Cytotechnology (2016) 68:771–781
DOI 10.1007/s10616-014-9828-7

ORIGINAL RESEARCH

Autologous transplantation of CD34$^+$ bone marrow derived mononuclear cells in management of non-reconstructable critical lower limb ischemia

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Received: 29 December 2013 / Accepted: 19 November 2014 / Published online: 16 December 2014

Abstract Patients with a decrease in limb perfusion with a potential threat to limb viability manifested by ischemic rest pain, ischemic ulcers, and/or gangrene are considered to have critical limb ischemia (CLI). Because of this generally poor outcome, there is a strong need for attempting any procedure to save the affected limb. The aim of this work is to evaluate the possibility to use stem cell therapy as a treatment option for patients with chronic critical lower limb ischemia with no distal run off. This study includes 20 patients with chronic critical lower limb ischemia with no distal run off who are unsuitable for vascular or endovascular option. These patients underwent stem cell therapy (SCT) by autologous transplantation of bone marrow derived mononuclear cells. 55 % of patients treated with SCT showed improvement of the rest pain after the first month, 60 % continued improvement of the rest pain after 6 months, 75 % after 1 year and 80 % after 2 years and continued without any deterioration till the third year. Limb salvage rate after STC was 80 % after the first year till the end of the second and third years. SCT can result in angiogenesis in patients with no-option CLI, providing a foundation for the application of this therapy to leg ischemia.

Keywords Bone marrow · G-CSF · Leg ischemia · Mobilization · CD34$^+$ cells

Introduction

Prevalence of peripheral arterial disease (PAD) is in the range of 3–10 %, increasing to 15–20 % in

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individuals over the age of 70 years. Patients with a decrease in limb perfusion with a potential threat to limb viability manifested by ischemic rest pain, ischemic ulcers, and/or gangrene are considered to have critical limb ischemia (CLI) (Hirsch et al. 2006).

Major amputation is linked with substantial morbidity and mortality, with a particularly high prevalence of co-morbid diseases (Dormandy et al. 1999). About half of patients who undergo above-knee amputation will die within 1 year after the procedure, and high percentage of patients are considered unfit for prosthetic rehabilitation. After 2 years, only 40 % of those fitted with prosthesis can ambulate, and even fewer are independent outside the home. Despite advances in vascular and endovascular techniques, 14–20 % of patients with chronic lower limb ischemia will not be eligible for distal arterial reconstruction due to occlusion of crural and pedal vessels (Attanasio and Snell 2009). Because of this generally poor outcome, there is a strong need for attempting any procedure to save the affected limb (Casamassimi et al. 2012; Inderbitzi et al. 1992).

Therapeutic angiogenesis by autologous bone marrow cell transplantation can improve blood supply in patients with critical limb ischemia (Mizuno et al. 2010). Bone marrow derived stem and progenitor cells have been identified as a potential new therapeutic option to induce therapeutic angiogenesis. Encouraging results of preclinical studies have rapidly led to several small clinical trials, in which bone marrow-derived mononuclear cells were administered to patients with limb ischemia. Clinical benefits were reported from these trials including improvement of ankle-brachial pressure index (ABPI), transcutaneous partial pressure of oxygen (TcPO2), reduction of pain, and decreased need for amputation (Lawall et al. 2010). The main goal of this study was to assess the influence of autologous mononuclear cell transplantation in the treatment of non reconstructable critical lower limb ischemia.

Patients and methods

This study included 20 patients with reconstructable chronic critical lower limb ischemia with no distal run off. All patients were admitted to the vascular surgery Unit, Tanta University Hospital, Tanta, Egypt in the period from January 2009 till January 2012. All recruited patients were exposed to complete history and physical examination, including measurement of ankle brachial pressure index (ABPI). Duplex study and CT angiography were also done for all patients. The age ranged from 42 to 83 years with mean age 62 years. Fourteen patients were males and 6 patients were females. The study was approved by the ethical committee of the Faculty of Medicine in Tanta University. A written informed consent was taken from all patients. The procedure was explained in details and in clear simple language to all recruited patients. All possible complications of therapy were explained to all patients with emphasis that the patient can withdraw from the study at any stage if he wishes.

