Effectiveness of Autologous Preparation Rich in Growth Factors for the Treatment of Chronic Cutaneous Ulcers

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FDA CLEARED INDICATIONS FOR USE
The Double Syringe (ACP) System is used to facilitate the safe and rapid preparation of autologous platelet-rich-plasma (PRP) from a small sample of blood at the patient's point of care. The PRP can be mixed with autograft and allograft bone prior to application to an orthopedic surgical site as deemed necessary by the clinical use requirements.

This article describes indications for use that are not cleared by the FDA.

Abstract: Autologous Preparation Rich in Growth Factors (PRGF), a small volume of plasma enriched in platelets, is a novel therapeutic strategy for the acceleration of the wound healing of a wide range of tissues because of the continuous release of multiple growth factors, including PDGF-AB, TGF-β1, IGF-I, HGF, VEGF-A, and EGF. In this article, we have characterized the PRGF preparation and designed a randomized open-label controlled pilot trial to evaluate the effectiveness of PRGF in the treatment of chronic cutaneous ulcers. Results showed that at 8 weeks, the mean percentage of surface healed in the PRGF group was 72.94% ± 22.25% whereas it was 21.48% ± 33.56% in the control group ($p < 0.05$). These results, with the limitations of a pilot study, suggest that topical application of PRGF is more effective than standard therapy in helping a chronic ulcer to heal. © 2007 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 84B: 415–421, 2008

Keywords: chronic cutaneous ulcers; platelet; growth factors; PRGF; randomized trial

INTRODUCTION

Cutaneous ulceration is a common clinical problem rising with the increasing median age of the population. The European Union allocates 2% of the yearly health budget to wound care[1] and it is estimated that in the United States the costs related to the care of patients with pressure ulcers is over $1.3 billion per year.[2] Absence of healing is not uncommon when predisposing factors, such as rheumatism, diabetes, peripheral vascular disease, or previous scars are present. In fact some comorbid conditions could be related to a deficiency of growth factors, such as platelet-derived growth factor (PDGF), epithelial growth factor (EGF), vascular endothelial growth factor (VEGF) in the ulcer site, resulting in the impairment of the healing process.[3] Additionally, matrix metalloproteinases (MMP's) have also been implicated with excessive extracellular matrix degradation in chronic venous ulcers with the resultant failure of completion of the healing process.[4,5]

The dynamic and efficient process of wound healing involves a complex dynamic series of events, including hemostasia, inflammation, granulation tissue formation, epithelialization, neovascularisation, collagen synthesis, and wound contraction. Blood platelets have a major role in initiation of cutaneous wound healing. They adhere, aggregate, and release numerous growth factors, adhesive molecules, and lipids that regulate the migration, proliferation, and functions of keratinocytes, fibroblasts, and endothelial cells. Some of the stored growth factors essential for wound repair include PDGF, transformed growth factor (TGF-β), VEGF, basic fibroblast growth factor, EGF, type-I insulin-like growth factor (IGF-I), and hepatocyte growth factor (HGF).[6–10] In fact, the potential therapeutic effects of some of these growth factors in promoting wound healing has been reported.[11,12] The key roles of growth factors in wound healing has stimulated significant research efforts aiming to test different platelet-derived products as therapeutic treatments to improve wound healing and to accelerate the closure of chronic wounds. Technology has evolved since the first applications of this concept. Initially a liquid product containing autologous platelet secreted molecules was

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applied in collagen gels. Thereafter, various types of platelet rich products known as platelet rich plasma (PRP), have been essayed in several pilot studies, case series, and clinical trial.[13–15] However, although most of the studies have reported significant improvements in the recovery of chronic ulcers, some did not observe any positive influence.[16] This is probably a consequence of the insufficient standardization and definition for the different platelet-derived products currently being tested that differ from a qualitative and quantitative point of view.

Preparation rich in growth factors (PRGF) consists of a limited volume of plasma enriched in platelets that is rapidly obtained from the patient and easily prepared. Some of the specific characteristics of this optimized and safe formulation include the absence of leukocytes and its activation by means of calcium chloride instead of thrombin that enables a more physiological release of the growth factors implicated in wound healing.[17] Using the same protocol, it is possible to obtain a highly elastic, biocompatible, and haemostatic fibrin, which is an excellent biomaterial to regenerate soft tissues. Autologous PRGF has been used in the treatment of multiple musculoskeletal disorders and in the regeneration and healing of a wide range of tissues.[18–21] The goal of this preliminary study is to test whether PRGF applications could be extended to the treatment of chronic ulcers. To address this issue, a full quantitative characterization of PRGF was carried out and a randomized open-label controlled pilot trial was designed to assess the effectiveness and safety of PRGF for the treatment of chronic cutaneous ulcers.

