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Fractional CO_2 laser: a novel therapeutic device upon photobiomodulation of tissue remodeling and cytokine pathway of tissue repair

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ABSTRACT: Minimally ablative fractional laser devices have gained acceptance as a preferred method for skin resurfacing. Notable improvements in facial rhytides, photodamage, acne scarring, and skin laxity have been reported. The aim of the present work was to compare how different CO_2 laser fluences, by modulating the secretory pathway of cytokines, are able to influence the wound-healing process, and how these fluences are associated with different clinical results. Eighteen patients, all with photodamaged skin, were treated using a fractional CO_2 laser (SmartXide DOT, Deka M.E.L.A., Florence, Italy) with varying laser fluences (2.07, 2.77, and 4.15 J/cm^2). An immunocytochemical study was performed at defined end points in order to obtain information about specific cytokines of the microenvironment before and after treatment. The secretory pathway of cytokines changed depending on the re-epithelization and the different laser fluences. Different but significant improvements in wrinkles, skin texture, and hyperpigmentation were definitely obtained when using 2.07, 2.77, and 4.15 J/cm^2, indicating fractional CO_2 laser as a valuable tool in photorejuvenation with good clinical results, rapid downtime, and an excellent safety profile.

KEYWORDS: cytokines, fractional CO_2 laser, immunohistochemistry, tissue repair

Introduction

Ablative lasers, such as the CO_2 and Er:YAG lasers, remain the gold standard for skin resurfacing and provide the greatest clinical improvements with the least number of treatments (1). However, because of the complete vaporization of the epidermis and the variable thermal damage to the dermis, healing time is prolonged, and treatments involve significant associated risks (2), for instance, delayed re-epithelization, persistent erythema, permanent hypopigmentation, and in some cases (above all in situations in which there are also problems linked to the patient's general conditions), even scars or keloids.

A particular CO_2 laser system has been developed in order to achieve good clinical results in micro-ablative fractional resurfacing. This device is able to cause a true "micro-ablation" of the epidermis and thermal effects of the dermis although

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the areas of ablated tissue are microscopic, thanks to its particular pulse shape (denominated SmartPulse, DEKA M.E.L.A., Florence, Italy); normal skin is preserved in the areas surrounding each microcolumn of light, and healing time as well as scarring risks are minimized compared with conventional ablative systems.

The histological and immunocytochemical evaluations of the effects of ablative fractional resurfacing CO_2 laser are closely linked to the experimental conditions and the energy and/or exposure time.

Although wound repair after surgery is a well-defined process, characterized by three consecutive phases, namely, inflammatory reaction, a proliferative process leading to tissue restoration, and finally, tissue remodeling, and during all three overlapping processes cells and soluble factors play well-defined roles (3), it is not the same for wound repair after ablative resurfacing CO_2 laser, and even more so with CO_2 fractional resurfacing.

Nowak et al. suggest in their study that superpulsed CO_2 may be an effective wound modulator by increasing basic fibroblast growth factor (bFGF) (R&D Systems, Minneapolis, MN) secretion and suppressing transforming growth factor-β1 (TGF-β1) (R&D Systems) secretion. Consequently, CO_2 laser promotes cell replication, but at the same time, it is potentially able to balance collagen organization against excessive fibrosis, thus avoiding aberrant wound healing (4). Besides, it has been observed, in an animal model, how the tissue concentration of two growth factors, such as the TGF-β1 and the bFGF, can have an impact on the quality of tissue repair. Very interesting data came from an in vivo model concerning several cytokines after laser skin resurfacing (5).

The aim of the present study was to investigate the secretory pathway of cytokines (Table 1) normally implicated in the early and late phases of wound repair after fractional CO_2 laser (SmartXide DOT, Deka M.E.L.A., Florence, Italy).

In order to evaluate whether the various parameters (energy, dwell time, and space between columns of light) and the "timing" of cytokine release could influence the results, the present study had two specific aims:

1. To assess the understand, at least in part, which are the main biological events characterizing the results of this particular device.
2. The clinical results using three different fluences (determined by 2.07, 2.77, and 4.15 J/cm^2; 1000-μsec dwell time (exposure time) and 500-μm spacing) with a fractional CO_2 laser (SmartXide DOT, Deka M.E.L.A.).

