Therapeutical potential of autologous peripheral blood mononuclear cell transplantation in patients with type 2 diabetic critical limb ischemia

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A B S T R A C T
Aim: The aim was to evaluate the therapeutic effectiveness of granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood mononuclear cells (PBMCs) in critical limb ischemia (CLI) of type 2 diabetic patients.
Method: Forty diabetic patients with CLI were enrolled and randomized to treatment and control groups. In the treatment group, the patients received subcutaneous injections of recombinant human G-CSF (30 MU/day) for 5 days to mobilize stem cells. PBMCs were collected and transplanted by multiple intramuscular injections of 1 ml in 1–1.5-cm depth into ischemic limbs.
Results: At the end of 12 weeks of follow-up, the baseline and end point results in transplant group were as follows: Fontaine score improved from 3.8±0.3 to 3±0.5 (P=.0001), ankle brachial pressure index increased from 0.68±0.24 to 0.87±0.24 (P=.001), transcutaneous oxygen increased from 33±14 mmHg to 44±10 mmHg (P=.0001), and 6-min walking distance improved from 280±82 m to 338±98 m (P=.0001). Pain score decreased from 8.2±1.3 to 5.63±1.6 (P=.001), and the number of patients with limb ulcers was reduced from 9/20 (45%) to 3/20 (15%) (P=.031). In the control group, Fontaine score, 6-min walking distance, and pain score were improved; ankle brachial pressure index and transcutaneous oxygen pressure were not improved. The number of patients with limb ulcers did not change in the control group. There are improvement in amputation rates, collateral vessel development, and number of limb ulcers healed.
Conclusions: These results indicate that the autologous transplantation of G-CSF that mobilized PBMCs in CLI diabetic patients is safe and effective in patient compliant reduction and improved perfusion.

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1. Introduction

Despite conventional treatment options, a substantial number of patients with thromboangiitis obliterans and diabetic limb ulcer experience limb loss. Physical disability, psychiatric problems, and high reamputation rates force investigators to identify novel therapeutic approaches in this area of medicine (Trautner, Haastert, Spraul, Giani, & Berger, 2001). Treatment with vasodilators, anticoagulants, and/or vascular bypass is indicated. However, despite these treatment options, a substantial number of patients undergo limb amputations. Recent studies in this field target cell-derived treatment strategies (Chen, Lee, Chiu, Shyu, Lee, Huang, & Li, 2006). Cell-derived treatment strategies aimed at revascularization and/or neovascularization for ischemic damage prevention.

Stem cell collection is made through either the bone marrow or peripheral route. Bone marrow aspiration carries the risk of anemia and also requires anesthetic medication. Peripheral stem cell collection is more risk free, and cells are collected by centrifuge.
A recent study compared therapeutic angiogenesis, ankle brachial pressure index (ABI), pain at rest, and transcutaneous oxygen pressure (TcPO₂) in patients with bilateral diabetic critical limb ischemia (CLI). One extremity was given bone-marrow-derived stem cells, and the other one was given peripheral blood derived mononuclear cells (PBMCNs). Bone-marrow-derived mononuclear cells showed better results in that extremity than the other one (Tateishi-Yuyama et al., 2002). However, recent studies established the potential benefit of autologous transplantation of PBMCNs in CLI diabetic patients (Huang, Li, Han, Xiao, Yang, & Han, 2005). After granulocyte-stimulating colony-stimulating factor (G-CSF) mobilization, the number of CD34 antigen-positive expressing hematopoietic progenitor cells increased, and these cells act as endothelial progenitor or angioblasts (Asahara, Murohara, Sullivan, Silver, van Der Zee, & Li, 1997). Recent and also ongoing studies showed positive effects of circulating progenitor cells on vascular healing. Rehman, Li, Orschell, and March (2003) suggest that angiogenesis-promoting effect of endothelial progenitor cells comes from endothelial cell activation by angiogenesis-stimulating factor secretion (Rehman et al., 2003). Endothelial progenitor cells collected from type 1 diabetics has impaired angiogenic capacity; the ones derived from type 2 diabetics had impaired proliferation and adhesion functions (Loomans et al., 2004; Tepper et al., 2002). The problems discussed explain insufficient vascular development in PBMCNs transplanted in diabetic patients. But in this aspect, future studies are needed. Studies also report the potential retinopathy side effect of endothelial progenitor cells (Sata, 2006).

