View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Archivio della ricerca- Università di Roma La Sapienza

Send Orders for Reprints to reprints@benthamscience.ae

Current Vascular Pharmacology, 2017, *15*, 000-000

RESEARCH ARTICLE

Wounds Difficult to Heal: An Effective Treatment Strategy

Raffaele Capoano[1], Rita Businaro[2,*], Maria Chiara Tesori[1], Claudia Donello[1], Federica Lombardo[1], Vincenza Rita Lo Vasco[3], Lorena Capriotti[2], Mariangela Corsi[2], Tania Di Raimo[2], Martina Leopizzi[2], Bruno Salvati[1] and Serafino Ricci[4]

[1]*Dept. of Surgical Sciences,* [2]*Dept. of Medico-Surgical Sciences and Biotechnologies,* [3]*Dept. of Sensory Organs,* [4]*Dept. of Anatomy, Histology, Forensic Medicine and Orthopaedics – "Sapienza" University of Rome, Italy*

Abstract: ***Objective*:** Treatment of wounds difficult to heal concerns 50% of the elderly population in Italy and is therefore a relevant social burden. The present study shows how the treatment with autologous leuco-platelets reduces the healing time of wounds improving the functional recovery.

***Patients and Methods*:** Patients (n=100) with ulcers of the legs were divided in two groups: 1) 50 patients treated with conventional therapies; 2) 50 patients treated with autologous leuco-platelet concentrate (LPC) and hyaluronic acid (HIAFF, Hyalofill-F®) as a scaffold.

***Results*:** After 2 months, a 49% reduction in wound area was observed in the second group and in about 65% wound reduction was achieved in 15 days (4 LPC dressings). In contrast, patients treated by conventional therapies, showed a longer healing time and a greater percentage of failures. Morphometric analysis of biopsy samples obtained from the edge as well as from the bottom of the lesions obtained from the LPC group, detected an abundant presence of neoformed capillaries, characterized by a cubic, "reactive endothelium", close to the site of LPC infiltration.

***Conclusions*:** These results suggest that healing was promoted not only by limiting bacterial infections but also by the release of chemotactic and proangiogenic factors from leukocytes and platelets, improving the neoformation of capillaries.

ARTICLE HISTORY

Received: October 19, 2016
Revised: February 23, 2017
Accepted: February 23, 2017

DOI:
10.2174/1570161115666170301122216

Keywords: Wound healing, leuco-platelet concentrate, hyaluronic acid, morphometry, neoangiogenesis.

INTRODUCTION

Difficult to heal wounds [1] frequently occur in the elderly and those with vascular or metabolic diseases (e.g. diabetes or obesity). Since population aging is a global phenomenon the presence of difficult to treat wounds is a worldwide social problem [2]. Most patients suffer from marked reductions of quality of life, including pain, physical discomfort, functional limitations, as well as psychological distress [3]. Most patients complain about the additional burden of treatment.

The problem of difficult to heal wounds is of great social relevance in our country where it involves >50% of the elderly population [4]. The treatment of difficult wounds requires in most cases 3 day dressings/week, involving the displacement of specialists or the patient himself, often needing the involvement of several skills (vascular surgeon and plastic surgeon, diabetologist, angiologist, infection specialist, nephrologist, etc) [5]. The functional recovery achieved by the intervention with 'integrated' polyspecialist skills, seems to have a favourable impact on the level of collective social costs, besides the 'Quality of Life' of the patient and his family [5-7].

Numerous studies in the literature have evidenced that the use of the advanced wound dressings allows to reach the best clinical and economic results in the process of recovery of difficult wounds. The advanced wound dressing assures a shorten time of treatment and, as a consequence, it requires a smaller number of applications compared with the traditional medications [8].

Considerable progress has been achieved after the introduction of regenerative medicine methods which propose to accelerate the *restitutio ad integrum* of damaged tissues, adding cells to biopolymers matrices capable to recruit and stimulate the differentiation of precursors, in order to repair and revascularise damaged tissues [9-11].

Biopolymers provide a mechanical support for the migration and cell proliferation. In contact with the wound, the three-dimensional fibres of hyaluronic acid that constitute a Hyalomatrix, form a resorbable scaffold that accelerates the migration of cells involved in dermal reparative processes.

*Address correspondence to this author at the Department of Medico-Surgical Sciences and Biotechnologies, Sapienza University of Rome, Corso della Repubblica 79, 04100 Latina; E-mail: rita.businaro@uniroma1.it

1570-1611/17 $58.00+.00 © 2017 Bentham Science Publishers

The addition of autologous cells, as described in detail below, triggers a series of processes that should lead to the formation of new blood vessels and new tissue, structurally and functionally analogous to the damaged tissue [12].

Among the various proposed preparations, the best, in terms of effectiveness and economy is the one that employs the platelet gels [9]. We proposed a therapeutic strategy to treat difficult wounds, using matrices of biopolymers to replace the dermis acting as mechanical support, seeded with autologous leukocytes and platelets. Several studies have been performed evaluating the role of platelet enriched preparations, especially for their high content of factors promoting soft tissue healing and neovascularization [13]. Most of them deal with animal experimental models [14, 15] whereas our results are based on patients.

