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Lower energy and pulse stacking. A safer alternative for skin tightening using fractional CO₂ laser¹

Marcos Matias Motta^I, Rafael Fantelli Stelini^{II}, Davi Reis Calderoni^{III}, Rovilson Gilioli^{IV}, Paulo Kharmandayan^V

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^IFellow Master degree, Postgraduate Program in Surgical Sciences, Faculty of Medical Sciences, Universidade de Campinas (UNICAMP), Brazil. Intellectual and scientific content of the study, design the protocol, surgical procedures, acquisition and interpretation of data, statistical analysis, manuscript writing.

^{II}MD, Department of Anatomic Pathology, Faculty of Medical Sciences, UNICAMP, Campinas-SP, Brazil. Intellectual and scientific content of the study, design the protocol, acquisition and interpretation of data, statistical analysis, critical revision.

^{III}PhD, Faculty of Medical Sciences, UNICAMP, Campinas-SP, Brazil. Intellectual and scientific content of the study, design the protocol, acquisition and interpretation of data, statistical analysis, critical revision.

^{IV}PhD, Molecular Biology and Genetics. Head, Multidisciplinary Center for Biological Investigation on Laboratory Animals Science, UNICAMP, Campinas-SP, Brazil. Surgical procedures, acquisition of data.

^VAssociate Professor, Head, Division of Plastic Surgery, Faculty of Medical Sciences, UNICAMP, Campinas-SP, Brazil. Intellectual and scientific content of the study, design the protocol, surgical procedures, interpretation of data, manuscript writing, critical revision.

ABSTRACT

PURPOSE: To evaluate the effect of different energies and stacking in skin shrinkage.

METHODS: Three decreasing settings of a fractional CO₂ laser were applied to the abdomen of Twenty five Wistar rats divided into three groups. Group I (n=5) was histologically evaluated for microthermal zones dimensions. Groups II and III (n=10 each) were macroscopic evaluated with freeware ImageJ for area contraction immediately and after 30 and 60 days.

RESULTS: No statistical significance was found within microthermal zone histological dimensions (Group I) in all settings studied. (Ablation depth: 76.90 to 97.18 μm; Coagulation depth: 186.01 to 219.84 μm). In Group II, macroscopic evaluation showed that all settings cause significant immediate skin contraction. The highest setting cause significant more intense tightening effect initially, contracting skin area from 258.65 to 179.09 mm². The same pattern was observed in Group III. At 30 and 60 days, the lowest setting significantly sustained contraction.

CONCLUSION: Lower fractional CO₂ laser energies associated to pulse stacking could cause consistent and long lasting tissue contraction in rats.

Key words: Lasers, Gas. Skin Aging. Laser Therapy. Rats.

Introduction

Treatment of photo aging with CO₂ laser resurfacing had been considered the gold standard for decades^{1,2}. However, although having very impressive results, it was nearly abandoned by doctors and patients over time due to its prolonged recovery time and high rate of complications. Among these complications stand out hypertrophic scars and dyschromia. With the concept of fractional photothermolysis^{2,3}, treating photo aging with CO₂ fractional laser became popular in recent years due to its potential to deliver consistent results coupled with shorter recovery time and low complication rates.

In fractional mode CO₂ laser creates an array of micro treatment zones in the epidermis and dermis surrounded by healthy tissue. This has been considered to be responsible for the significant shorter recovery time⁴. Histological evaluation showed that an increase in pulse duration results in a proportional increase in lesion depth⁵. Another study demonstrated that a higher penetration into the dermis causes a greater clinical efficacy in skin texture and rhytides⁶.

Complications from this modality are generally mild, temporary and easily managed. A retrospective study evaluating 490 treatments in 374 patients demonstrated an overall incidence of adverse effects of 13.6%⁷. The most frequent were acneiform eruption (5,3%), bacterial infection (2.2%), hyperpigmentation (1.2%) and prolonged erythema (0.8%). Although infrequent and lower than in traditional CO₂, they do exist and should be prevented whenever possible.