MATERIALS AND METHODS

PRGF Treatment Characterization

Before undertaking the application of PRGF in chronic ulcers, a full characterization of the PRGF was carried out to assess the variability between subjects. For this purpose, we determined platelet count and quantified the concentrations of some relevant factors that actively participate during the healing process. Briefly, 10 mL of blood were drawn from 25 informed healthy volunteer; platelets were counted in peripheral blood and in the PRGF. A platelet-rich fibrin matrix was formed by adding calcium chloride at a final concentration of 22.8 m*M* and clots were allowed to retract for 1 h at 37°C.[17] The released supernatants were assayed for PDGF-AB, TGF-β1, IGF-I, VEGF-A, HGF, and EGF, using commercially available enzyme-linked immunosorbent assay kits (Quantikine colorimetric ELISA kits, R&D, Minneapolis, MN).

Patients

Between September 2003 and July 2004, a randomized, open-label, standard care-controlled pilot clinical trial was conducted to estimate the effectiveness and safety of topical application of PRGF to accelerate wound healing in patients with chronic cutaneous ulcers. The study was conducted in accordance with the principles of Declaration of Helsinki 1996 version and Good Clinical Practice standards. The study protocol, informed-consent form, and the other study-related documents were reviewed and approved by Human Research Ethics Committee of Hospital Txagorritxu before initiation of the study. All patients gave written informed consent.

A minimum number of 14 patients completing the study were established to collect preliminary information on the effectiveness of PRGF for wound healing as a background experience to be explored in future studies. Patients of both sexes aged $\geq$18 years with at least one nonhealing ulcer (Wagner grade II or III) of less than 12 cm in diameter were candidates for inclusion in the study provided that the ulcers were chronic (i.e., had been present for $>$4 weeks) and patients were able to attend the clinical investigation unit for wound care during a 7-day screening period and then once a week for 8 consecutive weeks, and signed informed consent. Exclusion criteria were the following: arterial origin of the cutaneous ulcer; presence of systemic infection and/or clinical manifestations compatible with active local infection; history of insulin-dependent diabetes mellitus, active vasculitis, systemic lupus erythematosus, cryoglobulinemia, severe hematological abnormality, epilepsy, or solid tumor; current use of anticoagulants and/or treatment with immunosuppressant drugs; serum haemoglobin concentration $<$11 g/dL or hematocrit $<$34%; and any condition that may interfere the patient's participation in the study (e.g., total immobility, inadequate nutritional status). Women who were pregnant, nursing, or childbearing potential not using an acceptable form of birth control were excluded.

At baseline, a general medical history was obtained and physical examination performed. When several ulcers were present, the largest ulcer was designated as the target one. For each patient the following data were recorded: demographics (age, gender); origin of the lesion (venous ulcer, pressure ulcer, surgery, trauma); localization; ulcer duration; ulcer surface; presence or absence of pain; patient's general condition (categorized as excellent, good, intermediate, poor); and patient's mobility (categorized as no limitations, slightly limited, very limited). All this information is summarized in Table I.

Study Procedures

Patients were randomly assigned according to a computer-generated randomization table to wound care with PRGF (experimental group) or standard wound care (control group). The study protocol included a 7-day washout phase, a baseline assessment, and a treatment period of 8 weeks.

Baseline Assessment. In both groups, wounds were cleansed with normal saline and moist saline gauze dressings were used. Cleansing with normal saline solution was performed gently using gauzes and/or sponges with minimum mechanical force to avoid friction or rubbing against the

TABLE I. Baseline Data of the Intent-to-Treat Data Set

Data	PRGF Group ($n = 8$)	Standard Care Group ($n = 7$)
Men/women	4/4	4/3
Age, years, mean (SD)	45 (20)	61 (16)
General status, no. (%)		
Excellent/good	7 (87.5)	6 (85.7)
Intermediate	1 (12.5)	1 (14.3)
Poor	0	0
Functional mobility, no. (%)		
Full	6 (75)	5 (71.4)
Limited	2 (25)	2 (28.6)
Ulcer origin, no. (%)		
Venous	5 (62.5)	5 (71.4)
Pressure	2 (25)	2 (28.6)
Other	1 (12.5)	0
Localization, no. (%)		
Legs	6 (75)	5 (71.4)
Pelvic region	2 (25)	2 (28.6)
Duration of ulcer, weeks, mean (SD)	68 (61)	110 (164)
Ulcer area, cm^2, mean (SD)	5.5 (4.8)	8.9 (8.6)

ulcer bed. When infection of the ulcer bed was suspected, the dressing was removed and the patient underwent ulcer debridement, wound cleansing with physiological saline, and systemic antibiotic therapy.

