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Semi-Quantitative Histological Analysis of the Effect of Intense Pulsed Light (IPL) and Carbon Dioxide (CO_2) Intradermic Injection on Fibroblast and Collagen Proliferation in the Skin of Wistar Rats

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ABSTRACT

Background: In recent years, so-called "non-ablative rejuvenation" has been carried out with the use of lasers or intense pulsed light (IPL) to stimulate collagen production by dermal fibroblasts. Intradermal infusion of CO_2 stimulates fibroblasts and the synthesis of collagen and elastin, contributing to the retraction of the skin and tissue rejuvenation. **Objectives:** To evaluate the effects of IPL and the intradermal infusion of CO_2 on fibroblast proliferation and collagen in the skin of female rats. **Methods:** Sixteen adult female Wistar rats were divided into two groups of eight animals. Group 1 underwent IPL and group 2 underwent intradermal CO_2 infusion. There was a total of 8 weeks of treatment. We conducted a punch in each animal before any procedure (T0), another punch in the middle of treatment at 4 weeks post-procedure (T1) and a punch at the end of treatment at 8 weeks post-procedure (T2). The cells involved in inflammation, fibrosis and vascularization of the injured tissue by histopathology were analyzed. **Results:** There was statistically significant fibroblast proliferation and collagen proliferation noted when analyzing all 16 animals together and also when considering the two study groups separately. In both groups, the greatest proliferation of fibroblasts coincided with periods of increased collagen production. **Conclusion:** Both IPL and intradermal CO_2 infusion stimulated fibroblast and collagen proliferation in the skin of the rats studied.

Keywords: Collagen; Cosmetic Dermatology; Rejuvenation; Pulsed Light; Intense Pulsed Light; Intradermal Injection

1. Introduction

Collagen is the most abundant protein in the human body and is synthesized by several cell types, including fibroblasts. As people age, there is a reduction of the carrying capacity of the skin and subsequent atrophy and loss of elasticity secondary to the reduction in collagen production [1,2]. Various proposed techniques for the rejuvenation of skin involve increasing the proliferation of collagen via laser-based methods, pulsed light and intradermal injection of CO_2 [3,4].

Intense pulsed light (IPL) produces a non-coherent light beam that has a radiation spectrum that covers many wavelengths simultaneously. A polychromatic beam is captured by different chromophores associated with different wavelengths or colors. This, together with the other aspects mentioned, increases the therapeutic spectrum covered by IPL [5-7]. The non-ablative photorejuvenation with intense pulsed light works causing reversible thermal damage of collagen by light penetration into the dermis and direct heating of these structures, sparing the epidermis [5-7]. Thus, the contraction of collagen fibers and proper remodeling of the fibers after the inflammatory period is obtained. A polychromatic beam is captured by different chromophores associated with different wavelengths or colors. This, together with the other aspects mentioned, increases the therapeutic spectrum covered by IPL [8].

In recent years, so-called "non-ablative rejuvenation" involving the use of lasers or (IPL) to promote stimulation of collagen production by dermal fibroblasts has been increasingly conducted. The treatments typically are repeated monthly, and at least four sessions are necessary

to obtain efficient results [4,5,8].

Carbon dioxide (CO_2) treatment involves the subcutaneous or transcutaneous infusion of CO_2 to induce therapeutic effects that benefit microcirculation and tissue oxygenation [9,10]. Studies have demonstrated the beneficial effects of subcutaneous carbon dioxide therapy for various medical conditions, including functional disorders of blood flow, peripheral artery disease, microcirculatory disorders, delayed healing, multiple symmetric lipomatosis, cellulite and adiposity [2,9-12].

There are no published reports of adverse effects or complications associated with either local or systemic intradermal and subcutaneous CO_2 infusion [11]. The possible side effects are limited to low-intensity pain during application, small bruises of the punch and a local crackling sensation [9]. Studies using carbon dioxide for contrast angiography attest to the safety of this gas and have shown that it is not likely to promote clot. CO_2 can be used with intravascular bolus injections of up to 100 ml and continuous flows between 20 and 30 ml/second without adverse reactions [13-15].

When applied to the skin surface layer, carbon dioxide stimulates fibroblast synthesis of collagen and elastin, contributing to the retraction of the skin and resulting in the rejuvenation and the reduction of tissue laxity [9,10, 16,17].

A review of the literature concerning IPL and intradermal CO_2 injection as applied to fibroblast and collagen proliferation indicates existing gaps in knowledge about this subject [2]. After consideration of the relevance of the study of such techniques for the rejuvenation, prevention and healing of skin aging, it is obvious that additional research in this area is imperative.