Inclusion and exclusion criteria

Patients with chronic critical lower limb ischemia stage III or IV and with ABPI below 0.5 were included in which Duplex examination showing no flow in tibial and pedal arteries. Patients with angiography revealing no distal run off were included. Patients with acute ischemia and who are suitable for balloon angioplasty or vascular reconstruction were excluded. Other exclusion criteria included hematological abnormalities as anemia (Hb $<$ 10 g/dl), leukopenia (WBCs $<$ 4,000/cc), thrombocytopenia (platelets $<$ 100,000/cc), malignancies as leukemia and lymphoma, organ failure as liver cell failure and renal failure, and viral infections, such as HIV and hepatitis B.

Preparation of patients before bone marrow aspiration

Complete blood count (CBC), abdominal ultrasound, and examination of viral markers including hepatitis B and C and HIV viruses were performed in all patients before aspiration. The patients have received recombinant human Granulocyte Colony Stimulating Factor (rhGCSF) (GeneLeukim Injection from Shandong Geneleuk Biopharmaceutical Co., Ltd., Jinan, Shandong, China). Each 1 ml vial contained 600 μg Filgrastim given by subcutaneous injection in a dose of 5 μg/kg per day for 3–5 days to mobilize stem/progenitor cells. Meanwhile, a perfusion of 10,000 units/day heparin for 5 days by intravenous drop was used to avoid the possible risks of embolism because G-CSF induces the increase of circulating blood cells. CBC was done just before harvesting bone marrow by aspiration

and repeated daily to check the effect of G-CSF and final CBC just before harvesting bone marrow by aspiration.

Bone marrow aspiration

Under complete antiseptic precautions, a prophylactic dose of antibiotic (Cefotaxim (1 g via I.V. injection; purchased from Sanofi Company, Cairo, Egypt)) was given to all patients prior to bone marrow (BM) harvest. Two approaches were used to obtain a BM aspirate: the first one was from the anterior superior iliac spine; and the second one from the posterior superior iliac spine (which gives a higher amount of bone marrow aspirate). A volume of 100–150 cc of BM was aspirated from the iliac crest through anterior superior or posterior iliac spine of the patient then sent to the laboratory to separate the mononuclear cell fraction. All steps were performed under sterile conditions in a laminar flow hood (in the Clinical Pathology Department, Tanta University Hospital).

Preparation of human bone marrow mononuclear cells (BM MNCs)

The BM aspirate prepared above was diluted at a ratio of 4:1 with clinical buffer (Clini MACS PBS/EDTA buffer 1,000 ml, CE approved for clinical use catalogue number #700–25, from Miltenyi Biotec Company, Bergisch Gladbach, Germany). The diluted cell suspension was then carefully layered over 15 ml of Ficoll-Paque (GE Electric, Pharmacia, Piscataway, NJ, USA) in a 50 ml conical tube, and then centrifuged at 2,000 rpm for 20 min at 20 °C in a swinging out bucket rotor without brake. The upper layer was aspirated leaving the mononuclear cell layer undisturbed at the interphase containing lymphocytes, monocytes, and thrombocytes. The middle layer was carefully transferred to a new 50 ml conical tube. The cells were then washed twice with clinical buffer, mixed gently and centrifuged at 1,200 rpm for 15 min at 20 °C. Then the supernatant was carefully and completely removed. The cell pellet was resuspended in the appropriate amount of clinical buffer with the final volume of 300 µl of clinical buffer for up to 10^8 total cells.

Purification of stem cells by magnetic labeling

This purification was performed according to the previously described protocol (Lawall et al. 2010). The cells were kept cold and all the solutions used were at room temperature. The cells were passed through 30 µm nylon mesh (pre-separation filter) to remove cell clumps which might clog the column and the cell number was determined by using hemocytometer. The cell suspension was centrifuged at 1,200 rpm for 10 min and the supernatant was aspirated completely and the cell pellet was resuspended in 2 ml clinical buffer. CD_{34} Microbeads (150 µm; (Clini MACS CD_{34} microbeads, from Miltenyi Biotec Company, catalogue number #171-01) were added to the cell suspension, mixed well and refrigerated for 30 min. The cells were washed with the clinical buffer and centrifuged at 1,800 rpm for 20 min, then the supernatant was aspirated completely and the cells were suspended in 500 µl buffer. For magnetic separation, the column (MS column) was placed in the magnetic field of the Mini MACS separator. Cell suspension was applied to the column. Unlabelled cells that passed through were collected and the column was washed with Clinical buffer. Washing steps were performed by adding clinical buffer three times (3 × 500 µl clinical buffer), new clinical buffer was only added when the column reservoir was empty. The total effluent was collected and this was the unlabelled cell fraction. The column was removed from the Mini MACS separator (Miltenyi Biotec) and placed on a suitable collection tube. The clinical buffer was pipetted onto the column and the magnetically labelled cells were immediately flushed out by firmly pushing the plunger into the column.