The aim of this study is to evaluate the efficacy of the application of autologous transplantation of G-CSF mobilized PBMCNs in the treatment of CLI of diabetic patients.

2. Materials and methods

2.1. Study design

This was a prospective, randomized, open-label, controlled intervention study.

2.2. Study location

This clinical study was approved by the ethical committee board of the GMMA Hospital (1491-299-06, 30 November 2006) and Republic of Turkey Ministry of Health (14362/05.07.2007). Patients admitted to GMMA Internal Medicine, Yeditepe University Internal Medicine, GMM hematology section, from December 2008 to December 2009 were enrolled in this prospective controlled clinical trial. All participants gave written informed consent.

2.3. Population

The Fontaine classification stratified patients as class III–IV, type 2 diabetic patients with proven CLI.

2.4. Inclusion criteria

Diabetic patients with proven CLI but without hypercoagulable states or gangrene above the ankle and/or severe coronary, cerebral, and renal vascular diseases were eligible for participation in this trial. Causes of hypercoagulable states in patients’ history and medical records include medications (female hormones, estrogens, and birth control pills), posturgery phospholipid antibodies in blood (anti-cardiolipin antibodies, lupus anticoagulant), cancer, elevated blood homocysteine levels, and inherited protein deficiencies (antithrombin III, factor V Leiden, protein S, protein C). History and evidence in medical records for acute or chronic coronary event (coronary angiography, myocardial perfusion scintigraphy) were determined.

2.5. Exclusion criteria

Patients with proliferative retinopathy, malignant neoplasm, myocardial infarction, angina pectoris, cerebrovascular accident (<6 weeks), terminal renal and hepatic failure, anemia, leukopenia, and thrombocytopenia were excluded.

2.6. Initial patient visit

Patients with CLI symptoms were staged according to their Fontaine scores. Demographic characteristics, duration of diabetes, glyceria levels, hepatic and renal functions, inflammatory markers, and medications of stage III–IV patients were noted. Initial pain score, ABI, TcPO₂, 6-min walking distance (in patients without ulcer or superficial ulcer), and Doppler ultrasonography/digital subtraction angiography (DSA) /magnetic resonance angiography of leg ulcer were determined.

Forty diabetic patients with CLI were enrolled and randomized as 20 patients in the treatment group and 20 patients in the control group. Randomization was made via an internet-based system. Main clinical manifestations were observed 12 weeks after treatment. Mean age for treatment group and control group is as follows: 71±9 years and 70±8 years (P=.691), with a male/female ratio of 16/4 for the treatment group and 13/7 for the control group (P=.288). The baseline features (Fontaine score, ABI, TcPO₂, 6-min walking distance, number of limb ulcers, pain score, duration of diabetes, HbA1c levels) and clinical characteristics of the two groups were similar before the trial (Table 1).

2.7. Fontaine classification

The Fontaine classification is as follows: stage I: no symptoms, stage II: intermittent claudication subdivided into II-a (without pain on resting, but with claudication at a distance of greater than 200 m) and II-b (without pain on resting, but with a claudication distance of less than 200 m), stage III: nocturnal and/or resting pain, stage IV: necrosis and/or gangrene in the limb (Fontaine, Kim, & Kieny, 1954).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Baseline features and clinical characteristics of the patients enrolled</th>
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<tbody>
<tr>
<td></td>
<td>Treatment group</td>
</tr>
<tr>
<td>N</td>
<td>20</td>
</tr>
<tr>
<td>Age (mean ±S.D.)</td>
<td>71.9±9.2</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>16/4</td>
</tr>
<tr>
<td>Mean duration of diabetes (years)</td>
<td>13.5±8.5 (5-22)</td>
</tr>
<tr>
<td>Mean Hba1c (%)</td>
<td>7.4±1.5</td>
</tr>
<tr>
<td>Fontaine score</td>
<td>3.84±0.37</td>
</tr>
<tr>
<td>ABI</td>
<td>0.68±0.24</td>
</tr>
<tr>
<td>TcPO₂ (mmHg)</td>
<td>33.8±14.73</td>
</tr>
<tr>
<td>6-min walking distance (m)</td>
<td>280±82</td>
</tr>
<tr>
<td>Pain score</td>
<td>8.2±1.3</td>
</tr>
<tr>
<td>No. of patients with limb ulcer (%)</td>
<td>9/ 45%</td>
</tr>
<tr>
<td>No. of patients applied HBO (n%)</td>
<td>9/ 45%</td>
</tr>
</tbody>
</table>

