

Nonablative skin rejuvenation devices and the role of heat shock protein 70: results of a human skin explant model

Doris Helbig
Anne Moebius
Jan C. Simon
Uwe Paasch

University of Leipzig
Department for Dermatology, Venerology and Allergology
Philipp-Rosenthal-Str. 23
04103 Leipzig, Germany

Abstract. Nonablative thermal laser therapy with a 1540-nm laser induces controlled, spatially determined thermal damage, allowing subsequent collagen remodeling while preserving the epidermis. A photorejuvenation effect using nonthermal nonablative stimulation of cells with low energy and narrow band light has been termed photomodulation. Light emitting diodes (LEDs) are narrow band emitters that lead to photomodulation via stimulation of mitochondrial cell organelles. In a previous study, we demonstrated in a human skin explant model that heat shock protein 70 (HSP70) plays a pivotal role in the initiation of skin remodeling after ablative fractional photothermolysis. To test its importance in nonablative laser therapy and photomodulation, the spatio-temporal expression of HSP70 is investigated in response to a 1540-nm laser treatment and six different LED therapies. An Er:glass laser is used with a 1-Hz repetition rate, 30-J/cm² fluence, and a hand piece with a 2-mm spot size. Nonthermal nonablative treatment is performed using two LED (LEDA SCR red light: 635 nm, 40 to 120 W/cm², 40 to 120 J/cm²; LEDA SCR yellow light: 585 nm, 16 to 35 W/cm², 20 to 100 J/cm²; spot size 16 × 10 cm). Immediate responses as well as responses 1, 3, or 7 days postprocedure are studied; untreated skin explants serve as control. Immunohistochemical investigation (HSP70) is performed in all native, nontreated, and Er:glass laser- or LED-treated samples ($n=175$). Nonablative laser therapy leads to a clear time-dependent induction of epidermally expressed HSP70, peaking between one to three days post-treatment. In contrast, none of the various LED treatments up-regulated the HSP70 expression in our skin explant model. HSP70 is up-regulated by nonablative but thermal laser devices, but does not seem to play a significant role in the induction of skin remodeling induced by photomodulation. The maximum of HSP70 expression is reached later after Er:glass laser intervention compared to ablative fractional (AFP) treatment. © 2010 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.3449736]

Keywords: nonablative thermal laser therapy; nonablative nonthermal skin rejuvenation; photomodulation; light emitting diodes; heat shock protein 70; human skin explant model.

Paper 09551R received Dec. 11, 2009; revised manuscript received Apr. 26, 2010; accepted for publication Apr. 28, 2010; published online Jun. 14, 2010.

1 Introduction

Human integument forms the most visible indicator of age. Aging skin presents various morphologic changes such as wrinkles, skin atrophy, or thickening (both epidermal and dermal compartments), dyspigmentation, teleangiectasia, and loss of elasticity. Both chronological, intrinsic, and environmental, extrinsic influences are involved in the aging process of the skin.¹⁻³

With increasing age in the population, the demand for minimal invasive treatments to preserve or improve skin smoothness and tonicity is increasing. Various rejuvenation modalities have attempted to reverse the dermal and epidermal signs of photo- and chronological aging. There are different well-established ablative skin resurfacing options for the repair of rhytides and photoaged skin, including conventional and fractional ablative laser interventions using CO₂- or Er:YAG-lasers. Using this technique, controlled collateral dermal heating is achieved next to microscopic ablation zones (MAZ). This is followed by an up-regulation of heat shock

Address all correspondence to: Uwe Paasch, MD, University of Leipzig, Department for Dermatology, Venerology and Allergology, Philipp-Rosenthal-Str. 23 04103 Leipzig, Germany. Tel: 49-171-374-9870; Fax: 49-341-971-8691; E-mail: uwe.paasch@medizin.uni-leipzig.de

