

Histological investigation of picosecond laser-toning and fractional laser therapy

Shunji Nakano MD, PhD

Nakano clinic, Miyazaki, Miyazaki, Japan

Background and Aims: Rejuvenation therapy using picosecond pulse laser and picosecond pulsed fractional therapy with a fractional lens have been performed with clinical effects evaluated. However, no histological analysis of effects on photoaged skin exists. In this study, influence of laser-toning and fractional therapy using picosecond pulse laser on photoaging was histologically investigated.

Subjects and Methods: The flexor side forearm of a male, age 61, with photoaging was divided into three 20 cm² areas and irradiated with approximately 400 shots of 10-Hz laser, 8 mm spot size, and nine passes at an output of 0.7, 0.9, and 1.1 J/cm² using picosecond laser-toning therapy six times, every two weeks. Two weeks post final irradiation, 2 mm punch biopsies were taken from the irradiation fields. Fractional therapy using Micro Lens Array (MLA) attached picosecond fractional therapy was applied to the medial crural skin with marked photoaging of a male, age 63. Irradiation was applied at 0.5 and 0.7 J/cm² through two passes, with 3 mm punch biopsies taken from each irradiation field immediately after and again two months post-irradiation. Samples were subjected to hematoxylin and eosin (HE) and Elastica van Gieson staining and compared.

Results: In the picosecond laser-toning therapy sample, photoaging-induced dermis reconstruction occurred. The picosecond fractional therapy sample showed both epidermis and dermis reconstruction, with intrinsic aging and photoaging improvements.

Conclusions: Recovery of dermal and epidermal age related atrophy by picosecond laser-toning and picosecond fractional therapy was histologically confirmed. Picosecond fractional therapy demonstrated superior improvement.

Key words: Rejuvenation • Picosecond pulsed laser-toning therapy • Picosecond pulsed fractional therapy • photoaging • intrinsic aging

Introduction

Since 1985, many treatment methods have been considered for photoaged skin. Numerous chemical peeling methods were devised, and their effectiveness increased with increasing action on the dermis, but the rate of adverse events also increased¹⁾. In 1995, skin resurfacing methods using 10,600 nm wavelength carbon dioxide laser²⁾ and 2,640 nm erbium YAG laser³⁾ were developed to efficiently peel the skin to the papillary layer of the dermis; however, protracted pigmentation, vitiligo, and scarring were problematic. Later, non-ablative laser skin rejuvenation therapy^{5,6)} using 532, 585, 595, 755, 800, and 1,064 nm wavelength long-pulse lasers (msec), based on the selective photothermolysis theory⁴⁾, and intense

pulsed light (IPL) treatment using broadband light^{7,8)} were developed, demonstrating the dermis can be safely reconstructed by only irradiating the skin surface with laser and light⁹⁾. Furthermore, fractional laser (non-ablative/ablative) (µsec)¹⁰⁾ or fractional RF therapy¹¹⁾ was developed as an advanced skin resurfacing technology that was capable of safe and efficient rejuvenation effects by cauterizing the skin in a disseminated or dotted pattern to retain normal skin in the irradiation field. Subsequently, many treatments have been devised, such as 1,064 nm nanosecond laser therapy (nsec) applying low-output hollow irradiation, plasma treatment, and treatment with platelet-rich plasma (PRP). Additionally, multiple irradiation systems, such as monopolar/bipolar/unipolar RF treatments tighten not only the dermis, but also the subcutaneous fat¹²⁻¹⁴⁾. High-intensity focused ultrasound (HIFU) improves sagging and skin quality by targeting

Addressee for Correspondence
4-6-18 Tachibanadori, Miyazakisi,
Miyazaki, 880-0805 Japan
Tel: +81-985-22-2695 Fax: 81-985-22-2685
e-mail: info@clinic-nakano.com

Received date: November, 30th, 2019
Accepted date: February, 4th, 2020
J-STAGE Advance Publication Date: April 18th, 2020

not only the dermis and fascia, such as the superficial muscular aponeurotic system (SMAS), but by also targeting the subcutaneous fat.

