***In Vivo* and *Ex Vivo* Skin Reactions after Multiple Pulses of 1,064-nm, Microlens Array-type, Picosecond Laser Treatment**

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Background and Objectives

A picosecond-domain laser treatment using a microlens array (MLA) or a diffractive optical element elicits therapeutic micro-injury zones in the skin. This study examined the patterns of tissue reactions after delivering multiple pulses of 1,064-nm, MLA-type, picosecond neodymium:yttrium-aluminum-garnet laser treatment.

Materials and Methods

Multiple pulses of picosecond laser treatment were delivered to *ex vivo* human or brown micropig skin and analyzed histopathologically. A high-speed cinematographic study was performed to visualize the multiple pulses of picosecond laser energy-induced skin reactions in *in vivo* human skin.

Results

In the *ex vivo* human skin, a picosecond laser treatment at a fluence of 0.3 J/cm² over 100 non-stacking passes generated multiple lesions of thermally-initiated laser-induced optical breakdown (TI-LIOB) in the epidermis and dermis. In the *ex vivo* micropig skin, stacking pulses of 20, 40, 60, 80, and 100 at a fluence of 0.3 J/cm² generated distinct round to oval zones of tissue coagulation in the mid to lower dermis. High-speed cinematography captured various patterns of twinkling, micro-spot reactions on the skin surface over 100 stacked pulses of a picosecond laser treatment.

Conclusion

Multiple pulses of 1,064-nm, MLA-type, picosecond laser treatment elicit marked TI-LIOB reactions in the epidermis and areas of round to oval thermal coagulation in the mid to deep dermis.

Key words

Laser; Neodymium-doped yttrium aluminum garnet; Picosecond; Human skin; Thermally-induced laser-induced optical breakdown; Scar

INTRODUCTION

Picosecond-domain laser treatment using a microlens-array (MLA) optic or a diffractive optical element (DOE) can be used to generate numerous micro-injury zones of thermally-initiated laser-induced optical breakdown (TI-LIOB) in the epidermis and upper dermis.¹⁻⁴ These zones of TI-LIOB have been shown to stimulate the production of growth factors, chemokines, and cytokines to improve the appearance of enlarged pores, atrophic scars, and wrinkles.^{5,6}

Research has shown that different lasers elicit different reactions in the skin. Focused pulses of a picosecond alexandrite laser at the wavelength of 755 nm were found to generate a fractionated appearance of TI-LIOB primarily in the epidermis.⁷ Meanwhile, picosecond neodymium:yttrium-aluminum-garnet (Nd:YAG) lasers at the wavelength of 532 nm or 1,064 nm generated TI-LIOB lesions in both the epidermis and the upper dermis.^{1,2,8,9} Therein, in an experimental *ex vivo* skin model with a high melanin index, intraepidermal TI-LIOB lesions generated by picosecond laser treatments using an MLA or DOE optic were larger than those in skin with a higher melanin index.^{8,9} Moreover, most of the large cystic TI-LIOB lesions in the epidermis presented CD31-negative/Melan-A-positive immunoreactivity,⁹ while those in the upper dermis exhibited various patterns of immunoreactivity: CD31-positive/Melan-A-negative cystic cavities or CD31-negative/Melan-A-negative pseudo-cystic cavities with or without adjacent CD31-positive/Melan-A-negative microvascular components.⁹

In this pilot study, we aimed to investigate histologic patterns of immediate tissue reactions generated by multiple pulses of 1,064-nm, MLA-type, picosecond Nd:YAG laser treatment in *ex vivo* human and brown pig skin models. To do so, 20, 40, 60, 80, and 100 non-stacking or stacking pulses of picosecond laser treatment were delivered to the *ex vivo* human or brown micropig skin and, then, histopathologically analyzed. Furthermore, high-speed cinematographic study was performed to visualize multiple pulses of 1,064-nm, MLA-type, picosecond laser energy-induced skin reactions on the surface of *in vivo* human skin. Additionally, we evaluated the case of a patient with hypopigmented fibrotic linear scars that markedly improved without remarkable side effects after two sessions of treatment with 100 stacking pulses of a 1,064-nm, MLA-type, picosecond Nd:YAG laser.

