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Repetitive Er:YAG Laser Irradiation of Human Skin: A Histological Evaluation

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Background and Objective: Deep coagulation of skin collagen by Er:YAG laser repetitive pulses has been predicted by previous theoretical models and later demonstrated on animal skin. The goal of this study was to determine the effect of repetitive Er:YAG laser pulses on human skin and its response to this treatment.
Study Design/Materials and Methods: Lid skin of six female volunteers with blepharochalasis has been treated with laser at day 0, 7, and 21 before elective surgery—blepharoplasty. The treated skin was excised as part of the procedure and prepared for further histological examination. We used a 2,940 nm Er:YAG laser (Fidelis M320A by Fotona) with 'smooth' mode parameters: fluence from 0.50 to 2.00 J/cm^2; six pulses per packet; 550 μsecond/pulse, 250 millisecond/packet; single pass, no overlapping; spot size 5 mm; repetition rate 20 Hz.
Results: We observed deep collagen denaturation at laser fluences of 1.25 J/cm^2 and over; epidermal damage was proportional to fluence with total coagulation of the epidermal layer at fluences of 1.75 J/cm^2 and over. At day 7 after laser treatment we observed a complete regeneration of the epidermal layer and a regeneration zone within the dermis with prominent infiltration of CD68+ monocytes/macrophages. At day 21 after laser treatment we observed collagen remodeling and (myo-)fibroblast proliferation at tissue depths of up to 240 μm.
Conclusions: Repetitive Er:YAG laser irradiation is effective in deep denaturation and remodeling of human skin collagen in vivo, with less epidermal damage compared to standard Er:YAG laser skin resurfacing. Lasers Surg. Med. 35:146–151, 2004. © 2004 Wiley-Liss, Inc.

Key words: collagen denaturation; collagen remodeling; myofibroblast; non-ablative laser skin resurfacing

INTRODUCTION

The main goal of laser skin resurfacing is to achieve safe remodeling of the skin with as little as possible side effects (hypo- and hyperpigmentation, infection, scarring). Historically, CO_2 lasers were the first to be used for skin resurfacing, followed by Er:YAG lasers [1,2]. The latter produce a satisfactory remodeling of skin collagen, with less thermal damage compared to CO_2 lasers. In both, the problem of epidermal ablation with its implication of longer healing and possible side effects is present [3–12]. With the introduction of non-ablative and non-coherent light skin resurfacing methods a stimulus has been released to further improve what are nowadays largely used laser resurfacing techniques [13–16].

Spatial overlapping of consecutive laser exposures causes deep thermal damage, as studies of Er:YAG ablation and CO_2 skin resurfacing demonstrate [17,18]. This started the idea to produce deep collagen denaturation by stacking of repetitive Er:YAG pulses. Theoretical studies [19–21] as well as studies on animal skin searched for a possibility to deliver laser energy to deeper skin tissue without the unwanted superficial ablation [22].

The goal of our study was to determine whether repetitive laser irradiation of human skin with an Er:YAG laser could produce sufficient collagen denaturation and what would be the in vivo response of human skin to this treatment. In addition, we searched for a laser fluence that would produce enough dermal injury without complete epidermal ablation to cause new collagen synthesis.

MATERIALS AND METHODS

Patients

Six female volunteers participated in this study, aged between 52 and 63 years. All of them had Fitzpatrick skin type 2 and blepharochalasis, for which blepharoplasty was indicated. None of the patients had a history of previous skin disease or laser/surgical treatment of the involved area. The lid skin was treated with laser in local anesthesia with 2% Xylocaine at days 0, 7, and 21 before elective surgery—blepharoplasty. At the time of surgery the treated skin was excised as part of the procedure.

