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ORIGINAL ARTICLE

Long-term effects of repeated autologous transplantation of bone marrow cells in patients affected by peripheral arterial disease

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Long-term effects of autologous mononuclear bone marrow cell transplantation were studied in patients with severe peripheral arterial disease (PAD) and critical limb ischemia. Ten patients with end-stage disease were infused twice with autologous bone marrow cells and they completed the 12-month follow-up study. Substantial improvement of blood flow and increasing capillary densities were seen when compared with a concomitant control group comprising patients who did not enroll in the study. The ankle–brachial index (ABI) and pain-free walking distance improved significantly in treated patients. The improvement was sustained 12 months after treatment. These results confirm that the autologous bone marrow transplantation is an effective therapeutic strategy in critical limb ischemia.

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Introduction

Peripheral arterial disease (PAD) is a common circulatory problem in which narrowed arteries reduce blood flow, especially to the limbs, and critical limb ischemia represents the most dramatic clinical outcome. It is a disease with widely varying symptoms, ranging from no symptoms at all to pain on walking (intermittent claudication), continuous pain (pain at rest) in the foot, gangrene and ulcerations. At this stage, the patient is at high risk for amputation. However, because of the partial efficacy of pharmacological treatments, amputation is undertaken at the end stage of the disease as a unique solution to alleviate the symptoms.

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Each year, more than 100 000 individuals undergo lower limb amputation because of PADs.[1]

An effective clinical treatment for critical limb ischemia is revascularization of affected limbs, which occurs as a result of vasculogenesis and angiogenesis. Vasculogenesis requires the novel recruitment of exogenous stem cell progenitors, whereas angiogenesis involves the quiescent endothelial cell progenitors of the pre-existing vessels. Bone marrow consists of different progenitor cells, including endothelial progenitor cells, which can differentiate into endothelial cells and release angiogenic promoting factors.[2–4]

Preclinical studies have established that implantation of bone marrow-mononuclear cells (BM-MNC) into ischemic limbs increases collateral vessel formation in experimental models of hind limb ischemia.[5–8] In patients affected by limb ischemia, local intramuscular autologous bone marrow cell therapy has shown encouraging results with consistent revascularization of affected limbs.[9,10] A recent study showed that combined intra-arterial and intramuscular transplantation of autologous mononuclear bone marrow stem cells was a therapeutic option for patients with severe PAD.[11]

Although the underlying mechanism remains undefined, the beneficial effect of bone marrow transplantation raises the possibility that infused stem cells enhance angiogenesis by supplying endothelial progenitor cells as well as angiogenic cytokines and factors that activate progenitor cells resident in the tissue.[12,13]

Although initial trials with cell therapy showed encouraging results, follow-up studies have yet to show long-term benefits and whether this approach can be considered a plausible therapeutic strategy.

We report the effects of repeated autologous BM-MNC infusions in a group of 10 patients with severe PAD.

Methods

The study was designed as a non-randomized controlled trial and the primary end point was improvement of vascular function in the transplantation group.

Ten patients with advanced stage of PAD (six men and four women) were enrolled in this study. Enrolled patients

showed symptoms of the disease classified in phase III (21%) or in phase IV (79%) of the Leriche–Fontaine classification,[14] for which no curative alternatives exist and usually the patients undergo amputation of the limb, to avoid gangrene and subsequently death.

The inclusion criteria used for this study were:

- Diagnosis of severe PAD
- End-stage disease (III or IV stage of Leriche–Fontaine classification)
- Age >18 years
- Adequate neutrophil count $\geqslant 2000$/mmł, platelets $\geqslant 100\,000$/mmł and hemoglobin $\geqslant 10$ g/dl, adequate liver function (total bilirubin 2–14 mmol/l) and renal function (creatinine clearance mg/dl)
- Written consent

In parallel to the transplantation group, nine patients were recruited in the control group. They met all the criteria of the transplantation group, but were not included into the transplantation group because of personal reasons. They underwent the same follow-up examination; however, only four reached the 12-month follow-up, due to the fact that for the other five patients surgical interventions (amputation or arterial bypass) became essential for their survival.

The diagnosis of limb ischemia was confirmed by echography and computed tomography angiography (CTA) (see Table 1 and below). In 70% of the cases, vasculopathy was associated with diabetes mellitus. Half of the patients were smokers and were asked to stop smoking. All patients had rest pain, and showed intermittent claudication. Three patients had already undergone arterial bypass surgery. The ankle–brachial index (ABI) and pain-free walking for a standardized distance were used to classify the severity of PAD of the legs before and after treatment. The walking ability of patients was estimated taking into consideration the distance walked before suffering any pain, over a corridor of known length (25 m) in the hospital. Their lifestyle was regulated throughout the study. The drugs used (anticoagulants and anti-aggregants) were suspended just a week before the infusion. All patients were discharged from the hospital one day after transplantation. No complications or side effects were observed.