MATERIALS AND METHODS

Laser device and preparation of *ex vivo* human skin

A 450-picosecond, Nd:YAG laser device (PICOPLUS; Lutronic Corp., Goyang, Korea) at the wavelengths of 532 and 1,064 nm was used in this study. Using the device, a constant pulse width was maintained, regardless of the output fluence, using a master oscillator power amplifier configuration.¹⁰ This picosecond-domain laser system delivers laser energy to target tissue with beam profiles of a single flat top or a fractional optic array. With appropriate optics (e.g., MLA or DOE), a single pulse of picosecond laser energy can emit numerous microbeams. The size of the microbeams can be regulated by controlling the distance between the microlens and the distal end of the device's handpiece, with microbeam sizes of 150 μ m, 160 μ m, and 300 μ m at distances of 31 mm, 33 mm, and 48 mm, respectively.

This study was approved by the Institutional Review Board of International St. Mary's Hospital, Catholic Kwandong University College of Medicine (Incheon, Korea) and the ethics committee of the Catholic Kwandong University Institutional Animal Care and Use Committee. The methods were carried out in accordance with the approved guidelines. After obtaining written informed consent, human skin samples were obtained via one abdominoplasty surgery (54-year-old Korean female with Fitzpatrick skin type III) in order to histopathologically evaluate the patterns of immediate tissue reactions elicited by multiple pulses of 1,064-nm, MLA-type, picosecond laser treatment in an *ex vivo* human skin model. Additionally, fresh back skin tissue samples, which were obtained from a brown female micropig (8-month old, weighing 16 kg), were purchased from Medi Kinetics Co., Ltd. (MK Micropig®; Seoul, Korea). Each of the *ex vivo* human or micropig skin samples was prepared at a size of 10 cm × 10 cm; then, each skin sample was subsequently marked with black ink to outline 3-cm² grids for each experimental setting (a total of six grids/sample). Each grid was placed at least 0.5 cm from the others to minimize overlapping of laser-induced photothermal and photoacoustic effects between the grids.

Laser treatment and histopathological studies

The temperature of *ex vivo* human and micropig skin samples was maintained between 34-36°C on a heat plate during all experiments. Using an MLA-type handpiece, 1,064-nm picosecond Nd:YAG laser treatments were performed separately on each grid on the *ex vivo*

human skin with a spot size of 10 mm, which comprised 314 microbeams, and with the laser fluences of 0.1 J/cm^2 (peak power, 4.0 GW/microbeam) and 0.3 J/cm^2 (peak power, 12.0 GW/microbeam) over 100 non-stacking passes at a distance between the microlens and the surface of the skin of 31 mm. Additionally, picosecond laser treatments using the MLA-type handpiece at a wavelength of 1,064 nm were delivered to ex vivo brown micropig skin samples with a spot size of 10 mm, laser fluences of 0.1 J/cm^2 (peak power, 4.0 GW/microbeam) and 0.3 J/cm^2 (peak power, 12.0 GW/microbeam), and a distance setting of 31 mm over 1, 20, 40, 60, 80, and 100 stacking passes. All experiments were performed in triplicate.

Human and micropig tissue samples for each experimental grid were obtained 30 minutes after treatment, collecting the epidermis, dermis, and subcutaneous fat. The tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Then, serial tissue sections, which were cut along the longitudinal plane at a thickness of $5 \mu\text{m}$ for each condition, were prepared and stained with hematoxylin and eosin.

High-speed cinematography evaluation of laser-induced tissue reactions in *in vivo* human skin

To visualize laser-induced tissue reactions on the surface of *in vivo* human skin while delivering multiple pulses of 1,064-nm, MLA-type, picosecond Nd:YAG laser treatment, a high-speed digital video camera (Y4S2; IDT Vision, Pasadena, CA, USA) with a AF Micro Nikkor lens (Nikon Corp.) at a focal length of 105 mm and f/2.8G internal focusing-extra-low dispersion was utilized. The videos were captured at a rate of 2,000 Hz, an exposure time of 497 μsec , and a resolution of $1,024 \times 1,024$ megapixels under two light-emitting diode spotlights (120W). After obtaining written informed consent, normal skin on the

left forearm and a dermal melanocytosis lesion on the dorsum of the right hand in a 43-year-old male volunteer were treated with multiple pulses of 1,064-nm, MLA-type, picosecond laser. The patient reported no treatment history for dermal melanocytosis on the right hand or any pertinent medical or family history. Each lesion was cleansed with 70% ethanol, and topical anesthetic cream (eutectic mixture of 2.5% lidocaine HCl and 2.5% prilocaine, EMLA; Astra Pharmaceuticals, Westborough, MA, USA) was applied under occlusion for 1 hour. The normal skin and a dermal melanocytosis lesion were treated with a 1,064-nm picosecond Nd:YAG laser using an MLA-type handpiece with a spot size of 4 mm, which comprised 50 microbeams, and a laser fluence of 1.0 J/cm^2 (peak power, 40.1 GW/microbeam) over 100 stacking pulses at a frequency of 10 Hz. The distance setting between the microlens and the surface of the skin was 31 mm. Neither a dynamic cooling device nor air cooling device was used during the treatments. Video footage was recorded from the moment of starting laser irradiation until completing a total pulse count of 100 pulses on identical points along the patient's skin.

