

# Is the Fractional Laser Still Effective in Assisting Cutaneous Macromolecule Delivery in Barrier-Deficient Skin? Psoriasis and Atopic Dermatitis as the Disease Models

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## ABSTRACT

**Purpose** Most of the investigations into laser-assisted skin permeation have used the intact skin as the permeation barrier. Whether the laser is effective in improving cutaneous delivery via barrier-defective skin is still unclear.

**Methods** In this study, ablative (Er:YAG) and non-ablative (Er:glass) lasers were examined for the penetration of peptide and siRNA upon topical application on *in vitro* skin with a healthy or disrupted barrier.

**Results** An enhanced peptide flux (6.9 fold) was detected after tape stripping of the pig stratum corneum (SC). A further increase of flux to 11.7 fold was obtained after Er:YAG laser irradiation of the SC-stripped skin. However, the application

of Er:glass modality did not further raise the flux via the SC-stripped skin. A similar trend was observed in the case of psoriasiform skin. Conversely, the flux was enhanced 3.7 and 2.6 fold after treatment with the Er:YAG and the Er:glass laser on the atopic dermatitis (AD)-like skin. The 3-D skin structure captured by confocal microscopy proved the distribution of peptide and siRNA through the microchannels and into the surrounding tissue.

**Conclusions** The fractional laser was valid for ameliorating macromolecule permeation into barrier-disrupted skin although the enhancement level was lower than that of normal skin.

**KEYWORDS** atopic dermatitis · diseased skin · fractional laser · macromolecule · psoriasis · skin permeation

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## ABBREVIATIONS

|      |                             |
|------|-----------------------------|
| AD   | Atopic dermatitis           |
| AJ   | Adherent junction           |
| DEJ  | Dermal-epidermal junction   |
| FITC | Fluorescein isothiocyanate  |
| MTZs | Microscopic treatment zones |
| SC   | Stratum corneum             |
| TEWL | Transepidermal water loss   |
| TJ   | Tight junction              |

## INTRODUCTION

Skin disease is commonly observed as a human illness, affecting approximately 70% of the population worldwide. It is the fourth leading cause of disability globally (1). In the treatment of cutaneous disorders, topical application provides an appealing drug therapy method for nidus targeting, limitation of systemic toxicity, and noninvasive administration. However, the delivery of macromolecules is especially challenging due to

the skin's outer barrier. This barrier includes the stratum corneum (SC) and tight junction (TJ). Enhancement strategies are needed to overcome the skin's barrier function to promote topical macromolecule permeation. Over the last decade, the concept of fractional ablation or thermolysis has been developed as an active method to increase drug transport via the skin (2). Fractional approaches, such as laser, microneedle, radiofrequency, and microdermabrasion with a mask, have been employed to produce an array of microscopic defects surrounded by normal skin (3–5). The generated microchannels can be recovered quickly because each microchannel is small enough that it can be healed by the adjacent tissue (6).

The fractional laser is widely used to promote the absorption of drugs into the skin. It can create microchannels while precisely controlling the pore number and depth (7). The laser can be utilized for cutaneous drug delivery without cross-contamination risk (8). The fractional laser technique is found to be effective at enhancing the topical penetration of macromolecules, including proteins, dextran, and DNA (9,10). Both ablative and non-ablative lasers have commonly been used for assisting drug delivery. For example, the ablative Er:YAG laser emits light at 2940 nm, corresponding to the absorption peak of water, and leads to skin ablation with minimal thermal intervention (11). The non-ablative Er:glass laser emits a wavelength of 1550 nm that is capable of deep tissue interaction (12). In this technique, heat is mainly generated within the dermal tissue without removing the overlying skin surface.

Laser-assisted drug delivery is intended for application on diseased skin. The state of the disease is an important issue affecting topical drug permeation. Inflammatory skin diseases are always associated with impaired SC integrity and TJ function (13). Psoriasis, atopic dermatitis (AD), rosacea, lichen planus, and skin infections are examples of inflammatory disorders damaging the skin barrier function (14). Clinical use of the fractional laser for drug delivery has been limited until now (15). Most studies involving laser-assisted drug delivery focus on the *in vitro* and *in vivo* evaluation of the absorption level or dermatokinetics. Healthy skin is usually used as the diffusion barrier in these studies. Understanding the difference in drug absorption between intact and diseased skin is necessary. Drug permeation via disrupted skin is thought to be much higher than that via normal skin. Whether the fractional laser is still effective at enhancing topical drug delivery in lesional skin is unknown. This concern has not yet been thoroughly explored in the literature. The predominant aim of this study was to systematically determine whether the fractional laser can be used to increase peptide and siRNA penetration in diseased skin. Psoriasis-like skin and AD-like skin induced in mice were tested in this work. Some peptides show potential capability for treating skin aging, psoriasis, and AD (16). Gene silencing by siRNA opens a new perspective for managing psoriasis, skin allergies, and epidermolysis bullosa (17). Another aim of the present study was to determine the effect

of the fractional laser on the barrier function and structure of the SC and TJ in inflamed skin.

## MATERIALS AND METHODS

### Materials

A peptide conjugated with fluorescein isothiocyanate (FITC) with the sequence FITC-NH<sub>2</sub>-Pro-Arg-Leu-Leu-Tyr-Ser-Trp-His-Arg-Ser-His-Arg-Ser-His-COOH was synthesized by Biotools (New Taipei City, Taiwan). The siRNA sequences were 5'-fluorescein-UGCUGACUCCAAAG-3' and 3'-ACGACUGAGGUUC-5'. They were purchased from Genezyme (Miaoli, Taiwan). The molecular weights of the peptide and siRNA were 2335 and 4206 g/mol, respectively. The two macromolecules used in this study were simply the model peptide and siRNA for examining skin delivery with high stability (18,19). No bioactivity was found for either macromolecule.

### Lasers

An Er:YAG laser was purchased from Fotona (Ljubljana, Slovenia). The handpiece could deliver laser beams to produce microscopic treatment zones (MTZs). There were 8 microbeams with a diameter of 250 µm in a circle with an area of 0.785 cm<sup>2</sup>. The coverage of the microbeams in this area was 5%. The fluence used for the Er:YAG laser to ablate the skin was 3 J/cm<sup>2</sup>. An Er:glass laser was provided by Dinona (Daejeon, Korea). The spot diameter of the microbeam for producing MTZs was 100 µm. The spot density used was 529 spots/cm<sup>2</sup>. The laser fluence was set at 30 mJ with air cooling. The percentage of laser spot coverage in the irradiated area was 4.15%.

### Animals

A pathogen-free one-week-old pig was supplied by the Animal Technology Institute of Taiwan (Miaoli, Taiwan). An eight-week-old female nude mouse and an eight-week-old Balb/c mouse were provided by the National Laboratory Animal Center (Taipei, Taiwan). The animal experiments were performed in strict accordance with the recommendations in the Guidelines for the Care and Use of Laboratory Animals of Chang Gung University. The protocol was approved by the Institutional Animal Care and Use Committee of Chang Gung University.

### Induction of Psoriasis-like Skin in Mice

The induction method was carried out based on the protocol provided in our previous study (20). Briefly, the dorsal skin fur

of a Balb/c mouse was shaved. Imiquimod cream (Aldara®, 3 M, St. Paul, MN, USA) at a dose of 62.5 mg was topically applied on the back daily for 6 consecutive days. The dorsal skin was covered with Tegaderm® and non-woven cloth after the administration of Aldara®.

### Induction of AD-Like Skin

The epicutaneous sensitization protocol for inducing AD-like skin was the same as that used in our previous study (21). A Balb/c mouse was sensitized with 1 mg/ml ovalbumin (0.1 ml) by intraperitoneal administration every other day for 10 days. On day 8, the dorsal fur was removed and tape-stripped 6 times. Ovalbumin in saline (1 mg/ml) in a volume of 100 µl was pipetted onto a 1-cm<sup>2</sup> sterile gauze, which was topically applied to the back. The gauze was administered every day for 7 days.