Attaching a diffractive lens array to a picosecond alexandrite laser (psec), developed as a tattoo removal laser, and applying irradiation continuously to the entire skin, similarly to nanosecond laser toning¹⁵⁾, was clinically confirmed in 2012 to have rejuvenation effects¹⁶⁻¹⁹⁾. The picosecond pulse laser used in this study was a 532/1064 nm wavelength picosecond Nd:YAG laser (enlighten, Cutera, Inc., USA), developed in the US in 2014. This is a 750 psec/2 nsec variable type that is capable of toning at 1,064 nm. Further clinical improvement was confirmed by fractional irradiation using a Micro Lens Array (MLA) attachment. In this study, photoaged skin was irradiated using picosecond laser-toning and fractional therapy, and histological changes after irradiation were investigated.

Subjects and Methods

I. Picosecond laser-toning therapy

(1) Methods

After approval of the study design was granted by the ethics committee of this hospital, a 61 year old male volunteered to participate in the study. This study was performed in accordance with the Declaration of Helsinki. The objective and content of the study, and important points were sufficiently explained to the subject, and his written informed consent was obtained prior to the beginning of any study procedures.

The male subject periodically played golf between the ages of 20 and his current age of 61. Photoaging-induced, crepe-like wrinkles were observed on the flexor

side of the forearm, on both sides. The flexor side of the forearm was divided into three 20 cm² areas and irradiated with approximately 400 shots of 10 Hz laser with an 8 mm spot size, and nine passes at an output of 0.7, 0.9, and 1.1 J/cm² every two weeks, six times total (**Fig. 1a**). Irradiation was applied from a distance at which three distance gauges mostly contacted the skin surface. Two weeks after the final treatment, the skin was biopsied from the center of each irradiation field using a 2 mm punch. Tissue samples were subjected to HE and Elastica-van Gieson staining.

(2) Clinical results

Improvements to skin firmness were confirmed by a practitioner with palpation and visual examination of all irradiation fields. The effects became more favorable with higher outputs (**Fig. 1b**). No change was noted in the color tone.

(3) Histological results

[HE staining]

In the papillary layer of the dermis before treatment, collagen fibers were sparse without continuity and collagen bundles normally present in the subdermal reticular layer had decreased, reflecting moderate ultraviolet light-induced deterioration (**Fig. 2a**). In the laser-irradiated regions, continuous minute fibers were observed in the papillary layer of the dermis, and the growing fiber layer became thick in the papillary over the reticular layer with higher outputs (**Fig. 2 b,c,d**). In addition, thick collagen fiber bundles were increased in the subdermal reticular layer (**Fig. 2 b,c,d**). Outgrowth of capillary blood vessels was observed in the dermis, irradiated at 0.7 and 0.9 J/cm² (**Fig. 2b,c**). In the region irradiated at 1.1 J/cm², no

