Autologous Bone Marrow Cell Transplantation Increases Leg Perfusion and Reduces Amputations in Patients With Advanced Critical Limb Ischemia Due to Peripheral Artery Disease

Berthold Amann,* Claas Luedemann,* Richard Ratei,† and J. André Schmidt-Lucke*

*Department of Medicine, Franziskuskrankenhaus, Berlin Vascular Center, Berlin, Germany
†Cell Marker Laboratory, HELIOS Klinikum Berlin-Buch, Berlin, Germany

Bone marrow cell transplantation has been shown to induce angiogenesis and thus improve ischemic artery disease. This study evaluates the effects of intramuscular bone marrow cell transplantation in patients with limb-threatening critical limb ischemia with a very high risk for major amputation. After failed or impossible operative and/or interventional revascularization and after unsuccessful maximum conservative therapy, 51 patients with impending major amputation due to severe critical limb ischemia had autologous bone marrow cells (BMC) transplanted into the ischemic leg. Patients 1–12 received Ficoll-isolated bone marrow mononuclear cells (total cell number $1.1 \pm 1.1 \times 10^9$), patients 13–51 received point of care isolated bone marrow total nucleated cells ($3.0 \pm 1.7 \times 10^9$). Limb salvage was 59% at 6 months and 53% at last follow-up (mean $411 \pm 261$ days, range 175–1186). Perfusion measured with ankle-brachial index (ABI) and transcutaneous oxygen tension (tcpO$_2$) at baseline and after 6 months increased in patients with consecutive limb salvage (ABI $0.33 \pm 0.18$ to $0.46 \pm 0.15$, tcpO$_2$ 12 $\pm$ 12 to 25 $\pm$ 15 mmHg) and did not change in patients eventually undergoing major amputation. No difference in clinical outcome between the isolation methods were seen. Clinically most important, patients with limb salvage improved from a mean Rutherford category of 4.9 at baseline to 3.3 at 6 months ($p = 0.0001$). Analgesics consumption was reduced by 62%. Total walking distance improved in nonamputees from zero to 40 m. Three severe periprocedural adverse events resolved without sequelae, and no unexpected long-term adverse events occurred. In no-option patients with end-stage critical limb ischemia due to peripheral artery disease, bone marrow cell transplantation is a safe procedure that can improve leg perfusion sufficiently to reduce major amputations and permit durable limb salvage.

Key words: Bone marrow cell therapy; Critical limb ischemia; Peripheral vascular disease; Point of care

INTRODUCTION

Patients suffering from peripheral arterial occlusive disease that has progressed into critical limb ischemia (CLI) have few treatment options. Two methods are the mainstay of revascularisation: 1) open surgical techniques to remove the blockage or bypass it with vein or prosthetic graft or 2) endovascular treatment to reopen blocked arteries using a variety of catheter-based strategies that either crush or remove the blockage (balloon angioplasty, rotablation, atherectomy, etc.). For patients where these therapies have failed or who are no longer candidates for these therapies, 20% will die and 50–90% will have a leg amputation below or above the knee (major amputation) in 6 months (7). Estimates are that 150,000 such amputations are performed annually in the US. Alternative methods of revascularization are urgently needed to treat this patient population and reduce the number of amputations and the massive impact on the quality of life and life expectancy.

Research focused on the use of autologous cell therapy has shown promise and has led to several clinical studies where concentrated autologous bone marrow cells have been used as an angiogenic treatment for vascular patients who are not candidates for surgical or endovascular approaches (8,9,28,32).

While the mechanism of action has not been definitively elucidated, one intriguing hypothesis postulates that the improvements shown in perfusion and concomitantly in quality of life and walking capacity result from the effect of bone marrow cells on the small collateral vessels. The expansion of these vessels is the most important physiological repair mechanism in peripheral artery disease (34). These critical vessels can form direct
connections between branches of the major axial arteries. Their growth capacity is, however, suppressed in patients with severe atherosclerosis, especially in diabetes (14). We hypothesized that the delivery of a cell concentrate from the bone marrow containing a multiplicity of progenitor cell types along with cytokines and growth factors would stimulate neovascularization through collateral growth within the affected tissue, ultimately resulting in improved limb perfusion thereby reducing the need for leg amputations.

