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ORIGINAL RESEARCH REPORT

Induction of apoptosis by fractional CO_2 laser treatment

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Abstract

Introduction: Fractional CO_2-laser is considered a preferential method for skin resurfacing, but little is known about the molecular mechanisms underlying this surgical tool. In the present study, we investigated the possible role of apoptosis by the sequential analysis of lesional skin after laser treatment, with special attention to power. Moreover, we have analyzed if there is a correlation with clinical improvement. *Materials and methods:* We evaluated the effects of fractional CO_2-laser in twelve patients with photodamage skin Fitzpatrick types I to III. Apoptosis markers were assessed by an immunohistochemical study on skin samples of foream at 24 h, 72 h and 7 days after the irradiation with 15 W or 20 W. Moreover, clinical improvement was assessed by iconography. *Results:* Fractional CO_2-laser induced an inflammatory repair process mediated by activation of apoptotic pathway that was completed in 7 days. The expression of proapototic markers, as annexin-VII and Caspases-9 was increased 24–72 hours after irradiation and decreased after 7 days. While the expression of the anti-apototic marker Bcl-2 increased progressively during 7 days after treatment. *Conclusion:* Our study suggests that the skin's appearance may be enhanced by creating skin changes through apoptosis. Apoptosis, one of the major mechanisms of cell death, might play a key role in initiating the paracrine cascades that lead to cell proliferation.

Key Words: *annexin VII, Bcl-2, Caspases 9 minimally ablative fractional laser, tissue remodelling*

Introduction

Minimally ablative fractional laser devices have gained success as a preferential method for skin resurfacing. Notable improvements in facial wrinkles, photodamage, acne scarring and skin laxity have been reported (1,2).

The fractional resurfacing induces numerous microscopic thermal injury zones of controlled width, depth, and density that are surrounded by a reservoir of spared epidermal and dermal tissues, allowing rapid repair of laser-induced thermal injury (3). While the wound repair after surgery is a well-defined process characterized by an inflammatory reaction, a tissue restoration, and a tissue remodelling in which cells and soluble factors play well-defined roles (4), the wound repair after ablative resurfacing CO_2 laser is not yet a fully known process.

It has already been proved in an in vitro model on cell line fibroblasts obtained from facial skin samples after superpulsed CO_2 laser, that the secretion of growth factors, in particular trasforming-growth-factor-beta1 (TGFβ1) and basic fibroblast growth factor (bFGF) (5), represent important steps for a correct re-epithelization. bFGF inhibits collagen production and promotes organized collagen bundles. TGF β1 stimulates matrix proteins (such as collagens), inhibits protease production and enhances mitogenesis. Consequently, it increases fibroblast collagen deposition, which potentially leads to the formation of dense scarring.

Moreover, apoptosis, a ubiquitous way of cell death in both physiological and pathological conditions, is involved in the removal of inflammatory cells and in the evolution of granulation tissue into the scar tissue during skin wound healing (6). It is characterized by a number of well- recognized cytological and biochemical changes including: nuclear condensation, activation of caspase cysteine proteinases, DNA fragmentation at regular intervals resulting in DNA laddering, cell shrinkage, and membrane blebbing (7).

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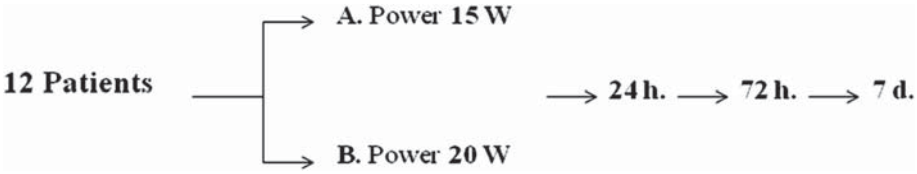

Figure 1. Study design schema.

In a previous study, we have documented the relevance of the secretory pathway of cytokines normally implicated in the early and late phases of wound repair after CO_2 fractional laser (8).

