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ORIGINAL ARTICLE

Carboxytherapy: Controls the inflammation and enhances the production of fibronectin on wound healing under venous insufficiency

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To examine the influence of carboxytherapy on wound healing under venous insufficiency, full-thickness excisional wounds were created on *Wistar* rats. We used three groups with 32 rats each: Group (I): daily cleaning with 0.9% saline solution; Group Sulfadiazine (II): 1% silver sulfadiazine; and Carboxytherapy (III): subcutaneous application of 0.3 mL of carbon dioxide. The predetermined periods of analysis were the 3rd, 7th, 14th, and 30th day. The slides were stained with haematoxylin and eosin and Picrosirius red and submitted for immunohistochemistry. Groups II and III presented a statistically significant decrease in relation to the presence of neutrophilic and lymphocytic infiltrates. The presence of collagen significant increased in groups II and III. However, group III presented better organisation. Only group I maintained the neovascularisation until the 30th day. The new epithelium statistically significantly increased in groups II and III. On immunohistochemistry, regarding fibronectin expression, only group III demonstrated a statistically significant increase since the beginning of the healing process. Thus, the use of carboxytherapy promotes the formation of a tissue better structured and that may be an important resource for the treatment of wounds under venous insufficiency, especially those of recurrent re-openings.

KEYWORDS

carbon dioxide, carboxytherapy, venous insufficiency, wound healing

1 | INTRODUCTION

Chronic venous insufficiency of the lower limbs occurs because of an imbalance of venous flow resulting in local oedema and hyperpigmentation[1] This condition promotes hypoxia in the tissues, which contributes to the inhibition of tissue repair, causing cutaneous ulceration.[2] To heal according to the normal repair times and mechanisms, the interaction of a complex network of cells, chemical signals, and extracellular matrix is necessary, but in this clinical condition, this interaction is flawed, so this process becomes chronic.[3,4]

Several methods exist to increase the availability of oxygen in these tissues in order to modulate the lesion environment to promote the healing[5]; it is also necessary that this occurs quickly to obtain a functional scar.[6] Among these methods stands out the use of carboxytherapy, which is the technique of controlled insufflation of carbon dioxide in the subcutaneous tissue, carried out safely and a low cost.[7] The increase of local CO_2 concentration promotes a reaction with H_2O, causing the liberation of H^+. This liberation modifies the local PH, becoming acid. This enhances the Bohr effect inside the red blood cells, diverting to the right the dissociation curve of hemoglobin oxygen and, as a result, increase the supply of oxygen to the tissue.[8] It is also causes an increase in the flow of proteins that are necessary to remodel the components of the extracellular matrix responsible for tissue repair.[9]

Thus, considering the basic mechanism of oxymetabolic action of carboxytherapy and the necessity of efficient therapeutic resources for the treatment of venous ulcerations of the lower limbs, the present study evaluated the action of carboxytherapy in skin lesions caused under venous insufficiency in *Wistar* rats.

Key Messages

- the carbon dioxide proposed in this study is a non-toxic, cost-effective, and safe treatment
- the objective was to evaluate the action of carboxytherapy in skin lesions caused under venous insufficiency in *Wistar* rats
- a total of 32 rats each were allocated as follows: Group (I)—daily cleaning with 0.9% saline solution; Group Sulfadiazine (II)—1% silver sulfadiazine; and Carboxytherapy (III)—subcutaneous application of 0.3 mL of carbon dioxide
- the carbon dioxide application improves neovascularisation and neoepithelisation, leading to the formation of better structured tissue

2 | MATERIALS AND METHODS

All procedures with the animals were approved by the Ethics Committee on the Use of Animals of the Federal University of Mato Grosso do Sul under protocol # 143/2007.

2.1 | Experimental design

A total of 96 adult, male rats (*Rattus norvegicus* albinus) of *Wistar* lineage—EPM, from the Central Animal House of the Institute of Biosciences of the Federal University of Mato Grosso do Sul (INBIO/UFMS), weighing between 250 and 300 (g) and with age between 78 and 90 days were used. Each animal was housed individually at a controlled temperature (22 ± 2°C) and a photoperiod of 12 hours (12 hours of light:12 hours of dark), with the supply of water and feed ad libitum.

The animals were submitted to ligation of the femoral vein and, posteriorly, to the cutaneous wound confection. They were then divided into three experimental groups (n = 32): Control Group (I): care in the postoperative period was performed daily cleaning of the wounds with 0.9% saline solution; Group Sulfadiazine (II): the wounds received topic application of 1% silver sulfadiazine; and Carboxytherapy (III): the wounds were treated with subcutaneous application of 0.3 mL of carbon dioxide, using an insulin syringe around the wound, applied immediately postoperatively and with consecutive 3-day intervals. The device used was the Carboxiderm® medical carbon dioxide insufflator with two gas outlet channels; micro-controlled with a graphical display for visualisation of treatment parameters; with a comfort system, automatic programming of filling of CO_2 tubing, and an alarm when there is an increase or decrease in the gas pressure; an output flow of 0.2 to 80 mL/min, with increments of 10 mL/min; and a maximum output pressure of 1.5 bar (with the output closed), registered in Anvisa under number 80286000005. The predetermined periods in which the analyses of the experiments were performed were on the 3rd, 7th, 14th, and 30th day.