We conducted a pilot study to evaluate the clinical efficacy, long-term outcome and safety of autologous bone marrow cells (aBMC) delivered by injection as a possible limb salvage procedure in a no-option patient cohort with CLI and very high amputation risk.

**MATERIALS AND METHODS**

**Patient Inclusion**

Fifty-one patients with end-stage CLI Rutherford grade 4–6 were treated with aBMC transplantation between May 2004 and February 2007. An institutional review committee approved the study protocol and all patients gave written informed consent before evaluation for inclusion. The baseline demographic and clinical characteristics of the patients are given in Table 1. Forty-five patients (88%) had undergone a mean of two unsuccessful attempts of operative and/or percutaneous revascularization at the ischemic limb; six patients (12%) were primarily technically not amenable to revascularization. Major amputation (above the ankle) had been recommended to 46 of the 51 patients (90%) by the treating vascular surgeons. Patients were included if ischemia was confirmed as the cause of their symptoms, and rest-pain and/or nonhealing ischemic wounds were present for more than 4 weeks without improvement in response to best standard care. Patients were excluded if they had concurrent illnesses with a life expectancy of less than 6 months, if they had active malignancy, or had bone marrow diseases like myelodysplastic syndrome.

**Diagnosis of Critical Limb Ischemia (CLI)**

CLI was diagnosed according to the Transatlantic Society Consensus Group (TASC) recommendations (31). All patients underwent baseline conventional intra-arterial digital subtraction angiography, repeated baseline measurements of resting ankle-brachial index (ABI), and transcutaneous partial oxygen tension (tcpO2). ABI was calculated as the quotient of the highest ankle pressure and highest brachial systolic blood pressure; the normal value is 0.95–1.2. If possible, absolute walking distance was also assessed with a standardized treadmill protocol (3.2 km/h, 12% slope). CLI was confirmed if there was angiographic proof of arterial occlusion and one of the following criteria was fulfilled: 1) ABI <0.6 or, if non-compressible ankle arteries due to Moenckeberg’s Media sclerosis precluded meaningful ABI determinations, 2) tcpO2 <30 mmHg at the forefoot in the supine position with 44°C electrode temperature (Radiometer Tina, Copenhagen, Denmark). The normal value of TcpO2 is 70–90 mmHg.

**Concomitant Treatments**

All medical therapies, including systemic antibiotic therapy, aspirin/clopidogrel, ACE inhibitors, beta blockers, and statins, were continued unchanged (5), and local wound therapy (consisting mainly but not exclusively of repeated manual debridement of necrotic tissue, moist wound therapy, in selected cases negative pressure therapy, and skin transplantation if indicated) was also continued and adjusted, if necessary. Necessary minor amputations were performed if perfusion had increased sufficiently to allow healing.

**Autologous Bone Marrow Preparation**

Two methods were used to concentrate the aspirated bone marrow.

**Remote Delayed Preparation.** For the first 12 subjects, 450–500 ml bone marrow was aspirated was aspirated under general anesthesia from both iliac crests and was anticoagulated with unfractionated heparin (100 U/ml, ratiopharm, Ulm, Germany). The cells present within the bone marrow aspirate were separated with a Ficoll density gradient centrifugation method (Ficoll-Paque Plus, Amersham Biosciences Europe, Freiburg, Germany). The resulting bone marrow mononuclear cell (BM-MNC) pellet was resuspended, and the BM-MNC suspension underwent three washing steps in isotonic saline (2). The pellet was resuspended, and Ringer’s lactate solution was added to achieve the desired final injection volume (55–85 ml).

**Immediate Bedside Preparation.** For the remaining subjects (patients 13–51) a fully automated bedside density gradient centrifugation method was used to prepare a concentration of bone marrow total nucleated cells (BM-TNC) from the aspirate (Harvest SmartPReP, Harvest Technologies, Plymouth, MA, USA). This method required one half the volume of aspirate (240 ml) and, due to the smaller volume, there was no need for general anesthesia. BM harvesting was performed under intravenous sedation with Propofol.

The final treating volume (55–85 ml) was adjusted with plasma depending on the area to be treated (whole leg, calf only, or foot).