A study comparing three different decreasing fluencies for treating photo aging, showed very similar results for the two lower fluencies compared to the highest one, in terms of biological response. Lower fluencies decrease the risk of scarring⁸.

Another way of getting deeper in the dermis is the use of multiple overlapping pulses (stacking)^{9,10}. Few histological studies describe the association between the applied parameters and the dimensions of the microthermal zones (MTZ) as well as the use of stacking for increasing their depth. One experimental study found a linear correlation between the depth of ablation and the energy used¹¹.

A feature of treatment with CO₂ laser is the immediate contraction of the tissue, and in traditional non-fractional CO₂ laser resurfacing this is well known^{12,13}. It was not possible to find in the literature an objective evaluation of the shrinkage of the

skin after application of fractional CO₂ laser, despite this fact is clinically observable.

Besides that, the use of lighter settings to achieve that skin contraction has not been addressed yet. We investigate if it is possible to achieve consistent tissue contraction with lower settings in association with pulse stacking.

Methods

The board of Ethical Committee of Animal Research approved the study, under the protocol number 3012-1.

Twenty-five male Wistar rats aging ten weeks were included in the study. They had free access to water and standard laboratory chow (3.100 kcal/kg) and they were kept on a 12-hour light/dark cycle. The room temperature was kept between 23 and 24°C.

Laser procedure

The animals were anaesthetized with an intra-peritoneal injection of 80mg/kg ketamine plus 10mg/kg xylazine and positioned on dorsal decubitus. Their abdomens were shaved and stamped with four 15 x15 mm squares. Then, the vertices of each square were tattooed for later area identification (Figure 1). In addition, an identification sequential number was tattooed on each animal's tail.

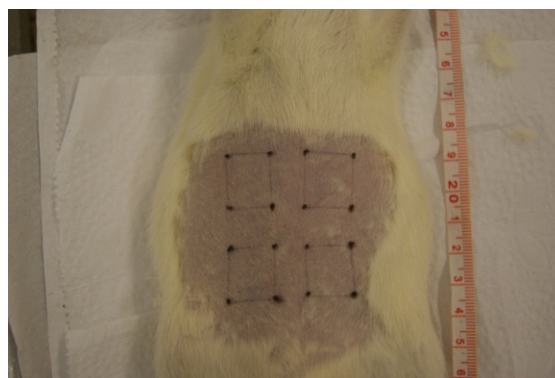


FIGURE 1 – Treatment areas on animal's abdomen.

Each square was assigned a number as shown in Figure 2. Each treatment area (except control area – area 1) underwent single-pass treatment with CO₂ laser equipped with a scanner specially developed for fractional skin resurfacing (Smartxide Dot; DEKA, M.E.L.A., Florence, Italy).

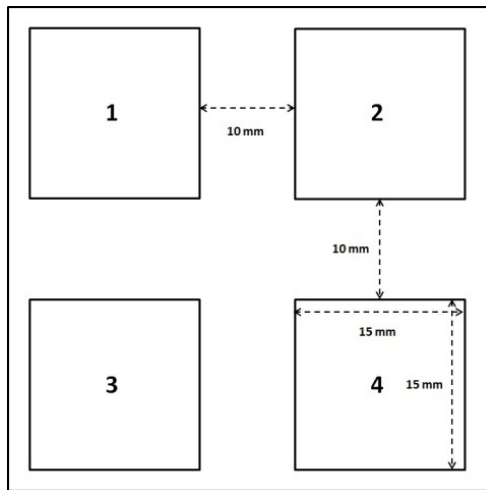


FIGURE 2 - Treatment areas: dimensions and arrangement.

The employed variable laser parameters (power, exposure time, stacking, fluence and energy per each MTZ) are summarized in Table 1. Other parameters were kept the same for all treated areas (500- μ m spacing and 120- μ m spot size).

TABLE 1 - Laser settings for each area.

