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Original Article

Effects of plasma rich in growth factors on wound healing in patients with venous ulcers

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ABSTRACT

Introduction: Significant evidence suggests that plasma-rich in growth factors (PRGF) favor the repair of chronic wounds, enabling a rapid return to functionality. However, components of PRGF and their effects on persistent ulcers and epithelial tissues are not well characterized. The goals of this research were to analyze the biological properties of platelet-derived factors, to examine their effectiveness on healing of venous ulcers, and to establish a correlation with clinical and sociodemographic data.
Methods: For the preparation of PRGF, the centrifugation technique was used, obtaining a 100 % autologous and biocompatible blood sample that was treated with sodium citrate and calcium chloride. The patients were attended weekly at the outpatient clinic for nursing consultation and wound dressing changes, with PRGF application every 15 days. The treatment protocols are described, and follow-up results are reported.
Results: Initially, the patients' ulcers ranged in sizes from 4 to 84 cm^2. After 12 weeks of treatment, there was a significant mean reduction of 46.2 % in ulcer area. At baseline, epithelial tissue was absent in all venous ulcers, but its presence grew significantly by the treatment period. However, the reduction of the area of the ulcers did not show significant correlation with the concentrations of the patient's growth factors.
Conclusions: Using the established protocol for PRGF isolating, it was possible to obtain a product with the presence of the six growth factors related to tissue regeneration and observed a positive response on wound healing following treatment of venous ulcers, with capacity to accelerate re-epithelialization and restore the skin functional integrity.

1. Introduction

The skin is essential in protecting the body against infections and water loss. Upon injury, immune and skin cells trigger a cascade of events to orchestrate the wound repair [1]. However, delayed or aberrant wound healing and tissue regeneration are common co-morbidities of major diseases including cancer, cardiovascular disease, and diabetes, as well as in patients with severe burn injuries [2].

Despite advances in the treatment of chronic ulcers, this condition continues to devastate the community of patients suffering

Abbreviations: PRGF, plasma-rich in growth factors; PRP, platelet-rich plasma; PRF, platelet-rich fibrin; PLG, platelet leukocyte gel; CP, platelet concentrate; PDGF, platelet-derived growth factor; TGF beta1, transforming growth factor-beta1; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; HGF, hepatocyte growth factor; EGF, epidermal growth factor; IGF-1, insulin-like growth factor-1; GF, growth factors.

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from micro and macrovascular afflictions [3]. Thus, we have observed that the continuous increase in health expenses, the development of new technologies and the changes in the epidemiological profile of populations have led to diversified needs for care.

Among the therapeutic and regenerative perspectives, biomaterials emerge as a promising approach and exhibit great potential in tissue repair and regeneration. The use of biomaterials for regeneration of tissues damaged by traumatic or pathological lesions is today well-established as a promising therapeutic approach, thanks to the ability of biomaterials with appropriate composition, textured structure and bio-competent mechanical performance, to instruct and guide endogenous or previously seeded stem cells toward appropriate differentiation and new tissue formation and remodeling [4]. In Regenerative Medicine, we can mention platelet-rich plasma (PRP), platelet-rich fibrin (PRF), platelet leukocyte gel (PLG), plasma rich in growth factors (PRGF), and platelet concentrate (CP) as important autologous biomaterials for the treatment of wounds [5].

The clinical use of plasma in the treatment of wounds is explained by the abundance and accessibility of growth factors contained in platelets, which promote tissue regeneration [6]. Thus, PRGF can be used as an intervention that allows the local application of autologous growth factors in order to stimulate the production of collagen and extracellular matrix, being an important alternative in the treatment of wounds, especially in cases where conventional treatments have not been successful [3]. Formulations containing plasma rich in growth factors (PRGF) are opening new avenues in the field of regenerative medicine [7].