Flow cytometry analysis

The purity of the $CD34^+$ cells harvested by magnetic labeling after the application of Mini MACS separator as described above was determined by flow cytometry. Aliquots of the fresh samples from the collected cells were stained with anti-human mAbs (BD Biosciences, Franklin Lakes, NJ, USA). The cells were incubated for 20–30 min at 4 °C in the dark with the anti-human CD34 (PE) and anti-human CD45 (Percep) mAbs using the concentrations recommended by the manufacturer. The cells were then washed twice using HBSS. Acquisitions were performed with a FACS Calibur (BD Biosciences) and data analysis was done by FlowJo software (BD Biosciences).

Treatment with cells

The patients were taken to the operating room and placed under general anaesthesia. The ulcers were surgically

debrided under sterile conditions to ensure a clean base with no scars, fibrotic or necrotic tissues. This allowed direct contact of bone marrow cells to a viable wound tissue bed. The cells were injected into the ulcer edge and the ulcer bed by using a 3 ml syringes with 19 gauge 1.5 needle. Thereafter, the wound surface was protected with ointment gauze (Biotulle (Betadine gauze (Povidone-Iodine) was purchased from Minapharm Company, Cairo Egypt)) and sterile dry gauze dressings (obtained from the local Pharmacy, Tanta Hospital University, Tanta, Egypt). This dressing was left on the wound for 24 h then removed, the wound was washed with saline 0.9 % only and a new dressing was used (Fig. 1).

Follow up

All patients were followed up for signs of improvement of circulation and tissue perfusion, including limb salvage/amputation, ulcer healing, disappearance of rest pain, increased pain-free walking distance, improvement of the ABPI. Angiography was also followed after 6 weeks, 3 and 6 months intervals after stem cell transplantation to detect the formation of new collateral vessels.

Statistical analysis

All data of the patients were entered into a database and analyzed using statistical software (SPSS Version 15.0 for Windows, SPSS, Chicago, IL, USA). Paired-samples t test was used to prove the differences between before and after intervention. Chi square test was performed for determination of the P value to compare the rates, and the probability ratio among the studied groups. Statistical significance was assumed at a value of $P < 0.05$. So P value above 0.05 is considered statistically non-significant.

Results

Level of arterial occlusion in patients

The main clinical presentation was rest pain in all patients. Gangrene was found in 5 patients, 3 of them in the toes and 2 in the forefoot. Ischemic ulcer was found in 4 patients. The level of occlusion was in the popliteal artery in 8 patients, femoral artery in 5 patients and external iliac artery in 2 patients.

Clinical responses to G-CSF

After G-CSF administration, the WBCs count was increased in the peripheral blood indicating the increase in mononuclear cell production and mobilization. The maximum WBCs count was 45,300/cc and the minimum was 19,000/cc (with mean value of 34,700).

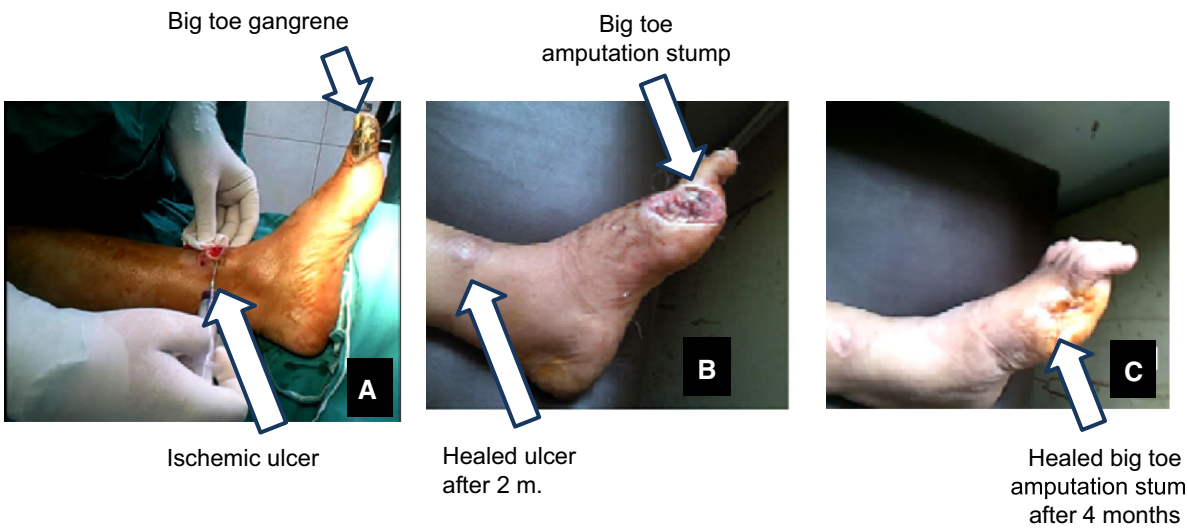

Fig. 1 **a** Injection of $CD34^{+}$ cells around ischemic ulcer in the left leg. **b** Healed ulcer after 2 months. **c** Healed big toe amputation stump after 4 months