Table 1. Cytokines involved in wound-healing processes and their major function

Cytokines	Major function
TGF-β	• Stimulates matrix proteins (such as collagen), inhibits protease production, and enhances mitogenesis. • Activates the chemotaxis of macrophages and granulocytes, and releases proinflammatory cytokines: interleukin-1, interleukin-6, and tumor necrosis factor-α.
bFGF	• Angiogenetic and mitogenic activity. • Provides the initial stimulation of endothelial cell migration and proliferation. • Inhibits collagen production and promotes organized collagen bundles.
EGF	• Re-epithelization activity (i.e., keratinocyte proliferation, keratinocyte marginalization, hyperproliferative wound epidermis, etc.).
PDGF	• Chemotactic activity for monocytes, macrophages, and neutrophils. • Mitogenic activity for fibroblasts and smooth muscle cells in vitro. • Stimulates fibroblasts to produce extracellular matrix and to contract collagen matrix.
VEGF	• Regulates vasculogenesis and angiogenesis.
Vimentin	• Fibroblast protein: involved in matrix and intercellular substance production.

bFGF, basic fibroblast growth factor; EGF, endothelial growth factor; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor.

Materials and methods

The present study included 18 healthy Caucasian female volunteers aged between 50 years and 60 years, all with photodamaged skin. The patients did not show evidence of any overt diseases. After the approval of the study protocol by the local ethics committee, written informed consent was obtained from each patient.

Clinical photographic documentation was carried out before the first session, and then repeated after 7 and 30 days, using a digital system (Anthology, Deka M.E.L.A). The pictures were 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.

In addition, a punch biopsy of 2 mm was taken at time 0 (control), immediately after the laser session, and 3 and 30 days after the last fractional CO_2 laser irradiation (SmartXide DOT, DEKA

Table 2. Primary monoclonal antibodies

Antibody	Clone	Isotype	Code	Dilution	Source
TGF-β	1D11	IgG1 mouse	MAB1835	1 : 10	R&D Systems
EGF		IgG1 mouse	E 2520	1 : 300	SIGMA-ALDRICH
VEGF	26503.11	IgG2b mouse	V 4758	1 : 20	SIGMA-ALDRICH
PDGF	35248	IgG1 mouse	MAB322	1 : 30	R&D Systems
bFGF	254625	IgG2a mouse	MAB1206	1 : 10	R&D Systems
Vimentin	LN-6	IgM mouse	V 2258	1 : 200	SIGMA-ALDRICH

bFGF, basic fibroblast growth factor; EGF, endothelial growth factor; Ig, immunoglobulin; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor.

Table 3. Results of immunolabeling scores at time 0, immediately after treatment, 3 days after treatment, and 30 days after treatment thereafter with 2.07, 2.77, and 4.15 J/cm^2 irradiation

		Immediately after			3 days after			30 days after		
	Time 0	2.07 J/cm^2	2.77 J/cm^2	4.15 J/cm^2	2.07 J/cm^2	2.77 J/cm^2	4.15 J/cm^2	2.07 J/cm^2	2.77 J/cm^2	4.15 J/cm^2
EGF	1	3	2	0	3	1	0	1	3	0
bFGF	0	1	0	1	1	2	1	1	3	0
PDGF	1	2	1	1	2	2	2	1	2	0
VEGF	0	1	1	0	1	1	0	1	1	0
TGF-β	0	1	0	0	2	3	0	1	1	0
Vimentin	1	3	0	0	3	2	0	1	1	0

EGF, bFGF, PDGF, VEGF, TGF-β, and vimentin immunoreactive cells were observed under light microscopy. A range from 0 to 3 was used for the score. The absence of immune reaction was scored as "0," a scarce immunoreactivity was scored as "1," an intermediate immunoreactivity was scored as "2," and an intense immunoreactivity was scored as "3."

bFGF, basic fibroblast growth factor; EGF, endothelial growth factor; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor.