Laser

We used a 2,940 nm Er:YAG laser (Fidelis M320A by Fotona, Ljublgana, Slovenia) with 'smooth' mode parameters: fluence 0.75, 1.0, 1.25, 1.50, 1.75, and 2.00 J/cm^2; six pulses per packet; 550 μsecond/pulse, 250 millisecond/

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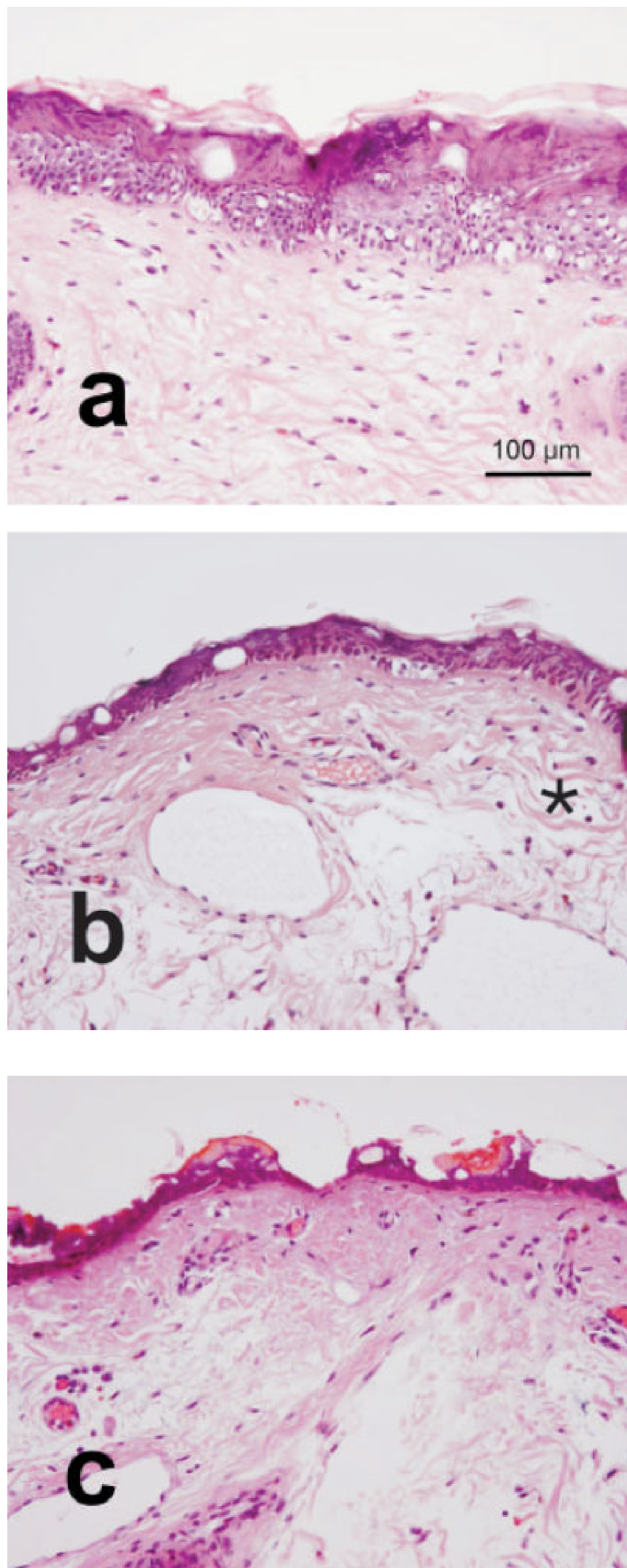


packet; one application to each location, no overlapping; spot size 5 mm; repetition rate 20 Hz. No cooling device was used.

Histology

The excised skin was fixed in 10% formalin and embedded in paraffin. Two-micrometer thick haematoxylin and eosin (H&E) stained sections were prepared for histological analysis. Special immunohistological staining was performed to show proliferating cells (anti Ki-67 antibody, Dako Glostrup, Denmark, clone MIB-1), monocytes/macrofages (anti CD68 antibody, Dako, clone PG-M1), myofibroblasts (anti smooth muscle actin–SMA antibody, Dako, clone 1A4) and basal membrane (anti collagen type IV antibody, Dako, clone CIV22). Immunohistochemistry was performed according to the standard routine procedure at the Institute of Pathology. The sections were analyzed by two pathohistologists experienced in the area of skin pathology, who were unaware of the laser parameters used.

