogenous and intensified PpIX fluorescence compared with less intense PpIX fluorescence in non-pretreated skin, illustrated in Figure 1C and 1D. Quantitatively, TMFI exposure significantly enhanced PpIX fluorescence

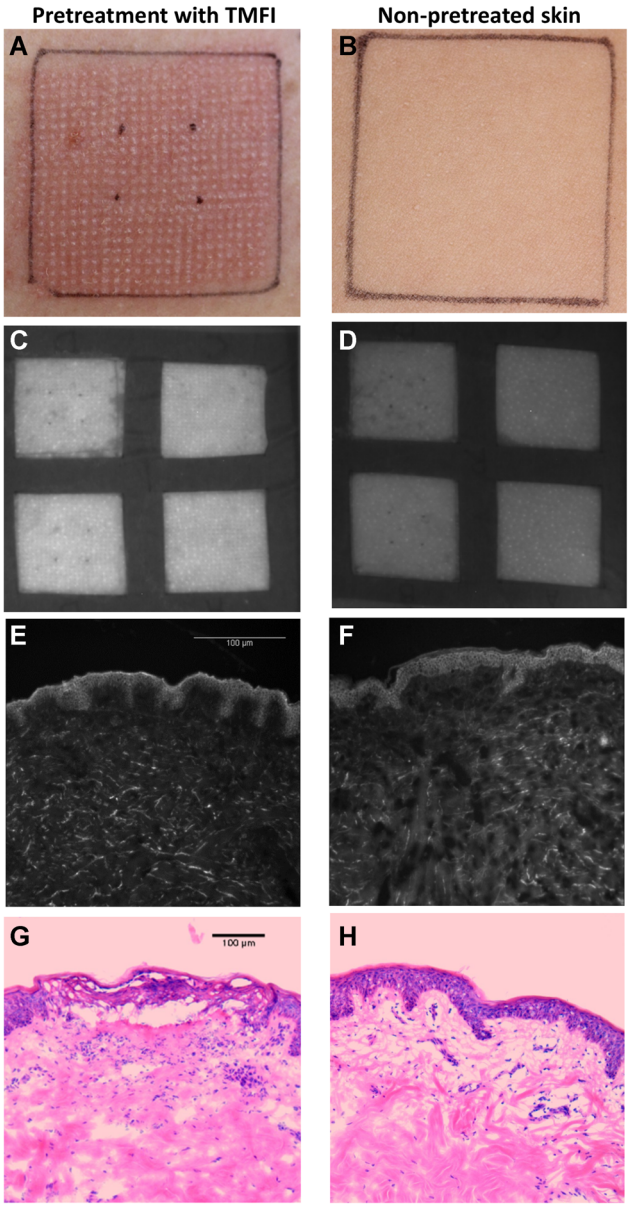

Fig. 1. Images illustrating impact of TMFI exposure on the skin and Protoporphyrin IX (PpIX) fluorescence. (**A**) The immediate erythema and edema following TMFI exposure and (**B**) non-pretreated skin. (**C**, **D**) Skin surface PpIX fluorescence photographs showing more intense fluorescence intensities at 3 hours in TMFI-pretreated skin (**C**) than un-pretreated skin (**D**) combined with ALA-cream (upper row) and ALA-gel (lower row) formulations. (**E**, **F**) Fluorescence microscopy illustrating enhanced epidermal PpIX fluorescence following TMFI pretreatment and ALA-cream (**E**) than ALA cream alone (**F**). (**G**, **H**) Hematoxylin-eosin (HE) stain showing subepidermal clefting and eosin coloring in the TMFI treatment zone (**G**) versus non-pretreated normal skin (**H**). ALA, 5-aminolevulinic acid; TMFI, thermo-mechanical fractional injury.

