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Drug Penetration Enhancement Techniques in Ablative Fractional Laser Assisted Cutaneous Delivery of Indocyanine Green

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Background and Objectives: Topical drug delivery can be increased by pretreatment of the skin with ablative fractional laser (AFXL). Several physical penetration enhancement techniques have been investigated to further improve AFXL-assisted drug delivery. This study investigated the influence of three of these techniques, namely massage, acoustic pressure wave treatment, and pressure vacuum alterations (PVP) on the distribution of the fluorescent drug indocyanine green (ICG) at different depths in the skin after topical application on AFXL pretreated skin.

Materials and Methods: In *ex vivo* human skin, test regions were pretreated with AFXL (10,600 nm, channel depth 300 μm, channel width 120 μm, density 15%). Subsequently, ICG was applied, followed by massage, acoustic pressure wave treatment or PVP. ICG fluorescence intensity (FI) was assessed after 1, 3, and 24 hours at several depths using fluorescence photography.

Results: FI was higher when using enhancement techniques compared to control (AFXL-only) up to 3 hours application time ($P < 0.05$). After 3 hours, mean surface FI was highest after acoustic pressure wave treatment (61.5 arbitrary units; AU), followed by massage (57.5AU) and PVP (46.9AU), respectively (for comparison: AFXL-only 31.6AU, no pretreatment 14.9AU). Comparable or higher FI was achieved already after 1 hour with enhancement techniques compared to 3–24 hours application time without. After 24 hours, no significant differences between enhancement techniques and AFXL-only were observed ($P = 0.31$).

Conclusion: Penetration enhancement techniques, especially acoustic pressure wave treatment and massage, result in improved drug accumulation in AFXL-pretreated skin and reduce the application time needed. Lasers Surg. Med. © 2019 The Authors. *Lasers in Surgery and Medicine* Published by Wiley Periodicals, Inc.

Key words: fractional laser; drug delivery; topical therapy; indocyanine green

INTRODUCTION

Topical treatments are still the cornerstone of dermatological therapy. However, the cutaneous bioavailability of most topically applied drugs, including corticosteroids, is relatively low with only 1–5% being absorbed in the skin [1]. Especially highly hydrophilic molecules and molecules with a molecular weight >500 Da are less suitable for topical use since they barely penetrate through the stratum corneum [2]. Several methods have been developed to increase penetration of topically applied drugs such as electroporation, iontophoresis, microdermabrasion, microneedles, mechanic pressure, sonophoresis, radiofrequency microporation, and laser techniques [3]. In the past years, ablative fractional laser (AFXL) pretreatment of the skin has been rapidly evolving as one of the foremost techniques to increase cutaneous drug delivery [4]. AFXL creates an array of microscopic ablation channels through which topical drugs can be delivered, while damage to the skin is minimized. Pretreatment with AFXL has proven to increase uptake of numerous topical substances, including photosensitizers, anesthetics, corticosteroids, methotrexate, and vismodegib [5–11]. In clinical trials, AFXL assisted photodynamic

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therapy has led to more effective treatments for actinic keratoses [12–15].

However, in AFXL assisted drug delivery, drug concentrations seem to increase slowly over time [6,7,16]. Therefore, relatively long application times may be needed to reach optimal drug concentrations, limiting clinical feasibility in outpatient or day care based treatments like photodynamic therapy. Furthermore, AFXL channels are believed to be filled with interstitial fluid or even a fibrin plug already shortly after AFXL treatment, preventing uptake of topical drugs in the channels [17–19]. In addition, in earlier clinical trials more deeply situated skin lesions such as nodular basal cell carcinomas have failed to respond to AFXL assisted photodynamic therapy, probably due to insufficient drug uptake in the deep dermis [20].

Various attempts have been made to increase and accelerate penetration of topical drugs through AFXL channels using physical penetration enhancement methods such as subsequent pressure vacuum alterations, transdermal acoustic pressure waves, and simple manual massage, with varying results [5,17,21]. However, to date, no studies have been performed quantitatively investigating the spatial distribution of topical drugs in deeper skin layers after application of these enhancement techniques on AFXL pretreated skin.

Primary aim of our present study was to investigate the influence of various enhancement techniques on the total accumulation of the fluorescent agent indocyanine green (ICG) at different depths in AFXL pretreated test regions in *ex vivo* human abdominal skin. Secondary aim was to assess the spatial distribution of ICG within the test regions at various time points after application of these enhancement techniques.

MATERIALS AND METHODS

Study Design

The study evaluated the accumulation of fluorescence of ICG in 5×5 mm test regions in *ex vivo* human abdominal skin samples pretreated with AFXL. After application of ICG, each test region was treated with one of three penetration enhancement techniques, manual massage, acoustic pressure waves or pressure vacuum alterations, or no penetration enhancement technique (control). ICG was left in place for 1, 3, or 24 hours. After the application time distribution and fluorescence intensity of ICG in the skin was assessed using digital surface fluorescence photography.

Skins Samples

Samples consisted of excised human abdominoplasty skin and were collected at the department of plastic surgery at the Slotervaart Hospital in Amsterdam. In total, six skin samples from six individual patients were used. The use of skin from the Slotervaart Hospital for these experiments was approved by AMC Medical Ethics Committee officials. As skin samples were anonymized, a formal review procedure of the protocol by the Medical Ethics Committee was not required. The skin was stored in a -20°C freezer shortly after excision and used within 1 month. The experiments were carried out on the same day following thawing of the skin sample. Before the start of the experiments excess subcutaneous fat was removed. The skin was tensioned with sutures in order to keep the skin in place and disinfected with alcohol (Orphilon Chlorhexidine 0,5% in alcohol 70%, Lage Zwaluwe, the Netherlands). The experiments, including the application time, were performed at room temperature (20°C). During the application time, the skin samples were kept under tension and entirely wrapped in aluminum foil to prevent light exposure and dehydration.