There were no differences in baseline features between the two groups. Between-group comparisons were performed using Mann–Whitney U test for continuous variables and χ² for categorical variables.
2.8. Pain score

We used numeric pain scale in this study. The pain score scale is from 0 to 10, 0 being no pain and 10 being maximum pain (Williamson & Hoggart, 2005).

2.9. ABI

The sensitivity and specificity of ABI for peripheral artery disease prediction were 99% and 99% (Bebe, 2001). The severity of occlusion is inversely proportional with result (Iwagura et al., 2002). An ABI>1.3 incompressibility of calcified arteries, 1.0–1.3: normal, 0.9–1.0: acceptable, 0.7–0.89: mild disease (intermittent claudication frequency), 0.5–0.7: moderate arterial disease (intermittent claudication), <0.5: severe peripheral vascular disease (pain at rest), <0.2: tissue loss (severe limb-threatening peripheral arterial disease is probably present) (Potier, Abi Khalil, Mohammedi, & Roussel, 2011).

2.10. Angiographic analysis

The patients were subjected to analysis of magnetic resonance angiography before and 12 weeks after treatment. The angiographic scores for the formation of new collateral vessels were assessed as 0 (no collateral development) and 1 (collateral development) (Graziani et al., 2007).

2.11. TcPO2

TcPO2 monitor consists of a combined platinum and silver electrode covered by an oxygen-permeable hydrophobic membrane, with a reservoir of phosphate buffer and potassium chloride trapped inside the electrode. TcPO2 is a safe, noninvasive, and accurate method for evaluation of ischemic extremity perfusion and oxygenation (Ruangsetakit, Chinsakchai, Mahawongkajit, Wongwanit, & Mutirangura, 2010).

Hyperbaric oxygen therapy

Galeazzi brand twin lock chamber pressure rooms were used for his study. Patients received 100% oxygen at 2.5 atm chamber pressure for 120 min. All of the patients enrolled in this study received approved medication (vasodilator medication, anticoagulation, antiaggregation, if needed antibiotics, glycaemia regulation, statin, wound care). In addition to these, all patients who have leg ulcer received hyperbaric oxygen (HBO) therapy. Baseline measurements were obtained 2 weeks later after HBO therapy (Niinikoski, 2003).

In the treatment group, the patients received subcutaneous injections of recombinant human G-CSF (30 MU/day) for 5 days. Their mean leukocyte counts were 25 000± 6000/ml on the day of apheresis (day 6).

2.12. PBMNC collection

The Haemonetics MCS3p cell separator with 871 coded apheresis kit was used.

2.13. Analysis of CD34+ cells

A Becton Dickinson FACSCalibur flow cytometer was used for the study. BD FACsFlow solution, BD Trucount Tubes kit, FACS Lysing Solution, Calibrite 3 solution, and BD Falcon 5-ml polystyrene tubes were also used. Cell suspension of 40–70 ml was obtained by apheresis. The number of cell CD 34+ cells retrieved was 24.8×10^6/ml.

2.14. PBMNC transplantation

Three hours later, each ischemic lower limb was injected intramuscularly at 40–50 sites, nearly 3 × 3 cm distance, 1–1.5 cm deep, with 1 ml CD34+ cell suspension. Five patients received bilateral and 15 patients received unilateral injections. Low-molecular-weight heparin was used for 5 days for thromboembolic prophylaxis.

2.15. Twelve-week follow-up period

Fontaine score, pain score, ABI, DSA/magnetic resonance angiography, TcPO2, number of patients with ulcer, and number of patients with amputation were measured. Healing of the ulcer was accepted when it showed a reduction in size in a 1-month treatment period; these patients were classified as responders.