M.E.L.A), using three different CO_2 laser fluences determined by 2.07, 2.77, and 4.15 J/cm^2. At the same end points, an immunohistochemical study to evaluate the cytokines' expression was performed.

Clinical evaluation

The clinical evaluation of efficacy was performed by dividing the treatment results into five groups: none, poor, fair, good, and excellent improvements. 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.

Immunohistochemistry

The immunohistochemical study was performed in accordance with the procedure used by Prignano et al. (6) through an alkaline phosphatase-anti-alkaline phosphatase method. Immunohistochemical staining using was performed to determine the expression of various cytokines before and after laser treatment at the defined end points (time 0, immediately after 2.07, 2.77, and 4.15 J/cm^2 irradiation, 3 days after 2.07, 2.77, and 4.15 J/cm^2 irradiation, and 30 days after 2.07, 2.77, and 4.15 J/cm^2 irradiation). All the mouse monoclonal antihuman antibodies used as primary reagents in the present study are listed in Table 2.

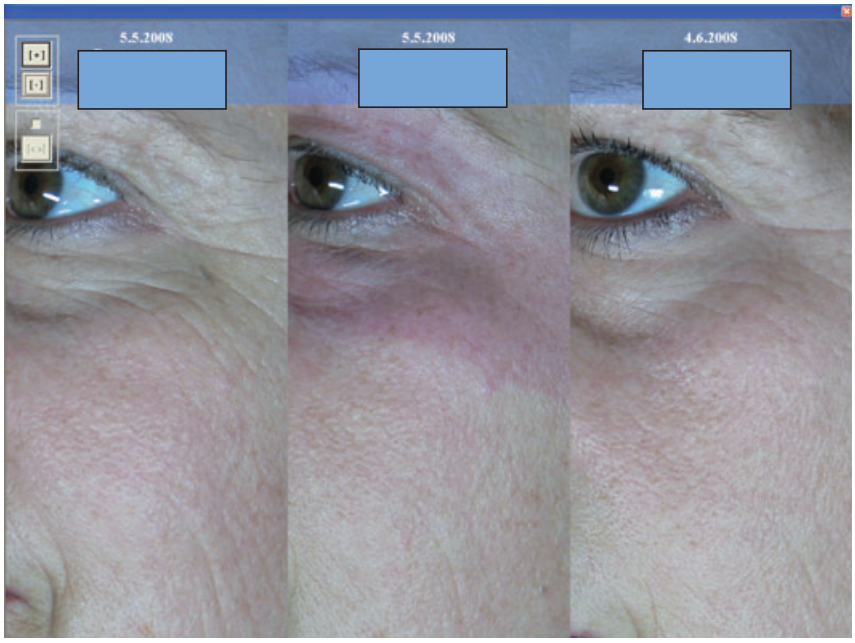
All sections were examined using a ZEISS microscope (Carl Zeiss SMT, Oberkochen, Germany). Two investigators (FP and FR) read all tissue sections and the immunoreactivity recorded in the score (Table 3). Discrepancies in the reading were resolved by a second parallel reading of the slides until consensus was reached.

The score recorded is the average of these observations. Absence of immunoreactivity had a score of "0," scarce immunoreactivity had a score of "1," intermediate immunoreactivity had a score of "3," and intense immunoreactivity had a score of "4."

Results

Clinical results

The clinical outcomes were evaluated for each patient in relation to improvements in skin texture and fine lines, and a reduction in irregular pigmentation based on a comparison with pictures taken before treatment (FIGS. 1 and 2).