	Power	Exposure Time	Stack	Fluence	Energy/MTZ
Area 1	-	-	-	-	-
Area 2	30W	900 μ s	1	3.74 J/cm ²	27 mJ
Area 3	20W	300 μ s	3	2.49 J/cm ²	18 mJ
Area 4	10W	300 μ s	3	1.87 J/cm ²	13.5 mJ

Groups for analysis

The twenty-five animals were distributed into three groups.

Group I (n=5) was euthanized by anesthesia overdose immediately after laser treatment. Treatment areas were collected for histological evaluation of laser channel's (MTZ) dimensions.

Group II (n=10) was euthanized 30 days after the procedure, and group III (n=10) was euthanized 60 days after the procedure. The same euthanasia method was used. Both groups II and III were macroscopically analyzed for contraction patterns. In these groups, no MTZ evaluation was carried out because at these time points it is impossible to identify them.

Histological analysis

Tissue specimens from group I animals were fixed in 10% buffered formalin then embedded in paraffin. Then, a vertically cut 5-10 μ m slice was obtained from the center of each treatment area and stained with hematoxylin and eosin (H&E). A calibrated microscope DM500 (Leica Microsystems, Wetzlar, Germany) equipped with a digital camera ICC 50HD (Leica Microsystems, Wetzlar, Germany) was used for image capture. Laser MTZ dimensions were measured using calibrated Leica Application Suite (LAS) Version 4.1.0 (Leica Microsystems, Wetzlar, Germany).

The entire slide was scanned for each sample. The three deepest MTZ found were considered for analysis, as they should approximately represent the MTZ central axis. Each of these three MTZ were recorded and for each one the following parameters were quantified: ablation depth, coagulation depth and coagulation width (measured at the surface).

Following these linear measurements, also a volume estimation of MTZ using a cone shape model previously described by Skovbølling *et al.*¹⁰, was calculated.

Macroscopic analysis

Standardized color photographs using a digital camera Alpha 230 (Sony) were obtained from all animals from groups II and III immediately before and after laser treatment as well as before the animal's sacrifice. Image analysis was carried out with freeware ImageJ (National Institutes of Health, USA. <http://www.imagej.nih.gov/ij>) for area calculation of each square.

Treatment areas from groups II and III were statistically analyzed in two distinct ways: evaluating each area's behavior along time points and evaluating all areas together at each time point compared to the control area.

Statistics

Origin[®] software was used for statistical analysis. In order to compare the evaluated parameters it was used ANOVA for repeated measures. Variables studied were ablation depth, coagulation depth, surface damage width, MTZ volume and area of laser application. They were converted into ranks due to lack of normal distribution. The level of significance used in this study was 5%.

Results

Group I – Histological analysis of MTZ

Ablation depth

It is shown in Table 2. We found no statistical significance between areas.

TABLE 2 – Ablation depth results for each region.

Region	Settings	Ablation Depth - Median	Range
1	No treatment	-	-
2	27 mJ – single pulse of 900 µs	89.08 µm	56-98 µm
3	18 mJ – 3 stacked pulses of 300 µs	97.18 µm	80-118 µm
4	13.5 mJ- 3 stacked pulses of 300 µs	76.90 µm	75-120 µm

Coagulation depth

A coagulation zone surrounded the ablation area, and its depth showed a decreasing pattern as the energy was reduced as shown in Table 3. No statistical significance was found.

TABLE 3 – Coagulation depth results for each region.

Region	Settings	Coagulation Depth - Median	Range
1	No treatment	-	-
2	27 mJ – single pulse of 900 µs	219.84 µm	127-241 µm
3	18 mJ – 3 stacked pulses of 300 µs	210.47 µm	166-274 µm
4	13.5 mJ- 3 stacked pulses of 300 µs	186.01 µm	185-219 µm

Surface damage width

This included ablation width plus the surrounding coagulation zone at the epidermis. Results are demonstrated in Table 4. No level of significance was found between areas.

TABLE 4 – Surface damage width results for each region.