Table 1 Rest pain improvement after SCT

Duration	Rest pain improvement		Visual analogue scale (VAS)		*P* value
	No.	%	Before CLS	After CLS	
1 month	11	55	7.3 ± 1.1	3.7 ± 1.6	0.047
6 months	12	60	7.3 ± 1.1	3.3 ± 1.7	0.038
First year	15	75	7.3 ± 1.1	3.2 ± 1.4	0.044
Second year	16	80	7.3 ± 1.1	3.4 ± 1.7	0.058
Third year	16	80	7.3 ± 1.1	3.4 ± 1.7	0.058

Effect of SCT on rest pain

By the use of Visual Analogue Scale (VAS), the severity of pain was assessed before and after SCT (Table 1). There was an improvement in the rest pain after SCT in 11 patients (55 %) after the first month. The VAS decreased from 7.3 ± 1.1 points to 3.7 ± 1.6 points (*P* value = 0.047). At 6 months duration after stem cell therapy, a number of 12 patients (60 %) showed continuous improvement of the rest pain with VAS being 3.3 ± 1.7 points (*P* value = 0.038). At the end of the first year, 15 patients (75 %) continued improvement of the rest pain with no need for analgesics after stem cell therapy with VAS being 3.2 ± 1.4 points (*P* value = 0.044). At the end of the second year 16 patients (80 %) continued improvement of the rest pain after 2 years and continued without any deterioration till the third year with VAS being 3.4 + 1.7 points (*P* value = 0.058). All patients with limb salvage showed improvement of the rest pain.

Effect of SCT on physical activity and pain-free walking distance

As shown in Table 2, the pain free walking distance increased after 6 months following stem cell therapy in 12 patients (60 %), it increased from 85.3 ± 81.7 m to 163.5 ± 127.6 m (*P* value = 0.028). At the end of the first year, 15 patients (75 %) showed slight improvement and the pain free walking distance was 169.6 ± 131.8 m (*P* value = 0.031). This improvement remained stable at the end of the second year 16 patients (80 %) showed stable improvement and the pain free walking distance was 156.6 ± 127.3 m (*P* value = 0.046). Finally after 3 years, the number of patients was the same as in the second year with improvement and the pain free walking distance being 149.8 ± 132.3 m (*P* value = 0.052). Ten out of the 16 patients (80 %) who had limb salvage showed improvement and the pain free walking distance.

Table 2 Pain-free walking distance after SCT

Duration	Patients		Pain-free walking distance (in meters)		*P* value
	n	%	Before SCT	After SCT	
6 months	12	60	85.3 ± 81.7	163.5 ± 127.6	0.028
First year	15	75	85.3 ± 81.7	169.6 ± 131.8	0.031
Second year	16	80	85.3 ± 81.7	156.6 ± 127.3	0.046
Third year	16	80	85.3 ± 81.7	149.8 ± 132.3	0.052

Table 3 Ankle brachial pressure index after SCT

Duration	Patients		ABPI		*P* value
	n	%	Before SCT	After SCT	
6 months	11	55	0.27 ± 0.18	0.71 ± 0.19	0.026
First year	12	60	0.27 ± 0.18	0.72 ± 0.18	0.031
Second year	14	70	0.27 ± 0.18	0.74 ± 0.12	0.038
Third year	16	80	0.27 ± 0.18	0.69 ± 0.22	0.048

ABPI after SCT

As shown in Table 3, eleven patients (55 %) showed improvement in the ABPI after SCT in the first 6 months. ABPI increased from 0.27 ± 0.18 to 0.71 ± 0.19 (*P* value = 0.026). At the end of the first year, ABPI continued improving in 12 patients (60 %). ABPI was 0.72 ± 0.18 (*P* value = 0.031). At the end of the second year ABPI continued improving in 14 patients (70 %). As compared to the values before treatment, ABPI follow up was 0.74 ± 0.12 (*P* value = 0.038). After the third year ABPI continued improving in 16 patients (80 %). ABPI follow up was 0.69 ± 0.22 (*P* value = 0.048).