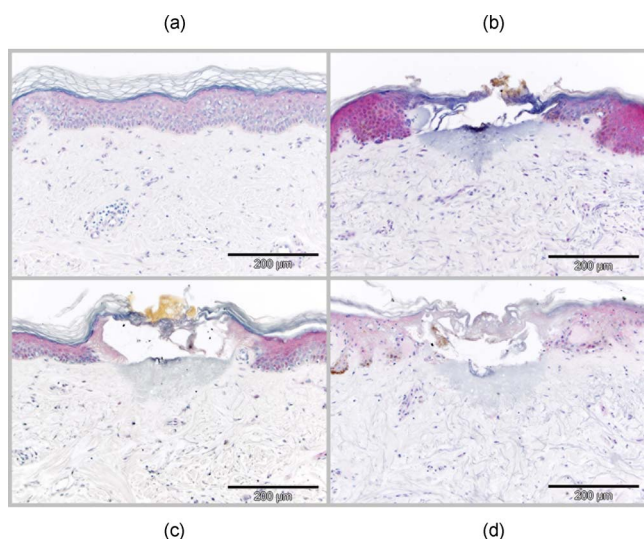


Fig. 1 Immunoreactivity (magnification 20 \times) before and after AFP, 64 mJ, 8 ms, and 8 W. (a) native (untreated skin), (b) day 0 (directly after AFP), (c) day 3 (three days after AFP), and (d) day 7 (seven days after AFP).

protein 70 (HSP70) expression, ultimately leading to reepithelialization and dermal remodeling⁴ (Fig. 1). Common side effects are pain, long-lasting erythema, infections, hypo- or hyperpigmentation, and sometimes scarring.⁵⁻⁷

Categories of thermal but nonablative devices include intense pulsed lights (IPLs), infrared lasers (1064, 1320, 1450, and 1540 nm), visible light lasers (532 and 585 nm), and radiofrequency.⁸⁻¹¹ They affect the wound-healing cascade by a thermal or photothermolysis type of injury. Hemoglobin, melanin, or water can be the target for different light sources (Fig. 2). Thermal but nonablative photorejuvenation involves selective thermal injury confined to the papillary and upper reticular dermis, which contains the majority of solar elastosis in photodamaged skin. Thermal photorejuvenation does not induce epidermal damage, which could lead to fibroblast activation and synthesis of new collagen.^{9,12-17} The skin surface is spared by selective energy deposition in the dermis and/or

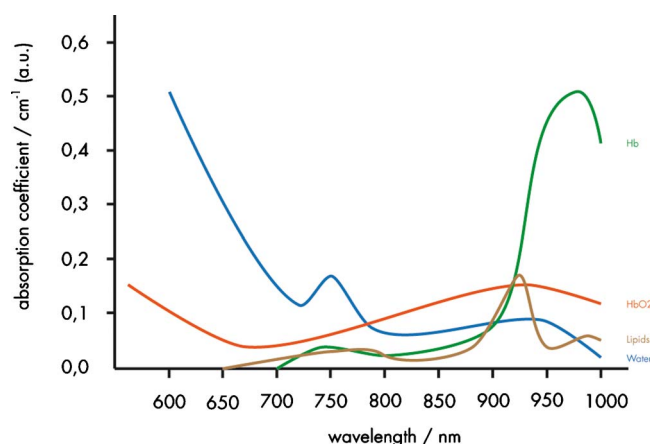


Fig. 2 Absorption spectra of the different targets.

application of active cooling. The 1540-nm laser has been shown to be safe and effective for these remodeling purposes.¹⁸⁻²²

The underlying molecular changes of both ablative and nonablative epidermal and/or dermal remodeling are not fully understood, but have been postulated to be induced by a time-dependent release of heat shock protein 70 (HSP70) among others.²³⁻³⁰

A photorejuvenation effect using nonthermal stimulation of cells with low energy and narrow band light has been termed photomodulation. Light emitting diodes (LEDs) are narrow band emitters that lead to photomodulation via stimulation of mitochondrial cell organelles, resulting in an up-regulation of the mitochondrial cytochrome electron transport pathway and associated mitochondrial DNA gene modulation. Cytochrome molecules within the mitochondrial membrane are synthesized from protoporphyrin IX. The proposed mechanism of LED is a modulation of fibroblast activity, resulting in smoother skin texture without inherent risks of other thermal photorejuvenation devices.^{31,32} But, the specific photomodulation parameter protocol for a particular cell target seems to be crucial for the results.^{33,34}

The role of HSP70 for these photomodulation effects has not been elucidated. For this reason, we analyzed the spatio-temporal expressions of HSP70 in response to six different 585- and 635-nm LED photomodulation regimens in comparison to a 1540-nm laser treatment in a human skin explant model.