METHODS

The study program was carried out in several distinct phases:

Selection and Recruitment of Patients

Patients were selected among those treated at Surgical Sciences Dept. (Policlinico Umberto I, School of Medicine, Sapienza University of Rome). Patients (n = 100) with ulcers of the legs were divided in two groups, locally treated with conventional therapies or with autologous leuco-platelet concentrate (LPC) and hyaluronic acid (HA) as a scaffold, and followed up until complete healing of the lesions. All patients, suffering of arterial hypoperfusion of lower limb, had extended cutaneous and soft tissues loss of the extremities with well controlled pain.

Exclusion criteria were steroid drugs. Before starting treatment, patient informed consent was obtained. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in approval by the institution's research review committee.

Photographic documentation, assessing ulcer and perilesional tissue state (exudate, infection and necrosis) was recorded. The following parameters: localization, size, time of onset, were also recorded. Sizes of the ulcers, surgical treatment and outcome are depicted in Table **1**.

Autologous Blood Collection and Preparation of the LPC

A multi-cell separator Haemonetics MCS + discontinuous flow ® (Haemonetics Corp., Braintree, MA, USA) was used to obtain blood components. A concentrate with a high number of leukocytes and platelets was obtained after 6 cycles of the average length of 76 min, using a disposable circuit for the collection of stem cells (Cod.971E).

LPC

The average platelet count was 6691 x 10^3/µl, producing a high yield, considering an average pre-apheresis count of 380 x 10^3/µl. The average pre-apheresis white blood cell count was 7.7 x 10^3/µl, while the LPC count was 54.7 x 10^3/µl. Of particular relevance this amount of leukocytes was obtained without any stimulation of the patient with growth factors (G-CSF), as was previously done by other groups, in order to improve the yield of mobilized peripheral blood cells by apheresis [16-18]. Figure **1** depicts the different phases of gel preparation. Blood counts performed during the following checks did not show any significant variations.

The LPC was stored at +4°C, while the plasma was immediately frozen at -80 °C and then thawed at + 4°C for 8-12 h to obtain the cryoprecipitate by centrifugation. The LPC was subsequently mixed with the cryoprecipitate in equal volume, to enrich it with fibrinogen and other proteins of the extracellular matrix. The enriched concentrate was divided into several aliquots and stored at -40°C. The gel was obtained by activating platelets by autologous thrombin, obtained from serum, using Sarstedt vacutainer tubes. Serum was mixed with calcium gluconate, with 2:1 ratio, and added to the enriched LPC to form the gel in a few minutes. To increase the consistency, the gel was placed in a "Petri" cap containing a sheet of hyaluronic acid (HIAFF, Hyalofill-F ®).

Treatment of Wounds

Every patient, after wound swab for microbiological investigations and cleansing, was subjected to infiltration in areas of necrosis. Curettage and debridement were performed to remove the necrotic tissue. The T.I.M.E. concept (Tissue. Infection. Moisture imbalance. Epidermal margin) was usually applied. It summarizes the four main components of wound bed preparation: Tissue management, Control of infection and inflammation, Moisture imbalance, Advancement of the epithelial edge of the wound. Initially the local treatment involved surgical or enzymatic debridement and antimicrobial therapies using iodoform gauze. Hyaluronic acid was applied on the ulcers as soon as possible. Secondary dressing with silver sulfadiazine was used for all the patients. Gel containing autologous leuco-platelet concentrate using hyaluronic acid (Hyalofill-F ®) as a scaffold, and Connettivina gauze ® as a secondary dressing, was then directly applied to the lesion in the form of gel. A part of the preparation not conjugated with calcium gluconate and thrombin, was infiltrated directly on the edge of the lesion, although necrotic or undermined, and at the bottom of the ulcer. During the dressing the direct contact of blood component with aggressive products such as hydrogen peroxide, betadine solution and/or potassium permanganate was avoided. Applications were stopped if Pseudomonas A infection occurred or the lesion was well underway toward recovery. The response to the therapy has been estimated taking into account the reduction in ulcer area and depth, and the presence of regenerative tissue. Image J software (NIH, USA) has enabled us to calculate the total area of the wound and granulation tissue, taking advantage of photographic documentation.