### Observation of the Skin Surface

Macroscopic observation of the skin surface with or without laser exposure was visualized using a handheld digital magnifier (Mini Scope-V, M and T Optics, Taipei, Taiwan). The skin surface was also visualized by optical microscopy (DMi8, Leica, München, Germany). A 200× magnification was employed to capture the images.

### Recovery of Laser-Irradiated Skin

The recovery of skin physiology after laser exposure was determined by transepidermal water loss (TEWL) (TM300, Courage and Khazaka, Köln, Germany) and skin blood flow (LAB Laser Doppler Flowmetry, Moor, Devon, UK). The duration for this determination was 24 h after the laser treatment.

### Histology

The mouse was sacrificed after the laser treatment. The treated skin area was excised for histological analysis. The skin samples were immersed in paraffin wax and then sliced. The sectioned skin was stained with hematoxylin and eosin. The skin morphology was imaged under a light microscope.

### mRNA Levels of TJ-Related Proteins

The mRNA amounts of filaggrin, involucrin, and integrin β1 in the skin were measured. Total RNA was extracted with TRIzol® reagent (Thermo Fisher, Waltham, MA, USA). cDNA was synthesized by reverse transcription using an iScript® cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Quantitative real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's

instructions. The primers used to analyze target mRNA were the same as those used in our previous study (22).

### Preparation of SC-Disrupted Skin

Full-thickness skin was excised from the dorsal region of the pig and nude mouse after sacrifice. SC-stripped skin was obtained by stripping the skin surface 20 times with adhesive tape (Scotch®, 3 M). Delipid skin was obtained by incubating the skin surface under a chloroform/methanol mixture (2:1) for 2 h (23). To prepare desebum skin, the SC side of the skin was washed with cold hexane (4°C) five times based on the procedure utilized previously (24). The deprotein skin was obtained by 40% ethanol/water treatment on the skin surface for 2 h. The proteins were denatured after this procedure (25).

### Skin Permeation Assessment of Peptide

A Franz diffusion cell was employed to detect peptide penetration via the skin. The skin was mounted between the donor and receptor with the SC facing up toward the donor. The receptor medium (5 ml) was pH 7.4 phosphate buffer. The donor medium (0.5 ml) was peptide (150 µM) in pH 7.4 buffer. The effective permeation area of the Franz cell was 0.785 cm<sup>2</sup>. The stirring rate of the stir bar and the receptor temperature were kept at 600 rpm and 37°C, respectively. At the determined intervals of 0, 2, 4, 8, 12, 12, 24, 36, and 48 h, 300 µl of receptor medium was withdrawn to analyze peptide accumulation. Fresh buffer was added to the receptor to maintain a constant volume. Peptide penetration into the receptor was quantified by a fluorescence spectrophotometer (F2500, Hitachi, Tokyo, Japan). Differential stripping and cyanoacrylate casting were used to determine peptide uptake in the hair follicles as previously described (26).

### Peptide and siRNA Distribution in the Skin

The skin was removed from the Franz cell after a 24-h application of peptide (150 µM) or siRNA (0.4 µM) in pH 7.4 buffer. After being washed with double-distilled water, the skin sample was directly positioned onto the stage plate of a confocal laser scanning microscope (TCS SP2, Leica). The skin thickness was scanned at 5 µm increments via the z-axis from the skin surface. The images were taken by summing 15 fragments (total 75 µm). The 3-D images of the skin were also processed by confocal microscopy software.

### Statistical Analysis

Significant differences in experimental group data were evaluated using the Kruskal-Wallis test. The post hoc test used to assess individual differences was Dunn's test. A 0.05 level of probability was taken as the statistical significance.

## RESULTS

### Laser Treatment on Pig and Nude Mouse Skin

A handheld digital magnifier and an optical microscope offered visualization of the changes caused by laser exposure on the pig and nude mouse skin surfaces. Figure 1a shows en face views of the skin with and without laser treatment. The morphology of the skin surface without treatment was found to be intact with no damage. The fractional laser was topically applied to the skin in precise alignment with

the microchannels in the skin (black arrows). At high magnification of the skin surface by bright field microscopy, a single micropore created by the Er:YAG laser was found in both the pig and nude mouse skin (white arrows in Fig. 1b). The pores showed a cylindrical shape on the disrupted SC. The diameter of the microchannels was approximately 250–300  $\mu\text{m}$ , approximating the irradiated spot size of the Er:YAG laser (250  $\mu\text{m}$ ). The Er:glass laser revealed smaller MTZs compared to the Er:YAG laser (red arrows in Fig. 1b). The microchannel was approximately 150  $\mu\text{m}$  in diameter.