In both methods, samples were taken for automated cell counts, and the number of CD34+ cells was determined using fluorescence-activated cell sorting (FACS Scan Flow cytometer, Becton Dickinson, San Jose, CA).
after staining with a monoclonal mouse-anti human CD34 antibody (Becton Dickinson).

**Autologous Bone Marrow Concentrate Transplantation**

Transplantation into the ischemic leg was performed under intravenous sedation with Propofol. Forty-five to 80 aliquots of about 0.75–1 ml were injected deep intramuscularly with a 23-gauge needle into the thigh, calf, or foot depending on the localization and extent of the arterial occlusions. Injections were commenced 4–5 cm proximal to the arterial obstruction and were continued distally spaced 1 cm apart in all patients. Injections were placed as near as possible (±1 cm) to the original albeit occluded arteries because the density of preformed collateral arteries is highest in the vicinity of the original arteries. If a wound was present, 4–10 injections of aBMC were given into the wound bed and the wound perimeter. If necessary, sharp wound debridement was performed. Patients who had not already received systemic antibiotics received periprocedural intravenous single-shot Sultamicillin 1.5 g to avoid infection with skin bacteria during the multiple intramuscular injections.

**Follow-up**

Minimum follow up was 6 months, and mean follow-up was 411 ± 261 days (range 175–1186). Patients were seen monthly up to 6 months and at least in half-year intervals thereafter. No patients were lost to follow-up. Physical examination, ABI, TcpO₂, walking distance, Rutherford categories, wound size, amputation status, and adverse events were documented. Digital photos of wounds were taken at each visit. The occurrence of malignant diseases, major adverse cardiovascular events, and other major adverse events was assessed. Conventional intra-arterial angiography was repeated at 24 weeks in 21 of 30 nonamputated subjects.
Statistical Analysis

All data are presented as mean ± SD. Statistical significance was accepted if $p$ was <0.05. Comparison of baseline versus follow-up continuous variables was performed with a paired $t$-test. Comparison of nonparametric data was performed with the Mann-Whitney test. Statistical analysis was performed with STATISTICA 7.1 (Statsoft Inc, Tulsa, OK, USA).

RESULTS

Procedural and Safety Results

For the Ficoll isolation procedure (patients 1–12), the total time for bone marrow aspiration, separation, and reinjection was 420 ± 32 min, and for the Harvest isolation 65 ± 11 min (patients 13–51). Red blood cells were depleted to 1 ± 0.5% hematocrit with Ficoll and to 5 ± 1.5% with the Harvest separation. Mean total number of injected BM-MNC with Ficoll isolation was $1.1 ± 1.1 \times 10^9$ cells (range $0.13–3.86 \times 10^9$) and $3.0 ± 1.7 \times 10^9$ (range $0.18–6.6 \times 10^9$) BM-TNC with the Harvest method. The mean total number of injected CD34$^+$ cells for Ficoll was $21.5 ± 9.1 \times 10^6$ and with the Harvest separation $24.9 ± 11 \times 10^6$.

Procedure-related complications occurred in 3/51 patients (6%): in one patient (#44) a colon puncture during bone marrow aspiration required surgical repair; one aspiration of gastric content during reinjection with subsequent aspiration pneumonia (#1). One patient (#8) had symptomatic anemia with vertigo with a fall in hematocrit from 33% to 26% after aspiration of 450 ml of bone marrow and was transfused one unit of packed red cells. All complications resolved without sequelae. Importantly, there were no local complications at the bone marrow puncture or reinjection sites. There was no additional swelling, pain, or discomfort at the injected leg. No infectious complications (local or systemic) due to cell injections occurred.

Clinical Outcome

Figure 1 shows major amputation-free survival. Improvement in perfusion and subsequent limb salvage was achieved in 30/51 patients (59%) at 24 weeks, and in 27/51 (53%) at the end of follow-up after mean 411 days. In patients with limb salvage, there was a significant improvement in mean and individual Rutherford categories as well as a reduction in wound size (Table 2). Eight below-knee and 16 above-knee amputations had to be performed. Two patients who underwent amputations and one who did not died during follow-up at weeks 3, 29, and 64. Two of these deaths (week 29 and 64) were cardiac and occurred in patients with known coronary artery disease; one patient with worsening gangrene declined amputation and died of septic multiorgan failure at week 3 posttreatment. Another three patients with known CHD developed myocardial ischemia and underwent percutaneous coronary revascularization at weeks 19, 23, and 50. No new cases of malignancy or unwanted neovascularization occurred.