Assessment of Neovascularization and Morphometric Analysis

Biopsy samples were collected periodically in order to assess the formation of new vessels by morphometric analysis and establish a temporal correlation with the time of recovery. During every curettage, biopsies were obtained from the edge of the lesion, even if necrotic or undermined, and from the bottom of the ulcer. Samples were fixed with 10% buffered formalin, then embedded in paraffin. Sections (2 µm) were then deparaffinised with xylene and hydrated

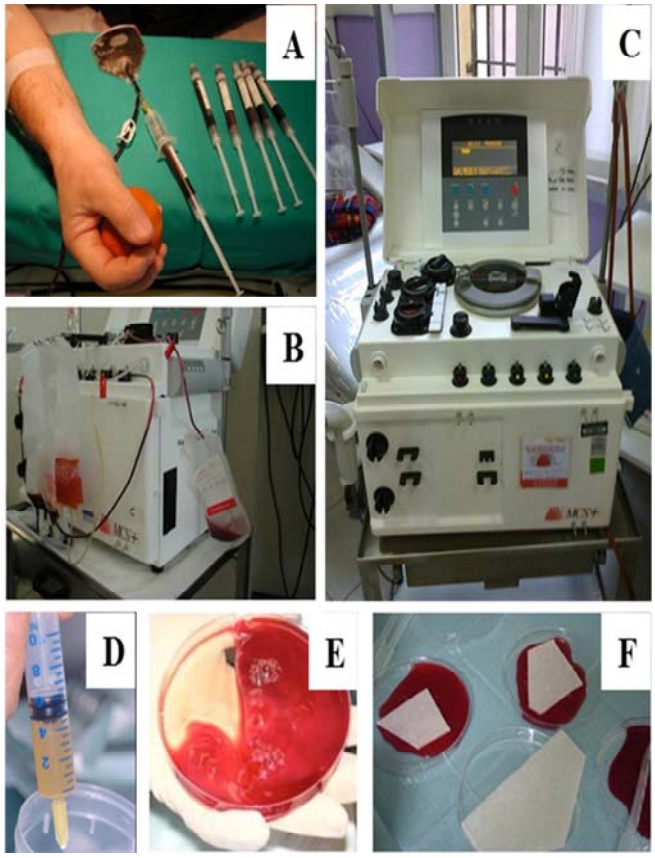

Fig. (1). Autologous blood collection and leuco-platelet concentrate preparation. **a**,**b**)withdrawal by multi-cell separator Haemonetics MCS + discontinuous flow. Serum plus calcium gluconate was added to the enriched leuco-platelet concentrate preparation to form the gel and to increase its consistency; the gel was placed in a "Petri" cap containing a sheet of hyaluronic acid (**c**-**e**).

through washes of ethanol and stained with haematoxylin/eosin. Morphometric analysis was performed using a Leica DM4000B microscope. For each sample neovessels were counted and related to the section area expressed in μm [2], as quantified by IAS2000 software.

Immunohistochemical Analysis

Wound samples obtained as described above, were fixed overnight in 10% neutral-buffered formalin, dehydrated and embedded in paraffin. Microtome sections (2 μm thick) were rehydrated and incubated for 15 min in 0.3% H_2O_2-methanol to block endogenous peroxidases. After extensive washing in PBS, sections were incubated with Ab anti-CD34 (Leica Biosystems, Novocastra, Surgipath cat. NCL-L-END), or with Ab anti-CD31 (Sino Biological cat. n.10148-H08H) or Antibody anti-VEGF (Vascular Endothelial Growth Factor) (Immunological Sciences AB-90040), rewashed and treated with a secondary antibody (LSAB+System-HRP, K0690, DAKO, Glostrup, Denmark). Sections were then incubated with DAB (3,3'-diaminobenzidine) and then extensively washed. The specificity of the reaction was assessed by incubating adjacent sections with isotype-matched irrelevant antibodies instead of the primary antibody. Images were obtained with an Olympus digital camera n.C5060 Wide Zoom on Leica Leitz DMRB Microscope (Wetzlar, Germany).

Usually 1 h before each dressing, which may consist of tissue removal (debridement) necrosectomy we administer analgesics because this process may elicit pain (pain or distress).

Statistical Analysis

Statistical analysis to evaluate the improvement of clinical results between groups was conducted using a chi-square or Fisher exact test, as shown in Tables **1** and **2**; $p<0.05$ two-tailed was considered significant.

RESULTS

Two groups of 50 patients each were included in this study in order to assess the effectiveness of autologous LPC compared to conventional therapies to promote healing of difficult wounds. In patients treated with a scaffold of hyaluronic acid supplemented with LPC we observed that 38 patients showed a reduction of the ulcer area of at least 25% after 2 to 4 LPC applications, over a period of 2-4 weeks, as shown in Table **1b**. In the same period we obtained the wound cleansing and a marked reduction in pain even during the medication (NRS: Numerical Rating Scale from 8 to 3). In the group treated with conventional local treatment only 24 patients (19 M and 5 F) showed a 25% reduction of ulcer area (Table **1a**). Over a period of 8 weeks we observed a superficialization of the ulcer bottom, after 2-8 LPC applications and a reduction of 50% of the wound volume; >60% of these patients achieved this result after 4-6 LPC dressings (Figs. **2**, **3**). In a 4 week period 8 patients showed only fluctuating improvements but had fever