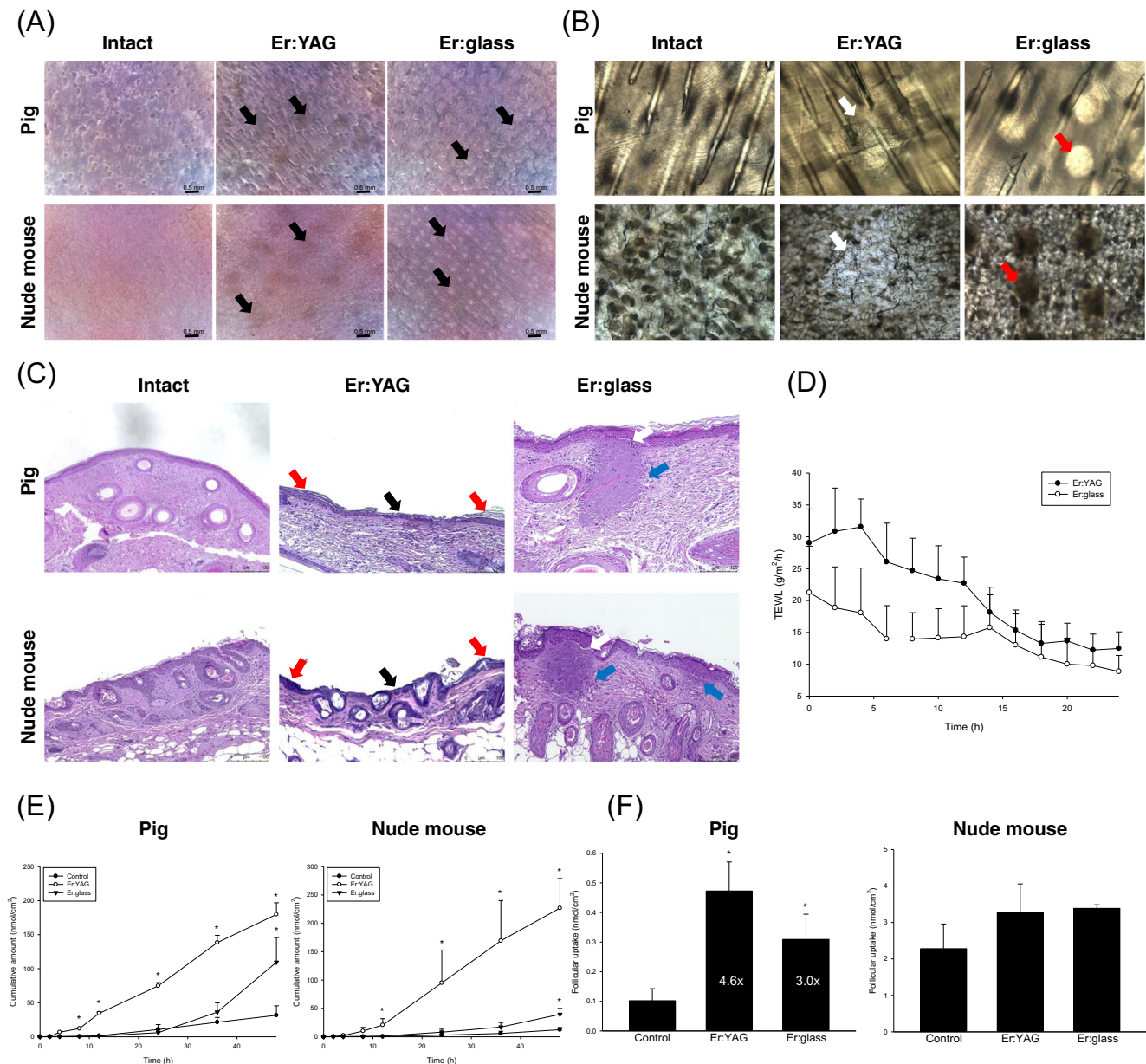


**Fig. 1** The Er:YAG and Er:glass laser treatment on pig and nude mouse skins: **(a)** the close-up images of skin surface captured by handheld magnifier; **(b)** the microscopic images of skin surface captured by optical microscopy; **(c)** the histology of cross section of skin; **(d)** the transepidermal water loss (TEWL)-time profile of nude mouse skin after laser treatment; **(e)** the cumulative amount-time profile of peptide permeation; and **(f)** the follicular uptake of peptide. The data inside the column are the enhancement ratio of the laser-treated group compared to the untreated control. \*, the data were significantly higher ( $p < 0.05$ ) compared to untreated control. The data are presented as the mean of 4 experiments  $\pm$  S.D.

Figure 1c shows cross sections of the pig and nude mouse skin. The untreated skin remained intact in all skin layers. The SC layer was compact and dense in the control skin. The Er:YAG laser removed the SC but left the viable skin intact (black arrows in Fig. 1c). The SC still adhered to the skin surface adjacent to the MTZ (red arrows in Fig. 1c). Following irradiation by the Er:glass laser, the histological images showed well-delineated fractional columns of thermal damage in the dermis discerned by intact surrounding tissue (blue arrows in Fig. 1c). There was isolated disruption in the dermal-epidermal junction (DEJ) of each microcolumn (white arrows in Fig. 1c). The SC of the Er:glass laser-treated MTZs remained anatomically intact with a small loss of cohesion. Figure 1d demonstrates the kinetics of TEWL after irradiation by the fractional laser on the dorsal skin of the nude mouse. TEWL is a parameter for detecting the barrier function of skin. The baseline of TEWL (untreated group) was approximately 10 g/m<sup>2</sup>/h. The ablative laser treatment resulted in SC removal and was accompanied by an immediate increase in TEWL to 29 g/m<sup>2</sup>/h ( $p < 0.05$ ). The barrier function of the Er:YAG laser-treated skin was recovered over a period of several hours, and the TEWL returned to the baseline at 18 h post treatment. Immediately after treatment by the non-ablative laser, the TEWL was 21 g/m<sup>2</sup>/h, suggesting less damage to the SC and TJ compared to that sustained from treatment with the ablative laser ( $p < 0.05$ ). Similar to the case of the Er:YAG laser, the TEWL of the Er:glass-treated skin at 18 h after irradiation reached the value before irradiation.

Figure 1e shows a time course of the cumulative amounts of peptide permeated per unit area from the pig and nude mouse skin. The untreated control represented a significant barrier to the peptide transport. The fractional laser treatment produced MTZs that increased the peptide permeation with statistical significance ( $p < 0.05$ ), with the Er:YAG laser showing greater enhancement. The peptide flux across the pig skin was 4.02 and 2.05 nmol/cm<sup>2</sup>/h for the Er:YAG laser and the Er:glass laser, respectively. The flux values for the nude mouse skin in the case of the ablative and non-ablative laser were 5.20 and 0.79 nmol/cm<sup>2</sup>/h, respectively. Figure 1f depicts the follicular accumulation in the pig and nude mouse skin after laser irradiation. Peptide uptake in the pig follicles was improved by the laser compared to that in the control ( $p < 0.05$ ). The uptake enhancement ratio by the laser is displayed inside the column in Fig. 1f. The laser did not enhance follicular peptide delivery in the case of nude mouse skin, although the mean value of the laser-treated group was higher than that of the control.

### Laser Treatment on SC-Disrupted Skin

We examined the permeation property of topically applied peptide on pig and nude mouse skin with pretreatment by SC stripping, lipid removal, sebum removal, and protein

degradation. The SC barrier could be disrupted in these ways. Figure 2a represents the peptide flux across the SC-stripped skin with and without laser assistance. Under the stripping pretreatment, the flux enhancement ratios relative to the intact skin were 6.9 and 22.0 for the pig and nude mouse skin, respectively. The Er:YAG modality further increased the flux 11.7- and 33.0-fold compared to that in the intact skin, respectively. This further enhancement was not detectable with the Er:glass laser. Lipid removal resulted in less flux enhancement compared to that in SC-stripped skin (Fig. 2b). The ablative and non-ablative lasers showed similar fluxes in the delipid pig skin, which was higher than the flux without laser irradiation. This phenomenon was not significant in the case of the nude mouse skin, although the mean flux value was higher in the laser-treated group than in the control group. Sebum removal was efficient at increasing the peptide fluxes in the pig and nude mouse skin by 3.1- and 4.6-fold, respectively (Fig. 2c). A further flux increase was detected by application of the fractional laser on desebum skin. Comparable flux was achieved for the desebum skin treated with the Er:YAG and Er:glass lasers. With respect to the skin with denatured protein in the SC, the Er:YAG laser did not promote peptide flux compared to the deprotein skin without laser treatment. A similar effect was observed for the Er:glass laser.

### Laser Treatment on Psoriasis-like Skin

The Balb/c mouse model was employed for the induction of inflammatory skin since it has been proven to be useful for simulating psoriasis and AD. Figure 3a demonstrates magnified images of psoriasiform skin. There were scaly lesions and erythema in the psoriasis-like skin. Dots on the skin surface proved the creation of MTZs by both lasers (black arrows in Fig. 3a). Microscopic images of the psoriatic skin surface showed silvery scales (Fig. 3b). The Er:YAG laser ablated the surface of the psoriasis-like skin with a diameter of approximately 300  $\mu$ m (white arrow in Fig. 3b). The Er:glass laser created an array of microchannels with clear morphology (red arrow in Fig. 3b). All MTZs were uniform and reproducible. At the microscopic level, in the cross section of psoriasiform skin, the desquamation of epidermal hyperproliferation is shown in Fig. 3c. The epidermal thickness was 4-fold greater in the psoriatic skin than the intact skin. Significant immune cell infiltration was detected in the dermis of the psoriasis-like skin. The Er:YAG laser removed the SC with no significant effects on the viable skin (black arrow in Fig. 3c). The non-ablative laser resulted in the creation of cone-shaped coagulation in the viable skin (blue arrows in Fig. 3c).