Ablative Fractional Laser

A fractional CO_2 laser (Ultrapulse®, DeepFx handpiece; Lumenis Inc., Santa Clara, CA) was used for AFXL pretreatment of the test regions. Test regions were pretreated at a pulse energy of 20 mJ/microbeam (600 Hz, pulse length: 80 μs, 5% density, 1 pulse, scanned area: 5×5 mm, microspot size: 120 μm, channel depth: ±300 μm), creating a square grid of 7×7 ablation channels. Channel depth at these settings had been verified using optical coherence tomography (OCT) before start of the study. Therefore, 2D and 3D images were acquired using a commercial swept source system (Santec Inner Vision 2000, Santec Corporation, Komaki, Japan) operating at 1,300 nm central wavelength at 50 kHz, with an experimentally determined axial resolution of 13 μm in tissue and lateral resolution of 25 μm.

Indocyanine Green

The fluorescent agent ICG (Cardiogreen, Sigma–Aldrich, St. Louis, MO) was used to study drug uptake in the skin through AFXL channels. ICG has a molecular weight of 751.4 Da and is slightly hydrophilic. ICG was used in a 0.08 mg/ml aqueous solution since quenching, that is, decrease in fluorescence intensity due to aggregate formation, is minimal at this concentration and fluorescence intensity increases linearly with increasing concentration up to 0.08 mg/ml [22,23]. At this concentration ICG has an absorption peak around 780 nm [24]. A total of 0.1 ml of ICG was occluded under a coverslip at each test region. During the application time ICG was kept under dark circumstances by covering the skin samples with aluminum foil. At the end of the application time, the cover slips were removed and excess ICG was wiped off. In aqueous solution, the emission spectrum of ICG peaks at 810–820 nm [25,26]. In plasma the emission peak shifts toward 820–834 nm [22,25]. The emission spectrum of ICG absorbed in dermal or epidermal tissue is not known.

Penetration Enhancement Techniques

Three penetration enhancement techniques were used: manual massage, acoustic pressure waves, and pressure-vacuum alterations. Massage was performed firmly for 1 minute in an area of 3×3 cm. To generate acoustic pressure waves the Legato^{II} Impact (Alma Lasers Ltd., Nuremburg, Germany) was used at 70% energy intensity and 50 Hz acoustic pressure pulse rate for 12 seconds in an

area of 3×3 cm. To generate pressure-vacuum alterations a self-made pressure-vacuum-pressure (PVP) device was used creating subsequent alternations in pressure for 3 minutes consisting of 1 minute relative positive pressure (atmospheric pressure + 1 bar), 1 minute relative negative pressure (atmospheric pressure − 0.7 bar), and relative 1 minute positive pressure (atmospheric pressure + 1 bar) based on an experimental device used by Erlendsson et al. [17].

Fluorescence Photography

Fluorescence of ICG in the skin was assessed using digital surface fluorescence photography performed under dark circumstances to minimize light exposure. A 780 nm continuous wave diode laser was used as excitation source and photographs were taken with a long pass cut-on 850 nm filter to block the excitation light and transmitting an adequate amount of the fluorescence light mounted on a full spectrum modified digital camera (Sony α 5000, 24 mm objective, F/6,5, ISO 800, 10 seconds shutter speed) with a fixed focal distance. In order to achieve uniform light distribution of the diode laser over the test region, a diffuser was used. A reference point was incorporated in the corner of each photograph in order to correct for frame-to-frame variations. The reference point consisted of a synthetic white diffusing bead on top of a fiber. The other end of the fiber was attached to an LED with an 810 nm peak emission generating a constant brightness at a fixed current/voltage.

Fluorescence images at skin surface, at 200 μm and at 400 μm depth were made. In order to enable imaging of deeper skin layers, the superficial skin layer was removed at the end of the application time by administering a pulse train with the CO_2 laser (Acuspot 712, kamami tonsil tip, Lumenis Inc.) at the following settings: Focused, Tonsil, spot size 2, depth 1, 25 (200 μm ablation depth) and 60 W (400 μm ablation depth). OCT was used to verify depth of the ablated skin layers at various laser settings (Fig. 1). The Acuspot 712 at the chosen settings provided a uniform removal of skin layers with minimal carbonization. Carbonized skin could be easily wiped off with a non-woven gauze.

Image Analysis

Fluorescence images were analyzed using ImageJ (ImageJ 1.50i, National Institute of Health, Bethesda, MD). Fluorescence intensity was assessed at the skin surface, at 200 μm (upper reticular dermis) and at 400 μm (lower reticular dermis) depth by measuring intensity on a 0–255 8 bit scale (0 representing black, 255 representing white).