2 Methods

35 skin samples, obtained during routine skin surgery, were used as skin explants. All subjects consented the use of their skin explants. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in approval by the institution's human research review committee.

The explants were treated once with an Er:glass laser ($n=5$, 1540 nm, Aramis, Quantel-Derma, Erlangen, Germany, spot size 2 mm) using a 1-Hz repetition rate, 30-J/cm² fluence, or a LED ($n=30$, LEDA SCR 635-nm red light or LEDA SCR 585-nm yellow light, Quantel-Derma, Erlangen, Germany, spot size 160 \times 100 mm). The light intensities were adjusted to 16 to 120 mW/cm² and 20 to 120 J/cm² (on time 0.25 s, off time: 0.10 s) resulting from various parameter combinations (Table 1) for immediate LED processing.

2.1 Routine Pathology Workup

One aliquot of the explants was fixed in 4% buffered formalin immediately after Er:glass laser or LED procedure whereas the others were subjected to cell culture medium [Dulbecco's Modified Eagle Medium (DMEM), enriched with streptavidin and 10% fetal calf serum] for one, three, or seven days at constant temperatures of 31 to 32 $^{\circ}$ C, corresponding to an average skin surface temperature. Another aliquot of the explant was fixed in 4% buffered formalin without laser procedure to serve as baseline control (neat). Following fixation in formalin, all skin explants were paraffin embedded and sectioned into 3- to 6- μ m-thick slices.

Table 1 Light emitting diode parameters chosen in the program of skin rejuvenation, stimulated and pulsed.

LED	Intensity (mW/cm ²)	Energy (J/cm ²)	Time (min)	Number of samples
585-nm yellow light	16	20	29.10	5
	30	45	35.00	5
	35	100	66.39	5
635-nm red light	40	40	23.20	5
	120	100	19.26	5
	100	120	27.46	5

2.2 Immunohistochemistry

The sections were rehydrated. Afterward, they were incubated with 0.5% phosphate buffered saline/bovine serum albumine [(BSA/PBS): 0.5% BSA prepared in PBS] for 5 to 10 min at 41 to 43 °C, washed twice in PBS, 10 min per wash and air-dried. Afterward, the sections were incubated in a wet chamber for another 30 min at room temperature. The reaction was stopped with 25 to 50 μ l of normal sheep serum, and the slides were washed twice in PBS, 10 min per wash. The primary antibody was anti-HSP70 [Catalogue number AM289-5M, specific for heat shock protein 70 (HSP70)], which recognizes both the constitutive (HSP73) and inducible (HSP72) forms of HSP70 (BioGenex, San Ramon, California; 1:1 PBS/0.1% Tween). Anti-HSP70 was added to the sections and incubated in a wet chamber for 45 min at 37 °C. To visualize mAb binding, a Dako Real Detection System (K5005, Dako, Hamburg, Germany, alkaline phosphatase/RED, rabbit/mouse) was used according to the manufacturer's protocol.

TGF β (Catalogue number: C-63504, PromoKine, Germany) was injected intradermally using a superfine needle syringe (Hamilton, Germany) at concentrations of 5 ng/ μ L (20 μ L) into control skin samples (positive controls), as shown previously by Ong et al.³⁵

2.3 Evaluation of the Intensity of Immunohistochemistry Staining

All tissue samples were stained at the same time using identical procedures. HSP70 expression was analyzed microscopically by two independent investigators using different magnifications (Olympus BX41, Germany, magnification 1.25, 4, 10, 20, 40, and 60) and documented using a calibrated digital camera system (Olympus DP72, Germany) together with the software evaluation package (Olympus Cell F, Germany).

