



Low-fluence and low-density CO₂ laser: histological analysis of collagen fiber changes in skin and its clinical repercussions in photorejuvenation

Juliana Merheb Jordão¹ · Mariana Fajgenbaum Feiges Stolar² · Sarah Sanches Melo³ · Giovana Liz Marioto de Campos³ · Lismary Aparecida de Forville Mesquita⁴ · Thelma Larocca Skare⁵

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Abstract

This study refers to clinical and histologic analysis of effects on photorejuvenation after one single treatment of fractional CO₂ laser with low fluence and low density. To analyze histologically the quantitative variation of collagen fibers type I and III, elastic fibers, and epidermal thickness on D84, besides clinical evaluation of amount, length, thickness, and depth of periocular wrinkles during the same period. This is an open, prospective, interventional study. There were 40 healthy female with age between 35 and 65 years. Twenty-six participants were randomly selected for D0 and D84 biopsy. A single session of fractional CO₂ laser was done in the whole face, using a 800- μ m tip, 5% density, and 10 mJ fluence with a single pass. On D0, D42, and D84, a clinical comparative analysis of amount, length, depth, and thickness of periocular wrinkles has been done. On histological analysis, a comparative quantitative evaluation of collagen fibers type I and III, elastic fibers, and epidermal thickness has been done on D0 and D84. The results of this study denoted a significant clinical improvement of amount (−32.17%; $p < 0.0001$), thickness (−33%; $p < 0.0001$), length (−35.84%; $p < 0.0001$), and depth of periocular wrinkles (−32.46%; $p < 0.0001$). A significant increase in the amount of collagen fibers type III was observed on D84 (+60.67%; $p = 0.0013$). Collagen fibers type I and elastic fibers did not have the same result, with a nonsignificant increase (+8.31%; $p = 0.3820$) and a decrease (−12.4%; $p = 0.0585$) respectively. Epidermal thickness has a tendency to significant variation ($p = 0.05553$). The results demonstrate that fractional CO₂ laser with low fluence and low density is a safe and efficient option for photorejuvenation of the face.

Keywords CO₂ laser · Ablative fractional laser · Photorejuvenation

Introduction

The carbon dioxide laser, better known as the CO₂ laser, is a fractional infrared long-pulse laser with a wavelength of 10.600 nm and high water absorption [1]. As it is a fractional

laser, it acts by creating numerous zones of thermal injury, known as microthermal zones (MTZs). The great advantage of this fractionation is the maintenance of approximately 60–90% of viable and healthy tissue around the MTZs, thus allowing their rapid healing with less downtime and fewer complications. MTZs are expected to close through the healing process from healthy skin areas 24–48 h after laser application [2].

High-power CO₂ lasers are considered the gold standard for photorejuvenation [2–4] and are also indicated for the treatment of atrophic and hypertrophic scars and stretch marks.

The great challenge of the high-power CO₂ laser is its prolonged downtime, involving intense erythema, edema, and crusting, which limit its acceptance by patients. The application of this laser with more conservative parameters is indicated for light photorejuvenation, as well as drug

✉ Juliana Merheb Jordão
dra.julianajordao@hotmail.com

¹ Head of Laser Therapy Dermatology Department, Hospital Universitário Evangélico Mackenzie, Curitiba, PR, Brazil

² L'Oréal Brazil, Rio de Janeiro, RJ, Brazil

³ Dermatology Department, Hospital Universitário Evangélico Mackenzie, Curitiba, PR, Brazil

⁴ Santa Casa de Misericórdia de Curitiba, Curitiba, PR, Brazil

⁵ Rheumatology Department, Hospital Universitário Evangélico Mackenzie, Curitiba, PR, Brazil

delivery, especially in combination with photodynamic therapy for actinic keratosis, and superficial basal and squamous cell carcinoma. It is also an option for low-level laser therapy (LLLT).

Among the most commonly observed complications of using the high-fluence CO₂ laser are acneiform eruption (5.3%), bacterial infection (2.2%), hyperpigmentation (1.2%), and prolonged erythema (0.8%) [5]. Thus, it is clear that the use of conservative parameters would minimize the risk of complications.

There has been much discussion about the optimal parameters to achieve satisfactory results without compromising patient safety. Unfortunately, there have been few studies on the use of conservative parameters with CO₂ lasers. Fluence or energy is one of the most discussed parameters in regard to depth, results, and safety. Saluja et al. believe that higher parameters correspond to greater laser penetration in the dermis and better clinical results in photorejuvenation [6].