RESULTS

Histopathologic findings of post-treatment skin reactions

Ex vivo human skin specimens were obtained after 100 non-stacking passes of the 1,064-nm picosecond Nd:YAG laser using an MLA-type handpiece at the fluence settings of 0.1 J/cm^2 and 0.3 J/cm^2 . At a fluence of 0.1 J/cm^2 , no remarkable TI-LIOB lesions were noted in the epidermis; however, there were a few pseudo-cystic lesions in the mid dermis at irregular intervals surrounded by thermal tissue coagulation. Meanwhile, at a fluence of 0.3 J/cm^2 ,

Fig. 1. Thermally-initiated laser-induced optical breakdown (TI-LIOB) lesions in *ex vivo* human skin. (A) The picosecond laser treatment at a fluence of 0.3 J/cm^2 generated multiple TI-LIOB lesions over 100 non-stacking passes in the epidermis and in the upper and mid dermis. (B) Multiple TI-LIOB lesions in the epidermis. (C) Cystic and pseudo-cystic cavities with adjacent vascular components in the upper dermis. Hematoxylin and eosin stain. Original magnification (A) $\times 40$, (B) $\times 200$, and (C) $\times 400$.

100 non-stacking passes generated multiple TI-LIOB lesions in the epidermis and in the upper and mid dermis (Fig. 1A, B). TI-LIOB lesions in the upper dermis exhibited cystic and pseudo-cystic cavities with adjacent vascular components (Fig. 1C), whereas those in the mid dermis showed discrete pseudo-cystic cavities without adjacent vascular components.

Ex vivo brown micropig skin specimens were obtained after stacking 20, 40, 60, 80, and 100 passes of the 1,064-nm picosecond Nd:YAG laser using an MLA-type hand-piece at the fluence settings of 0.1 J/cm² and 0.3 J/cm². At a fluence of 0.1 J/cm², the micropig skin specimens treated with 40, 60, 80, and 100 stacking pulses showed remarkable TI-LIOB lesions in the basilar layer of the epi-

Fig. 2. Picosecond laser-induced TI-LIOB lesions in *ex vivo* brown micropig skin. (A) The picosecond laser treatment at a fluence of 0.3 J/cm² over 60 stacking passes generated multiple TI-LIOB lesions in the epidermis and in the mid to deep dermis (asterisks). (B) Multiple TI-LIOB lesions in the basilar epidermis. (C) Round to oval zones of tissue coagulation (asterisk) in the mid to lower dermis. Hematoxylin and eosin stain. Original magnification (A) $\times 40$, (B) $\times 400$, and (C) $\times 100$.

Fig. 3. High-speed cinematography images. *In vivo* human normal skin was treated with a 1,064-nm, MLA-type, picosecond laser with a spot size of 4 mm and a laser fluence of 1.0 J/cm² over 100 stacking pulses at a frequency of 10 Hz. High-speed cinematography images were captured at the (A) 1st, (B) 12th, (C) 26th, (D) 59th, (E) 91th, and (F) 100th pulses. White circles indicate the margins of MLA-type picosecond laser pulses. Arrows indicate petechial skin reactions.

dermis and in the basement membrane. Histopathologic patterns in the epidermis at a fluence setting of 0.3 J/cm^2 were comparable to those at 0.1 J/cm^2 (Fig. 2A, B). Similarly, 20, 40, 60, 80, and 100 stacking pulses at a fluence of 0.3 J/cm^2 generated remarkable round to oval zones of tissue coagulation in the mid to lower dermis (Fig. 2A, C).

High-speed cinematography study using *in vivo* human skin

Normal forearm skin was treated with a 1,064-nm picosecond Nd:YAG laser using an MLA-type handpiece with a spot size of 4 mm and a laser fluence of 1.0 J/cm^2 over 100 stacking pulses at a frequency of 10 Hz. The first pulse thereof elicited a group of strong, heterogeneous twinkling micro-spots on the surface of the normal skin (Fig. 3A). The degree of twinkling gradually became weaker from the fourth to the eleventh pulses. Macroscopic skin reactions became weak and homogeneous from pulses 12 to 25 (Fig. 3B). Picosecond laser-induced twinkling reactions on the skin surface were unnoticeable from pulses 26 to 58 (Fig. 3C). Pulses 59 to 90 exhibited weak and heterogeneous twinkling reactions on the skin

surface that disappeared from pulses 91 to 100 (Fig. 3D-F). Petechial skin reactions started to appear after the first pulse and gradually became more obvious and bigger with additional pulses. The progression of petechial reactions seemed to be affected by the degree of macroscopic twinkling reactions.

