Immunohistochemical investigation of wound healing in response to fractional photothermolysis

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Abstract. Despite growing clinical evidence of ablative fractional photothermolysis (AFP), little is known about the spatiotemporal molecular changes within the targeted compartments. Six subjects received three different single AFP treatments using a scanned 250 μm CO_2-laser beam. Spatiotemporal changes of skin regeneration were estimated by immunohistochemical investigation (HSP70, HSP72, HSP47, TGFβ, procollagen III, CD3, CD20, and CD68) in skin samples 1 h, 3 days, and 14 days postintervention. The remodeling was uniformly started by regrowth of the epidermal compartment followed by partial to complete replacement of the microscopic ablation zones (MAZ) by newly synthesized condensed procollagen III. From day 3 to 14, the number of macrophages as well as giant cells surrounding the MAZ increased. TGFβ expression was highest 1 h to 3 days following AFP. HSP70 and HSP72 expressions were highest 3–14 days postintervention in the spinocellular layer leading to an upregulation of HSP47. AFP performed by a scanned CO_2-laser results in an early epidermal remodeling, which is followed by a dermal remodeling leading to a replacement of the MAZ with newly synthesized (pro)-collagen. During this, an inflammatory infiltrate with $CD3^+$ and $CD20^+$ cells surrounds the MAZ. The count of macrophages and giant cells involved in the replacement of the necrotic zones seems to be crucial for wound healing. [DOI: 10.1117/1.3275479]

Keywords: ablative fractional photothermolysis; thermoablative resurfacing; CO_2 laser; 10,600 nm; immunohistochemistry; HSP70; HSP72; HSP47; TGFβ; procollagen III; CD3; CD20; CD68; skin rejuvenation.

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1 Introduction

In an aging population, the demand for minimally 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 fractional ablative laser interventions in order to reduce side effects, such as infection, erythema, scarring, and hypopigmentation of the treated area.[1–7] Ablative skin resurfacing by fractionated CO_2- or Er:YAG-laser ablates the epidermal compartment as well as parts of the dermal compartment depending on the amount of energy applied[8] and the skin surface temperature.[9] Using this technique, controlled collateral dermal heating is achieved next to microscopic ablation zones (MAZ). The controlled thermal stress to the epidermis and the dermal compartment is followed by a wound-healing response ultimately leading to reepithelization and dermal remodeling.

In a previous study, we could show a clear time-dependent post–ablative fractional photothermolysis (post-AFP) heat shock protein 70 (HSP70) and TGFβ expression profile performed by a scanned 250-μm CO_2-laser beam with ablative single-pulse energies set to 50, 64, and 300 mJ (150 ablation zones per cm^2), proving our designed skin explant model (publication submitted). HSP70 and TGFβ demonstrated slight baseline expression patterns followed by a marked upregulation within 1 h, peaking between 1 and 24 h post-AFP, and a significant decline within the following 7 days. Using this skin explant model, one aliquot of the explants was fixed in 4% buffered formalin immediately after the laser 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 1, 3, or 7 days at constant temperatures of 31–32 °C corresponding to an average skin surface temperature before immunohistochemical staining. Another non-laser-treated aliquot of the explant served as the baseline control.

Despite the easy practicability and good mimicking of the *in vivo* initial responses to laser light, later dynamic tissue changes cannot be examined well with the skin explant

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model. For this reason, we performed the following study to analyze the targeted epidermal and dermal responses following three different fractionated CO_2-laser treatment regimens with the aim of comparing histological responses in an *in vivo* situation.

One possible mediator of laser-assisted remodeling is the induction of heat shock protein 70 (HSP70). Heat shock proteins are stress proteins and tend to be upregulated in all cell types if exposed to thermal stress by increasing temperatures (+4–6 °C above their physiologic temperature) or other forms of physical and chemical stress.[10–17] During this, HSP facilitates various aspects of protein maturation (molecular chaperones). Increasing levels of HSP following thermal stress enhances the ability of cells to deal with the resultant accumulation of abnormally folded proteins, either facilitating the refolding of damaged proteins or participating in the synthesis of new proteins to replace those irreparably damaged. Therefore, they are fundamentally involved in the protection against UV- or other stress-factor-induced cell damage, cell reparation, and the wound-healing process.[4,18] Additionally, HSP70 plays a role in inducing the expression of growth factors, such as transforming growth factor β (TGFβ), which is a key element in wound healing and fibrogenic processes.[1,3,19,20] TGFβ has also been shown to induce HSP70 heat independently.[21] This is known from the high rate of protein synthesis upregulated by TGFβ during wound healing.[21–24] The various latent TGFβ forms will also be induced via physiochemical means, such as UVB,[25] heat,[26] altered pH, a large group of proteases and enzymes as well as high-energy ionizing radiation.[19,20]

The most important HSPs are the family members of HSP70 (72 kDa HSP=HSP72 and 73 kDa HSP=HSP73) with a 95% sequence homology[16] and HSP47. Both, the 72 and 73 kDa HSP are present in the cytoplasm and the nucleus of keratinocytes, fibroblasts, and adipocytes.[2,27] HSP73 is synthesized constitutively in all mammalian cells and therefore is often referred to as the constitutive HSP70. The synthesis of HSP72 is usually restricted to the cell experiencing stress and is therefore often referred to as the inducible form of HSP70.