Among the growth factors released by platelets isolated from plasma, some stand out in tissue repair, including platelet-derived growth factor (PDGF), transforming growth factor-beta1 (TGF beta1), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF, also known as FGF-2), hepatocyte growth factor (HGF), epidermal growth factor (EGF), and insulin-like growth factor-1 (IGF-1) [8]. In the treatment of chronic wounds, these growth factors favor the repair of injuries, allowing a faster return to functionality, possibly by stimulating vascularization, improving blood supply and availability of nutrients necessary for tissue regeneration [9].

Topical administration of growth factors has displayed potential in wound healing, but variable efficacy, high doses, and costs have hampered their implementation [2]. Although this is a promising treatment, there are still questions and reservations regarding its use. Sufficient evidence describing clear biological interventions is still lacking in published studies. Therefore, there is a need for a better description of treatment protocols, including the composition of the biological products and their respective clinical outcomes, e.g., wound healing time and rate [10]. In addition to conducting clinical studies with different platelet concentrations, one need to characterize the specific physiochemical factors that may be present in PRGF, in addition to the underlying mechanisms, as well as their respective beneficial effects on wound healing [10,11].

Previous studies have attempted to characterize and classify the various platelet concentrates using different preparation techniques (e.g., centrifugation speed and added anticoagulant), sample contents (leukocytes and/or growth factors) and application methods, yet without a clear consensus [12]. The goals of this research are (i) to analyze the biological properties of platelet-derived factors, (ii) to examine their effectiveness on healing of venous ulcers, and (iii) to establish a correlation with clinical and sociodemographic data.

2. Materials and methods

2.1. Ethical approval

Our research protocol complied with the Declaration of Helsinki and was approved by the Research Ethics Committee of the *Universidade Federal Fluminense* in Rio de Janeiro, Brazil (number 1.210.139). After signing a free informed consent form, the patients with venous ulcers who met the inclusion criteria were subjected to a weekly treatment regimen with PRGF.

2.2. Study design

This prospective study included 18 patients with venous ulcers treated at the Wound Repair Outpatient Clinic of Antonio Pedro University Hospital Health Center, Fluminense Federal University, RJ, Brazil.

Inclusion criteria: Older than 18 years of age, without gender distinctions; diagnosis of chronic venous insufficiency; Ankle Brachial Index (ABI) $\geq$ 0.9 to 1.3; presence of pulses in the lower limbs through palpation, specifically focusing on the dorsalis pedis and posterior tibialis; venous ulcer size between 2 cm^2 and 100 cm^2; presence of wounds with an evolution time of more than 12 weeks; hematocrit >34 %; hemoglobin >11 g/dL; and platelet count >150,000/mm^3.

Exclusion criteria: Pregnant or breastfeeding; alterations in prothrombin activation time or partial thromboplastin time; using corticosteroids; receiving immunosuppressive treatment or suffering from an immunosuppressive disease; suspected malignancy of the ulcer; non-adherence to the treatment plan; having received a blood transfusion in the preceding 3 months; and having a circular ulcer.

The patients were attended weekly at the outpatient clinic for nursing consultation and wound dressing changes, with PRGF application every 15 days. Wound valuations through manual planimetry and photographic records were performed at three time points, i.e., 1st, 6th and 12th weeks of treatment.

2.3. Collection and preparation of plasma rich in growth factors (PRGF)

In this study, for the preparation of PRGF, we used a centrifugation technique previously described by Anitua et al. (2008) [13]. From each patient, we obtained a 100 % autologous and biocompatible blood sample that was treated with sodium citrate and calcium chloride as anticoagulant and activator, respectively.

We collected 20 ml of blood in coagulogram tubes containing 3.2 % sodium citrate and centrifugated the samples at 1200 rpm for 10 min at room temperature, followed by the isolation of the plasma in new tubes. Subsequently, 1.5 ml of plasma were aliquoted in three microtubes for platelet counts and growth factor concentration analyses. To the rest of the plasma, 0.4 ml of 10 % calcium gluconate was added for platelet activation and subsequent application in the ulcer.

2.4. Platelet count

Platelet counts were performed on 0.5 ml of each plasma sample, using the automated method in a Cell-DYN hematological system (Abbott, USA).