In the present study, we investigated the possible role of apoptosis by the sequential analysis of lesional skin after fractional CO_2 laser treatment, with special attention to power of the laser.

Materials and methods

The study included twelve healthy Caucasian female volunteers aged between 50 and 60 with a photo-damaged skin. After approval of the study protocol by the local ethics committee, written informed consent was obtained from each patient.

A punch biopsy of 4 mm was taken 24 h, 72 h and 7 days after the CO_2 fractional laser irradiation with three different CO_2 laser power: 15 watt and 20 watt. The patients were divided into two groups (A and B) based on potency applied: 15 watt (group A, 6 patients) and 20 watt (group B, 6 patients) while maintaining constant dwell time (1000 μsec) and spacing between the columns of light (500 μ) (Figure 1). The shape of the delivered energy irradiation with the scanner was a square-shaped stamp (15 × 15 mm). In addition, clinical photographic documentation was carried out at baseline and repeated after 7 days, 30 days, and one year. The pictures were taken using a digital system (Anthology, DEKA M.E.L.A, Florence) and standardized using the same camera, the same shooting setting, a twin-flash, the same ambient light, and a chin-holder to achieve the same distance.

The clinical treatment protocol consisted of 2–4 sessions at 4-week intervals and the areas treated were the upper lip and the peri-orbital area. All treatments were performed with a flexible optical fibre-based CO_2 laser equipped with a scanner specially developed for fractional skin resurfacing (Smartxide Dot, DEKA, M.E.L.A., Florence).

We investigated the influence of different energies (15 watt, 20 watt,) on the expression of apoptotic marker: Caspasis 9, Annexin VII and Bcl-2.

Immunohistochemistry

Immunohistochemical staining using an alkaline phosphatase-anti-alkaline phosphatase (APAAP) method was performed to determine the expression of all the markers listed in Table I Biopsies were frozen at −80°C immediately after surgery; 6 μm cryostat serial sections were cut and immediately fixed with ice-cold acetone for 4 minutes. The sections were incubated with MoAb for 60 minutes at room temperature. After washing in 0.01 M phosphate-buffered saline (PBS), pH 7.4, for 10 minutes, the sections were incubated for 40 minutes with rabbit anti-mouse immunoglobulin antiserum (RAM; DakoCytomation, Glostrup, Denmark), and then processed with APAAP complex (Dako Cytomation) twice. Binding of the complex was revealed by hexazotized new fuchsine as chromogenic substrate (Merk, Darmstadt, Germany). The sections were then counterstained with Mayer's haematoxylin. Control sections were incubated with normal mouse IgG.

All sections were examined using a ZEISS microscope. Two investigators (FP and FR) read all tissue sections and the immunoreactivity recorded in the score (Table II). Discrepancies in the reading were resolved by a second parallel reading of the slides until consensus was reached. Absence of immuno-reactivity had a score of "0", scarce immunoreactivity, a score of "1", intermediate immunoreactivity, a score of "2", and intense immunoreactivity, a score of "3".

Clinical evaluation

The clinical evaluation of efficacy was performed by dividing the treatment results into five groups: none, poor, fair, good and excellent improvement. The evaluations were performed individually for each of the following parameters: reduction of visible fine lines, improvements in skin texture, and clearance of irregular pigmentation. The clinical outcomes were examined at 7 days, 30 days, and 12 months after the treatment

Table I. Antibodies used in the study.

Antibody	Clone	Isotype	Dilution	Source
Anti annexin-VII	203-217/6	Mouse IgG1	1:50	Sigma-Aldrich
Anti Bcl-2	Bcl-2-100	Mouse IgG1	1:50	Sigma-Aldrich
Anti caspases-9	LAP6	Mouse IgG1	1:50	R&D Systems

and were based on the comparison of the digital pictures.

Results

A total of 12 female patients 50 to 60 years of age, with clinically significant photodamage of the skin, were enrolled in the study and provided forearm skin samples. Patients tolerated the treatment well without any need for anesthesia. The procedure resulted in initial "frosting" of the treated skin, as expected. At initial follow-up visits, all patients were found to have erythema at the treatment sites that gradually resolved over the first 1–2 weeks after the laser procedure.