Region	Settings	Surface damage width - Median	Range
1	No treatment	-	-
2	27 mJ – single pulse of 900 µs	412,33 µm	305-450 µm
3	18 mJ – 3 stacked pulses of 300 µs	430.70 µm	273-581 µm
4	13.5 mJ- 3 stacked pulses of 300 µs	406.14 µm	372-454 µm

MTZ volume

We calculated MTZ volume using the cone volume formula (cone shape model) and the results are shown in Table 5. Again, there was no statistical significance.

TABLE 5 – Microthermal zones (MTZ) Volume for each region.

Region	Settings	MTZ volume - Median	Range
1	No treatment	-	-
2	27 mJ – single pulse of 900 µs	8.48 x 10 ⁻³ mm ³	4-13 x 10 ⁻³ mm ³
3	18 mJ – 3 stacked pulses of 300 µs	11.2 x 10 ⁻³ mm ³	7-14 x 10 ⁻³ mm ³
4	13.5 mJ- 3 stacked pulses of 300 µs	8.62 x 10 ⁻³ mm ³	6-10 x 10 ⁻³ mm ³

Group II – Macroscopic analysis – 30 days

Before treatment, there was no statistically difference between all areas.

When following each area along time points, it was observed that immediately after treatment all areas except the control (area 1) were significantly smaller than before laser shot. Thirty days after the procedure, area 1 (control) was larger than it was before the experiment (p=0.001). Areas 2 and 3 showed no significant difference to pre-treatment dimensions. On the other hand, area 4 was smaller than before treatment (p=0.029) (Figure 3).

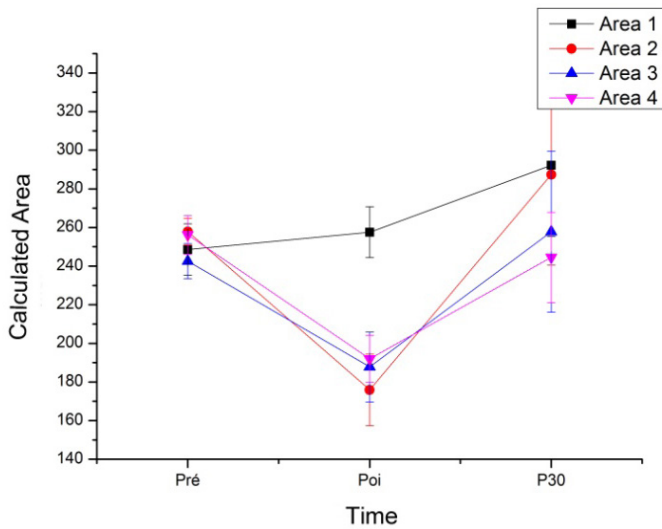


FIGURE 3 - Group II: Linear dimensions x Time points.

Analyzing areas together at each time point demonstrated that immediately after laser, all treatment areas were significantly smaller than the control area. In addition, area 2 was smaller than area 3 ($p=0.029$) and area 4 ($p=0.017$). After thirty days, area 4 was significantly smaller than area 1 ($p=0.0001$) and area 2 ($p=0.004$), and was the only area that remained smaller than the observed in the pre-treatment period (Table 6).

TABLE 6 – Calculated linear dimensions for Group II.

Region	Pre-Treatment	Immediately after	30 days after
1	249.58 mm ²	262.87 mm ²	291.99 mm ²
2	258.65 mm ²	179.09 mm ²	296.92 mm ²
3	242.92 mm ²	179.89 mm ²	254.83 mm ²
4	258.05 mm ²	190.81 mm ²	243.49 mm ²

Another way of evaluating the contraction effect, was measuring the variation in size of each area from immediately post treatment to day 30.

In group II we observed that after being treated with the laser all areas had a positive variation, that is, all areas increased in size after thirty days. However, area 2 had a significant higher increase than area 1 ($p<0.0001$), area 3 ($p=0.005$) and area 4 ($p<0.0001$) (Figure 4).