Minor Amputations, Wound Healing (Table 2)

After perfusion had sufficiently improved to allow a minor amputation, a total of 17 minor amputations (6 forefoot and 11 toe amputations) were performed in the 30 patients with 24-week limb salvage. Complete wound healing at 24 weeks, including operative sites from minor amputations, was achieved in 15 of 21 patients with ischemic wounds. In the three patients with major amputation after 24 weeks, the wound was never fully closed and became infected, necessitating major amputation at weeks 25, 41, and 55, respectively, after treatment. The need for analgesics was greatly reduced by 62% of the baseline analgesic dose.

Perfusion Parameters

Figure 3 shows ABI and transcutaneous oxygen tension in responders (limb salvage) and nonresponders (major amputation) after 24 weeks. All patients with durable limb salvage showed a rise in ABI beginning 2 months after aBMT and sustained at 24 weeks. Mean baseline ABI in responders was higher than in nonresponders ($0.33 ± 0.18$ vs. $0.12 ± 0.14$, $p = 0.005$) and rose in responders from $0.33 ± 0.18$ at baseline to $0.46 ± 0.15$ ($p = 0.005$). The apparent increase in the nonresponder group is due to earlier amputation of the legs with lowest ABI and not a therapy effect. The course of transcutaneous partial oxygen tension shows a similar pattern. Responders had a higher baseline tcpO$_2$ than nonresponders ($12 ± 12$ vs. $1 ± 3$ mmHg, $p = 0.003$), and tcpO$_2$ values in responders started rising at week 4 and stabilized on a higher level up to 24 weeks after aBMT ($12 ± 12$ vs. $25 ± 15$ mmHg at 24 weeks, $p = 0.001$).

Walking Distance

Figure 4 shows the increase of the total walking distance from 0 to 24 weeks with a standardized treadmill protocol (3.2 km/h, 12% slope) for individual patients. Because there was no normal distribution, we calculated median values; at baseline, the median walking distance was 0 m (0–150 m), which increased to median 40 m (0–459 m) at 24 weeks.
Angiography

In only three patients new collaterals could be convincingly documented after 24 weeks despite increased perfusion parameters and clinical improvement. Therefore, we abandoned routine 6-month control angiography at the end of 2006.

<table>
<thead>
<tr>
<th>Rutherford category* (mean)</th>
<th>0 Weeks</th>
<th>24 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Category 2</td>
<td>4.9 ± 0.74</td>
<td>3.3 ± 0.72†</td>
</tr>
<tr>
<td>Category 3</td>
<td>0</td>
<td>2 (7%)</td>
</tr>
<tr>
<td>Category 4</td>
<td>0</td>
<td>17 (57%)</td>
</tr>
<tr>
<td>Category 5</td>
<td>9 (30%)</td>
<td>8 (27%)</td>
</tr>
<tr>
<td>Category 6</td>
<td>14 (47%)</td>
<td>3 (10%)</td>
</tr>
<tr>
<td>Category 6</td>
<td>7 (23%)</td>
<td>0</td>
</tr>
</tbody>
</table>

Wound area (cm²) in 21 patients with Rutherford categories 5/6 at baseline

<table>
<thead>
<tr>
<th>Wound area (cm²) in 21 patients with Rutherford categories 5/6 at baseline</th>
<th>11.6 ± 20</th>
<th>4.4 ± 11†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minor amputations</td>
<td>17 (80%)</td>
<td></td>
</tr>
<tr>
<td>Complete wound closure</td>
<td>15 (71%)</td>
<td></td>
</tr>
<tr>
<td>Analgetic dose</td>
<td>100%</td>
<td>38% (0–100)†</td>
</tr>
</tbody>
</table>

Data are frequencies (percentage) or mean ± SD (range). Analgetic dose at baseline in mg was set to 100%.