HSP47 expression has been found to be localized in the endoplasmatic reticulum of fibroblasts, where it interacts essentially with the synthesis and transport of the pro-α1(I) and pro-α2-(I)-chains of procollagen I. In the absence of HSP47, collagen microfibrils and basement membrane formation are impaired because of the failure in molecular maturation of types I and IV collagen.[3,28–33] The upregulated expression of HSP47 in the dermis is directly proportional to the rate of collagen formation.[8,34–37]

The three mammalian isoforms of TGFβ 1, 2, and 3 have been shown to have a wide range of effects in a cell/tissue-context-dependent manner. In wound responses, TGFβ promotes chemotaxis for fibroblasts and induces extracellular matrix and procollagen formation that may result in scar formation.[38] Furthermore, TGFβ induces myofibroblast differentiation, which has been suggested to lead to wound contraction.[39]

Following these studies, diverse light sources or lasers were used to induce wound healing or dermal remodeling.[1,6,22–24,40,41] Laser light exposure increased the expression of HSP70 within the epidermis around the "microscopic thermal injury zones" (2–48 h after therapy) and in dermal structures, particularly around blood vessels, hair follicles, and sebaceous glands using an 815-nm diode laser (1.5 W, 3 s pulse duration, fluence of 145 J/cm^2)[23,24] or a non-ablative fractional 1500 nm diode laser system (5 mJ/microscopic treatment zones (MTZ), 1600 MTZs/cm^2).[37] HSP47 expression is upregulated 4–7 days post–laser intervention and is persistent over three months,[8,34,35,37] leading to increased procollagen and collagen I and III deposition.[40,42,43] Middle energy doses (of ~30 J/cm^2) and repeated treatments had the greatest effect on collagen formation, whereas higher energies (of ~50 J/cm^2) lead to marked ablative epidermal changes in porcine skin.[44] TGFβ 1 and TGFβ 2[40] are activated or induced by different lasers[1,6] 2 days following laser irradiation, while TGFβ 3 expression was increased after 14 days, concomitant with an increased inflammatory infiltrate.

2 Materials and Methods

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected by the approval of the institution's human research review committee (Registration No. 277-08). All subjects gave consent prior to participation in this open-case control study.

Six healthy subjects with Fitzpatrick skin types II and III and clinically evident photodamage were treated with a fractionated CO_2-laser (10,600 nm, Exelo$_2$, Quantel-Derma, Erlangen, microbeam spot size: 250 μm) in order to assess epidermal and dermal remodeling in neck skin. The design of the handpiece is a scanner that can be adjusted to squares at various sizes (each axis can be 5 or 10 or 15 or 20 mm long, e.g., 5×5, 5×10, 5×15, 5×20 mm; 10×5, 10×10 … maximum 20×20 mm). It is created spot after spot in a burst sequence by automatic movement of the scanner. Therefore, the pattern produced looks like a stamp although generated via scanning. The density per square centimeter can be chosen as 25/50/100/150/200/250/300/350/400 microspots per square centimeter. The distance center of the spot to center of the next spot results in 2.11, 1.49, 1.05, 0.86, 0.75, 0.67, 0.61, 0.56, and 0.53 mm. Each patient was treated once with three different AFP treatment regimens in areas of 20 mm×20 mm. The ablative energies per MAZ were adjusted to 50, 100, or 300 mJ/cm^2 per microbeam resulting from various combinations of power and pulse duration (50 mJ/cm^2: 5 ms and 10 W/cm^2, 100 mJ/cm^2: 5 ms and 20 W/cm^2; 300 mJ/cm^2: 10 ms and 30 W/cm^2). The density of the MAZ was set to 200, 150, or 100/cm^2. During treatment, airflow cooling was adjusted to level 5 (Zimmer Cryo 6, Zimmer MedizinSysteme GmbH, Neu-Ulm, Germany).

Patient age ranged between 35 and 66 years with a mean of 54.3 years. The four male and two female healthy volunteers who enrolled in the study had no known contraindications to laser therapy, and no history or current viral or bacterial infection, and no skin disease or previous laser therapy of the area to be treated.