PRGF Ulcer Application Procedure. Preparation and application of the PRGF was performed by qualified technicians who had been previously trained by personnel of the Biotechnology Institute where the PRGF procedure was developed. 18–27 mL of venous blood were withdrawn by venous puncture from an antecubital vein a few minutes before wound care. Blood was collected on sterile tubes (4.5 mL) containing 3.8% (w/v) trisodium citrate, then centrifuged at 460 g for 8 min (PRGF System®, B.T.I. Biotechnology Institute, Vitoria-Gasteiz, Spain). The 1-mL fractions located immediately above the erythrocytes were collected from each tube and transferred to sterile tubes. Fifty microliters of $CaCl_2$ at 10% (w/v) were added per 1-mL fraction of platelet-enriched plasma and small volumes (100–200 uL) of the activated plasma were sequentially injected into the margins of the ulcer. Additionally the remaining plasma was allowed to clot *ex vivo* and the newly developed fibrin matrix was placed on the bed of the ulcer. Then the area was covered with a moist saline gauze dressing. In all cases, the time elapsed between veinpuncture and treatment ulcer was less than 2 h.

Standard Care. Patients underwent ulcer debridement and wound care with physiological saline (sodium chloride 0.9%) warmed to room temperature as cleansing agent and sterile gauzes as secondary dressing.

Each week, during the 8-week study period, ulcer evaluation was performed, patients underwent treatment procedure (PRGF or standard care) and adverse effects reported spontaneously by patients or after questioning in a general way were recorded. Pictures of the ulcers were obtained with a digital camera. Patients attended the research unit of the hospital until either complete healing (full epithelization) or 8 weeks of treatment.

Efficacy Variable

The primary endpoint of the study was the percentage of surface area healed. This variable was selected according to recommendations of the National Group for the Study and Assessment of Pressure Ulcers and Chronic Wounds (http://www.gneaupp.org) based on the National Pressure Ulcer Advisory Panel (NPUAP) (http://www.npuap.org).[22] The Mouseyes software program (version 2.1) was used to calculate wound areas derived from digital camera image files.[23] The percentage of surface area healed was estimated as ''initial ulcer surface − final ulcer surface/initial ulcer surface × 100''.

Statistical Analysis

Platelet counts and growth factor concentrations are expressed as mean + SD. Correlations were sought using Pearson correlation coefficient (r). Patients' data were analyzed by intent-to-treat. Between and within group comparisons of efficacy variables were carried out using the Mann–Whitney U-test and the Wilcoxon signed-rank test for paired samples, respectively. To determine the effect of patient's related confounding variables (age, sex, baseline general status, functional mobility) or ulcer-related variables (duration of ulcer [<4 weeks vs. ≥ 4 weeks], type of ulcer [venous vs. non-venous], localization, ulcer area [$<4\ cm^2 \geq 4\ cm^2$], and classification [Braden scale score]) on outcome, a stratified analysis for each variable using the Mann–Whitney U-test and the Kruskal–Wallis tests according to the number of categories of the explanatory variable was performed. Categorical data were compared with the chi-square (χ^2) test. The Statistical Package for the Social Sciences (SPSS, version 12.0) was used for the analysis of data. A p-value of 0.05 or less was considered statistically significant.

