Autologous peripheral blood mononuclear cells from selective filtration for treatment of chronic lower limb lesions: Results at 4 years

Matteo Bucalossi,[1,2,3] Fabrizio Mariani[1,2,3]

[1]The Compression Therapy Study Group (CTG), Firenze; [2]Surgical Operations Unit, Valdisieve Hospital, Pontassieve (FI); [3]European Training Centre in Phlebology-European Union of Medical Specialists UEMS, Angiomedica Vein Clinic, Colle Val d'Elsa-Siena, Italy

ABSTRACT

A selective filtration technology has recently been introduced on the market to produce an autologous concentrate of Peripheral Blood Mononucleate Cells (PBMNC) with indication for use for human cell therapy. PBMNCs are indicated in the treatment of chronic lower limb injuries. The purpose of this study is to assess the efficacy and tolerability of this treatment in a group of patients who presented skin ulcers of the lower limbs, with various aetiology, not responders to traditional and advanced topical therapies. The study is prospective. Eight patients were treated with a total of 22 skin ulcers with variable etiology. Autologous PBMNCs were implanted along the course of the reference tibial arteries and in the peri-lesional area. All patients were subjected to a cycle of three infiltrations, performed in the operating room, on a monthly basis, except one patient who was subjected to a fourth implant, because the operator considered that a further one would accelerate the already regenerative state of the skin. Of the 22 ulcers treated with this method, 14 have reached complete recovery within a month of the end of the three implants, while 8 have gone towards a clear improvement. All patients showed good compliance to treatment and no minor or major adverse effects were reported. Fifty per cent of the treated patients were followed up four years after the end of treatment, and only one patient had a recurrent skin ulcer, but elsewhere from the initial one. The PBMNCs, produced with selective filtration system, have been shown to be an effective treatment of chronic lesions with different etiology of the lower limbs. The healing of all skin lesions treated quickly, the sharp reduction of pain, and the absence of recurrence at four years suggest a lasting clinical effect over time.

Correspondence: Matteo Bucalossi, The Compression Therapy Study Group (CTG), Via Kyoto 51, 50126 Firenze, Italy.
Tel.: 338-6892045.
E-mail: matteobucalossi@tiscali.it

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INTRODUCTION

The cutaneous ulcers of the lower limbs represent the outcome of different pathological processes and constitute an increasingly current pathology, both for their frequency and for the therapeutic problems that they involve.

They affect about 1% of the adult population of developed countries (with over 3% of those over seventy) and its characteristics are to be considered a real social scourge, involving, in addition to the direct interested, also all the surrounding family environment.[1]

Moreover, the figures of the studies are often underestimated, in fact it is not infrequent that the first therapeutic approach is carried out by the patient himself, exploiting experiences of friends or neighbours. In case of failure or worsening of the clinical picture, the general practitioner is consulted and, only in the third instance, the patient is sent to centres specialized in vulnology.[2]

To this day, the therapies proposed for the treatment of cutaneous ulcers have been many and often indigestible, but a common characteristic is that of giving alternate or even unsatisfactory results.

Autologous cell therapy is an innovative and promising approach to the regeneration of damaged tissues, in particular new scientific evidence indicate the total peripheral blood mononuclear cells, consisting of monocytes/macrophages and lymphocytes populations, as cells with high angiogenic and arteriogenic capacity and

vasculogenic[3–5] and in general fundamental in the processes of regeneration of the same tissues.[6-8]

The mononuclear cells from peripheral blood, indicated in the scientific literature as PBMNC (Peripheral Blood Mononuclear Cells), represent an autologous cell concentrate for the treatment of patients with critical ischemia with complex skin ulcers of the lower limbs.[9,10]

Recently a meta-analysis has indicated PBMNC as the only autologous cell concentrate capable of significantly reducing amputations in patients with critical ischemia.[11] Our observation includes chronic ulcers that affect the lower limbs, in fact these lesions are frequently resistant to normal therapies for numerous factors, including: i) circulatory, venous or arterial disorders that are preferentially affecting the lower limbs; ii) the leg is one of the most exposed to the action of traumas, in fact these, on a limb already compromised by circulatory insufficiency, can cause the development of cutaneous lesions to torpid course; iii) the foot and the lower third of the leg are inspected or cleaned less frequently than other parts of the body and therefore are more easily the site of infections and, in turn, cause chronic skin lesions; iv) the incidence of diabetic disease, associated with micro- and macroangiopathy of the lower limbs, is increasing significantly among the population of developed countries.

