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Systematic evaluations of skin damage irradiated by an erbium:YAG laser: Histopathologic analysis, proteomic profiles, and cellular response

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ABSTRACT

Background: The erbium:yttrium–aluminum–garnet (Er:YAG) laser is used for surgical resurfacing. It has ablative properties with water as its main chromophore.
Objective: This study attempted to establish the cutaneous effect and cellular response to Er:YAG laser irradiation using different fluences (7.5 and 15 J/cm^2).
Methods: Female nude mouse was used as the animal model in the study. Physiological parameters were examined and histology was evaluated at 4, 24 and 96 h after laser exposure. A proteomic analysis and immunoblotting were also used to determine the mechanisms of the laser's effect on the skin.
Results: Both fluences were associated with a significant increase in transepidermal water loss (TEWL), erythema (a*), and the skin pH at 4 and 24 h. In contrast, at 96 h, the levels of these parameters had generally decreased to the baseline. The histology examined by light microscopy and transmission electron microscopy (TEM) showed vacuolization, hydropic degeneration and epidermal necrosis of laser-irradiated skin. The higher fluence (15 J/cm^2) exhibited more-severe disruption of the skin. Bulous and scarring were observed in skin treated with the higher fluence during the recovery period. p53 and p21 proteins were significantly activated in skin following exposure to the laser. However, proliferating cell nuclear antigen and cytokeratin expressions were downregulated by the low fluence (7.5 J/cm^2).
Conclusion: Both proliferation and apoptosis occurred when the laser-irradiated the skin.

1. Introduction

The range of laser applications in medicine and surgery is rapidly expanding. Laser–skin resurfacing is an effective treatment option for many patients with cutaneous photodamage, wrinkles, rhytides and acne scarring [1,2]. Skin remodeling is initiated by controlled injury to the skin [3]. The erbium:yttrium–aluminum–garnet (Er:YAG) laser is a commonly used ablative device. This laser, with a wavelength of 2940 nm, produces laser irradiation in the near-infrared portion of the electromagnetic spectrum. This wavelength corresponds to the main peak of water absorption. This feature causes minimal residual thermal damage to skin [4,5]. Besides the treatment of skin disorders, Er:YAG lasers enhance topical/transdermal drug delivery via the skin route [6–8]. Complications are infrequent with careful use but include: erythema, hyperpigmentation, hypertrophic or atrophic scarring, acne flare-ups, pruritus, wound infection, milia and contact dermatitis [9,10].

One important but usually neglected issue with laser resurfacing is the safety and recovery of the skin. Moreover, the exact characterization of skin–laser interactions is not well understood. The aim of this study was to establish the histopathologic effects and cellular profiles of the skin after an Er:YAG laser pulse was applied. The mechanisms of skin damage-induced by the laser were elucidated based on data obtained in this work. We used a nude mouse animal model to address the complications associated with the use of an Er:YAG laser for skin rejuvenation. Animal

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studies are important, because it will take decades to determine whether the use of such devices is harmful to humans [11].

The structural and ultrastructural alteration of the skin after laser treatment was examined by light microscopy, transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The safety of the laser and recovery of the skin barrier were examined by physiologic parameters using bioengineering techniques, including transepidermal water loss (TEWL), skin redness (a*), skin lightness (L*) and pH. Determination of skin tissue damage by the laser may represent protein denaturation and sensitization [12]. Hence a proteomic analysis was performed on the skin after laser treatments. Furthermore, changes in biomarkers of the skin such as p53, p21, 14-3-3, proliferating cell nuclear antigen (PCNA) and cytokeratin were also revealed by Western blotting. The skin damage caused by the laser depends primarily on the penetration depth of the radiation used. Two laser fluences, 7.5 and 15 J/cm^2, were used in this study to compare the disruption produced by different penetration depths.

2. Materials and methods

2.1. Laser assembly

The modulated Er:YAG laser (Contour, Sciton Laser, Palo Alto, CA, USA) has a wavelength of 2940 nm and a pulse duration of 100 μs. An articulated arm was used to deliver the laser beam onto nude mouse skin. A square scanning handpiece was used to vaporize the skin in a 1.5 × 1.5 cm^2 area. The dorsal skin of a nude mouse was treated with the laser at settings of 7.5 or 15 J/cm^2, with 10% overlap and zero coagulation.

2.2. Animal model

The female nude mouse (ICR-Foxn1nu strain, 8 weeks old) was used as the *in vivo* animal model, in accordance with a protocol approved by the Institutional Animal Care and Use Committee at Chang Gung University. After being housed in a pathogen-free animal facility and fed a base diet and water, the dorsal skin of the animals was treated by the laser. The bioengineering characterization was determined 4, 24 and 96 h after laser treatment. Skin specimens were also obtained from animals at various post-operative durations. These biopsies were used for histopathology, Western blot analysis and proteomic analysis.