2.1 *Routine Pathology Workup and Immunohistochemical Investigations*

Biopsy specimens were obtained from all three treatment areas 1 h, 3 days, and 14 days after laser intervention. One additional biopsy per subject was performed at baseline (before therapy) to serve as the control. All specimens were fixed in 4% buffered formalin, embedded in paraffin, sectioned into 4–6 μm thick slices and stained with hematoxylin and eosin. For immunohistochemistry, the primary antibodies anti-HSP70 (Catalog No. AM289-5M, BioGenex, Canada; 1:1 in PBS (phosphate buffered saline)/0.1% Tween), anti-HSP72 (Catalog No. SPA-810, Stressgen, USA; 1:50 in PBS/0.1% Tween), anti-HSP47 (Catalog No. SPA-470, Stressgen, USA; 1:500 in PBS/0.1% Tween), anti-procollagen III (Catalog No. BP8034, Acris Antibodies, Germany; 1:2000 in PBS/0.1% Tween), anti-CD3 (Catalog No. A0452, Dako, Germany; 1:50 in PBS/0.1% Tween), anti-CD20 (Catalog No. M0755, Dako; 1:200 in PBS/0.1% Tween), anti-CD68 (Catalog No. M0814, Dako, Germany, 1:50 in PBS/0.1% Tween), and anti-TGFβ (Catalog No. MAB240, R&D Systems, Germany; 1:100 in PBS/0.1% Tween) were applied to the sections and incubated in a wet chamber for 60 min at room temperature after deparaffinization. Subsequently, sections were washed three times for 5 min in PBS/0.3% Tween. The reaction was stopped with the Super Sensitive Link Label IHC Detection System (Catalog No. QA900-9L BioGenex, Canada) and washed another three times for 5 min in PBS/0.3% Tween. In order to visualize the samples, the Dako New Fuchsin Substrate System (Catalog No. K0698, Dako, Germany) was used according to the manufacturer's protocol. At least, slides were counterstained with hematoxylin (Meyer).

2.2 *Evaluation of the Intensity of Immunohistochemical Staining*

All tissue samples were stained at the same time with the same procedure. All expression patterns were analyzed by two independent investigators experienced in skin histology of laser-treated skin under the microscope (Olympus BX41, Germany) using different magnifications (1.25, 4, 10, 20, 40, 60). They were 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 ranged from 0=undetected, 1=low density, 2=medium density, 3=dense, to 4=very dense as described previously by Souil et al.[24]

Semiquantification of HSP47-positive cells was based on the average cell number of 10 high-power fields per section using a 10× eyepiece and 10× objective lens and scored as follows: −=no positive cells, +=<10% positive cells of constituent cells, ++=10–25% positive cells of constituent cells, +++=26–70% positive cells of constituent cells, ++++ >71% positive cells of constituent cells. Intensity of individual cell staining was expressed as follows: −=no, +=weak, ++=moderate, and +++=strong as used by Kuroda et al.[45]

3 Results

Six healthy subjects were treated with three different ablative fractional laser regimens (see section 2) on their neck skin using a 10,600 nm CO_2 laser equipped with a scanned 250-μm beam (Exelo$_2$, Quantel-Derma, Germany) to investigate the clinical and histological changes over 14 days.

As shown in Figs 1(a)–1(d), 2(a)–2(d), 3(a)–3(d), and 4(b)–4(d), AFP led to immediate ablation of the dermis and parts of the epidermis. Increasing energy levels using paired combinations of pulse duration and power resulted in bigger MAZ. In general, the lesion depth increased more than the width. The ablative zones were surrounded by a coagulation zone with the same trend depending on the energy levels [see Figs. 4(b), 5(b), 6(b), and 7(b)].

Remodeling started by a regrowth of the epidermal compartment and was followed by dermal remodeling depending on the energy applied [see Figs 4(b)–4(d), 5(b)–5(d), 6(a)–6(d), and 7(a)–7(d)]. Rapid skin closure (up to day 3) has been seen in those skin samples in which the cornified layer had been destroyed by the laser treatment. 14 days after laser intervention, the MENDs were replaced by a normal epidermis and the MAZs were replaced by newly synthesized condensed (pro)-collagen for the 50 mJ group [Figs 6(b)–6(d)]. Day 14 was characterized by small subepidermal cleftings in the 100-mJ group and regressing epidermal invagination in the 300-mJ group. In addition, the space within the MAZ was partially to completely replaced by newly synthesized condensed procollagen III. Constitutive expressions of HSP70 and HSP72 as well as TGFβ were highest in the spinocellular layer as compared to moderate expression in the basal cell layer and minor expression in the dermal papillary layer and around sebaceous glands, hair follicles, and blood vessels. HSP70 and HSP72 as well as TGFβ expressions were very weak or absent in the MAZ, stratum corneum, and adipose tissue.

The HSP70, HSP72 and TGFβ staining intensities over time are shown in Figs. 1(a)–1(d), 2(a)–2(d), 3(a)–3(d), and 4(a)–4(d). The HSP72 and TGFβ staining intensities were lower than that of HSP70 with no significant differences for the expression of TGFβ over time.

