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CGF IN THE TREATMENT OF THE MIXED ULCERS OF THE LOWER LIMBS

--Manuscript Draft--

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Corresponding Author:	Bruno Amato, Ph.D., M.D. University of Naples Federico II Naples, ITALY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Naples Federico II
Corresponding Author's Secondary Institution:	
First Author:	Bruno Amato, Ph.D., M.D.
First Author Secondary Information:	
Order of Authors:	Bruno Amato, Ph.D., M.D.
Order of Authors Secondary Information:	
Abstract:	Background: Concentrated Growth Factors (CGF) is a concentration of second generation autologous growth factors compared to platelet rich plasma (PRP) and represents a multifactorial stimulation system that can be used for the management and treatment of chronic skin ulcers. Aim oft he study: The aim of the work is to evaluate the additional benefits of the CGF compared to the standard of dressing and his effects on the dynamics of the healing process. Methods: Autologous CGFs were obtained from 100 patients with chronic mixed ulcers (venous ulcers in patients with II stage claudication) of the lower limbs in a multicentric controlled randomized study. Results: The results showed a significant advantage in the use of CGF in association with cleansing and selective compression in the healing time and stabilization of mixed ulcers of the lower extremities. Conclusions: These results support the CGF's clinical use for improving clinical outcomes in mixed ulcers of the legs.
Suggested Reviewers:	Roberto Cirocchi, MD Professor, University of Perugia roberto.cirocchi@unipg.it Expert
	Nicola Avenia, MD Professor, University of Perugia nicolaavenia@libero.it Expert
Opposed Reviewers:	

CGF IN THE TREATMENT OF THE MIXED ULCERS OF THE LOWER LIMBS: RANDOMIZED CONTROLLED MULTICENTRICAL CLINIC STUDY

Introduction

Concentrated Growth Factors (CGF) is a concentration of "second generation" autologous growth factors compared to platelet rich plasma (PRP) and represents a multifactorial stimulation system that uses all phases of the blood and can be used for the management and treatment of chronic skin ulcers.

The CGF has been shown to have an immediate hemostatic action and to be biocompatible, to accelerate epithelial regeneration (stimulating angiogenesis and promoting the synthesis of collagen, so it promotes tissue healing) and finally a high antimicrobial effect for the high concentration of leukocytes. (1-4)

Considerable progress in the management of chronic skin wounds has led to high rates of recovery (5-7). Nevertheless, further progress is needed to improve not only the effectiveness of treatment protocols but also the comfort and safety of patients.

The CGF represents an evolution of the PRP whose use is progressively spreading in the clinical field: both are based on the liberation of platelet growth factors, mainly contained in alpha granules, which exert a stimulating action on the reparative processes of skin lesions. Despite this, the studies published in the literature are few and often with modest cases or with protocols of use significantly different from center to center (8-10).

Materials and methods

The aim of the work is to evaluate the additional benefits of the CGF compared to the standard of dressing, consisting of "advanced" medications, and the effects of these on the dynamics of the healing process.

A multicenter randomized controlled trial was then performed on patients with venous ulcers and concomitant chronic obstructive arterial disease not candidate for revascularization (it is therefore about patients with venous ulcers with particularly slow and difficult healing times) consisting of the application of CGF in addition at a standard weekly medication, for a duration of 12 weeks (or less in case of ulcer healing) and followed by a 6 months follow-up. None of the patients enrolled underwent surgery for venous or arterial disease, because they were considered non-candidates or because the treatment was refused by the patient.

The observational study was preliminarily approved by the Istitutional Review Board Independent Ethics Committee (IRB-IEC) as the Ethics Committee of the Inter-University Center of Phlebo-lymphology (CIFL), with a positive opinion (protocol: ER: ALL.2016.03.A). The study was conducted through the recruitment of patients from 10 Italian vascular centers under the control of authors as members of the "SIMCRI Group for the study of CGF in the treatment of Mixed Ulcers", and coordinated by the group of researchers of the CIFL.

It should be noted that both comparative study groups have applied procedures already in clinical use. In particular, CGF is a modern method of preparing platelet gel that enriches the advanced medication of growth factors present in platelet granules (11-16).

END-POINTS OF THE STUDY

The primary end-point was identified in the reduction of the surface and volume of the ulcerative lesion within 12 weeks of treatment.

Secondary end-points were:

1. the presence and variation of the symptom of ulceration pain.