The protocol was approved by the Ethics Committee of the Seconda Universita' of Naples. Written informed consent for participation was obtained from all patients.

Computed tomography angiography

Computed tomography angiography confirmed the diagnosis of PAD. Four-channel multi-detector row CT scans were acquired with a CT scanner (Light speed Qxi; GE Medical Systems, Milwaukee, WI, USA) under a protocol approved by our institutional review board. Informed consent was obtained from all patients. Patients were placed in a supine position on the CT table to allow transport of the entirety of their body, inferior to the xyphoid process, to pass through the CT gantry. An anteroposterior scout view was first acquired to encompass the entire body below the xyphoid process. Subsequently, helical CT was performed without intravenous contrast medium from the apex of the diaphragm to the ankles, with a 5-mm nominal section thickness, pitch of 1, 5:1, 30-mm rotation table feed, and a gantry rotation period of 0.8 s. The X-ray tube potential was 120 kV and the current was 300 mA. An 18- to 22-gauge catheter was placed into a superficial vein within the antecubital fossa, forearm or dorsum of the hand.

To optimize contrast enhancement, the scan delay was determined with the aid of a semiautomatic bolus-tracking system (Smart Prep, GE Medical Systems, Milwaukee, WI, USA). A region of interest was positioned in the infra-renal abdominal aorta and a threshold of 130 HU was selected.

CT angiography (CTA) was performed from the infra-renal abdominal aorta to the pedal arteries after administration of a single bolus of 2 mg/kg of body weight (Ultravist 370, Schering, Berlin, Germany) at a flow rate of 3.5 ml/s. The rate of the contrast medium injection was adjusted at the discretion of the physician on the basis of the quality of intravenous access.

Postprocessing into three-dimensional volume renderings, maximum intensity projections and curved planar reformations were performed on a dedicated workstation (Advantage Windows 4.0; GE Medical Systems). In all cases, CTA scans at baseline confirmed the large involvement of the lower-extremity peripheral arterial system, whereas at 1-year follow up examinations after treatments, there were not significant modifications of the number of arterial vessels with a caliber greater than 10 mm (data not shown).

Bone marrow transplantation

Bone marrow (about 10^9 cells in 200 ml) was extracted from the iliac crest under anesthesia in a sterile manner and anticoagulated. The mixture was passed through filtering devices to remove large particulate matter such as fat, bone chips and/or clots (Fenwal Bone Marrow kit collection 4R2104, Fenwal Inc Blood Technologies, Lake Zurich, IL, USA) and transplanted immediately into femoral artery through peristaltic pump at 120 ml/h. Forty-five days after the first infusion, the treatment was repeated following the same procedure.

Measurement of blood flow

Microcirculatory disturbances were evaluated by laser Doppler measurements (Periflex PF2B, Perimed AB, Stockholm, Sweden) and videocapillaroscopy[15] (Videocap DS Medica srl, Milan, Italy, optical $\times 200$) before and after the treatment at two different stages: 6 months and 12 months after the infusion.

All tests were performed at a stable room temperature (25 °C) after the patient was allowed 30 min of acclimatization while resting in the supine position. We started the measurements at least 5 min after placement of the probe to ensure a stable flux measurement. The probe was positioned on the third distal dorsum of the foot or on the first toe of the limb, which underwent the infusion of stem cells. Each position was marked with a dermographic pen or was photographed to place the probe in the same position in the subsequent controls.