The integrity of the psoriatic skin was measured by TEWL as summarized in Table I. TEWL was significantly increased from 8 to 35 g/m<sup>2</sup>/h by imiquimod treatment for prompting psoriasis-like skin. Both fractional lasers enhanced the TEWL

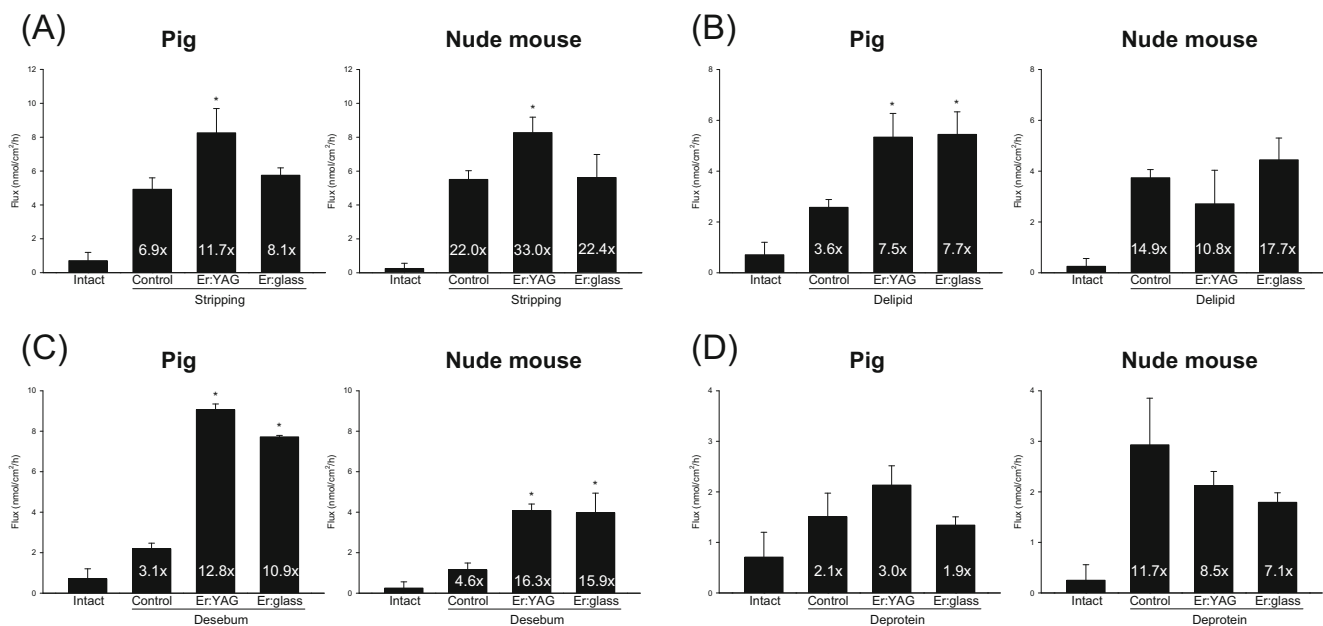


**Fig. 2** The Er:YAG and Er:glass laser treatment on stratum corneum (SC)-disrupted skins: **(a)** the comparison of peptide flux (nmol/cm<sup>2</sup>/h) via SC-stripped skin; **(b)** the comparison of peptide flux (nmol/cm<sup>2</sup>/h) via delipid skin; **(c)** the comparison of peptide flux (nmol/cm<sup>2</sup>/h) via desebum skin; and **(d)** the comparison of peptide flux (nmol/cm<sup>2</sup>/h) via deprotein skin. The data inside the column are the enhancement ratio of the SC-disrupted group compared to the intact skin control. \*, the data were significantly higher ( $p < 0.05$ ) compared to untreated control. The data are presented as the mean of 4 experiments  $\pm$  S.D.

of healthy skin to 37~40 g/m<sup>2</sup>/h. The TEWL of psoriasisform skin was increased to 41~42 g/m<sup>2</sup>/h by both lasers. Erythema was calculated using laser Doppler flowmetry that measured the movement of erythrocytes. The lasers increased blood flux in the case of healthy skin as shown in Table II. However, this increase did not reach statistical significance because of the large inter-subject variation. The elevation of blood flux from 132 to 362 indicated the production of erythema in the psoriatic skin. The exposure of psoriatic skin to lasers did not further increase the erythema. As with SC, TJ in the epidermis also presents a barrier to macromolecule delivery. Some TJ-related proteins were quantified at the mRNA level using PCR analysis as depicted in Fig. 3d. The non-ablative laser downregulated filaggrin and involucrin in the intact skin but the ablative laser did not. The integrin  $\beta$ 1 level was not changed by exposure to either laser. Imiquimod treatment significantly reduced all TJ-related proteins examined in this study. Treatment with both fractional lasers on psoriasisform skin significantly decreased the expression of filaggrin, accompanied by no influence on involucrin or integrin  $\beta$ 1. As shown in Fig. 3e, the degradation of the intact skin barrier function of the Balb/c mouse by the Er:YAG and Er:glass lasers enhanced the peptide flux by 25.3- and 14.5-fold, respectively. In the psoriatic skin, peptide permeation was increased by the ablative laser over the control group. Nevertheless, the enhancement level by this laser in the psoriasis-like skin (3.0-fold) was less than that in the intact skin. The non-ablative laser showed no enhancement of peptide flux after irradiation on the psoriasisform skin.

### Laser Treatment on AD-Like Skin

The Balb/c mouse skin was challenged by ovalbumin to produce dry and scaly skin as demonstrated in Fig. 4a. Some deep furrows could be seen in the AD-like skin. The gross appearance of the laser-irradiated AD-like skin showed an array of dots on the surface (black arrows in Fig. 4a). Close-up images of the AD-like skin surface also clearly showed MTZs after laser exposure (white and red arrows in Fig. 4b). The skin section displayed a thickened epidermis from ovalbumin sensitization (Fig. 4c). The infiltration of immune cells in the dermis was significant. Like in the other skin models, the Er:YAG laser removed the SC without viable skin damage (black arrow in Fig. 4c). A coagulation zone and DEJ disruption were visualized in the Er:glass laser-treated AD-like skin (blue and white arrows in Fig. 4c).

The TEWL of AD-like skin was approximately 2 times greater than that of healthy skin, as shown in Table I. TEWL was significantly increased from 19 to 45 g/m<sup>2</sup>/h by the Er:YAG laser. Er:glass laser irradiation on AD-like skin generated a higher TEWL (37 g/m<sup>2</sup>/h) than the control. Ovalbumin treatment did not significantly increase the blood flux (Table II). Neither the Er:YAG laser nor the Er:glass laser increased the blood flux of AD-like skin. The TJ-related proteins were examined to evaluate whether the laser destroyed the epidermal barrier of the AD-like skin. As demonstrated in Fig. 4d, the ablative laser significantly downregulated integrin  $\beta$ 1. Filaggrin and integrin  $\beta$ 1 expression were decreased by the non-ablative laser. Increased peptide flux was achieved by