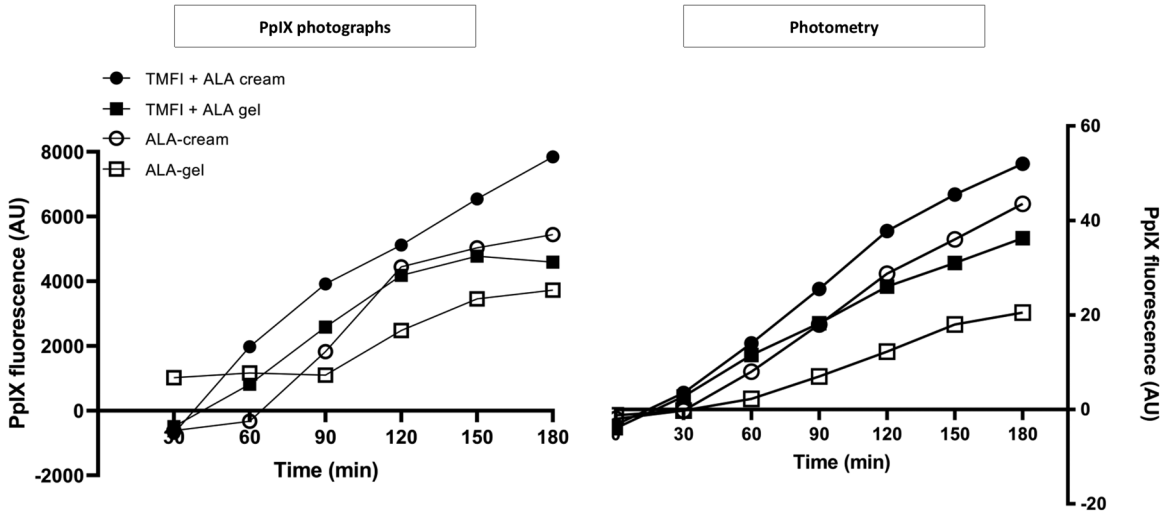

Fig. 2. Skin surface protoporphyrin IX (PpIX) intensities (median) accumulating over time in test areas exposed to TMFI, 5-ALA-gel, and cream vehicles incubated for 3 hours. Fluorescence units are expressed in arbitrary units (AU). ALA, 5-aminolevulinic acid; TMFI, thermo-mechanical fractional injury.

intensities compared with incubation with both ALA cream and ALA gel formulations without any pretreatment ($P < 0.01$) and were documented with PpIX fluorescence photography and PpIX fluorescence photometry, as listed in Table 1. Thus, at 3 hours of photosensitizer incubation, combination of TMFI-ALA-cream resulted in higher median PpIX fluorescence intensities than ALA cream alone (photometry 52 vs. 44 AU, $P < 0.001$; fluorescence photography 7848 vs. 5441 AU, $P < 0.01$). In ALA-gel-incubated skin, TMFI pretreatment increased PpIX fluorescence compared with no TMFI pretreatment (photometry 36 vs. 21 AU, $P < 0.001$; fluorescence photography 4591 vs. 3723 AU, $P < 0.043$).

The extent to which TMFI pretreatment increased PpIX fluorescence ranged from 48% to 136% after 3 hours of ALA-gel incubation compared with 20–44% after ALA-cream incubation ($P =$ ns). Overall, the ALA-cream vehicle achieved higher median PpIX fluorescence intensities than ALA-gel vehicle, both in combination with TMFI pretreatment ($P < 0.017$) and in non-pretreated skin ($P < 0.001$), Table 1. PpIX fluorescence intensities were slightly higher at 3 hours than 2 hours of ALA incubation when quantified with photometry ($P = 0.04$), whereas comparisons of fluorescence photographs showed no difference between incubation times ($P =$ ns).

PpIX Fluorescence Microscopy

Epidermal PpIX fluorescence reached overall higher intensities (range 261–421 AU) than dermal PpIX fluorescence (range, −41 to 92 AU), Figure 3. In the epidermis, the combination of TMFI and ALA-cream induced higher PpIX fluorescence than ALA-cream alone (medians, 421 vs. 293 AU; $P = 0.034$), whereas TMFI and ALA-gel did not enhance fluorescence intensities compared with ALA-gel alone (medians, 264 vs. 261 AU; $P = 0.791$). Furthermore, epidermal PpIX fluorescence reached higher intensities by TMFI-ALA-cream compared with TMFI-ALA-gel (medians, 421 vs. 264 AU; $P = 0.03$). In non-TMFI-pretreated skin, ALA-cream and ALA-gel induced similar median fluorescence values at 293 and 261 AU, respectively.

In the superficial, mid, and deep dermis, PpIX fluorescence intensities reached significantly lower values than in the epidermis (ALA-cream and ALA-gel range −41 to 92 AU). TMFI exposure did not increase fluorescence intensities in neither superficial, mid nor deep dermis.