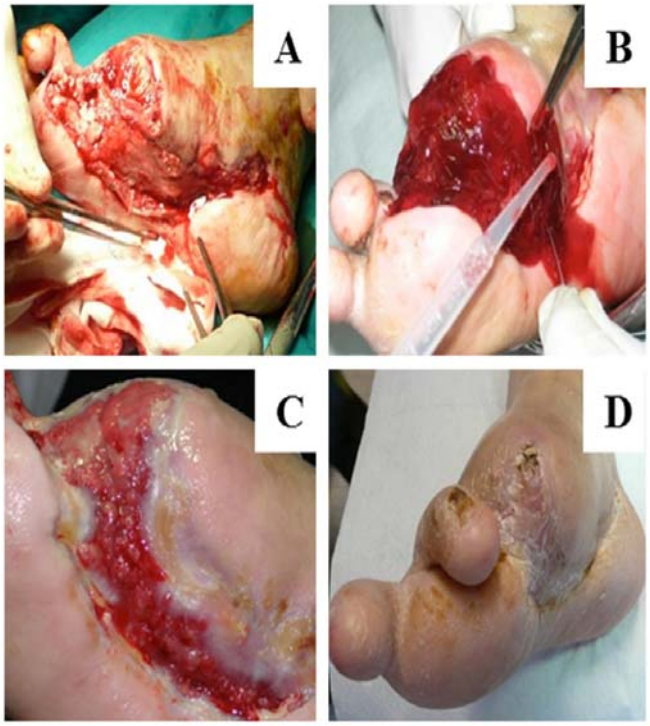

Fig. (2). **a**,**b**) Debridement of ischemic lesion of the leg; **c**,**d**) Hyaluronic acid used as dermal substitute (Hyalofill-F, HyaloMatrix) and treatment with infiltration of PRP (Platelet Rich Plasma); **e**,**f**) Regenerative and healing outcome: reduction of the area of the lesion (new epidermal tissue of the borders and growth of the bottom of the ulcer) until complete healing.

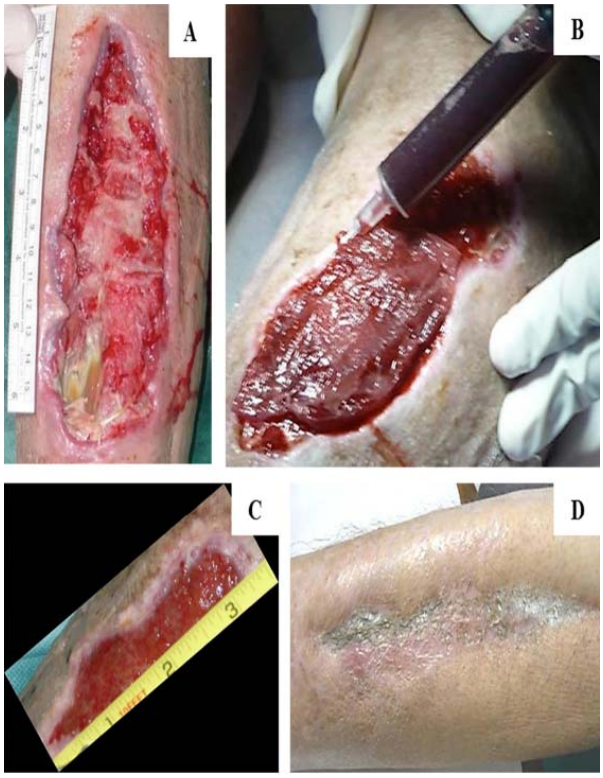

Fig. (3). Severe limb ischaemia treated with femoropopliteal bypass and wound care: transmetatarsal amputation, debridement, use of leuco-platelet concentrate and wound dressings (H.A.). Heal as final result. **a**) Post-traumatic ischaemic limb; **b)** Surgical exploration of damaged tissues; **c)** Bone stabilization and autologous saphenous vein graft; **d)** NPWT Negative Pressure Wound Therapy.

with humid gangrene and had to undergo amputation: 6 of them have been treated with conventional therapy and 2 with the LPC. In two LPC-treated cases it was necessary to amputate above the knee (AK: see Table **2**) for the worsening clinical, systemic and local conditions.

Major amputations were necessary in 11 other cases: 9 patients in the "conventionally treated" group and 2 patients of the “LPC treated” group for the appearance of septic fever and gangrene beyond 4 weeks. Fourteen segmentary amputations occurred in both groups, as shown in Table **2a** and Table **2b**.

We also successfully treated those patients at high risk of "major" amputation for large necrotic lesions and severe limb ischaemia (ABI, Ankle Brachial Index < 0.5): we limited amputations to metatarsophalangeal joints maintaining functional autonomy (Fig. **2**).

So the wound healing and the limb salvage was obtained in 81 patients; 46 in the “LPC-treated” group and 35 in the “conventional” one: healing was achieved between 6 and 22 months; the median time to heal for all wounds was 96 days [interquartile range (IQR) = 84-106]; all the third quartile is composed of patients treated with conventional therapy.

In the LPC treated group the wound healing was obtained after 6 - 22 weeks; the frequency of dressings was from 3 and 15 days, during the treatment. More than half of the patients recovered complete functional capacity within the first 12 months (28 patients).