It is possible to experimentally evaluate the histological effects of intense pulsed light and the intradermal injection of CO_2 into skin [2,4,5,8]. Therefore, in the present study, our aim was to evaluate the effect of IPL and intradermic CO_2 injection on fibroblast proliferation and collagen in the skin of female Wistar rats.

2. Methods

This study was an experimental, comparative, non-controlled trial that provides a skin semi-quantitative histological analysis of 16 adult female Wistar rats with a body mass ranging between 160 and 200 grams. The animals received food and water *ad libitum* before and during the experimental period and underwent alternating cycles of 12 h light and 12 h darkness.

The animals were kept in collective cages with rectangular dimensions of 49 × 34 × 16 cm (length × width × height) with eight animals in each. The environment was quiet, and the temperature was maintained between 21˚C and 25˚C, according with guidelines for the use of laboratory animals [18,19].

The rats were divided into two groups, each consisting of 8 animals, as described below:

- Group 1 (G1): underwent to intense pulsed light (IPL);
- Group 2 (G2): underwent intradermal injection of CO_2.

Before any procedures, the animals were anesthetized with 10% ketamine injectable solution at a dose of 10 mg/kg associated with a 1 mg/kg intramuscular xylazine dose [18-21]. All animals were anesthetized and shaved on the dorsal region to create a "punch" of approximately 6 mm in diameter to remove a circular piece of skin in the left inferior-lateral portion of the back for histological study. The skin piece was standardized as a control at time zero (T0) that corresponded to intact skin from pretreatment and without intervention. These (T0) biopsies were also called as controls non-treated skins.

After a week of completing the initial (T0) punch, the animals were subjected to treatment according to the group divisions:

- G1 (IPL): Eight animals in this group were treated with intense pulsed light at a wavelength of 550 to 900 nanometers in the average pulse. Each animal was submitted to six passages. The light was applied to the right supero-lateral region of the animal's back just after shaving. The treatment was applied once every two weeks for eight weeks;
- G2 (CO_2): The eight animals in this group were treated with an intradermal CO_2 injection at a flow of 80 ml/min. The total volume infused by the application of CO_2, in milliliters, was limited to the formula weight of the animal (in kg) × 5. The treatment was applied in the right supero-lateral region of the animal's back just after shaving once a week for eight weeks.

After the fourth week of treatment, the animals underwent a new punch of approximately 6 mm in diameter to remove a circular piece of skin from the intervention site (right superolateral portion of the back of the animal). The piece was used for the histological analysis of the middle portion of the treatment (T1).

At the end of eight weeks of treatment, the animals underwent a new punch of approximately 6 mm in diameter to remove circular piece of skin from the intervention site (right superolateral portion of the back of the animal) for the end treatment (T2) histological analysis.

After eight weeks of treatment and the acquisition of all of the punches for histological analysis, the animals were euthanized by chemical methods (overdose of pentobarbital) [18-21].

The specimens were fixed in 10% buffered formalin

for 24 hours. For the routine histopathology techniques, the specimens were embedded in paraffin and 4 μm thick sections were taken with a rotating microtome. We analyzed the cells involved in inflammation, fibrosis and the vascularity of the injured tissue histopathologically. For the analysis of inflammatory cells, we used universal histochemical staining (hematoxylin-eosin). For the analysis of fibrosis, a Masson's trichrome stain was used.

To assess fibroblast and collagen proliferation, a semi-quantitative analysis was carried out estimating the amount of newly formed collagen and fibroblasts in an organized fashion as seen on the blades. The slides were classified into four grades (G0, GI, GII and GIII) according to the percentage of the slide on which new fibroblasts and organized collagen [22] had formed (**Table 1**). The semi-quantitative analysis was proposed in accordance with the classic technique of quantitative morphometry [22-27].

The slides were coded so as to not identify the treatment groups or time of biopsy. A pathologist evaluated the slides at random, not knowing the groups or the corresponding treatment times. The fibroblast and collagen proliferation ratings were recorded in a spreadsheet, along with the identity of the blade used, and were then subsequently decoded.

The data is presented as absolute values, percentages and proportions. A nonparametric analysis of variance test (Kruskal-Wallis) was used to compare the study groups. When the test indicated a significant difference, we used a Dunn post-test to compare the two groups. As a measure of accuracy, 95% confidence intervals were employed. A $p < 0.05$ was considered significant. All statistical analysis was performed using the GraphPad Prism, version 5.0.1 statistical software package (GraphPad Software, San Diego, CA, USA). This study was approved by the Ethics Committee on Animal Use, protocol 241/2009.