RESULTS

Day 0

We observed a range of thermal injury in the epidermal and dermal layer immediately after laser treatment. Thermally injured keratinocytes were eosinophillic and clumped, indicating coagulative necrosis. Some less injured cells displayed vacuolar cytoplasmic degeneration.

The extent of epidermal injury increased with increasing laser fluence. At least some basal keratinocytes were preserved following treatment with fluence of 1.0 and 1.25 J/cm^2 while full thickness epidermal coagulative necrosis with partial or total ablation of the epidermis was noted at fluences of 1.5 J/cm^2 and above. Notably, no ablation below basal membrane was observed at fluence 1.75 J/cm^2 (Fig. 1 and Table 1), but it was mostly destructed at 2.0 J/cm^2.

Treated dermis displayed eosinophillic degeneration of collagen fibers (thermal coagulation), which appeared pink and homogeneous. Cells in the dermis did not show prominent signs of coagulative necrosis, except for some cells beneath the basal membrane. Dermal injury was inconspicuous and was noted only focally following treatment with 1.0 J/cm^2, while it was readily observed at fluences of

Fig. 1. Progressive changes of the epidermis and dermis immediately after laser treatment. **a**: Fluence 1.00 J/cm^2. Mostly upper epidermis is injured and basal keratinocytes are preserved. Dermal injury is inconspicuous (H&E, original magnification = 200×). **b**: Fluence 1.25 J/cm^2. Epidermal injury is almost complete, but some basal keratinocytes are preserved. Prominent collagen denaturation zone is seen; compare with the untreated skin on the right side of the figure (asterix) (H&E, original magnification = 200×). **c**: Fluence 1.50 J/cm^2. There is a full thickness epidermal injury with partial denudation of the epidermis. An extensive collagen denaturation is seen in the dermis (H&E, original magnification = 200×).

TABLE 1. Changes of the Epidermis and Dermis at Days 0, 7, and 21 After Laser Treatment With Different Fluences

Fluence (J/cm^2)	0.75	1.00	1.25	1.50	1.75	2.00
Day 0						
Epidermal injury	/	Mostly upper half, basal keratinocytes preserved	Upper half to full thickness, some basal keratinocytes preserved	Full thickness, partial ablation of epidermis, but not below basal membrane	Full thickness, ablation of epidermis, but not below basal membrane	Complete ablation of epidermis, basal membrane mostly destructed
Depth of dermal injury (μm)	/	Focally up to 5	80 (±12)	120 (±8)	140 (±10)	150(±19)
Day 7						
Epidermal regeneration	Complete	Complete	Complete	Complete	/	/
Depth of dermal regeneration (μm)	No	No	220 (±18)	280 (±31)	/	/
Day 21						
Depth of dermal regeneration (μm)	/	No	No	120 (±13)	240(±27)	/

Estimated by five measurements per spot. Slash mark (/) means no specimen was obtained or evaluated for the given setting.

1.25 J/cm^2 and above, and its depth increased progressively with increasing laser fluence (Table 1 and Fig. 1).

Day 7

The epidermis was completely regenerated, regardless of the fluence used (range 0.75–1.50 J/cm^2). There was an increased proliferation of keratinocytes, as shown by Ki-67 labeling. Only following treatment with a laser fluence of 1.25 and 1.50 J/cm^2 a zone of regeneration in the dermis was noted (Table 1). In the regeneration zone, there were small areas of homogeneous pink degenerated material (remnants of degenerated collagen), especially beneath the basal membrane. The most prominent feature in the regeneration zone was increased cellularity, the vast majority of cells being CD68 positive macrophages/monocytes (Fig. 2). In the regeneration zone, there was an up to threefold increase in the number of the CD 68 positive cells compared to the zone of same depth in the untreated skin sample. No neutrophillic granulocytes indicating infection were noted in any specimens. The estimation of the depth of dermal regeneration zone was based on increased cellularity beneath regenerated epidermis (Table 1).