The results, compared with those obtained with a control group of 50 patients matched for sex, age and pathogenesis, who were treated with drugs, including bioactive ones (HA) without autologous LPC, showed a longer healing time with a greater loss of substance and a higher number of major amputations (15 *vs* 4), with a lower "limb salvage" percentage (72 *vs* 92%) (Tables **2a**, **2b**). Statistical analysis conducted with chi-square or Fisher exact test showed a significant correlation between an improved clinical result and the autologous LPC treatment (Tables **1**, **2**).

The histological analysis of biopsy samples (Fig. **4**) showed abundant newly formed capillaries, characterized by a cubic, "reactive endothelium" near the site of LPC infiltration, with an average number of 16 neocapillaries/mm^2 (Figs. **4A**-**C**). Morphometric techniques applied to sequential tissue samples obtained from the edges of ulcers showed a rise in neo-angiogenesis in patients treated with the autologous LPC compared with those who received traditional therapy. In Fig. (**4A**, **B**, **C**) neovessel formation at the ulcer border is depicted: note the rounded agglomerates, devoid of lumen, and several surrounding structures

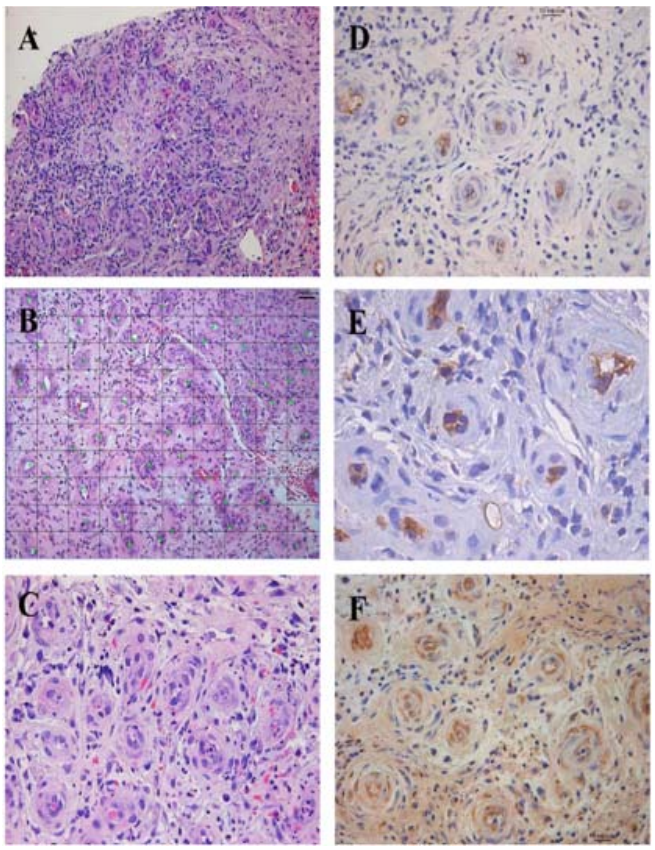

Fig. (4). Transmitted light images of haematoxylin-eosin stained sections of ulcer biopsy samples and immunohistochemical analysis **A**) abundant capillary formation in the close proximity of the injected leukocytes (100x); **B**) a representative count of neocapillaries (100x); **C**) Clusters of neoformed vases in the healing ulcers (200x); **D**) EPC (Endothelial Precursor Cells) lining neoformed capillaries are stained by an anti-CD34 Antibody (200x); **E**) EPC stained by an anti-CD31 Antibody are present in capillaries undergoing cavitation (400x); **F**) An abundant presence of Vascular Endothelial Growth factor (VEGF) was detected in the LPC infiltrated lesion (200X).

Table 1a. Patients treated with conventional local treatment §.

N° pts -Gender	Age	Diabetes	Size range (cm^2)*	Wound Treatment	Area of the lesion reduced by 25% (**)
36M	45-84	12	8-112	Weekly debridement and twice-daily dressing changes	17 pts (47.7%)
14F	36-78	3	3-91		5 pts (35.7%)

Sizes before(*) and after (**) 2-4 weeks of local treatment;
Pts = patients, M = males, F = females

Table 1b. Patients treated with leuco-platelet concentrate §.

N° pts -Gender	Age	Diabetes	Size range (cm^2)*	Wound Treatment	Area of the lesion reduced by 25% (**)
36M	36-84	9	12-96	After debridement weekly LPC application and infiltrations (2-4)	28 pts (77.7%)
14F	41-76	4	3-84		10 pts (71.4%)

Sizes before(*) and after (**) 2-4 weeks of local treatment;
(§) Chi-square = 9.375, p = 0.022
LPC = Leuco-Platelet Concentrate, Pts = patients

Table 2a. Clinical results local treatment.
#Patients treated with conventional local treatment.