The purpose of this study is to report and analyse the experience gained, at the specialized Vulnologic Center of Valdisieve Hospital, the use of autologous PBMNC cell therapy produced by a point of care selective filtration system, with indication for use for human cell therapy, in a group of patients with chronic lower limb ulcers, various aetiology, resistant to common topical therapies, including advanced medications, and also classified as “difficult”, present for at least six months on an ongoing basis.

MATERIALS AND METHODS

Patients

The clinical experience with PBMNC was conducted by us on a group of patients with chronic lower limb ulceration, with various aetiology, refractory to any topical therapy and present for more than six months.

The protocol included an accurate history of the patient, a careful specialist examination with echocolor-doppler artery-venous examination, and measurement, with photograph of the ulcer, so as to classify in detail both the aetiology and the initial size of the lesion.

From October 2013 to January 2015, 22 skin ulcers were treated, with various aetiology, of 8 patients, 4 both male and female, with an average age of about 86 years (range 73-94 years).

The cutaneous ulcers recruited within the study are divided into 12 arterial, 3 mixed arterial-venous, 6 diabetic, and 1 iatrogenic type, as a result of a reconstruction of the calcaneal tendon (Table 1).

All injuries had been present for at least six months on an ongoing basis and had not responded to the most common standard treatments. Going into more detail, among arteriopathy patients two could not be revascularized due to the general conditions in place, while in the other two the lesion hesitated even after endovascular treatment of revascularization. The patient with iatrogenic injury could not avail itself of negative pressure therapy for a difficult home management, while diabetic patients presented a glycaemic picture out of control, that has been reported with adequate therapy in the normal with two months of therapy. All patients had been treated in the previous months with advanced medications, including polyurethane foams and hydrofibers, which however had not given any substantial improvement, so the injuries were in a phase of “tired”. All ulcers, about five days before each treatment, were subjected to culture testing with antibiogram, so, based on the pathogen present, was started a targeted antibiotic therapy, which had an average duration of about ten days. At the first swab all lesions were found positive for bacteria and/or fungi, while at the third about half showed no identifiable pathogen.

The size of the skin ulcers was very uneven, in fact the surface of the skin lesions varied from 5 to 168 cm²; ulcers with size <5 cm² have been excluded from the study (Table 2).

Patients were subjected to a cycle of three treatments that were carried out about every 30 days from each other, while only in a particularly critical patient a fourth treatment was carried out about two months after the third implant.

Table 1. Etiopathogenesis of treated ulcers.

Arterial	12
Mixed	3
Diabetic	6
Iatrogenic	1

Table 2. Size of treated ulcers.

5 - 10 cm²	10
10 - 20 cm²	5
20 - 30 cm²	3
30 - 50 cm²	1
50 - 100 cm²	2
100 - 168 cm²	1

Exclusion criteria have been: renal insufficiency and heart or liver failure, neoplastic pathologies in place, HIV and Hepatitis B or C, systemic infection in progress, severe anemia or hematopoietic diseases, such as platelet dysfunction syndrome and thrombocytopenia.

CONCENTRATION OF PBMNC

The sterile and disposable selective filtration system used MonoCells, now marketed under the name Hematrate Blood Filtration System Cook Regentec, is a CE marked Medical Device (class IIB) Point of Care for intra-operatory use, which allows to process 20-120 ml of peripheral blood in a short time without leaving the operating field, producing an autologous cell concentrate of PBMNC with indication of use for human cell therapy. The cell concentrate obtained has been extensively characterized[12] and produces an average concentration of mononuclear cells of 200 million per 120 mL of processed blood.