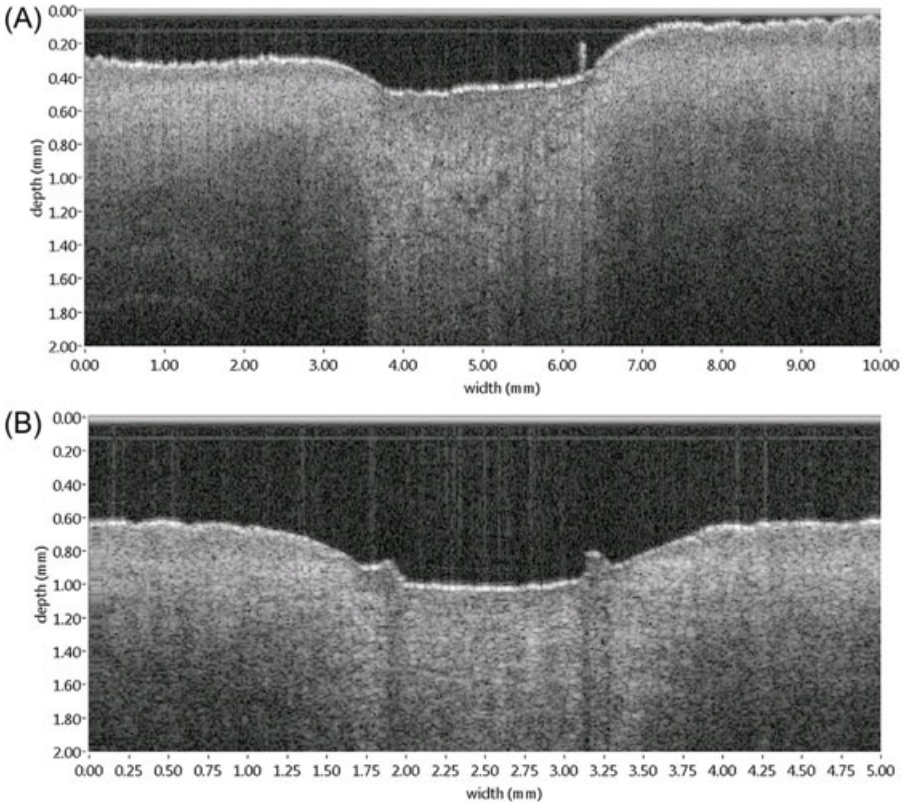

Fig. 1. OCT image of one of the skin samples after removal of the superficial skins layer with the non-fractional CO_2 laser. The depth in these images is given in millimeters in air. (**A**) 200 μm ablation depth. (**B**) 400 μm ablation depth.

Mean fluorescence intensity of entire test regions. A circular area, at a standardized size slightly smaller than the test region diameter (for the measurements in deeper skin layers, slightly smaller than the diameter of the full surface ablated area) was drawn. Mean fluorescence intensity within this circular area was measured. Images were corrected for autofluorescence of the skin by subtracting fluorescence intensity of the control image (no pre-treatment, no ICG) measured at the skin surface, at 200 μm and at 400 μm depth. Frame to frame variations were corrected for by the reference point.

Assessment of ICG distribution. In order to assess diffusion of ICG from the ablation channels into the skin between the channels, surface fluorescence intensity was measured at the 36 points located at the exact center of the areas between four ablation channels of one of the skin samples. These points represent the single pixels farthest from the ablation channels. The dispersion between the obtained 36 values also functioned as a measure for heterogeneity of ICG distribution within each test region. When ICG is equally distributed over the test region, little variation in fluorescence intensity at the 36 points is expected.

Furthermore, we plotted surface fluorescence intensity at each pixel across the diagonal of the individual test regions (Fig. 2). This enabled us to visualize the differences in fluorescence intensities between the center of the ablation channels, the coagulation zone surrounding the channels and the intact skin between the channels.

Statistical Analysis

The statistical analysis of the outcomes was performed using Statistical Package for the Social Sciences 23 (SPSS, Chicago, IL). All data were entered in the SPSS database. Means, medians, and interquartile ranges (IQR) were calculated. Wilcoxon signed rank test was used to compare paired data. The significance level was set at $P < 0.05$.

RESULTS

Visual Assessment of Fluorescence

In non-laser pretreated skin, fluorescence was weak, displaying an equal distribution (Fig. 3). In CO_2 laser pretreated skin, fluorescence was particularly concentrated around the ablation channels in what we, based on earlier studies, assume to be the laser coagulation zone, after 1 h application time [19]. With increasing application time, higher fluorescence intensities were seen in the areas between the ablation channels. Fluorescence was most intense at the test regions which had been treated with the enhancement techniques. Fluorescence at the test regions treated with acoustic pressure waves had an inhomogeneous patchy appearance.

Removal of the superficial layer of the skin with the non-fractional CO_2 laser, enabled us to visualize the skin at different depths in the reticular dermis dependent on the laser settings (Fig. 3B and C). At 200 μm depth the pattern of ablation channels was visible, except for the test regions that had not been treated with any of the penetration enhancement techniques, due to the absence of significant fluorescence. At 400 μm the channel pattern could not be identified, which is in agreement with the channel depth of 300 μm. In some images however, the bottom of the coagulation zone could still be seen.

Assessment of Fluorescence Intensity

Mean fluorescence intensity of entire test regions. Mean fluorescence intensities are displayed in Table 1. Fluorescence intensity was significantly higher at the skin surface and at 200 and 400 μm depth for all laser pretreated test regions compared to the non-laser pretreated test regions (12.94–89.97 arbitrary units [AU] vs. 1.98–23.50 AU; $P < 0.05$), regardless of the application time, and for all regions treated with any of the penetration enhancement techniques compared to the regions that were pretreated with the CO_2 laser alone (19.45–89.97 AU vs. 12.94–34.21 AU; $P < 0.05$, Fig. 4A) after 1 and 3 hours application time. After 24 hours application time, the only significant difference was found between acoustic pressure waves and CO_2 laser alone at 200 μm depth (mean 68.09 vs. 54.94; $P < 0.05$). No significant differences were found between the penetration enhancement techniques after 24 hours application time.