There were no remarkable differences between the expression of HSP70 or HSP72 in groups differing in laser energy used at the different time points studied. The intensities of HSP70 and HSP72 expression in the spinocellular and basal cell layer differed significantly when comparing values obtained before AFP to values 1 h, 3 days, and 14 days ($p<0.05$) post-AFP, but not between the different time points, postintervention.

HSP47 was expressed by dermal fibroblasts. There was a slight increase in HSP47 distribution and intensity over time without significant differences between the different treatment areas [see Figs. 5(a)–5(d)].

One hour after laser treatment, no CD3+ and only a few CD20+ (figures not shown) and CD68+ cells [see Fig. 7(b)] could be detected around the blood vessels. By day three, a subtle inflammatory infiltrate (CD3+, CD20+, and CD68+ cells) was present around the vascular structures and there were some macrophages (CD68+ cells) surrounding the MTZs [see Figs. 7(c)]. 14 days postintervention, there was a regressing inflammatory infiltrate around the blood vessels,

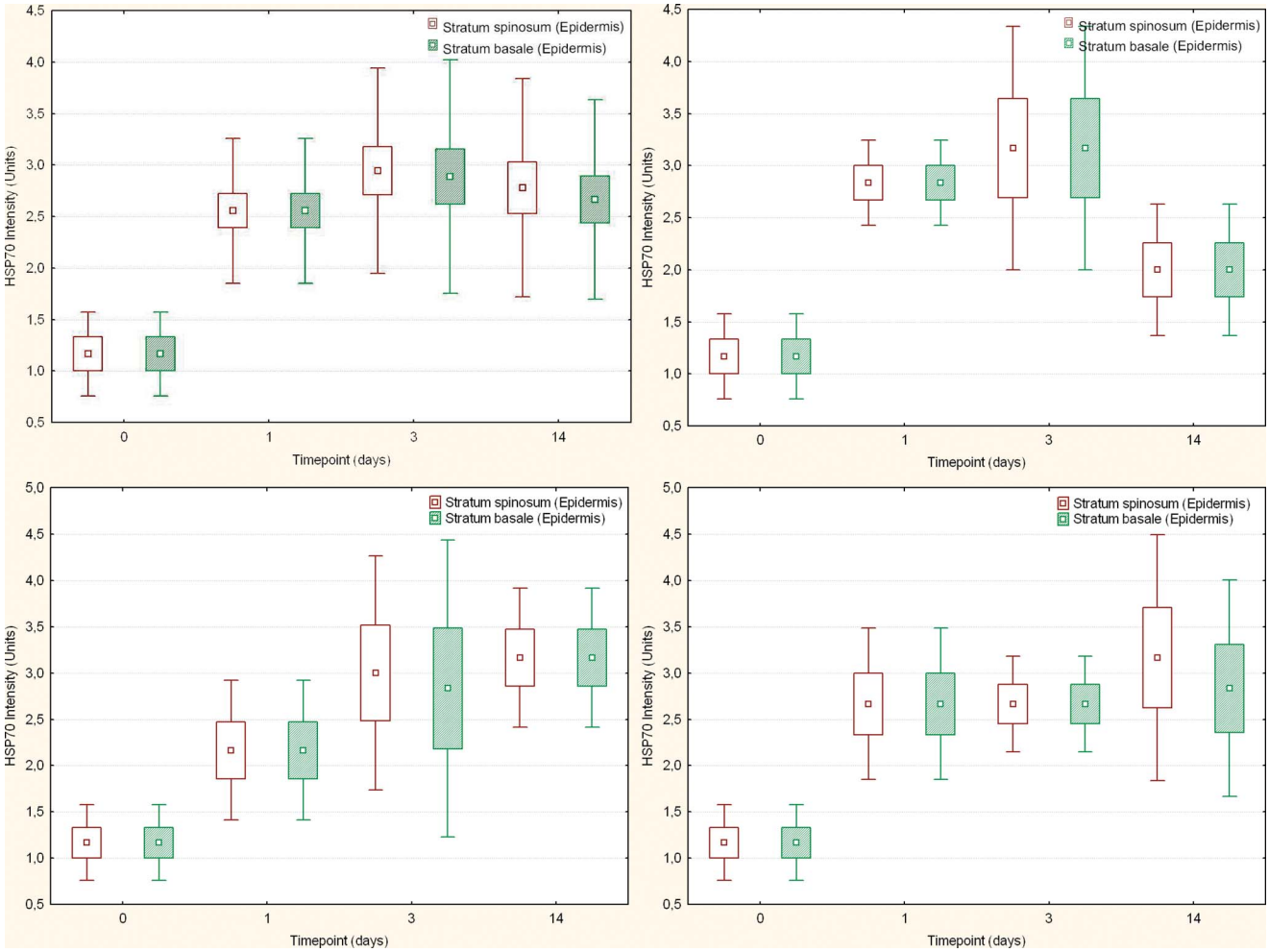

Fig. 1 Effects of AFP on HSP70 staining intensity over time (mean, standard error: 1): (a) all samples (n=60), (b) 50 mJ group (n=18), (c) 100 mJ group (n=18), and (d) 300 mJ group (n=18).

but accumulations of macrophages and some giant cells around the MTZ forming granulation tissues or granulomas were still seen in the 300-mJ group [see Fig. 7(d)].