2.2 | Surgical procedure for the ligation of the femoral vein

The animals were anaesthetised intramuscularly, with a solution with 1: 1 of xylazine (20 mg/mL) and ketamine (50 mg/mL), in the proportion of 0.1 mL/100 g^{-1} body weight (b.w.). The effectiveness of the procedure was evidenced by the absence of the corneal-eyelid reflex to the digital stimulus and absence of the pain reflex to the tail clamping.

The animals were then placed on an operative table in crural decubitus, and epilation, asepsis, and sterilisation of the right inguinal region were performed. After that, an oblique incision (20 mm) was made on the skin and subcutaneous tissue with a # 15 blade, following the skin fold of the right inguinal region. Delicate divulsion was performed to identify and expose the right femoral nerve tract with posterior ligation of the femoral vein with polyamide 4.0.[10]

Venous insufficiency was verified by comparing perimetry before and after the procedure, performed in the right crural region at three levels (proximal, medial, and distal). Once venous insufficiency was confirmed, the animals were submitted to the wound confection.

2.3 | Rat wound models

The animals were again anaesthetised and placed on a surgical table. Epilation, asepsis, and sterilisation of the right inguinal region were performed, and a circular skin fragment was excised using a metallic punch, 10 mm in diameter, in the centre of the epilated area, remaining exposed to lateral crural muscular fascia. Haemostasis was obtained by digital compression using sterile cotton gauze for approximately 2 minutes. The lesion was hygienised with 0.9% saline solution and received occlusive dressings. Finally, the animals were returned to their original cages for anaesthetic recovery and subsequent submission to postoperative care as previously described in the experimental design. Wounds were observed daily regarding the presence of necrosis on the border, bleeding from superficial or deep planes, formation of crusts, eczema of stasis, and/or the presence of secretions suggestive of infectious process.

2.4 | Tissue harvesting and staining

The animals were submitted to euthanasia in a CO_2 chamber after the predetermined periods of analyses previously described in the experimental design. Samples (3 mm

diameter) of tissue were collected using a metal punch, starting from the original border of the lesion, with subsequent fixation in 3 mL of 10% buffered formalin, sufficient for total immersion. The samples were sent to the Department of Pathological Anatomy of the State University of São Paulo (UNESP), Botucatu Campus, and were then submitted to dehydration in ethyl alcohol (70%, 90%, and 100%, consecutively), diaphanization in xylol (P.A.), and histological paraffin impregnation at a temperature of 59°C. The blocks were sectioned on a microtome, and the slides were mounted and stained with Haematoxylin and Eosin (HE); then, when dense connective tissue was observed, the following step was taken—the Picrosirius red colouring was added, so it was analysed under a Nikon optical microscope, model Eclipse E400, with monochrome lenses coupled to the Samsung image capture model SCC 131, to obtain images of the interest areas. All the slide analyses were blindly performed by the author.

In the slides stained with HE, the following parameters were observed according to Santos et al,[11] with modifications: neovascularisation was analysed using 40× magnification and inflammatory cells (neutrophils and lymphocytes), fibroblastic proliferation, and reepithelialisation in 100× magnification. For this, three cross-sectional rankings were assigned: (0) absent, (+) moderate presence, and (++) accentuated presence, represented by scores of 0, 1, and 2, respectively. It was considered an area of interest for inflammatory reaction to that with presence of inflammatory cells, such as neutrophils, fibroblasts, and macrophages. For the reepithelialisation observation, the assigned scores were: (0) absence of reepithelialisation, (+) partial reepithelialisation, and (+) complete reepithelialisation, represented by score 0, 1, and 2, respectively.

In the slides stained with Picrosirius red, a quantitative analysis was performed for the presence of collagen fibres in 40× magnification. For this, according to Melo et al,[12] with modifications, three cross-sectional rankings were assigned: (0) absent, (+) moderate presence, and (++) accentuated presence, represented by scores 0, 1, and 2, respectively. It was considered an area of interest for the study of the presence of collagen fibres to those stained in red and strongly birefringent.

2.5 | Immunohistochemistry

The samples were sent to the Department of Histopathology of the Federal University of São Paulo (UNIFESP), and the blocks were prepared as described above and were then sectioned in a rotary microtome, adjusted to 3 μm thickness; the slides were mounted on glass with organosilane (3-aminopropyltriethoxysilane)-based adhesive and antibodies to the glycoprotein extracellular matrix, fibronectin. The sections were then deparaffinised and subjected to antigenic recovery and were subsequently immersed in two passages of oxygen peroxide (H_2O_2, 10 v) of 15 minutes each for inactivation of the endogenous peroxidase. Subsequently, they were incubated with anti-fibronectin primary antibodies, and the washes between the steps were performed in Tris-HCl buffer solution (pH 7.4). Subsequently, the sections were incubated at room temperature for 15 minutes in a humid chamber, with the respective secondary antibodies and the streptavidin-biotin-peroxidase complex; 0.03% diammonobenzidine was used as a chromogen for reaction development, and counterstaining was performed with Mayer haematoxylin. All the slide analyses were conducted blindly by the author.