Histologically, fractional CO_2 laser treatment resulted in epidermal damage; consistent with epidermal damage, we observed epidermal expression of markers of apoptosis.

Immunohistochemical results

By immunohistochemistry, we studied the effects of fractional CO_2 laser treatment on apoptotic markers at varying pulse energies (15 and 20 W) at various time intervals (24 h, 72 h, 7 days) post-treatment.

Figure 2 (panel A) shows the expression and the localization of Caspases-9 (a), Annexin VII (b) and Bcl-2 (c) proteins after 15 W irradiation. An early increase (within 24 hours) was observed in the Annexin VII and Caspase-9 expression; the expression of the anti-apoptotic, heat-regulated molecule Bcl-2 remained unaltered at the same time point. At 72 hours after irradiation the Caspase-9 and Annexin VII expression remained increased throughout the epidermis and nearby areas; expression of these molecules returned to baseline after 7 days. Interestingly, the expression of Bcl-2, an anti-apoptotic marker, increased, when the expression of the two pro-apoptotic markers decreased.

After 20 W irradiation (Figure 2, panel B), no change in expression was found, except for a more strong expression of the protein in the dermis.

Table II shows the immunolabeling scores for each condition.

Table II. Immunolabeling scores.

	24 h.		72 h.		7 d.	
Marker	15W	20W	15 W	20W	15W	20W
Anti annexin-VII	1	2	3	3	1	1
Anti caspases-9	1	2	3	3	1	1
Anti Bcl-2	1	1	2	3	3	3

Clinical results

The clinical evaluation of efficacy was performed 7 days, 30 days and 12 months after the treatment, dividing the treatment results into five groups: none, poor, fair, good and excellent improvement. The clinical outcomes were evaluated for each patient in relation to improvements in skin texture and fine lines, and in reduction of irregular pigmentation based on a comparison with pictures taken before treatment.

The six patients belonging to group A (treated at 15 W) showed a rapid recovery time (2–4 days), a good reduction of visible fine lines, a good improvement in the skin texture, and a fair clearance of irregular pigmentations (Figure 3).

The six patients belonging to group B (treated at 20 W) had a slightly longer healing time compared to group A (3–5 days); more pain during each single treatment with the same clinical results at 15 W (Figure 4).