RESULTS

PRGF Characterization

To provide broad information of the product obtained using the earlier described procedure, a full characterization of the main growth factors released from activated PRGF was carried out in 25 subjects matched by age with the studied group, 51 ± 17 years, (range: 20–80). The PRGF elaborated as described earlier resulted in a significant enrichment in platelet number, 2.67 ± 0.6-fold increase comparing with peripheral blood. On the contrary, leukocyte content was below the detection limit of the coulter, confirming the absence of leukocytes in the PRGF, which improves the homo-

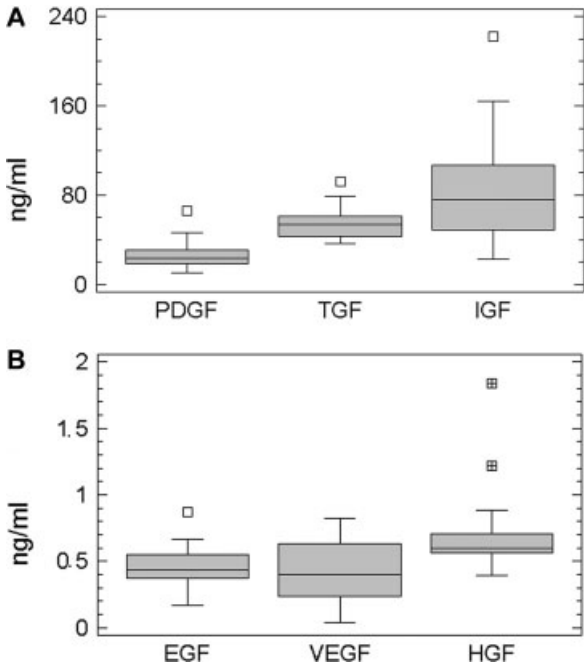

Figure 1. Growth factor concentrations determined in the PRGF from 25 healthy donors. (A) the most concentrated growth factors (ng/mL), including TGF-β1, PDGF-AB, and IGF-I and (B) less concentrated factors (ng/mL), including VEGF, HGF, and EGF. Boxes show the range between the 25th and 75th percentiles with a horizontal line at the median value.

geneity of the product and reduces donor-to-donor variability.

Platelets counts and growth factor content were examined for a general description of this product. The content of growth factors released from the activated PRGF was also measured for each donor. This data is particularly important since these growth factors are directly implicated in the wound healing process. Results showed that activation of PRGF with calcium chloride induced the release of different growth factors from platelet alpha-granules [Figure 1(A,B)]. Interestingly, it was found a direct correlation between platelet number and some of the released growth factors, such as PDGF, TGF-β1, VEGF, and EGF (Table II). This strong relationship indicates that platelets are the main source of these growth factors. On the other hand, no correlation was observed for HGF and IGF-I. Another focus of interest is to study the correlation between the age of the patients and the growth factor content. As shown in Table II, IGF-I is inversely correlated with the age ($r = -0.5477$, $p = 0.0046$), that is, IGF-I levels decrease as patients get older.

TABLE II. Correlations (Pearson Coefficients) Between Age, Growth Factors, and Platelets Measured in the PRGF Prepared from the Peripheral Blood of 25 Healthy Volunteers

	Platelet Count in PRGF	TGF-β1	PDGF	IGF-I
Age	−0.0448	−0.2225	−0.0824	−0.5477*
PDGF	0.6751**	0.8731**		−0.2800
TGF-β1	0.8231**		0.8731**	−0.3483
EGF	0.6084*	0.4065***	0.2184	−0.2249
VEGF	0.5713*	0.4645*	0.4299***	−0.5887*
HGF	0.2170	0.0746	0.0163	−0.1436

* $p < 0.01$,
* $p < 0.001$,
*** $p < 0.05$.

Clinical Outcome of PRGF and Control Groups

In this randomized open-label controlled pilot trial, fourteen patients (7 men, 7 women) with a mean (standard deviation, SD) age of 53 (20) years (range: 23–76 years) were included in the study, 7 patients were assigned to the PRGF group, and 7 to the standard care group. One patient from the PRGF group was excluded from the analysis because a new cutaneous lesion causing invasion of the index ulcer developed in the course of a respiratory infection at week 2. In the PRGF group, 1 patient discontinued the study at week 4 because of atopic dermatitis and use of topical medication incompatible with the clinical trial, and another patient was withdrawn at week 5 because of chronic venous insufficiency surgery (an elective operation that was already foreseen at the study entry). In the standard care group, 3 patients discontinued the study: 1 patient had a systemic infection that required inpatient care at week 5, 1 patient discontinued after week 4 because he considered that hydrocolloid dressings used prior to the study were more effective than the standard care, and 1 patient was lost to follow-up because of a change in the place of residence. Therefore, 5 patients in the PRGF group and 4 in the standard care group completed the study.