The slides was evaluated according Martins et al,[13] with modifications for intensity and distribution pattern, with 100× magnification. The expression intensity of fibronectin was graded as (0) absent, (+) weak, and (++) strong, represented by scores 0, 1, and 2, respectively. The distribution pattern was considered diffuse when it extended throughout the connective tissue and focal when expressed in certain areas of the tissue.

2.6 | Statistical analysis

For the statistical analysis of the results, the Kruskal-Wallis test, followed by Student-Newman Keuls test, was applied in order to compare the three study groups for the variable of interest in each period. The results were considered significant when $P \leq 0.05$.

3 | RESULTS

3.1 | Macroscopic analysis

The operative procedures of the ligation of the femoral vein and the temporal sequencing of the postoperative follow up caused the installation of venous hypertension, which is considered the trigger point of venous vascular insufficiency. Venous insufficiency can be demonstrated by perimetry of the inferior limb before and after the confection of the femoral vein ligature that demonstrated a statistically significant increase. The values of MEAN ± SD of the right leg was 7.02 ± 0.13 before and 8.28 ± 0.19 after 30 days and in the left leg was 7.01 ± 0.12 before and 7.29 ± 0.12 after 30 days.

In the macroscopic analysis of the wounds, during all the postoperative follow-up periods, there was no evidence of bleeding, border necrosis, crust formation, stasis eczema, or secretion suggestive of infectious process. In the follow-up periods, no animal presented complete scarring of the cutaneous lesion. After 30 days, it was observed that the wounds were healed in all the groups.

3.2 | Microscopic analysis

Microscopic analysis of the slides stained with HE showed the presence of neutrophilic and lymphocytic infiltrates (Tables 1 and 2), demonstrating time-dependent

TABLE 1 Neutrophil scores in the different experimental groups (control—I, sulfadiazine—II, and carboxytherapy—III) during the predetermined time points (3rd, 7th, 14th, and 30th)

	3rd			7th			14th			30th		
Scores	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**
0 (0 = absent)	0	0	0	0	0	0	0	6	8	0	6	8
+ (1 = moderate)	0	0	0	2	0	3	3	2	0	6	2	0
++ (2 = accentuated)	8	8	8	6	8	5	5	0	0	2	0	0
Total of animals	8	8	8	8	8	8	8	8	8	8	8	8
Mean of scores	2.0	2.0	2.0	1.8	2.0	1.6	[a]1.6	[b]0.3	[b]0.0	[a]1.3	[b]0.3	[b]0.0
P	1.00			0.184			<0.001			0.001		

Note: Kruskal-Wallis test followed by Student-Newman-Keuls test. Absence of letters or equal letters indicates non-significant difference. Different letters indicate a statistically significant difference: [a, b] comparison between groups (I, II, and III); [c, d] comparison between the periods (3, 7, 14, and 30 days).
I ([c] 3 × 7 × 14 × [d] 30): *P*-value = 0.018.
II ([c] 3 × [c] 7 × [d] 14 × [d] 30): *P*-value = <0.001.
III ([c] 3 × [c] 7 × [d] 14 × [d] 30): *P*-value = <0.001.

characteristics. Groups II and III presented a statistically significant decrease in relation to the control group on the 14th and 30th day.

Histological analysis (Figure 1) showed that the migration of fibroblasts increase significantly at the beginning of the repair process, when all groups presented a higher amount of fibroblasts on the 7th day. Besides that, group III showed a statistically significant increase in relation to groups I and II. On the 14th and 30th days, the presence of fibroblasts decreased significantly in groups II and III (Table 3).

In the microscopic analysis of the slides stained with Picrosirius red, the presence of collagen showed time-dependent characteristics, with higher production on the 14th and 30th days. Group III showed a significant increase in the amount of collagen in relation to the groups I and III (Table 4). It was also possible to observe a greater synthesis of collagen in groups II and III. However, group III presented better organisation of the lamellae (Figure 2).

Neovascularisation predominated the 7th and 14th days. From this period until the 30th day, only group I maintained this process. In groups II and III, a decrease in neovascularisation was observed (Table 5). The formation of a new epithelium occurred more clearly from the 14th to the 30th day, with a statistically significant increase in groups II and III in relation to group I (Table 6).

On immunohistochemistry, regarding fibronectin expression, it was demonstrated that, in group III, there was a statistically significant increase in relation to groups I and II since the beginning of the healing process (Table 7). It was also possible to observe a better formation/structuring of the framework and the carboxytherapy group (Figure 3).

