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Biostimulation of venous chronic ulcers with platelet-rich plasma gel and biocompatible membranes of chitosan and alginate: A pilot study

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ABSTRACT

Venous ulcers are a common disease caused by circulatory insufficiency, having as characteristics slow healing and therefore a difficult treatment. Consequently, this disease has a high impact at personal, professional and social levels, representing large costs for both patients and healthcare system. The aim of the present pilot study was to evaluate the effectiveness of an alternative treatment for this specific type of wound, based on the use of autologous platelet-rich plasma gel (PRP) combined with biocompatible porous membranes produced with the polysaccharides chitosan (C) and alginate (A). The applicability, security and healing efficacy of this treatment was evaluated in four patients suffering from venous ulcers with an at least 6-month of evolution that did not heal after conventional treatments. The clinical evolution analysis during the treatment was performed by periodic measurement of the lesions area for an average of 5.4 months. The results attained showed that the use of this treatment led to completed reduction in the ulcers area as well as to pain decrease. Hence, the combination between autologous PRP gel and chitosan-alginate (CA) porous membranes could be a useful alternative to treat chronic skin ulcers in humans.

1. Introduction

Chronic ulcers are skin lesions involving epidermis, dermis, adipose tissue and muscular fascia, having diverse etiologies that include vascular, neurological or endocrine disorders. Venous ulcers originate as a chronic inflammatory response to a combination of vein wall architecture and cellular abnormalities, as well as varicose vein formation factors. These lesions are characterized by overproduction of proinflammatory cytokines and alterations in its healing mechanisms. These wounds result from changes in collagen synthesis and increased levels of metalloproteinases, which contribute to collagen degradation [1]. Frequently, this type of lesions are directly associated to deterioration in the patient quality of life, due mainly to pain in lower limbs and wound discharge, resulting in social isolation, emotional stress and depression [2].

Chronic ulcers are an important problem also because of the risk of infection and sepsis, which can lead to hospitalizations associated with an increase in patient mortality. In addition, these lesions are often implicated in rises of direct costs related to health services, estimated at $ 11 billion per year for the United States [3,4]. Indirect costs are also increased, e.g. due to medically-related employee absence to work and work-loss due to disability. Therefore, the development of new treatments for chronic ulcers is attractive both from the point of view of improvement of patient life-quality and from potential reduction of the costs related to the problem.

Typical management of venous ulcers is based on the treatment of venous stasis associated with rest and compression bandaging of the leg to favour healing. Another strategy is based on the application of local drugs and dressings to keep the wound bed moist and clean, topical antibiotics and alginate, foam, hydrocolloid, hydrofiber, and hydrogel dressings are frequently used for this purpose. There is no evidence to support the use of systemic antibiotics in case of infected ulcers [5,6].

Abbreviations: PRP, platelet-rich plasma gel; C, chitosan; A, alginate

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Recently, new therapeutic strategies are being established, which include: skin replacement using biological skin substitutes, treatment with laser, hyperbaric oxygen, electrical stimulation, negative pressure dressings and use of growth factors, especially when patients do not respond to conventional treatments or if the ulcers are recurrent [7].

Polymeric materials of natural origin have been used in the production of dressings and other biomaterials due their biocompatibility and biodegradability properties [8]. The combination of chitosan (C) and alginate (A), particularly, can result in dressings in the form of membranes with adequate mechanical properties in a wide range of relative humidity conditions [9]. Porous CA membranes may be obtained mixing both polysaccharides in controlled conditions and depending on the production method, satisfactory liquid uptake capacity values can be achieved [10]. Furthermore, CA membranes may accelerate cutaneous wound healing in the earlier phases and improve the quality of the scar tissue [11]. These membranes can be combined with natural and synthetic bioactive agents such as growth factors [12], antimicrobials [13,14], antibiotics [15] as well as anti-inflammatory and wound healing compounds [16,17], and in this work, they were associated with platelet-rich plasma (PRP).

PRP is a platelet concentrate with both mitogenic and chemotactic properties thanks to degranulation of alpha-granules, which allows the release of growth factors such as platelet derived growth factor, transforming growth factor β, vascular endothelial growth factor, epidermal growth factor, platelet derived endothelial growth factor and insulin-like growth factor. Further, secretion of molecules such as fibrinogen, fibronectin, vitronectin, osteocalcin, thrombospondin-1 and interleukin-1 is observed when PRP contacts an injured site. These proteins favour cell differentiation and proliferation, promoting additionally the formation of a new extracellular matrix, which plays an important role in re-epithelization and neovascularization process [18,19]. Several researchers have evaluated the efficacy of PRP in the treatment of wounds, showing promising results [19–23], however, further studies are needed to standardize clinical protocols and understand the mechanisms of wound regeneration mediated by the PRP.