After 1 and 3 hours application time, the highest fluorescence intensity was observed at the test regions treated with acoustic pressure waves (21.28–89.97 AU), followed by massage (27.37–70.68 AU) and PVP (19.45–61.84 AU). Overall, differences were statistically significant ($P < 0.05$) but due to greater dispersion of data at the acoustic pressure wave regions after 3 hours application time, although giving the highest mean fluorescence intensity, no statistical significance could be found between acoustic pressure waves and massage or PVP. After 1 hour application time, high dispersion between the measurements at the acoustic pressure wave regions also led to partially non-significant differences compared to the other penetration enhancement techniques in the deeper skin layers (200 and 400 μm depth).

At the skin surface, a higher fluorescence intensity was observed after 1 h at the test regions treated with massage (38.43–64.81 AU) and acoustic pressure waves (mean 47.51–72.40 AU) compared to CO_2 laser pretreatment alone after 3 hours application time (mean 26.42–34.05 AU; $P < 0.05$). At both 200 and 400 μm depth, fluorescence intensity was significantly higher for the test regions treated with PVP (25.51–45.95 AU), massage (27.37–55.83 AU), and acoustic pressure waves (32.00–69.91 AU) after 1 hour application time compared to CO_2 laser pretreatment alone after 3 hours application time (12.94–26.49 AU; $P < 0.05$). Fluorescence intensity after 24 hours application time for the test regions that received CO_2 laser pretreatment alone (29.42–81.65 AU) was not significantly different compared to the test regions treated with the enhancement techniques after 1 and 3 hours application time, except for PVP after 1 and 3 hours application time at the skin surface (25.86–61.84 AU) and 1 hour application

