A preclinical study testing “Focused Multiple Laser Beams”, a new concept of irradiation with the 1064 nm Nd:YAG laser for skin rejuvenation

A new irradiation method “Focused Multiple Laser Beams”

Masatoshi Horiguchi, MD

Nariaki Miyata, MD

Hiroshi Mizuno, MD

1Department of Plastic and Reconstructive Surgery, Juntendo University School of Medicine, Tokyo, 113-8421, Japan

2Miyata Plastic Surgery & Skin Clinic, Tokyo, 105-0003, Japan
Author contributions to this manuscript:

Masatoshi Horiguchi: Study conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing

Nariaki Miyata: Conception and design, data analysis and interpretation

Hiroshi Mizuno: Conception and design, financial support, data analysis and interpretation, final approval of manuscript

Address correspondence to:

Hiroshi Mizuno, MD, PhD
Professor and Chief
Department of Plastic and Reconstructive Surgery,
Juntendo University School of Medicine
2-1-1 Hongo, Bunkyo-ku, Tokyo 1138421, Japan
Tel: +81-3-3813-3111
Fax: +81-3-5689-7813
E-mail: hmizuno@juntendo.ac.jp
ABSTRACT

Introduction: In order to avoid epidermal heat damage, we developed a novel irradiation method termed “Focused multiple laser beams (FMLB)”, which allows long pulse Nd:YAG laser beams to be irradiated from several directions in a concentric fashion followed by focused into the dermis without epidermal damage. This method is completely different from simple optical focusing with lens on the point that the optical fiber can rotate mechanically. This study aimed to assess whether FMLB achieves the desired dermal improvement without epidermal damage. Materials and Methods: The dorsal skin of New Zealand White rabbits was irradiated with FMLB. One hour and 1, 2, 3 and 4 weeks later, macroscopic and histological analysis were performed. Real-time PCR analysis of type I and III collagen expression was performed at 2 and 4 weeks. Results: Control exhibited skin ulcers healed with scar whereas FMLB remained intact macroscopically. Histologically, FMLB group showed increase of dermal thickness at 4 weeks while epidermis remained intact. Real-time PCR demonstrated that both type I and III collagen increased at 2 weeks while decreased at 4 weeks. Conclusions: FMLB can deliver the target laser energy to the dermis without significantly affecting the epidermis.

Key Words: Non-ablative laser, Real-time PCR, Silver impregnation staining, Type I
collagen, Type III collagen
INTRODUCTION

Laser treatment of facial wrinkles is one of the most popular and standard procedures used to rejuvenate facial skin. Non-ablative lasers, including 1064 nm Nd:YAG and 1320 nm Nd:YAG, are particularly preferred for this purpose, especially in Asian populations, because they limit the chance of various side effects such as post-inflammatory hyperpigmentation (1). It is generally accepted that non-ablative laser treatment improves wrinkles by heating the dermis, which denatures the dermal collagen. This induces an inflammatory cascade that stimulates the dermal fibroblasts and eventually promotes neocollagenesis (2).

Despite its effectiveness, non-ablative laser treatment for facial rejuvenation sometimes causes thermal injuries that manifest as prolonged erythema and pigmentation disturbances. To prevent this, most of the present laser irradiation systems have devices to cool the overlying epidermis. These devices include cryogen sprays at the head of the handpiece. While several studies have sought to optimize cryogen spray-mediated refrigeration of the epidermis (3-8), there is a continuing risk of thermal injury that cannot be eliminated as long as the laser beams penetrate the epidermis perpendicularly from a single direction. We hypothesized that this problem could be eliminated by using a dispersing device that causes the individual laser beams to land on multiple areas of the
epidermis before converging at a predetermined point in the dermis. Theoretically, the
dermis, but not the epidermis, would receive the full energy of the laser treatment, thus
achieving the clinical aim without the risk of inducing epithelial injury. To our knowledge,
studies on such a strategy have not been reported previously.

To test this strategy, we recently developed a new laser irradiation system that
we designated Focused Multiple Laser Beams (FMLB). The present rabbit model study
was performed to determine whether FMLB effectively heats the dermis while protecting
the epidermis.