Next, a dermal melanocytosis lesion on the dorsum of the right hand was treated with an MLA-type picosecond laser at a spot size of 4 mm, a laser fluence of 1.0 J/cm^2 over 100 stacking pulses at a frequency of 10 Hz. The first pulse thereof generated noticeable twinkling reactions on the skin surface (Fig. 4A), albeit weaker and more homogeneous than those in normal skin. Twinkling skin reactions disappeared from pulse 32 to 95 (Fig. 4B-E). Pulses 62 to 64 and 96 to 100 transiently generated marked macroscopic skin surface reactions (Fig. 4F). Post-laser petechial skin reactions were unremarkable.

In vivo case study

A 33-year-old Korean female patient visited our clinic presenting with two post-trauma, hypopigmented atrophic and fibrotic linear lesions on the forehead and right

Fig. 4. High-speed cinematography. *In vivo* human skin with a dermal melanocytosis lesion was treated with a 1,064-nm, MLA-type, picosecond laser with a spot size of 4 mm and a laser fluence of 1.0 J/cm^2 over 100 stacking pulses at a frequency of 10 Hz. High-speed cinematography images were captured at the (A) 1st, (B) 32th, (C) 62th, (D) 65th, (E) 96th, and (F) 100th pulses. White circles indicate the margins of MLA-type picosecond laser pulses.

upper eyelid that had persisted over 20 years (Figs. 5A, 6A). The patient reported no pertinent medical and family history. Prior treatment history for the lesions included non-ablative 1,540-nm fractional erbium-glass laser, ablative 10,600-nm fractional carbon dioxide laser, invasive bipolar radiofrequency using penetrating microneedles, and chemical dermabrasion treatments. However, treatment outcomes were unsatisfactory, and she had not sought treatment in the most recent 12 months.

After obtaining written informed consent, the lesions were treated with two sessions of treatment with multiple pulses of a 1,064-nm, MLA-type, picosecond Nd:YAG laser at 2-week intervals. We cleansed the atrophic and fibrotic lesions with 70% ethanol and applied EMLA cream under occlusion for 1 hour. Then, 100 stacking pulses of 1,064-nm picosecond Nd:YAG laser treatment were emitted using an MLA-type handpiece with a spot size of 4 mm, a laser fluence of 0.8 J/cm² (peak power, 32.0 GW/microbeam), a frequency of 10 Hz, and a distance setting

between the microlens and the surface of the skin of 31 mm. Neither a dynamic cooling device nor air cooling device was used during the treatments. Immediately after treatment, the treated areas were cooled with icepacks, and a hydrocolloid dressing (DuoDERM® Extra Thin [Convatec Inc, Princeton, NJ, USA]) was applied. No prophylactic corticosteroids (systemic or topical) or antibiotics were used. The patient was recommended to avoid excessive scrubbing and sun exposure. Photographs were taken at an identical setting under normal light, polarized light, and ultraviolet exposure using an imaging tool (Mark-Vu®; PSI PLUS, Suwon, Korea).

Immediately after each treatment, marked petechial patches with mild pinpoint bleedings were noted in the hypopigmented atrophic and fibrotic linear scar lesions. Pinpoint bleedings were well controlled with mild compression with sterile dry gauze. No noticeable oozing, bleeding, and crust formation were encountered. Post-treatment petechial skin reactions were spontaneously

Fig. 5. Linear fibrotic and hypopigmented scars (arrows) treated with 100 stacking passes with a 1,064-nm, MLA-type, picosecond laser. Photographs were taken under normal light exposure at (A) baseline, (B) 2 weeks after the first session of picosecond laser treatment, and (C) 4 months after the second session of picosecond laser treatment.

Fig. 6. Linear fibrotic and hypopigmented scars (arrows) treated with 100 stacking passes with a 1,064-nm, MLA-type, picosecond laser. Photographs were taken under polarized light exposure at (A) baseline, (B) 3 weeks after the first session of picosecond laser treatment, and (C) 6 months after the second session of picosecond laser treatment.

dissolved in 5 days. At 3 weeks after the first session of MLA-type picosecond laser treatment, scar lesions had improved without noticeable post-treatment crusts, erythema, and pigmentation (Figs. 5B, 6B). At 6 months after the second session of MLA-type picosecond laser treatment, the lesions had further improved without remarkable major side effects (Figs. 5C, 6C).