N° pts -Gender	Age	Diabetes	Clinical Result	Limb salvage (%)
36 M	45-84	**12**	**10 major amputations** ***(7AK, 3BK)*** ***9*** *segmentary amputations* ***All wounds and stumps healed***	72.2
14 F	36-78	**3**	***5 major amputations (5AK)*** ***5*** *segmentary amputations* ***All wounds and stumps healed***	71.4

AK = Above knee, BK = Below knee

Table 2b. Clinical results leuco-platelet infiltration.
#Patients treated with leuco-platelet infiltrations.

N° pts -Gender	Age	Diabetes	Result	Limb salvage (%)
36 M	45-84	9	***3 major amputations (1AK and 2 BK)*** ***8 segmentary amputations*** *All wounds and stumps healed*	91.6
14 F	36-78	4	***1 major amputation (1AK)*** ***6 segmentary amputations*** *All wounds and stumps healed*	92.8

# Fisher exact test p = 0.044
AK = Above knee, BK = Below knee

where cavitation, critical to establish a blood flow, is taking place. Note the cubic appearance of endothelial cells within the vessels taking shape. The endothelial precursor cells (EPC) of the neo-vessel are interconnected and are arranged to establish an apical-basal polarity, according to Datta *et al*. [19]. EPCs involved in vessel regeneration are stained by an anti-CD34 Ab (Fig. **4D**) in the early stages and by an anti-CD31Ab (Fig. **4E**) in later forms. Furthermore, an abundant presence of VEGF was detected in the area of the lesion very close to neovessels and the LPC infiltrate (Fig. **4F**).

Our data show that the presence of neoangiogenesis correlates with clinical outcome preceding wound healing.

DISCUSSION

The severity of the disease, the costs incurred both in the time of hospitalization, and the subsequent rehabilitation, has

led researchers to explore new therapeutic options to optimize the treatment of advanced ulcers of the extremities. Encouraging results have come from the use of stem cells obtained from bone marrow and/or precursors of endothelial cells present in peripheral blood [20-22]. Several different protocols have been proposed and developed in different trials worldwide, some of them taking advantage of bone marrow-derived cells, others of peripheral blood derived cells to induce therapeutic angiogenesis, in order to provide a local perfusion sufficient for wound healing. The proposed ways of cell administration included intra-muscular and intra-arterial injection or a combination of both [23]. Considering the totality of the studies, we may conclude that a local supplementation of proangiogenic haematopoietic progenitors consisting of immature and differentiated myelomonocytic cells is needed to allow the revascularization of ischaemic tissue [24]. Another interesting source of stem cells is represented by mesenchymal cells obtained from adipose tissue [10]. The fundamental contribution of these cells is not limited to the recruitment of new elements for vascular growth, but consists in the secretion of cytokines and growth factors, which promote vascular growth by paracrine mechanisms [25]. In this connection platelets seem to play a central role: activated platelets produce extremely high levels of Platelet Derived Growth Factor (PDGF) and stromal-derived factor-1 (SDF-1), also known as CXCL12, which induces the mobilization of CXCR4+VEGFR1+ cells, accelerating revascularization: the levels of these molecules are increased by pro-inflammatory mediators, and by alterations of the extracellular matrix following mechanical forces and hypoxia [17, 26-28].

Moreover, the literature [29-33] suggests the role of some cytokines in triggering a 'cascade' of events that promotes the expression of phenotypic characteristics of mature endothelial cells in undifferentiated progenitor elements, increasing the availability of cell populations able to repair the endothelium and thus improve vascular function [16]. Among the activated components of the cryoprecipitate, fibronectin, a glycoprotein of the extracellular matrix, promotes cellular adhesion and migration to the site of the lesion, forming locally the ideal "micro-environment" for the differentiation of endothelial cells and the formation of new capillaries. As reported by Asahara and coworkers [16], EPCs isolated from peripheral blood, seeded onto plates coated with fibronectin, grew with typical characteristics of endothelial cells. More recent studies have also identified a subpopulation of EPCs (CD34 +, CD45-), derived directly from peripheral blood monocytes, which can play a key-role in neoangiogenesis *in vivo* [34]. Our histochemical results prove the presence of these cells in neoforming vessels.

Another important aspect of platelet concentrate is the potential anti-bacterial effect, determined both by the presence of proteins of the HPAP (Human Antimicrobial Peptides Platelet), such as fibrinopeptides A and B, thymosin beta-4, platelet basic protein 3, RANTES, tissue activation peptide, and platelet factor 4, and the higher concentration of leukocytes present in situ (7 times higher than in circulating blood). The effectiveness of the Platelet Rich Plasma was evaluated against Escherichia coli, Staphylococcus aureus, Candida albicans, as well as Cryptococcus neoformans and Staphylococcus MSSA and MRSA (methicillin-sensitive and methicillin-resistent staphylococcus aureus), with positive or promising results [35, 36]. The antibacterial activity, probably associated to the infiltrated preparation, suggests the topical use of haemocomponents, earlier, during the phase called "TIME". In this phase necrotic tissue, fibrin or marked exudation is present, favouring the development of bacterial colonies. Patients with diabetes are primarily affected with infected lesions, with 'necrotizing' evolution, involving tegumentary structures such as tendons and ligaments: in these situations it is very important to minimize the surgical debridement, which might irreparably compromise the functions of these structures; in these cases we perform the cleansing with an ultrasonic dissector, to allow a faster regrowth of the surrounding tissues and to obtain a functional and anatomic saving.