4 | DISCUSSION

In healthy individuals, tissue repair occurs through the deposition of scar tissue at the site, which has different physiological characteristics from healthy cutaneous tissue.[14] Regarding wound healing resulting specifically from chronic venous insufficiency, it is known that the repair process occur slowly, and it is insufficient because the migration of cells is inefficient, which causes the chronicity of this condition as the region cannot be reepithelialised,[15] resulting in dysfunctional tissue in the affected area.[3] The literature on the improvement of this condition does not present concise and recent findings, so we consider the choice of the carboxytherapy in this study because the carbon dioxide used in this process is non-toxic, cost-effective, and safe[16,17] and

TABLE 2 Lymphocyte scores in the different experimental groups (control—I, sulfadiazine—II, and carboxytherapy—III) during the predetermined time points (3rd, 7th, 14th, and 30th)

	3rd			7th			14th			30th		
Scores	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**
0 (0 = absent)	0	0	0	0	0	0	0	3	7	1	8	8
+ (1 = moderate)	5	2	3	7	8	8	3	5	1	4	0	0
++ (2 = accentuated)	3	6	5	1	0	0	5	0	0	2	0	0
Total of animals	8	8	8	8	8	8	8	8	8	8	8	8
Mean of scores	1.4	1.8	1.6	1.1	1.0	1.0	[a]1.6	[b]0.6	[b]0.1	[a]1.1	[b]0.0	[b]0.0
P	0.317			0.368			<0.001			0.003		

Note: Kruskal-Wallis test followed by Student-Newman-Keuls test. Absence of letters or equal letters indicates non-significant difference. Different letters indicate a statistically significant difference: [a, b] comparison between groups (I, II, and III); [c, d] comparison between the periods (3, 7, 14, and 30 days).
I (3 × 7 × 14 × 30): *P*-value = 0.186.
II ([c] 3 × [c] 7 × [d] 14 × [d] 30): *P*-value = <0.001.
III ([c] 3 × [c] 7 × [d] 14 × [d] 30): *P*-value = <0.001.