2.5. Identification of growth factors

To identify the growth factors in our samples, we used the XMAP technology, through commercial kits from MILLIPLEX®. Twenty-

five microliters of each sample were used in duplicates and fluorescence was detected using the Bio-Plex Magpix® system (BioRad, USA). Emitted fluorescence spectra were converted to concentrations of EGF, FGF2, PDGFab, PDGFbb, VEGF and TGF-beta from standard curves using Exponent 3.0 software.

2.6. Treatment procedure

Each patient's wound was irrigated with 0.9 % saline and application of PRGF on the wound bed creating a thin layer (1–2 mm) [14]. After 5–8 min, the wound was covered with dry sterile gauze and hydrated with NDERM® (Viemed) cream. The dressing was fixed using cotton bands and finished with compression therapy using an elastic bandage.

Every 24 h the dressing was changed according to the home protocol. The wound was irrigated with 0.9 % saline, dressed with sterile petrolatum gauze ADAPTIC® (Systagenix) and supported by dry sterile gauze and hydrated with NDERM® (Viemed) cream. The dressing was fixed using cotton bands and finishing with compression therapy using an elastic bandage.

The mechanical debridement was performed only at the outpatient clinic, when necessary. All patients received kits containing dressing materials (i.e., the sterile petrolatum gauze ADAPTIC® (Systagenix), sterile dry gauze, NDERM® (Viemed) cream, cotton bands, and compression materials, in addition to written guidance offered in an informative leaflet about daily home dressing changes and the correct application of elastic compressive bandages.

2.7. Statistical analysis

Statistical analysis was performed with SPSS program, version 22.0, using the Wilcoxon, Friedman, Spearman's correlation, Shapiro-Wilk and ANOVA tests, considering a significance level of 5 % (0.05).

Descriptive analysis was based on graphs, frequency distributions, cross tables and calculation of descriptive statistics (proportions, minimum, maximum, mean, median, standard deviation, and coefficient of variation - CV). The variability of the distribution of a quantitative variable was considered low when CV < 0.20; moderate when 0.20 ≤ CV < 0.40 and high when CV ≥ 0.40. The distribution of frequencies in classes of a quantitative variable was obtained following the determination of the number of classes by the Sturges formula ($n_c = 1 + 3.32\ logn$) and the range of classes was defined by por $h = Range/n_c$.

3. Results

All 18 patients completed the study, and their characteristics are summarized in Table 1. No treatment-related adverse events were reported, and no patients withdrew from the study.

We analyzed the general area covering each participant's ulcers, verifying that not all participants had a single ulcer. Thirty-one ulcers were observed in 18 patients, and the number of ulcers per participant ranged from one to five, with a mean of 1.7.

Initially, the ulcer sizes ranged from 4 to 84 cm^2, with a median of 22.0 cm^2. After 12 weeks of treatment, there was a significant mean reduction of 46.2 % in ulcer areas, Table 2, ($p = 0.001$, Wilcoxon test).

At baseline, epithelial tissue was absent in all venous ulcers, but its presence grew significantly at the end of treatment (mean 58.1 %, $p < 0.001$ – Wilcoxon test). Devitalized tissue decreased throughout the study with a significant reduction at the end of 12 weeks (mean 11 %, $p = 0.001$ – Wilcoxon test). In relation to the granulation tissue, there was an increase in the first six weeks, but the percentage decreased significantly in the last six weeks (mean 38.2 %, $p = 0.046$ – Wilcoxon test), with the increase in epithelization tissue.

Table 1
Characteristics of patients.