2.3. Bioengineering evaluations of the skin

At 4, 24 and 96 h after laser irradiation, transepidermal water loss (TEWL), colorimetric parameters and the pH of the treated skin were measured. TEWL was recorded using a Tewameter® (TM300, Courage & Khazaka, Köln, Germany). Measurements were taken at a stable level 30 s after application of the TEWL probe to the skin. The TEWL was automatically calculated and expressed in g/m^2/h. A spectrocolorimeter (CD100, Yokogawa Electrical, Tokyo, Japan) was used to measure the skin erythema (a*) and lightness (L*) as recommended by the CIE (Commission Internationale de l'Eclairage). When recording color values, the measuring head was held perpendicular to the dorsal skin, and the aperture was fitted with an applicator, to avoid compression of the subcutaneous capillaries. The reading was obtained within a few seconds on the display. The skin surface pH was determined by Skin-pH-Meter® pH 905 (Courage & Khazaka). An adjacent untreated site was used as a baseline standard for each determination. The temperature and relative humidity in the laboratory were kept at 26 °C and 55%, respectively. The sample number for each experiment was six (n = 6).

2.4. Histological examination by light microscopy

At 4, 24 and 96 h after laser treatment, the macroscopic condition of the skin was observed using a digital camera (E4500, Nikon, Tokyo, Japan). Then the animals were sacrificed, and specimens of the exposed area were taken for histological examination. Each specimen was fixed in a 10% buffered formaldehyde solution at pH 7.4 for at least 48 h. Each section was dehydrated using ethanol, embedded in paraffin wax, and stained with hematoxylin and eosin (H&E). The antibody specific for PCNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was also used for immunohistochemical staining. The biopsies were examined and evaluated under light microscopy with a digital camera (DX71, Olympus, Tokyo, Japan).

2.5. Ultrastructural examination by TEM and SEM

Excised skin samples were cut into appropriate-sized cubes and immediately fixed at 4 °C in 2% paraformaldehyde and 2.5% glutaldehyde in 0.2 M cacodylate and 7% sucrose buffer for 15 min, post-fixed with 2% osmium tetroxide for 24 h, and washed three times as described before. Specimens were then dehydrated in graded concentrations of ethanol. Samples for TEM were embedded in an epon–epoxy mixture and sectioned. Thin sections were double-stained with uranyl acetate and lead citrate and examined by TEM (H-6000, Hitachi, Tokyo, Japan). For SEM, specimens were affixed with gold–palladium in an ion coater and examined by SEM (S-5000, Hitachi). All analyzes were performed in a blinded fashion.

2.6. Two-dimensional electrophoresis and image analysis

Skin tissue samples (250 μg) extracted from nude mouse were thawed and diluted in IPG sample buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 65 mM dithiothreitol (DTT) and 1% IPG buffer (a carrier ampholyte mixture matching the pH range used) to a volume of 350 μl. After rehydration for 12 h at 30 V, IEF was conducted automatically with a total of 75 kVh. Following IEF separation and equilibration, electrophoresis was carried out on 12% acrylamide gels (Bio-Rad, Hercules, CA, USA) at 40 mA. Proteins were visualized by silver staining and then scanned using an Imagescanner (Amersham Bioscience, Buckinghamshire, UK). Protein spots were quantified using the Non-linear Progenesis software (J&H Technology, Taipei, Taiwan). The amount of each protein spot was presented as its volume. Spot volumes were normalized as a percentage of the total volume of all the spots appearing in a gel. The average value of each protein spot was calculated from three independent experiments. All experiments were repeated three times to confirm the reproducibility of the protein profiles.

2.7. In-gel-digestion of proteins and MALDI-TOF/MALDI-TOF–TOF MS

Silver-stained spots were excised and in-gel-digested with trypsin according to procedures described previously [13]. Briefly, gels were destained with 1% potassium ferricyanide and 1.6% sodium thiosulfate (Sigma, St. Louis, MO, USA). Then the proteins were reduced with 25 mmol/l NH_4HCO_3 containing 10 mmol/l DTT (Amersham Bioscience) at 56 °C for 30 min and alkylated with 55 mmol/l iodoacetamide (Amresco, Solon, OH, USA) at room temperature for 30 min. Then, the proteins were digested with 20 μg/ml trypsin (Promega, Madison, WI, USA) at 37 °C overnight. After digestion, the tryptic peptides were acidified with 0.5% TCA and loaded onto an MTP AnchorChip™ 600/384 TF (Bruker-Daltonik, Billerica, MA, USA). The MALDI-TOF MS analysis was performed on an Ultraflex™MALDI-TOF mass spectrometer