3. Results

In the eight animals subjected to IPL treatment, when fibroblast proliferation between the times of onset (T0), the middle (T1) and the end (T2) of treatment was compared, it initially appeared that non-treated skin exhibited a grade 0 classification in 100% of the animals. By the middle of treatment (T1), five of the eight animals (62.5%) presented grade II and III fibroblast proliferation, and at the end of treatment (T2), six of the eight (75%) of the animals presented grade I and II proliferation. This evolution of the histological proliferation of fibroblasts was statistically significant when the beginning and the end of treatment were compared (T0 vs. T2, $p < 0.05$, **Table 2**).

For the non-treated skin controls, grade 0 fibroblast proliferation was also observed in 100% of the animals that received the intradermal injections of CO_2. After 4 weeks of treatment, all animals in this group presented no changes in histological grade, as they maintained 100% presentation of grade 0 fibroblast proliferation. However, by the end of treatment (T2), all animals (100%) exhibited grade I and II fibroblast proliferation. The evolution of histological fibroblast proliferation was also statistically significant in the CO_2 group when the beginning and the end of treatment were compared (T0 vs. T2, $p < 0.001$, **Table 2**).

In regards to collagen proliferation, both study groups exhibited grade 0 proliferation in non-treated control skin at time zero in 100% of the samples. By the middle of treatment (T1), 87.5% (7/8) of the animals in the IPL group exhibited proliferation of grade I and grade II collagen, and 87.5% (7/8) the CO_2 group animals exhibited histological grade I collagen proliferation. At the end of treatment (T2), in the intense pulsed light-treated animals, 62.5% (5/8) exhibited grade II and III proliferation, and 25% (2/8) exhibited grade I collagen proliferation. In the CO_2 group, 50% (4/8) exhibited grade I proliferation and 50% (4/8) grade II collagen proliferation after the treatment (**Table 2**).

There was a statistically significant difference in the degree of collagen proliferation between the time points, both for the animals in the light-pulse treated group (T0 vs. T2, $p < 0.001$) and the animals in the CO_2 group (T0 vs. T2, $p < 0.001$). These data are summarized in **Table 2**.

When all 16 animals were analyzed together (G1 and G2), there was a statistically significance different in fibroblast and collagen proliferation when comparing the beginning and the end of treatment (T0 vs. T2, $p < 0.001$),

Table 1. Classifications for the semi-quantitative analysis of fibroblast and collagen proliferation.

Grade	Fibroblastic proliferation (% of blade)	Collagen proliferation (% of blade)
Grade 0	0% to 5%	0% to 5%
Grade I	5% to 25%	5% to 25%
Grade II	25% to 50%	25% to 50%
Grade III	More than 50%	More than 50%

Table 2. Expression of fibroblast proliferation and collagen proliferation according to the period analyzed and separated by study group.

	Time 0: T0 (Onset treatment)				Time 1: T1 (4 weeks of treatment)				Time 2: T2 (8 weeks of treatment)			
	Grade 0 0% - 5%	Grade I 5% - 25%	Grade II 25% - 50%	Grade III >50%	Grade 0 0% - 5%	Grade I 5% - 25%	Grade II 25% - 50%	Grade III >50%	Grade 0 0% - 5%	Grade I 5% - 25%	Grade II 25% - 50%	Grade III >50%
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
Intense pulsed light (G1) (N = 8 animals)												
Fibroblastic proliferation	8 (100%)	-	-	-	3 (37.5%)	2 (25%)	-	3 (37.5%)	2 (25%)	3 (37.5%)	3 (37.5%)	-
Collagen proliferation	8 (100%)	-	-	-	1 (12.5%)	5 (62.5%)	2 (25%)	-	1 (12.5%)	2 (25%)	3 (37.5%)	2 (25%)
CO_2 Intradermic Injection (G2) (N = 8 animals)												
Fibroblastic proliferation	8 (100%)	-	-	-	8 (100%)	-	-	-	-	5 (62.5%)	3 (37.5%)	-
Collagen proliferation	8 (100%)	-	-	-	1 (12.5%)	7 (87.5%)	-	-	-	4 (50%)	4 (50%)	-

Comparisons using the Kruskal-Wallis with Dunn post-test:

G1: Pulsed light (fibroblastic proliferation)		
T0 vs. T1 $p < 0.05$	G2: CO_2 (fibroblastic proliferation)	T0 vs. T1 $p > 0.05$
	T0 vs. T2 $p < 0.05$	**T0 vs. T2 $p < 0.001$**
	T1 vs.T2 $p > 0.05$	T1 vs. T2 $p < 0.001$
G1: Pulsed light (collagen proliferation)		
T0 vs. T1 $p < 0.05$	G2: CO_2 (Collagen proliferation)	T0 vs. T1 $p < 0.05$
	T0 vs. T2 $p < 0.001$	**T0 vs. T2 $p < 0.001$**
	T1 vs. T2 $p > 0.05$	T1 vs. T2 $p > 0.05$

as shown in **Table 3**. **Figures 1 - 4** illustrate the proliferation of collagen at different time points (T0, T1 and T2) in the IPL group animals and the CO_2 group animals.