MATERIAL AND METHODS

Principle of the Focused Multiple Laser Beams system

The laser fiber in the tip of the handpiece is attached at an adjustable angle to the inside
wall of a cone-shaped cylinder that has a diameter of 5 mm and can spin at a speed of 180
rpm. As a result, the laser fiber spins around in a concentric fashion while emitting its
radiation, causing numerous beams with relatively low energy to strike the epidermis at
a wide angle from multiple directions (Fig. 1). The beams then converge under the
epidermis at a predetermined depth in the dermis: the depth reflects the depth of the
wrinkle and is determined by the angle of the fiber in the cylinder. Thus, the dermis
becomes heated to the point that collagen denaturation occurs, whereas the epidermis under the target dermis receives less thermal energy than would be delivered if the laser beam was focused perpendicularly on the epidermis from a single point. The heat of the epidermis would thus rapidly dissipate before the next irradiation arrives.

Animal Preparations and Laser Irradiation

This study was approved by the Juntendo University Institutional Animal Care and Use Committee (Approval #: 240190). Ten-month-old female New Zealand White rabbits (Oriental Koubo, Tokyo, Japan) were used for the study. All rabbits were given an intramuscular injection of ketamine (35 mg/kg) and xylazine (5 mg/kg) to initiate anesthesia before the treatment.

The non-ablative laser system used in the study was STREAK: this is a long-pulsed Nd:YAG system that emits 1064 nm wave lengths (Altech, Yamanashi, Japan). The irradiation parameters were set to a pulse duration of 200 μs, a fluence of 350 J/cm², and a repetition rate of 30 Hz. For FMLB, the usual handpiece on the laser system (which delivers a single beam at 90° to the epidermis) was replaced with a special handpiece (Fig. 2). The depth of laser beam convergence was set at 1.5 mm under the epidermis. The back of each rabbit was shaved, after which the left dorsal skin was treated with FMLB and
the right side was irradiated with the standard treatment (a perpendicular beam from one direction). Prior to irradiation, the area that was to be irradiated was marked by India ink tattoos. Both sides were irradiated with the same laser system, albeit with different handpieces.

Macroscopic Observation

The irradiated area was macroscopically observed every day and photographs were taken 1 hour and 1, 2, 3 and 4 weeks after irradiation. All photographs were taken in the same operating room with identical lighting conditions.

Histological Assessment

Skin biopsy specimens were harvested from both sides of the back 1 hour and 1, 2 and 4 weeks after irradiation by using a 6 mm punch biopsy kit. All specimens were fixed in formalin solution, embedded in paraffin, and sectioned. To detect gross microscopic changes, structural changes in the collagen fibers, and the extent of the reticular fibers, the sections were stained with hematoxylin and eosin, Elastica van Gieson, and the silver impregnation method, respectively, followed by light microscopy.
Real-time PCR

Gene expression of type I and III collagen in the 2 and 4 week biopsy samples were measured by real-time PCR. Total RNA was extracted from the samples by using the Qiagen RNeasy Mini Isolation Kit (Qiagen, Valencia, CA), according to the instructions of the manufacturer. The cDNA was prepared by using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). The mRNA levels of alpha 2 type I collagen (Cat. # 4351372), pro-alpha 1 type III collagen (Cat. # 4331182), and 18S ribosomal RNA (Cat. # 4319413E) were determined by using the TaqMan Gene expression Assay (Applied Biosystems, Carlsbad, CA). The endogenous control, 18S ribosomal RNA, was used to normalize target gene expression. The random primers for alpha 2 type I collagen and pro alpha 1 type III collagen were used according to the manufacturer’s guidelines (Applied Biosystems). For the PCR, the primers, probes, and cDNA were combined with TaqMan Fast Advanced Master Mix (Applied Biosystems) and amplification was performed by the Applied Biosystems 7300HT Fast Real Time PCR System. The thermocycler parameters were 50°C for 2 min, 95°C for 10 min, and 30 cycles at 95°C for 15 s and 60°C for 1 min. Quantitative analysis of gene expression was performed by using the comparative CT (ΔΔCT) method. All experiments were performed in triplicate.
Statistical Analysis

All statistical analyses of the real time PCR data were conducted with GraphPad prism (San Diego, CA). The FMLB- and standard-treated skin groups were compared in terms of collagen gene expression by using Student’s t-test. Results were considered to be statistically significant when P<0.05.