2.16. Statistical analysis

Data analysis was performed using Statistical Package for Social Sciences for Windows (version 15.0). Data are expressed as number, percentage, mean, median, and standard deviation. Between-group comparisons were performed using Mann-Whitney U test for continuous variables and χ² for categorical variables. Within-group comparisons were performed using Willcoxon signed-rank test. A P value less than .05 was considered an indication of statistical significance.

3. Results

Twelve weeks after PBMNC administration, the scale of pain score decreased, 6-min walking distance increased, Fontaine score decreased, and ABI score and TcPO2 improved. In addition to these, the number of patients with ulcers was reduced from nine to three. The results for the control group, respectively, were as follows: Fontaine score decreased, ABI and TcPO2 were not changed, 6-min walking distance decreased, 6-min walking distance increased, and ABI score and TcPO2 improved.

Table 2
Comparison of the parameters in groups at baseline and at the end of 12 weeks

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment group</th>
<th>Control group</th>
<th>P</th>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>After 12 weeks</td>
<td>Control</td>
</tr>
<tr>
<td>------------------------------------</td>
<td>-----------</td>
<td>----------------</td>
<td>---------</td>
</tr>
<tr>
<td>Fontaine score</td>
<td>3.84±0.37</td>
<td>3.05±0.52</td>
<td>.001</td>
</tr>
<tr>
<td>ABI</td>
<td>0.68±0.24</td>
<td>0.87±0.24</td>
<td>.001</td>
</tr>
<tr>
<td>TcPO2 (mmHg)</td>
<td>33.81±14.73</td>
<td>44.31±10.03</td>
<td>.010</td>
</tr>
<tr>
<td>6-min walking distance (m)</td>
<td>14 patients 280±82</td>
<td>11 patients 338±98</td>
<td>.001</td>
</tr>
<tr>
<td>Pain score</td>
<td>8.2±1.3</td>
<td>5.6±1.6</td>
<td>.001</td>
</tr>
<tr>
<td>No. of patients with ulcer (n/%)</td>
<td>9/ 45%</td>
<td>3/15%</td>
<td>.031</td>
</tr>
<tr>
<td>No. of patients with collateral vessel (n/%)</td>
<td>9/ 45%</td>
<td>5/ 25%</td>
<td>.138</td>
</tr>
</tbody>
</table>

All parameters showed significant improvement in the treatment group. Within-group comparisons were performed using Wilcoxon signed-rank test; between-group comparisons were performed using Mann-Whitney U test for continuous variables and χ² for categorical variables.
distance increased, pain score decreased, but there were no differences in ulcer numbers (Table 2).

In this clinical trial, we observed that all clinical manifestations in the treatment group were significantly improved after autologous transplantation of PBMNCs, including outcome of limb pain, 6-min walking distance, limb ulcers, ABI, Fontaine score, and TcPO2 ($P=.001$–0.003) (Graphs 1, 2).

After 12-week follow-up, three (15%) patients from the treatment group and five (25%) patients from the control group underwent limb amputation ($P=.429$). The number of ischemic limbs with new collateral vessel development in the treatment group was higher than in control patients (45% vs.25%, $P=.138$) (Table 2). No significant statistical difference was found between the control and treatment populations in amputation rates and collateral vessel development.

4. Discussion

Our present study is the second randomized controlled study with a larger number of patient group that investigates the therapeutic effectiveness of G-CSF mobilized PBMNC in CLI of diabetic patients.

At the end of the study, the main manifestations including clinical limb ischemia score, ABI, transcutaneous oxygen pressure, 6-min walking distance, pain score, and number of ulcer healed improved with significant statistical difference in the patients of the treatment group. However, no significant statistical difference was found between the control and treatment populations in amputation rates and collateral vessel development.

Interventional and surgical revascularization is the treatment of choice in critical limb ischemic patients. Despite the technical advances in revascularization procedures, a substantial number of patients with widespread and distal vascular disease in whom revascularization is not possible with these techniques remain. The uses of pharmacologic drugs also give limited benefit to these patients.