Patient characteristics	*N*	Percent
Demographic data		
Age Mean (years)	62.0 [41–83]	
Sex (F/M)	6/12	33.3/66.7
Body Mass Index Mean (kg/m^2)	30.0 [20–53]	
Smoking (habit of)	4	22.2
Comorbidities		
Chronic venous insufficiency	18	100.0
Hypertension	13	72.2
Diabetes mellitus	9	50.0
Reference ulcer		
Common sites	Malleolus	72.2
Ulcers per Patient Mean	1.7 [1–5]	
Duration of current ulcer	>10 years	77.7
Surface at Baseline Mean (cm^2)	29.6 [4–84]	

The amount of exudate from the ulcers was observed in 25–75 % of the gauzes in the first week, less than 25 % of the gauze after six weeks of treatment, and the total absence of exudate being observed in the healed ulcers. Thus, a significant decrease in the amount of exudate in the ulcers was observed after 12 weeks. Figs. 1 and 2 show before and after pictures of the venous ulcers of 16 patients.

3.1. PRGF characterization

The characterization of the PRGF products obtained showed an average number of platelets of (125.2 ± 61.2) × 10^3 per mm^3, $p = 0.05$. The specific growth factors (GF) contained in the isolated PRGF were also assessed. The mean concentrations of GF were PDGF-AB (1.5 ± 0.62 ng/ml, $p = 0.061$), PDGF-BB (1.3 ± 0.59 ng/ml, $p = 0.074$), TGF-β (1.4 ± 0.49 ng/ml, $p = 0.320$), VEGF (0.9 ± 0.83 ng/ml, $p = 0.0001$), EGF (1.5 ± 0.88 ng/ml, $p = 0.001$) and FGF2 (1.0 ± 0.88 ng/ml, $p = 0.0001$).

However, the absolute reduction (cm^2) and the relative reduction (%) of the area of the ulcers in the total period of 12 weeks did not show significant correlation with the concentrations of the patient's growth factors ($p > 0.05$, Spearman's correlation $r < 0.3$, Table 3).

4. Discussion

Leg ulcers generally tend to become chronic and difficult to cure. In addition, in many patients, conventional treatments are ineffective, increasing the comorbidity associated with this condition [15]. Scientific studies show that 88 % of wounds heal when underlying diseases are treated and when good care is established. For the remainder (12 %), advanced strategies should be considered for healing to occur. Advanced strategies aim to influence the bioactive environment of wounds, such as reducing pH values, applying extracellular matrix, metal-protease binding matrix or increasing the level of growth factors have been used [16]. The time of evolution of these wounds and consequently the suffering of patients motivates the search for new methods of treatment. In this perspective, PRGF could be considered as a therapy option indicated for the treatment of wounds, mainly when conventional strategies do not yield satisfactory results. There is growing evidence regarding platelet-based autologous therapies that support their use in promoting cutaneous regeneration [17].

Table 2
Analysis of the ulcer area of the 18 patients in three moments.

	Evaluation (cm^2)			Analysis		
	Initial	6 weeks	12 weeks	Initial-6 weeks	6–12 weeks	Initial-12 weeks
Minimum	4	2	0	Average	Average	Average
Maximum	84	66	70	Reduction	Reduction	Reduction
Median	22.0	14.0	11.0	26.4 %	33.3 %	46.2 %
Mean	29.6	21.8	18.3			
SD[b]	23.9	21.2	20.7	*p*-value[a]	*p*-value[a]	*p*-value[a]
CV[c]	0.81	0.97	1.13	**0.003**	**0.006**	**0.001**

[a] Wilcoxon Test.
[b] Standard deviation.
[c] Coefficient of variation.