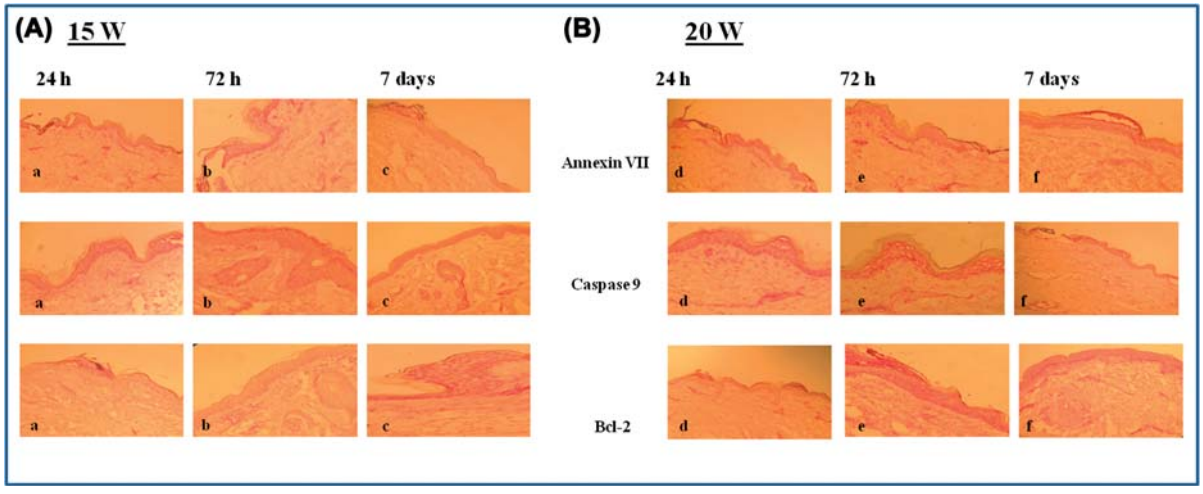

Figure 2. Representative immunohistochemistry (alkaline phosphatase-anti-alkaline phosphatase) stained skin sections of tissue harvested 24 hours, 72 hours and 1 week following delivery of 15 W (panel A) and 20 W (panel B) fractional CO_2 laser irradiation (magnification $\times 150$).

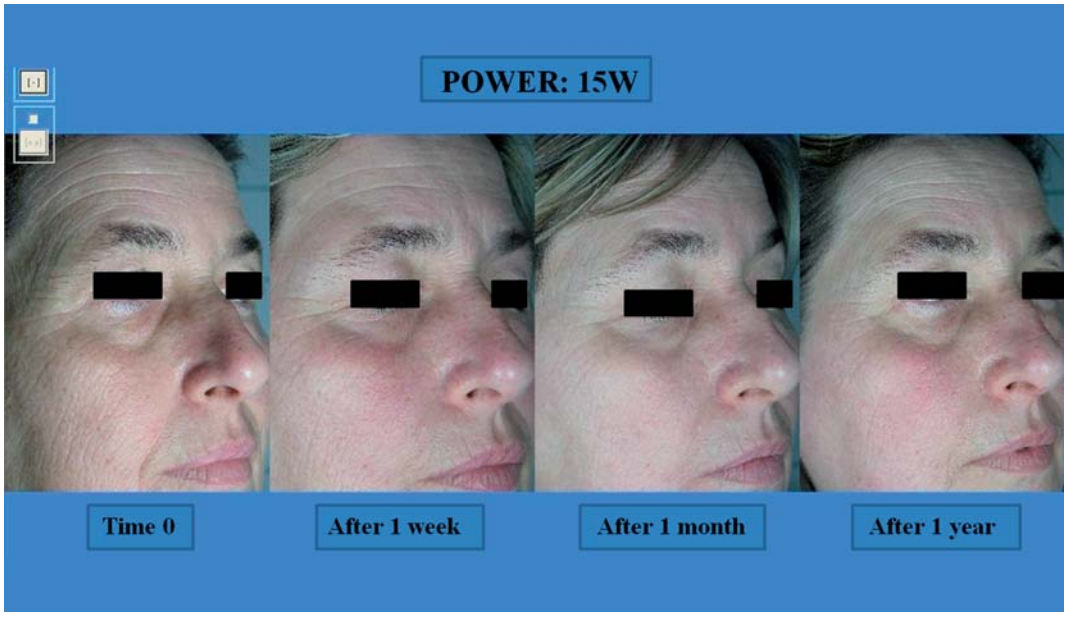

Figure 3. 15 W treatment: a quick recovery time (2–4 days), a good reduction of visible fine lines, a good improvement in the skin texture and a fair clearance of irregular pigmentations.

Discussion

The aim of this study was to assess the response of skin to fractional CO_2 laser in photorejuvenation and to confirm its efficacy always reported in previous studies (2,9).

In a previous study, we had focused on the roles of cytokines and growth factors (such as FGF7, FGF10 and TGFβ) on tissue regeneration after laser injury. However, it is unclear which cell types and molecular mechanisms initiate these signaling cascades. In this study, we hypothesized that dying cells in the treated tissues send signals to stimulate the proliferation of stem or progenitor cells that start the process of tissue regeneration. As apoptosis, one of the major mechanisms of cell death plays a role in the healing mechanisms riepithelization (10) we wanted to investigate if it could play a role also in the healing process after CO_2 treatment.

Apoptosis is characterized by specific biochemical and morphological features that culminate in the shrinkage of the cell to apoptotic bodies that are engulfed by neighboring macrophages.