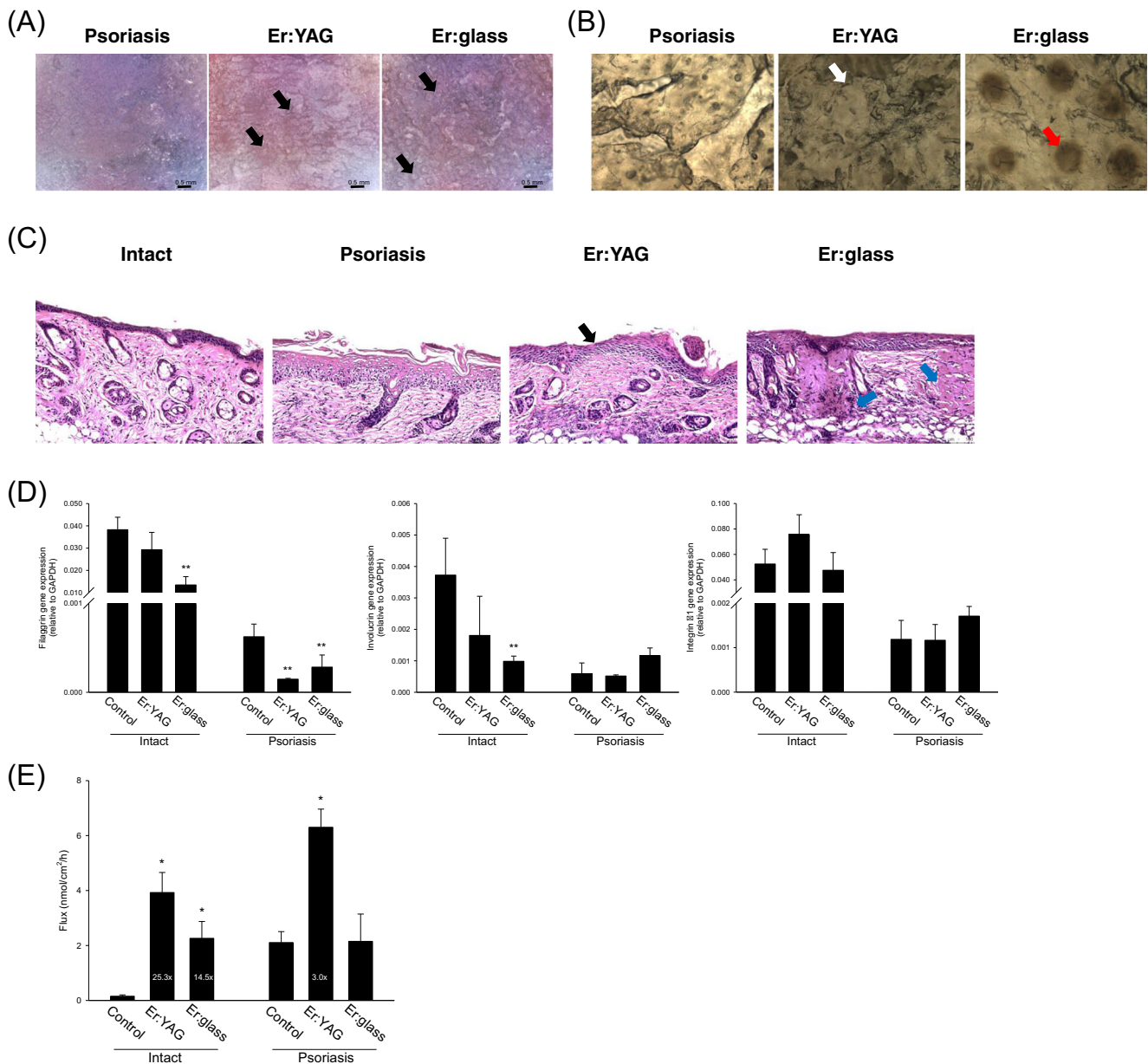


**Fig. 3** The Er:YAG and Er:glass laser treatment on psoriasis-like skin: **(a)** the close-up images of skin surface captured by handheld magnifier; **(b)** the microscopic images of skin surface captured by optical microscopy; **(c)** the histology of cross section of skin; **(d)** the mRNA level of filaggrin, involucrin, and integrin  $\beta 1$ ; and **(e)** the peptide flux (nmol/cm<sup>2</sup>/h). The data inside the column are the enhancement ratio of the laser-treated group compared to the untreated control. \*, the data were significantly higher ( $p < 0.05$ ) compared to untreated control. \*\*, the data were significantly lower ( $p < 0.05$ ) compared to untreated control. The data are presented as the mean of 4 experiments  $\pm$  S.D.

**Table I** The Transepidermal Water Loss (TEWL, g/m<sup>2</sup>/h) of Healthy, Psoriasis-Like, and Atopic Dermatitis-Like Skin after Treatment of Fractional Lasers

| Treatment | Healthy skin     | Psoriasis-like skin | Atopic dermatitis-like skin |
|-----------|------------------|---------------------|-----------------------------|
| Control   | 8.47 $\pm$ 1.85  | 35.40 $\pm$ 8.45    | 19.38 $\pm$ 4.16            |
| Er:YAG    | 39.70 $\pm$ 3.59 | 41.53 $\pm$ 9.24    | 45.22 $\pm$ 6.85            |
| Er:glass  | 36.55 $\pm$ 8.07 | 40.67 $\pm$ 6.65    | 36.52 $\pm$ 7.05            |

Each value represents the mean  $\pm$  SD ( $n = 6$ )

**Table II** The Blood Flux (arbitrary unit) of Healthy, Psoriasis-Like, and Atopic Dermatitis-Like Skin after Treatment of Fractional Lasers

| Treatment | Healthy skin       | Psoriasis-like skin | Atopic dermatitis-like skin |
|-----------|--------------------|---------------------|-----------------------------|
| Control   | 132.17 $\pm$ 23.19 | 361.77 $\pm$ 89.85  | 157.03 $\pm$ 37.39          |
| Er:YAG    | 196.91 $\pm$ 56.66 | 305.84 $\pm$ 93.67  | 205.58 $\pm$ 66.77          |
| Er:glass  | 200.40 $\pm$ 61.61 | 315.79 $\pm$ 94.25  | 173.39 $\pm$ 50.75          |

Each value represents the mean  $\pm$  SD ( $n = 6$ )