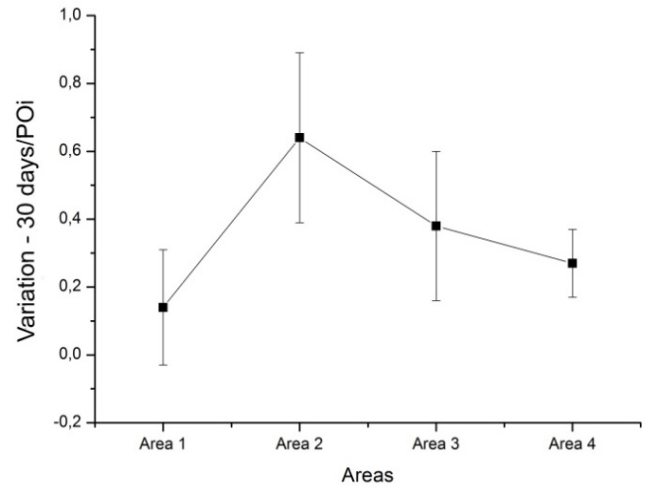


FIGURE 4 – Linear dimensions variation from immediately after laser to 30 days after.

Group III - Macroscopic analysis – 60 days

Treatment areas were statistically analyzed the same way as group II.

When following each area along time points, it was observed that immediately after treatment all areas except the control (area 1) were significantly smaller. Sixty days after the procedure, control area ($p=0.0001$), area 2 ($p=0.003$) and area 3 ($p=0.025$) were larger than they were before the experiment. On the other hand, area 4 was still smaller than it was before treatment, but without statistical significance. We can observe this behavior in Figure 5.

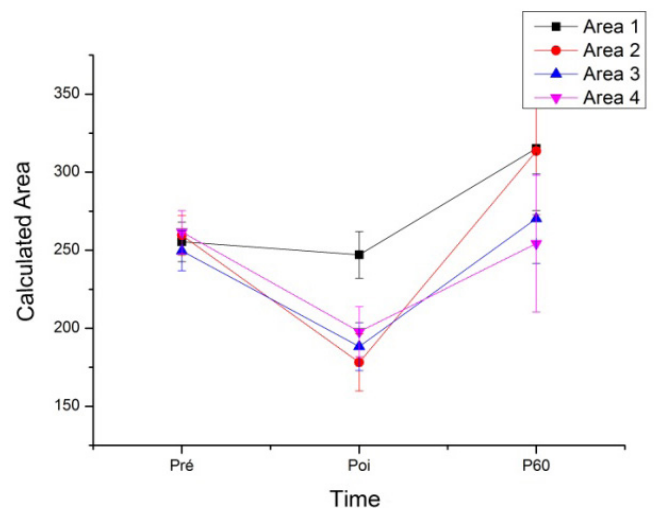


FIGURE 5 – Group III: Linear dimensions x Time points.

Analyzing areas together at each time point demonstrated that immediately after laser, all treatment areas were significantly smaller than the control area. In addition, area 2 was significantly smaller than area 4 (p=0.001). After sixty days, area 4 was significantly smaller than area 1 (p=0.030) and area 2 (p=0.008) (Table 7).

TABLE 7 – Calculated linear dimensions for Group III.

Region	Pre-Treatment	Immediately after	60 days after
1	256.63 mm ²	246.05 mm ²	323.52 mm ²
2	256.95 mm ²	179.61 mm ²	307.75 mm ²
3	247.59 mm ²	191.17 mm ²	267.78 mm ²
4	261.18 mm ²	198.19 mm ²	256.43 mm ²

Measuring the variation in size of each area from immediately post treatment to day 60 showed that area 2 had a significantly greater variation than area 1 (p<0.0001) and area 4 (p=0.001). Its variation was also greater than area 3, but without statistical significance. In addition, area 4 was not significantly different from the control (area 1). We can observe these results in Figure 6.