Table 1 Clinical characteristics of patient's cohort (19) affected by severe PAD

Patient		*Gender*	*Age (years)*	*Site of occlusion*	*Other*	*ABI T_0*	*ABI T_{12}*	*Pain-free walking distance T_0*	*Pain-free walking distance T_{12}*
P1	L1 sx	M	75	Deep femoral artery	D	0.7	0.76	40	50
	L1 dx			Deep femoral artery		0.62	0.72		
P2	L2 dx	M	38	Superficial femoral artery	S	0.75	0.69	100	1000
	L2 sx			Posterior tibial artery		1.16	1		
P3	L3 sx	M	55	Anterior tibial artery	D, H	1.07	1.07	200	500
	L3 dx			Anterior tibial artery		1.07	1.15		
P4	L4 sx	F	74	Superficial femoral artery	D,H	NM	0.86	ND[a]	ND[a]
P5	L5 dx	M	77	Deep femoral artery	D, H, S	0.45	0.52	30	500
P6	L6 dx	M	64	Deep femoral artery	D, S, H	NM	0.92	50	200
	L6 sx			Popliteal artery		NM	0.78		
P7	L7 dx	F	58	External iliac artery	H	0.73	0.71	200	1000
	L7 sx			External iliac artery		0.69	0.78		
P8[b]	L8 sx	F	54	Superficial femoral artery	D, S, H	0.31	0.58	100	500
P9[b]	L9 dx	M	59	Superficial femoral artery	S, H	0.91	0.68	50	900
	L9 sx			Femoro-popliteal artery		NM	0.9		
P10[b]	L10 dx	F	49	Femoro-popliteal artery	D	0.60	0.66	75	200
	L10 sx			Femoro-popliteal artery		0.64	0.75		
Control group									
P11	L11dx	F	72	Deep femoral artery	H	NM	NM	ND[c]	ND[c]
P12	L12dx	M	76	Superficial femoral artery	D, H	0.72	0.58	100	100
P13	L13sx	M	69	Femoropopliteal artery		0.72	0.60	150	150
P14	L14dx	M	73	Popliteal artery		0.50	0.37	200	200
P15	L15sx	F	67	Femoropopliteal artery	H	NM	NM	50	ND[c]
P16	L16dx	M	74	Deep femoral artery	D	NM	NM	25	ND[c]
P17	L17dx	M	66	External iliac artery	D, H	0.60	0.52	150	ND[c]
P18	L18dx	M	79	Femoropopliteal artery		0.78	0.62	200	200
P19	L19sx	F	71	Deep femoral artery	D, H	0.50	NM	25	ND[c]

Abbreviations: ABI = ankle–brachial index; D = diabetes; F = female; H = hypertension; M = male; ND = not determined; NM = non-measurable; PAD = peripheral arterial disease; S = patients have had smoking habits.
Ten patients (P1–P10, 17 ischemic legs) underwent BMT; ABI index and pain-free walking distance were assessed before and after BM-MNC infusion. The remaining nine patients (P11–P19) belonged to the control group and they were followed-up similar to the transplantation group.
[a]Pain-free walking distance was not determined as Patient 4 underwent amputation of the right leg before the study.
[b]Patients who underwent arterial bypass surgery before starting the trial.
[c]Patients underwent amputation before the scheduled control at 12 months.

The laser Doppler (LD) measurement procedure was performed immediately afterwards as follows: LDBO (laser Doppler—clinostatic position) recorded the blood flow for 5 min with the patient in clinostatic position; LDBP (laser Doppler with lowered leg) recorded blood flow after positioning the limb in supine position activating venoarteriolar response; LD44° (laser Doppler—clinostatic position, probe temperature 44 °C) recorded blood flow after increasing the temperature of the probe to 44 °C to remove the myogenic vessel tone; LDPF (laser Doppler peak flux after induced ischemia)-LDPT (laser Doppler peak time after induced ischemia) recorded blood flow after a mixed hyperemia test by increasing the probe temperature to 44 °C and inducing limb ischemia by arm cuff positioned below the knee and inflated to a pressure of 240 mm Hg for 3 min.

Subcutaneous infiltration of lidocaine and papaverin, to obtain a blockade of neurogenic tone and maximum vasodilatation, was not performed as in other studies because of the severity of the PAD.

The videocapillaroscopy was performed using Videocap 100 apparatus (DS Medica srl, Italy, optical $\times 200$) and the images were taken at nail wall of the fourth finger of the non-dominant hand of every patient, on the skin of tibia anterior region of the limb (at least 4 cm^2), which underwent the transplantation of stem cells, on the dorsum of the skin foot (of 4 cm^2) and on the nail wall of the toe.

The positions were labeled with a dermographic pen or were photographed.

All tests were performed at a stable room temperature (25 ± 1 °C).

Using Fagrell classification, we calculated the visible number of capillaries for mm^2, the number of dilated capillaries and the number of neocapillaries positioning the probe at the same sites where the images were taken at 6 and 12 months after the treatment.