In this sense, the objective of this pilot study was to evaluate the clinical efficacy of the simultaneous application of autologous platelet rich plasma and porous chitosan-alginate membranes in the treatment of four patients with venous ulcers Further, the individual responses of the patients regarding the perception of pain, ulcer infection and allergic reaction to the treatment were also assessed.

2. Materials and methods

This study was done between January 2016 and February 2017. All subjects enrolled in this research responded voluntarily to an informed consent formulary previously approved by the Ethics Committee of the Antonio Nariño University, registered with the number 2015066.

2.1. Type of study

This prospective pilot study, included patients who came from wound clinics of two hospitals in Bogotá, Colombia. The subjects of this study were patients suffering from at least one chronic ulcer with more than six months of evolution, aged between 43 and 76 years. The patients were classified according to their risk by the Norton Scale and reassessed at each treatment session, regarding general condition and ulcers area.

The treatment and follow-up were carried out at the Antonio Nariño University in Bogotá. The treatment consisted in the application of autologous platelet-rich plasma gel on the wounds. After that, the ulcers were immediately dressed with porous chitosan and alginate membranes. At the beginning of each session, the surface of the ulcer to be treated was washed with physiological saline solution, and then the injured area was measured, and its morphology was assessed. The ulcers were evaluated for signs of superinfection, presence of granulation tissue, fibrin and tissue remnants.

The treatment was applied with intervals of at least 15 days and maximum of 90 days, depending on each patient evolution. Clinical evolution, however, was evaluated every seven days. In each new application of the treatment, the PRP and the membrane dressings were left in contact with the ulcers for 7 days. After that, the lesions were washed with saline solution and covered with Cuticell (BSN medical®), to keep the wound moist and protected from dust.

The patients received strict instructions about wound cleaning procedures during the treatment period. Wound washing was performed daily by the patients themselves, at their own homes, by using neutral soap and potable drinking water, followed by coating with Cuticel dressing (BSN medical®). Ulcer progression was analysed by photographs (Canon Power Shot SX530HS) and each patient follow up was performed periodically.

Compression stockings were not used during the treatment due to the possibility of crushing both the PRP and the porous membrane. However, instructions were given to the patients to make active pauses and to lift the lesioned leg at least once every two hours during the study. At the end of the treatment (when ulcers were completely closed), the use of compression stockings was recommended to the patients.

2.2. Patient inclusion and exclusion criteria

The following criteria were used to select the patients for the study: formal agreement manifested by signing an informed consent form; age above 18 years; hematological test with normal platelet counts; occurrence of at least one sub poplite or malleolar ulcer, superficial or deep, with no commitment of the fascia, with more than six months of evolution and no greater than 20 cm^2; ultrasound doppler duplex or triplex results compatible with superficial venous insufficiency (SVI) or chronic venous insufficiency (CIV); ankle brachial index (ABI) between 0.8 and 1.5; absence of severe arteriopathy with compromise of major arterial vessels and/or ischemic index less than 0.7 on arterial doppler ultrasound.

The exclusion criteria were mainly related to the pathologies that might compromise the patient healing capacity, such as clotting disorders; chronic infectious diseases; uncontrolled severe hypertension; ABI below 0.8 or above 1.5; renal insufficiency defined by creatinine level greater than 1.2 mg/dl; systemic administration of corticosteroids or chemotherapy in the 3 preceding months; vasculitis or connective tissue diseases; history of cancer; treatment with chemotherapy or radiotherapy; myeloid or lymphoproliferative syndrome with hemoglobin below 11 mg/dl; or wound with bone exposition.

2.3. Blood collection and production of platelet-rich plasma gel

Blood samples were collected by venipuncture from the cephalic vein. Eleven tubes of 4.5 mL with sodium citrate (Vacutainer® Ref 369714; BD Biosciences) were collected from each patient. The protocol employed by Gómez et al. was used to obtain the PRP gel [24]. Briefly, the blood samples were centrifuged (ThermoscientificSorvall ST16 centrifuge) for 10 min at 240 *g* at 20 °C. Thereby from 49.5 mL of blood, it was possible to obtain 8 mL of PRP. For the preparation of the gel, the platelet-rich plasma of each patient was transferred to a Petri dish (90 × 14 mm) and 10% calcium gluconate was added in a proportion 1/20 (v/v), and incubated at 37 °C, to allow that the fibrinogen contained in the plasma to be converted into fibrin, forming a stable gel. This is stable about an hour without losing its consistency, what favours its adhesion to the membrane. In general, the clot was obtained 30 min after addition of platelet activator on PRP. To avoid microbiological contamination, the PRP was processed inside a biological safety cabinet. The PRP gel obtained was transferred directly from the Petri dish to the wound surface. The porous chitosan and alginate membranes were cut depending on the area of the ulcers and were applied on the