Some authors have advocated the use of low-energy CO₂ lasers. Yuan et al. believe that high energies can exceed the balance between modulation and suppression in the healing response [7]. Therefore, instead of stimulating neocollagenesis and tissue contraction, high energies may impair this effect. The same authors observed no difference in clinical outcomes between 10 and 20 mJ.

Motta et al. histologically evaluated the effects of CO₂ laser application on rats at 3 different fluencies to analyze whether the use of low and high energies would yield similar results [5]. There was no statistically significant difference between the different fluencies in ablation depth, coagulation depth, or MTZ density or diameter. Prignano et al. conducted a similar study on the forearm and face of single-session patients [2]. Due to the similarities in clinical, histological, and electron microscopy results between the different fluencies, the authors concluded that there was no advantage to using higher fluencies and that higher fluencies only increased the risks of the procedure.

Given the controversial analyses about the ideal parameters to achieve good clinical and histological results in balance and the safety of the procedure, it is necessary to perform a CO₂ laser study using minimal energy and low density to clarify both how reduced energy could act in neocollagenesis and its safety.

Materials and methods

This study was conducted at the author's private practice from 14 March 2016 to 14 June 2016 and was approved by the Research Ethics Committee of the Evangelical Beneficent Society of Curitiba.

This was a prospective, interventional, open study.

Subject selection and analysis

Forty adult female participants were selected for this study. Of these, 26 were randomly selected for biopsy on day 0 and underwent repeated biopsy on day 84.

Only participants who consented to participate in a voluntary act by signing the informed consent form (ICF) were included.

Participants eligible for inclusion in this study met all of the following criteria: healthy female participants from 35 to 65 years old; absence of dermatological conditions that contraindicated the application of a CO₂ laser; skin phototypes I–IV according to the Fitzpatrick classification; and presence of wrinkles in the periorbital and frontal areas with a degree equal to or from 2 to 4 according to the *Skin Aging Atlas* [8].

The exclusion criteria were as follows: pregnant or nursing women; participants with a dermatological pathology that contraindicated the application of the CO₂ laser; participants who had undergone a dermatological treatment on the face in the last 3 months; participants who had undergone facial plastic surgery in the last 6 months; participants who had used nutraceuticals in the last 3 months; and smokers.

After inclusion in the study, the participants were evaluated by the main investigator in terms of the degree of skin roughness and lightness, uniformity of skin tone, and skin smoothness and general appearance using a visual analog scale (VAS), a subjective continuous scale from 0 to 10. Then, the participants were evaluated in terms of the degree of roughness of the frontal and periorbital regions using the *Skin Aging Atlas* [8]. These evaluations were repeated on day (D)6, D42, and D84.

Standardized photographs of the right, left, and front profiles of participants' faces were recorded using *Reveal Imager*® photographic equipment (Canfield Scientific, Inc., Fairfield, NJ).

To evaluate the postlaser healing time and possible adverse effects, participants returned to the researcher's office every day during the first 7 days of study (D0 to D6), when clinical evaluations and recordings were performed.

Subsequently, on D42 and D84 after the first visit, they were clinically reevaluated using the VAS and the *Skin Aging Atlas*.

Participants were instructed not to expose themselves to the sun during the study period. Free sunscreen, neutral soap, and neutral moisturizing serum were provided free of charge and standardized; the patients were instructed to use these products 3 times a day to provide comfort and hydration after the procedure, thus benefiting the healing process. These were the only cosmetics allowed during the study. All participants were also given 200 mg of acyclovir in 5 daily doses for 7 days as prophylaxis for herpes simplex infection after the procedure.

CO₂ laser

During the first visit, at D0, participants underwent a full-face CO₂ laser session (Sculptor CO₂, Vyndence Medical, Brazil) using an 800- μ m tip and a 5% density; a fluence of 10 mJ in one stride was used, resulting in what is considered a low-intensity procedure. The CO₂ laser procedure was performed after skin sanitization and the application of anesthetic ointment containing lidocaine and tetracaine all over the face.

Histological evaluation

Biopsies were performed at D0 (baseline) and at D84 on 26 randomly selected participants. The procedure was performed in the preauricular region close to the capillary insertion. After applying local anesthetics, a sample was collected using a 3-mm punch. The D84 biopsy was performed on the contralateral side to avoid visible scars.

The obtained samples were sent for analysis by a single dermatopathologist.