The use of stem or precursor cells has achieved impressive results and has demonstrated high potentials, as emphasized in the literature [37], but costs represent a major limitation to their extensive use and further technological development. We have to make some considerations about their clinical application in the treatment of acute and chronic soft tissue lesions. The improvement of reconstructive techniques and regenerative medicine has contributed to reduce, in recent years, the time needed for healing, to decrease the cases of non-healing, the number of overall major amputations and segmental amputations, thus influencing positively the subsequent costs of temporary or permanent disabilities. An Italian observational study has already shown that new therapies, using among others, hyaluronic acid, help to reduce the lesion (40 *vs* -34%, $p<0.05$) and to achieve complete healing in less time than that required using traditional dressings; costs also being lower [4, 38]. From these data it follows that, although the costs per unit are on average higher, the total final cost is beneficial. Our approach, probably directing the recruitment of EPC, already induced by hypoxic conditions, at the lesion level, promoted increased neoangiogenesis and reduced bacterial infections. Limitations to this study are that we did not assess whether there is a different LPC treatment efficacy depending on the aetiology of the ulcer. Moreover factors released by LPC, responsible for improving wound healing, are still not identified.

CONCLUSIONS

The possibility of improving local perfusion with reconstructive or restorative surgery of the vascularity of the limb becomes the determining factor for clinical success and to avoid major amputation. Neoangiogenesis is a prerequisite for determining tissue repair: new vessels deliver oxygen to the injury site as well as nutrients and humoral factors necessary to allow the healing of the wounds. The intensity and the speed of this phenomenon is critical to get the regeneration and then heal wounds. The approach proposed in this study appears to meet these requirements and seems to be an attractive alternative to traditional methods. In fact, the infiltration of leukocytes and platelets stimulates angiogenesis even in the early phase of treatment. A significantly higher vascular density was observed in the LPC-treated group compared with conventional therapy-treated group. The use of hyaluronic acid as a scaffold in the gel formulation of the "Platelet Rich Plasma" in the activated form undoubtedly

helps regeneration but in a later stage only and therefore the results obtained in the present study are directly dependent on LPC delivered within the wounds.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

Declared none.

REFERENCES

[1] Montfrans CV, Stok M, Geerkens M. Biology of chronic wounds and new treatment strategies. Phlebology 2014; 29: 165-7.

[2] Sen CK, Gordillo GM, Roy S, *et al*. Human skin wounds: a major and snowballing threat to public health and the economy. Wound Repair Regen 2009; 17: 763-71.

[3] Augustin M. Cumulative life course impairment in chronic wounds. Curr Probl Dermatol 2013; 44: 125-9.

[4] Apollonio A, Antignani PL, Di Salvo M, Failla G, Guarnera G, Mosti G, Ricci E;SUV Study Group.. A large Italian observational multicentre study on vascular ulcers of the lower limbs (Studio Ulcere Vascolari). Int Wound J. 2016;13:27-34.

[5] Krasner DL, Rodeheaver GT, Sibbald RG. Interprofessional wound caring and the IWC Model Ostomy Wound Manage 2007; 53: 6, 8.

[6] Nehler MR, McDermott MM, Treat-Jacobson D, Chetter I, Regensteiner JG. Functional outcomes and quality of life in peripheral arterial disease: current status. Vasc Med 2003; 8: 115-26.

[7] Arai H, Ouchi Y, Yokode M, *et al*. Members of Subcommittee for Aging. Toward the realization of a better aged society: messages from gerontology and geriatrics. Geriatr Gerontol Int 2012; 12: 16-22.

[8] Boateng J, Catanzano O. Advanced Therapeutic Dressings for Effective Wound Healing--A Review. J Pharm Sci. 2015;104:3653-80.

[9] Langer A, Rogowski W. Systematic review of economic evaluations of human cell-derived wound care products for the treatment of venous leg and diabetic foot ulcers. BMC Health Serv Res 2009; 9: 115.

[10] Businaro R, Corsi M, Di Raimo T, *et al*. Multidisciplinary approaches to stimulate wound healing. Ann N Y Acad Sci 2016 Jul 19. doi: 10.1111/nyas.13158.

[11] Kasuya A, Tokura Y. Attempts to accelerate wound healing. J Dermatol Sci 2014; 76: 169-72.

[12] Clark RA, Ghosh K, Tonnesen MG. Tissue engineering for cutaneous wounds**.** J Invest Dermatol 2007; 127: 1018-29.