Table 2 Evaluation of blood flow in the study group at T_0 (before infusion), T_6 and T_{12} (after infusion)

	Basal test (*PU* ± *s.d.*)	*6 months later* (*PU* ± *s.d.*)	P-*value*	*1 year later* (*PU* ± *s.d.*)	P-*value*
LDBO	27.68 ± 12.41	50.18 ± 43.62	0.05	59.90 ± 34.3	0.001
LDBP	22.91 ± 12.69	37.56 ± 30.11	0.07	54.04 ± 55.47	0.01
LD44°	74.59 ± 41.38	141.53 ± 101.35	0.01	164.42 ± 100.34	0.002
LDPT	222.40 ± 168.26	386.53 ± 190.43	0.01	233.84 ± 117.82	NS
LDPF	144.78 ± 75.94	280.67 ± 168.72	0.005	311.69 ± 201.55	0.003

Abbreviations: LD44° = laser Doppler—clinostatic position, probe temperature 44 °C; LDBO = laser Doppler—clinostatic position; LDBP = laser Doppler with lowered leg (Venoarteriolar Reflex Activation); LDPF = laser Doppler peak flux after induced ischemia; LDPT = laser Doppler peak time after induced ischemia; NS = not significant; PU = perfusion units.
Data are expressed as perfusion units (PU) ± s.d. All reported probability values were two-tailed.

Results are represented as mean ± s.d. All reported probability values were two-tailed.

Results

Clinical characteristics

Nineteen patients with severe PAD (Leriche–Fontaine III or IV,[14]) were recruited and underwent echographic and CT angiography to verify the diagnosis of PAD. All patients showed an extensive peripheral arterial occlusion (see Table 1 for details) and 10 of them were subjected to BMT. After the transplant, they were followed-up at 6 and 12 months and the results are summarized in Table 1. In the control group, only four patients reached the 12-month follow-up, due to the fact that surgical intervention for others became essential for their survival. The ABI index and the pain-free walking distance assessed ischemic status of all patients, and as shown in Table 1, the ABI index and pain-free walking distance were significantly increased in 8 of 10 of the treated patients (80%) at 12-month follow-up.

Effects of BM-MNC implantation on perfusion and endothelial function

Amelioration of clinical symptoms prompted us to evaluate the blood flow rate of treated legs after the BM-MNC infusion by means of a laser Doppler perfusion scanner. Table 2 summarizes the results of laser Doppler flowmetry under the defined conditions.

Quantitative analysis of the laser Doppler imaging revealed that the BM-MNC implantation had a significant increase in blood flow at rest (LDBO) in the ischemic limbs, either at T_6 and T_{12}.

Relative blood flow was increased around twofold after 12 months from the treatment (LDBO: T_0 27.68 vs T_{12} 59.90, *P*-value 0.001), showing an enhancement at T_6.

Restoration of perfusion has also been evident from the blood flow measured for the lowered leg (LDBP: T_0 22.91 vs T_{12} 54.04, *P*-value 0.03). The significance of this measure was lower; however, we should take into account that the increase of LDBP is abnormal in a healthy patient, whereas it is considered a compensatory mechanism in a patient affected by vasculopathy. Therefore, the increase of LDBP confirmed the beneficial effect of infusion.

Next, we considered the response in hyperemia conditions. Increasing local skin temperature leads to an increase in blood flow avoiding the myogenic vessel tone, providing an effective method to assess the presence of microcirculatory modifications. As shown in Table 2, a significant increase in the hyperemia condition was observed (LD44° T_0 74.59 vs T_{12} 164.02, *P*-value 0.001). We then measured the response to the reactive hyperemia test and we observed a significant increase in the peak flow (LDPF, Table 2), both at T_6 and at T_{12}, as an expression of the maximal vasodilatation to the combined stimulus of high temperature (44 °C) and induced transient hypoxia, due to reduced vascular resistance.

We also measured the significant increase in the peak time (LDPT) at T_6; however, this value was not confirmed at T_{12}. It is known that this parameter is prone to variability without concomitant blockage of neurogenic tone.[16]

Videocapillaroscopy[15] allowed us to evaluate the effect of BM-MNC infusions on capillary densities, the presence of enlarged capillaries and, more important, to evaluate the appearance of neoangiogenesis. As reported in Table 3, the number of capillaries is increased either at the level of tibia, foot and toe, as well as in distal districts, such as the fourth finger of the hand.

It is worth noting that the main visible effect of BM-MNC infusions is seen in the increase of neocapillaries in the tibia, foot and toe of the treated leg, as shown in Figure 1. We also evaluated the effect on capillary densities according to the Fagrell classification; the BM-MNC infusions resulted in a significant decrement in the Fagrell index, corroborating the positive effect of the infusion in the proximal districts, such as tibia (from 2.75 to 1.44), foot (from 2.71 to 1.44) and at the level of the toe (from 2.60 to 1.53).

Discussion

In this study, we report the follow-up of 10 patients (17 ischemic legs) with advanced PAD treated with autologous bone marrow cell transplantation. Compared with already existing studies, we used two consecutive infusions done 45 days apart into the femoral arteries and we followed the patients up to 12 months after treatment. This is a novel aspect about BM-MNC transplantation in patients affected by PAD.