The **Figures 4-7** demonstrate the evaluation of collagen in group intense pulsed light (G1) and CO_2 (G2) at three time points analyzed: onset (T0), middle (T1) and final (T2) treatment.

4. Discussion

Intense pulsed light (IPL) is a source of light energy that has many applications. IPL is composed of different wavelength, *i.e.*, all or part of the light spectrum, while the laser has a single wavelength [3]. IPL reaches the skin surface and allows, through the principle of selective photothermolysis, the correction of various skin lesions and facial blemishes resulting from photoaging, as well as stains and pigmentation issues [5].

As IPL is defined by not being composed of coherent light, it can interact with a variety of chromophores, and its energy can, therefore, be more quickly dissipated. Thus, IPL heating is more superficial compared to similar use of lasers. The difference provides better security and safety, especially in most advanced skin types [28].

A recent study evaluated the action of IPL on stimulating the proliferation of collagen in human skin damaged by sun and concluded that, in addition to collagen deposition, the clinical improvement observed after treatment may be secondary to the reduction of perifolicullar inflammatory infiltrate [4,8]. Other reports indicate some degree of the appearance of newly formed collagen in the upper dermis after IPL treatment, thus suggesting the possibility that stimulated dermal fibroblasts are the source of this increased collagen expression [6,29].

The results of the present study indicate that eight weeks after the application of intense pulsed light once every other week, there were significant differences on fibroblast proliferation and collagen in the skin of the animals studied. The moments of the greatest prolifera

Table 3. Expression of fibroblast proliferation and collagen proliferation according to the period analyzed and separated by study group.

	Time 0: T0 (onset treatment)				Time 1: T1 (4 weeks treatment)				Time 2: T2 (8 weeks treatment)			
	Grade 0 0% - 5%	**Grade I 5% - 25%**	**Grade II 25% - 50%**	**Grade III >50%**	**Grade 0 0% - 5%**	**Grade I 5% - 25%**	**Grade II 25% - 50%**	**Grade III >50%**	**Grade 0 0% - 5%**	**Grade I 5% - 25%**	**Grade II 25% - 50%**	**Grade III >50%**
	N (%)	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**
Intense pulsed light (G1) + CO_2 intradermic injection (G2) (N = 16 animals)												
Fibroblastic proliferation	16 (100%)	-	-	-	11 (68.75%)	2 (12.5%)	-	3 (18.75%)	2 (12.5%)	8 (50%)	6 (37.5%)	-
Collagen proliferation	16 (100%)	-	-	-	2 (12.5%)	12 (75%)	2 (12.5%)	-	1 (6.25%)	6 (37.5%)	7 (43.75%)	2 (12.5%)

Comparisons using the Kruskal-Wallis with Dunn post-test:

Fibroblastic proliferation
T0 vs. T1 $p > 0.05$
T0 vs. T2 $p < 0.001$
T1 vs. T2 $p < 0.05$
Collagen proliferation
T0 vs. T1 $p < 0.001$
T0 vs. T2 $p < 0.001$
T1 vs. T2 $p > 0.05$

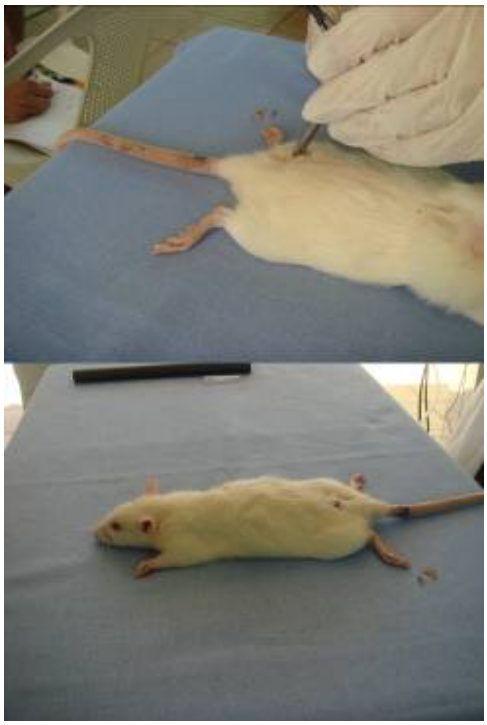

Figure 1. Experimental procedures on Wistar Rats. A "punch" of approximately 6 mm in diameter to remove a circular piece of skin in the left inferior-lateral portion of the back, designed as (T0) that corresponded to intact skin from pre-treatment and without intervention.