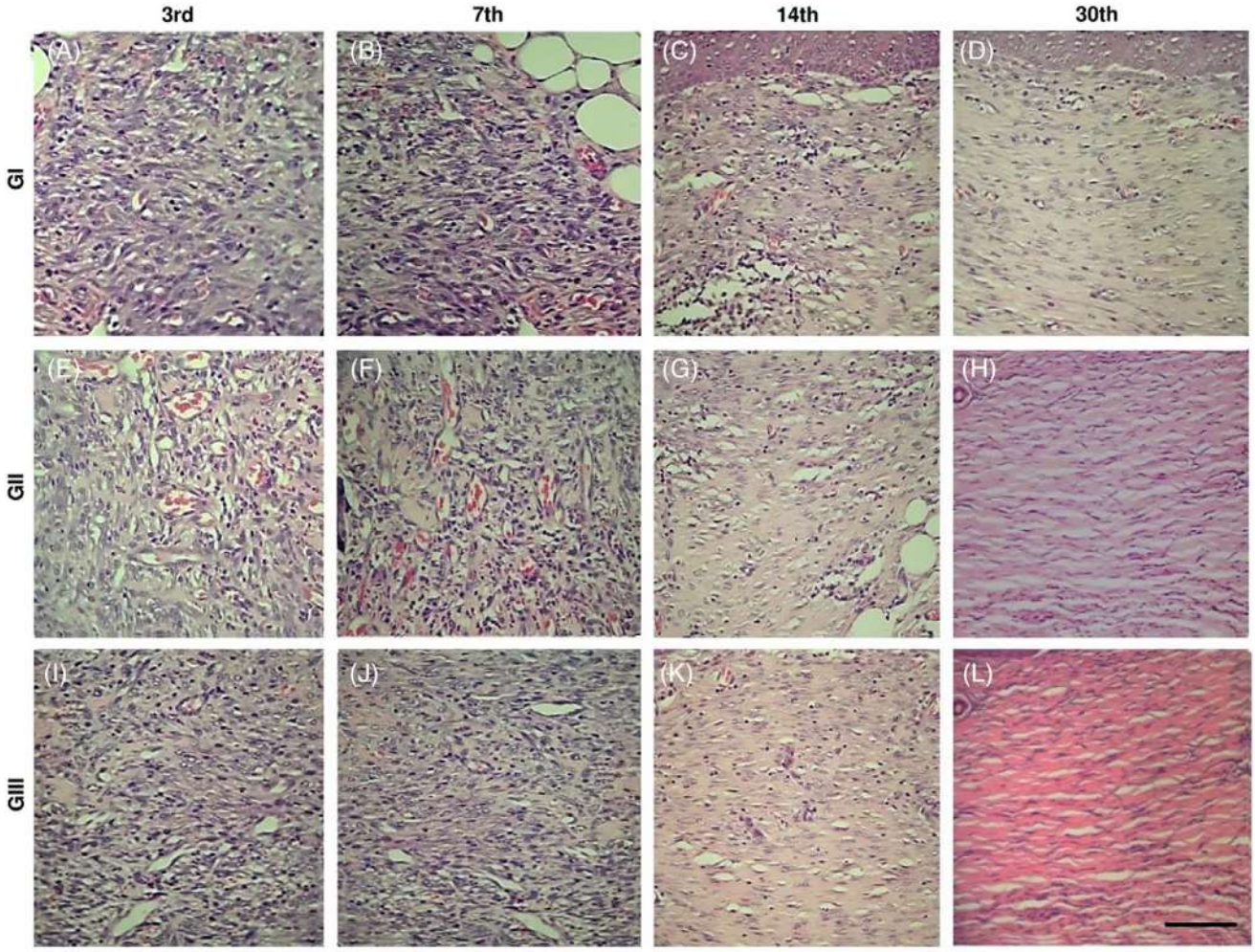

FIGURE 1 Microscopic analysis with haematoxlyn and eosin staining on the 3rd and 7th days, showing a large number of inflammatory cells and fibroblasts: Control group (A/B), sulfadiazine group (E/F), Carboxytherapy group (I/J); and on the 14th and 30th days with few inflammatory cells and fibroblasts that are more organised: Control group (C/D), sulfadiazine group (G/H), Carboxytherapy group (K/L). Bar = 40 μm

respects the principle *primum non nocerae* (first of all not to prejudice), aiming to stimulate, or at least not inhibit, cell replication.[18]

The present study confirmed the decrease of the inflammatory process after treatment with carboxytherapy, which promotes an increase of cell proliferation. It was also observed at the beginning of this process that group III presented a higher presence of neovascularisation in relation to the other groups and, in the end, also demonstrated a decrease of this event sooner than the other groups. Some studies suggested that the vasodilation promoted by carboxytherapy may be mediated in part by nitric oxide (NO), and

TABLE 3 Fibroblasts scores in the different experimental groups (control—I, sulfadiazine—II, and carboxytherapy—III) during the predetermined time points (3rd, 7th, 14th, and 30th)

	3rd			7th			14th			30th		
Scores	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**
0 (0 = absent)	8	8	8	4	2	0	0	0	0	0	0	0
+ (1 = moderate)	0	0	0	4	6	8	5	3	1	5	3	0
++ (2 = accentuated)	0	0	0	0	0	0	3	5	7	3	5	8
Total of animals	8	8	8	8	8	8	8	8	8	8	8	8
Mean of scores	0.0	0.0	0.0	[a]1.1	1.5	[b]2.0	[a]1.4	0.8	[b]0.3	[a]1.,1	[b]0.1	[b]0.0
P	1.000			0.003			0.004			0.019		

Note: Kruskal-Wallis test followed by Student-Newman-Keuls test. Absence of letters or equal letters indicates non-significant difference. Different letters indicate a statistically significant difference: [a, b] comparison between groups (I, II, and III); [c, d] comparison between the periods (3, 7, 14, and 30 days).
I ([c] 3 × [d] 7 × [d] 14 × [d] 30): *P*-value = <0.001.
II ([c] 3 × [d] 7 × + [d]14 × [c] 30): *P*-value = <0.001.
III ([c] 3 × [d] 7 × [c] 14 × [c] 30): *P*-value = <0.001.

TABLE 4 Collagen scores in the different experimental groups (control—I, sulfadiazine—II, and carboxytherapy—III) during the predetermined time points (3rd, 7th, 14th and 30th)

	3rd			7th			14th			30th		
Scores	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**
0 (0 = absent)	8	8	8	4	2	0	0	0	0	0	0	0
+ (1 = moderate)	0	0	0	4	6	8	5	3	1	5	3	0
++ (2 = accentuated)	0	0	0	0	0	0	3	5	7	3	5	8
Total of animals	8	8	8	8	8	8	8	8	8	8	8	8
Mean of scores	0.0	0.0	0.0	0.5	0.8	1.0	1.4	1.6	1.9	[a]1.4	1.6	[b]2.0
P	1.000			0.083			0.130			0.033		

Note: Kruskal-Wallis test followed by Student-Newman-Keuls test. Absence of letters or equal letters indicates non-significant difference. Different letters indicate a statistically significant difference: [a, b] comparison between groups (I, II, and III); [c, d] comparison between the periods (3, 7, 14, and 30 days).
I ([c] 3 × [c]7 × [d] 14 × [d] 30): *P*-value = <0.001.
II ([c] 3 × [c] 7 × [d] 14 × [d] 30): *P*-value = <0.001.
III ([c] 3 × + [c] 7 × [d] 14 × [d] 30): *P*-value = <0.001.