(A) (B) (C)

FIG. 1. **(A) Before treatment. (B) Marked edema immediately after treatment. (C) Reduction in fine lines 1 month after treatment.**

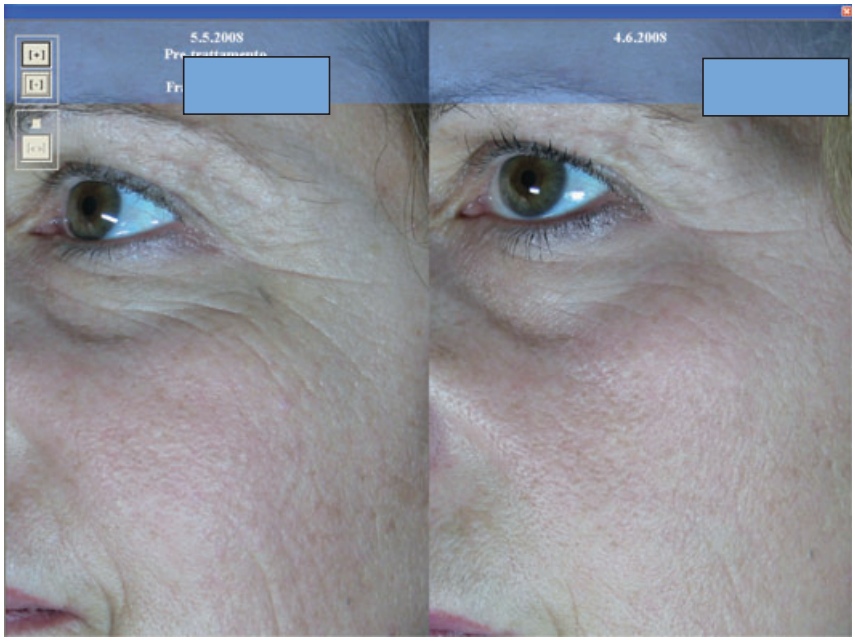
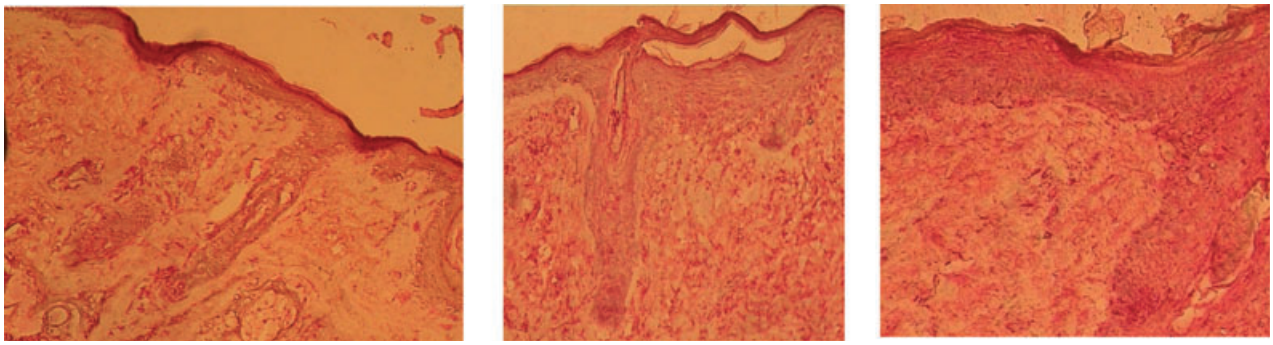


FIG. 2. **Before and 1 month after treatment.**

The six patients belonging to group A (treated at 2.07 J/cm^2) showed a rapid recovery time (2–4 days), a good reduction of visible fine lines, a good improvement in skin texture, and a fair clearance of irregular pigmentations.