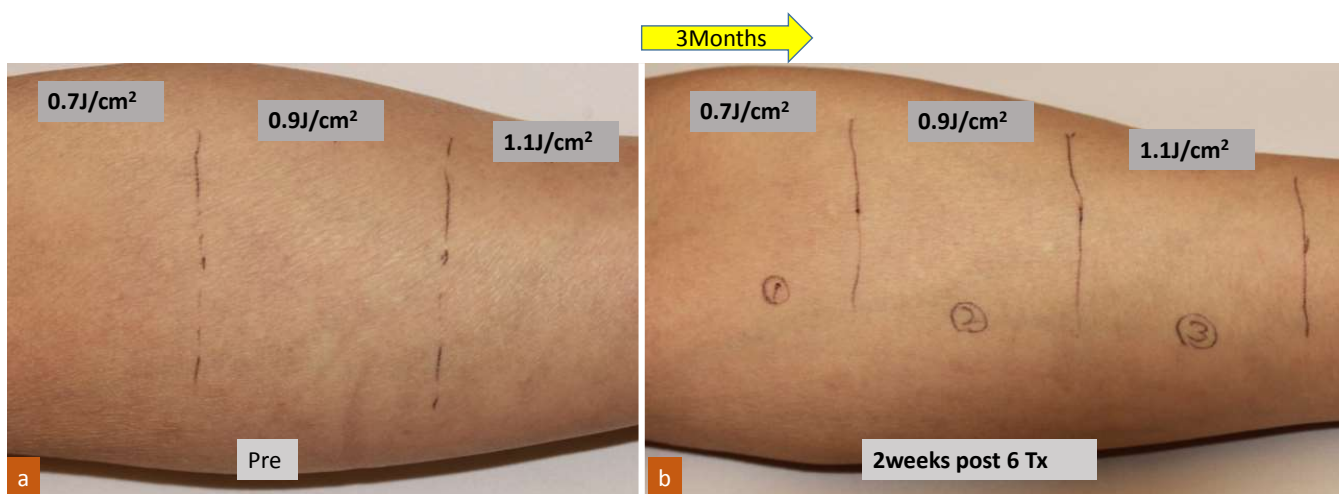


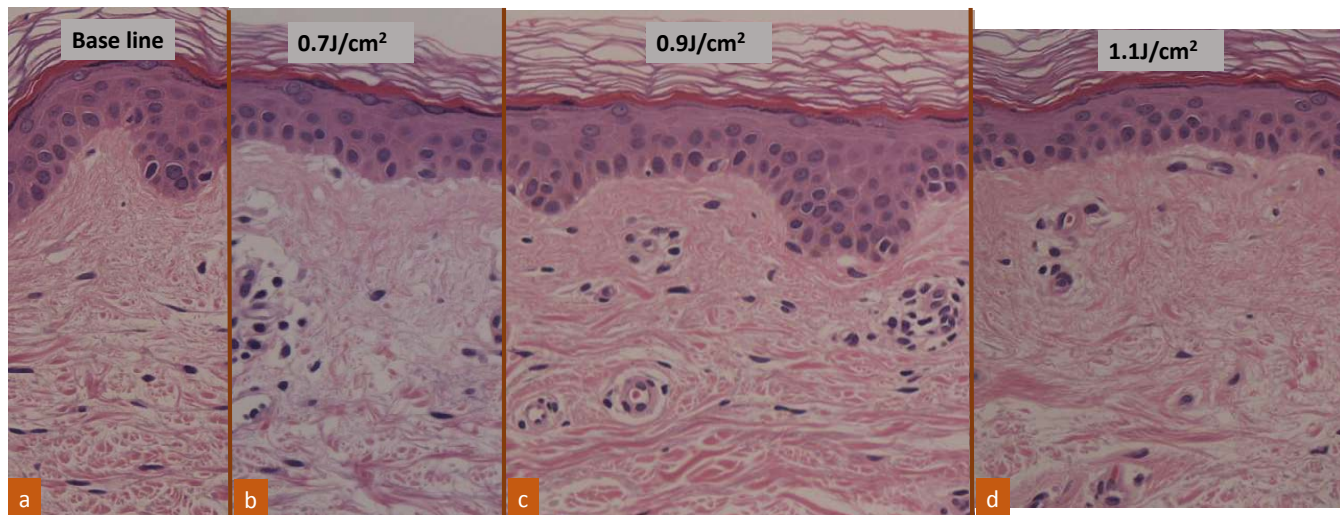
Fig. 1: **a:** Fine wrinkles are seen at the forearm
b: Clinical findings shows the remarkable improvement in texture and tightening at the forearm of irradiation site as laser intensity increased.

outgrowth of capillary blood vessels was observed in the newly formed, thick collagen fibers on HE staining (**Fig. 2d**), but it was noted on EV staining (**Fig. 3d**).