[13] Lubkowska A, Dolegowska B, Banfi G. Growth factor content in PRP and their applicability in medicine. J Biol Regul Homeost Agents. 2012;26:3S-22S

[14] Mohammadi R, Mehrtash M, Mehrtash M, Hassani N, Hassanpour A. Effect Platelet Rich Plasma Combined with Chitosan Biodegradable Film on Full Thickness Wound Healing in Rat Model. Bull Emerg Trauma. 2016;4:29-37

[15] Esat Duymus M, Temel S, Ozer H, *et al.* Comparison of the Effects of Platelet rich Plasma Prepared in Various Forms on the Healing of Dermal Wounds in Rats. Wounds. 2016;28:99-108.

[16] Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997; 275: 964-7.

[17] Massberg S, Konrad I, Schürzinger K, *et al.* Platelets secrete stromal cell-derived factor 1alpha and recruit bone marrow-derived progenitor cells to arterial thrombi *in vivo*. J Exp Med 2006; 203: 1221-33.

[18] Fadini GP, Agostini C, Avogaro A. Autologous stem cell therapy for peripheral arterial disease meta-analysis and systematic review of the literature. Atherosclerosis 2010;209:10-7.

[19] Datta A, Bryant DM, Mostov KE. Molecular regulation of lumen morphogenesis. Curr Biol.;21:R126-36

[20] Falanga V. Stem cells in tissue repair and regeneration. J Invest Dermatol 2012; 132: 1538-41.

[21] Liu FP, Dong JJ, Sun SJ, *et al*. Autologous bone marrow stem cell transplantation in critical limb ischemia: a meta-analysis of randomized controlled trials. Chin Med J (Engl) 2012; 125: 4296-300.

[22] Kawamoto A, Katayama M, Handa N, *et al.* Intramuscular transplantation of G-CSF-mobilized CD34(+) cells in patients with critical limb ischemia: a phase I/IIa, multicenter, single-blinded, dose-escalation clinical trial. Stem Cells 2009; 27:2857-64.

[23] Lawall H, Bramlage P, Amann B. Treatment of peripheral arterial disease using stem and progenitor cell therapy. J Vasc Surg 2011; 53: 445-53.

[24] Jin DK, Shido K, Kopp HG, *et al.* Cytokine-mediated deployment of SDF-1 induces revascularization through recruitment of CXCR4+ hemangiocytes. Nat Med 2006; 12: 557-67.

[25] Khan S, Villalobos MA, Choron RL, *et al.* Fibroblast growth factor and vascular endothelial growth factor play a critical role in endotheliogenesis from human adipose-derived stem cells. J Vasc Surg. 201 pii: S0741-5214(16)30174-4.

[26] Ceradini DJ, Kulkarni AR, Callaghan MJ, *et al*. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nat Med 2004; 10: 858-64.

[27] Jhamb S, Vangaveti VN, Malabu UH. Genetic and molecular basis of diabetic foot ulcers: Clinical review. J Tissue Viability 2016; pii: S0965-206X(16)30041-9.

[28] Martínez CE, Smith PC, Palma Alvarado VA. The influence of platelet-derived products on angiogenesis and tissue repair: a concise update. Front Physiol 2015; 6: 290.

[29] Johnson C, Sung HJ, Lessner SM, Fini ME, Galis ZS. Matrix metalloproteinase-9 is required for adequate angiogenic revascularization of ischemic tissues: potential role in capillary branching. Circ Res 2004; 94:262-8.

[30] Profumo E, Buttari B, Saso L, Capoano R, Salvati B, Riganò R. T lymphocyte autoreactivity in inflammatory mechanisms regulating atherosclerosis. Scientific World J 2012; 157534

[31] Profumo E, Buttari B, Petrone L, *et al.* Redox imbalance of red blood cells impacts T lymphocyte homeostasis: Implication in carotid atherosclerosis. Thromb Haemost 2011; 106:1117-26

[32] Asahara T, Takahashi T, Masuda H, *et al.* VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. EMBO J 1999; 18: 3964-72.

[33] Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 1996; 86 :353-64

[34] Timmermans F, Van Hauwermeiren F, De Smedt M, *et al.* Endothelial outgrowth cells are not derived from CD133+ cells or CD45+ hematopoietic precursors. Arterioscler Thromb Vasc Biol 2007; 27: 1572-9.

[35] Lei H, Xiao R, Tang XJ, Gui L. Evaluation of the efficacy of platelet-rich plasma in delivering BMSCs into 3D porous scaffolds. J Biomed Mater Res B Appl Biomater 2009;91: 679-91.

[36] Bielecki T, Gazdzik TS, Arendt J, Szczepanski T, Król W, Wielkoszynski T. Antibacterial effect of autologous platelet gel enrich with growth factors and other active substances. *In vitro* study. J Bone J Surg Br 2007; 89: 417-20.

[37] Cerqueira MT, Pirraco RP, Marques AP. Stem Cells in Skin Wound Healing: Are We There Yet? Adv Wound Care (New Rochelle) 2016;5:164-175

[38] Foglia E, Restelli U, Napoletano AM, Coclite D, Porazzi E, Bonfanti M, Croce D. Pressure ulcers management: an economic evaluation. J Prev Med Hyg 2012;53:30-6