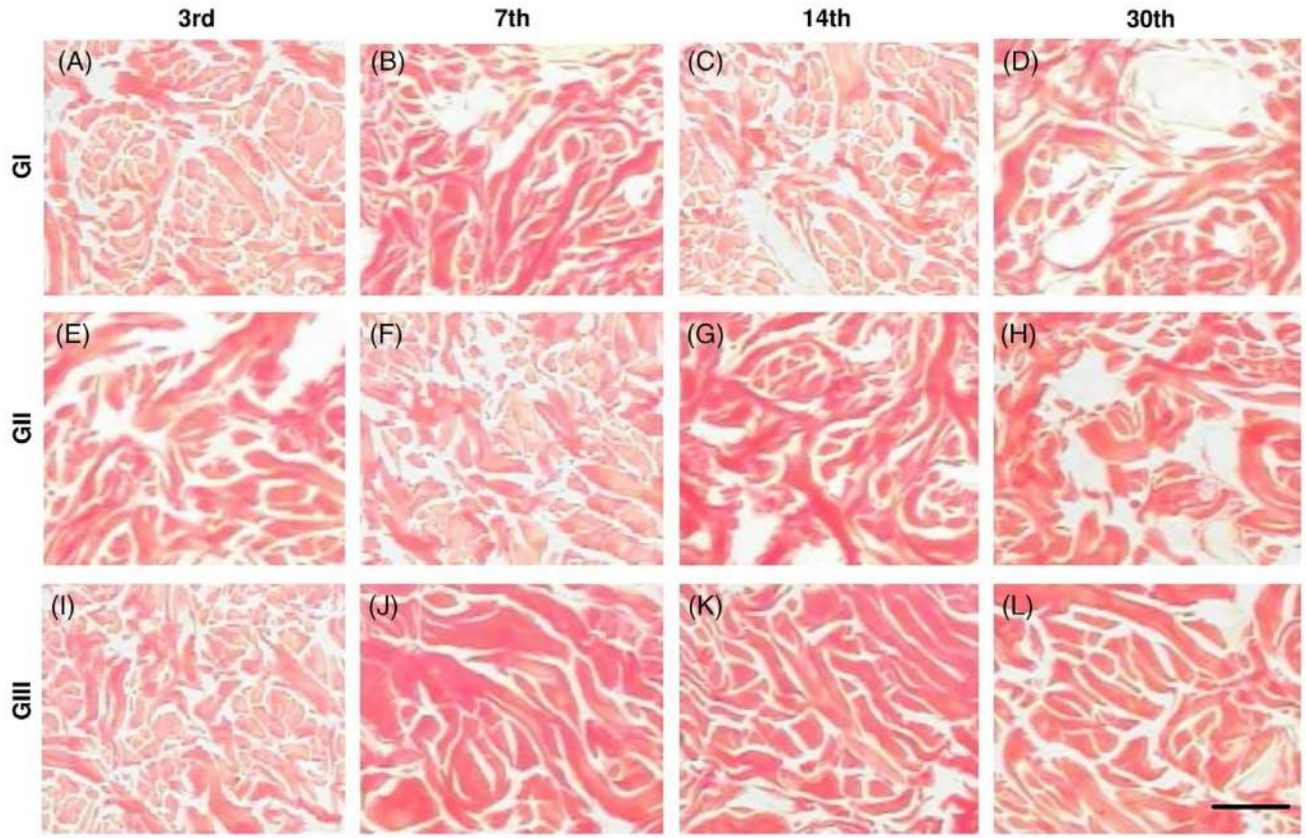

FIGURE 2 Microscopic analysis with Picrosirius red staining in the group control (A, B, C, D) showing a random arrangement of collagen fibres; sulfadiazine group (E, F, G, H) with a collagen organisation similar to normal skin but with more density; carboxytherapy (I, J, K, L) with a collagen organisation in lamellae and great organisation and density. Bar = 40 μm

this enhances the neoangiogenic properties of carbon dioxide, inducing the expression of angiogenic factors, vascular endothelial growth factor (VEGF), or basic fibroblast growth factor (FGF) and inhibiting endothelial cell apoptosis.[19–22] The presence of NO is important in the early and late phases of wound healing.[23] Therefore, this oxygenation provided by the carboxytherapy, especially in the wounds that result from complications of chronic venous insufficiency,[24] has great importance for the homeostasis of the biochemical environment that allows the installation of a chemical gradient that is more attractive to the fibroblasts, favouring a greater synthesis of collagen.[25]

The proliferative phase relies on the important role of fibronectin, which has the function of adhesion to fibrin, collagen, and other types of cells.[26] It acts as a glue to consolidate the fibrin clot that protects the environment in the first instance,[25] and it works as a framework for the adhesion of the other cells, favouring the formation of a better scaffold for the deposition of granulation tissue.[27] This is an important role in the healing of wounds, especially for the chronic

TABLE 5 Neovascularisation scores in the different experimental groups (control—I, sulfadiazine—II, and carboxytherapy—III) during the predetermined time points (3rd, 7th, 14th, and 30th)