Figure 2. Experimental procedures on Wistar Rats. The CO_2 intradermal infusion treatment was applied in the right supero-lateral region of the animal's back just after shaving once a week for eight weeks.

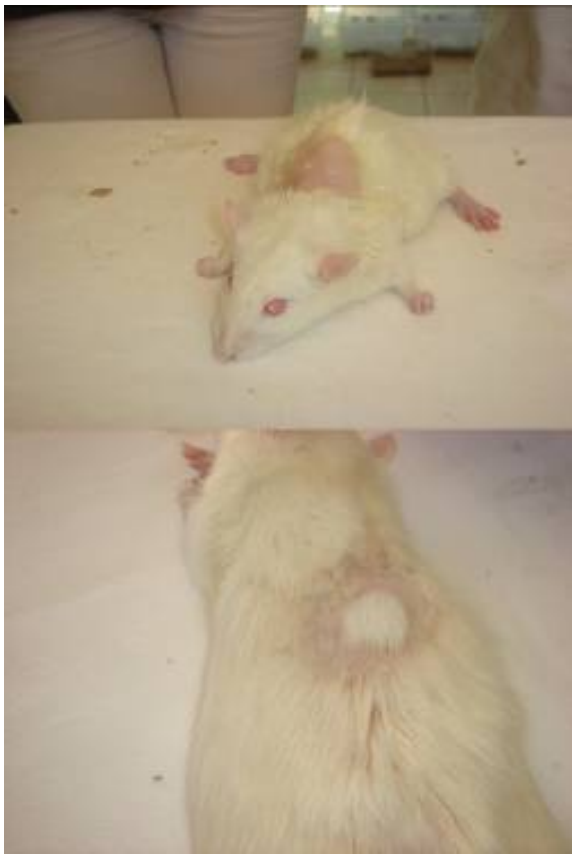

Figure 3. Experimental procedures on Wistar Rats. Each animal was submitted to six passages of IPL. The light was applied to the right supero-lateral region of the animal's back just after shaving. The treatment was applied once every two weeks for eight weeks. The bottom image shows the final aspect of the treated skin just before the sacrifice.

tion of fibroblasts coincide with the periods of increased collagen production. These results support the hypothesis that the source of the increased expression of collagen is the stimulation of dermal fibroblasts, probably due to the principle of selective photothermolysis. The concept underlying the principle is that the absorption of light by water causes a photothermal effect and a consequent inflammatory response that stimulates the fibroblastic activity [3].

With respect to the intradermal injection of CO_2, the hypothesis of action is that carbon dioxide, when applied subcutaneously, results in the mechanical destruction of fat cells. In addition, the CO_2 promotes local vasodilatation and a subsequent increase in tissue oxygenation and, when applied in the most superficial layer of the skin, will stimulate fibroblasts and the synthesis of elastin and collagen. These processes contribute to the retraction ofthe skin and result in skin tissue rejuvenation and a reduction in sagging [2,9-12].

The easily recognized skin undergoes changes with advancing age, as the appearance of furrows, atrophy, ptosis and laxity alters its appearance. Changes in the connective tissue, which acts as the structural foundation for the epidermis, outline these changes externally and