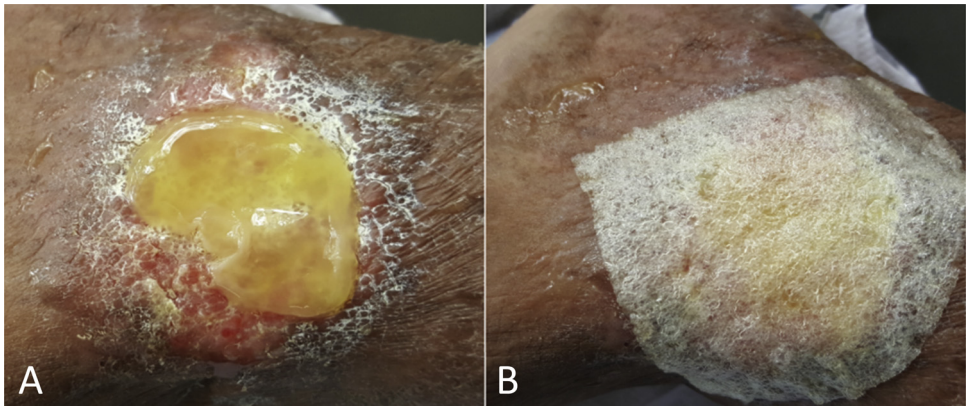

Fig. 1. Typical aspect of the wound subjected to the treatment. A) Wound coated with PRP gel; B) chitosan-alginate membrane set upon the wound coated with the PRP gel.

PRP gel (Fig. 1). Finally, the porous CA membrane was immobilized with medical adhesive tape (3M™), on its edge.

2.4. Production of the membranes

The porous membranes were produced using chitosan (Sigma-Aldrich, lot # 109K0043 V, deacetylation degree of 96%), sodium alginate of medium viscosity from *Macrocystispyrifera* (Sigma-Aldrich, lot # 058K0126), Kolliphor® P188 (Sigma-Aldrich, lot #SLBL0262 V), glacial acetic acid, calcium chloride dihydrate and sodium hydroxide (Merck). The water used throughout the work was distilled and deionized in a Milli-Q system (Millipore).

Porous chitosan-alginate membranes were prepared based on the procedures described previously [10,25]. Chitosan solution at 1% (w/v) (180 mL, in 2% v/v acetic acid) was mixed with 360 mL of sodium alginate solution at 0.5% in water containing 0.4 g of the surfactant Kolliphor® P188, at 25 °C, under stirring first at 500 rpm, and afterwards, at 1000 rpm for 10 min. Then, 26.0 mL of NaOH aqueous solution at 2 M and 7.2 mL of $CaCl_2$ aqueous solution at 2% (w/v) were added to the suspension. The mixture was then transferred to four polystyrene Petri dishes (15 cm in diameter) and dried at 60 °C for 6 h. Following, the resulting membranes were immersed in 150 mL of 2% (w/v) $CaCl_2$ aqueous solution for 30 min and then washed twice with 200 mL of deionized water. A final drying procedure was performed at room temperature for 24 h and the membranes were sterilized by exposure to 30% ethylene oxide and 70% carbon dioxide for 8 h at 40 °C at a relative humidity of 30–80% at Acecil Central de Esterilização Comércio e Indústria (Campinas, SP, Brazil).

The porous membranes showed the properties summarized in Table 1. Their typical aspect is shown in Fig. 2, in which the inner porous structure is depicted, as well as the integrity of the biomaterial surface (visual aspect photography taken with a digital Nikon camera, COOLPIX S3300 (Fig. 2, panel A), and scanning electronic microscopy images obtained using a Leica scanning electron microscope, LEO 440i after sample coating with a thin gold layer (Fig. 2, panel B and C)).

Table 1
Physicochemical characteristics of the porous chitosan-alginate membranes used in the study.