A total of 14 ulcers were assessed (64% venous leg ulcers, 29% pressure ulcers, and 7% other). Both groups were comparable at entry. Percentage of healing surface, calculated from ulcer area variations during the study period are shown in Figure 2. Results show that the percentage of surface area healed in the PRGF group was significantly higher than in the standard care group during all the evaluation points of the study. In fact, the mean percentage of surface healed at 8 weeks was 72.94 (22.25)% in the PRGF group and 21.48 (33.56)% in the standard care group ($p <$

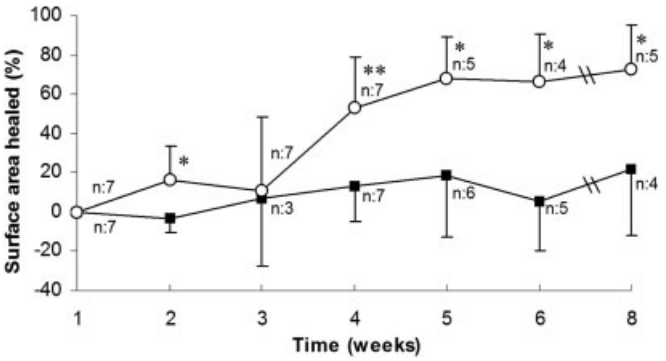

Figure 2. Percentage of surface area healed in the PRGF group (empty circles) versus standard care group (full squares). *$p < 0.05$, **$p < 0.01$.

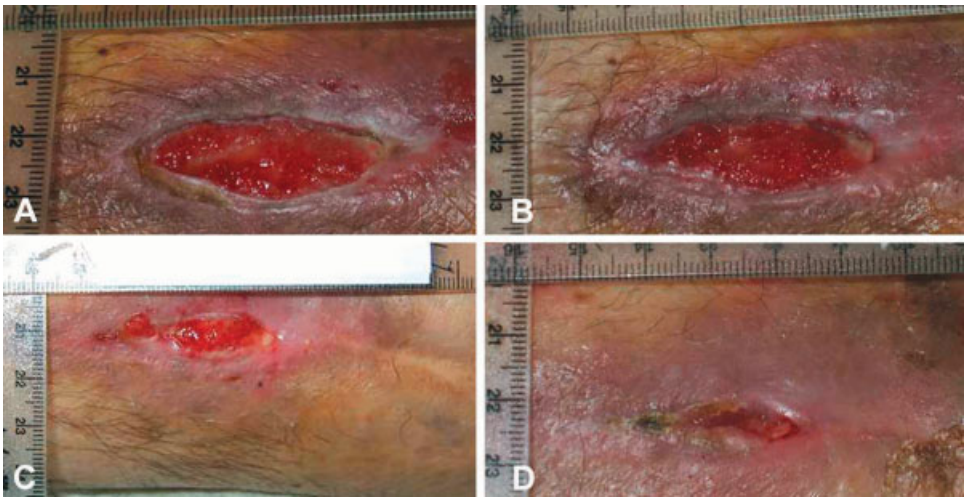

Figure 3. Evolution of a typical skin ulcer treated with PRGF: debrided ulcer before treatment (A), after 1 (B), 4 (C), and 8 (D) weeks, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

0.05). Figure 3 shows a representative case of an ulcer treated with PRGF. At the end of the study, complete healing was observed in only one ulcer from a patient in the PRGF group.

In the stratified analyses the patient's variables or ulcer-related variables had no significant effect on outcome in any of the study groups.

A total of four adverse events (ulcer bed infection 3, anemia 1) possibly related to the study treatment occurred in 3 patients. Super-infection of the ulcer bed developed in 1 patient from the PRGF group and in 2 patients from the standard care group, in 1 of which in combination with laboratory abnormalities. This patient required admission to the hospital and was treated with antimicrobials and oral protein–energy supplements. In all cases, however, ulcer bed infection resolved in about 8 days with oral antibiotic treatment. Differences between the treatment groups in relation to adverse events were not found ($p = 0.476$).

DISCUSSION

In the present article, we have evaluated the effectiveness of PRGF in the treatment of chronic ulcers, which suppose a major cost for the public health and substantially impair the patients' quality of life. The hypothesis for this study is a consequence of the increasing interest in the use of platelet-derived growth factors in cutaneous wound healing,[8] particularly in the management of pressure ulcers, chronic venous leg ulcers,[24] and diabetic neuropathic foot ulcers.[25]