*Rutherford categories: category 2, moderate claudication; category 3, severe claudication; category 4, rest pain; category 5, minor tissue loss; category 6, gangrene of parts of the foot or leg.

†p = 0.0001 (two-sided test) baseline versus 24 weeks.

DISCUSSION

This pilot study shows that the transplantation of BMC in a critically ischemic leg can increase blood flow, support wound healing, reduce pain, and ultimately avoids leg amputation in a substantial part of otherwise incurable patients. Patients suffering from CLI exhibit a severe reduction in blood flow due to occlusion or severe stenosis in one or more leg arteries. A network of collateral vessels, adjacent to these major vessels, has the capability to increase in size and thereby serve to restore some blood flow (21,35). This natural capability, however, is impaired in CLI patients, especially those suffering from diabetes and hypertension (4,6,13).

Previous studies support the therapeutic value of BMC in ischemic diseases. They have been shown to naturally home to ischemic tissue and are functional in an hypoxic environment (3,20,26). Bone marrow mononuclear cells are a potent source of proangiogenic cytokines and chemokines and thus can act as supportive paracrine effector cells adjacent to the growing collateral vessels (18). Also, marrow monocytes mediate the inflammatory response that is the initial step of collateral artery formation in the setting of arterial obstruction (22). Thus, we initially chose to treat our first 12 patients with a Ficoll-isolated mononuclear BMC concentrate. Both Ficoll and Harvest cell concentrates contain CD34+ hematopoietic stem cells and AC133+ endothelial progenitor cells, which have been shown to incorporate...
Figure 2. (A) A 63-year-old male diabetic with Rutherford cat. 5, chronic wound for 3 months, two failed crural bypasses, TcpO$_2$ 5 mmHg, ABI 0.3. (B) Four months after aBMT: complete wound healing, tcpO$_2$ 37 mmHg, ABI 0.63. Rutherford cat. 3. (C) A 58-year-old male, Rutherford cat. 5, two failed bypasses (crural + pedal), tcpO$_2$ 0 mmHg, ABI >2 (media sclerosis). (D) Three months after aBMT wound healing, tcpO$_2$ 52 mmHg. Rutherford cat. 3. (E) A 72-year-old diabetic male, Rutherford cat. 6, two failed crural bypasses, tcpO$_2$ 1 mmHg, ABI 0.0. (F) Six months after aBMT stable minor transmetatarsal amputation, Rutherford cat. 3, tcpO$_2$ 57 mmHg, ABI 0.4.
artery growth may also explain the consistently positive results of cell-based therapies in peripheral artery disease in contrast to indifferent or negative results of single growth factor applications (19).

We converted our processing method from Ficoll to the Harvest bedside system without any negative effect. The relative biologic potential of both the Ficoll and Harvest cell compositions had been evaluated in an ischemic hind limb with BM cells isolated by the Harvest point of care device displaying similar or greater angiogenic activity compared to Ficoll-isolated BM-MNC (15).

Should other studies also prove this therapy to be effective, adoption by hospitals other than large academic centers will require a simple method for processing the bone marrow. We found Ficoll separation to require a GMP facility, which is not available to many hospitals, and a total treatment time of 7–8 h. The Harvest system allowed for processing at the bedside and reduced total time for the treatment to 1 h.

The results of our study suggest that aBMT has the potential to treat severely ischemic limbs with imminent major amputation. While long-term limb salvage was 53%, it should be understood that 90% of the patients had been scheduled for major amputation before aBMT. It was also possible to perform minor, limb-sparing amputations with complete wound healing in more than half of the nonamputated patients. From a clinical point of view, it is especially important that there was a significant improvement in the mean Rutherford category from 4.9 to 3.3. This means that in the patients with

into the growing collateral vasculature and ensure stability of the enlarged vessel (1,33).