4 Discussion

Fractional ablative laser intervention currently marks the latest development in order to reduce downtime and side effects while trying to preserve efficacy of conventional ablative laser skin resurfacing. However, optimal laser settings still need to be defined to reproduce best clinical results under various skin conditions. For this reason, we analyzed the epidermal and dermal responses following three different fractionated CO_2-laser treatment regimens with the aim of comparing histological responses in six volunteers.

Here we demonstrate that AFP performed by a scanned CO_2-laser results in an early epidermal remodeling which is followed by a dermal remodeling leading to a replacement of the MAZ with newly synthesized procollagen III. AFP does result in microwounding accompanied by a collateral thermal damage with increasing lesion dimensions depending on the energy applied. Three days post-treatment, the ablative zones were partially to completely replaced by invaginating epidermal cells. Complete healing was seen after 14 days in the 50-mJ group. There were small subepidermal cleftings in the 100-mJ group and regressing epidermal invaginations in the 300-mJ group over newly synthesized condensed (pro)-collagen on day 14.

In our study, HSP70 and HSP72 expressions were upregulated as early as 1 h following AFP in neck skin, reaching peak levels at 3 and 14 days postintervention. On day 14, all staining intensities were above baseline values. This early increase is in contrast to other human studies,[35,36] where upregulations of HSP70 or HSP72 in human forearm skin were first detected 2 days post-treatment, but are consistent with studies in animals[8,10,46] and our own results in a skin explant model. The highest expression levels of HSP70 and HSP72 were observed in the epidermal spinocellular layer; expression was minor in the dermal papillary layer and around sebaceous glands, hair follicles and blood vessels similar to the report of Simon et al.[18]

The progressions of HSP70 and HSP72 in the different treatment groups from day 3 to day 14 [see Figs. 1(c), 1(d), 2(c), and 2(d)] seem to be artifacts due to the small sample count. Epidermal HSP70 and HSP72 expression differed significantly between the time points before treatment and 1 h, 3 days, and 14 days after.