Limb salvage and major amputation

The total number of saved limbs without major amputation after SCT after the first, second, and third year was 16 patients (80 %). Major amputation was done for 4 patients (20 %) during the 6 months due to

extensive necrosis, resistant infection and persistent rest pain.

Effect of SCT on healing of ischemic ulcer

As presented in Fig. 2, four patients (out of 20) were presented with toe gangrene in the SCT group. Two patients (50 % of the 4 patients) had gangrene in one toe and showed good healing after toe amputation. Two patients (out of 20) with forefoot gangrene did not show any improvement after SCT and ended with major amputation. Three patients (75 %), out of the four who presented with ischemic ulcer, showed healing of the ulcer in the first 6 months after SCT and continued till the end of the third year. The fourth patient ended with major amputation.

Effect of SCT on angiography

As shown in Fig. 3, angiography was done after SCT in 12 patients (60 %). No angiography was performed for the remaining 8 patients (40 %) including the 4 patients having undergone major amputations and 4 patients having refused to do angiography because all symptoms improved. Among the 12 patients who had follow up angiography, 9 patients (75 %) showed angiogenesis and 3 patients (25 %) did not show angiogenesis despite clinical improvement.

Adverse effects of SCT

The complications of SCT included injection site pain having occurred in 5 patients (25 %), it was mild pain and was treated with non-steroidal anti-inflammatory drugs. Small intramuscular hematoma occurred in one patient (5 %) and resolved spontaneously after 2 weeks. Three patients (15 %) developed mild edema in the injected leg which improved after 3 weeks. Low grade fever appeared in 2 patients (10 %) despite routine antibiotic use in all patients after injection. This fever resolved spontaneously within 4 days after injection. Extensive necrosis and gangrene with no response to stem cell therapy were encountered in 4 patients (20 %) and major amputation was performed. No procedure-related mortality or thrombo-embolic complications were observed after stem cell therapy (Fig. 3).

Discussion

The main challenge in the therapeutic approach to critical limb ischemia is to establish an effective reperfusion of the ischemic limb. Conventional arterial reconstruction by a bypass surgery can solve the problem of a long segment occlusion while percutaneous trans-luminal angioplasty can be done in short segment lesions. Peripheral vascular disease with arterial occlusion with poor distal run off is considered unsuitable for both bypass and angioplasty. Indeed, several variables still remain to be elucidated for stem cell therapy, including the type of cells to be used, the infusion route, and more importantly, the stage of patients to be treated (Casamassimi et al. 2012).

Growth of new vessels develops by proliferation of endothelial cells in vascular extremities as well as by BM-mobilized HSCs, which are transformed into endothelial progenitor cells. These cells contribute to generation of new endothelial cells and vascularization. Therefore, some studies used mononuclear BM cells and others used differentiated mesenchymal stem cells. In our study presented here, patients with

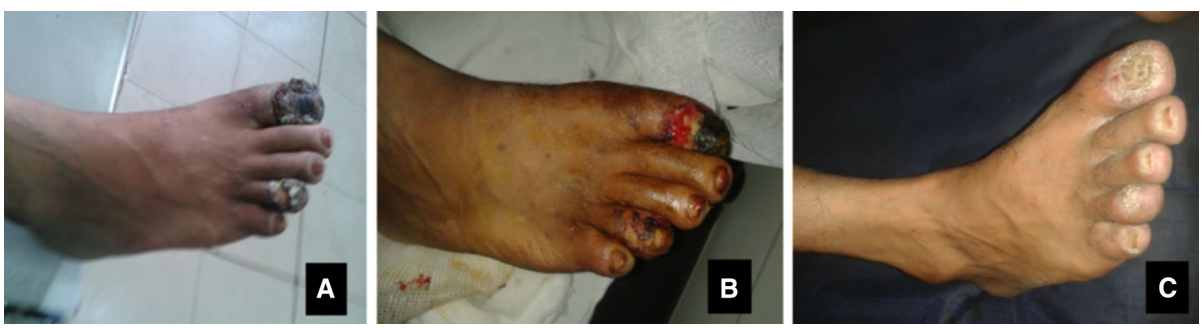

Fig. 2 **a** Before injection of $CD34^{+}$ cells. **b** One month after injection. **c** 3 months after injection with complete healing