For subjects 13 to 51 we transplanted the whole nucleated cell fraction from the marrow. This composition contained a small portion of the erythrocyte layer as well as a high level of platelets, both of which are not present in a Ficoll BMC preparation. This fraction has been shown to be very bioactive (24). Platelets have been shown to augment collateral vessel formation when in the presence of mononuclear cells (17). The pleiotropic effects of autologous bone marrow cells on collateral

Figure 3. (A) Ankle-brachial index in responders with limb salvage (black filled squares, n = 21 with measurable ABI) and nonresponders with major amputation (empty squares, n = 17 with measurable ABI). *p < 0.05 for baseline responders versus nonresponders and **p < 0.05 for responders baseline versus 24 weeks responders. (B) Time course of transcutaneous partial oxygen tension up to 24 weeks after aBMT in responders with limb salvage (black filled squares, n = 30) and nonresponders with major amputation (empty squares, n = 21). *p = 0.01 for baseline responders versus nonresponders and **p = 0.0045 for baseline responders versus responders at 24 weeks.

Figure 4. Individual total treadmill (3.2 km/h, 12%) walking distance in meters after 24 weeks in patients with limb salvage. Median at baseline 0 m and after 24 weeks median 40 m.
limb salvage, not only amputation was avoided, but the leg also was pain free and usable.

Because this was an uncontrolled study, we cannot totally exclude the possibility of some spontaneous improvement. However, a meta-analysis showed for similar patient populations without any operative or interventional revascularization option and severe critical limb ischemia a 1-year amputation rate of 95% (36). Additionally, we found that limb salvage was only possible if an increase in perfusion measured with ABI and tcpO2 took place. However, patients with limb salvage had better baseline perfusion than the eventually amputated patients. Because there were no data about the magnitude of the effect of aBMT in this severely ischemic population, we choose broad inclusion criteria and included basically all patients with CLI and no revascularization option. This means that some nonviable legs, defined according to Rutherford (27), with no measurable flow at the ankle (zero ABI) and tcpO2 values of zero were treated in this study. Future randomized controlled trials should probably not include these patients with definitely nonsalvageable limbs. From our results these can tentatively be identified by an ABI of less than 0.1 and clinical signs and symptoms of a “dead” leg.

With tcpO2, there was a considerable overlap between responders and nonresponders; however, only two patients with a baseline tcpO2 of 0 mmHg improved sufficiently for limb salvage whereas most of the amputees had tcpO2 baseline values of less than 5 mmHg. This confirms results by Wyss et al. (37) and Faglia et al. (10), who found amputation rates of 95% in CLI patients with a tcpO2 lower than 20 mmHg. We rarely observed an increase of angiographically visible collaterals and found no correlation between angiographic results and clinical status or perfusion parameters at 24 weeks. This may be explained with the diameter of new or enlarged collaterals being smaller than 0.2 mm, the angiographic visibility threshold. Multiple regression analysis did not show any influence of age, diabetes, smoking habits, vascular and nonvascular comorbidities, or BMC isolation method on amputation status at 24 weeks.

A recent report postulated that the amputation rate is higher in the presence of noncompressible arteries in patients with diabetes (30); however, we found no difference in response to aBMT nor in the amputation rate between patients with and without noncompressible arteries.

Bone marrow aspiration and reinjection were generally well tolerated. There was no procedure-related mortality. During follow-up, we encountered a total of five major cardiac events, two of them fatal. Taking into account the advanced stage of atherosclerosis and the severe comorbidities of the study population, this rate compares rather favorably with the MACE rate in other CLI series (12,16,23). A recent publication raised concern about a suspected increase of long-term adverse events in patients with thromboangiitis obliterans treated with aBMT, especially the occurrence of unwanted neovascularization and plaque instability (25). However, we did not encounter any clinically relevant neovascularization, arteriovenous fistulas, clinically manifest retinopathy, nor new malignancies. Because recent animal studies in ApoE knockout mice study suggested an increase in plaque size and vulnerability after bone marrow transplantation into ischemic hind limbs (11,29), we looked carefully at all coronary events during follow-up. The event rate was low, and all five coronary events happened well after 3 months after aBMT, which makes a direct plaque-destabilizing effect of aBMT unlikely.

To our knowledge, this is the largest published study in patients with end-stage arteriosclerotic peripheral arterial disease treated with aBMT for limb salvage. The encouraging results of our study have to be confirmed in a double-blind, placebo-controlled trial that is currently under way (NCT00434616). We conclude that aBMT does increase arterial perfusion in a substantial part of CLI patients and thus could possibly avert major amputations in many patients with failed revascularization and critical, limb-threatening ischemia.

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