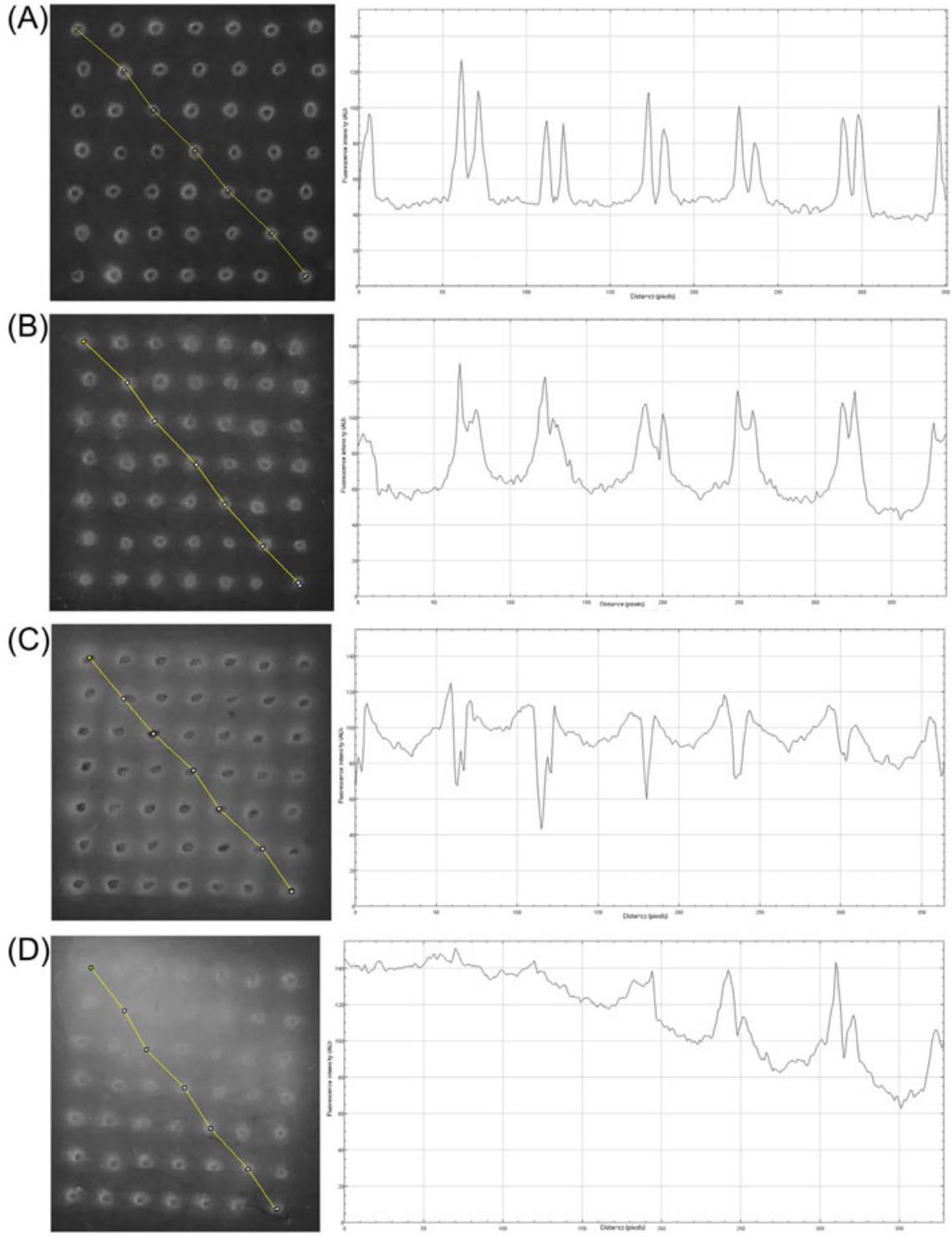

Fig. 2. Diagonal surface fluorescence intensity plots after 3 hours application time. The ablation channels can be recognized by the indentations between two peaks in fluorescence intensity, which represent the ICG saturated coagulation zones. (**A**) Fractional CO_2 laser alone. (**B**) Fractional CO_2 laser + pressure vacuum alterations (PVP). (**C**) Fractional CO_2 laser + massage. (**D**) Fractional CO_2 laser + acoustic pressure waves.

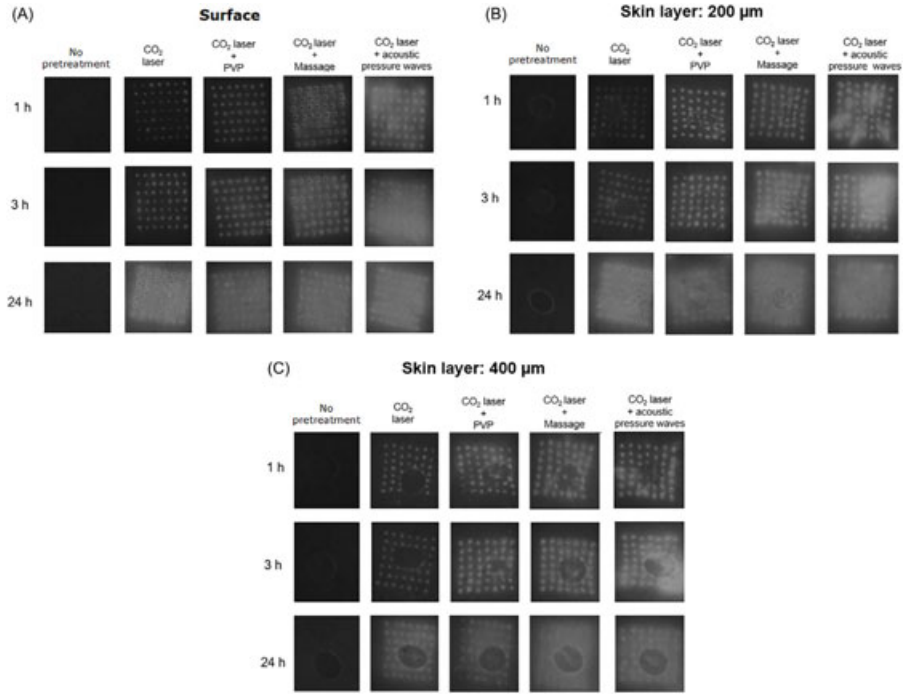

Fig. 3. Fluorescence photographs of one of the skin samples at (**A**) the skin surface, (**B**) 200 μm depth and (**C**) 400 μm depth after application of indocyanine green with various application times. Imaging of the deeper skin layers was performed after removal of the superficial skin layers with a non-fractional CO_2 laser. On the images this ablated zone is represented by the circular area in the center of the test regions. Fluorescence is most pronounced in the test spots treated with acoustic pressure waves, followed by massage, PVP and fractional CO_2 laser without the use of an enhancement technique, respectively. Fluorescence after application of indocyanine green on the intact skin was generally weak.

time at 200 μm depth (25.51–44.53 AU), which rendered lower fluorescence intensity ($P < 0.05$).

Assessment of ICG distribution. As described above, fluorescence intensity was measured at the 36 points at the skin surface located at the exact center of the areas between four ablation channels of one of the skin samples. After 1, 3, and 24 hours application time, median fluorescence intensity at the test regions treated with any of the penetration enhancement techniques was significantly higher compared to CO_2 laser alone

TABLE 1. Mean Fluorescence Intensities (Minimum-Maximum) in Arbitrary Units (AU) After Various Pretreatment/Penetration Enhancement Regimens and Application Times at Various Depths in the Skin

	Time	Surface (AU)	200 μm (AU)	400 μm (AU)
No pretreatment	1 hour	10.29 (8.13–14.34)	9.41 (2.54–16.85)	9.1 (1.98–12.50)
	3 hour	14.88 (6.90–22.62)	11.80 (4.67–18.44)	10.21 (4.66–19.18)
	24 hour	14.85 (9.16–23.50)	10.34 (3.57–19.44)	8.91 (4.89–19.13)
CO_2 laser alone	1 hour	22.17 (15.76–32.59)	19.92 (12.94–26.49)	17.75 (13.40–22.07)
	3 hour	31.58 (26.42–34.05)	27.76 (18.11–34.21)	22.00 (18.44–28.67)
	24 hour	55.59 (37.33–71.89)	54.94 (37.52–81.65)	43.56 (29.42–78.21)
CO_2 laser + PVP	1 hour	34.46 (25.86–45.82)	36.46 (25.51–44.53)	32.33 (20.49–45.95)
	3 hour	46.89 (37.52–61.84)	48.07 (36.05–58.79)	37.46 (19.45–50.90)
	24 hour	60.06 (44.50–75.85)	59.38 (46.24–79.91)	47.80 (32.18–71.70)
CO_2 laser + massage	1 hour	47.79 (38.43–64.81)	48.63 (44.09–55.83)	38.64 (27.37–46.68)
	3 hour	57.45 (45.21–69.41)	58.88 (44.74–70.68)	49.03 (33.52–62.87)
	24 hour	63.73 (54.97–72.66)	67.22 (52.06–78.09)	54.78 (45.49–61.95)
CO_2 laser + acoustic pressure waves	1 hour	60.86 (47.51–72.40)	57.84 (36.23–69.91)	50.20 (32.00–68.55)
	3 hour	61.45 (21.28–81.33)	64.84 (31.38–89.97)	54.06 (23.51–78.99)
	24 hour	65.52 (27.37–83.44)	68.09 (33.68–89.16)	56.53 (30.37–82.68)

