Therapeutic Outcomes of Transplanting Autologous Granulocyte Colony-stimulating Factor-mobilised Peripheral Mononuclear Cells in Diabetic Patients with Critical Limb Ischaemia

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Key words
- peripheral arterial disease
- critical limb ischemia
- stem cell transplantation

Abstract

The efficacy and safety of transplanting autologous mesenchymal stem cells (MSCs), from granulocyte-colony-stimulating factor (G-CSF)-mobilised peripheral blood, was investigated in diabetic patients with critical limb ischaemia (CLI).

After 3 months, the transplanted group of patients (n=7) showed a significant improvement in ischaemia manifestations, including pain and neurological signs, wound healing and the rate of lower-limb amputation, compared to the control group of patients (n=14). Pain was significantly reduced in the transplanted group compared to controls (P=0.014). The ankle-brachial index (ABI) and the pulse strength within ischaemic tissues of the transplanted group were significantly improved (P=0.035 and P=0.01, respectively).

Importantly, 50% of the control group (7/14 patients) faced major amputation of a limb at the study's conclusion, compared to none of 7 patients in the transplanted group (P=0.047).The safety of transplantation was confirmed by observing no adverse reactions among the transplanted group, including infection and immunological rejection.

Hence, this study provides further evidence that transplantation of autologous peripheral blood MSCs, mobilised by G-CSF, induces angiogenesis and improves the wound healing process in diabetic patients with CLI.

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Introduction

Peripheral arterial disease (PAD), including critical limb ischaemia (CLI), is one of the devastating complications of diabetes, where patients are frequently poor candidates for surgical re-vascularisation and amputation is the likely outcome.

Currently, no satisfactory treatment options are available for CLI. In diabetic patients, the proliferation, adhesion, and incorporation of circulating endothelial progenitor cells (EPCs) into vascular structures are impaired (Huang et al., 2005, Loomans et al., 2004, Tepper et al., 2002). Ischaemic injury usually induces formation of collateral vessels via migration and proliferation of endothelial cells from pre-existing vasculature (Iba et al., 2002, Jackson et al., 2001, Kienstra et al., 2008, Kwon et al., 2008, Lechner and Habener 2003). Several clinical trials have shown that transplantation of functional EPCs, to induce angiogenesis and wound healing in ischaemic tissues, may be a successful novel therapeutic for patients with diabetic CLI (Barcelos et al., 2009, Huang et al., 2005, Ishida et al., 2005, Kajiguchi et al., 2007, Zafarghandi et al., 2010).

An alternative approach is to utilise mesenchymal stem cells (MSCs), which can be obtained from the iliac crest bone marrow, expanded in vitro and then transplanted into the ischaemic organ, including skin, gastrointestinal tract and muscle (Battiwalla and Hematti 2009). MSCs can also be found in the peripheral blood, but at a very low concentration. However, it has been demonstrated that MSCs can be mobilised from peripheral blood mononuclear cells (PBMCs) using granulocyte colony-stimulating factor (G-CSF) (Hicks et al., 2007). Such a strategy produces sufficient numbers of cells for transplantation by leukapheresis (Yang et al., 2005).

The current clinical trial aimed to assess the safety and efficiency of therapeutic transplantation of autologous peripheral blood-derived MSCs to induce angiogenesis in diabetic patients with CLI.

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Parameter	Control group (n = 14)	Transplantation group (n = 7)	P-value*
age (mean ± SD), years	64.2 ± 7.8	63.5 ± 7.8	0.847
smoking (% of patients per group)	21.4 (n = 3)	42.9 (n = 3)	0.610
diabetes duration (mean ± SD), years	14.2 ± 8.5	16.5 ± 8.7	0.574
peripheral neuropathy score† (mean ± SD)	5.9 ± 2.6	6.7 ± 2.2	0.529
wound duration (mean ± SD), months	3.4 ± 3.0	5.0 ± 3.9	0.333
wound size (mean ± SD), cm^2	15.8 ± 17.0	14.2 ± 4.1	0.817
ulcer score‡ (mean ± SD)	2.9 ± 0.7	2.8 ± 0.8	0.847
Hb (mean ± SD), mg/dL	11.6 ± 1.5	11.5 ± 1.2	0.806
Cr (mean ± SD), mg/dL	1.2 ± 0.2	1.1 ± 0.01	0.555
history of CVA§, %	7.1 (n = 1)	14.3 (n = 1)	1.000
hyperlipidemia, %	42.9 (n = 6)	71.4 (n = 5)	0.361
hypertension, %	42.9 (n = 6)	71.4 (n = 5)	0.361

Hb, Hemoglobin; Cr, Creatinine

*No significant differences found between the 2 groups in outside factors affecting the wound healing process, including smoking, hemoglobin, creatinine levels, and degree of neuropathy. P-values were determined using Mann-Whitney, Chi-square and Fisher's exact tests

† Peripheral neuropathy scored by using monofilament examination

‡ Ulcers were scored using Wagner's ulcer classification

§ Cerebrovascular accident (CVA) in the patients has occurred more than 3 months prior to treatment (7–11 months earlier)

Table 1 Demographic and pathological profile of diabetic patients prior to treatment.