[Elastica van Gieson staining]

Collagen fibers and elastic fibers are stained red and black, respectively. No difference was observed between

the control and skin irradiated at 0.7 J/cm² (**Fig. 3a,b**). Elastin appeared in the papillary layer in the region irradiated at 0.9 J/cm² (**Fig. 3c**). In the region irradiated at 1.1 J/cm², the number of thick elastic fibers in the papillary layer increased and dendritically growing elastin fibers extended toward the epidermis (**Fig. 3d**).



61years male was treated with picosecond laser, enLIGHTen(1064nm,750psec,0.7-1.1J/cm²). 400shots were radiated for each three divided area on forearm by different Intensity. 6times at 2weeks apart. Biopsies taken after 2weeks after final radiation. Specimens were stained HE stain, Azan-Mallory stain and Elastica van Gieson stain .

Fig. 2: Hematoxylin-Eosin stain (HE stain)

a: Dispersed collagen fibers are seen at the whole dermis. **b:** Narrow continuous collagen are recognized in the upper dermis and collagen bundles are appeared in the reticular dermis. **c:** In the upper dermis, cleared collagen fibers recognized and increased in thickness. **d:** Collagen fibers and bundles increased prominently .

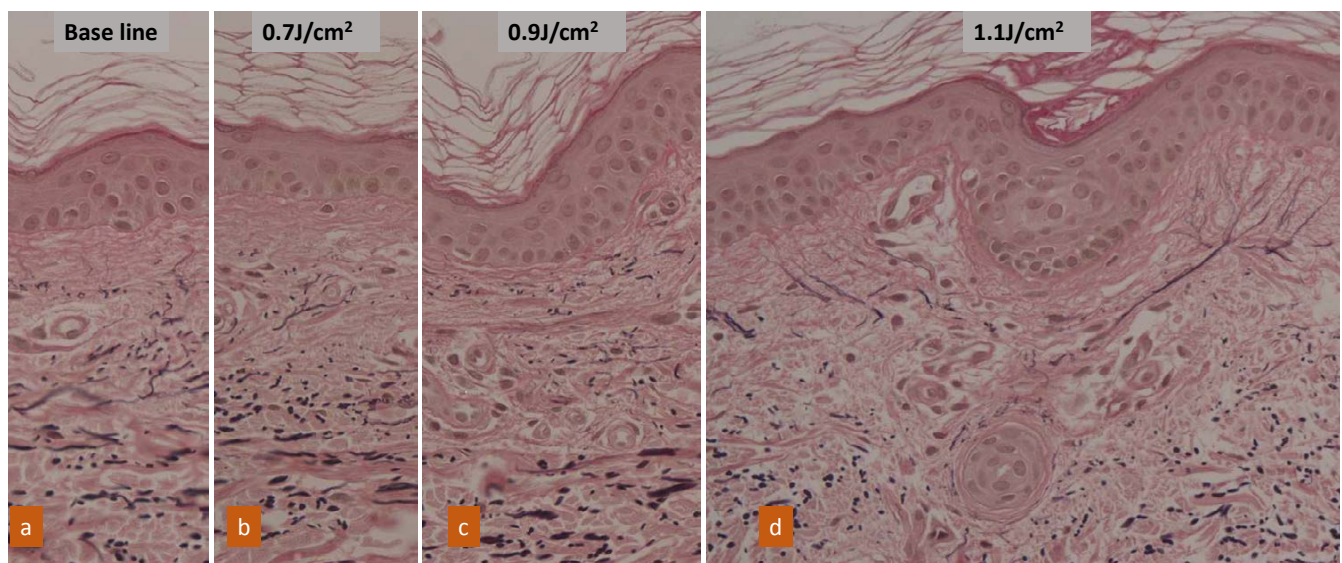


Fig. 3: Elastica van Gieson stain (EV stain)

Elastica van Gieson staining stain elastin fibers. Normally, elastic fibers in the papillary dermis are present either as bundles of microfibrils oxytalan fibers, or as cross-linked elastin and elongate to epidermis. In the reticular dermis elastin appear to be fragmented in sections(a,b). Elastin appears in the papillary layer in the region irradiated at 0.9 J/cm² (c). Elastin fibers obviously synthesized dendritically are observed in specimen(d).