DISCUSSION

In this pilot study, we documented histologic patterns of immediate tissue reactions elicited by multiple pulses of 1,064-nm, MLA-type, picosecond Nd:YAG laser treatment in *ex vivo* human and brown pig skin models. Picosecond laser irradiation using an MLA or a DOE optic theoretically induces plasma-mediated tissue reactions via multiphoton-initiated laser-induced optical breakdown (MI-LIOB) or TI-LIOB.^{1,6,11} Research has shown that very tightly focused beams of laser energy at picosecond pulse durations generate chromophore-independent tissue reactions of MI-LIOB at power densities exceeding 10^{11} W/cm².^{6,11} Meanwhile, chromophore-dependent, thermionic emission-induced reactions generated by picosecond laser treatment at power densities below 10^{11} W/cm² promote tissue ablation and photo-disruption.^{6,11} Because most commercially available picosecond laser devices use power densities below 10^{11} W/cm², picosecond laser-induced tissue reactions have been suggested to result from thermionic emission-induced plasma formation.^{6,11}

Picosecond lasers have been used for treating various types of scars with satisfactory clinical outcomes.^{4,5,12-14} In the present study, we demonstrated that stacking or non-stacking multiple-pulses of a focused picosecond laser at a wavelength of 1,064 generate large cystic or pseudocystic laser-induced tissue reactions in the epidermis and dermis. After dozens of stacking pulses of picosecond laser treatment, round to oval zones of tissue coagulation formed in the mid to lower dermis. The patterns thereof were histopathologically similar to thermal coagulation reactions after invasive radiofrequency treatment with penetrating microneedle depths targeting the deep dermis.¹⁵ Accordingly, we suggest that picosecond-induced tissue reactions can effectively induce wound repair and remodeling in the epidermis and in the upper and mid to deep dermis. Nonetheless, further investigations to evaluate histopathologic findings in *in vivo* human skin after multiple picosecond laser treatments with a stacking technique are needed to confirm our findings.

In this study, high-speed cinematography revealed distinctive picosecond laser pulse-induced tissue reactions

in normal human skin *in vivo* over 100 pulses at 10 Hz. The first phase of tissue reactions showed twinkling microbeams in the upper part of the normal skin that could have resulted from interactions between laser energy and chromophores, including melanin and hemoglobin, in the epidermis and upper dermis and/or foreign materials on the skin surface. The second phase of non-twinkling skin reactions that followed could have resulted from deeper penetration of laser energy at the wavelength of 1,064 nm and the accumulation of photo-thermo-mechanical tissue reactions in the mid to deeper dermis. We suspect that as most of the epidermal and upper dermal factors that absorb, reflect, and scatter laser energy were eliminated over the first phase, thereby allowing the laser energy to penetrate deeper into the skin.

Additional high-speed cinematography study of an *in vivo* skin lesion of dermal melanocytosis showed different patterns of laser-induced tissue reactions over 100 pulses at 10 Hz, compared with normal skin. The degree of twinkling surface reactions seemed weaker, and the number pulses that reacted with the chromophores of the epidermis and papillary dermis seemed to be less in the dermal melanocytosis lesion, compared with normal skin. We suggested that the location and number of chromophores, particularly melanin, could have affected the patterns observed in the cinematography study. Notwithstanding, picosecond laser-induced skin reactions can be influenced by various factors, including skin type, thickness, hydration, and underlying disease.

In the high-speed cinematography study, the videos were captured at a rate of 2,000 Hz, an exposure time of 497 μ sec, and a frame interval of 5 msec; however, the pulse interval of picosecond laser treatment was 100 msec. Thus, our study was analyzed under the supposition that the laser irradiation and video were temporally synchronized. Nonetheless, because the lifespan of light emitting from the irradiated target chromophores is expected to last only hundreds of μ sec, video capture images in our pilot study may not fully reflect the patterns of *in vivo* human skin reactions over multiple pulses of picosecond laser treatment.

In conclusion, multiple pulses of 1,064-nm, MLA-type, picosecond laser treatment were found to generate marked TI-LIOB reactions in the epidermis and areas of round to oval thermal coagulation in the mid to deep dermis. Furthermore, dozens of stacking passes thereof resulted in satisfactory therapeutic outcomes without remarkable major side effects in an Asian patient with old, hypopigmented, fibrotic linear scars. We believe that our data on the distinctive patterns of skin reactions gener-

ated by multiple-pass, MLA-type picosecond laser treatments may provide practical information of use to clinicians administering treatments with picosecond lasers.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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