Patients and Methods

Study design and patients

A randomised, controlled and parallel clinical trial was designed to assess the safety and practical efficacy of transplanting autologous G-CSF-mobilised PBMCs in diabetic patients with CLI.

21 eligible volunteers attending the diabetes clinics of Shariati and Rasul Akram Hospitals, Tehran University of Medical Sciences, Tehran, Iran, between December 2008 and December 2010, were randomly assigned into either a group for transplantation (7 patients) or the control group (14 patients).

Eligible diabetic patients were between 18 and 75 years old, had an unilateral or bilateral diabetic CLI, together with a history of angioplasty failure (or else could not benefit from angioplasty). All volunteers were expected to continue their cooperation throughout the study and needed to be negative for retinopathy, cystic infection, kidney dysfunction (i.e., a creatinine level = 2.5 mg/dL), severe heart failure (based on echocardiography), pregnancy or lactation, severe allergy, malignancies or a history of immunosuppressive therapy, coagulation disorders, acute or severe dyspnea, and a history of myocardial or brain infarction within the past 3 months.

Any patients with intracerebral haemorrhage, myocardial infarction or stroke within the past 3 months were excluded (due to risk of deterioration of health condition associated with bleeding for the purposes of the separation of stem cells).

Foot ulcers were scored by Wagner's ulcer classification system. Human experimentations were approved by the institutional ethics committee boards and conducted in accordance with the Declaration of Helsinki; written informed consent was obtained from all volunteers of this study.

Table 1 shows demographic profile of patients in both groups and their symptoms prior to treatment.

Assessment of vascularity

To evaluate the vascular status of a patient's diabetic limb, the ankle-brachial index (ABI) was determined using the Sonoline G40 Ultrasound System (Siemens Medical Solutions AG, Erlangen, Germany), as described previously (Ono et al., 2003). ABI measurements of less than 0.9 were considered a marker of severe claudication and debilitating peripheral arterial disease. CLI classed as severe PAD was defined in this study according to criteria described previously (Dorros et al., 2001), namely: i) persistent and recurring rest pain requiring analgesia, and an ankle systolic pressure of 50 mmHg and/or toe systolic pressure of 30 mmHg, and/or ii) non-healing wounds, ulceration or gangrene of the foot with ankle systolic pressure of 50 mmHg or toe systolic pressure of 30 mmHg.

Angiographic analysis of peripheral vascular status in the dorsalis pedis and tibialis posterior arteries of the diabetic limb was performed before and after treatment using a Light Speed 16 Scanner (GE Medical Systems, Milwaukee, Wisconsin, USA). The peripheral vascular status was ranked as 0 for a non-palpable pulse, 1 for a weak pulse, and 2 for a natural pulse. The skin of the foot was examined for any redness, especially between the toes and under the metatarsal. The patient's limbs were also carefully examined for callus formation, bone deformity, limited joint mobility and balance disorders.

Assessment of the ulcers

Wagner's ulcer-classification system was used to assess the limb ulcer status, according to guidelines described previously (Oyibo et al., 2001, Tateishi-Yuyama et al., 2002). Ulcer severity was scored from 0 to 5: grade 0, high-risk foot with no ulceration; grade 1, superficial ulcer without involvement of deep layers; grade 2, deep ulcer (cellulitis) in muscles and ligaments; grade 3, osteomylitis with ulceration or abscess; grade 4, localised and partial foot gangrene; grade 5, gangrene of entire foot.

Peripheral neuropathy and pain examinations

To detect peripheral neuropathy in the diabetic limb, a 5.07–10 gram monofilament examination was performed and the impairment of the patient's contact sensation was scored, as previously described (de Win et al., 2002). Briefly, a 10-gram monofilament was placed on 10 different points of the skin of the foot for 1–2 s and impairment of the patient's contact sensation was assessed.

The pain-free walking distance test was performed by walking with a constant speed on the same road.