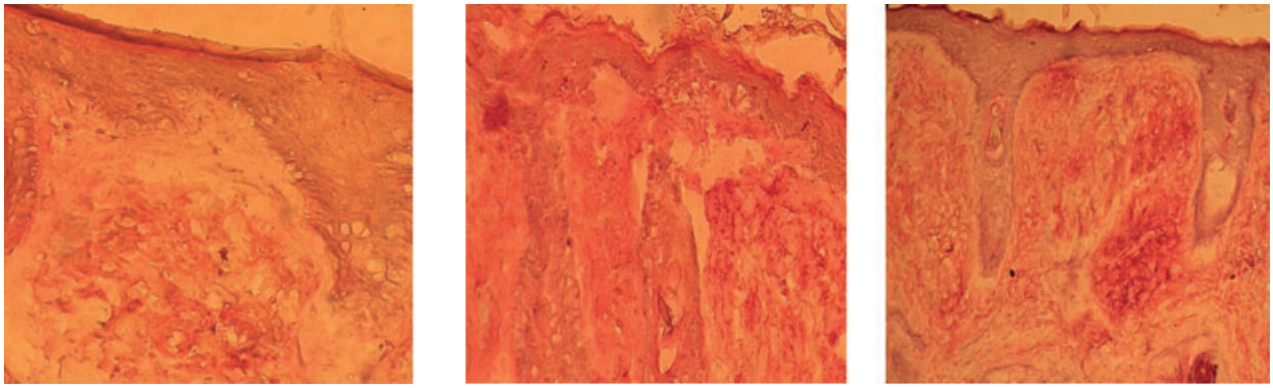
The six patients belonging to group B (treated using 2.77 J/cm^2) had a slightly longer healing time compared with group A (3–5 days) but more consistent clinical results. In particular, the present authors observed an excellent reduction in visible wrinkles, a good improvement in skin texture, and a fair clearance of irregular pigmentations.

The six patients belonging to group C (treated at 4.15 J/cm^2) showed more consistent clinical results with very good skin tightening, excellent reduction



(A) (B) (C)

FIG. 3. Immunohistochemistry (alkaline phosphatase-anti-alkaline phosphatase): (A) Time 0 (control): endothelial growth factor (EGF) is expressed within the epidermal layers and in part of the dermis (magnification ×150). (B) Immediately after laser treatment at 2.07 J/cm^2: intense staining for EGF within the epidermis and in the dermis (magnification ×150). (C) Three days after laser treatment at 2.07 J/cm^2: intense staining for EGF within the epidermis and the dermis with a complete re-epithelization (magnification ×150).



(A) (B) (C)

FIG. 4. Immunohistochemistry (alkaline phosphatase-anti-alkaline phosphatase): (A) Time 0 (control): platelet-derived growth factor (PDGF) is moderately expressed in the epidermis and in the dermis (magnification ×150). (B) Immediately after laser treatment at 2.77 J/cm^2: positive staining for PDGF but with higher intensity within the dermis closer to the columns of laser disruption (magnification ×250). (C) Three days after laser treatment at 2.77 J/cm^2: absence of PDGF expression within the epidermis, and moderate expression within the dermis (magnification ×150).

of visible wrinkles, and a fair clearance of irregular pigmentation. However, longer recovery times were observed, and a greater sensation of pain was recorded compared with groups A and B.

Immunohistochemical results

The application of different laser fluences (2.07, 2.77, and 4.15 J/cm^2) in the present study resulted in a change in growth factor profiles (FIGS. 3 and 4). The expression of the cytokines and of the protein vimentin (SIGMA-ALDRICH, Milan, Italy) is reported in Table 3, and their modifications are reported in FIGS. 5–7.

Discussion

The aim of the present study was to verify the photobiomodulation of tissue remodeling and cytokine pathway of tissue repair upon fractional CO_2 laser. The present authors did not address the presence and quantity of collagen synthesis because it cannot predict if the tissue repair will be toward a eutrophic or hypertrophic scar (or even worse, toward keloids). Therefore, at variance with other works (4), the present authors concentrated their scientific interest toward the synthesis of growth factors and cytokines at different end points. Also, the expression of heat shock protein reported

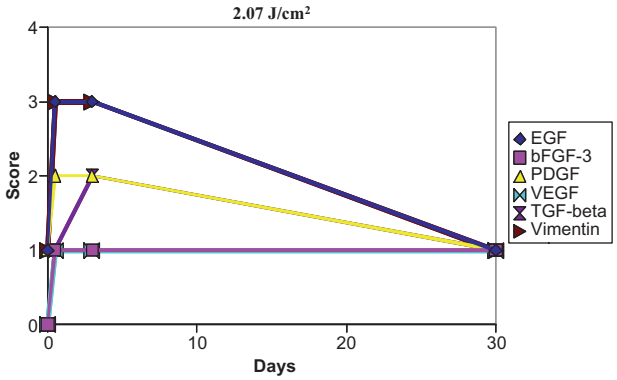


FIG. 5. The graph shows the results of immunolabeling scores at time 0, immediately after treatment, 3 days after treatment, and 30 days after treatment with 2.07 J/cm². bFGF, basic fibroblast growth factor; EGF, endothelial growth factor; PDGF, platelet-derived growth factor; TGF-beta, transforming growth factor-β; VEGF, vascular endothelial growth factor.