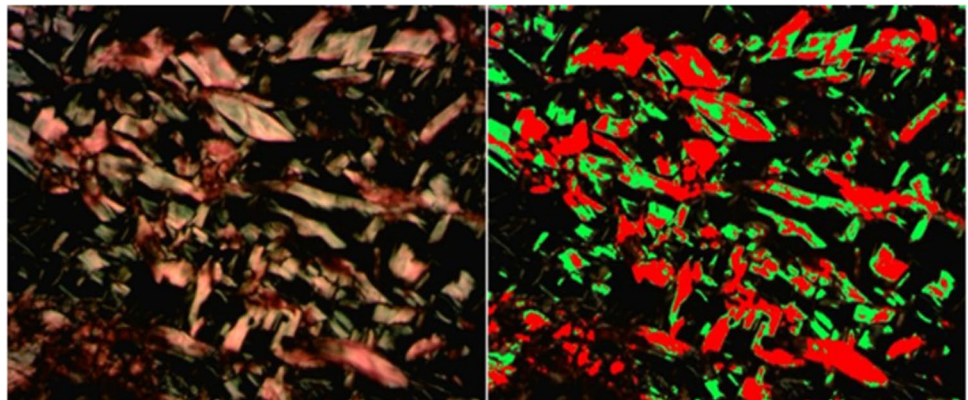
Picrosirius staining was performed (Fig. 1) for the qualitative and quantitative evaluation of collagen fibers at D0 and D84. The material was analyzed under polarized light, which allows the differentiation between type I and III collagen fibers and ensures the differentiation between new and old fibers. Fiber quantification was performed using *Image-Pro Plus*® software.

Weigert staining was also performed for the qualitative and quantitative evaluation of elastic fibers. The material was analyzed under an optical microscope, and images were captured for computerized quantification to differentiate the brown coloration of the elastic fibers and evaluate its prevalence as a percentage.

Statistical methodology

Student and Wilcoxon *t*-tests were applied using R Studio software version 1.1.442 for data analysis.

Fig. 1 Manually programmed mask on computer for reading and quantifying collagen fibers represented by red coloring



Results

The CO₂ laser was applied in a single session to 40 adult female participants aged 35–65 years, with an average age of 49.17 and a standard deviation (d.p.) of 7.48. All subjects fully participated in the study.

After the CO₂ laser session (Fig. 2), mild edema, erythema, and crusting were observed, with much less downtime compared to after high-fluence CO₂ laser. No participants presented any adverse effects.

On D3, patients could return to work using sunscreen and makeup, as edema, erythema, and crusting were barely noticeable. On D5, all participants presented only small areas of dry skin and very little crusting.

In the quantitative clinical analysis of wrinkles performed using the VAS score, it was observed that between D0 and D84, there was a reduction of 32.17% ($p < 0.0001$).

The clinical analysis of the thickness, length, and depth of wrinkles by the VAS showed a similar trend. Between D0 and D84, there was a reduction of 33%, 35.84%, and 32.46%, respectively ($p < 0.0001$).

The clinical results regarding the improvement in skin quality and reduction in the thickness, depth, and length of periorbital wrinkles can be seen in Fig. 3.

The quantitative histological analysis of type I collagen fibers showed a nonsignificant increase between D0 and D84.

For type III collagen fibers, there was a significant quantitative increase of 60.67% between D0 and D84, according to Graph 1 and Fig. 4.

The quantitative histological analysis of elastic fibers showed an inverse result, with a reduction at D84 compared to D0.

Discussion

The exact mechanism of success of the ablative fractional laser remains unknown. It is believed that collagen remodeling is induced by the production of MTZs. The expression



Fig. 2 CO₂ laser postoperative evolution between D0 and D5

of some metalloproteinases increases after CO₂ laser application, thus promoting the removal of old collagen fibers [9].

Thermal injury stimulates the physiological healing response. Type III collagen is the dominant form in wound granulation tissue. After application of the CO₂ laser, there is an increase in the messenger RNA (mRNA) expression of procollagen types I and III, peaking on the 21st day and remaining elevated for up to 6 months. As the healing process progresses, more rigid type I collagen is deposited, while the amount of type III collagen decreases to levels similar to those in healthy skin [10–14].

This justifies the higher concentration of type III collagen fibers in the D84 biopsy in our study. It is expected that type



Fig. 3 Subject clinical developments number 3 between D0 and D84. Photos in normal light and polarized light filter, subsequently

III collagen fibers will later be replaced by mature type I fibers.