RESULTS

FMLB did not damage the epidermis or skin appendages, as shown by macroscopic observations

One hour after standard treatment, the center of the irradiated area exhibited whitening and the surrounding area displayed erythema and swelling. One week later, the central part developed skin necrosis and ulcer formation. Four weeks after standard irradiation, the ulcers had decreased in size due to skin contracture and epithelialization, but mild scar formation and loss of hair follicles were observed. By contrast, 1 hour after FMLB treatment, only the center exhibited slight reddening; other macroscopic changes were not observed at any time point thereafter. No loss of hair growth was ever detected (Fig. 3).
Characteristic microscopic change early after FMLB

The biopsy sample taken 1 hour after FMLB irradiation followed by stained with hematoxylin and eosin exhibited a circle of structural denaturation in the dermis. The center of this circle was located 1.5 mm below the epidermis and thus was the point where the laser beams converged (Fig. 4).

The skin thickened after FMLB but retained a normal structure, as shown by hematoxylin and eosin staining

One hour after standard irradiation, the epidermis exhibited no appreciable change but the entire dermis layer exhibited edematous denaturation of collagenous fibers. One week later, there were necrotic changes that associated with fibrin-like structures, edematous denaturation of collagenous fibers, and inflammatory cell infiltration throughout the epidermis and dermis. Two weeks after standard irradiation, there was marked fibroblast proliferation between the edematous collagen fibers. Moreover, at 2 and 4 weeks, the epidermis was thickened due to epithelialization and the skin appendages were damaged, while the dermis continued to exhibit edematous denaturation of collagenous fibers. By contrast, appreciable changes in the epidermis and dermis were not detected 1 hour or 2
weeks after FMLB. However, 4 weeks after FMLB, moderate knoll-like thickening in the dermis was observed. The area of dermal thickening change spread from the point where the laser beams had converged (Fig. 5).

FMLB did not induce appreciable changes in dermal collagenous structures, as shown by Elastica van Gieson staining

To investigate the effect of irradiation on the structure of the dermal collagen fibers, the standard- and FMLB-treated skin biopsies were subjected to Elastica van Gieson staining. One hour after standard irradiation, the collagen fibers were swollen and their alignment was disarrayed. The extent of these irregular structures gradually decreased over the following 2 weeks. Four weeks after standard irradiation, the collagen fibers exhibited dense alignment and elastic fibers appeared between the collagen fibers. By contrast, the structure and alignment of the dermal collagen fibers were maintained at all time points after FMLB irradiation and elastic fibers among the collagen fibers were not observed at 4 weeks (Fig. 6).

FMLB associated with a marked decrease in reticular fiber numbers, as shown by silver impregnation staining
Two weeks after standard irradiation, reticular fibers were found among the collagen fibers. Similar numbers of reticular fibers seemed to be present at 4 weeks. By contrast, while the FMLB-treated skin had similar numbers of reticular fibers at 2 weeks as the standard-treated skin, these numbers dropped dramatically at 4 weeks in the FMLB-treated skin (Fig. 7).

FMLB associated with a dramatic drop in type I and III collagen gene expression at 4 weeks, as shown by real-time PCR

Two weeks after irradiation, FMLB associated with a 2.1-fold higher gene expression of type I collagen compared to the standard treatment. However, at week 4, FMLB associated with a significant 2.6-fold lower expression of this gene compared to standard treatment (p<0.05). Similarly, 2 weeks after irradiation, FMLB associated with a 1.5-fold higher gene expression of type III collagen compared to the standard irradiation. However, 4 weeks after irradiation, FMLB associated with a significant 2.6-fold lower expression of this gene compared to standard treatment (p<0.05) (Fig. 8).