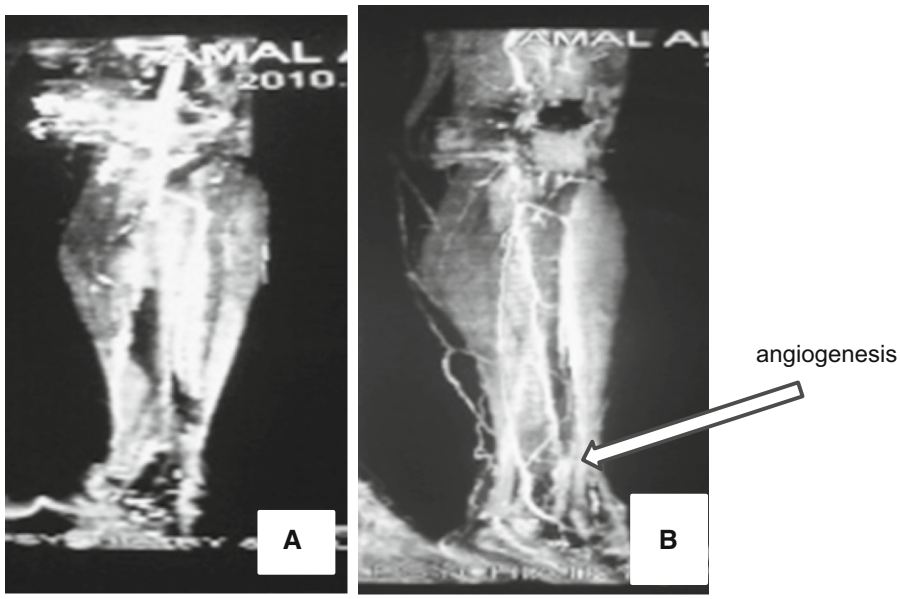

Fig. 3 **a** Arteriogram of a patient with critical Left lower limb ischemia with popliteal artery occlusion with no distal run-off before $CD34^+$ cell transplantation. **b** Good collaterals and angiogenesis after 6 weeks following $CD34^+$ transplantation

chronic critical lower limb ischemia with no distal run off who were unsuitable for vascular or endovascular option underwent SCT by autologous transplantation of BM-derived mononuclear cells after mobilization of stem cells with G-CSF. Our results showed that 55 % of patients treated with SCT showed improvement of the rest pain after the first month, 60 % continued improvement of the rest pain after 6 months, 75 % after 1 year and 80 % after 2 years and continued without any deterioration till the third year. Limb salvage rate after STC was 80 % after the first year till the end of the second and third years, indicating that stem cell based therapy can result in angiogenesis in patients with no-option CLI.

Similar to our results, intravascular injection of mononuclear BM cells laterally through a 4 Fr sheet in 24 patients with CLI, 11 of 14 defects were healed (78 %) and Fontaine grade of ischemia changed from median grade 3.5 to median grade 2 associated with improvement in the collateral vessel development (Chochola et al. 2008). The utility of BM derived mononuclear cells to produce angiogenesis in 33 Indian patients with Buerger's disease was evaluated, where major amputation was done for 3 patients (12 %). The mean ABI improvement after 6 months in the salvaged limbs was 0.14 (Motukuru et al. 2008). A similar study investigated BM-mononuclear cell transplantation in 7 patients with critical lower limb ischemia (3 with Buerger's disease, 4 with arteriosclerosis obliterans undergoing chronic hemodialysis). Three out of 7 patients responded to the therapy. The objective criteria of improvement were ABI, laser thermography, transcutaneous oxygen tension and angiography. Interestingly, the numbers of circulating $CD34^+$ and $CD133^+$ cells persistently increased for 1 month after the treatment only in the responders (Kajiguchi et al. 2007). Similarly, neovasculogenesis in CLI diabetic patients was evaluated after infusion of unfractionated autologous BM-derived mononuclear cell generating a significant increase in the vascular network in ischemic areas and promoting remarkable clinical improvement (Ruiz-Salmeron et al. 2011).

Besides BM-derived mononuclear cells, MSCs are a source of pericyte progenitors and angiogenic regulahtors and thus represent preferential stimuli for the development of blood vessels (Rastegar et al. 2010). Therefore, several studies have utilized these cells in treatment of leg ischemia. For instance, treatment with intravenous infusions (3 pulses) of expanded autologous MSCs in 1 patient with critical limb ischemia due to systemic sclerosis who developed acute gangrene of the upper and lower limbs reduced the area of necrotic skin and associated with revascularization of the patient's extremities, indicating that this approach may foster the recovery of the vascular network, restore blood flow, and reduce skin necrosis in this patient population (Guiducci et al. 2010).