Property	Value ± Standard Deviation
Thickness of dry membranes (mm)	0.47 ± 0.02
Thickness of wet membranes (after 24 h in water at 37 °C) (mm)	0.95 ± 0.01
Swelling in saline solution (after 24 h at 37 °C) (g/g)	30.0 ± 0.8
Mass loss in saline solution (after 7 days at 37 °C) (%)	14.0 ± 0.5
Tensile strength (MPa)	3.9 ± 0.6
Elongation at break (%)	2.2 ± 0.3

2.5. Wound assessment

To quantitatively assess wound healing kinetics, both width and length of the ulcer was made by measuring, always using with the same ruler pattern (photographs were taken with Canon Power Shot SX530HS, 16.0 megapixels). Images were analysed with the free access software Image J (1.45 version). To calculate the wound area, a calibration was performed with the first 5 cm of the ruler pattern used in all photographs.

3. Results

3.1. Overall assessment

All patients were in good mental health and attended the treatments accompanied by a family member. In all of them the treatment consisted of application of autologous PRP gel and porous dressing chitosan-alginate membranes. The overall results are summarized in Table 2 and details on each case are given subsequently.

In this pilot study four female patients, with average age of 54.8 years (ranging from 43 to 76 years), suffered from refractory venous ulcers at their inferior limbs. All of them, previously received treatment with conventional therapy that consisted of local coverage and control of infection with systemic antibiotic therapy, showing no clinical recovery. Three patients had a single lesion and one patient had two lesions on the same leg, therefore five ulcers were treated in total. The lesions ranged in size from 1.7 to 13.14 cm^2, with initial mean area equal to 3.73 cm^2 ± 5.3 and having a previous evolution time of 6 months to 10 years.

The recovery percentage was 100% in four of the five injuries. In all these patients, wounds remained healed during 6 months of follow up. Although one of the lesions had a cure ratio of only 18.6%, however, it was completely re-epithelialized at the end of treatment. Formation of granulation tissue was promoted in all patients since the first application of the PRP gel and of the CA membrane. These results were reached after a mean of three treatment sessions (ranging from 2 to 5), after the wounds healed in an average of 5.4 months (varying from 2 to 9 months).

Additionally, all patients reported a decrease in pain intensity since the beginning of the treatment. The patients rated pain according to a visual pain analogous scale of 1–10. We also found that exudate amount and bad smell of wounds decreased significantly after the first treatment.

Nevertheless, two patients presented localized minor wound

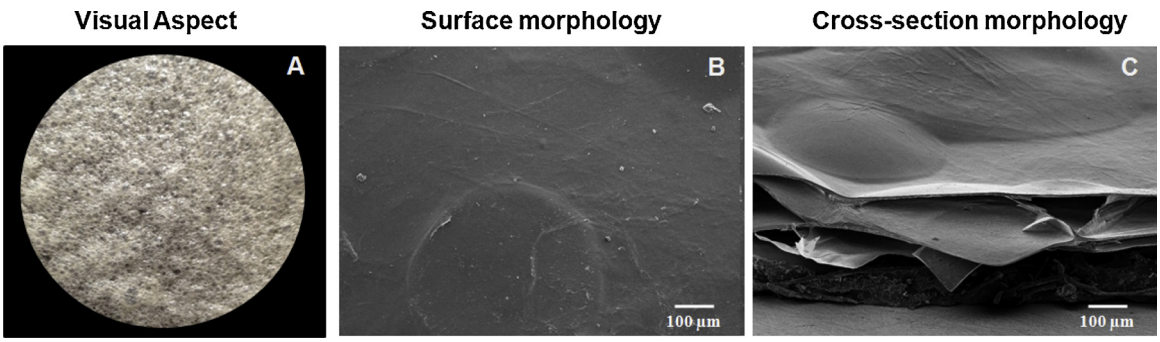

Fig. 2. Visual aspect and morphological characteristics of porous chitosan-alginate membrane at microscopic scale.

Table 2
Patient information and comparison of their response during the study.

General Parameters	Patient Information				
	1	2		3	4
		Lesion 1	Lesion 2		
Age	49	51		76	43
Basal platelet count (pl/µl)	319.000	358.000		260.000	228.000
Initial wound area (cm^2)	2.049	0.938	0.839	13.142	1.696
Time of evolution prior to treatment (months)	6	8	6	120	8
Number of PRP-membrane applications	3	2	3	3	5
Time for wound/ulcer/ injury healing (months)	9	2	6	4	6
Final ulcer area(cm^2)	0	0	0	10.70	0
Recovery (%)	100	100	100	18.6	100

infection during the treatment; probably these were related to poor home care of the injuries After the use of topical antibiotics, prescribed by the attending physician, the infections were cured, these patients continued with the subsequent applications of the treatment proposed in the study.