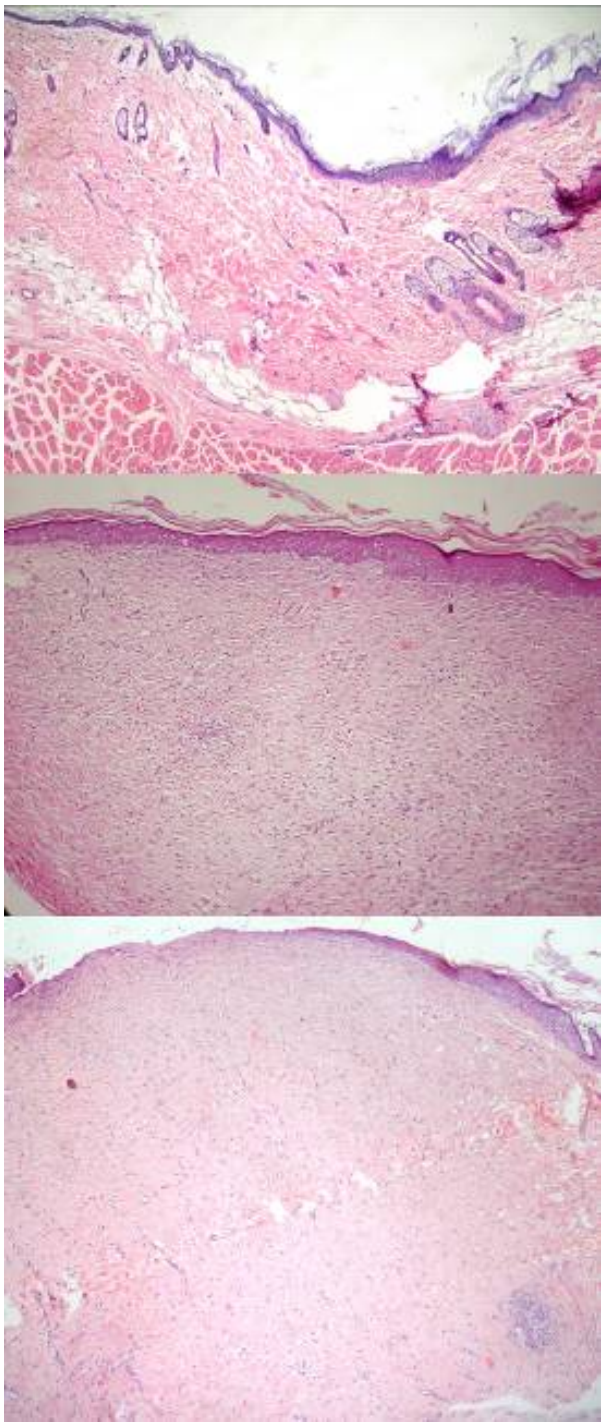

Figure 4. Evaluation of collagen in group intense pulsed light (G1) at three time points analyzed: baseline (T0), half (T1) and final (T2) treatment, from up to down, respectively. Hematoxylin-eosin (100×).