II. Picosecond fractional therapy

(1) Methods:

The medial side of the crus with crepe-like wrinkles of a 63-year-old male with photoaging (**Fig. 4a**) was irradiated with two passes of the laser at an output of 0.5 and 0.7 J/cm² (**Fig. 4b**). Irradiation was applied from a distance at which three distance gauges mostly contacted the skin surface. Two months after treatment, the skin was biopsied from the center of each irradiation field using a 3 mm punch (**Fig. 4c**). The tissue samples were subjected to HE and Elastica van Gieson staining.

The experiment was performed in accordance with the Declaration of Helsinki after obtaining the subject's written informed consent.

(2) Clinical results:

After treatment, crepe-like wrinkles disappeared from both regions irradiated at 0.5 and 0.7 J/cm² (**Fig. 4c**). The skin surface felt moist on observation by a practitioner.

(3) Histological results

[HE staining]

Before treatment (control), growth of the stratum corneum, thinning of the epidermis, and loss of epidermal rete ridges were noted, demonstrating intrinsic aging (**Fig. 5a**). In the papillary layer of the dermis, sparse collagen fibers had no continuity, collagen bundles normally present in the subdermal reticular layer had decreased, and dilated capillary blood vessels were observed in the dermis, reflecting marked ultraviolet light-induced deterioration (**Fig. 5a**). In the sections at two months post-irradiation at 0.5 J/cm², which is unlikely to cause bleeding in the skin immediately after irradiation, the epidermis was thickened and epidermal rete ridges were clearly observed (**Fig. 5d**). Dense growth of continuous minute fibers was observed in the papillary layer of the dermis right below the epidermis, and a thick fiber layer had grown in the papillary over the reticular layer (**Fig. 5d**). In addition, thick collagen fiber bundles increased in the subdermal reticular layer.



Fig. 4: a: Base line shows crepe-like wrinkles on the medial side of the crus. Area cutanea does not recognize.

b: Immediately after irradiation using MLA. Spot-like intradermal bleeding is more frequent on the right. Biopsy sites are seen.

c: 2 months after irradiation using MLA. Wrinkles are disappeared and area cutanea has been recovered. Biopsy sites are still red.

[Elastica-van Gieson staining]

No elastin was noted in the superficial layer of the dermis before irradiation (**Fig. 5b**), and the amount of elastin had decreased in the middle to deep layers. In the skin irradiated at 0.5 and 0.7 J/cm², elastin and fiber extension were noted in the superficial to deep layers of the dermis (**Fig. 5e,f**). Minute fibers in the papillary layer were dendritically ascended toward the epidermis in the skin irradiated at 0.7 J/cm² (**Fig. 5f**). Outgrowth of capillary blood vessels was observed in the dermis irradiated at 0.5 and 0.7 J/cm² (**Fig. 5d,e,f**).

Discussion

Skin aging is either intrinsic or due to photoaging²⁰. In intrinsic aging, thinning of the epidermis, reduction of epidermal rete ridges, and reduction of the extracellular matrix accompanying deterioration of fibroblast function, i.e., volume loss due to the reduction of collagen fibers, hyaluronic acid, and proteoglycan are observed. In photoaged skin, turnover of the stratum corneum is impaired, leading to thickening, and natural moisturizing factors (NMF) decrease, leading to dry skin. Decomposition of collagen fibers and elastic fibers is promoted by the enhanced expression of matrix metalloproteinase-1 (MMP-1), and capillary blood vessels are dilated due to the re-