Safety

TMFI was well-tolerated, resulting in mild LSRs and participant-assessed pain at low intensities (median, 3; range, 1–6) that cleared within minutes. Immediate skin responses included mild erythema (median, 2; range, 1–2) and edema (median, 1; range, 0–1), visualized in Figure 1A and 1B. Mild erythema and edema persisted at the 3 hours assessment but were cleared at day 14 follow-up. Supporting clinical evaluations, noninvasively quantified redness increased immediately and 3 hours after TMFI (median, 28–29%) compared with untreated skin (median, 23%; $P < 0.001$). At day 14 follow-up, two participants developed mild postinflammatory hyperpigmentation in all ALA-test areas that were unrelated to TMFI exposure. The TMFI device was easy to operate.

DISCUSSION

This study assessed the potential of TMFI pretreatment to enhance PpIX fluorescence at the skin surface and in the skin following standardized applications of 20% ALA in gel and cream vehicles. Quantified by two different fluorescence measurement techniques, skin surface PpIX fluorescence intensities significantly and consistently enhanced throughout the 3-hour incubation period when the

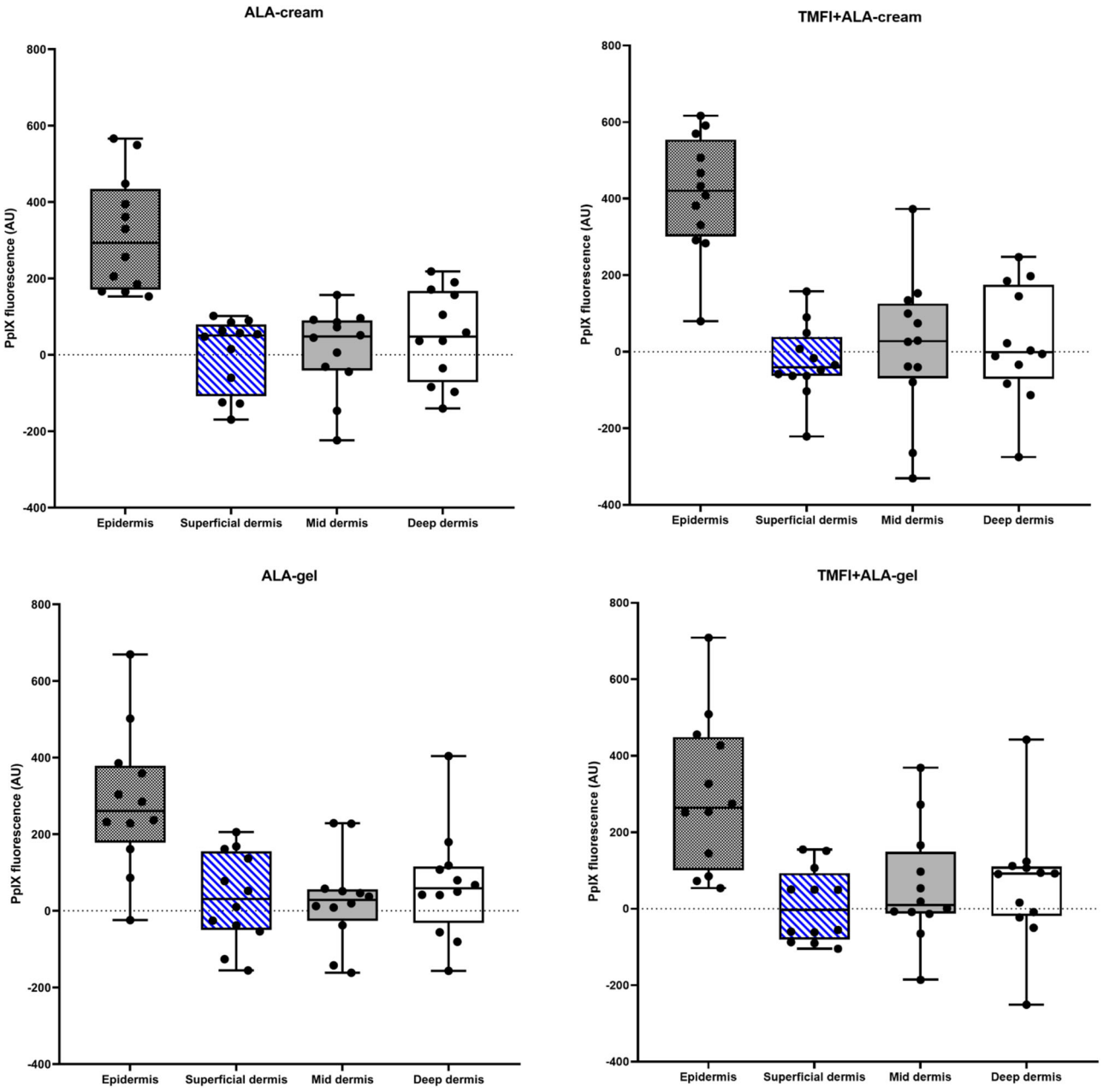

Fig. 3. Box-and-whisker plot of Protoporphyrin IX (PpIX) fluorescence intensities (arbitrary units, AU) after 3 hours ALA cream and ALA gel vehicle incubation, quantified in regions of interest (ROI): Epidermis, superficial dermis, mid dermis, and deep dermis. Epidermal PpIX fluorescence was more intense than dermalPpIX fluorescence. TMFI pretreatment enhanced epidermis PpIX fluorescence in the skin exposed to ALA cream. Dermal PpIX fluorescence intensities were not significantly different after correction for autofluorescence. Plot illustrates the median, interquartile range, and minimum and maximum fluorescence values.

skin was exposed to TMFI before application of photosensitizer. Furthermore, PpIX fluorescence microscopy demonstrated higher epidermal PpIX fluorescence intensities in the skin exposed to TMFI and 20% ALA-cream formulation compared to ALA-cream without TMFI pretreatment. These findings confirm TMFI as a novel and tolerable pretreatment technique that increases the accumulation of ALA-induced PpIX to superficial skin layers before PDT treatment.

TMFI-mediated thermal disruption of the skin barrier has been explored *in vitro* and *in vivo* for hydrophilic drugs that poorly penetrate the intact skin. Evaluated