Differentiation between the ischaemic and the neuropathic pain was performed based on the physical signs or angiographic patterns of ischaemia, including resting pain and lack of peripheral pulses or dystrophic nails.

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Recovery index	Control group (n = 14)	Transplanted group (n = 7)	P-value*
major amputation, %	50 (n = 7)	0.0 (n = 0)	0.047
improvement in walking ability, %	28.6 (n = 4)	85.7 (n = 6)	0.024
improved blood flow, %	21.4 (n = 3)	100 (n = 7)	0.010
ABI measurement (mean ± SD)	0.65 ± 0.25	0.92 ± 0.15	0.035
peripheral neuropathy score† (mean ± SD)	6.77 ± 2.04	8.14 ± 1.21	0.140
wound size (mean ± SD), cm^2	18.16 ± 23.20	8.50 ± 6.15	0.290

* P-values were determined using Mann-Whitney, Chi-square and Fisher's exact tests

† Peripheral neuropathy scored by using monofilament examination

Table 2 Recovery indications at 3 months post-treatment.

Separation of stem cells

All patients (in both groups) received a single daily dose of 5 pg/kg of human recombinant G-CSF (PDgrastim, Pooyesh Darou Co., Tehran, Iran) subcutaneously (SC) for 5 days. To prevent embolism, patients received a SC injection of 5 000 units of heparin (Alborz Darou Pharmaceutical Co., Tehran, Iran) every 12 h for 5 days.

The day after the last dose of administered G-CSF (day 6), 100 mL of circulating blood containing PBMCs was collected from each patient using a Gambro-BCT COBE Spectra Apheresis System (CaridianBCT, Inc., Lakewood, CO, USA); blood samples were concentrated to 8×10^7 mononuclear cells/mL.

PBMCs were stored overnight at 2–4 °C. Under sterilised and anesthetised (intrathecally) conditions, mobilised PBMCs were injected into the impaired lower limb muscles at 60 sites ($1.5–2.0 \times 10^7$ cells per site) approximately 3.0 × 3.0 cm distant and 1.0–1.5 cm deep. The control group of patients received injections of sterile phosphate-buffered saline (PBS) instead of cells.

Other standard care was similar in both groups of patients: dressing, debridement, antibiotic therapy and offloading from the clinic. The progress of recovery and side- effects of treatment was assessed carefully for at least 3 months. All patients suffering from diabetic foot infection (in both groups) received appropriate antibiotics and debridement throughout the study. Antibiotics were changed, when required.

Statistical analysis

Quantitative and qualitative data were analysed using the Mann-Whitney *U* test and the Chi-square and Fisher's exact tests, respectively (SPSS version 15, SPSS Inc., Chicago, IL, USA). Differences between mean values were considered statistically significant if $p < 0.05$.

Results

All patients in the transplanted group (n = 7) were found to be free of severe pain from 2 to 3 months after the completion of treatment. At 3 months post-treatment, improvement in foot inflammation and ulcers was observed in 86% of the transplanted group (6/7 patients), compared to improvement in only 29% of the control group (4/14 patients). Wound size in the transplanted group was smaller than for the control group, but the difference was not statistically significant (**Fig. 2**).

The ankle-brachial index, used to evaluate the vascular health of a patient's diabetic limb, was found to be markedly increased in the transplanted group (mean of 0.92 ± 0.15) compared to the control group (mean of 0.65 ± 0.25), thereby indicating significant improvement (P = 0.035) in restoration of blood circulation following transplantation. CT angiography confirmed the formation of new collateral vessels; this change was more obvious for the micro-vascular network than for the macro-vascular network (**Fig. 1**).

6 out of the 7 transplanted patients (86%) showed marked improvement in their ability to walk without pain; the remaining patient in the transplanted group showed little change in the ability to walk without pain. In contrast, only 4 patients in the control group (29%) experienced relief from walking pain by 3 months; the walking ability of the other 10 patients in the control group deteriorated over time. Hence, relief from pain was deemed to be significant (P = 0.024) following transplantation.

Critically, 7 out of 14 patients in the control group (50%) required their diseased leg to be amputated above the ankle by the conclusion of this study; no patient in the transplanted group faced amputation (P = 0.047).

In addition, no significant post-treatment reactions were observed in the transplanted group of patients over a total of 2 years of follow-up.

Diabetic patients with CLI were transplanted with autologous blood-derived mesenchymal stem cells to investigate the potential of this strategy as an effective therapeutic for patients at risk of limb amputation. This investigation followed patients for at least 3 months post-treatment; the results are shown in **Table 2**.