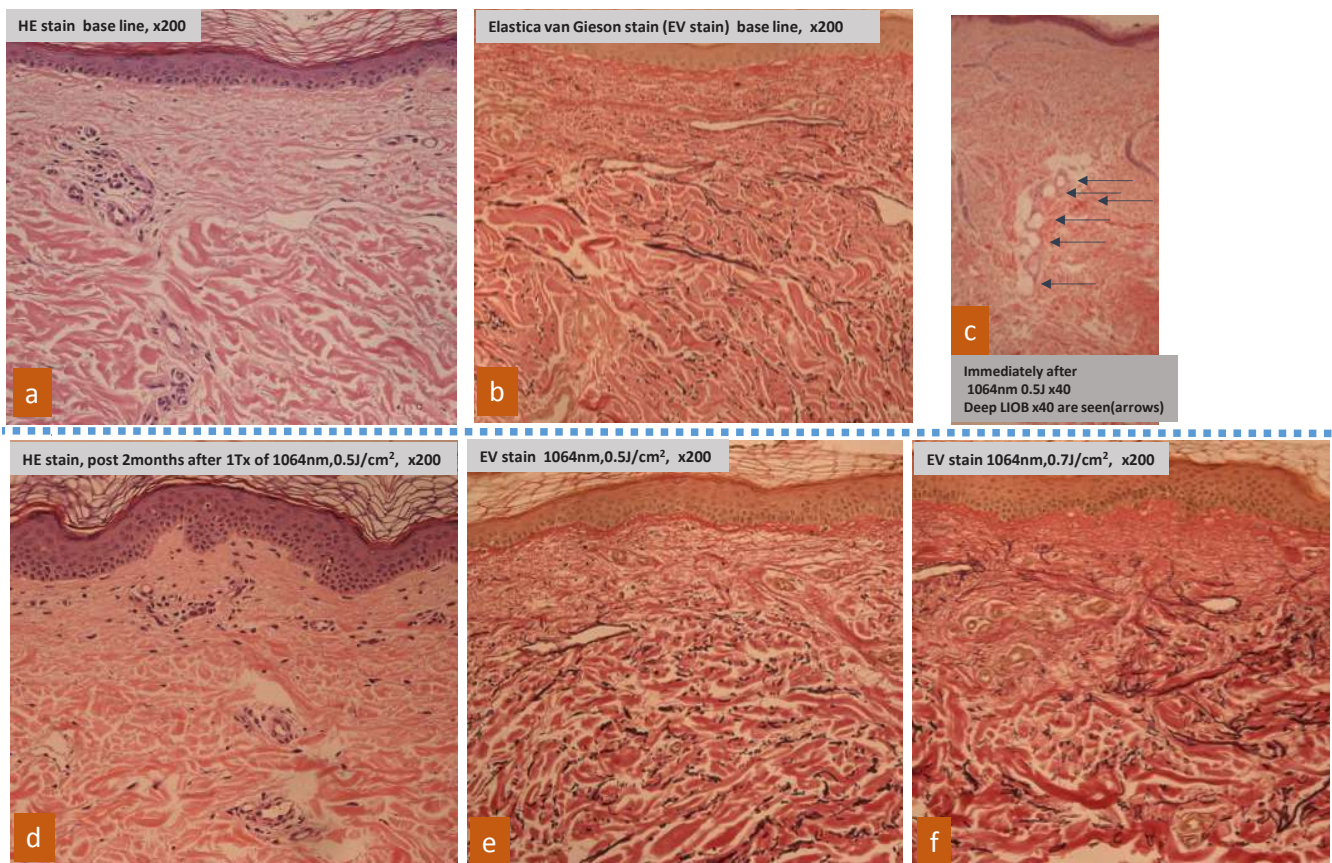


Fig. 5: **a:** Baseline. HE staining: The epidermis is thin and epidermal rete ridges are lost. The dermal components have markedly decreased and capillary blood vessels are dilated, showing marked telangiectasia with an increase in the number and diameter of small blood vessels. **b:** Baseline. Elastin is stained black by EV staining. Reduction of the volume is observed. **c:** HE staining: immediately after picosecond fractional therapy. LIOS is present in the deep layer of the dermis (arrows). **d:** HE staining: 2 months after irradiation at 0.5 J/cm². Thickening of the epidermis and regeneration of epidermal rete ridges are noted. Dermal collagen fibers increase in number. Capillary blood vessels are also observed in the deep layer of the dermis. **e, f:** EV staining: 2 months after irradiation. Elastin markedly increases compared with that at the baseline (b). In the region irradiated at 0.7 J/cm², dendritically extending fibers and those extending toward the epidermis are noted in the dermis. In addition, many capillary blood vessels are present in the superficial to middle layers of the dermis.

duction of thrombospondin-1 (TSP-1) expression and promotion of VEGF expression, leading to poor blood flow²¹⁾. Moreover, the dermis becomes thinner and more susceptible to external pressure due to sunlight exposure-induced aging, such as an increase in advanced glycation end products (AGEs) with an increase in reactive oxygen and a further decrease in fiber components due to MMP-1, promoted by inflammatory cytokine secretion²²⁾.

Kim confirmed the presence of a cytokine network between the epidermis and dermis by demonstrating²³⁾ the reduction of melanogenesis with a decrease in cytokines influencing melanocyte regeneration and new melanin formation, such as the reduction of α -MSH, tyrosinase, tyrosinase-related protein, and NGF release from the dermis, after toning of chloasma with a 1,064 nm laser for 6 nsec. This cytokine network has been investigated as a wound healing mechanism²⁴⁾, and a mutual complementary relationship between the epidermis and dermis is constructed through the cytokine network. When impaired, epidermal cells, pigment cells, dermal vascular plexus, platelets, monocytes, and dermal fibroblasts release many cytokines and regenerate cells in cooperation with autocrine and paracrine factors.