drugs include verapamil hydrochloride, diclofenac sodium, botulinum toxin A, and ALA [10–13,18]. A recent study by Shavit and Dierickx determined skin surface PpIX fluorescence in normal skin after TMFI, combined with commercially available photosensitizers in different concentrations and vehicle formulations [13]. Photosensitizers included 7.8% ALA in microemulsion-gel (Ameluz®), 20% ALA in alcohol solution (Levulan Kerastick®), 16.8% MAL in a cream (Metvix®), and 20% ALA in a self-produced gel [13]. In accordance with our results, the authors found that TMFI enhanced PpIX fluorescence at 3 hours using 20% ALA in a gel formulation. In the present study, TMFI-20% ALA-cream achieved higher PpIX fluorescence intensities than TMFI-20% ALA-gel but was not compared to ALA in a low-viscosity vehicle formulation. The observed difference may rely on vehicle formulation characteristics as all factors associated with conventional drug diffusion are the same for ALA cream and gel vehicles, except for the partition coefficient. In general, hydrophilic molecules are less prone to partition from aqueous vehicles compared to lipophilic vehicle formulations [6]. Thus, being a hydrophilic molecule, ALA (logP −1.5) may easily partition from a lipophilic cream vehicle than from a gel vehicle resulting in faster diffusion from the vehicle and thereby generate higher PpIX fluorescence in the skin incubated with ALA cream than ALA gel. A lipophilic cream vehicle may potentially facilitate higher epidermal retention for increased probability of intracellular entry, although both ALA cream and gel were supplied in excess on the skin [19]. As a potential confounder, the applied cream vehicle contained cetrimide, not only an emulsifier but also a surfactant that may enhance ALA penetration of the cream vehicle compared to the gel vehicle.

By disruption of skin integrity, different physical pretreatments are available to facilitate cutaneous uptake of photosensitizers [3,6,8]. Hence, pretreatment modalities may focus on stratum corneum removals, such as curettage and microdermabrasion, or may target full epidermis by generating microscopic channels of ablated or coagulated tissue using fractional ablative or nonablative lasers [8,9], thus targeting the permeability barrier of the full epidermis [7]. Microneedling is a cost-effective technique that uses mechanical force to create transient micropores to enhance PpIX fluorescence accumulation [20]. In head-to-head comparisons and *in vitro* studies, the ablative fractional laser is the only pretreatment technique to provide deep and intense delivery of photosensitizers in deep dermal compartments, and is also associated with more pronounced LSR [3,9,21]. With the current settings, TMFI produced nonablative coagulation zones with low trauma to the skin barrier, resulting in enhanced PpIX accumulation in superficial skin layers, which is consistent with PDT's current Food and Drug Administration (FDA) indication for superficial AK. However, offering high tolerability and minimal downtime posttreatment [22], TMFI holds potential as a novel tool to enhance ALA-induced PpIX accumulation.