The mean of fasting blood-sugar (FBS) values at commencement of the trial was 143.1 ± 42.9 for the control group and 158 ± 49 for the transplant group (P = 0.49). 3 months following transplantation (or saline injections, as was the case with the control group), the mean of FBS values was 102 ± 24.4 for controls and 118 ± 21.3 for the transplant group (P = 0.14). Hence, there was no significant difference in blood-sugar levels between the 2 groups during the course of the study.

Discussion

Previous studies have shown that G-CSF-mobilised PBMCs play an important role in the healing process for patients with diabetic CLI.

It has been suggested that G-CSF can improve angiogenesis and wound healing in ischaemic tissues by mobilising EPCs into peripheral blood (Seiler et al., 2001) and inducing their differentiation and incorporation into the endothelial cells lining the blood vessels (Minamino et al., 2005). It remains controversial whether the actual administration of G-CSF itself plays a role in the process of healing observed in ischaemic tissues. It has been suggested that G-CSF can help wound healing by triggering the migration of progenitor endothelial cells to peripheral blood (Cho et al., 2003), but consistent with results from previous studies (Wang et al., 2005, Yang et al., 2005), no significant

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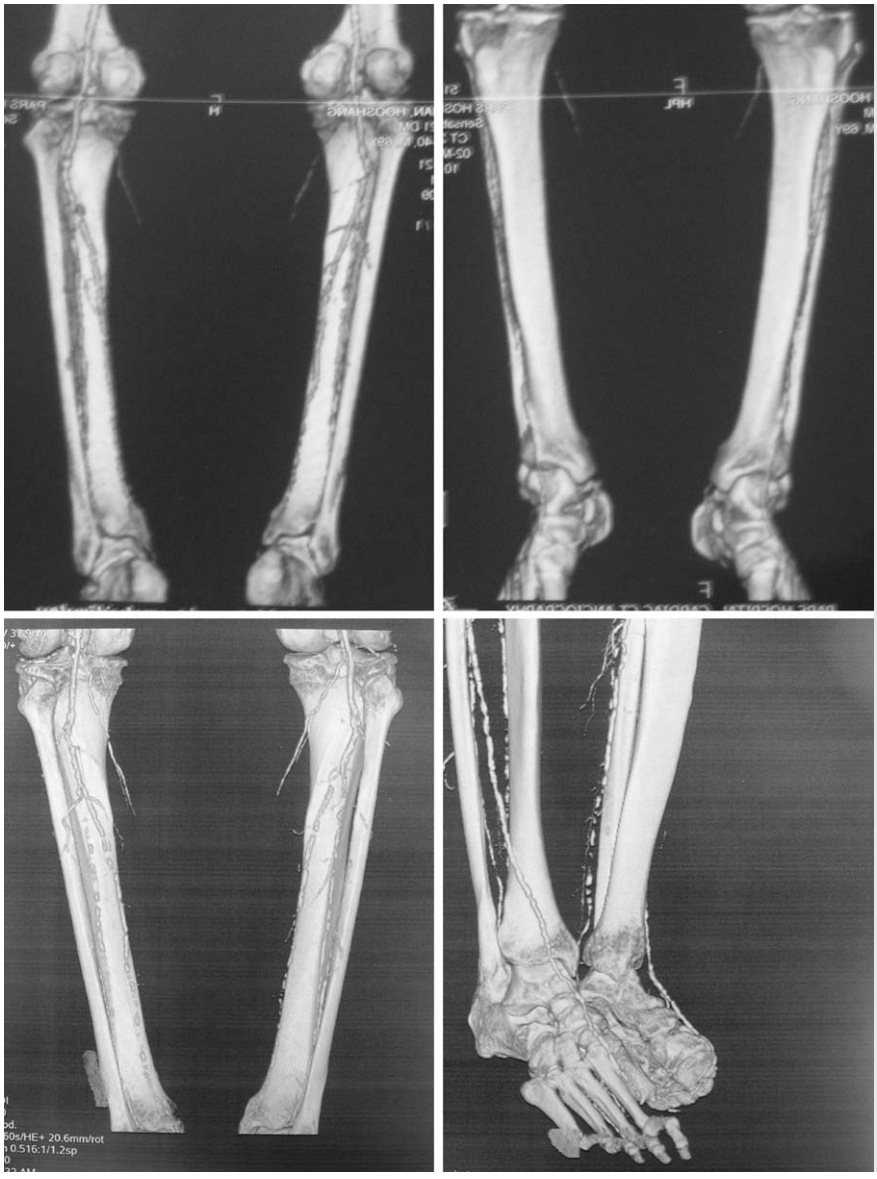

Fig. 1 CT angiographs from a patient with CLI before (top) and after (bottom) treatment. Top left and bottom left show the posterior-anterior view; top right and bottom right show the anterior-posterior view.