	3rd			7th			14th			30th		
Scores	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**
0 (0 = absent)	8	8	6	0	0	0	0	0	0	4	1	8
+ (1 = moderate)	0	0	2	7	7	0	5	8	8	4	7	0
++ (2 = accentuated)	0	0	0	1	1	8	3	0	0	0	0	0
Total of animals	8	8	8	8	8	8	8	8	8	8	8	8
Mean of scores	0.0	0.0	0.3	[a]1.1	[a]1.1	[b]2.0	[a]1.4	[b]1.0	[b]1.0	0.5	[a]0.9	[b]0.0
P	0.619			<0.001			0.037			0.007		

Note: Kruskal-Wallis test followed by Student-Newman-Keuls test. Absence of letters or equal letters indicates non-significant difference. Different letters indicate a statistically significant difference: [a, b] comparison between groups (I, II, and III); [c, d] comparison between the periods (3, 7, 14, and 30 days).
I ([c] 3 × [d] 7 × [d] 14 × + [c] 30): *P*-value = <0.001.
II ([c] 3 × + [d] 7 × [d] 14 × [d] 30): *P*-value = <0.001.
III ([c] 3 × [d] 7 × [d] 14 × [c] 30): *P*-value = <0.001.

TABLE 6 Reepithelialisation scores in the different experimental groups (control—I, sulfadiazine—II, and carboxytherapy—III) during the predetermined days (3rd, 7th, 14th, and 30th)

	3rd			7th			14th			30th		
Scores	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**
0 (0 = absent)	8	8	8	6	6	6	2	0	0	0	0	0
+ (1 = partial)	0	0	0	2	2	2	6	0	0	6	0	0
++ (2 = complete)	0	0	0	0	0	0	0	8	8	2	8	8
Total of animals	8	8	8	8	8	8	8	8	8	8	8	8
Mean of scores	0.0	0.0	0.0	0.3	0.3	0.3	[a]0.8	[b]2.0	[b]2.0	[a]1.3	[b]2.0	[b]2.0
P	1.000			1.000			<0.001			<0.001		

Note: Kruskal-Wallis test followed by Student-Newman-Keuls test. Absence of letters or equal letters indicates non-significant difference. Different letters indicate a statistically significant difference: [a, b] comparison between groups (I, II, and III); [c, d] comparison between the periods (3, 7, 14, and 30 days).
I ([c] 3 × [c] 7 × [d] 14 × [d] 30): *P*-value = <0.001.
II ([c] 3 × [c]7 × [d] 14 × [d] 30): *P*-value = <0.001.
III ([c]3 × [c]7 × [d] 14 × [d]30): *P*-value = <0.001.

TABLE 7 Fibronectin scores in the different experimental groups (control—I, sulfadiazine—II, and carboxytherapy—III) during the predetermined time points (3rd, 7th, 14th, and 30th)

	3rd			7th			14th			30th		
Scores	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**
0 (0 = absent)	3	2	0	0	0	0	0	0	0	0	0	0
+ (1 = weak)	5	6	1	8	8	0	8	6	0	8	4	0
++ (2 = strong)	0	0	7	0	0	8	0	2	8	0	4	8
Total of animals	8	8	8	8	8	8	8	8	8	8	8	8
Mean of scores	[a]0.6	[a]0.8	[b]1.9	[a]1.0	[a]1.0	[b]2.0	[a]1.0	[a]1.3	[b]2.0	[a]1.0	1.5	[b]2.0
P	<0.001			<0.001			<0.001			<0.001		

Note: Kruskal-Wallis test followed by Student-Newman-Keuls test. Absence of letters or equal letters indicates non-significant difference. Different letters indicate a statistically significant difference: [a, b] comparison between groups (I, II, and III); [c, d] comparison between the periods (3, 7, 14, and 30 days).
I ([c] 3 × [d] 7 × [d] 14 × [d] 30): *P*-value = 0.022.
II ([c] 3 × 7 × 14 × [d] 30): *P*-value = 0.016.
III (3 × 7 × 14 × 30): *P*-value = 0.392.

ones.[25] In this study, the presence of fibronectin in group III was predominant in relation to the others in all the analysed periods, and it was possible to affirm that carboxytherapy promotes a better restructuring of the basal membrane through the greater synthesis and organisation of the collagen, which promotes a scar with better aesthetic appearance and with greater elasticity and mechanical resistance.[28] It is also emphasised that the epithelial tissue was formed in a shorter period of time when compared with the other groups.

It is known that chronic ulcer tissue has a disorganised microenvironment, with the presence of inflammatory cells and free radicals that degrade the extracellular matrix,[14] against these characteristics, and considering the results of the present study, it is possible to affirm that the