One such opinion is therapeutic angiogenesis. Recent studies demonstrated the beneficial effects of peripheral progenitor cells in CLI even in totally occluded patients. At present, there is a great deal of interest in cell-based treatment strategies for the repair of ischemic damage. (Kalka et al., 2000; Iwagura et al., 2002).

Huang, Li, Han, Xiao, Yang, and Han (2004) reported that transplantation of G-CSF mobilized autologous PBMNCs improves limb ischemia in patients with arteriosclerosis obliterans of lower extremities (Huang et al., 2004). Following these, they designed a new study and investigated potential benefits of G-CSF mobilized PBMNCs in CLI of a diabetic patient group (Huang et al., 2005). In this pilot clinical trial, they showed that many clinical manifestations in the transplanted patients were significantly improved after autologous transplantation of PBMNCs, including outcome of lower limb pain, pain-free walking distance, diabetic foot ulcers, and angio-graphic scores. They reported minor amputation rates and better collateral vascular development than our study. The likely basis for this result is that they retransplant critical patients on day 40.

Cardiac, infectious, renal, hepatic, metabolic, and clinical parameters were measured before and after cell transplantation, and both were noted to be in normal limits. We did not detect infection or any abnormality in vital signs both during and after the trial.

The autologous transplantation of bone marrow mononuclear cells was also found to be safe and effective for achievement of therapeutic angiogenesis (Tateishi-Yuyama et al., 2002); however, because it is a noninvasive procedure and there is a lack of anesthesia need, peripheral stem cell collection is the choice for most of the investigators.

G-CSF used for peripheral stem cell mobilization increases the cost. But it has been suggested that G-CSF by itself is possible to improve neovascularization (Seiler et al., 2001) and wound healing in ischemic diseases because of its ability to mobilize endothelial progenitor cells into peripheral blood (deLalla et al., 2001). In the first study, angiographic collateral degree increased significantly after treatment with GM-CSF, and it was statistically unchanged in the placebo group after treatment in the GM-CSF but not in the placebo group. But in this study, GM-CSF had been applied in an intracorony manner. In our study, application was perivascular. There was no evidence that show beneficial effect of G-CSF itself applied perivascularly. Because of the potential risks of G-CSF, patients who have proliferative retinopathy were excluded, and low-molecular-weight heparin was used for thromboembolic prophylaxis.

The main findings in recent studies reported that implantation of PBMNC into ischemic limbs shows its benefit by two mechanisms. First, it promotes angiogenesis and endothelial progenitor cell migration to the ischemic region; second, it secretes several angiogenic factors that promote neovascularization and vascular cell repair (Iba et al., 2002). Several investigators reported that angiogenic factors such as vascular endothelial growth factor (Iseri, Piezcek, Schainfeld, Blair, Haley, & Ashara, 1996), basic fibroblast growth factor (Wanataba, Smith, Sun, Smart, Delcarpio, & Roberts, 1998), and hepatocyte growth factor (Taniyama, Morishita, Aoki, Nakagami Yamatoko, & Yamazaki, 2001) promote collateral vascular development in limb and myocardial ischemia models.
experimentally. Recent studies demonstrated a limited number of bone-marrow-derived endothelial progenitor cells in peripheral circulation (Asahara et al., 1997). Endothelial progenitor CD34+ cells need to be mobilized before peripheral collection.

This is the first randomized controlled study following Huang’s pilot study. We thought that improvement in limb perfusion is responsible for clinic and laboratory healing. Collateral vascular development around occluded vessels also remains an important factor. In our study, we detected more collateral development in the treatment group. Future studies are needed in a large number of patients to determine the mechanism of action of angiogenic factors in collateral vessel development. Also, patients should be followed for longer (couple of years) to detect possible side effects of cell-based therapies.

In conclusion, angiogenesis is an important process during pathologic conditions such as wound healing in diabetic patients with CLI. In our data, there was a clear trend for many clinical manifestations in the transplanted patients to be significantly improved after autologous transplantation including outcome of limb pain, walking distance, tissue oxygen content, ankle brachial index, and ulcers. We also observed more new collateral vessels, but there was no significant statistical difference.

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References