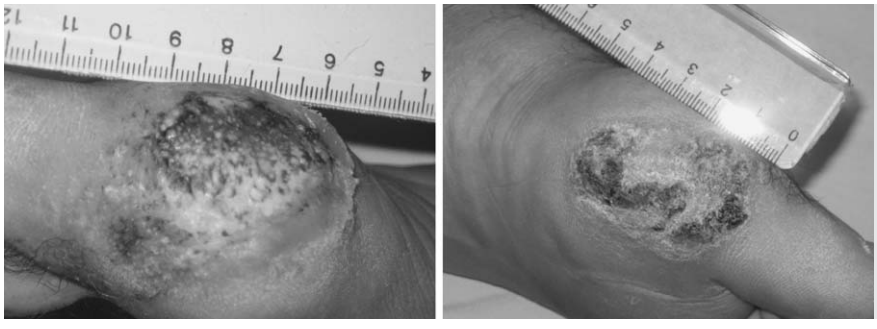

Fig. 2 Photos showing improvement in wound healing of diabetic foot ulcers before (left) and 3 months after transplantation (right).

improvement of the main clinical manifestations of ischaemia was observed in the control group that received G-CSF alone in our study.

Consistent with the findings of our study, Iba et al. found that transplantation of G-CSF-mobilised PBMCs directly into ischaemic tissues can introduce a significant number of functional EPCs, thereby enhancing neovascularisation as well as releasing angiogenesis-activating substances that induce formation of new collateral vessels (Iba et al., 2002). It has been also shown that transplantation of G-CSF-mobilised PBMCs can improve blood glucose metabolism (Yang et al., 2005).

A limitation of this study is the small cohort of patients investigated. This was due to the limited number of volunteers as well as the exclusion of a large portion of volunteers deemed unsuitable upon medical examination. We excluded patients with a number of critical diseases that might affect the results, including cystic infection, kidney diseases, severe heart failure, severe allergy, coagulopathy disorders, acute or severe dyspnea, and a history of stroke and heart attack in the past 3 months. Also, to

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avoid any potential deterioration in retinal pathology, diabetic patients with retinopathy were excluded from this study for a number of reasons. Firstly, the "diabetic paradox" stipulates that patients with diabetes experience poor angiogenesis in ischaemic organs and enhanced neovascularisation in retinal complications; the paradox is attributed to the differing mechanisms regulating angiogenic factors in the retina and the body's circulatory system (Duh and Aiello 1999, Fadini and Avogaro 2006). Secondly, transplantation of G-CSF-mobilised PBMCs has been predicted to trigger proliferative diabetic retinopathy (PDR) (Ishikawa and Asahara 2004).

The results from this current study showed that transplantation of G-CSF-mobilised PBMNCs can induce angiogenesis and improve the recovery process in diabetic patients with CLI. We observed significant improvement in pain levels, the ankle-brachial index, blood flow, wound size, and amputation rate.

Apart from a mild erythema at the injection site, which was relieved after a few days, no other adverse reactions were observed in treated patients for up to 24 months following treatment, including infection at the site of injection, immunological rejection, tumorogenesis, detriment to the function of liver or kidney, and other routine blood and urine parameters. This is consistent with previous reports of stem cell transplantation (Burt et al., 2010, Huang et al., 2005), although some adverse effects during the process of mobilising stem cells have been reported (Yang et al., 2005).

The first patient treated in the transplanted group, at the commencement of the study, showed substantial hair growth on the treated limb and reported "a cold feeling" in the limb. The induction of angiogenesis in this ischaemic limb was confirmed with CT angiography.

Measurements of toe and ankle systolic pressure and CT angiography prior to treatments showed arterial occlusions in all patients, hence explaining the CLI in their diabetic feet.

Patients in both groups were monitored for blood parameters affecting the wound-healing process, including hyperlipidemia, creatinine, haemoglobin and hypertension. After adjusting for age and smoking, we found no significant differences in these factors before and after transplantation.

Aside from the small sample size (as mentioned above), the main limitations of our study were its short duration (of following patients post-treatment) and any unintended on the part of the physicians when assessing the lesions of the patients. Nevertheless, the results of this cross-disciplinary study clearly demonstrate the therapeutic benefit of transplanting autologous stem cells, which undoubtedly increases quality of life and is less costly on the health system than the ongoing management issues associated with diabetic patients with CLI.

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Conflict of Interest: None.

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