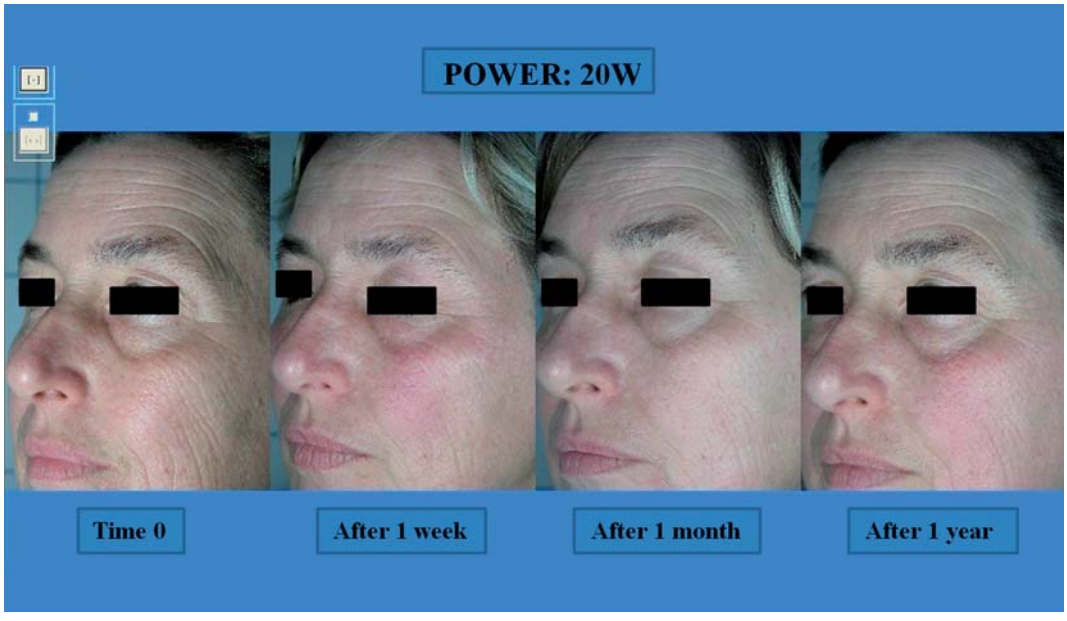

Figure 4. 20 W treatment: a longer recovery time with a longer-lasting erythema, reduction of visible fine lines, a good improvement in skin texture and a fair clearance of irregular pigmentations.

The first microscopic evidence of injury is said to occur after 24 h. This finding is consistent with our observations, where staining for apoptotic markers (Caspases-9 and Annexin VII) were positive 24 hours after laser irradiation.

Caspases are a family of proteins containing a nucleophilic cysteine residue that participates in the cleavage of aspartic acid-containing motifs (11). To date, at least 11 (interleukin-1converting enzyme (ICE) and 10 family members) human caspase members have been identified (12). Caspase-9 is a member of the initiator caspase group, and is generally thought to be responsible for initiating the caspase activation cascade during apoptosis.

The effect of fractional CO_2 laser on apoptosis can also be modulated by the balance between pro and antiapoptotic proteins. Among the molecules that regulate apoptosis are several members of the inhibitor of apoptosis (IAP1) family, which prevent cell death by acting as endogenous suppressors of caspase activity (13). One of the most important regulators of this pathway is the Bcl- 2 family of proteins. Bcl-2 family members act by regulating the efflux of apoptogenic proteins from mitochondria (14). High levels of bcl-2 in a cell which lead to increased bcl-2 dimers are antiapoptotic (15). On the contrary, low bcl-2 levels and high bax levels resulting in bax dimers are proapoptotic (16).

We have shown that the level of caspases-9 and annexin VII proteins increase during the first 72 h, confirming that fractional CO_2 laser initially induces proapoptotic events. Then, after 7 days, the proapoptotic protein level decreases, whereas Bcl-2 has shown to prevent apoptosis. The balance between anti and proapoptotic proteins that determines survival or death, favors an inhibition of the apoptotic process, 7 days after irradiation. Furthemore, the Bcl-2 protein level can increase also immediately after fractional CO_2 laser exposure.