It was found in recent studies that the efficacy of combinatorial cell therapy based on infusion of autologous BM-derived mononuclear cells (as a source of EPCs progenitors) and MSCs is higher than that of the single therapy. For instance, in phase I and II clinical trials on patients with CLI, the use of a combination cell product (mesenchymal stem cells in conjunction with a source of endothelial progenitor cells) was found to be safe and efficient and optimized the clinical results obtained with the use of endothelial progenitor cells alone in term of improvement in walking time and ankle-brachial index with a significant increase in blood flow in the ischemic legs, and quality of life (Lasala et al. 2010).

One mechanism that might mediate the induced improvement in the clinical responses to our G-CSF mobilizes SCT is the presence of progenitors of WBCs, in particular it has been found that the flow rate of fractionated granulocytes and mononuclear cells, as well as unfractionated mixed WBC from leg ischemia patients was impaired. However, amputation of the ischemic leg or pharmacological intervention (with Pentoxyphylline infusion) improved the filterability of granulocytes from severe ischemic patients (Nash et al. 1991). This could explain the improved clinical response in our patients with leg ischemia after injection of the unfractionated bone marrow cells which might release WBC progenitors with normal flow rate due to G-CSF mobilization of BM.

The route of administration of stem cells has been found to be critical for its efficacy. Intravenous stem cell delivery for regenerative tissue therapy has been increasingly used in both experimental and clinical trials. However, recent data suggest that the majority of administered stem cells are initially trapped in the lungs (Zonta et al. 2010). After intra-artery and intravenous infusion, MSCs were detected primarily in the lungs and then secondarily in the liver and other organs. When sodium nitroprusside was used, more labeled MSCs cleared the lungs resulting in a larger proportion detected in the liver. Most importantly, the homing of labeled MSCs to the marrow of long bones was significantly increased by the pretreatment with vasodilator. These results indicate multiple homing sites for injected MSCs and that the distribution of MSCs can be influenced by administration of vasodilator (Fischer et al. 2009; Gao et al. 2001; Omlor et al. 2010). Therefore, there are numerous doubts about the best route of stem cell administration to achieve implantation into the injured site. With this regard, the comparison of various administration routes of MSCs in a porcine model of myocardial infarction showed that the mean number of engrafted cells within the infarct zone was significantly greater after intracoronary infusion than either intramyocardial or endocardial injection. Fluorescent cells were not observed in healthy zones of the myocardium or in healthy animals (Moscoso et al. 2009). Another study found that intra-arterial (in the renal graft) administration route of MSCs achieved higher immunomodulating effects than intravenous route in experimental rat kidney transplantation after bilateral nephrectomy (Zonta et al. 2010). The effects of $CD34^{+}$ stem cells delivered by different routes on cardiac function were compared in rats with ischemic cardiomyopathy reproduced by ligation of left anterior descending coronary artery. It was found that intravenous and trans-epicardial delivery of hematopoietic stem cells (HSC) can significantly improve cardiac function, and both methods may be safe and effective for the treatment of AMI (Zhang et al. 2008). Therefore, one explanation for the efficacy of our treatment protocol for leg ischemia might be the route of administration, where we injected BM cells into and around the diseased area rather than a systemic administration. Under this setting, the injected cells are localized in the injured site which might contain some growth factors that mediate the differentiation of the injected cells into endothelial cells and as a consequence enhance angiogenesis.

Onodera et al. (2011), compared the use of BM mononuclear cells and G-CSF-mobilized peripheral blood mononuclear cells in treatment of no-option critical limb ischemia. Their results suggest that there was no significant difference in long-term prognosis between patients treated with BMMNC and those treated with M-PBMNC (Zhang et al. 2008). Lara-Hernandez et al. (2011) studied the safety and efficacy of therapeutic angiogenesis in critical limb ischemia patients. They reported no adverse effects and limb salvage rate of 74.4 % after 1 year (Zhang et al. 2008). In our study, however, we have measured several parameters before and after therapeutic angiogenesis, including rest pain by visual analogue scale, walking distance before and after therapy in meters, ankle brachial pressure index, limb salvage over all versus amputation and degree of ischemic ulcer healing. Moreover, differently from these studies in which

G-CSF was administered for 5 days to mobilize stem cells into peripheral circulation, G-CSF in our study was administered for only 3 days. This period of time is just enough for bone marrow progenitor proliferation with very limited mobilization to peripheral tissues. As such, the clinical improvement that has been noticed in our patients is probably due to the injection of autologous $CD34^+$ bone marrow derived mononuclear cells and not due to G-CSF administration itself.