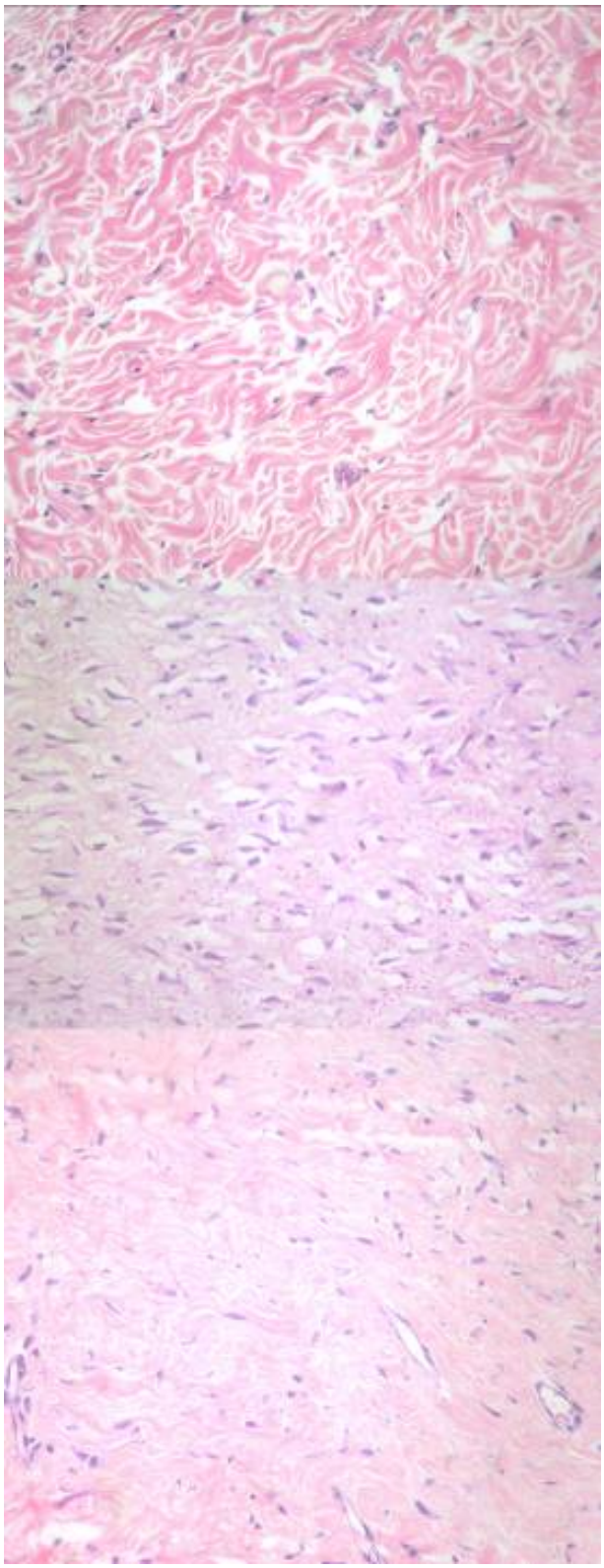

Figure 5. Evaluation of collagen in group intense pulsed light (G1) at three time points analyzed: baseline (T0), half (T1) and final (T2) treatment, from up to down, respectively. Hematoxylin-eosin (400×).

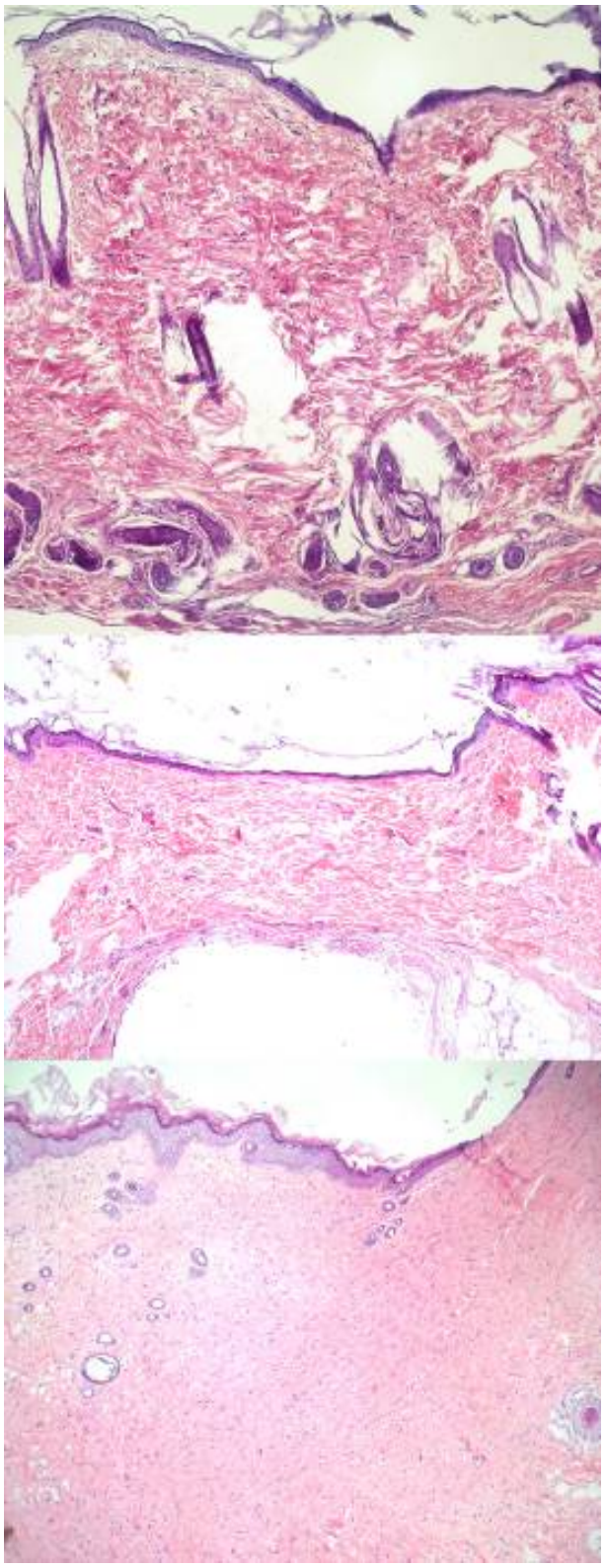

Figure 6. Evaluation of collagen in group CO_2 injection (G2) at three time points analyzed: baseline (T0), half (T1) and final (T2) treatment, from up to down, respectively. Hematoxylin-eosin (100×).