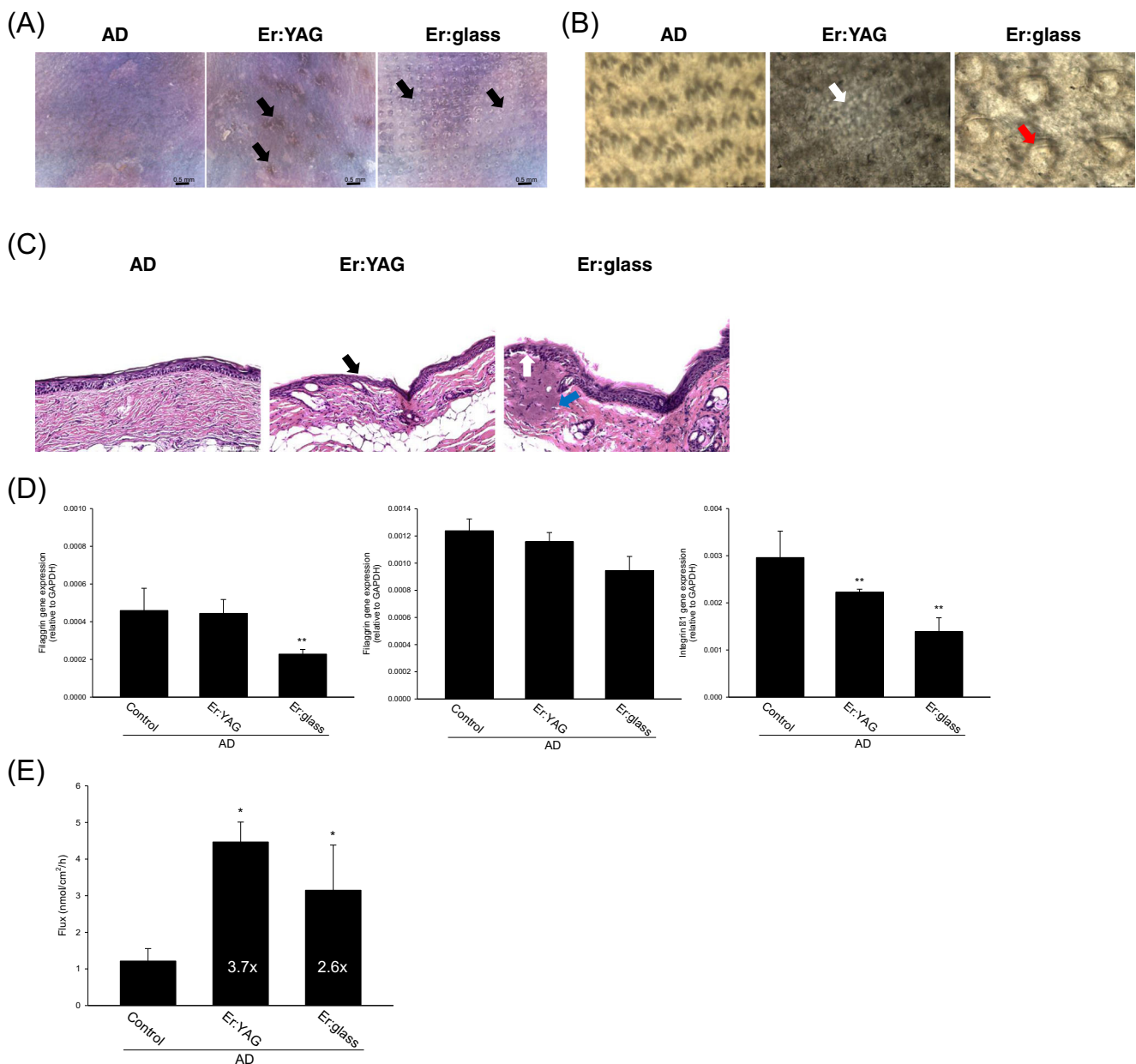


**Fig. 4** The Er:YAG and Er:glass laser treatment on atopic dermatitis (AD)-like skin: **(a)** the close-up images of skin surface captured by handheld magnifier; **(b)** the microscopic images of skin surface captured by optical microscopy; **(c)** the histology of cross section of skin; **(d)** the mRNA level of filaggrin, involucrin, and integrin  $\beta 1$ ; and **(e)** the peptide flux (nmol/cm<sup>2</sup>/h). The data inside the column are the enhancement ratio of the laser-treated group compared to the untreated control. \*, the data were significantly higher ( $p < 0.05$ ) compared to untreated control. \*\*, the data were significantly lower ( $p < 0.05$ ) compared to untreated control. The data are presented as the mean of 4 experiments  $\pm$  S.D.

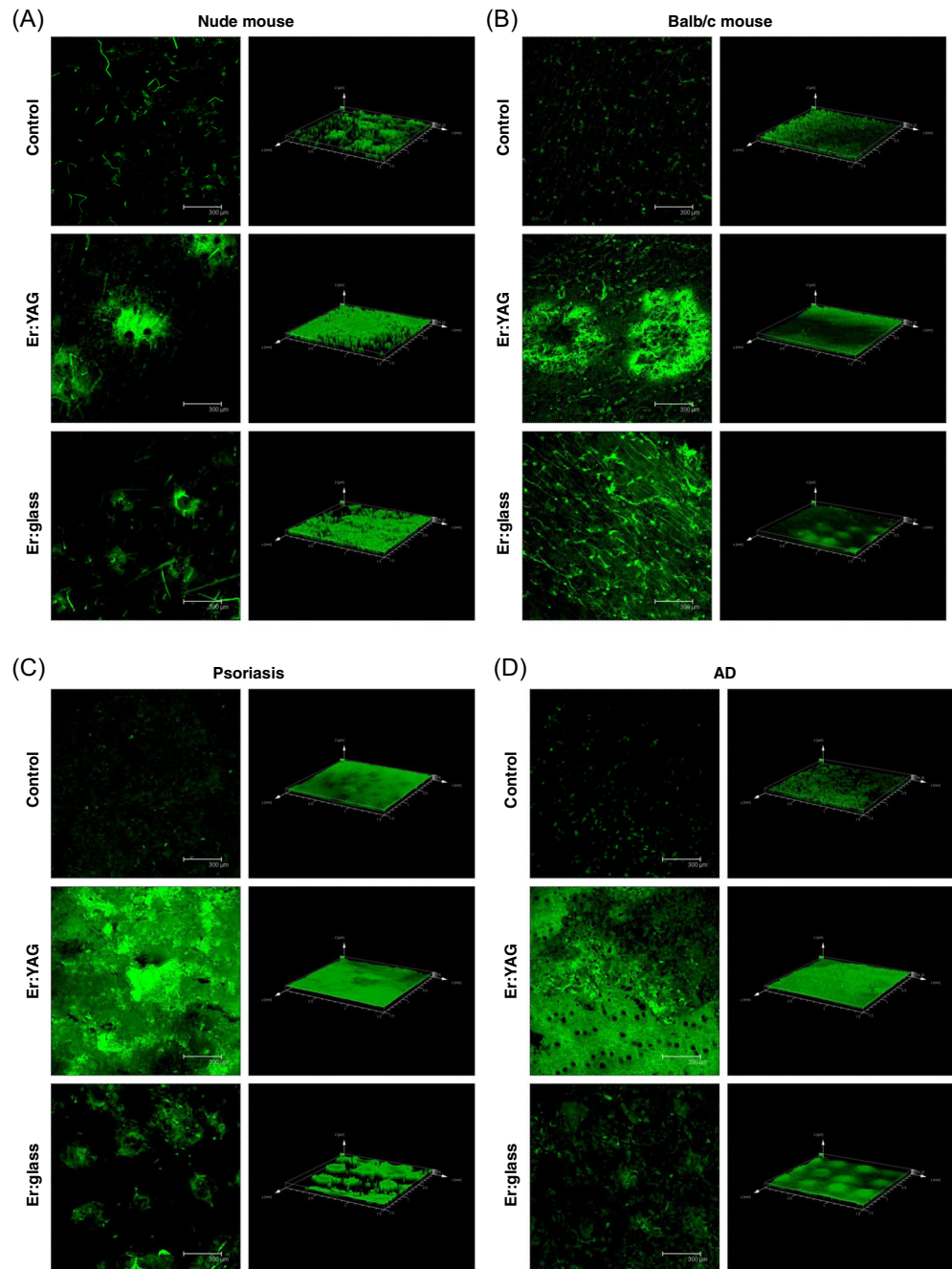
treatment of the AD-like skin with fractional lasers (Fig. 4e). The fluxes of the Er:YAG and the Er:glass groups were 3.7- and 2.6-fold higher than those of the control skin.

#### Peptide and siRNA Distribution in the Skin

A permeation experiment was also conducted by observing macromolecule distribution in the skin. Figure 5 shows confocal images of intact skin and inflamed skin with peptide absorption. The left panel of the figure is a planar image

summarizing 15 separate sections. The right panel is the 3-D skin structure of the merged photographs. After the topical application of peptide, faint fluorescence was detected in the nude mouse skin without laser treatment (Fig. 5a). After irradiation with the Er:YAG laser, a deposit of fluorescence with disc-like shapes was observed. The diameter of the fluorescence-stained microchannels was approximately 320  $\mu$ m, which was greater than the irradiation dot diameter of the Er:YAG laser. This suggested radial diffusion of the permeant. Er:glass laser treatment led to the formation of a

**Fig. 5** Confocal microscopic images of skin with topical peptide application: **(a)** intact nude mouse skin; **(b)** intact Balb/c mouse skin; **(c)** psoriasis-like skin; and **(d)** atopic dermatitis (AD)-like skin. The summary of 15 fragments from the skin surface to the depth of  $75\ \mu\text{m}$  is displayed in the left panel of this figure. The 3D structure of the summary of 15 fragments is displayed in the right panel of this figure.



well-defined array of microchannels. The discs produced by the non-ablative laser had diameters of approximately  $150\ \mu\text{m}$ . A similar trend was visualized in intact Balb/c mouse skin (Fig. 5b).