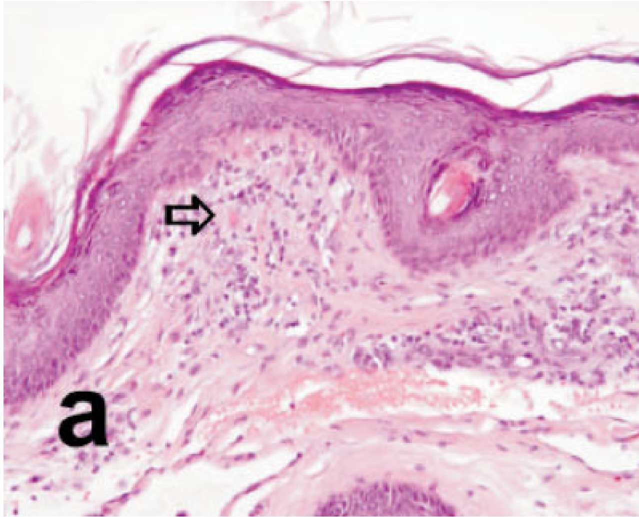
Day 21

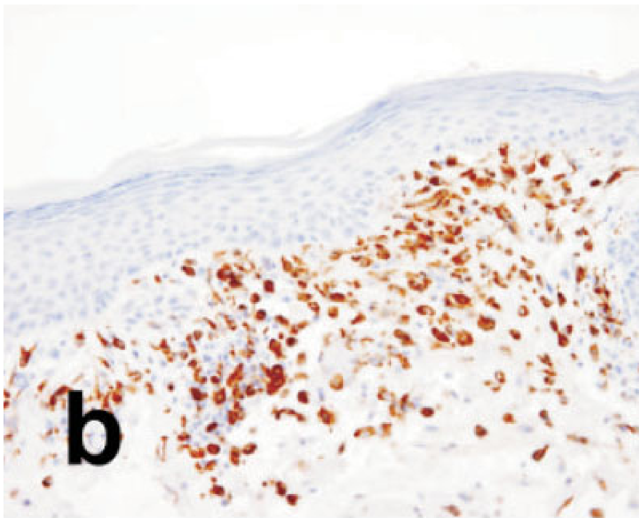
Following treatment with laser fluences of 1.50 and 1.75 J/cm^2 but not at lower fluences, a subepidermal regeneration zone was noted. It consisted of edematous tissue with basophillic hue. Stellate appearing cells with relatively abundant eosinophillic cytoplasm and large nuclei with fine chromatin structure that displayed immunohistochemical positivity to SMA were noted (Fig. 3). Immunohistochemical staining to collagen IV revealed an intact basal membrane, which was less intensively stained compared to untreated areas (Fig. 3).

DISCUSSION

Laser skin resurfacing constitutes to be a popular procedure in rejuvenating sun damaged and aged skin [2]. To reduce potential complications of ablative skin resurfacing intensified research effort has been invested on nonablative laser resurfacing. The objective is to achieve selective, heat-induced denaturation of dermal collagen that leads to subsequent synthesis of new collagen with as little as possible damage to the epidermis [13]. Various types of non-ablative lasers have been used [23]. While skin resurfacing with intense pulsed light or radiofrequency waves causes no epidermal damage at all, the term non-ablative resurfacing has a different meaning with Er:YAG resurfacing—the epidermis is in reality damaged, but not removed, and acts as a wound dressing [13–16].