On confocal microscopy, both fibers and fibroblasts appeared in a single horizontal and parallel direction after this process. Parallel alignment is suggestive of skin tensile stress. This traction is essential for improving skin sagging and partly justifies the improvement of fine wrinkles [15].

Unlike collagen fibers, elastic fibers had their concentration reduced between D0 and D84. This result corroborates those of other studies, such as that by Moetaz El-Domyati et al., which demonstrated an increase in the synthesis of collagen types I, III, and VII and tropoelastin, which is considered a precursor of elastic fiber, after 6 sessions of 2940-nm Er:YAG laser irradiation. The amount of elastin was reduced after treatment [16].

Quantitatively, the amount of elastin increases with aging. The observation of reduced elastin indicates a reduction in abnormal and old fibers. The paradoxical increase in tropoelastin demonstrates new elastin production and a reduction in elastotic material corresponding to a qualitative improvement in elastic fibers [17].

In our study, we did not quantify tropoelastin, but the quantitative reduction observed may correspond to the

Graph 1 Average type III collagen fibers per area (μM^2) in histopathological analysis through picrosirius staining, comparing the quantitative evolution between D0 AND D84

Percentage of variation from baseline D0

Colagen fibers average % : % of evolution / Baseline											
Group	Time	N	Nmiss	Mean	SD	95% CI	SEM	Min	Max	Median	Q1 ; Q3
Group 1 Serum	D84	24	0	41.51	97.37	[0.39 ; 82.62]	19.88	-58.84	430.36	22.63	-10.32 ; 56.28
Group 2 Vehicule	D84	24	0	64.97	101.20	[22.24 ; 107.70]	20.66	-69.42	297.73	41.39	-10.33 ; 93.63

Bar charts

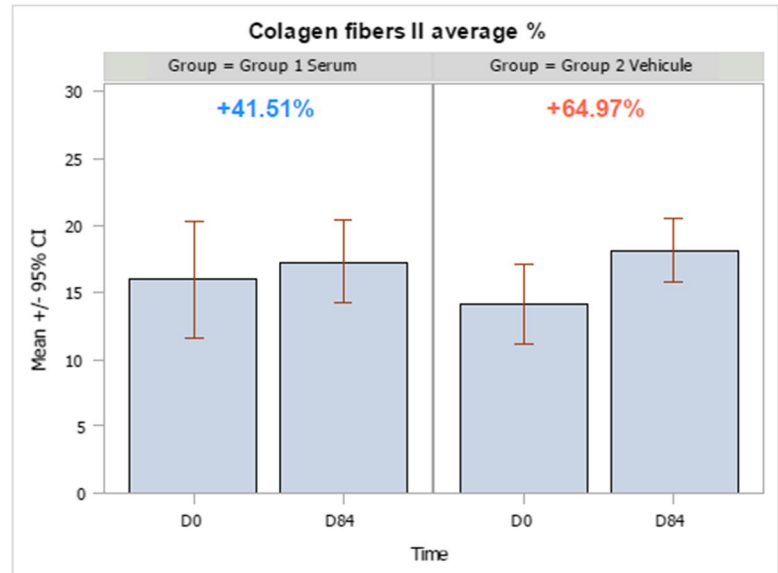
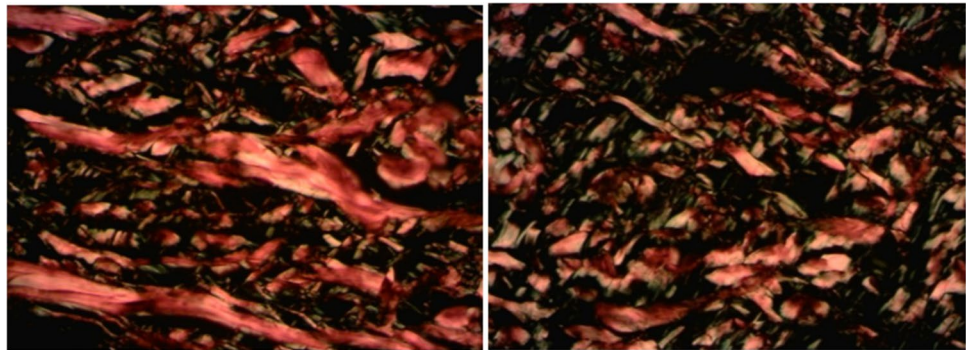


Fig. 4 The left figure represents biopsy in D0, picrosirius staining. There is observation of predominance of thick red collagen fibers, representing prevalence of type I (ripe) collagen fibers. While in the right figure, in D84, a larger prevalence of type III (recent) collagen fibers is observed, with thin thickness and predominance of green coloring, therefore indicating neocolagenesis



replacement of these fibers by their precursor and future new elastic fibers.