In the last few decades, several crucial roles of growth factors and platelets have been identified in tissue repair. For example, PDGF has been suggested to have two major but distinct roles in wound healing, an early role to stimulate fibroblast proliferation and a later function to induce the myofibroblast phenotype.[26] VEGF due to its angiogenic effects play a key role in wound healing. In fact, application of neutralizing VEGF-A antibodies caused a significant reduction of wound angiogenesis, fluid accumulation, and granulation tissue formation in a pig wound model. Additionally, several studies have reported that abnormal expression of IGFs and their receptors lead to impaired wound healing.[27,28]

One major limitation, however, to the therapeutic use of growth factors to assist in tissue repair and in the management of chronic ulcers is to find efficacious alternatives for efficient growth factor delivery. Platelet rich preparations might overcome this limitation by combining the potential benefits of platelets and 3D fibrin matrices hence creating a biotechnological product for the continuous release of growth factors.[15] Several studies using different platelet-rich products have reported significant improvements in the recovery of chronic ulcers. For instance, Martí-Mestre et al. reported healing of chronic vascular ulcers using a platelet rich product in 12 of a series of 14 patients in a mean of 2.93 months (range: 0.5–7 months).[29] In another report, Mazzucco et al. observed that treatment of dehiscent sternal wounds with platelet gel reduced the complete healing rate (3.5 vs. 6 weeks) and hospital stay in 21 days when comparing with conventional treatment.[30] However, some other studies testing platelet rich products have no reported beneficial effects in the treatment ulcers. For example, Stacey et al. compared 42 patients receiving a platelet lysate preparation with 44 patients having conventional treatment. Results showed that their platelet lysate product had no influence on the healing of chronic ulcers.[16] These discrepancies may lead to a general controversy about the potential benefits of platelet-rich products. Therefore, it is necessary to perform a full characterization of the main properties of each platelet-rich preparation.

To address this issue and thus describe the important original features identifying PRGF from other PRP products, we have made a full characterization of the PRGF obtained from healthy subjects. As shown, PRGF contains a moderate

elevated platelet concentration (2–3-fold increase) that has been reported to induce an optimal biological benefit.[31] Of note, PRGF does not contain leukocytes, improving the homogeneity of the product and reducing donor-to-donor variability. This is important since neutrophils are an important source of MMP-8 and -9 and secrete other proteases, such as elastases[4] that are destructive for growth factors and release reactive oxygen species deleterious for cell survival. Furthermore, PRGF is activated by calcium chloride enabling a more physiological and sustained release of a wide range of growth factors, including PDGF-AB, TGF-β1, VEGF-A, EGF, HGF, and IGF-I. The quantification of the growth factors released from activated PRGF, revealed a strong direct correlation among PDGF-AB, TGF-β1, VEGF-A, and EGF and platelet number. This is a consequence of the major presence of these growth factors in platelets' alpha-granules.[6] Furthermore, an interesting constant proportion between PDGF-AB and TGF-β1 is released from PRGF in the absence of leukocytes.

Another important consideration of the PRGF elaboration procedure is that using the same cost-efficacy protocol, we obtain a highly elastic and haemostatic fibrin from the same patient's blood. This autologous fibrin in an excellent biomaterial to regenerate surrounding soft tissues. Even though studies made in fibrinogen knockout mice have revealed that fibrinogen is not an essential component for healing,[32] it provides an excellent vehicle for local growth factor delivery, functioning as a provisional matrix that would support the ingrowth of neovasculature and the migration of cells into the dead space.

After the characterization of the product, we evaluated the effectiveness of PRGF in the treatment of chronic ulcers. Results of this pilot randomized, controlled trial indicates that wound care using PRGF is effective in the management of chronic cutaneous ulcers compared with standard care with normal saline. In fact, statistically significant differences were found between both groups from the 2nd week post-treatment until the end of the study (8th week). In addition, PRGF resulted to be a safe treatment. Although one of the four cases of infection of the ulcer bed occurred in the PRGF group, it was more related to the evolution of the lesion than to the PRGF treatment itself. The remaining adverse events were observed in the control group but differences between the study groups were not significant. Given the heterogeneity of ulcers included in the study, it may be argued that response to treatment may be influenced by differences in baseline characteristics of cutaneous lesions. However, the stratified analysis considering both patient- and ulcer-related variables did not reveal significant differences.

In summary, results from this pilot randomized controlled trial, although obtained in a small group of patients, strongly support safety and effectiveness of PRGF formulation as a therapeutic treatment for accelerating the healing process of different chronic ulcers in a reduce period of time. Future clinical trials with larger samples and larger end-points will be necessary to unequivocally establish the full potential of PRGF.

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