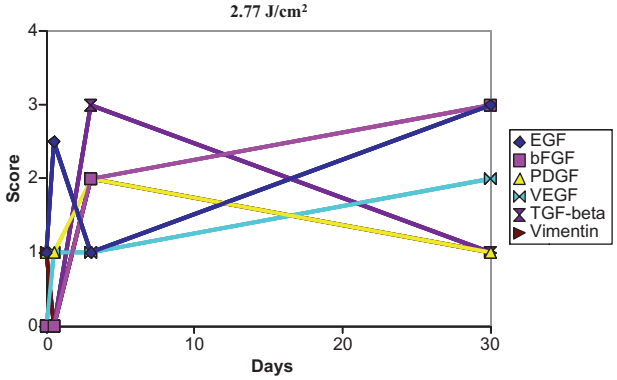


FIG. 6. The graph shows the results of immunolabeling scores at time 0, immediately after treatment, 3 days after treatment, and 30 days after treatment with 2.77 J/cm². bFGF, basic fibroblast growth factor; EGF, endothelial growth factor; PDGF, platelet-derived growth factor; TGF-beta, transforming growth factor-β; VEGF, vascular endothelial growth factor.

by other authors (7) had no relevance for our investigation, as it is mainly a sign of severe cell damage.

Growth factors and cytokines (mainly those of tissue repair) play a multiple and specific role in skin-repairing processes. There are cytokines, such as platelet-derived growth factor (PDGF) (R&D Systems) and endothelial growth factor (EGF) (SIGMA-ALDRICH), that are important in the early, inflammatory phase of wound healing. PDGF is chemotactic for monocytes, macrophages, and neutrophils, and mitogenic for fibroblasts and smooth muscle cells in vitro (8). PDGF stimulates fibroblasts to produce extracellular matrix and to contract it. The levels of PDGF in dermal nonhealing wounds were strongly reduced compared with

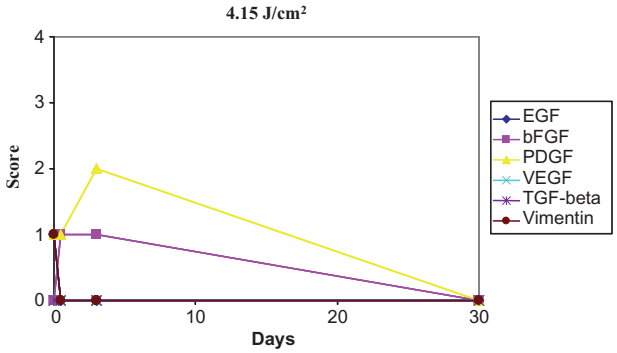


FIG. 7. The graph shows the results of immunolabeling scores at time 0, immediately after treatment, 3 days after treatment, and 30 days after treatment with 4.15 J/cm². bFGF, basic fibroblast growth factor; EGF, endothelial growth factor; PDGF, platelet-derived growth factor; TGF-beta, transforming growth factor-β; VEGF, vascular endothelial growth factor.

surgically created acute wounds, further supporting an important role of PDGF in normal healing. In addition, overexpression of PDGF is always increased in hypertrophic scars and keloids (9).

EGF is involved in all steps of re-epithelization, playing a role that is in accordance with the reparative steps (keratinocyte proliferation, keratinocyte marginalization, hyperproliferative wound epidermis, etc.) (10,11). bFGF provides the initial stimulation of endothelial cell migration and proliferation (11,12). TGF-β, after trauma or injury, is able to activate the chemotaxis of macrophages and granulocytes, and to release proinflammatory cytokines: interleukin-1, interleukin-6, and tumor necrosis factor-α (13). The vascular EGF (SIGMA-ALDRICH) is implicated in the regulation of vasculogenesis and regulation of angiogenesis during wound healing. The results of the present study have clearly demonstrated that the secretions of cytokines upon fractional CO_2 laser treatment correlate only in part with the secretory pathway after tissue injury.