3.2. Case presentations

Case 1: A 49-year old woman, with a venous ulcer with six months of evolution in the lower part of the right leg. At the beginning of the treatment, the ulcer had an area of 2.049 cm^2 with abundant exudate, and according to analogous pain intensity scale (1–10), the patient perceived pain at level eight, for which she took 1 g of acetaminophen every day. After the first treatment, the patient did not need to take analgesics because the pain subsided. This patient went through three treatments and five months after starting the first treatment, the wound was about 50% closed. Nine months after starting the first treatment, the wound was completely closed (Fig. 3, Patient 1).

Case 2: A 51-year old woman, who started the treatment with a wound of 0.938 cm^2 in area and eight months of evolution. After starting the first treatment, a second wound with 0.839 cm^2 of area was exacerbated. The patient received five treatment applications in total, of which two were on the first ulcer and three on the second one. At the end of the treatment the patient perceived that the pain intensity decreased from six to two, according to the analog pain scale of 1 to 10. The first lesion showed 100% closure two months after starting the treatment, while the second lesion showed 100% closure nine months after the first treatment (Fig. 3, Patient 2).

Case 3: A 76-year old female, with lymphadenitis and overweight, with an ulcer in the lower part of the left leg, lasting about ten years, with 13.142 cm^2 in area and mild exudate production, with no bad smell. The patient reported that before treatment the pain was intermittent (5 according to a scale of analog pain). After three PRP-CA sessions the wound shows a complete re-epithelialization and no pain was reported by the patient (Fig. 3, Patient 3).

Case 4: A 43-year-old female, suffering insufficient saphenous vein of the left leg with an ulcer in the internal malleolar region with an area of 1.696 cm^2. The patient mentioned having pain on a scale of 8 at the beginning of treatment, and it decreased to 5 after the first treatment. At the end of the treatment, the pain was referred as zero according to the analog pain scale of 0–10. The patient was treated for five times, the ulcer totally closed at 6 months after starting therapy and the patient reported no pain. Safenectomy was done four months after the wound closed (Fig. 3, Patient 4).

4. Discussion

The American Venous Forum has proposed the following definition for lower- limb venous ulcer: “a full-thickness defect of skin, most frequently in the ankle region, that fails to heal spontaneously and is sustained by chronic venous disease” (based on venous duplex ultrasound testing) [6]. These ulcers have a prevalence of 0.7%–2.7%, increasing significantly with age, and being the origin of many chronic leg ulcers. The estimated annual burden of health care costs in the USA associated to patients suffering venous leg ulcers is around $15 billion, due to the medical resources expenses and to work loss costs [26].

This pilot study had two main goals: to evaluate the safety and to assess the efficacy of autologous PRP gel used in association with the application of biocompatible porous chitosan-alginate membranes for the treatment of chronic venous ulcers. Our findings support that the combined use of PRP and porous CA membranes is safe and effective, showing no adverse reactions and being a no surgical and economic treatment to chronic venous leg ulcers. The wound healing process involves many events: homeostasis, associated to coagulation, at the same time due to platelet aggregation, inflammation, cell proliferation and finally remodeling [27]. The oxidative stress caused by pain cause cortisol release and increase in cytokine levels, delaying healing [28]. PRP may decrease cytokine release and modulate inflammation, interacting with macrophages to improve tissue healing. One of the most striking results of this study was the reduction of pain in wounds from the first therapy, which probably contributes to the acceleration of healing, and leading to a decrease in analgesic intake by patients. Part of the exudate released was absorbed by the porous membrane, since this biomaterial shows significant swelling in the presence of aqueous fluids, as shown in Table 1. Possibly, the removal of the exudate from the lesion area contributed significantly for the reduction in the bad smell intensity.

Additionally, in this study no adverse reactions to the PRP or to the membranes used were observed, however an allergic reaction to the Micropore paper tape was detected in one of the patients.