There has been much discussion about the optimal parameters to achieve good results safely with as little downtime as possible.

For skin rejuvenation, high-fluence and high-density lasers are usually used. Most professionals believe that only CO₂ lasers with high parameters could yield good results. The major disadvantage of a very aggressive CO₂ laser is its frequent association with adverse effects and, of course, a prolonged postoperative period. The intensity of erythema and delay of complete reepithelialization can lead a patient to remain at home for up to 2 weeks [18, 19].

Currently, the demand for minimally invasive treatments to improve skin quality, texture, and tone is increasing. Patients concerned about their appearance but also unwilling to withdraw from their social or professional activities for posttreatment recovery have often requested effective solutions with minimal downtime.

It is unclear how different parameters act on dermal remodeling. Moreover, there is no way of knowing which depth, coagulated tissue volume, and percentage of skin reached would be ideal for achieving good results [9, 14].

Fluence or energy is undoubtedly the most discussed parameter. Some authors believe that increased fluence leads to increases in the depth and MTZ diameter without compromising the structure or viability

of interlesional tissue. Marqa et al. observed a linear increase in the column depth with fluence elevation between 5 and 40 mJ [20].

Schmitt et al. conducted a high-fluence CO₂ laser study *ex vivo* with histological analysis and PCR to evaluate the molecular effects of the laser. The authors observed a better response with higher fluence, with the upregulation of genes associated with healing and the immune response and the downregulation of metalloproteinase genes [21].

Hantash et al. observed that in cases of constant density, either by the number of strides or by the number of MTZs selected, fluence would determine the intensity of tissue contraction [22]. Another author observed in an experimental study a linear relationship between the ablation depth and the energy used. However, the column diameter and coagulation zone thickness increased only to a fluence of 16 mJ, reaching stability at higher fluences [23].

Some authors observed no statistically significant difference between 3 different CO₂ LFA fluences in ablation depth, coagulation depth, MTZ density, or diameter [2, 5].

Helbig et al. observed that PCT production did not show significant variations among a variety of energies [14].

The density of the shots is also an important variable. It can be increased by selecting the concentration of MTZs or by increasing the number of passes.

In one study, an old scar was treated with different MTZ densities. A better response was observed in areas with higher densities. However, the use of high densities is believed to cause, in addition to longer healing periods, an increased risk of scarring [17].

Ross et al. demonstrated that immediate contraction and residual thermal damage are linearly correlated to the increasing number of passes when using low fluences, thus achieving satisfactory results with conservative parameters [24].

In our study, the combination of low density with low fluence may have allowed rapid recovery without associated adverse effects.

The size of the chosen spot size also varies with the treatment objectives. It is known that by using a smaller spot size, such as 120 µm, it is possible to reach a depth of approximately 2 mm [25].

Due to the more conservative treatment proposal, with reduced downtime, we used a larger and more superficial spot size of 800 µm in our study.

The number of sessions is also debatable. Most authors propose 3–5 sessions to achieve good results. In our study, we applied a single session. However, we believe that increasing the number of sessions would bring progressive and positive effects in terms of rejuvenation.

Conclusions

The application of a low-fluence, low-density ablative CO₂ fractional laser in a single session proved to be safe and efficient for face photorejuvenation. There was a significant reduction in the clinical parameters of wrinkles and an increase in the production of type III collagen fibers on biopsy, indicating the occurrence of neocollagenesis.

Comparative studies of the application of a CO₂ laser with high and conservative parameters are needed with clinical and histological analyses to evaluate the difference in results in wrinkle reduction and collagen and elastic fiber remodeling.

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Declarations

Ethics approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the Bioethics Committee of the Faculdade Evangélica Mackenzie (n° 53058216.6.0000.0103).

Conflict of interest Dr. Jordão, Dr. Melo, Dr. de Campos, and Dr. Skare have stated explicitly that there are no conflicts of interest in connection with this article.

Dr. Fajgenbaum Feiges Stoliar reports personal fees from L'Oréal Brazil, from null, during the conduct of the study, and personal fees from L'Oréal Brazil, from null, outside the submitted work.

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