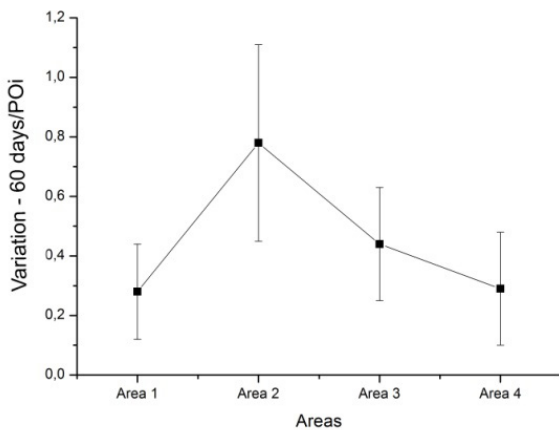


FIGURE 6 - Linear dimensions variation from immediately after laser to 60 days after.

Discussion

The pursuit of good results in treating photo aging and other disorders with fractional CO₂ laser has been studied widely in recent years¹⁴⁻¹⁶. Since the papers of Hantash *et al.*^{1,2} which demonstrated fractional photothermolysis concept applied to the

CO₂ laser, this modality has virtually replaced traditional CO₂ laser resurfacing. Another study¹⁷ demonstrated that molecular pathways, in both traditional and fractional CO₂ laser wound healing, are very similar. Since that, it was possible to treat photo aging without a high incidence of dyschromia and hypertrophic scarring.

In the fractional modality, an array of microthermal zones (MTZ) is created within the epidermis and dermis. These MTZ's consist of a central ablation area surrounded by a thin annular area of coagulated tissue. Ross *et al.*¹⁸, although studying traditional CO₂ laser, had already discussed the importance of coagulation zone in tissue contraction. In between each MTZ there is healthy tissue, which is said to be responsible for the faster recovery time⁴.

This column pattern allows fractional CO₂ laser to reach deeper areas into the dermis than traditional CO₂ laser. Saluja *et al.*⁶ demonstrated that a deeper coagulation results in better clinical efficacy in skin texture and rhytides. Also, a persistent collagen remodeling for at least three months after treatment was immunohistochemically demonstrated in humans when deep dermis was reached². Another study⁵, demonstrated in ten patients that lesion depth was proportional to increases in pulse duration.

Increasing depth is also possible by means of overlapping pulses at the same site (stacking). Oni *et al.*¹⁰ demonstrated that a 15mJ double pulse resulted in similar MTZ as a single 30mJ pulse. In this article, they also suggest that double pulsing could result in improved outcomes, as the amount of unaffected tissue between MTZ is reduced. This would increase dermal injury leading to increased collagen synthesis.

Few histological studies describe the relationship between laser settings and MTZ dimensions, as well as the use of stacked pulses. Besides that, there are many different devices, which are difficult to compare. One study used an ex vivo porcine model to characterize laser columns at different energy settings with a constant pulse length in single and stacked pulses¹¹. In this study, they found a linear correlation between ablation depth and energy. Column diameter was not related to energy levels, and coagulation zone thickness increased until 16mJ only.

In our study, we found no significant difference between the three settings studied in terms of ablation depth, coagulation depth, superficial tissue damage, and calculated cone-shaped MTZ volume. These findings are very interesting especially when thinking about the MTZ calculated volume. This represents the amount of tissue that is actually injured by the laser and will lead to collagen synthesis¹⁰. After reducing the power in 33,3% (from area two to area three) and splitting the 900µs exposure time

into three 300 μ s stacked pulses (resulting in a 33.3% decrease in fluence and energy), we found a 32% increase on the MTZ volume. Even when power was reduced by 66.6% (from area two to area four) and the 900 μ s exposure time was again split into three 300 μ s stacked pulses (which resulted in a 50% reduction in fluence and energy) the results showed an increase of 1% on MTZ volume.

Although no statistical significance was found, these results could indicate that is possible to achieve similar amounts of tissue damage into the dermis with less aggressive power settings and with the use of pulse stacking. A technical limitation affecting these data is the difficulty in obtaining the MTZ real central axis on histology^{1,11}. Even a minor angling during technical procedures may result in a non-real central axis. Zelickson *et al.*¹⁹ proposed a computational method for calculating MTZ depth based on horizontal sectioning. On their work, they found slightly larger MTZ comparing to traditional vertical sectioning. We tried to minimize these limitations by scanning every MTZ on each slide for the three deepest ones and averaging them.