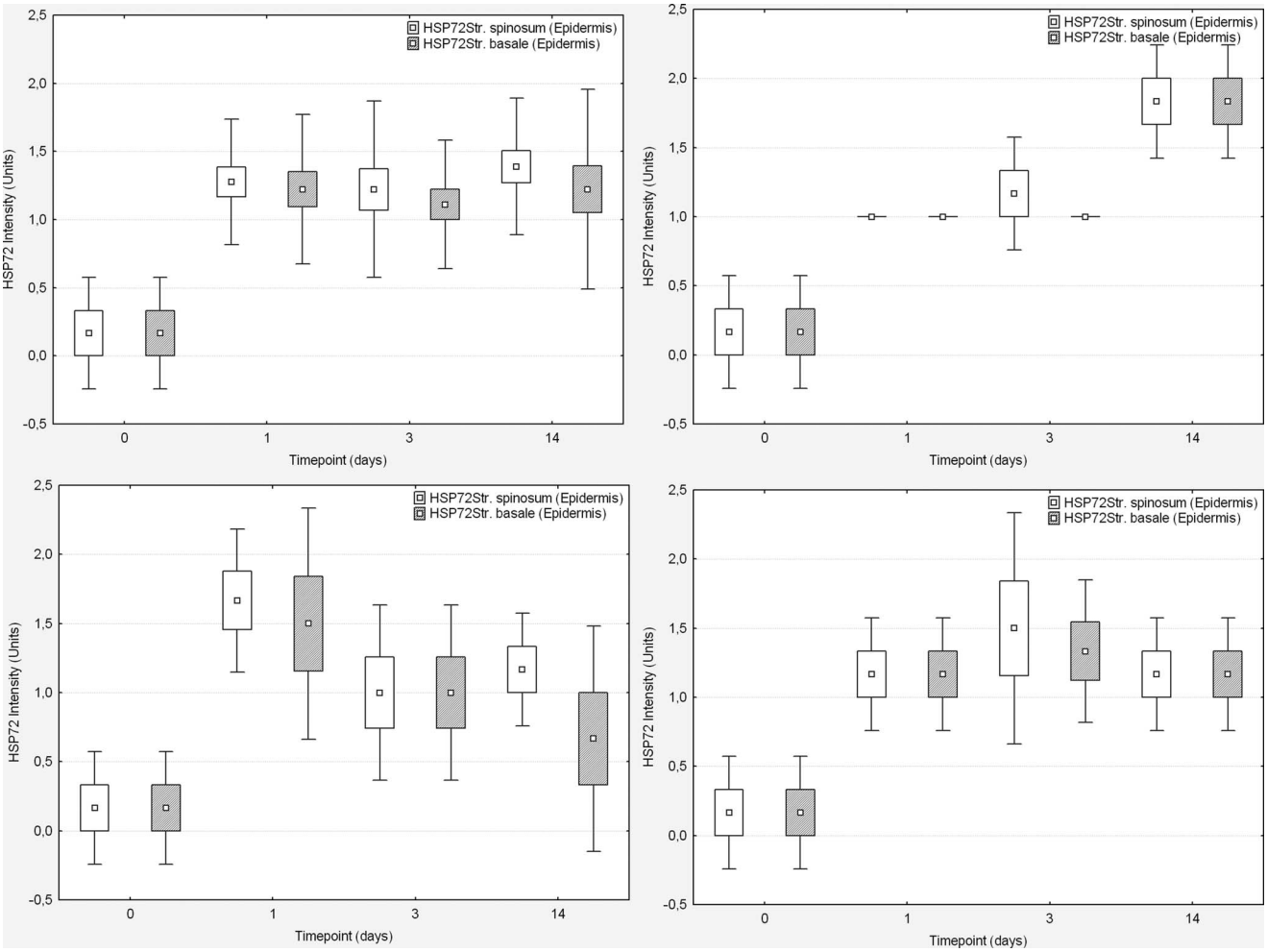

Fig. 2 Effects of AFP on HSP72 staining intensity over time (mean, standard error: 1): (a) all samples (n=60), (b) 50 mJ group (n=18), (c) 100 mJ group (n=18), and (d) 300 mJ group (n=18).

We could not detect significant differences in the distribution and intensity of HSP47 expression during the observation period of 14 days among the groups treated with different energies. Specifically, we observed a slight, albeit insignificant, increase, especially in the HSP47 distribution over time in all treatment groups with a peak at 3–14 days postintervention. Hantash et al.[35] detected an increased expression of HSP47 seven days post-treatment, which persisted over three months. This expression of HSP47 further depended on the skin surface temperature.[14]

TGFβ expression was weak at all time points but highest in the spinocellular layer 1 h to 3 days postintervention with declining values little over baseline in the following 14 days. Light-induced TGFβ labelings were observed in some dermal structures. This upregulation can promote chemotaxis for fibroblasts forming extracellular matrix and collagen, which may result in dermal remodeling or scar formation.[7,21,38,39,42,43,47] Types I and III procollagen mRNA levels peaked at day 21 after CO_2-laser resurfacing of photodamaged human skin and remained elevated for at least six months.[40] During this dermal remodeling, younger patients seemed to form more new collagen compared with older patients with photodamaged skin[48] and denatured collagen appeared to be metabolized differently according to the depth of damage. Superficially denatured collagen was degraded within two weeks, whereas denatured collagen of >600 μm beneath the skin surface was associated with granulomatous inflammation.[49] These results are consistent with our findings of granulomatous infiltrates with macrophages (CD68+ cells) and giant cells surrounding the MAZs 3–14 days postintervention in the treatment areas where the highest energies were applied (300 mJ/cm^2). These energies led to ablation and necrotic zones extending into the deeper dermis. The inflammatory infiltrate is involved in replacing the MAZ, and its extension seems to be crucial for determining whether wound healing is efficient or delayed and limits the dermal ablation and remodeling depth.

Although ablative CO_2-laser therapies suffer from increased complication rates relative to nonablative lasers, a number of studies had suggested a better clinical efficacy for the treatment of deep rhytides, probably due to a prolonged wound healing. AFP is able to remove relatively deep dermal tissue, such as solar elastosis, through transepidermal elimination that conventional CO_2 and ER:YAG laser resurfacing were incapable of reaching without causing side effects. In our study, most of the samples showed maintained epidermal