Briefly, 120 mL of peripheral blood is taken from the central veins of the anticoagulated forearm and mixed with a 10% ACD solution. The sample is loaded into the upper bag and filtered by processed gravity (about 10 min). PBMNCs are retained by the filter and recovered through a back-wash with 10 mL of saline. The collected cells are immediately transferred sterilely into ten 1cc syringes with 21 G needle and infiltrated.

PBMNCs implant

The PBMNCs implantation was performed in the operating room (patients admitted to normal or day hospital) always after careful debridement.

The cell implantation was performed under ultrasound guidance, after sedation of the patient, with peri-vasal treatment along the course of the tibial arteries. About 0.25 cc of cell concentrate obtained at about 1 cm from each other and at about 1 cm of depth have been infiltrated for each implant site. The cells were also implanted in the peri-lesional tissue to be treated, infiltrating at about 1 cm outside the ulcer and with a needle at 45 degree, to infiltrate the cell concentrate under the ulcer itself. In addition, 1 cc of cell concentrate was infiltrated into the wound bed.

Protocol of the management of the lesion

The final dressing was performed flat with non-adhesive gauze and betadinated, then in all cases a galvanized and compressive bandage was then performed, with a light cohesive band, to remain for at least seven days following the treatment.[13]

The dressings have had a weekly frequency and have been characterized by the only change of the gauze in contact with the lesion and the new positioning of the bandage to the treated inferior limb.

The control of the medication after seven days then allowed to make an objective assessment on pain reduction case by case, in addition to the prevention of a possible onset of local infection.

In addition, at each check, an eco-color-doppler arterial examination of the popliteal-tibial tract to the treated lower limb was performed to assess the onset of a possible new network of collateral, connected to the main circle, along the course of the infiltrations carried out in the operating theatre.

Clinical outcome

The primary end point considered is the complete healing rate of the wound.

Secondary end points were the measurement of pain, through administration of VAS scale, the healing time of the wound itself and the rate of wound recurrence.

The follow up was performed at 1,3,4,6 and 12 months. A second follow-up, out of five patients, was performed after 4 years.

RESULTS

Of the 22 cutaneous ulcers of the treated lower limbs, already at the end of the third infiltration, that is after the third month of treatment, 14 were brought to healing (about 64%), while 8 ulcers (about 36%), are clearly improved (Table 3).

Of the 8 ulcers improved with the three procedures defined at the beginning of the study, 3 came to spontaneous recovery after a month from the last treatment, 4 ulcers needed, after a month, a homologous graft of skin to reach complete recovery, which occurred after six months from the beginning, while the last one, belonging to the arterial group, needed a fourth treatment, associated at the same time with homologous skin grafting, and healed after about 12 months from the beginning of treatments. The first clinical case reported represents a male patient with an arterial ulcer present for about 5 years, who already after the first treatment was able to rest in bed instead of in the armchair, while the final healing took place through a graft of homologous

Table 3. Results of ulcers treated at 3 next months.

	Heal	Improved
Arterial	7	5
Mixed	1	2
Diabetic	5	1
Iatrogenic	1	-

skin after the third cycle of infiltration (Appendix, Photo 1-4). The second, instead, depicts a female patient with a mixed ulcer, present for about 1 year, which has reached spontaneous recovery after about a month from the last treatment (Appendix, Photo 5-9), while the third represents a mixed wound, with already lymphatic impairment, hesitant for about 6 months, which has also reached healing with homologous graft (Appendix, Photo 10-11).