2. reduction of bacterial load during treatment.

Patients and inclusion criteria:

100 patients with chronic mixed ulcers of the lower limbs (whose presence dates back to at least 3 months) were recruited in the various Vascular Units participating in the study: the patients suffered for an ulcerative pathology of the lower limbs with a situation of chronic venous insufficiency and of chronic obliterative arteriopathy of the lower limbs of non-critical grade, for which no direct revascularizing therapy was indicated (arteriopathy at the II stage of the Rutherford classification, with an ankle / arm pressure index (ABI) between 0.90 and 0.50 at the affected limb.

All patients were evaluated with echo-color-doppler at the entrance to the protocol.

As a criterion for the correct management of the lesion, the principles of wound bed preparation have been applied. (17-19), for which, during the weekly medications, an accurate cleansing of the ulcer from necrotic material was carried out, under sterile conditions.

All the patients participating in the study were informed of the aims of the study and at the end of the information phase, an informed consent was given, then kept in the records by each participating center.

Once the informed consent was acquired, a study form based on a single model shared by all the participating centers was opened on behalf of the patient.

Preliminary exclusion criteria:

The exclusion criteria foreseen by the recruitment of patients were the following: recent outcomes of cardiac infarct, dialysis, serious infection of the lesion (with extension to the surrounding tissues), reduced life expectancy, cachexia.

The drugs aimed at treating the patient's basic pathology or co-morbidities are not considered exclusion criteria, except in cases where a significant change in therapy is required during the course of the study. In this case the reason for the exclusion from the study was reported.

Exclusion criteria during the study:

1. extension or onset of severe ulcer infection
2. amputation or revascularization surgery.

Ulcers examination

In order to make the characteristics of the lesion in terms of surface, presence of fibrinous material (slough) or granulation tissue comparable and quantifiable, photographic images of the lesions were acquired, subjected to the evaluation of two external, neutral observatories: at the entrance to the protocol and for each medication (both in the treatment and group phases in the monthly follow-up). Complete ulcer healing was defined by both clinical and photographic documentation of complete skin epithelialization.

Preparation of the CGF

CGF (concentrated growth factors) represents a concentration of growth factors that is produced by the patient's blood without the addition of exogenous substances through the use of a special centrifuge Phase Separator (Medifuge MF 200® - Silfradent, Italy).

After taking 10 ml of blood from an arm vein, under aseptic conditions, the blood centrifugation / separation process follows an automatic program with alternate speeds that prevent platelet degranulation. The centrifugation duration is 14 minutes, with the following program:

- 2 minutes at 2700 rotations per minute (rpm) (average value RCF 453.6), to obtain the separation of blood components;

- 4 minutes at 2400 rpm (average value of relative centrifugal force RCF = 358,4), to obtain cell lysis;

- 4 minutes at 2700 rpm (average value of relative centrifugal force RCF = 453.6), to obtain the aggregation of the components of the blood;

- 3 minutes at 3000 rpm (average value of relative centrifugal force RCF = 559,9), to obtain homogenization of blood components.

Other characteristics of centrifugation are:

1. Acceleration in about 29/30 sec. from 0 to 2700 rpm with Fg 25 (to avoid damage to the blood components);

2. Stop in 33 sec. from 3000 to 0 rpm with Fg 87/88 (to avoid mixing the blood fractions obtained);

3. Use of glass test-tube (CE certificate 0476) ("red cap") allows to obtain a solid product with characteristics of gel, for elastic and suturing membranes. After centrifugation a stratification of the product is observed: (1) an upper layer, which represents the liquid phase of the plasma, called platelet-poor plasma (PPP - Platelet Poor Plasma); (2) a lower layer, consisting mainly of red blood cells (RBC - Red Blood Cells); (3) an intermediate layer, dense and gelatinous, which represents the CGF.

The CGF and the RBC attached to it are extracted from the test tube and separated by means of a scissors, taking care to make the cut a few millimeters below the CGF / RBC interface (20,21).

4. Use of glass tube with red cap with addition of 18/20 units Sodium Heparin ("Sodium Heparin") to keep all phases in liquid state: allows to obtain different fractions (serum, fibrin-rich plasma and platelet-poor, fibrin-rich and platelet-rich plasma, leukocyte layers, mononuclear cells and fraction

with erythrocytes) in the liquid state, therefore not gelled. This tube "Sodium Heparin" allows to have the different fractions in liquid form at room temperature for about 8 hours at a temperature of 4 ° C for about 40 hours. In addition, Sodium Heparin aggregates the platelets for a better release of growth factors.