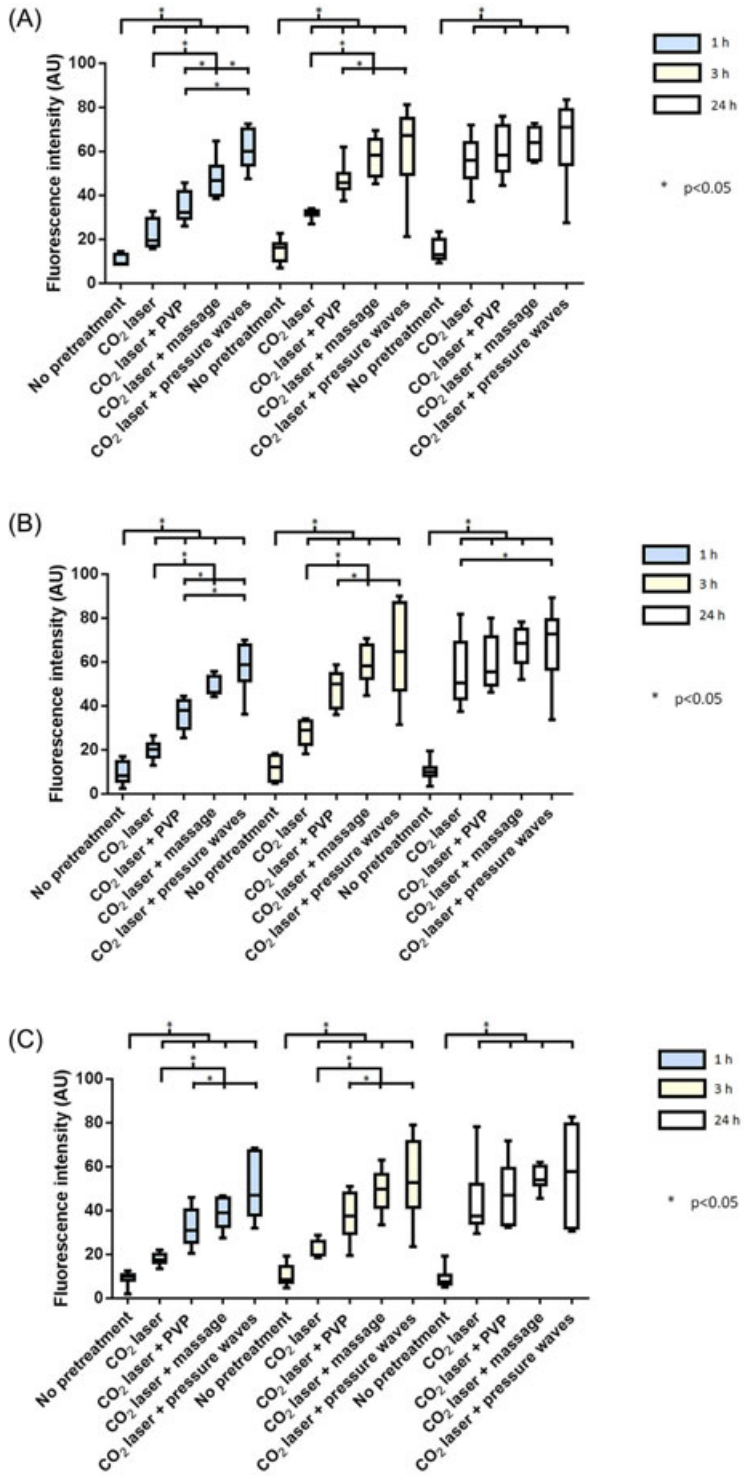

Fig. 4. Boxplots showing fluorescence intensities of indocyanine green in arbitrary units (AU) after various pretreatment / penetration enhancement regimens and application times. Fluorescence intensities are presented as median with ranges and interquartile ranges. (**A**) At the skin surface. (**B**) At 200 μm depth. (**C**) At 400 μm depth.