The expression densities of HSP70 in skin explants were ranged from 0=undetected, 1=low density, 2=medium density, 3=dense, to 4=very dense, as described previously by Souil et al.²⁹

3 Results

35 fresh human skin explants were obtained from the trunk ($n=12$, 34.3%), arms ($n=8$, 22.9%), legs ($n=10$, 28.6%), and face and neck region $n=5$, 14.3%). Patient age ranged from

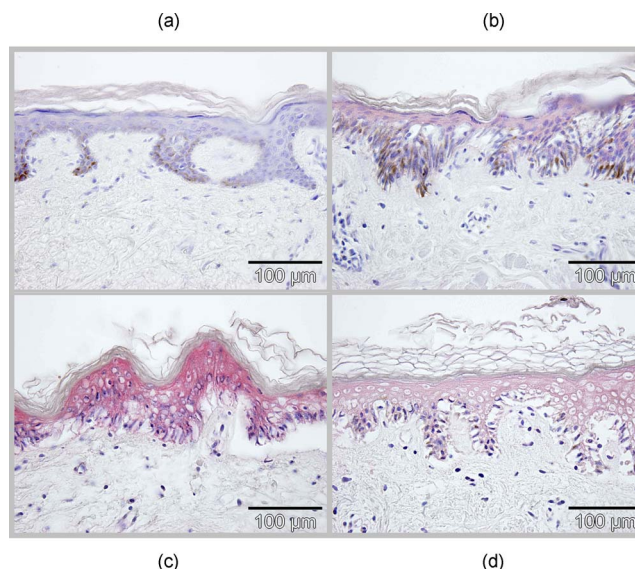


Fig. 3 Immunoreactivity (magnification 40 \times) before and after Er:glass laser therapy (1-Hz repetition rate, 30 J/cm² fluence): (a) native (untreated skin), (b) day 0, (c) day 3, and (d) day 7.

42 to 72 years (mean 59.9 years \pm 11.2). 23 of the 35 patients (65.7%) were male and 12 (34.3%) were female.

175 aliquots were generated for experimentation. 20 of the 175 aliquots were subjected immediately after surgery to 1540-nm Er:glass laser treatment (Aramis, Quantel-Derma, Erlangen, Germany, spot size 4 mm), and 120 aliquots were exposed to LED intervention by a 635-nm red or 585-nm yellow light (LEDA SCR 635 red or LEDA SCR 585 yellow, Quantel-Derma, Germany, program: skin rejuvenation, stimulated and pulsed) to measure overall expression of HSP70 over seven days in response to the different treatment protocols (see Sec. 2). The different intensities or energies applied are shown in Table 1. 35 aliquots served as controls without treatment.

Overall, the highest constitutive expression of HSP70 was observed in the epidermal compartment and was minor within the dermal papillary layer, around sebaceous glands, hair follicles, and blood vessels. HSP70 expression was very weak or absent within the corneal layer and adipose tissue.

Nonablative thermal laser intervention with a 1540-nm Er:glass laser resulted in a uniform up-regulation of HSP70 protein expression in the epidermal layers immediately (about 60 min) after laser procedure, with maximal expression one to three days postintervention (Fig. 3). The intensities did not differ clearly between the time points due to the small sample count.

Various LED treatments resulted in no up-regulation of HSP70 protein expression in the skin explants. The intradermally injected TGF β led to an up-regulation of the HSP70 expression compared with a control sample, thus validating the positive control. The original localization of skin explants as well as the age of the donors did not influence HSP70 expression.

4 Discussion

Fractional laser skin treatment has shown high clinical efficacy for the ameliorization of photodamaged and scarred skin, and low postoperative side-effect rates compared with conventional nonfractionated laser therapies.³⁶ But transient erythema, edema, and xerosis of treated skin are expected side effects, although severe or long-standing complications are rare. Despite the limited recovery period after fractional ablative laser resurfacing, patients are often inconvenienced by skin erythema and edema that prevent them from immediately pursuing their activities of daily living. For this reason, different nonablative lasers and light sources, including the 1540-nm Er:glass laser and LEDs, have subsequently been introduced in the treatment of skin rejuvenation with the aim of reducing down-time.^{18–21,31,37}

HSP70 is suggested to play a significant role in ablative and nonablative (but thermal) laser interventions and during wound healing.^{23–25,27–30,38–40} Its role during photomodulation after LED treatment remains unclear.