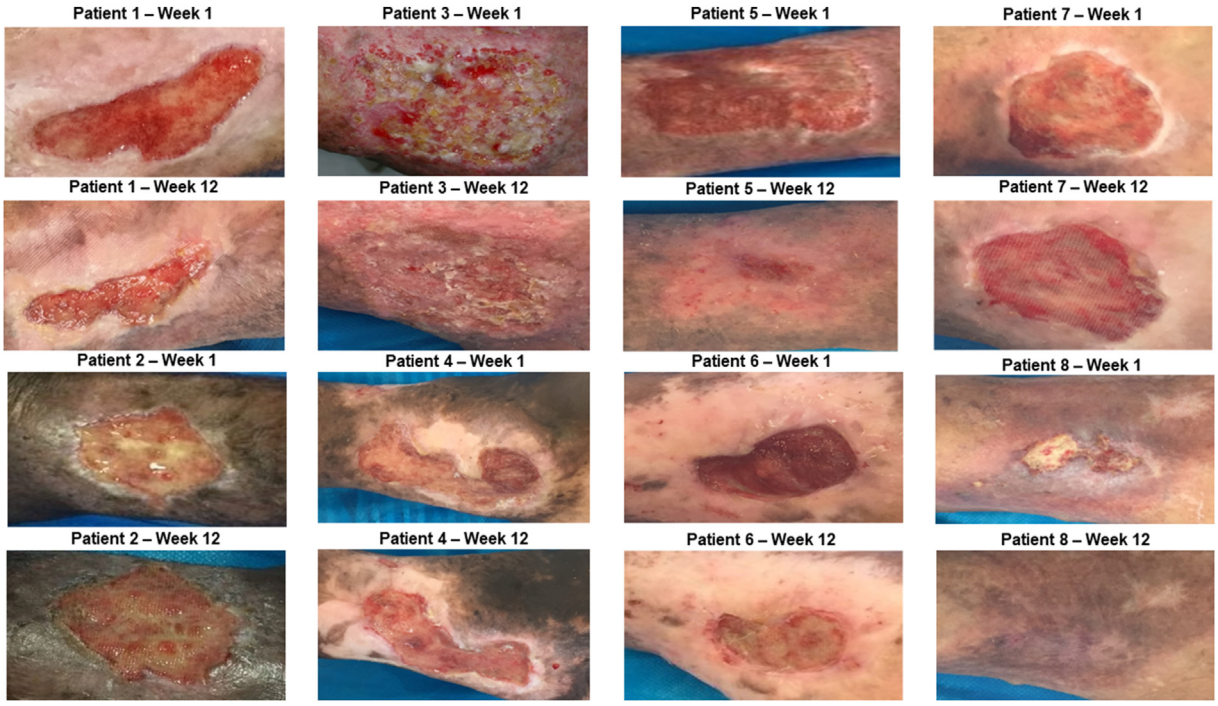

Fig. 1. Images of venous ulcers of patients 1 to 8 at two time points, i.e., before treatment (week 1)) and at the end of treatment (week 12). P1 (W1): ulcer with predominance of granulation tissue; P1 (W12): ulcer with areas of granulation and epithelialized perilesional; P2 (W1): ulcer with predominance of devitalized tissue; P2 (W12): ulcer with predominance of granulation tissue and smaller devitalized area; P3 (W1): ulcer with predominance of devitalized tissue; P3 (W12): ulcer with granulation tissue and healed areas; P4 (W1): ulcer with predominance of devitalized tissue; P4 (W12): ulcer with predominance of granulation tissue and healed areas; P5 (W1): ulcer with predominance of granulation tissue; P5 (W12): ulcer with predominance of healed area; P6 (W1): ulcer with predominance of granulation tissue; P6 (W12): epithelialized perilesional area; P7 (W1): ulcer with predominance of devitalized tissue; P7 (W12): ulcer with predominance of granulation tissue; P8 (W1): ulcer with predominance of devitalized tissue; P8 (W12): healed ulcer.

Regarding the protocol for obtaining PRGF, we used a method that allows a low-cost preparation in an outpatient setting, previously described by Anitua et al. (2008) [13]. The ease and speed of obtaining samples are favorable characteristics, but not all techniques developed are accessible. Considering the lack of consensus in the literature for a more effective protocol, a simple and easily reproducible technique was chosen, since it would be carried out in outpatient settings and the process of obtaining and applying it would occur during the consultation with a nursing staff.