The effect of PRP combined with chitosan membranes on wound healing in rats has been evaluated previously, and although both PRP and the chitosan membrane alone promote re-epithelialization, neovascularization, and a collagen amount increase, resulting in

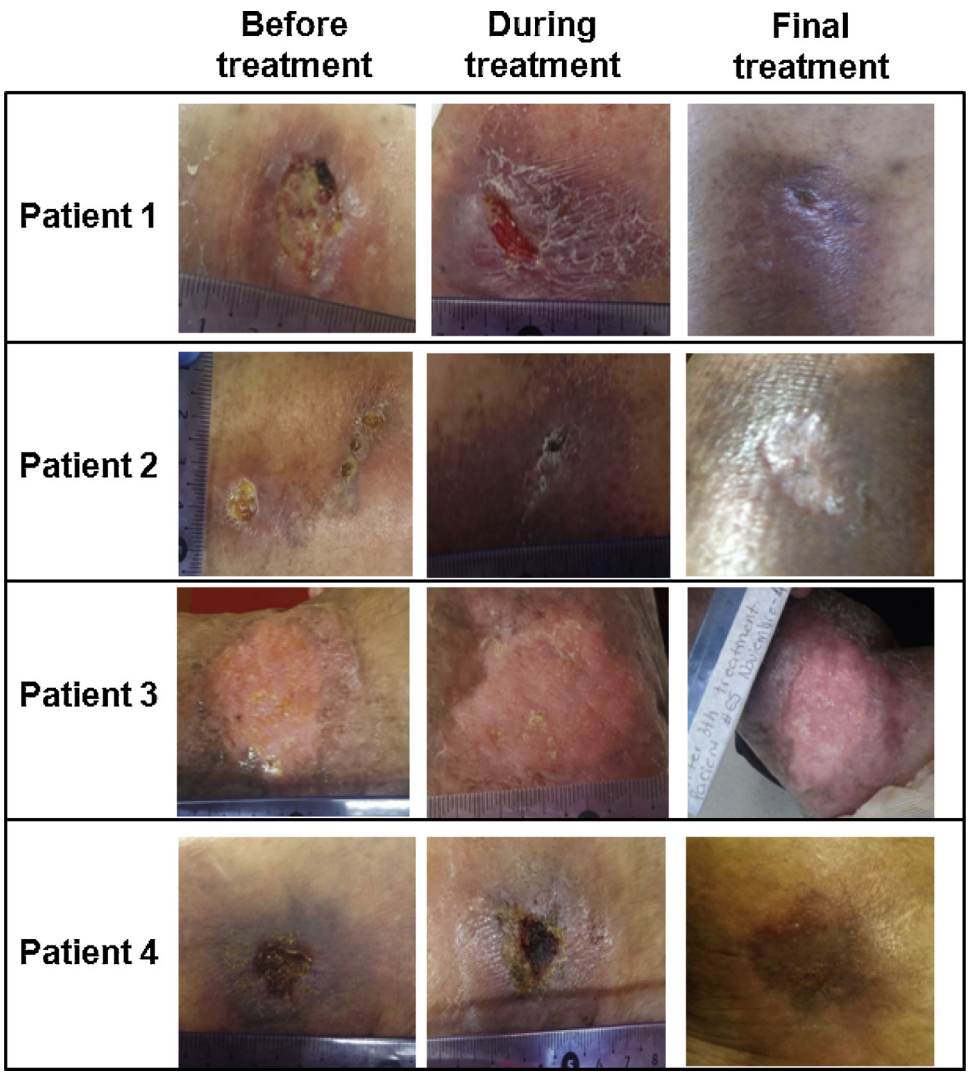

Fig. 3. Evolution of patients at different times of treatment.

appropriate wound contraction and wound closure percentage, the combination of both showed the best results, with significant differences [29]. Although in this report only four patients were treated, these results indicate also, that the combination of both PRP and chitosan membranes was effective, probably as a result of the sum of the well-known beneficial effects of the growth factors of PRP combined with the induction of granulation tissue formation accompanied by angiogenesis (also stimulated by chitosan). This approach could then be established as an alternative protocol for treating human chronic venous leg ulcers. Additionally, the proposed approach could contribute to reduce many of the traditional costs associated with these ulcers, due to the decrease not only of expenses related to health services and consumables, but also to the reduction of indirect costs by shortening the time needed for healing. However, studies with a larger population size and longer follow-up periods are necessary to confirm treatment efficacy and to allow establishing clinically useful standardized protocols.

5. Conclusion

In conclusion, autologous platelet rich plasma used with porous chitosan-alginate membranes may favor the healing of chronic ulcers in humans. Its beneficial effects are noticed from the first session, improving the clinical evolution of the wound as well as the patient perception of pain. These results suggest that this type of treatment is safe, effective and inexpensive. However, more studies are needed to understand the mechanisms underlying the response of chronic wounds to PRP-CA to reach its wide use in primary care.

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Declarations of interest

None.

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