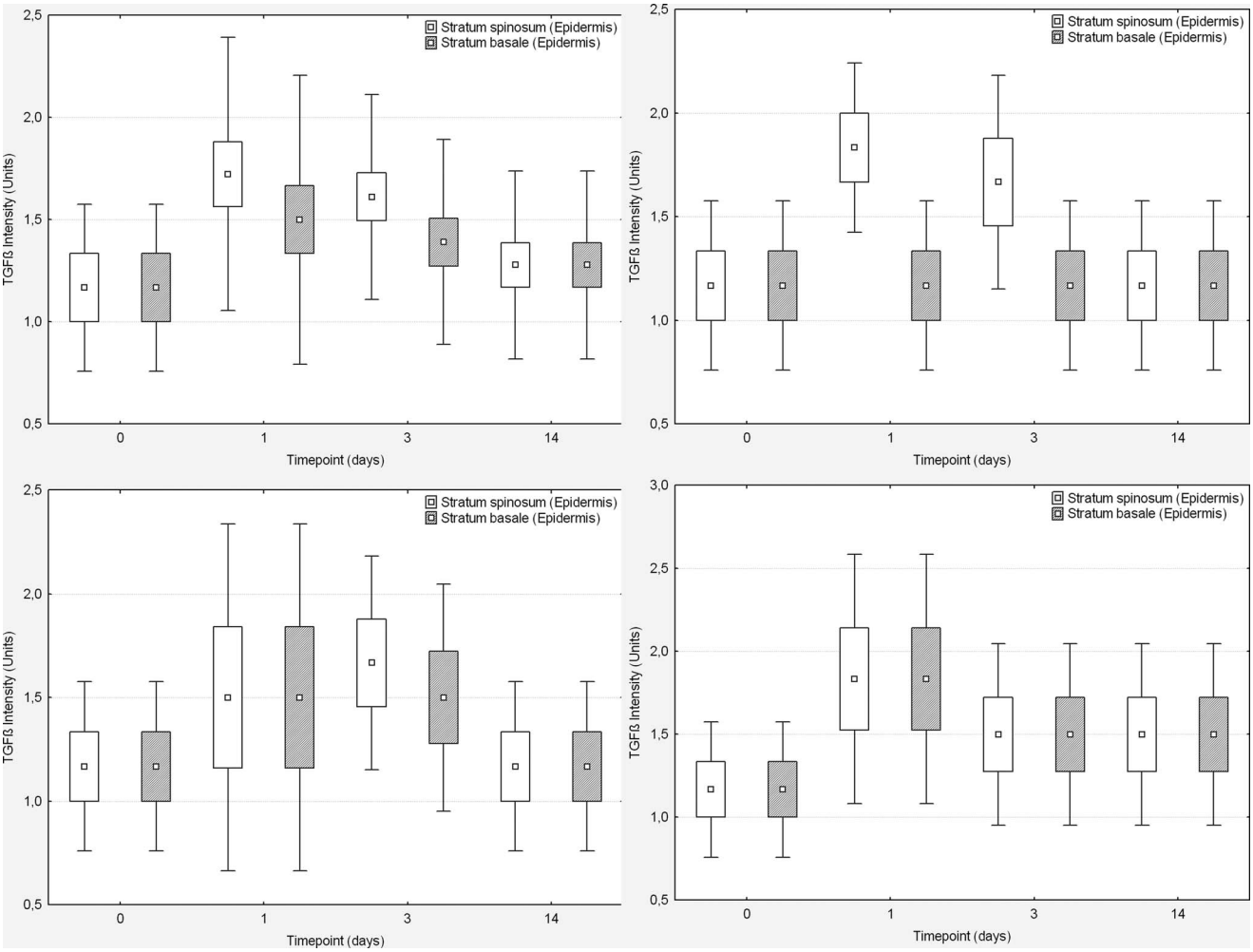

Fig. 3 Effects of AFP on TGFβ staining intensity over time (mean, standard error: 1): (a) all samples (n=60), (b) 50 mJ group (n=18), (c) 100 mJ group (n=18), and (d) 300 mJ group (n=18).

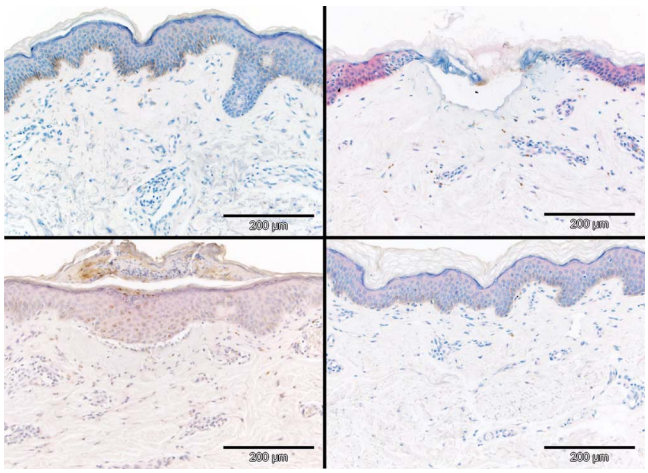

Fig. 4 HSP70 immunoreactivity (magnification 20×) before and post-AFP [50 mJ/cm^2 (5 ms, 10 W)]: (a)=native (untreated skin), (b) 1 h post-AFP, (c) 3 days post-AFP, and (d) 14 days post-AFP.