However, for the secondary endpoints, concerning the rate of recurrence with four-year follow-up of the treated injuries for the eight patients completed the procedure, it was no longer possible to trace one patient, two died in the selected period, while the other five were revalued by the operator himself, who performed an arterial eco-color-doppler of the peripheral vessels. The diagnostic test showed a very important result, that in four patients were still detected some collateral circles formed at the end of the initial treatments, while in patient affected by iatrogenic lesion were not visible, maybe because he had no basic vascular pathology. Patients who were revalued after 4 years were carriers of 9 of the 22 initial ulcers, accounting for about 41% of all lesions initially treated, were all in good health and two were part of the dysmetabolic group, one respectively with arterial ulcers, one with mixed arterial and venous wound and one with of the iatrogenic wound. In the four years under consideration, only one patient in the dysmetabolic group stated that she had reappeared a lesion, but distant from the one initially treated, due to a transient diabetes no longer compensated and already healed elsewhere, while none of the others had been affected by a new ulcer in the selected period (Table 4). The clinical cases presented as follow-up at 4 years include three patients of two different groups, respectively one of arterial (Appendix, Photo 12-14) and two diabetic (Appendix, Photo 15-20), of which the first of the latter group underwent to a fourth infiltration before reaching full recovery.

By performing a more detailed analysis of the secondary endpoints, by Visual-Analog Scale (VAS) assessing pain before and after treatment, all patients showed already after seven days from the beginning of the treatment a reduction of the symptomatology evaluated as referred night pain, while it was strongly reduced after the three cycles for more than 2/3 of the initial value (Figure 1) Regarding the healing times, we observed an acceleration of the process since 14 ulcers have already healed at the third month of treatment, that is at the end of the last cycle, while the other 8, 3 are healed within a month, 4 after three and only one after more than six months from the end of the cycle of treatments established (Figure 2).

In all treated patients, the surgeon who performed the eco-color-doppler along the course of the infiltrations, noted a constant increase in detectable subcutaneous neo-vessels with the power-doppler and color-doppler function of the ultrasound, therefore suggesting an increased flow in areas considered hypoxic at the start of treatment.

There were no side effects or adverse reactions or major or minor ones in any of the treated patients with autologous PBMNC.

Table 4. Etiology of ulcers with follow-up at 4 years.

	Heal	Recurrence
Arterial	2	-
Mixed	2	-
Diabetic	4	1
Iatrogenic	1	-

DISCUSSION

The ulcer, by its nature, does not tend spontaneously to heal, but with the correction of the pathological

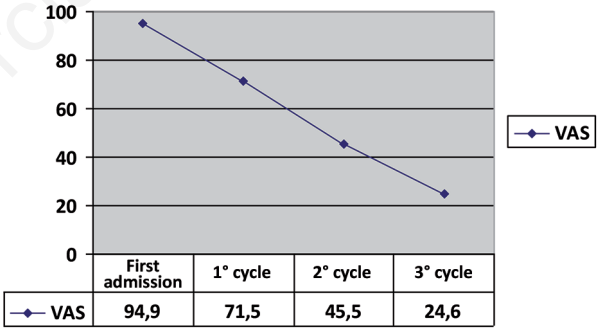

Figure 1. The graph shows the values of the VAS scale for each cycle.

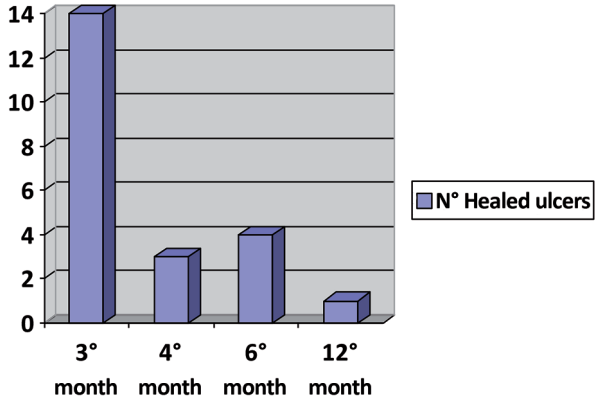

Figure 2. The graph represents the number of ulcers healed at 12 months.

conditions, which are often the cause of its formation, and with the use of appropriate therapies, the lesion tends to heal and be filled by an epithelial connective tissue, of different appearance to the surrounding healthy skin.