The regenerative properties of blood are related to the presence of platelets, leukocytes, mononuclear cells and growth factors. The platelets contain in fact numerous active substances that play an important role in tissue regeneration through the recruitment and activation of mesenchymal cells, such as osteoblasts, fibroblasts and endothelial cells. The active substances are located in different sub-cellular structures: alpha-granules, dense granules, lysosomes and micro-peroxisomes. Alpha-granules contain chemiotactic and mitogenic growth factors important in tissue regeneration, including PDGF (Platelet-Derived Growth Factor), TGF (Transforming Growth Factor))-beta 1 and -beta 2, VEGF (Vascular Endothelial Growth Factor), EGF (Epidermal Growth Factor), IGF (insulin-like growth factor) and FGF (Fibroblast Growth Factor) (22,23).

Randomization

Patients were randomized, according to a computer-generated procedure, by placing them in a group treated with CGF + fatty gauze (Group A) or in a second group (Group B) for which a standard treatment (control group) was provided.

Randomization was implemented by a single coordinator for randomization and it is indicated in the patient record.

Local ulcer therapy

Standard medication (group A):

The medication was carried out with the following protocol:

1. cleansing with physiological solution at a temperature between 34 and 37 ° C;

2. compress with Dakin's solution (sodium hypochlorite diluted 0.05%) for 15 min .;

3. application of the CGF gel on the wound;

4. coverage of CGF with gauze containing ac. hyaluronic acid;

5. subsequent coverage of the ulcer surface with an advanced absorbent and impermeable medication (Allevyn Adhesive ®);

6. application of a containment sock (day and night) and of an elastic knee sock 18 mm. Hg at the ankle (personal measure), only during the day.

Standard medication (group B):

The medication was carried out with the same protocol, devoid of only point 3, that is the application of CGF.

The medications were weekly for a total of twelve weeks, followed by a follow-up of a further twelve weeks during which a standard weekly medication will be applied to all patients, if the healing of the ulcers has not occurred; in case of recovery, elastic compression already in place has been prescribed (with monthly check)

Follow up

For both groups the medications and follow-up were carried out only at the participating centers, by the person responsible for the application of the protocol.

Failure to comply with the prescribed medication timing for an advance or delay of more than one day, or the accumulation of deviations from the scheduled medication cadence above a total of two days throughout the duration of the protocol, were considered lack of adherence to the protocol and reason for exclusion from the study.

Pain evaluation

Pain evaluation was performed at enrollment and at each medication change, evaluating pain intensity using the Wong-Baker scale, and recording its reported intensity on the card, in addition to site characteristics, duration, concomitant, and associated symptoms.

However, patients are given pain treatment through home care with non-steroidal anti-inflammatory drugs, as needed. (Tab. I)

Evaluation of the bacterial load

The evaluation of the bacterial load was performed, with semi-quantitative buffer, in all the patients enrolled in the study during the first medication (T0) and the subsequent medications after 3-6-9 weeks (T3-T6-T9).

Bacterial growth values of 4+ or higher were considered positive for wound infection, while lower values were considered indicative of non-infected wound.

STATISTICAL ANALYSIS OF RESULTS

Statistical analysis

All statistical analyses derived from this study were performed using Medcalc statistical software. Visual histograms and analytical methods (Student's t-test) were used for determination of normal distribution. The statistical significance threshold was set to * $P < 0,05$. Continuous variables were expressed as mean ± standard mean error.

Paired values for planimetric data, including wound area (as cm^2), were compared with paired samples t-test among consecutive times of this study period.

Results

Recruited patients (64 women and 36 males), mean age 68 +/- 8 years, had the onset of mixed ulcers (venous ulcers associated with arteriopathy) from an average period of 26 +/- 8 weeks, already undergoing treatment of medications for at least 4 weeks, with no tendency to heal. All patients were classified as grade 4 CEAP classification, with regard to chronic venous insufficiency, and as stage 2 of the Rutherford classification for arteriopathy. At the time of recruitment, they presented an area of ulcerative lesion of at least 4 cm^2, with confirmation echo-doppler sonography of the mixed venous and arterial pathology.