Right from the start, the positive staining of EGF (time 0) in all our cases, even in the absence of laser stimuli, demonstrated that it was linked to photoexposition, also with heterogeneous intensity. The expression of both EGF and PDGF in the early phases after treatment (both after 2.07 and 2.77 J/cm² treatment, but with a higher intensity after 2.07 J/cm²) correlates with their specific functions: PDGF is chemotactic for most of the cells of the dermis and is highly expressed in traumatic fluids; EGF has a reparative role and can enhance re-epithelization.

A relevant fact is that only after 2.07-J/cm² irradiation was there an increase of vimentin positive cells in the dermis, which could be interpreted as

the "ideal stimuli" for the increase of PDGF, which in turn – or as a consequence – induces fibroblasts to proliferate. Vimentin is a protein of fibroblast cytoskeleton (6), and its expression is a marker of fibroblasts' activation and collagen synthesis.

The irradiation with 4.15 J/cm^2 has proved to be less effective in modulating the secretory pathway of the studied cytokines, as – in our hands – it proved to be rather suppressing than modulating; after 30 days no cytokines are expressed (see Table 3).

The presence of TGF-β and bFGF is a later phenomenon than PDGF in the biologic steps of re-epithelization after laser treatment. TGF-β was present in our specimens 3 days after treatment; its main activity is to stimulate cell proliferation and differentiation (5). It also enhances epithelial cell migration from the surrounding areas. It is expressed at its highest intensity after 3 days, when migration of epithelial cells is essential for replacing the "deprived" posttreatment areas. The presence of bFGF is also in line with its biological function: to induce proliferation and migration of endothelial cells. The concentration of both cytokines is essential in regulating healing. TGF-β regulates fibroblast collagen deposition; if produced in excess, it would lead (potentially) to the formation of dense scars (its presence has been largely documented in hypertrophic scars and keloids). The presence of bFGF later on in our experiments, in contrast with TGF-β, which slowly reduces (from the 3rd to the 30th day), proves that laser-induced wounds are "much more physiological," as the regular healing process warrants an adequate quantity of bFGF in order to maintain correct homeostasis. EGF, with its tendency to induce keratinocyte proliferation and differentiation, is once again represented in the last end points of the present study. EGF and vascular EGF together, in the very last phases (after 30 days), could be interpreted as a restoration of correct epithelial differentiation and neoangiogenesis. These two events correlate with a reduction of TGF-β and a stable expression of bFGF, two additional hypothetical expressions of physiological scarring.

The presence of vimentin positive cells in the dermis underlines the value of the remaining pool of cells within the dermis able to produce matrices and intercellular substances.

The number of vimentin positive cells correlates mainly with the intensity of energy (see Table 3) applied; at the moment, the present authors are not able to comment on these data; however, it may become clearer with the treatment ongoing.

In conclusion, the results of the present study demonstrate that a multitude of growth factors and cytokines are present at the wound site. After skin irradiation, the dynamics of these vary over time, and the quality and the sequential secretion of these cytokines are extremely important for achieving good quality wound repair. Already with 2.0 J/cm^2 irradiation, it was possible to achieve important biological results.

The originality of the present work is that of having established a correlation between the time-defined end points of laser energy and the cytokines released in a human model. Given the extreme "operate flexibility" of our fractional CO_2 lasers, in order to obtain better and more consistent results that meet the social needs and compliance of the patient, it is important to investigate the influence of the different treatment parameters on biological response, clinical treatment efficacy, and recovery time.

Further studies are currently under way, using increased levels of fluences, particularly 4.15 J/cm^2, for evaluating whether the brilliant photorejuvenation results obtained with this parameter, and demonstrated in previous studies, could be related to a different cytokine pathway and timing.

Disclosure

The authors have no conflicts to declare.

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