Although we have not performed experiments to fully understand the mechanisms mediating the beneficial effects of our treatment protocol to the patients, several mechanisms can be suggested based on related previous studies. With this regard, the mechanism(s) of hematopoietic stem cells-induced angiogenesis have been suggested to be either due to an indirect effect of angiogenic factors secreted by stem cells such as vascular endothelial growth factor (VEGF) or by the direct role of the endothelial progenitor cells (EPC) fraction, present in these stem cell preparations, to form new vessels (Burt et al. 2008). Further, injection of G-CSF has been reported to induce a fourfold increase in the production of the total numbers of white blood cells in the peripheral blood and 1.5-fold increase in the numbers of mononuclear cells coincided with increases in the numbers of $CD133^+$ and $CD34^+$ cells by 18-fold, where the level of $CD133^+$ cells peaked on the third day and $CD34^+$ cells peaked on the fourth day upon G-CSF injection (Reddy et al. 2013). Indeed, EPCs have direct angiogenic action, supporting angiogenesis through their ability to secrete paracrine mediators. In this respect, several studies have shown that these cells release interleukins, growth factors, and chemokines that altogether regulate CD14-positive cells, accelerating vascular network formation, and enhance healing processes (Jarajapu and Grant 2010). Furthermore, ischemia itself induces production of growth factors, cytokines, and hormones, which promotes proliferation, differentiation, and mobilization of MSCs and EPCs to form new vessels. In addition, the growth factors can stimulate EPCs sprouting from preexisting blood vessels (Botti et al. 2012). G-CSF itself can also induce activation, proliferation and consequent migration of endothelial cells, since it has been found that G-CSF induces angiogenesis-related process of endothelial cells, including proliferation and migration (Bussolino et al. 1991).

Taken the above studies together, we suggest similar events in our setting after treatment with 3 days of G-CSF. We suggest that cells, in either the injected exogenous BM or sorted $CD34^+$ or stimulated in the patient blood, were able to home to the injured tissues. In line with this suggestion a recent study using bioluminescence reported that injection of freshly isolated BM cells to injured skeletal muscle after ischemia–reperfusion injury associated with cell homing to injured muscle as measured for up to 7 days (Corona and Rathbone 2014). This is likely to be a working hypothesis given that BM cells have been confirmed to contain plenty of EPCs and secrete abundant angiogenic factors, including VEGF and basic fibroblast growth factor (bFGF) (Suzuki and Iso 2013). Furthermore, in response to hypoxia, cytokines such as hypoxia-inducible factor alpha-1 (HIF-1α), VEGF and angiopoietin are released which are known to recruit progenitor cells (Asahara et al. 1999; Smadja et al. 2006; Ho et al. 2006). Our suggestion is also in line with other studies which reported that BM-derived mononuclear cell therapy induced distal angiogenesis after local injection into critical leg ischemia where the newly formed vessels were positive for endothelial cell markers (CD31, CD34, and von Willebrand factor and negative for markers of lymphatic vessels (Podoplanin)). This study further showed extensive endothelial cell proliferation within the new vessels as evidenced by immunohistochemical staining for Ki-67 and c-kit (Duong Van Huyen et al. 2008). In sum, we can suggest that the mechanisms of the beneficial effects of our treatment protocol consisting of short treatment with G-CSF and exogenous administration of BM stem cells is mediated by both G-CSF itself as well as by the administered cells. Both can induce the stimulation of EPC to differentiate into endothelial cells by growth factors. We have established an animal model of leg ischemia to address the proposed mechanisms. Our study, however, form a foundation to further preclinical and clinical studies to dissect the underlying mechanisms as well as to improve the treatment protocol.

Conclusion

SCT can result in angiogenesis in patients with non-reconstructable critical lower limb ischemia, providing a foundation for the application of this therapy to leg ischemia, indicating that stem cell based therapy

can result in angiogenesis in patients with no-option CLI. Transplantation of autologous bone marrow derived mononuclear cells is safe and feasible. Long term follow up is required for standardization of therapy with the possibility of repetition for recurrence of ischemic manifestations. Based on our results these studies merit validation by randomized controlled studies in patients with less critical limb ischemia.

Acknowledgment This work was funded by a grant from the Research Development Fund, Tanta University, Egypt.

Conflict of interest The authors indicate no potential conflicts of interest.

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