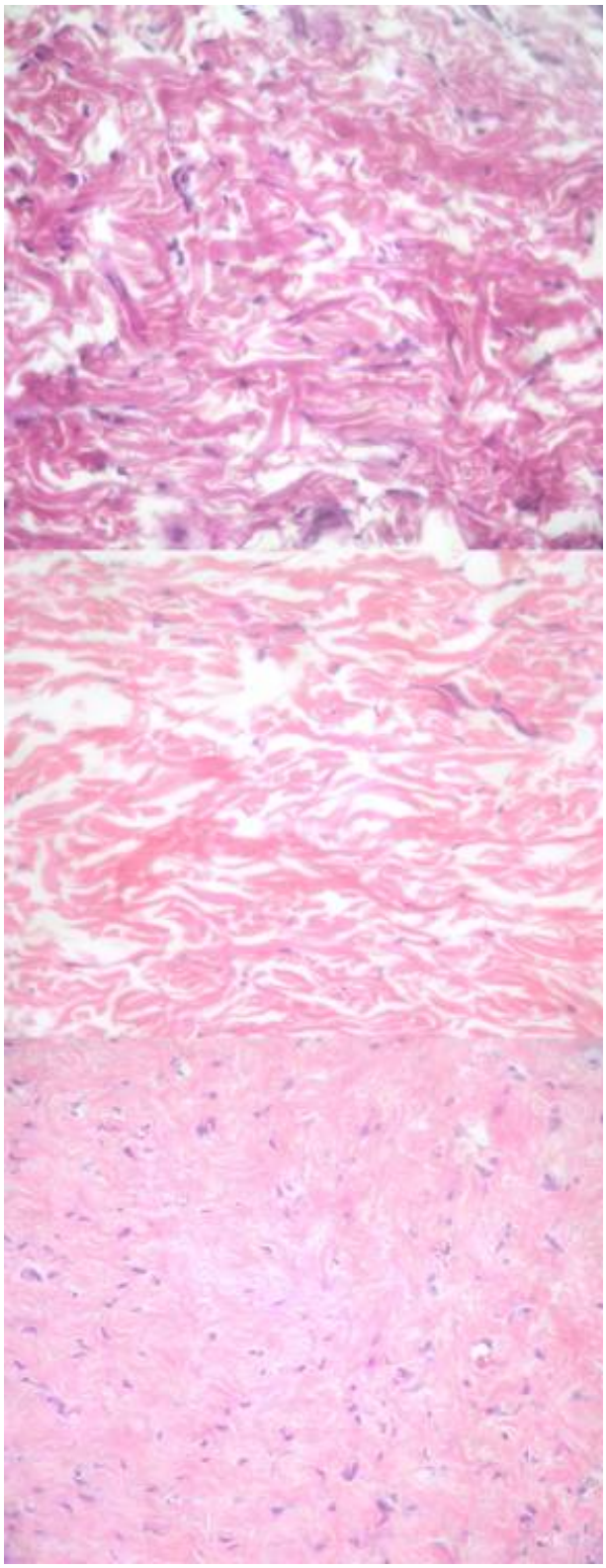

Figure 7. Evaluation of collagen in group CO_2 injection (G2) at three time points analyzed: baseline (T0), half (T1) and final (T2) treatment, from up to down, respectively. Hematoxylin-eosin (400×).

are reflected in the corneum stratum [1]. The modifications to the lifelong unit-elastic collagen establish a substantial morphological basis for the understanding of the biochemical and biomechanical changes of the skin with age [1]. Thus, the intradermal injection of CO_2 provides a greater exchange rate (increase in blood flow) and improves tissue oxygenation; in addition, it may stimulate dermal fibroblasts and may increase collagen and elastin synthesis [9-12].

A recent study evaluated CO_2 injection into the skin of ten Wistar male rats. The results of the study indicated a marked increase in collagen after infusion of carbon dioxide into the skin of animals. Furthermore, intradermal injections appeared more effective than subcutaneous injections in reducing wrinkles [2].

Based on those results, we studied the stimulation of fibroblast and collagen proliferation via the application of intense pulse light and intradermal CO_2 in the skin of sixteen rats. It should be emphasized that the proliferation of collagen is a dynamic process and depends mainly on the stimulation of fibroblasts. Therefore, it is possible that a longer period of exposure to the treatments could lead to a greater activation of dermal fibroblasts and even greater collagen proliferation.

The results of the current study allow us to conclude that both the intradermal injection of CO_2 and intense pulsed light promotes fibroblast and collagen proliferation in the skin of animals. However, other questions are raised by these results. Is it possible that various mechanical or traumatic stimuli (punctures, local heat) can also stimulate fibroblast and collagen proliferation? Can the results observed in rat skin be repeated in human skin?

Because of the paucity of data (especially experimental study data) published in medical journals about this subject, this work represents a milestone in rejuvenating dermatopathology procedures. Thus, further studies in this area utilizing similar methodologies are needed. Dermatology now offers unlimited possibilities in the use of skin-rejuvenating procedures. The gathering of scientific evidence is the best way to establish new methods for cosmetic dermatology.

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