From the many clinical observations and diagnostic results, it seems that factors recur constantly in the pathogenesis of chronic ulcers of the lower limbs: the reduced inflow or outflow of blood and/or the presence of a systemic pathology in place. The presence of one or a combination of these factors is undoubtedly indispensable for the origin of chronic lower limb ulcers.[13]

The fundamental role of monocytes-macrophages in the healing of lesions has now been described in numerous papers.[6,14–17] In particular, several studies[17,18] indicate the monocytes-macrophages as a new and interesting therapeutic target on which to act to reactivate the healing of chronic lesions and the same concept is deepened in its cellular and molecular aspect.[19–21]

It has also been shown that macrophages control collagen transcription during the early stages of wound healing and that dermal fibroblasts, stimulated by paracrine factors released by macrophages activated in the M2 phenotype, show an increased proliferation index.[16,22]

In vitro and in vivo evidence indicate a fundamental role of monocytes-macrophages in the processes of tissue regeneration, in fact they show how these types of cells are able to create space in the extracellular matrix and encourage the diffusion of progenitor cells (including vascular cells). [8,23]

Monocytes are present in human tissues as circulating cells in peripheral blood and as resident cells in all tissues in the form of macrophages, which are highly plastic cells capable of interacting with multiple cell populations and tissue-resident stem cells.[19,24] Furthermore, monocytes secrete interleukin-1, which facilitates the activation of T cells by monocytes-macrophages, promoting the multiplication of fibroblasts and therefore, consequently, the wound healing.[25,26] The macrophages residing in the tissues are in a quiescent form called Mφ,but are activated by bacteria or inflammatory cytokines in the form M1. In the lesions that heal the M1 phenotype is converted into the regenerative form M2 which causes remodelling of the lesion and its healing. In chronic wound, on the contrary, polarization does not take place and the lesion remains in an inflammatory state which prevents its healing.[15,27] It has been shown that by implanting a cell concentrate of monocytes in an inflamed tissue, M1 macrophages are “polarized” in the form of M2, with the beginning of the regenerative phase.[28,29] It is not clear whether the mechanism of this switch includes the involvement of circulating precursors or the re-education of cells in situ, but it has been shown that some specialized subsets of T lymphocytes (Th1, Th2, Tregs) are able to activate the polarization of macrophages and exhibit a flexibility and plasticity never seen before.[30]

The term therapeutic angiogenesis was first proposed in 1993 by Hockel to describe interventions aimed at inducing the growth of blood vessels in areas of hypo vascularization and it has long been known that in the patient with obstructive artery pathologies, physiologically, there are two different forms of compensatory vascularity, angiogenesis and arteriogenesis.

Angiogenesis means the development of a new capillary circle by gemmation (sprouting) from pre-existing capillaries in response to local tissue hypoxia and is mediated by the release of cytokines (VEGF and other cytokines). The resulting capillaries are very small, with a diameter of about 10-20 um, which alone cannot compensate for the lack of blood flow due to arterial occlusion (Hagen’s law - Poiseuille).

Arteriogenesis, in contrast, is the transformation of pre-existing arterioles into functional collateral arteries, which can compensate for the reduction in flow. The original diameter of a small arteriole can increase up to 20 times during the arteriogenesis process and this is triggered by an increase in the “shear stress” on the endothelium which, in turn, causes an increase in adhesion molecules (MCP-1) for circulating monocytes that accumulate around the arteries in a proliferative state and provide the release of cytokines and growth factors, thus indicating that monocyte-macrophages play a fundamental role in this process.[31,32] It has also been reported, in addition to the increase of monocytes-macrophages, also an increase of T lymphocytes around the new collateral, indicating therefore a fundamental importance also of this type of cells in the growth of the new vessels.[33–35] Despite this, the physiological mechanism of arteriogenesis compensation through the recruitment of monocytes does not work in a large number of patients, as the reduced perfusion does not allow monocytes and lymphocytes to reach the damaged tissue in sufficient quantities. Particularly interesting is the correlation shown in numerous studies between risk factors of critical ischemia and low number of circulating EPC-monocytes; particularly this mechanism is compromised in patients with diabetes, smokers and with hyper-lipidaemia, in which the concentration of the measured EPC-monocytes is strongly diminished.[36,37]