Epidermal damage triggered by fractional CO_2 laser treatment activated a wound healing response.

The clinical outcomes were an excellent reduction in visible fine lines, a good improvement in skin texture, and a fair clearance of irregular pigmentation. The treatment with 20 W did not affect the results more specifically than with 15 W, as the pain upon treatment was more intense and erythema lasted for a longer period of time. Therefore, we are in favour of 15 W treatment.

Our results suggest that the skin's appearance may be enhanced by creating skin changes through apoptosis. Apoptosis, one of the major mechanisms of cell death, might play a key role in inducing the paracrine cascades that lead to cell proliferation.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1. Laubach HJ, Tannous Z, Anderson RR, Manstein D. Skin responses to fractional photothermolysis. Lasers Surg Med 2006;38:142–149.
2. Gotkin RH, Sarnoff DS, Cannarozzo G, Sadick NS, Alexiades-Armenakas M. Ablative skin resurfacing with a novel microablative CO_2 laser. Drugs Dermatol. 2009;8: 138–144.
3. Manstein D, Herron GS, Sink RK, Tanner H, Anderson RR. Fractional photothermolysis: a new concept for cutaneous remodeling using microscopic patterns of thermal injury. Lasers Surg Med 2004; 34:426–438.
4. Eming SA, Krieg T, Davidson JM. Inflammation in wound repair: molecular and cellular mechanism. J Invest Dermatol. 2007;127:514–525.
5. Nowak KC, McCormack M, Koch RJ. The effect of superpulsed carbon dioxide laser energy on keloid and normal dermal fibroblast secretion of growth factors: a serum-free study. Plastic Reconstr Surg. 2000;105:2039–2048.
6. Greenhalgh DG. The role of apoptosis in wound healing. Int J Biochem Cell Biol. 1998;30:1019–1030.
7. Hengartner MO. The biochemistry of apoptosis. Nature. 2000;407:770–776.
8. Prignano F, Campolmi P, Bonan P, Ricceri F, Cannarozzo G, Troiano M, Lotti T. Fractional CO_2 laser: a novel therapeutic device upon photobiomodulation of tissue remodeling and cytokine pathway of tissue repair. Dermatol Ther. 2009; 22:S8–15.
9. Hantash BM, Bedi VP, Sudireddy V, Struck SK, Herron GS, Chan KF. Laser-induced transepidermal elimination of dermal content by fractional photothermolysis. J Biomed Opt. 2006;11:041115.
10. Prignano F, Bianchi B, Domenici L, Rossi R, Romagnoli P, Pimpinelli N, et al. Early apoptosis plays an important role in the healing mechanism of cutaneous basal cell carcinomas after photodynamic therapy. Br J Dermatol. 2003; 149:205–206.
11. Peter ME, Krammer PH. The CD95(APO-1/Fas) DISC and beyond. Cell Death Differ. 2003;10:26–35.
12. Degterev A, Boyce M, Yuan J. A decade of caspases. Oncogene. 2003;22:8543–8567.
13. Wang Z, Cuddy M, Samuel T, Welsh K, Schimmer A, Hanaii F, et al. Cellular, biochemical, and genetic analysis of mechanism of small molecule IAP inhibitors. J Biol Chem. 2004;279:48168–48176.
14. Akhtar RS, Ness JM, Roth KA. Bcl-2 family regulation of neuronal development and neurodegeneration. Biochim Biophys Acta. 2004;1644:189–203.
15. Chiou SK, Rao L, White E. Bcl-2 blocks p53-dependent apoptosis. Mol Cell Biol 1994;14:2556–2563.
16. Raybaud H, Odin G, Fafet A, Santini J, Monteil RA. Genic alterations in oral and head and neck squamous cell carcinomas: analysis of international literature. Pathol Biol. (Paris) 2003;51:176–184.