Among the basic characteristics of these patients, it should be noted that 15 of the recruited patients made use of oral antidiabetics and 5 of insulin therapy, while the use of tobacco was reported by 57 patients. Coronary artery disease was found in 31 patients and moderate renal failure in 12 patients. These conditions were found without significant differences in the two randomized groups. (Table II)

The treatment of weekly medications, according to the procedures for group A and group B of patients, associated with elastic-compression, was performed for 12 weeks in recruited patients.

In group A (patients treated with CGF, plus medication and elastic compression) the complete wound healing was found in all patients (53/53) during the first 12 weeks of observation: in 11 patients. (20.7%) after 5 weeks, in 14patients (26.4%) in 6 weeks, in 10 patients (18.8%) in 7 weeks, in 8 patients (15.0%) in 8 weeks, in 7 patients (13.2%) in 9 weeks, in 2 patients (3.7%) in 10 weeks, and in 1 patient (1.8%) in 11 weeks. The ulcers healing in group B (47 pts. treated with medication and elastic compression only) occurred in the first phase of the study (12 week interval from first observation) only in 32 out of this 47 patients (68% of cases), with the following timing: in 2 patients (4.2%) in the first 5 weeks; in 1 patient (2.1%) in 6 weeks, in 3 patients (6.3%) in 8 weeks, in 5 patients (10.5%) in 9 weeks, in 7 patients (14.8%) in 10 weeks, in 6 patients (13.9%) in 11 weeks, in 8 patients (18.6%) in 12 weeks, while in 15 patients (31.9%) wound healing was achieved over 12 weeks.

The timing of the healing times in the two groups of treated patients is described in Table III.

(Table III)

In the comparisons that were performed between the two groups in consecutive weeks, the surface of the ulcers did not show a significant reduction in the control after the first week ($p = 0.375$), while it was possible to evaluate significant reductions in all consecutive comparative measurements occurred between the first and second week ($p = 0.004$), and then between weeks subsequent ($p < 0.001$). (Table IV).

The overall evaluation of pain intensity by means of the Visual Analogue Scale (VAS), using the Wong-Baker scale, initially homogeneous in the two study groups (5.38 +/- 1.35 in Group A and 5.16 +/- 1.94 in group B) showed a significant improvement ($p < 0.001$) of pain symptoms in Group A patients compared to those of Group B, starting from the second week of treatment (Group A = 2.88 +/- 0.83, Group B = 5.26+ /-1.14), continued and increased in the following weeks (Table V), with the disappearance of the pain that preceded the healing of the ulcer on average 3.4 weeks. (Table V)

The evaluation of the degree of wound infection in the two groups of treated patients is described in Tab.VI. (Table V

No complication or undesirable effects of GCF have been observed in any of the Group A patients, such as skin irritation, pain or allergic reactions.

Cost benefit analysis

In the context of the cost / benefit analysis applicable to the use of the CGF for the treatment of difficult ulcers of the lower limbs, the average cost of the CGF application procedure in the study carried out was first evaluated. The costs relating to the application of the CGF consist of the following phases and have been evaluated as follows:

1. Peripheral blood collection (10 cc) in a dedicated sterile test tube: the collection was carried out by the nursing staff in charge of the ulcers' Peripheral blood collection (10 cc) in a dedicated sterile

test tube: the collection was carried out by the nursing staff in charge of the ulcers' surgery and includes the costs of the collection set and the special sterile test tube: 3.15 euro.

2. Specific centrifugation with phase separator centrifuge ((Medifuge MF 200® - Silfradent, Italy): the cost of centrifugation is represented by the depreciation of the appliance and can be considered of about 2.80 euro (use for 1500 procedures in average).

3. Aseptic preparation of CGF for topical application (canvas, gloves and other sterile handling materials): 1.55 euro.

The total cost of the procedure for applying the CGF as an additional treatment of ulcers of the lower limbs was therefore € 7.50.

In the analysis, then, of the time required for the procedure, there were found on average 3 minutes for collection, 14 minutes for centrifugation and a further 3 minutes for preparation and application on the wound, for an average total of 20 minutes. This time was not additional to the total medication time, as the preparation of the patient's medication took place at the same time as the centrifugation periods, so that the additional time required by the procedure for applying the CGF was about 10 minutes.

The onset of an ulcer that delays her healing is a condition of discomfort for each patient, both from the point of view of painful symptomatology and the impediments that limit their daily habits and social and work life.