Originally, laser light with an msec-nsec pulse width was used in non-ablative laser skin rejuvenation therapy. Liu et al. reported that when a long pulse laser (msec: photothermal effects) was used in mice, type I collagen increased, whereas when a nanosecond pulse laser (nsec: photothermal and photoacoustic effects) was used, the outgrowth of type I (not readily degraded, and retain tension such as toughness and elasticity) and type III collagen (thin fibers retaining ductility and flexibility) was observed¹¹⁾. Orringer et al. reported that type I procollagen messenger RNA was expressed after irradiation with a long pulse dye laser and 1,320 nm long pulse Nd:YAG laser, and type III procollagen, matrix metalloproteinases, and primary cytokines were produced later²⁵⁾, demonstrating that dermal regeneration is induced by long pulse laser irradiation alone, through the skin surface.

On the other hand, the clinical effects by irradiation with a picosecond pulse laser, 1,064 nm Nd:YAG laser for 750 psec as picosecond laser-toning therapy have been frequently reported at academic meetings, but no detailed histological investigation has been performed. In the skin treated by picosecond laser-toning therapy in our study, rejuvenation of the entire dermis with reconstitution was confirmed. The dermal thickness increased and collagen bundles were regenerated in the reticular layer, thin collagen in the papillary increased and extended over the reticular layer of the dermis, elastic fibers became extended, and capillary blood vessels increased in the superficial layer of the dermis, confirming the effects on photoaging. The effects may increase with a higher output, but an output not causing bleeding in the skin is recommended. Clinically, whitening effects are noted, and

attention should be paid to complications such as vitiligo. However, as the 750 psec pulse width is shorter than the thermal relaxation time (50 nm) of melanosomes and longer than the stress relaxation time (200-400 psec) of melanin granules, damage of melanocytes may be low unless the laser is frequently applied at a high output causing bleeding.