In a previous study, we could show a clear time-dependent HSP70 expression profile post-AFP (ablative fractional treatment) performed by a scanning 250- μm CO₂ laser beam, validating our human skin explant model (Fig. 1).⁴¹ The ban on animal testing in the European cosmetic industry has increased the urgency to develop innovative alternative skin models to replace the use of laboratory animals.^{42–44} Various skin models are now available but they often lack a dermal compartment, and only a few methods have been validated by relevant regulatory authorities such as the European Centre for Validation of Alternative Methods (ECVAM).^{45–47} Despite the disadvantages of our human skin explant model such as lack of vascularization and reepithelialization, we investigated the spatio-temporal expression of HSP70 in response to a 1540-nm Er:glass laser treatment and six different LED therapies. As expected, nonablative thermal laser intervention with the 1540-nm Er:glass laser resulted in a uniform epidermal up-regulation of HSP70 expression. The maximum HSP70 expression was one to three days postintervention and therefore was slightly delayed compared with the AFP-induced HSP70 expressions. This could be explained by the missing ablative part of the action.

Light-emitting diode therapy has been reported to accelerate cutaneous wound healing after various injuries, including surgical procedures as well as radiation. Therefore, it has been further combined with thermal-based rejuvenation treatments^{48–51} leading to enhanced wound healing. These devices successfully modulate the activity of fibroblasts and eventually melanocyte activity, inflammatory response, blood flow, or even stem cell activity.^{31,32} It is important to recognize the specificity of the photomodulation parameter protocol for a particular cell target, because it is possible with an identical LED light source and energy fluence to see either an increase or decrease of collagen synthesis in cell culture.^{33,34} The therapy is painless, safe, and large LED panel arrays can be assembled so that large skin areas can be treated in a few minutes.

Different clinical trials using the 590-nm wavelength LED and a very specific pulsing time code (total output 0.1 to 0.9 J/cm², 2 pulses per cycle with 100 cycles delivered over 35 s, on time for each pulse is 250 ms, off time is

100 ms, eight treatments delivered over 4 weeks) were highly effective for the stimulation of collagen synthesis and clinical improvement of photoaged skin with a reduction of elastosis, erythema, and pigmentation in 60 to 90% of patients.⁵² Results peaked between 4 and 6 months and declined slowly over the following 6 to 12 months after completion of treatment. In contrast, application of continuous LED light had minimal effect.⁵¹ A randomized, placebo-controlled clinical trial comparing an 830 nm (55 mW/cm², 66 J/cm²), 633 nm (105 mW/cm², 126 J/cm²) or both LED treatments showed significant reductions of wrinkles (maximum 36%) and increases of skin elasticity (maximum 19%) compared to baseline and sham treatment measured by profilometric evaluation and cutometer.³² Histologically, a marked increase in the amount of collagen and normal elastic fibers was observed two weeks post-treatment, with the most significant changes perifollicular and in the papillary and upper reticular dermis. Staining was performed with Verhoeff-van Gieson stain⁵³ Alcian blue stain³¹ and immunohistochemistry including anti-collagen I.⁵¹

However, changes in the collagen network appeared even deeper than 500 μm , extending to almost the entire dermis. These changes were not restricted to the areas affected by thermal damage, as shown in other studies.^{54,55} This is probably due to an increase of connexin Cx43 mRNA after LED therapy, thus enhancing cell-cell communication between fibroblasts synchronizing their cellular responses to the photobiostimulation effects.⁵³ Further, Cx43 up-regulation is observed in smooth muscle cells and endothelial cells in the dermis suggesting mediating transendothelial migration of leukocytes through gap junctional intercellular communication during wound healing.^{56–60}

Our investigations to the induction of HSP70 in skin explants after the treatment with the 585-nm LED (energies of 20 to 45 J/cm²) and the 635-nm LED (energies from 40 to 120 J/cm²) in a pulsed mode showed no up-regulation in any of the treatment options. In contrast, Er:glass laser intervention led to an up-regulation of HSP70 with maxima one to three days postintervention.

In conclusion, HSP70 is important for the induction of skin remodeling after nonablative thermal treatment devices, but seems to play no significant role for photomodulation. We could not find parameters of LED treatment changing intensities in a pulsed code leading to reliable up-regulations of HSP70.

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