Previous study has shown a therapeutic effect of bone marrow transplantation in the gastrocnemius of the

Table 3 Capillary density analysis in treated ischemic legs

	Basal test (*n* ± *s.d*)	*6 months later* (*n* ± s.d.)	P-*value*	*12 months later* (*n* ± s.d.)	P-*value*
Capillary density					
Hand	14 ± 7	14.93 ± 7	NS	20.67 ± 7	NS
Tibia	11 ± 8	12.80 ± 8	NS	13.67 ± 8	NS
Foot	17.12 ± 11	20.20 ± 12	NS	28.78 ± 12	NS
Toe	16.94 ± 10	16.47 ± 10	NS	30.44 ± 10	NS
Capillaries enlarged					
Hand	5.65 ± 6	3.20 ± 6.4	NS	2.78 ± 6.44	NS
Tibia	4.24 ± 4	3.73 ± 4	NS	4.78 ± 4	0.09
Foot	5.12 ± 6	3.67 ± 6	NS	6.11 ± 6	NS
Toe	4.88 ± 4	4.53 ± 4	NS	7 ± 4	NS
Neoangiogenesis					
Hand	0 ± 0.0	0.07 ± 0.0	NS	0 ± 0.0	NS
Tibia	0.59 ± 1.3	2.27 ± 1.3	0.01	1.33 ± 1.37	NS
Foot	0.53 ± 1.7	2.53 ± 1.7	0.01	3.67 ± 1.74	NS
Toe	0 ± 0.0	0.87 ± 1.1	0.0052	1.11 ± 0.0	0.03

Abbreviation: NS = not significant.
Data are expressed as mean ± s.d. All reported probability values were two-tailed.

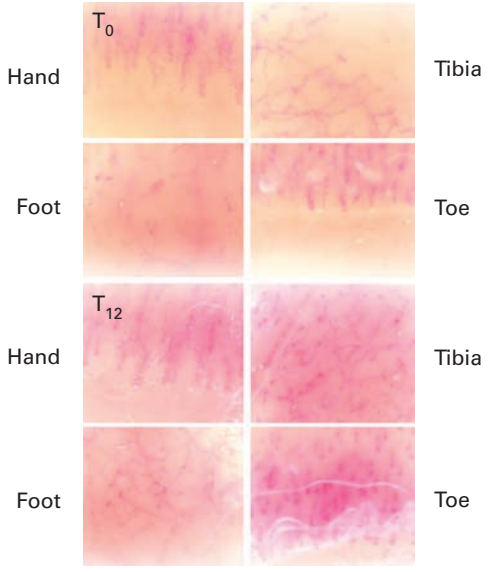

Figure 1 Videocapillaroscopy images of non-dominant hand of tibia, foot and toe of ischemic legs before and after treatment.

affected limb at 6-month follow-up.[9] Recently, both intramuscular and intra-arterial transplantation of bone marrow cells had been proved therapeutically beneficial and the follow-up study was performed at 13 months,[11] indicating that the beneficial effect is maintained when a massive dose of cells are infused.

The main result of this study is that two-fold transplantation of autologous BM-MNCs significantly ameliorated blood flow in all treated legs, as assessed by substantial increases of ABI index, pain-free walking distance and by the formation of new capillaries, as evaluated by videocapillaroscopy. Moreover, the BM-MNC infusion had the positive effect of salvaging the limb; none of our patients underwent amputation, compared with the control group, which had a deep impact on the quality of the life of these patients.

More important, the beneficial effects of the treatment are maintained at 12 months. Therefore, we can conclude that our experimental procedure can be considered clinically feasible and as a minimally invasive therapeutic approach for patients affected by PAD.

Cell transplantation offers the potential for treatment and cure for a range of diseases including ischemic disorders (PAD, myocardial infarction, cerebral ischemia). Bone marrow-derived cells are actually required for adult revascularization in individuals affected by angiopathies. It is not known which is the most efficient subpopulation of bone marrow able to induce beneficial effects in ischemic disorders. Evidence indicates that progenitor cells generated from monocytic or non-monocytic cells are both effective, suggesting that angiogenesis is not a prerequisite for a certain subpopulation, but is the result of cross talk between different cells and pathways.

In addition, elucidation of these mechanisms finely regulates angiogenesis, and our data pave the way for therapeutic exploitation of bone marrow cells in defining the optimal protocol for the treatment of patients affected by PAD. The encouraging results should encourage research toward the fulfillment of this purpose and should be validated by large numbers of studies.

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