Healing of a mixed ulcer of the lower limbs may take several weeks, and several trials have indicated the average healing time over 24 weeks, with the frequent risk of early recurrences (24-27)

The social costs of treatment of ulcers must also consider the costs of home self-medications, medical examinations and possible hospitalizations for specific procedures.

Furthermore, most patients belong to the category of geriatric patients and may have numerous co-morbidities that influence the course of the wound and interfere with the healing pathway (28,29)

A recent controlled critical review, produced with the Cochrane Collaboration methodology (30), indicates that there is limited evidence to support the fact that dressing modality and their frequency in the management of venous or mixed leg ulcers could be clinically significant on the time of healing and on the his stability: probably the limited number of trials, the lack of meta-analysis in this field and the poor quality in methodology does not allow to formulate recommendations with adequate levels of evidence, nor to define the comparative validity of the various types of treatment proposed.

The therapeutic effects of the application of autologous growth factors of blood derivation have found indead a wide space of application in various sectors of medicine and surgery (from odontostomatology, to orthopedics, or plastic surgery and also vascular surgery), both in vitro and in vivo, with various mechanisms: induction of cell proliferation, stimulation of inflammatory cells, increase of collagen deposition and angiogenesis, through the enhancement of enzymatic fibrinolysis and stimulation of protein synthesis (31-33).

Further analytical and interpretative assessments of the role of autologous growth factors are beyond the scope of this article, but further informations can be derived from future studies in this field.

Undoubtedly, personalized compression therapy (especially with low extensibility bandages, possibly through the execution of multi-layer type), in the context of mixed ulcers, and also for those exclusively of venous origin, represents a fundamental step in treatment, and is reported as a high-grade recommendation in all the guidelines produced in the vascular field.

Conclusions

The results of this multicentric study on the effect of CGF in the treatment of mixed ulcers of the lower limbs showed a significant advantage in the use of CGF in association with cleansing and selective compression in the healing and stabilization of such ulcers. Also the symptom pain was significantly reduced comparatively in the group of patients who were treated with CGF, and

furthermore the cost / benefit analysis of the use of this product indicates significant advantages in favor of its application.

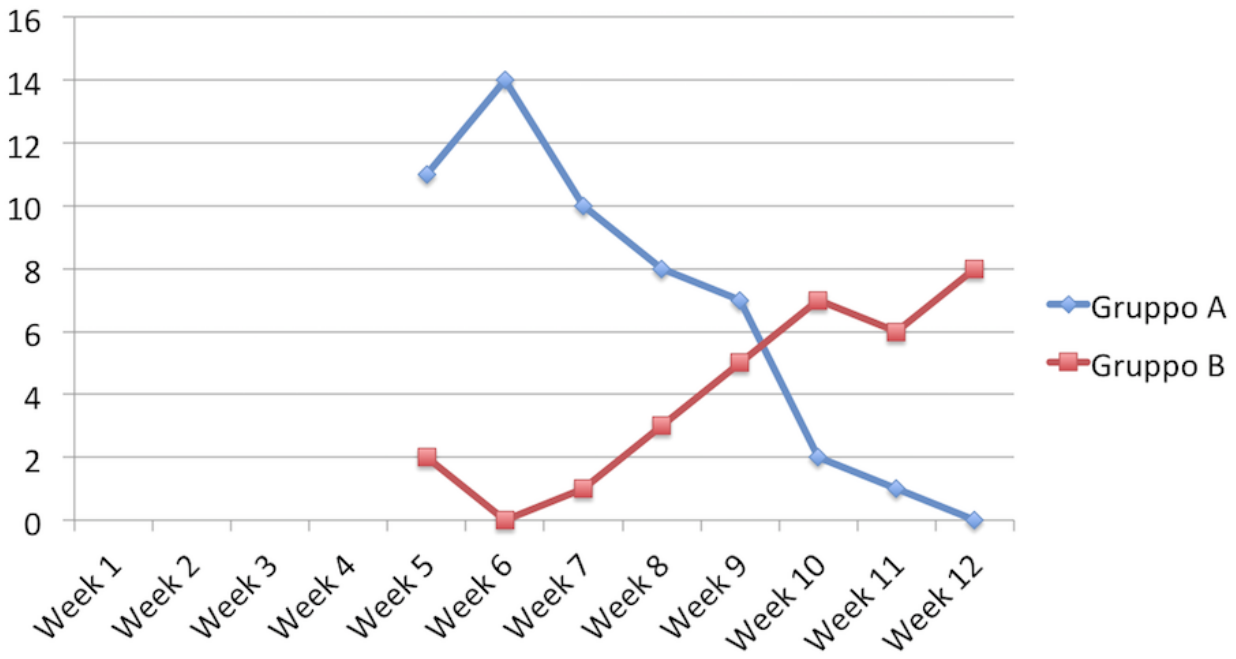
In the treatment of mixed ulcers, considered difficult to cure, CGF has therefore proved useful because it is effective in obtaining a faster, less painful, longer lasting and cheaper healing of the ulcer. For these reasons, the extension of treatment with CGF to all chronic ulcerative manifestations with a tendency to chronicization is considered valid by the authors.