($P < 0.001$, except for PVP after 24 hours application time), indicating enhanced penetration of ICG into the tissue between the ablation channels. Especially after 3 hours, significant differences between the enhancement techniques were observed. Highest median fluorescence intensity was measured at the test regions treated with acoustic pressure waves (100.00), followed by massage (86.50) and PVP (58.00; $P < 0.001$; Fig. 5). After 24 hours

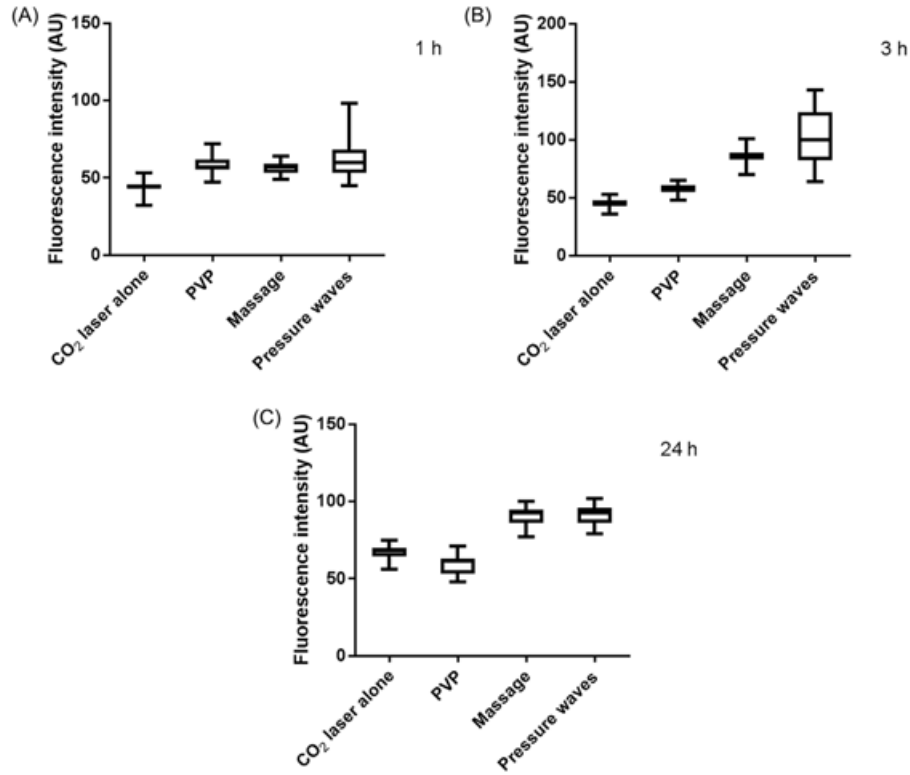

Fig. 5. Boxplots showing surface fluorescence intensity at 36 points located centrally between four ablation channels in the fractional laser grid. Fluorescence intensities are presented as median with ranges and interquartile ranges. (**A**) 1 hour application time. (**B**) 3 hour application time. (**C**) 24 hour application time.

application time, differences were smaller. Dispersion of fluorescence intensity between the 36 points of interest throughout the test region was relatively small for massage and PVP with small minimum-maximum range and interquartile range (IQR) regardless of application time, indicating a homogeneous distribution of ICG. In contrast, dispersion of fluorescence intensity after 1 and 3 hours application time was much larger for the test region treated with acoustic pressure waves (e.g., after 3 hours application time: minimum-maximum range: 79.00 for acoustic pressure waves vs. 31.00 for massage and 17.00 for PVP; IQR: 41.25 for acoustic pressure waves vs. 6.00 for both massage and PVP), in line with the patchy appearance of ICG distribution already visible with the naked eye. After 24 hours application time minimum-maximum range and IQR at the test region treated with acoustic pressure waves had decreased to a level similar to that of the other test regions (23.00 and 9.75, respectively vs. 23.00 and 8.75 for massage and 23.00 and 9.75 for PVP).

When the surface fluorescence intensity at each pixel across the diagonal of the test regions treated with CO_2 laser alone was plotted, fluorescence intensity after 3 hours application time was more than twice as high in the coagulation zones compared to the surrounding intact skin (Fig. 2A). At the test regions treated with PVP, only a slightly higher "baseline" fluorescence intensity in the intact skin was observed (Fig. 2B) compared to the test regions treated with CO_2 laser alone. At the test regions where massage was applied, fluorescence intensity in the intact skin was approximately twice as high compared to the test regions treated with CO_2 laser alone, and approached the intensities found in the coagulation zones (Fig. 2C). Corresponding with the patchy appearance of fluorescence at the test regions treated with acoustic pressure waves, the diagonal fluorescence intensity plot was very irregular, showing very high fluorescence intensities at one side of the test region to an extent where individual ablation channels and coagulation zones could not be identified from the graph anymore, and relatively low fluorescence intensity at the other side (Fig. 2D).

DISCUSSION

Our result show that efficacy of AFXL assisted delivery of ICG can be increased by the use of penetration enhancement techniques, such as massage, acoustic pressure waves, and pressure vacuum alterations. Although acoustic pressure waves appeared to give the highest peak accumulation of ICG in AFXL pretreated skin, ICG distribution in both the coagulation zones and the intact tissue surrounding the ablation channels was more homogeneous after massage. A comparable or even higher accumulation can be achieved already after a much shorter application time of 1 hour when using penetration enhancement techniques compared to 3–24 hours

application time without the use of penetration enhancement techniques. When application time is sufficiently long (24 hours), no additional effect of penetration enhancement techniques is observed. This may either be due to saturation of the tissue with ICG or depletion of ICG from the AFXL ablation channels. In both scenarios a similar amount of ICG is absorbed by the skin regardless of the penetration enhancement technique used.