In this study, we used an Er:YAG laser with non-ablative resurfacing settings. Absorption of Er:YAG radiation ($\lambda = 2{,}940$ nm) is very strong in tissue water, $\mu = 1{,}000\ mm^{-1}$. At fluences greater than 1.00 J/cm^2 ablation of epidermis occurs, and residual thermal damage does not exceed 20–50 μm [9]. Theoretical models of repetitive pulse Er:YAG laser exposure (e.g., sequence of ten 300 μsecond long pulses at a fluence of 0.6 J/cm^2, repetition rate 50 Hz) predicted an increase of collagen denaturation up to 195 μm





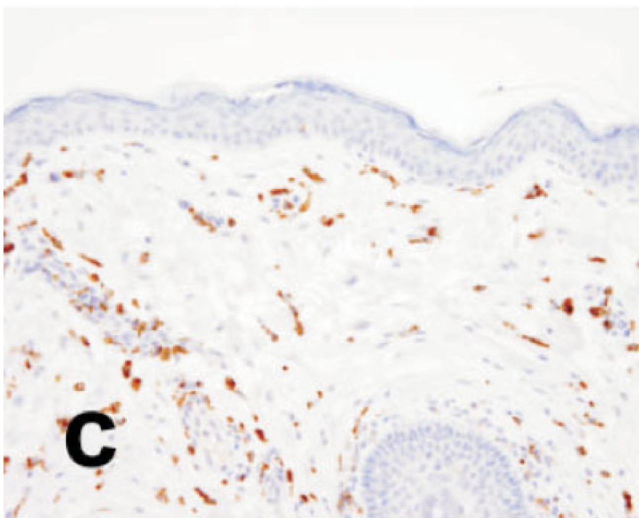


Fig. 2. Changes at day 7 following laser treatment with 1.25 J/cm^2. **a**: The epidermis is completely regenerated. An increased cellularity in the dermal zone is noted. Some small foci of degenerated collagen (arrow) are noted in the upper dermis (H&E, original magnification = 200×). **b**, **c**: The increased cellularity of the regeneration zone is principally due to an increased number of CD68 positive monocytes/macrophages; compare treated (b) and untreated (c) skin (immunohistochemistry, original magnification = 200×).

deep [20,21]. Our laser settings were chosen on the base of results of studies of repetitive Er:YAG exposure of animal skin, which correlated well with the predictions from theoretical studies. In the first study by Majaron et al. [22] from 2000, collagen denaturation deeper than 200 μm with only partial epidermal ablation was observed, with laser fluence of 0.8 J/cm^2. Stacking of more than 5 pulses and change of repetition rate from 10 to 33 Hz produced minor effects on coagulation depth and epithelial thickness. In the second study by Majaron et al. [6] from 2001, maximum dermal coagulation without complete epidermal ablation was noted at the following settings: fluence 1.6 J/cm^2, 10 pulses/packet, single pulse duration 550 μsecond, repetition rate 20 Hz. Shortening of pulses to 150 μsecond or the addition of a cooling device diminished dermal denaturation. In both studies, rising of fluence caused more epidermal damage, and in concordance with the theoretical model, less dermal coagulation.

Our first aim was to analyze an immediate effect of laser treatment and to determine the most appropriate fluence range. In our study, there was no significant collagen denaturation at fluences of 1.00 J/cm^2 or less, but it increased progressively with increasing fluence with the most prominent increase between 1.25 and 1.50 J/cm^2. Epidermal damage also increased with increasing fluence. Complete ablation of the epidermis was noted at fluence of 1.75 J/cm^2 and basal membrane was mostly destructed at fluence of 2.00 J/cm^2. These two fluences are not appropriate for nonablative resurfacing of the periocular skin, but might be appropriate for use on other anatomic locations, where the epidermis is thicker. Addition of a cooling device does not seem to be a viable alternative, because it does not offer spatial selective protection of the epidermis [6].