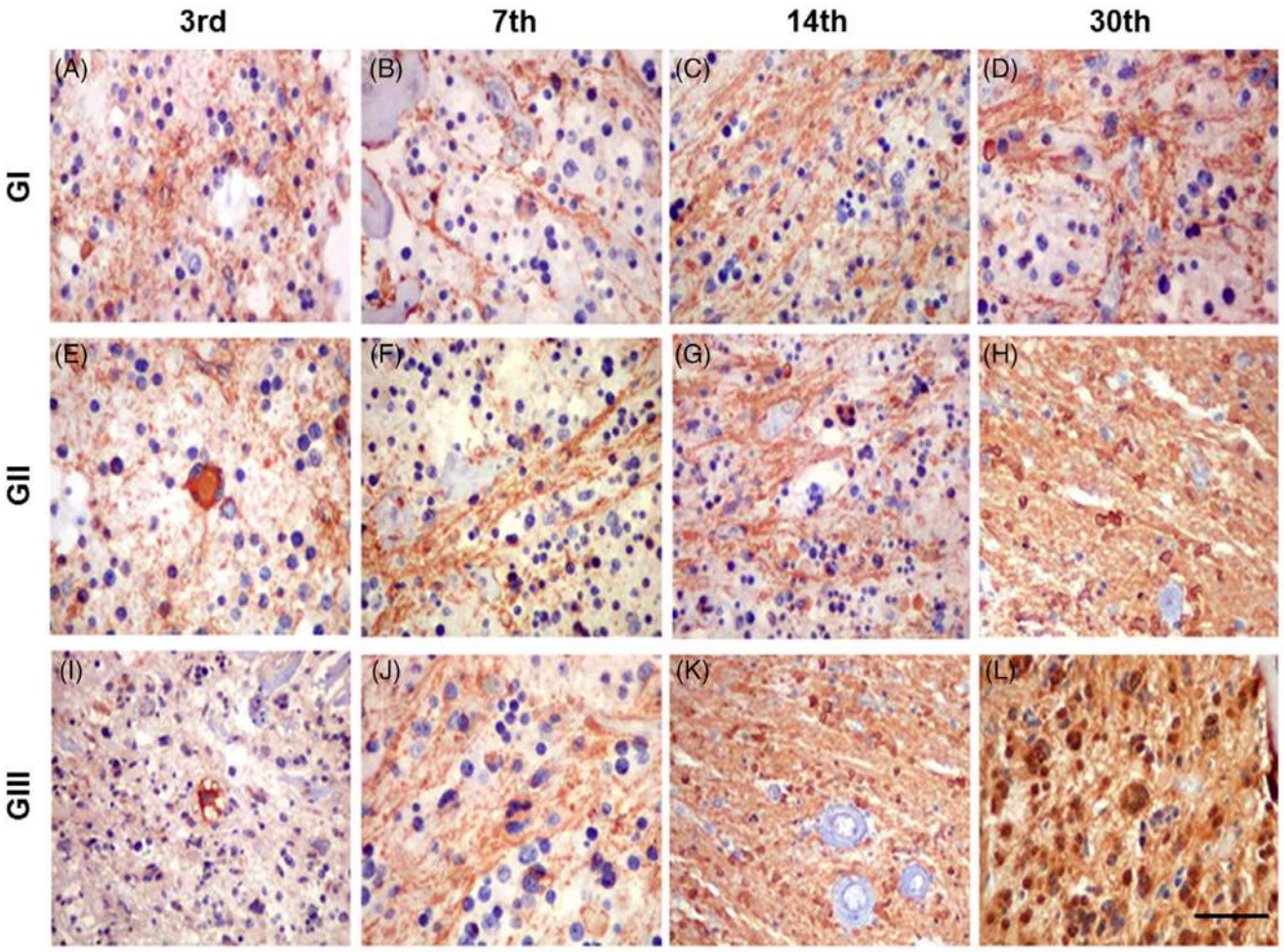

FIGURE 3 - Microscopic analysis of immunohistochemistry in control group (A, B, C, D) with random arrangement of fibronectin; in sulfadiazine group (E, F, G) with random arrangement of fibronectin and also (H) with presence of fibronectin dense layer; in carboxytherapy group (I/J) with random arrangement of fibronectin and also (K/L) a better formation/structuring of the framework of fibronectin. Bar = 40 μm

carboxytherapy acted as an inflammatory modulator capable of improving the organisation of the cellular environment at the lesion site and still provided efficient epithelial tissue recovery. However, the complete way in which this modulation happens and what interferes in this process since the beginning of the healing is still not fully understood, so it needs to be further studied.

The group that used 1.0% silver sulfadiazine was chosen as a positive control for the analyses in this study because of its wide use in the treatment of wounds caused by venous insufficiency.[15] However, the process of neoepithelialisation occurred more efficiently with carboxytherapy; the total rate of deposition of the extracellular matrix and the remodelling of deposited collagen are factors that determine the healing process, but when the inflammatory process is inhibited (as occurs in the use of 1.0% silver sulfadiazine), precarious matrix deposition occurs, leading to the delayed proliferation of epithelial cells.[25,29]

Thus, this study inferred that the local vasomotor effect and the organised deposition of the healing components in the wounds that were submitted to carboxytherapy, compared with the other groups, enhanced the efficiency of the carbon dioxide application for neovascularisation and neoepithelisation, leading to the formation of better-structured tissue. Therefore, carboxytherapy may be an important resource for the treatment of venous stasis wounds, especially those of recurrent re-openings.

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