To our knowledge, this is the first study comparing several penetration enhancement techniques in AFXL assisted drug delivery. The concept of pressure vacuum alterations was introduced in 2015 by Erlendsson et al. [17], who showed that active filling using a PVP device identical to the device used in our current study secures filling of AFXL channels and induced a deeper, greater, and more rapid delivery of polyethylene glycol in a Franz cell model. Waibel et al. [21] used a transdermal acoustic pressure wave device to enhance delivery of aminolevulinic acid in an *in vivo* study, showing increased penetration depth of aminolevulinic acid. In contrast, Choi et al. [27] did not find any additional effect of sonophoresis in AFXL assisted delivery of aminolevulinic acid. In one of the very first studies on the subject of AFXL assisted drug delivery skin massage did not seem to improve uptake of methyl aminolevulinate in the skin [5]. However, no comparative studies have been performed so far.

For most clinical applications, such as AFXL assisted photodynamic therapy or topical anesthesia, reaching optimal drug concentrations within a limited time span is essential. The use of penetration enhancement methods may help achieving this goal. However, we noticed some differences in execution time and practical feasibility between the three penetration enhancement techniques. The fastest execution time was provided using acoustic pressure waves, 14 seconds compared to 1 minute for massage and 3 minutes for PVP, making acoustic pressure waves more suitable for the treatment of large areas. Although average ICG accumulation appeared to be relatively high after treatment with the acoustic pressure wave device, distribution appeared to be less homogeneous and a relatively large variation between measurements in different samples was observed, indicating a limited reproducibility. Although slightly more time consuming, massage is still a quick and easy technique, that resulted in homogeneous and reproducible ICG delivery in our study, where massage was performed by the same investigator. However, the efficacy of massage may highly depend on massage technique and duration, making it less reproducible in clinical practice. PVP on the other hand is a standardized technique that can be easily performed using standardized parameters, but is limited by practical and safety issues, since very firm pressure to the skin is necessary to generate +1 bar. Prolonged application of −0.7 bar vacuum leads to the formation of petechiae and possibly even suction blisters. Besides, one should notice that acoustic pressure wave treatment requires a complex and expensive device, whereas PVP and massage are simple and inexpensive techniques.

We observed that after a short application time a large fraction of the available drug is probably located in the coagulation zones surrounding the ablation channels. The role of the coagulation zone in fractional laser drug delivery is not well understood yet. This shell of disintegrated tissue might act as a barrier for drug penetration although it has also been advocated that the coagulation zone functions as a reservoir for sustained release of a drug into the viable tissue [19,28,29]. Our findings are in line with those observed by Banzhaf et al. [19] in a recent study using fluorescence confocal microscopy with fluorescein as indicator drug. They described low fluorescence intensity in viable skin surrounding the coagulation zones, which increased over time but did not reach the same levels as in the coagulation zones within 4 hours. In our study, fluorescence intensity was almost equal over the entire treated area after 24 hours, which might be considered as a confirmation of the theory of sustained release through the coagulation zones.

Main limitations of this study are the *ex vivo* setting, the large number of test regions in a relatively small number of skin samples and the lack of histological controls. The skin samples all had been frozen for several days before being used for the experiments, because it was not possible to perform the experiments in freshly excised skin for logistic reasons. Surface fluorescence photography remains a relatively crude method offering limited options for quantitative measurements. On the other hand, the current setting enabled us to compare multiple parameters in a standardized and reproducible way, whereas histological evaluation is sensitive to sectioning errors and unwanted alterations in tissue dimensions during fixation procedures [30,31].

In addition, in our study, depths of up to 400 μm are investigated. However, issues with limited delivery by AFXL are most prominent at 1 mm or deeper, for example, when applying AFXL assisted drug delivery in the treatment of high-risk basal cell carcinoma [20]. The effect of penetration enhancement techniques on drug accumulation at larger skin depths still has to be investigated.

Another limitation is formed by the limited clinical applications for ICG in dermatology. In this study, ICG was mainly used as an indicator drug and one should be aware that absorption parameters for ICG on AFXL pretreated skin might not apply for drugs with different pharmacological properties. ICG is a hydrophilic substance (log P: −0.29) with a relatively large molecular weight (775 Da). Taking into consideration all drugs that have been used in fractional laser assisted drug delivery research so far, ICG shares some properties with, for example, methotrexate, which is also hydrophilic (Log P: −1.85) and has a molecular weight of ±500 Da [4,10,32]. Our findings can therefore be primarily extrapolated to this type of drugs. Methotrexate is an immunosuppressive but also a cytostatic drug that hardly penetrates through the intact stratum corneum and its AFXL assisted delivery may therefore create perspectives for future local treatment of both inflammatory skin disorders and skin tumors [10,32]. Previous studies have shown a potentially

AFXL channel depth dependent uptake of hydrophilic substances, as deeper AFXL channels tend to be rapidly filled with interstitial fluid, providing a more favorable environment for hydrophilic than for hydrophobic drugs [7,17,33]. Other substances that are frequently used in AFXL assisted drug delivery either have a very different molecular weight, such as methyl aminolevulinate (145 Da), or are lipophilic molecules, such as lidocaine (Log P: 2.44) and are hence less comparable to ICG.

In conclusion, PVP, acoustic pressure waves and massage increase accumulation of ICG in AFXL pretreated skin. A shorter application time is needed to reach similar or even higher concentrations of ICG when combining one of these penetration enhancement techniques with AFXL pretreatment compared to AFXL pretreatment of the skin alone. The use of an acoustic pressure wave device seems most effective regarding to peak accumulation, while massage is a simple and effective alternative giving homogeneous drug accumulation in the skin. PVP is also effective but is limited by practical drawbacks. Further studies are needed to confirm these findings *in vivo* with drugs with various different pharmacological properties.

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