Another point that interferes in MTZ dimension is hair distribution on the animal's abdomen. On histology we identified different patterns of skin appendages and dermis thickness comparing the upper skin abdominal specimens (areas one and two) with the lower ones (areas three and four). This could partially explain the fact that area two, although receiving the highest power with a single pulse, showed the smallest MTZ values. On their work, Oni *et al.*¹⁰ described in humans that a higher skin content of hair follicles, as well as blood vessels and sebaceous glands, absorbs part of the laser energy. The same study¹⁰ also propose that using double pulsing the ablative column could continue beyond these structures due to less absorption within them. In single pulse modality the ablative column is usually terminated when there is an appendage in its path.

Another study comparing facial and abdominal skin²⁰ found that MTZ were shallower in the face due to the higher water content of skin appendages. In our study, areas receiving stacked pulses (areas three and four) showed similar ablation depth values to area two, which received a single pulse although with a higher power.

Tissue contraction after traditional CO₂ laser resurfacing is well known. Ross *et al* demonstrated in an experimental study, with traditional CO₂ laser resurfacing, that immediate contraction and residual thermal damage have a linear correlation to increasingly number of passes when using lower fluences¹².

In fractional CO₂ laser resurfacing, this tissue shrinkage also occurs and is related to the radial micro-contraction of multiple MTZs. This was observed both in ex-vivo and in-vivo studies^{1,2}.

Our study evaluated tissue contraction after fractional CO₂ laser both immediately after and at thirty and sixty days post procedure. To accomplish this purpose we used freeware ImageJ for area calculation, which has been successfully used in previous studies^{21,22}.

Immediately after laser treatment we observed that area two, which received higher fluence and energy within a single pulse, contracted significantly more than areas three and four in group II and significantly more than area four in group III. This is in agreement with Hantash *et al.*¹ who in their first study on characterization of a fractional CO₂ laser device, they hypothesized that with a constant treatment density, pulse energy would predict tissue shrinkage. The same correlation between higher energies and immediate tissue tightening was found in clinical studies^{5,23}.

On the other hand, when examining tightening effect at days thirty and sixty we noticed that although the animals grew, areas three and four were smaller than area two. That is, although showing a greater tightening effect just after treatment, the area receiving the highest energy apparently lost most of that effect after thirty to sixty days. In fact, this area approximated area one dimensions, which received no treatment. We hypothesized that the higher energy extrapolated the modulating and suppressing balance in wound healing response as suggested by Yuan *et al.*²⁴. Thus, instead of stimulating neo-collagenogenesis and tissue contraction, higher energies would impair it.

This objective experimental analysis suggests that it is not necessary to use higher energies to obtain consistent skin tightening. Some clinical studies had similar results. A randomized split-face trial in Asians²⁴ treating acne scars, showed no difference in clinical outcomes after using 10 mJ or 20mJ at fixed densities. Prignano *et al.*⁸ in a clinical study using the same equipment as the one used in the present study, found that there is no advantage of using a higher fluence. They compared fluences at 2.07, 2.77 and 4.14 J/cm² and both histological and clinical data demonstrated the lower fluences had very similar responses. With the fluence at 4.14 J/cm², besides the lack of clinical advantage, histology demonstrated a larger amount of collagen fibers, which could represent more risks of scarring. In this way, it may possible to improve clinical results with lower energy and the use of pulse stacking.

Conclusion

Lower fractional CO₂ laser energies associated to pulse stacking could cause consistent and long lasting skin shrinkage in rats.

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Correspondence:

Prof. Dr. Paulo Kharmandayan
Rua Tessália Vieira de Camargo, 126
Cidade Universitária "Zeferino Vaz"
Caixa Postal: 6111 13083-887 Campinas - SP Brasil
Tels.: (55 11)95020-2080 / (55 19)3521-7092
Fax: (55 19)3521-8043
pkharmand@hotmail.com

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