In the evaluation of the PRGF samples, it should be noted that there was a high variability in the concentration of growth factors, as well as in the platelet count, showing an interpersonal variability in the release of growth factors and indicating that the composition of the PRGF is individual. Even if the platelet count is not so high, it is possible to obtain a product with the main growth factors involved in healing, which is very positive, since the combination of growth factors present in platelets is mentioned as more important than the concentration of individual factors, because the application of multiple growth factors is more similar to the natural healing process [15,18].

Platelet-rich plasma (PRP) includes the combination of seven growth factors transported in the same concentrations found in normal clots (200–400 μg/ml) [19]. Although they are released by platelet alpha granules, these mediators have defined moments of activity. One of the hypotheses put forward is that platelet granules are not uniform, and that certain factors, especially α-granule cargoes, may be differentially packaged and thus released "thematically" [20]. Its clinical application was endorsed by evidence that several major growth factors are contained at high levels in PRP preparations [21]. However, for some reasons, such as low handling efficiency and fundamental individual differences, it has been indicated that it is difficult to reproducibly control the quality of PRP preparations at similar levels. To overcome these drawbacks, it was developed the plasma rich in growth factors (PRGF), by

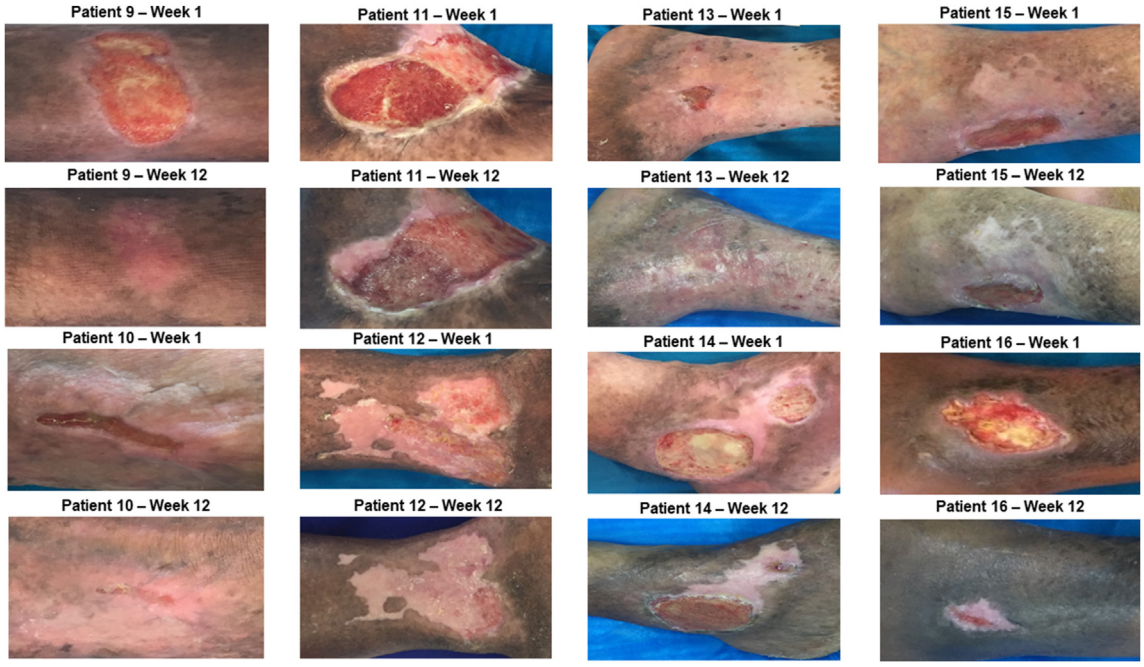

Fig. 2. Images of venous ulcers of patients 9 to 16 at two time points, i.e., before treatment (week 1) and at the end of treatment (week 12). P9 (W1): ulcer with predominance of granulation tissue; P9 (W12): healed ulcer, P10 (W1): ulcer with predominance of granulation tissue; P10 (W12): ulcer with healed perilesional area; P11 (W1): ulcer with predominance of granulation tissue; P11 (W12): ulcer with predominance of epithelization tissue; P12 (W1): ulcer with predominance of epithelization tissue; P12 (W12): ulcer with predominance of healed area; P13 (W1): ulcer with predominance of granulation tissue; P13 (W12): healed ulcer; P14 (W1): ulcer with predominance of devitalized tissue; P14 (W12): ulcer with predominance of granulation tissue, with healed areas; P15 (W1): ulcer with predominance of granulation tissue; P15 (W12): ulcer with epithelialization and granulation tissue; P16 (W1): ulcer with predominance of devitalized tissue; P16 (W12): ulcer with predominance of healed area.