Assessed by PpIX fluorescence microscopy, TMFI in combination with 20% ALA in cream formulation augmented epidermal PpIX fluorescence intensities whereas dermal intensities remained low and were unaffected by TMFI pretreatment. The difference between epidermal and dermal PpIX fluorescence intensities results from a significantly low density of fibroblasts, responsible for the conversion of ALA to PpIX. Furthermore, delayed diffusion of ALA from the epidermis to dermis as well as elimination of ALA by dermal blood and lymphatic vessel structures affects the kinetics of ALA concentration and contributes to low dermal PpIX fluorescence [6].

A major study limitation is that interventions were performed on normal skin on the back as opposed to facial skin or diseased skin with localized hyperkeratoses that may influence TMFI coagulation zone dimensions and ALA-induced PpIX fluorescence. TMFI settings can, therefore, not be directly extrapolated to all skin regions but should be adjusted according to skin thickness and skin constitution in treatment areas. Likewise, longer TMFI pulse durations or deeper penetration depths as well as different ALA vehicle formulations may have produced different PpIX fluorescence kinetics or distribution in epidermal and dermal structures.

As an active photosensitizer for PDT, PpIX is a relevant, well-established surrogate marker for cutaneous and systemic ALA pharmacokinetics [1,19,23]. Hence, the scope of the study was not to determine ALA concentrations in epidermal or dermal structures but to investigate ALA-induced PpIX accumulation with and without TMFI pretreatment. The strengths of the study include standardized study interventions and assessment of endpoints, including three different techniques to quantify PpIX fluorescence. Although a limited number of participants were included in the study, the intra-individual design permitted a direct comparison of study interventions evaluated in 144 test areas.

In conclusion, using the given standardized settings and vehicle formulations, TMFI exposure combined with 20% ALA in cream and gel formulations effectively enhanced PpIX fluorescence intensities at the skin surface and in the epidermis combined with 20% ALA cream. Thus, TMFI present as a novel, tolerable pretreatment technique with the potential to enhance PpIX accumulation in conjunction with PDT procedure.

ACKNOWLEDGMENTS

The authors thank Catharina M. Lerche, MSc (Pharm), PhD for preparing photosensitizer formulations and medical laboratory technologist Diana Hoeeg for sectioning biopsies.

AUTHOR CONTRIBUTIONS

C.F., K.T.B., P.A.P., and M.H. had full access to all study data and take responsibility for data integrity and accuracy of data analysis. Study concept and design: C.D., K.T.B., and M.H.; acquisition, analysis, or interpretation of data: all authors; drafting of the manuscript: C.F., M.H.,

and K.T.B.; critical revision of the manuscript for important intellectual content: all authors; statistical analysis: C.F. and P.A.P.; obtained funding: M.H.; administrative, technical, or material support: all authors; study supervision: C.F., K.T.B., P.A.P., and M.H.