**DISCUSSION**

This study showed that FMLB significantly protected the epidermis from laser-induced
heat damage while causing the laser energy to converge at the designated point in the dermis. At the macroscopic level, the epidermis and its appendages remained intact after FMLB even though the energy that was used in this treatment was the same as that employed with the standard treatment, which consisted of one beam directed perpendicularly to the epidermis. Unlike FMLB, the standard treatment led to serious macroscopic epidermal damage. At the histological level, appreciable changes were not observed 1 hour or 1 or 2 weeks after FMLB, but moderate knoll-like thickening of the dermis was observed 4 weeks after FMLB. By contrast, the epidermis and the dermis exhibited necrosis, scarring, and hair loss 4 weeks after the standard treatment. Elastica van Gieson staining showed that the dermal collagenous structures did not change appreciably after FMLB, whereas after standard treatment, the collagen fibers swelled and became disarrayed, and at 4 weeks exhibited dense alignment; elastic fibers between the collagen fibers also appeared at this time point. Silver impregnation staining showed that while standard and FMLB treatment yielded equivalent numbers of reticular fibers at 2 weeks, these numbers dropped at 4 weeks in the FMLB group only. Real-time PCR showed that standard and FMLB treatment elevated type I and III collagen gene expression, but only the FMLB group showed a dramatic decrease in the expression levels of these genes at 4 weeks.
Generally, non-ablative lasers are preferred for skin rejuvenation in Asian populations because they are less likely than the ablative lasers (e.g., carbon dioxide and erbium:YAG lasers) to induce unwanted side effects such as post-inflammatory hyperpigmentation (1,9). To date, many studies have shown that non-ablative lasers are safe and effectively induce skin rejuvenation (10-13). However, despite the lower risks of non-ablative lasers, it is still essential to use cooling devices such as cryogen spray to prevent epidermal heat damages. Many researchers have investigated the optimal conditions of the cryogen spray, including nozzle-to-skin distance, spurt duration, the temperature of the handpiece’s tip, and the degree of skin compression (3,6,14). However, despite such optimization, the risk of epidermal heat damage remains. The main reason for the excessive heating of the epidermis during non-ablative laser treatment is that the laser beams all come from a single direction and hit the skin perpendicularly to the epidermis. We hypothesized that if the individual laser beams would be dispersed such that they overall hit a wide area of the epidermis before converging at the desired location in the underlying dermis, this would reduce epidermal heat damage while simultaneously achieving the thermal heat in the dermis that is needed to induce collagen denaturation. To test this notion, we developed the new laser irradiation system that we called Focused Multiple Laser Beams (FMLB). To the best of our knowledge, previous studies that revise
the angle, dispersion, and convergence of laser irradiation have not yet been reported. We found that FMLB did not damage the epidermis or the appendages in the superficial dermal layer at both the macroscopic and microscopic levels. By contrast, the standard treatment induced necrosis, scarring, and hair loss.

As opposed to ablative laser resurfacing such as carbon dioxide and erbium: YAG lasers, non-ablative laser resurfacing has been shown to induce selective dermal injury with minimal damage to the epidermis. Therefore, few studies have actually assessed the epidermal changes that arise after non-ablative laser irradiation that increases dermal thickness (15,16). Liu et al reported that 30 J/cm² of long pulse 1,064-nm Nd:YAG induced marked increase of epidermal thickness after 1 week of irradiation in Kunming mouse model (15). However, serious damages including tissue degeneration, necrosis and scar formation in the epidermis and appendages could be induced by higher doses of irradiation. In our study, the laser fluence in both the FMLB and standard treatments was 350 J/cm². Indeed, necrotic changes occurred after standard treatment. However, the epidermis and appendages remained intact after FMLB, which suggests that FMLB can target the energy required for skin rejuvenation to the dermis while inducing less epidermal damage.

In our study, dermal thickening was observed 4 weeks after FMLB. Such dermal
thickening is generally considered to be induced by the thermal injury in the dermis, which initiates a cascade of inflammatory events that induces fibroblast proliferation and up-regulation of collagen expression (17). This thermal damage of the dermal collagen is characterized by decreased staining intensity and an increase in the homogeneous appearance of collagen fibers (18). The fibroblasts largely synthesize type III collagen in the early stage of the wound healing process; later, type I collagen is predominantly synthesized. Alam et al. suggested that when the horizontal reticular fibrous array is seen in the dermis after irradiation, it reflects thermal damage-induced scarring rather than an improvement in collagen deposition (16). Thus, type III collagen is considered to be important for the appropriate remodeling of skin collagen. Indeed, many studies have used histological or biochemical measurements of the changes in type III collagen synthesis as an index of skin rejuvenation treatment efficacy (15,19,20). In our study, edematous denaturation of collagenous fibers and inflammatory cell infiltration in the dermis were observed 1 week after standard treatment. By contrast, FMLB did not induce appreciable histological dermal changes 1 hour or 1 or 2 weeks after irradiation. Moreover, silver impregnation showed that 2 weeks after standard and FMLB treatment, the numbers of reticular fibers (which are composed of type III collagen) were elevated. While these numbers dropped at 4 weeks in the FMLB group, they persisted in the
standard treatment group. Consistent with these silver impregnation findings, real-time PCR showed that while both standard and FMLB treatment elevated type I and III collagen gene expression at 2 weeks, FMLB associated with a dramatic drop in both expression levels at 4 weeks. By contrast, the high expression levels persisted in the standard treatment group. Based on the general consensus described above and our findings in this study, we speculate that unlike the standard treatment, FMLB did not induce a protracted wound healing process in the dermis, thus facilitating collagen synthesis without generating appreciable dermal changes.