Table 3
Correlation analysis between total wound reduction at 12 weeks and total concentration of growth factors.

Correlation coefficient	Total concentration of growth factors					
	PDGF-AB	PDGF-BB	VEGF	EGF	FGF2	TGFβ
Absolute reduction in 12 weeks	−0.15 ($p = 0.547$)	−0.24 ($p = 0.339$)	−0.14 ($p = 0.587$)	−0.17 ($p = 0.504$)	−0.19 ($p = 0.455$)	−0.27 ($p = 0.283$)
Relative reduction in 12 weeks	−0.03 ($p = 0.893$)	−0.10 ($p = 0.704$)	−0.14 ($p = 0.575$)	−0.06 ($p = 0.829$)	−0.31 ($p = 0.215$)	−0.12 ($p = 0.638$)

modifying the procedure of PRP preparation [21]. Gene therapy is under development for more efficient growth factor delivery; a single application will induce constitutive growth factor expression for weeks [22].

It has been previously reported that platelet growth factors are intimately involved with wound healing [11]. However, the specific factors that are deficient in stagnant or difficult to heal wounds are not known. An analysis done on fluids from chronic wounds showed that there was no difference in the levels of PDGF, EGF, FGF-β and TGF-β between ulcers in the healing process and those in the stagnation phase of healing [18].

In this research, the average initial size of the ulcers was 29.6 ± 23.9 cm^2 and the average percentage of area reduction after 12 weeks of treatment was 46.2 %. The size of the ulcers after treatment was significantly smaller than the initial ulcer size ($p = 0.001$, Wilcoxon test). However, the reduction in ulcer area after 12 weeks did not show a good correlate with PRGF growth factor concentrations.

In effectiveness studies, the healing rate is usually evaluated. However, in clinical practices, tissue improvement and reduction of the affected area are more realistic goals in the short term, especially in chronic wounds that present greater difficulty in healing [23]. Positive responses are just as important as healing. In the present study, 83 % of the ulcers showed reduction of the injured area, with complete healing in 17 % of the cases, showing significant improvements and desirable results.

The participation of a center specialized in the treatment of wounds, with qualified professionals, intensive patient education and regular care for ulcers is a differential for obtaining positive responses in the tissue repair process. Overcoming the factors that contribute to delayed healing are key components of a comprehensive approach to wound care and present the primary challenges to the treatment of chronic wounds [24].

5. Conclusions

Current trends indicate a growing interest among healthcare specialists and the public in the use of regenerative medicine-based therapies for skin regeneration and cutaneous wound healing [25]. In this way, using the established protocol for obtaining PRGF, it was possible to obtain a product with the presence of six growth factors of relevance to tissue regeneration, with a potential capacity to accelerate re-epithelialization and restore the skin functional integrity.

A positive response was identified in relation to the healing of treated venous ulcers, even though without correlation with the individual concentration of growth factors in the biomaterial.

Although the healing rate is used to assess the effectiveness of treatments in clinical practice, tissue improvement and area reduction are realistic short-term goals, especially in chronic wounds. In this case, it is relevant to consider the positive responses in the tissue repair process. Despite their many confirmed advantages, further studies on the use of growth factors for the treatment of venous ulcers are warranted, and additional randomized controlled trials with larger patient populations are needed to further establish their efficacy.

Declaration of competing interest

We declare that this material is original research, has not been previously published and has not been submitted for publication elsewhere while under consideration and there is no conflict of interest.

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