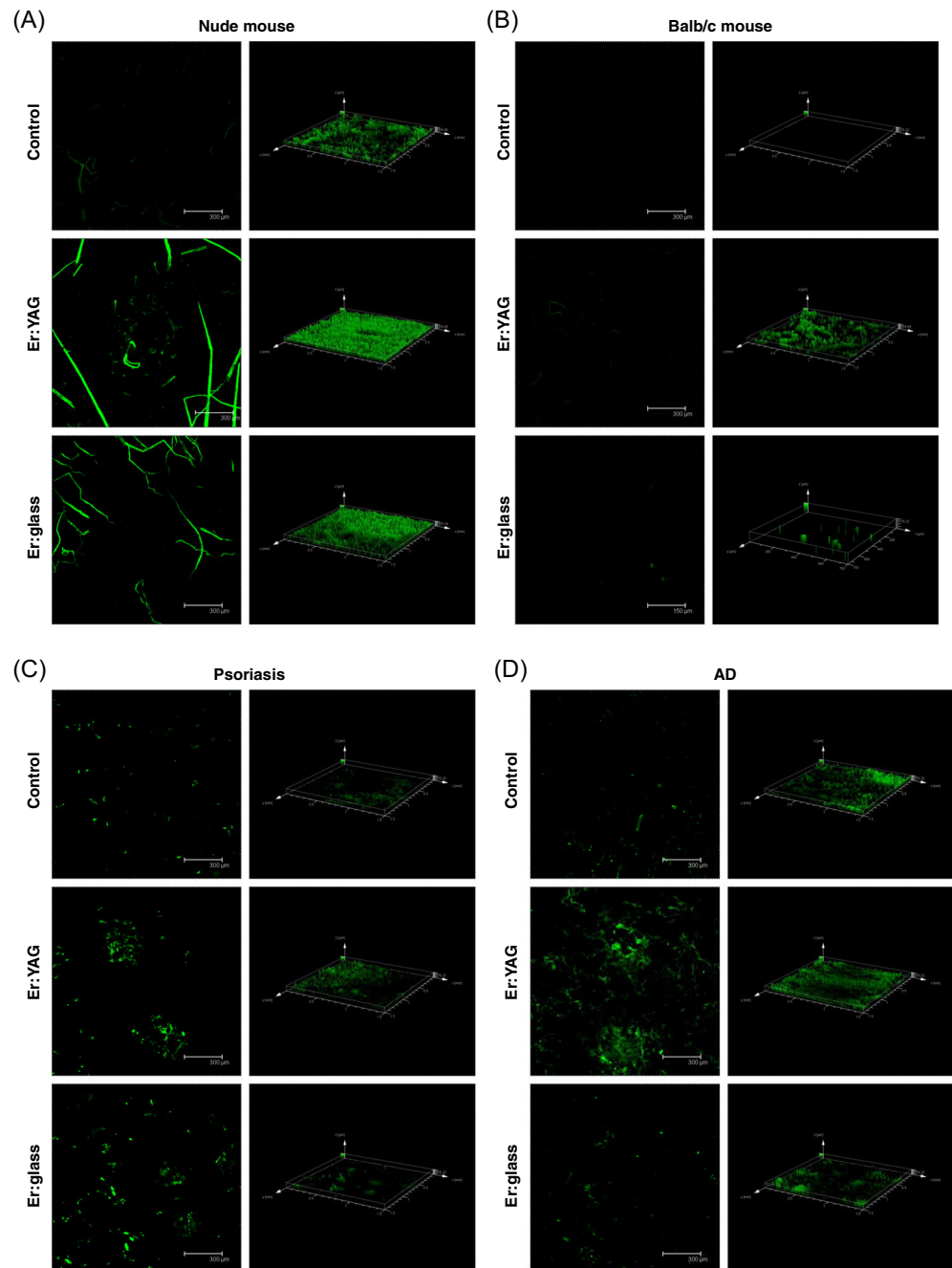
The array of peptide deposition in the laser-treated skin is clearly observable in the 3-D mode. In the 3-D image of Er:YAG laser-treated Balb/c mouse skin, the peptide was mainly located in the margins of the MTZs. A pale fluorescence signal was observed in the center of the micropores. On the other hand, the peptide filled the MTZs created by the Er:glass laser (Fig. 5b). This was because the ablative laser

perforated the MTZs to form holes with a void space. Thus, the permeant partitioned and diffused into the rim. The fluorescence signal of both the psoriasis- and AD-like skin was more intense than that of healthy skin in the absence of laser perforation (Fig. 5c and d). The peptide showed more facile penetration into the barrier-defective skin than into the intact skin. The fluorescence of the Er:YAG laser-treated inflamed skin was not limited to the MTZs but was distributed extensively throughout the skin. 3-D images of the Er:glass laser-treated lesional skin showed that the peptide deposits were oriented parallel to the skin surface.

An siRNA with a larger molecular size than the peptide was also utilized as a model permeant in this experiment. As shown in Fig. 6a, a weak siRNA signal was observed on the superficial layer of the nude mouse skin. A single micropore stained with siRNA in the center of the Er:YAG laser-treated nude mouse skin was found. The filaments in the images of the laser-treated nude mouse skin could be the hair shafts stained with siRNA. This indicated a possible penetration pathway of hair follicles for laser-assisted siRNA delivery. Topical application of siRNA onto the control Balb/c mouse skin resulted in essentially no

detectable fluorescence (Fig. 6b). Application of the fractional laser revealed minimal enhancement of siRNA absorption in the Balb/c mouse skin. The impaired skin showed stronger siRNA fluorescence compared to that in the intact Balb/c mouse skin (Fig. 6c and d). The green siRNA was found inside the laser-generated microchannels of both the psoriasiform and AD-like skin, demonstrating increased siRNA absorption into the diseased skin under the assistance of the fractional laser. The larger molecular size of the siRNA produced less deposition inside the microchannels than the peptide.

**Fig. 6** Confocal microscopic images of skin with topical siRNA application: **(a)** intact nude mouse skin; **(b)** intact Balb/c mouse skin; **(c)** psoriasis-like skin; and **(d)** atopic dermatitis (AD)-like skin. The summary of 15 fragments from the skin surface to the depth of 75  $\mu\text{m}$  is displayed in the left panel of this figure. The 3D structure of the summary of 15 fragments is displayed in the right panel of this figure.



## DISCUSSION

Using a fractional laser is an efficient way to overcome the cutaneous barrier function to enhance topical macromolecule delivery. The permeation kinetics of permeants in diseased skin are quite different from those in healthy skin. Permeability is thought to be increased in barrier-disrupted skin. Whether the fractional laser can further promote macromolecule permeation into diseased skin is uncertain. In this study, the laser-assisted permeation of a peptide and siRNA was investigated on animal skin with an intact or damaged barrier. Skin was artificially manipulated to develop SC-disrupted models to mimic diseased skin. These models included SC-stripped, delipid, desebum, and deprotein skin. Psoriasis- and AD-like skin were also developed as barriers to simulate the clinical therapeutic status. The experimental results showed that both the ablative and non-ablative lasers were able to ameliorate macromolecule transport in the impaired skin models, although the enhancement level was lower than that in normal skin. We demonstrate for the first time the use of a fractional laser for enhancing macromolecule penetration via diseased skin and provide an in-depth characterization of barrier function changes by inflammation and lasers.

Permeation of the peptide via the intact skin was negligible because of its inability to penetrate the SC and TJ. This could be attributed to the peptide being highly hydrophilic and ionized. Its large molecular size also retarded passive delivery of the peptide to the intact skin. The fractional laser overcame the rate-limiting barrier of the intact skin. The Er:YAG laser was valid for the controlled removal of the SC with a minimal ablation depth and thermal effect as observed in histological images. The remnant SC was also weakened by the generation of a photomechanical wave from the Er:YAG laser (27). The ablation process led to the exposure of the epidermis to peptide transport. Once the peptide left the vehicle, the permeant directly partitioned into the viable skin. This partitioning into the epidermis was facile for peptide diffusion because of its hydrophilic property. The raised TEWL after laser exposure demonstrated that the barrier function was compromised. The TEWL value of the nude mouse skin immediately after the Er:YAG laser treatment was greater than that after treatment with the Er:glass laser. This resulted in less permeation enhancement by the non-ablative laser than by the ablative laser. The wavelength of the non-ablative laser was weakly absorbed by the SC (28). The Er:glass laser energy mainly penetrated the deeper skin strata with minor disruption to the superficial layer. The peptide still needed to pass the SC of the skin treated by the non-ablative laser. The macromolecule permeation enhancement by the non-ablative laser could be due to the thermal damage to the dermis and a leak in the DEJ. The basal lamina is located between the epidermis and the dermis. It is a part of the DEJ, serving as a semipermeable membrane between the two skin layers (29).

Although the SC and epidermis were anatomically intact after non-ablative laser irradiation, this laser may ultrastructurally disrupt the superficial layer by creating a transient pathway as the permeant transport route (30,31). The fractional laser's ablation or thermolysis in a limited region was sufficient to largely increase cutaneous delivery. Because of the small area of fractional treatment, the untreated skin around the MTZs served as a reservoir for quick healing (6). Our data showed that the barrier function of the intact skin could be regained within one day after fractional laser application.