Tables

TAB. I: Wong-Baker scale for the evaluation of the degree of pain.

Patients serie n.100	Group A n.53	Group B n.47	
M/F	M = 18 F = 35	M = 16 F =31	
Median age	M = 62 +/-9 F = 71 +/-12	M = 68 +/-5 F = 69 +/-10	
Ulcer size (cm2)	24 +/-16	22 +/- 9	

TAB. II - Clinical features of the two groups of patients with mixed ulcers, after randomization.



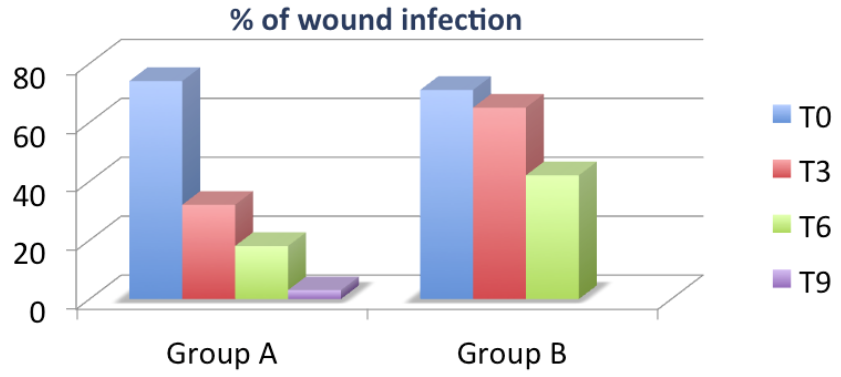
TAB. III - Time of wound healing (n. of ulcers / n. of weeks)

% reduction in wound area	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12
Group A (ulcers n. 53)	12	23	28	35	46 (42/53)	52 (28/53)	58 (18/53)	64 (10/53)	72 (3/53)	85 (1/53)	94 (1/53)	100
Group B (ulcers n. 47)	10	12	15	19	22 (45/47)	28 (44/47)	36 (44/47)	41 (41/47)	49 (36/47)	57 (29/47)	65 (23/47)	71 (15/47)
P- value for comparisons	P= 0.014	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001

Tab. IV: Median reduction of wound area for repeated comparisons through the study period in patients treated with CGF medication + compression (*Group A*) and standard medication + compression (*Group B*)

% reduction of pain	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12
Group A (n.53)	**5.38**	2.12	1.82	1.14	0.76 (42/53)	0.54 (28/53)	0.47 (18/53)	0.40 (10/53)	0.46 (3/53)	- (1/53)	- (1/53)	-
Group B (n.47)	**5.16**	4.78	4.55	3.81	3.22 (45/47)	2.65 (44/47)	2.52 (44/47)	2.41 (41/47)	1.96 (36/47)	1.88 (29/47)	1.57 (23/47)	1.45 (15/47)
P- value for comparisions		P= 0.01	P= 0.01	P= 0.01	P= 0.01	P= 0.01	P= 0.01	P= 0.01	P= 0.01			

Tab. V: Median reduction of pain for repeated comparision through the study period in patients treated with CGF medication + compression *(Group A)* and standard medication + compression *(Group B)*



% Grade of infection	T0	T3	T6	T9
Group A	**74%**	**32%**	**18%** (28/53)	**4%** (3/53)
Group B	**65%**	**58%**	**42%** (44/47)	**34%** (36/57)
P*	**P = 0.01**	**P = 0.01**	**P = 0.01**	**P = 0.01**

Tab. VI: Percentage of infection in ulcers of Group A (CGF) and Group B (controll) at first observation time (T0) and after 3 (T3), 6 (T6) and 9 (T9) weeks.