In fractional therapy using picosecond pulse comprising a normal hand piece with a 10 mm lens attached to a Micro Lens Array (MLA) with 200 convex lenses, the output on the skin surface increases to 2.5 times the output displayed on the device. Free electrons taken from atoms, i.e., plasma, are considered to act on the skin. When a picosecond pulse laser with a very high peak power emitted through MLA reaches the target areas (the skin, melanosomes, moisture, and oxidized hemoglobin), multi-photon ionization and avalanche ionization (repeated impact ionization by electrons and ions) occur, and plasma is formed in "optical breakdown" and exhibits a non-thermal action such as cavitation. When a cavity is formed, the condition is not influenced by the laser, being a blocked state (**Fig. 5c**)²⁶⁾. In the epidermis or dermis, spherical cavities, termed laser-induced optical breakdown (LIOB), are sporadically formed. When the output is high, degenerated red blood cells flow in LIOB, which is observed as bleeding in the skin²⁷⁾. This may influence a layer deeper than that reached by picosecond laser-toning therapy because the peak power is larger. Subsequently, remodeling of the dermis may be induced following the mechanism of wound healing, such as the activation of fibroblasts and the regeneration of capillary blood vessels.

Many studies on the clinical effects of picosecond fractional therapy have been reported, but few studies have included detailed histological investigations, similar to studies on toning treatment using picosecond laser-toning therapy²⁶⁾. In the present study, in addition to marked photoaging, signs of intrinsic aging not observed on the medial side of the forearm, epidermal atrophy, and the disappearance of epidermal rete ridges were histologically noted (**Fig. 5a**). When histological changes were investigated two months after irradiation in the region irradiated at 0.5 J/cm² with little bleeding in the skin and region irradiated at 0.7 J/cm² in which bleeding in the skin was clearly observed, epidermal rete ridges markedly lost in intrinsically aged skin were regenerated, thinning of the epidermis was improved (**Fig. 5d,e,f**), area cutanea was observed clinically (**Fig. 4c**), and production of moisturizing components, including NMF, was recovered- demonstrated by a moist feeling, suggesting functional recovery of keratohyalin granules in the granular layer. In addition, there was an increase in the extracellular matrix reduced by photoaging, i.e., outgrowth of capillary blood vessels in the papillary layer over the deep layer of the dermis, and increases in collagen fibers

and elastic fibers were observed. As observed in conventional non-ablative laser skin rejuvenation, type III and type I collagen fibers likely increased. Type VII collagen fibers maintaining the epidermis-dermis junction markedly decrease in intrinsic aging, causing the loss of epidermal rete ridges. Regeneration of epidermal rete ridges was observed, suggesting that type VII collagen fibers were also regenerated and this was marked when there was bleeding in the skin. Moreover, whitening effects were observed, suggesting that the stimulation of melanogenesis from the dermis was reduced. Vitiligo may not be of concern because cautery is applied in a disseminated dot pattern, leaving sufficient normal tissue in this fractional therapy.

Conclusions

Picosecond laser-toning and fractional therapy were both confirmed to effectively induce skin rejuvenation. In addition to improving the photoaged epidermal hypofunction and reconstruction of photoaged dermal extracellular matrix, improvement of intrinsic aging-induced thinning of

the epidermis was observed in the skin treated by picosecond fractional therapy, and similar activation of the epidermis in the skin treated by picosecond laser-toning therapy was also suggested as it caused remodeling of the dermis. The reported half-life of collagen fibers is 15 years²⁰. Therefore, steadily increasing the amount of collagen fibers is important for rejuvenation. Although the half-life of elastic fibers is unclear, no change, such as erythema, is noted in the skin in most cases after treatment using picosecond laser-toning therapy, demonstrating a superior QOL. To prevent vitiligo, it may be appropriate to apply the laser once a month or at a level not causing bleeding in the skin. When more marked changes are desired, fractional therapy by picosecond fractional therapy is recommended. The risk of vitiligo decreases because normal skin remains. This method may be superior, exhibiting more marked rejuvenation effects than those of picosecond laser-toning therapy. There may be downtime accompanied by bleeding in the skin depending on the output, but histological improvement was superior when bleeding occurred in the skin after treatment.

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