It remains unclear precisely how FMLB with the same amount of laser energy as the standard treatment induced dermis thickening while avoiding thermal damage to the epidermis and superficial dermal layer. Further studies that investigate the molecular activity in the dermis and characterize the local environment (e.g., its temperature and changes in dermal thickness) are needed. Nevertheless, our study results suggest that FMLB may have several advantages over the standard non-ablating laser modality in terms of reducing wrinkles in esthetic procedures clinically. Future clinical trials that explore the usefulness and limitations of FMLB are warranted.
REFERENCES

14. Klavuhn KG, Green D. Importance of cutaneous cooling during photothermal


FIGURE LEGENDS

Figure 1. Schematic depiction of Focused Multiple Laser Beams. The laser fiber is attached at an adjustable angle to the inside wall of a cone-shaped cylinder that spins around in a concentric fashion and causes the laser fiber to deliver multiple relatively low energy beams that land on a wide area of epidermis at a wide angle before converging at a predetermined point in the underlying dermis. Consequently, the laser energy is spread out over the epidermis and can thus dissipate readily, and only the dermis receives the full energy that is delivered by the laser fiber.

Figure 2. The gross appearance of the Focused Multiple Laser Beams (FMLB) irradiation system. For FMLB, a special handpiece is attached to the 1064 nm Nd:YAG laser system.

Figure 3. Representative images of the macroscopic appearance of the irradiated areas on the dorsal skin of the rabbits 1 hour and 1, 2, 3, and 4 weeks after they underwent Focused Multiple Laser Beams (FMLB) or the standard treatment. After FMLB, appreciable changes, including hair loss, were not observed at any time point after irradiation (a–e). One hour after standard treatment, central whitening with surrounding erythema and swelling was observed. By week 1, skin necrosis and ulcers had developed (f, g). At 4
weeks, scar formation and loss of hair follicles were evident (h–j).

Figure 4. A representative image of the characteristic histological change 1 hour after Focused Multiple Laser Beams (FMLB) treatment. The dermis contained a circular area of structural denaturation, the center of which (arrow) was 1.5 mm from the surface of the epidermis. This is consistent with the initial setting. (Bar=1 mm).

Figure 5. Representative images of hematoxylin- and eosin-stained skin after Focused Multiple Laser Beams (FMLB) or standard treatment. After FMLB, the dermal thickness increased while the epidermis and skin appendages remained intact (a–d). After standard treatment, dermal edematous denaturation followed by skin necrosis and ulcer formation was observed. Thereafter, epidermal hyperplasia and loss of skin appendages were observed (e–h). (Bar=200 μm).

Figure 6. Representative images of Elastica van Gieson-stained skin after Focused Multiple Laser Beams (FMLB) or standard treatment. After FMLB, disruption of the dermal collagenous structures was not detected during the 4 week observation period (a–d). After standard treatment, the collagen fibers were swollen and their alignment was
disarrayed 1 hour after irradiation (e). The extent of such irregular structures gradually decreased. Four weeks after standard treatment, the collagen fibers were densely aligned and there were elastic fibers among the collagen fibers (f–h). (Bar=50 μm).

Figure 7. Representative images of silver impregnation-stained skin after Focused Multiple Laser Beams (FMLB) or standard treatment. After FMLB, the reticular fiber numbers initially rose at 2 weeks and then dramatically dropped 4 weeks after irradiation (a, b). After standard treatment, the reticular fiber numbers rose at 2 weeks and were sustained at 4 weeks (c, d). (Bar=50 μm).

Figure 8. Real-time PCR of type I and III collagen gene expression in skin after Focused Multiple Laser Beams (FMLB) or standard treatment. Two weeks after irradiation, FMLB associated with greater gene expression of type I and III collagen than standard treatment, although these differences between the groups did not achieve statistical significance. At 4 weeks, these expression levels persisted in the standard treatment group but dropped dramatically in the FMLB group. As a result, the FMLB groups had significantly lower type I and III collagen gene expression at 4 weeks than the standard treatment group (both p<0.05).