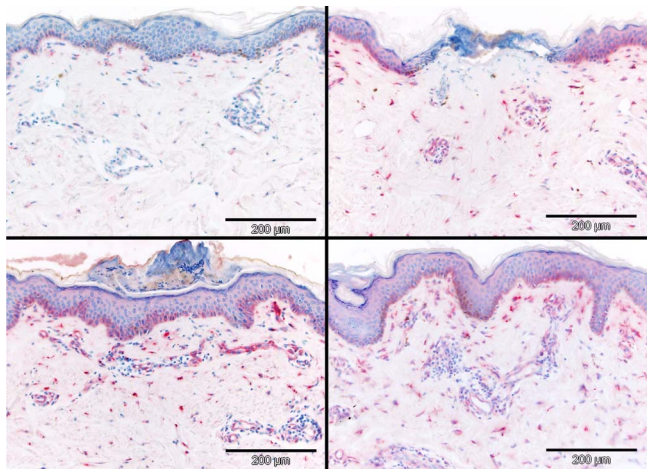

Fig. 5 HSP47 immunoreactivity (magnification 20×) before and post-AFP [50 mJ/cm^2 (5 ms, 10 W)]: (a) native (untreated skin), (b) 1 h post-AFP, (c) 3 days post-AFP, and (d) 14 days post-AFP.

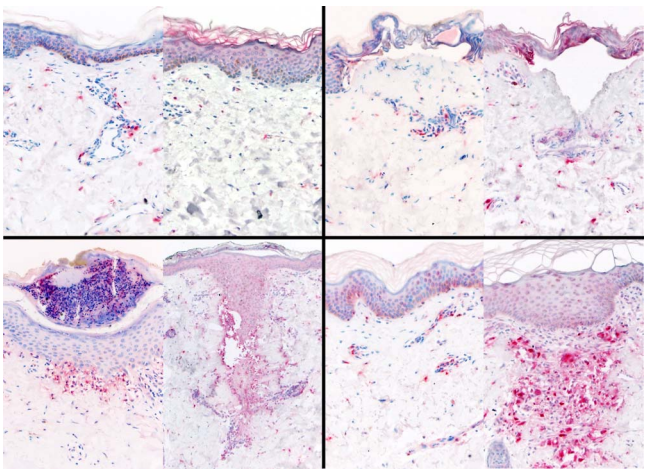

Fig. 6 Procollagen III immunoreactivity (magnification 20×) before and post-AFP [50 mJ/cm^2 (5 ms, 10 W)]: (a) native (untreated skin), (b) 1 h post-AFP, (c) 3 days post-AFP, and (d) 14 days post-AFP.

rooves probably due to the practiced skin cooling. Repetitive treatments may not undergo application intervals corresponding to the time needed for wound healing. Melanophages as a sign of postinflammatory hyperpigmention, one of the most common side effects of AFP, could not be observed within the two weeks of our histological investigation.

In conclusion, we could demonstrate initial epidermal changes in the form of upregulation of HSP70 and TGFβ followed by dermal remodeling resulting in newly synthesized collagen. The extent of dermal damage and granulomatous infiltrate depends on the laser energy applied and seems to be the limiting factor for dermal remodeling. The use of 50 or 100 mJ caused no granulomata in our patients. One patient of the 300-mJ group developed an extended granulomatous inflammation seen in histological investigation that does not prevent normal wound healing. Clinical improvements were observed in all treatment areas with a slight tendency to better results in the 300-mJ group after 14 days. Future studies should be conducted to analyze the wound-healing process in human skin over longer periods following various protocols of AFP in line with clinical read out (e.g., profilometry) in order to establish a safe and effective treatment regimen.

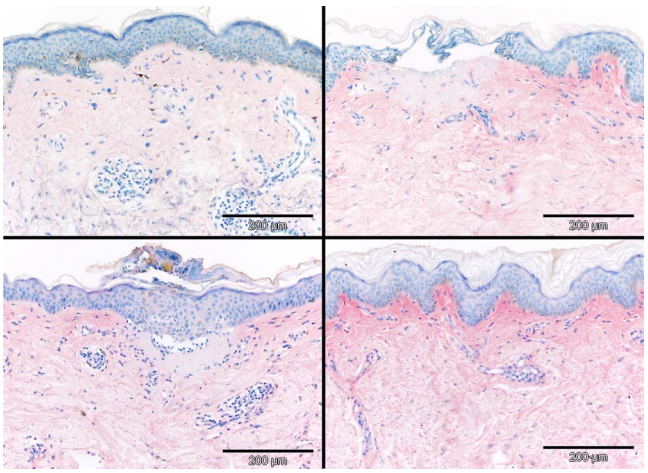

Fig. 7 CD68 immunoreactivity (magnification 20×) before and post-AFP [right side: 300 mJ/cm^2 (10 ms, 30 W); left side: 50 mJ/cm^2 (5 ms, 10 W)]: (a) native (untreated skin), (b) 1 h post-AFP, (c) 3 days post-AFP, and (d) 14 days post-AFP.

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