To determine in vivo response to collagen denaturation, we analyzed treated skin 7 days after laser exposure. Consistently with changes at day 0, a dermal regeneration zone was noted at fluences 1.25 and 1.50 J/cm^2. Two important features characterized the regeneration zone, a prominent increase in the number of dermal macrophages and small areas of degenerated collagen. Because the estimation of the depth of dermal regeneration zone was based on the increased infiltration of macrophages, it was most probably overestimated, and the actual depth was similar to day 0. Compared to day 0, only small areas of degenerated collagen were seen (mostly beneath basal membrane) indicating its degradation and clearance from the tissue. Three consecutive phases of response to tissue

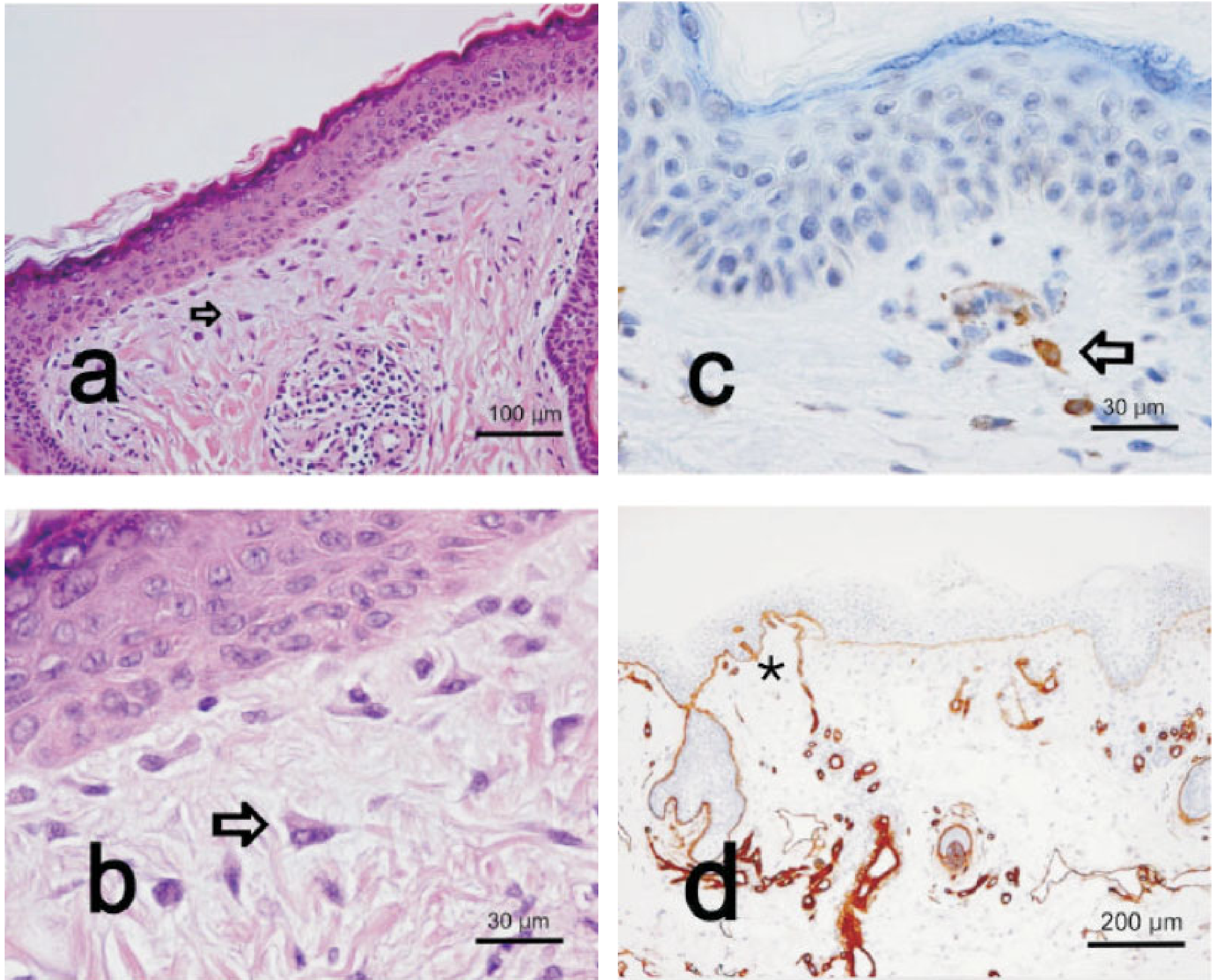


Fig. 3. Changes at day 21 following treatment with 1.50 J/cm^2. **a**, **b**: A subepidermal regeneration zone is seen, which consists of edematous tissue with stellate appearing cells (arrow) (H&E; original magnification = 200× and 600×, respectively). **c**: The cells in the regeneration zone display (at least focally) positive immunostaining to SMA (arrow) (immunohistochemistry, original magnification = 600×). **d**: Immunostaining to collagen type IV reveals intact basal membrane, which is less intensely stained in the treated (right) compared to untreated (left) area (asterix); note a regeneration zone beneath the epidermis (immunohistochemistry, original magnification = 100×).

damage (wound healing) have been traditionally described, an inflammatory phase, a proliferation phase, and a remodeling phase. The principal cell of the inflammatory phase is macrophage, which is responsible for degradation of damaged tissue (wound debridement) and stimulates influx and proliferation of fibroblasts by production of cytokines [24,25]. At day 7, epidermis was completely regenerated, regardless of the fluence applied. There were no histological signs of infection (no neutrophillic granulocytes were seen).

Clinical effectiveness of laser skin resurfacing is based on the induction of synthesis of new collagen and other components of extracellular matrix. Therefore, histologically, the goal of laser treatment is induction of a proliferative phase in tissue repair (transition from inflammatory to proliferative phase). At day 21 after treatment, we noted a regeneration zone in the dermis that consisted of oedematous tissue with stellate appearing fibroblasts/myofibroblasts, while no increase in the number of macrophages was noted. This finding is consistent with induction of a proliferative phase. Some stellate cells were SMA positive, indicating myofibroblastic differentiation [26]. Myofibroblasts play an important role in proliferative phase of tissue injury and participate in active production of extracellular matrix components including collagen I and III [27,28]. At day 21 a regeneration zone was noted at laser fluences of 1.50 and 1.75 J/cm^2, but not at 1.25 J/cm^2. The explanation for this finding could be: (1) a threshold collagen denaturation might be necessary to induce a proliferative phase, and (2) the synthesis of extracellular matrix could be induced

without remarkable histological changes at this time after treatment. In the later case, more sensitive methods should be used to detect synthesis of new collagen, such as in situ hybridization [29].

Taking together, in our study, no remarkable collagen denaturation is achieved at 1.00 J/cm^2 or below and no dermal regeneration is noted. Despite observed collagen denaturation at 1.25 J/cm^2 it is not clear, whether this is sufficient to induce regeneration of the dermis. We observed a prominent regeneration of the dermis (proliferative phase of tissue repair) at 1.50 and 1.75 J/cm^2. However, laser fluences of more than 1.50 J/cm^2 produced a significant damage to the epidermis, which is comparable to complete ablation. Also, damage to the basal membrane has been noted at 2.00 J/cm^2. Therefore these fluences, especially 2.00 J/cm^2 could not be considered non-ablative. The most appropriate fluence for further testing seems to be around 1.50 J/cm^2, probably in the range from 1.25 to 1.75 J/cm^2. The use of a cooling device is probably of no benefit, as shown on rat skin.

We did not notice any enhanced collagen denaturation around hair follicles, as observed in the treatment of rat skin [22].

CONCLUSIONS

Stacking of repetitive Er:YAG laser pulses on human lid skin produces deep collagen denaturation while still partially preserving the superficial epidermis. Complete epidermal regeneration occurred at day 7 with fluences of up to 1.50 J/cm^2. A clear regeneratory response of the treated skin with new collagen formation and remodeling has been documented, shown mainly by prominent macrophage/monocyte and myo-fibroblast infiltration at day 7 and 21, respectively, with clearance of denaturated collagen.

We believe repetitive irradiation of human skin with a 2,940 nm Er:YAG laser to be a potentially useful method for clinical laser skin resurfacing. By yielding comparable histological results to established treatment regimes, its main advantages would be shorter healing times and fewer side effects. These hypotheses remain to be proved in further clinical trials, and a safe and effective range of laser fluences needs to be clinically determined.

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