Although hair follicles occupy only 0.1% of the skin surface, they are essential for the selective transport of hydrophilic molecules and macromolecules (32). Previous studies (33,34) found that the fractional laser affected the epithelial structure of follicles to increase shunt permeant delivery. This study showed a preferential uptake of peptide in the hair follicles with laser-assisted diffusion. The follicular route may be an important permeation pathway for the laser-assisted delivery of peptides.

Some approaches for controlling skin barrier damage are used for drug absorption measurement. The approaches for controlling impairment include SC stripping and delipid, desebum, and deprotein skin (35). The advantage of these models is to provide a more reproducible and simpler procedure than the models that simulate diseased skin (36). Tape stripping is a commonly used model to eradicate the SC barrier. It can be a model for dermatitis (37). The cornified cell envelope of cross-linked protein and lipid renders the barrier effect of the SC. SC removal obviously contributed to the enhanced peptide penetration compared to that in the intact pig and mouse skin. The peptide flux after removal of the SC lipid or protein did not surpass that of tape stripping. This suggests that both lipids and proteins were the permeation barriers for peptide absorption. Delipid skin can resemble a model for psoriasis, AD, xerosis, and ichthyosis (38). The protein-denatured skin model reflects the diseased condition of AD and photoaging (39). The delipid skin exhibited a higher peptide flux than the deprotein skin, indicating the lipid as the rate-limiting barrier for peptide passage. Since a peptide is hydrophilic, this molecule cannot readily penetrate the lipophilic domain of the SC. The intracellular protein and corneocyte envelope are the major routes for peptide transport. The ablative laser ablation on SC-stripped skin elicited more peptide delivery than the stripped skin without ablation. This demonstrates that the Er:YAG laser modulates the epidermal barrier to increase peptide permeation, as the ablative laser generates a photomechanical wave with local propagation, leading to loosening of the epidermal structure (40). The inability of the Er:glass laser to enhance macromolecule delivery via the SC-stripped skin indicated the potential involvement of superficial skin disruption in the non-ablative modality-assisted skin permeation, although no clear SC injury was histologically observed. The Er:glass laser increased the

peptide flux in the delipid skin but not in the deprotein skin. In the SC lacking lipid, the laser affected only proteins for assisting cutaneous permeation. We demonstrated that the Er:glass laser mainly created aqueous protein channels for permeant passage enhancement. A previous study (15) also suggests that non-ablative laser-denatured skin protein can generate hydrophilic microchannels. The pilosebaceous gland releases sebum to spread onto the skin surface. Sebum is an important determinant in permeant partitioning into the SC (41). Sebum removal increased peptide permeation compared to that in intact skin, indicating a laborious partitioning of hydrophilic peptides into the SC. Significant peptide penetration enhancement was detectable in desebum skin exposed to the fractional laser exposure. This enhancement was greater than that in the other SC-disrupted skin models. Thus, the sebum was not the laser's main target. This is reasonable since both lasers generated irradiation corresponding to the absorption peak of water.

Although the SC-disrupted skin as a surrogate for diseased skin provided valuable information about macromolecule absorption, this information could result in a misleading conclusion because of the structural and physiological differences between SC-disrupted skin and inflamed skin. We further developed models of psoriasis and AD, which are considered reliable models of barrier deficiency. Psoriatic SC is characterized by an immature cornified cell envelope and lipid synthesis (42). The abnormality leads to barrier dysfunction. The content and chain length of SC ceramides in AD skin are reduced compared those in normal skin (43). The reduced SC barrier of lesional skin could reflect greater peptide penetration compared to that in intact skin. TJs and adherent junctions (AJs) are present beneath the SC, mainly in the granular layer. The junctions form a paracellular barrier to regulate the passage of molecules (44). Filaggrin and involucrin are TJ-related proteins, which are also associated with the cornified envelope in the SC (45). Integrin  $\beta 1$  is a kind of AJ confined to the basal layer for regulating cell adhesion and stratification. The expression of these proteins is lessened in psoriasis and AD (46,47). Our data confirmed this down-regulation. It can be inferred that the increased peptide permeation in the inflamed skin resulted from barrier dysfunction of both the SC and the epidermal layers.

The laser perforation was helpful for improving macromolecule absorption through the inflamed skin. The enhancement level on the diseased skin was less than that on the healthy skin because the barrier capacity of the skin had been already compromised by the inflammatory status. Further disruption of barrier function by the laser enhanced the cutaneous permeation at a finite level. SC hydration was decreased in the psoriatic and AD skin (42). The increased TEWL data in our study confirmed the insufficient water contents in the psoriasis- and AD-like skin. The effect of laser perforation was reduced in the inflammatory skin since the laser's main target

for structural disruption is water. Our result showed that laser treatment did not further increase the TEWL value of the psoriatic skin. Since the SC barrier effect was already diminished in the inflamed skin, the laser may have affected the junctions in the epidermis to increase the macromolecule delivery to the diseased skin. Some TJ-related proteins were decreased after laser applications to the psoriasis- and AD-like skin. Acute barrier perturbation can reportedly lower the mRNA levels of profilaggrin and involucrin (48). With respect to the psoriasis-like skin, peptide permeation was significantly increased by the Er:YAG laser but not by the Er:glass laser. This result correlated with the trend in the SC-stripped skin. Both lasers could elevate the TEWL of the AD-like skin. This result was quite different from that of the psoriasisform skin. This led to enhanced macromolecule transport via the AD-like skin after exposure to both lasers.

In addition to the vertical transport into the deeper skin layer via MTZs, horizontal permeant diffusion from the microchannels to the surrounding tissue can be observed (49,50). The confocal images showed that the diameter of the peptide penetration area was larger compared to the dot diameter of the ablative and non-ablative lasers (250 and 100  $\mu\text{m}$ ). This indicates radial diffusion of the peptide to the lateral region. The peptide was extensively distributed even in the Er:YAG laser-perforated inflamed skin with no borderline of the MTZs. The peptide delivery procedure can be vertical diffusion into the MTZs along a concentration gradient, followed by radial diffusion from the MTZs to the peripheral area. Delivery of siRNA to the skin is a significant challenge (51). The molecular size of therapeutic siRNA is generally greater than that of peptides. The laser could assist siRNA absorption into the skin. The fluorescence signal in the laser-treated skin from siRNA administration was darker than that from the peptide. The overall low permeation of siRNA could be attributed to the large molecular size, leading to difficult partitioning into the MTZs regardless of the barrier defect throughout the skin layers. The microchannels could be discerned in the laser-irradiated skin receiving the siRNA application. A limitation of our study regarding macromolecular distribution was the restriction of observations to the upper 75  $\mu\text{m}$  layer of the skin. This was because the confocal laser can penetrate only a limited cutaneous depth for clear visualization. Further study is needed to examine the macromolecular biodistribution in the deeper skin strata, which is an important target for many anti-psoriatic and anti-AD drugs.

## CONCLUSIONS

Understanding drug absorption via barrier-deficient skin is an important step toward the development of innovative enhancement approaches for topical therapy. We examined the effect of the fractional laser on macromolecule delivery

to inflamed skin. The flux of peptide through lesional skin after ablative or non-ablative laser exposure was increased compared to that after passive transport. However, the enhancement level was less than that achieved by using intact skin as the permeation barrier. The ablative laser generally demonstrated higher macromolecule permeation than the non-ablative modality. The laser-assisted delivery of peptides and siRNA could enable topical absorption for broader and deeper penetration. The macromolecules were mainly deposited inside the microchannels created by the laser according to the confocal images. The findings provide the first report that the fractional laser can still effectively ameliorate cutaneous delivery via barrier-defective skin.

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