REFERENCES

1. Morton CA, Szeimies R-M, Basset-Seguin N, et al. European Dermatology Forum guidelines on topical photodynamic therapy 2019 Part 1: Treatment delivery and established indications—Actinic keratoses, Bowen's disease and basal cell carcinomas. J Eur Acad Dermatol Venereol 2019;33(12): 2225–2238.
2. Kennedy JC, Pottier RH. Endogenous protoporphyrin IX, a clinically useful photosensitizer for photodynamic therapy. J Photochem Photobiol B 1992;14(4):275–292.
3. Haedersdal M, Sakamoto FH, Farinelli WA, Doukas AG, Tam J, Anderson RR. Pretreatment with ablative fractional laser changes kinetics and biodistribution of topical 5-aminolevulinic acid (ALA) and methyl aminolevulinate (MAL). Lasers Surg Med 2014;46(6):462–469.
4. Togsverd-Bo K, Idorn LW, Philipsen PA, Wulf HC, Hædersdal M. Protoporphyrin IX formation and photobleaching in different layers of normal human skin: Methyl- and hexylaminolevulinate and different light sources. Exp Dermatol 2012;21(10):745–750.
5. Tierney E, Petersen J, Hanke CW. Photodynamic diagnosis of tumor margins using methyl aminolevulinate before Mohs micrographic surgery. J Am Acad Dermatol 2011;64(5):911–918.
6. Prausnitz MR, Elias PM, Franz TJ, Schmuth M, Tsai JC, et al. Skin barrier and transdermal drug delivery. In: Bolognia J, Jorizzo JL, Schaffer JV, editors. Medical Therapy. Philadelphia, Saunders: Elsevier; 2012. pp 2065–2073.
7. Andrews SN, Jeong E, Prausnitz MR. Transdermal delivery of molecules is limited by full epidermis, not just stratum corneum. Pharm Res 2013;30(4):1099–1109.
8. Champeau M, Vignoud S, Mortier L, Mordon S. Photodynamic therapy for skin cancer: How to enhance drug penetration? J Photochem Photobiolog B Biol 2019;197:111544.
9. Bay C, Lerche CM, Ferrick B, Philipsen PA, Togsverd-Bo K, Haedersdal M. Comparison of physical pretreatment regimens to enhance protoporphyrin IX uptake in photodynamic therapy: A randomized clinical trial. JAMA Dermatol 2017;153(4):270–278.
10. Elman M, Fournier N, Barnéon G, Bernstein EF, Lask G. Fractional treatment of aging skin with Tixel, a clinical and histological evaluation. J Cosmet Laser Ther 2016; 18(1):31–37.
11. Sintov AC, Hofmann MA. A novel thermo-mechanical system enhanced transdermal delivery of hydrophilic active agents by fractional ablation. Int J Pham 2016;511(2): 821–830.
12. Artzi O, Koren A, Niv R, Mehrabi JN, Mashiah J, Friedman O. A new approach in the treatment of pediatric hypertrophic burn scars: Tixel-associated topical triamcinolone acetonide and 5-fluorouracil delivery. J Cosmet Dermatol 2019;19:131–134.
13. Artzi O, Koren A, Niv R, Mehrabi JN, Friedman O. The scar bane, without the pain: A new approach in the treatment of elevated scars: Thermomechanical delivery of topical triamcinolone acetonide and 5-fluorouracil. Dermatol Ther 2019;9(2):321–326.
14. Shavit R, Dierickx C. A new method for percutaneous drug delivery by thermo-mechanical fractional injury. Lasers Surg Med 2019;52(1):61–69.
15. Rosen R, Marmur E, Anderson L, Welburn P, Katsamas J. A new, objective, quantitative scale for measuring local skin responses following topical actinic keratosis therapy with ingenol mebutate. Dermatol Ther 2014;4(2):207–219.
16. Nissen CV, Wiegell SR, Philipsen PA, Wulf HC. Short-term chemical pretreatment cannot replace curettage in photodynamic therapy. Photodermatol Photoimmunol Photomed 2016;32(3):146–152.
17. Wiegell SR, Wulf HC. Photodynamic therapy of acne vulgaris using 5-aminolevulinic acid versus methyl aminolevulinate. J Am Acad Dermatol 2006;54(4):647–651.
18. Bar-Ilan E, Koren A, Shehadeh W, Mashiah J, Sprecher E, Artzi O. An enhanced transcutaneous delivery of botulinum toxin for the treatment of Hailey-Hailey disease. Dermatol Ther 2020;33(1):e13184.
19. Rick K, Sroka R, Stepp H, et al. Pharmacokinetics of 5-aminolevulinic acid-induced protoporphyrin IX in skin and blood. J Photochem Photobiol B Biol 1997;40:313–319.
20. Mikolajewska P, Donnelly RF, Garland MJ, et al. Microneedle pre-treatment of human skin improves 5-aminolevulinic acid (ALA)- and 5-aminolevulinic acid methyl ester (MAL)-induced PpIX production for topical photodynamic therapy without increase in pain or erythema. Pharm Res 2010;27(10):2213–2220.
21. Haedersdal M, Katsnelson J, Sakamoto FH, et al. Enhanced uptake and photoactivation of topical methyl aminolevulinate after fractional CO_2 laser pretreatment. Lasers Surg Med 2011;43(8):804–813.
22. Kokolakis G, von Grawert L, Ulrich M, Lademann J, Zuberbier T, Hofmann MA. Wound healing process after thermomechanical skin ablation. Lasers Surg Med 2020; 52(8):730–734.
23. Szeimies RM, Landthaler M. Photodynamic therapy and fluorescence diagnosis of skin cancers. Recent Results Cancer Res 2002;160:240–245.