SUPPORTING INFO

AUTHORS:

Bruno Amato[1,2], Michele Angelo Farina[3], Silvana Campisi[4], Marino Ciliberti[5], Vincenzo Di Donna[6], Anna Florio[7], Antonino Grasso[8], Rosario Miranda[9], Francesco Pompeo[10], Eleonora Farina[3], Raffaele Serra[2-11], Francesca Calemma[1], Aldo Rocca[1], Rita Compagna[7-2]

Affiliations

1 Department of Clinical Medicine and Surgery – University Federico II of Naples, Italy – via S. Pansini, 5 - 80131 Naples, Italy
2 Interuniversity Center of Phlebolymphology (CIFL), International Research and Educational Program in Clinical and Experimental Biotechnology, Catanzaro, Italy
3 Vascular Surgery Unit, Villa Del Sole Hospital - Via Nazionale Appia, 35, 81100 *Caserta,* Italy
4Transfusion Immunohematology Service – S.Andrea Hospital – via Grottarossa 1035, 00192 Rome, Italy
5 Ulcer Service Program – ASL NA3 Sud - Corso Alcide De Gasperi , 167- *Castellammare Di Stabia* – Naples, Italy
6 *Medical Center Regeneration* Home - via della Macina, 60/A, Corato, Foggia,Italy
7 Department of Cardio-thoracic and Respiratory Sciences - University of Campania "Luigi Vanvitelli", Naples - via S. Pansini, 5 - 80131 Naples, Italy
8 Vascular Surgery Unit, University of Catania - "Policlinico - Vittorio Emanuele" P.O. G. Rodolico - Via S. Sofia, 78 *Catania, Italy*
9 Angiology Service ASL NA 3 Sud –Nola District, via Fontanarosa – 80035 Nola, Napoli , Italy
10 Vascular Surgery Unit, Neurologic Mediterranean Institute Neuromed, - Via Atinense, 18, 86077 Pozzilli, Isernia, Italy
11 Department of Medical and Surgical Science, University Magna Graecia of Catanzaro, Catanzaro, Italy

E-mail:
BA= bruno.amato@unina.it;
MAF= angiofar@libero.it
SC= silvana.campisi@virgilio.it
MC= <marinodoct@gmail.com>
VDD= vivido@vodafone.it
AF= anna.florio@unicampania.it;
AG= grasnin@hotmail.com
RM= rosmir@tiscali.it
FP= pompeofrancesco@yahoo.it
EF= eleonoraf77@hotmail.com
RS= profraffaeleserra@gmail.com;
FC=francescacalemma@yahoo.it;
AR=aldorocca88@gmail.com;
RC=rita.compagna@libero.it;

Correponding author:
Aldo Rocca MD, Department of Clinical Medicine and Surgery – University Federico II of Naples,

Italy – via S. Pansini, 5 - 80131 Naples, Italy

E-mail: aldorocca88@gmail.com;

Declarations:

List of abbreviations:

CGF = Concentrated Growth Factors
PRP = Platelet Rich Plasma
IRB-IEC = Istitutional Review Board Independent Ethics Committee
CIFL = Inter-University Center of Phlebo-Lymphology
RPM = rotations per minute
RCF = Relative Centrifugal Force
PPP - Platelet Poor Plasma
RBC = Red Blood Cells
PDGF = Platelet-Derived Growth Factor
TGF = Transforming Growth Factor
VEGF = Vascular Endothelial Growth Factor
EGF = Epidermal Growth Factor
IGF = Insulin-like Growth Factor
FGF = Fibroblast Growth Factor

Ethics approval and consent to participate

All patients underscribed an informed consent to be included in the study. Ethics committee approval approved the study (Ethics Committee of the Inter-University Center of Phlebo-lymphology (CIFL), with a positive opinion (protocol: ER: ALL.2016.03.A).

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request

Competing interests

The authors declare that they have no competing interests

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Authors' contribution

BA, MAF and RS: conception and design, acquisition of the data, gave the final approval of the version to be published
FC, AR, EF: drafted the manuscript, gave the final approval of the version to be published
SC,MC,VDD,AF,AG,RM and FP: critical revision, interpretation of data, gave the final approval of the version to be published

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