The results observed on chronic lesions treated with PBMNC in our center can be related both to the angiogenic and arteriogenic capacity of PBMNC, and to the ability of polarization in M2 that can change the inflammatory state of the tissue.

The observed data are in line with what previously observed both in the diabetic foot[38,39] and in chronic lesions with other etiology[40,41] in studies that used the PBMNC produced with selective filtration system. This new Point of Care device for intra-operatory use, represents an

innovative technology for the isolation and concentration of PBMNC cells, bringing several important advantages over centrifugation-based technologies, preserving both membrane integrity, but especially the release of active molecules on which the biological mechanism of action is based (paracrine effect). In addition, the ease and speed of use, high reproducibility (the method is not operator – dependent) make it extremely handy.

Patients affected by peripheral arterial disease wounds and those with mixed ulcers all had antiaggregating in their home therapy, to which was added a low molecular weight heparin therapy, with prophylactic dosage, up to the third treatment, while the diabetic group has definitely benefited from the best adaptation of anti-diabetic therapy associated with both insulin and oral. The only patient who did not change the home therapy was the one with iatrogenic ulcer, while everyone definitely benefits the targeted antibiotic therapy throughout the course of treatment.

The original fact of our observations is that in the five patients who were followed up to 4 years, the collateral circles were still detectable and none of the patients had shown a recurrence in the treated limb.

These data are a simple case reports of patients with ulcers with mixed etiology, although all non-healing at 6 months: no statistical consideration is clearly possible on an extremely limited number of cases and referred to a group not homogeneous by definition.

CONCLUSIONS

Chronic ulcers of the lower limbs, due to their high frequency and prolonged disability, is a pathology now considered social, requiring from doctors and patients themselves the utmost attention that, in practice, it consists of prevention carried out scrupulously over time, to limit the evolution and appearance of local and systemic complications.

Both the overcoming of the concept of disease as an expression of localized process, and the significant cognitive deepening of recent years, have contributed to the framing of the skin ulcers of the lower limbs as an expression of an organism pathology and no longer as an entity in its own right.

The treatment of cutaneous ulcers is quite complex, in consideration of the multiplicity of the mechanisms that determine its appearance and feed its chronicization.

In addition, there are factors external to the injury that affect the healing of the same, including the age of the patient and his general condition, in fact in our experience the totality of cases is over-70, so with high percentages of co-morbidity.

Autologous cell therapy with PBMNC presents a favourable safety profile with a very low rate of adverse events and no increase in serious events, in line with reports from numerous meta-analyses,[42–44] and has an excellent positive risk-benefit ratio, so it can be a viable therapeutic option, especially for those patients not responding to standard treatments.

Therefore, the lack of significant side effects, low invasiveness, and repeatability, allow to consider optimal the tolerability of cell therapy with PBMNC in the treatment of lower limb ulcers. This characteristic is of extreme importance for a therapeutic modality that is destined to be utilized by a mainly old population like the these one. Moreover, many of the patients treated in the study were carriers of other concomitant pathologies, yet they have shown to tolerate well the possible stress brought by such therapeutic method.

In conclusion, these eight case reports on the treatment with autologous PBMNC of ulcers with different aetiology, suggest the effectiveness and excellent tolerability in the treatment of lower limb ulcers, with a significant improvement in the quality of life of patients undergoing such treatment.

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