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Transplantation of autologous bone marrow mononuclear cells with VEGF gene improves diabetic critical limb ischaemia

Przeszczep jednojądrzastych autologicznych komórek szpiku kostnego inkubowanych z genem VEGF poprawia rokowanie w krytycznym niedokrwieniu kończyn dolnych spowodowanym cukrzycą

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Abstract

Introduction: The aim of this study was to assess the safety and efficacy of combined autologous bone marrow mononuclear cell and VEGF165 gene therapy in patients with diabetes mellitus suffering from critical limb ischaemia (CLI).

Material and methods: The administration of mononuclear cells (MNCs) and naked VEGF165 plasmid was performed in 16 limbs of 16 patients with rest pain and ischaemic ulcers due to diabetes. MNCs and plasmid were injected into the muscles of the ischaemic limbs. The levels of VEGF in serum and the ankle-brachial index (ABI) were measured before and after treatment. The Visual Analogue Scale (VAS) was used to evaluate pain sensation. CT angiography was performed before and after three months of therapy.

Results: Mean (± SD) plasma levels of VEGF increased non-significantly from 257 ± 80 pg/L to 391 ± 82 pg/L ($p > 0.05$) two weeks after therapy. The ABI improved significantly from 0.26 ± 0.22 to 0.49 ± 0.30 ($p < 0.001$) three months after therapy. A decrease in rest pain was observed in all patients; mean VAS decreased from 6.3 ± 1.4 to 1.2 ± 1.1 after three months ($p < 0.002$). Angiograms showed the development of collateral vessels in 12 limbs. Ischaemic ulcers healed in 12 limbs. Amputation was performed in four patients only, because of advanced wound infection. However, the level of amputations was lowered below knee level in these cases. Complications were limited to transient leg oedema in two patients and fever in two patients.

Conclusions: Intramuscular bone marrow MNCs autotransplantation combined with the administration of phVEGF165 gene is safe, feasible and effective for patients with diabetes and CLI. **(Endokrynol Pol 2013; 64 (2): 129–138)**

Key words: *diabetic foot, gene therapy, VEGF, bone marrow mononuclear cells*

Streszczenie

Wstęp: Celem pracy była ocena bezpieczeństwa i skuteczności skojarzonej terapii autogennymi jednojądrzastymi komórkami szpiku kostnego oraz terapii genowej plazmidem VEGF165 u chorych z krytycznym niedokrwieniem kończyn dolnych spowodowanym cukrzycą.

Materiał i metody: U 16 chorych z bólami spoczynkowymi oraz niedokrwiennym owrzodzeniem kończyny dolnej w przebiegu cukrzycy zdecydowano o podaniu komórek jednojądrzastych i plazmidu VEGF165. Komórki jednojądrzaste szpiku oraz plazmid były podawane drogą iniekcji domięśniowych do mięśni niedokrwionej kończyny. Do oceny wyniku zastosowanej terapii określano poziom VEGF w surowicy oraz wskaźnik kostka-ramię. Do określenia stopnia odczuwania bólu została użyta skala wzrokowo-analogowa (VAS). Do wykazania w naczyniach wykonywano CT angiografię przed i po 3 miesiącach terapii.

Wyniki: Średnie (± SD) stężenie VEGF w osoczu wzrastało nieistotnie statystycznie z 257 ± 80 pg/l przed terapią do 391 ± 82 pg/l ($P > 0,05$) po 2 tygodniach od zakończenia leczenia. Wskaźnik kostka–ramię wzrósł istotnie statystycznie z poziomu 0,26 ± 0,22 przed terapią do 0,49 ± 0,30 ($p < 0,001$) po 3 miesiącach terapii. Zmniejszenie bólu spoczynkowego obserwowano u wszystkich pacjentów, średnia wartość VAS zmniejszyła się z 6,3 ± 1,4 przed terapią do 1,2 ± 1,1 po 3 miesiącach ($p < 0,002$). Angiogramy wykazały rozwój naczyń krążenia obocznego w 12 kończynach. Niedokrwienne owrzodzenie zostało całkowicie wyleczone w przepadku 12 chorych. Amputacje przeprowadzono tylko u 4 pacjentów z powodu zaawansowanego zakażenia rany, jakkolwiek poziom amputacji obniżono w tych przypadkach poniżej kolana. Powikłania były ograniczone do przemijających obrzęków podudzi u 2 chorych i gorączki u 2 pacjentów.

Wniosek: Domięśniowa autotransplantacja komórek jednojądrzastych szpiku kostnego w połączeniu z podaniem phVEGF165 genu jest bezpieczną oraz skuteczną metodą leczenia pacjentów z cukrzycą i krytycznym niedokrwieniem kończyn dolnych. **(Endokrynol Pol 2013; 64 (2): 129–138)**

Słowa kluczowe: *stopa cukrzycowa, terapia genowa, VEGF, jednojądrzaste komórki szpiku kostnego*

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Introduction

Diabetes mellitus (DM) is a common chronic disease with a significant morbidity and mortality rate. One devastating complication of diabetes is peripheral arterial disease (PAD) including critical limb ischaemia (CLI) — the most extreme form of diabetic angiopathy, which may result in limb loss. The prevalence of PAD in patients with the diabetic foot syndrome exceeds 80% [1, 2].

At present, there is no available permanent cure for diabetic CLI [1–5]. Several investigations have indicated that in patients with diabetes, circulating endothelial progenitor cells (EPCs) exhibit impaired proliferation, adhesion, and incorporation into vascular structures. The adverse metabolic stress factors are associated with a reduced number and dysfunction of EPCs [6–8]. The severity of the disease necessitates amputation in more than a quarter of all patients [9–11]. Treatment goals for CLI include reducing the number of cardiovascular risk factors (i.e. quitting smoking). Local care is the second essential element of the treatment. In a large proportion of these patients, the anatomical extent and the distribution of PAD make the patients unsuitable for operative or percutaneous revascularisation [9, 12, 13]. Thus, the disease frequently follows an inexorable downhill course [1–4, 8, 9, 13]. Recent progress in molecular biology has led to the development of a new strategy to treat a variety of cardiovascular diseases [14–19]. The use of gene- based or cell-based therapy to induce therapeutic angiogenesis has opened up new possibilities for CLI [20–23].

The transplantation of bone marrow- or blood-derived EPCs has been shown to accelerate blood flow restoration, neovascularisation, and the healing of diabetic mouse skin [8–10]. Therefore, therapeutic angiogenesis induced by transplantation of functional EPCs into ischaemic tissues may represent a novel approach to diabetic patients with CLI. Based on our previous study and data from literature, we designed a human clinical trial of gene therapy using Vascular Endothelial Growth Factor (VEGF) gene and stem cells as an unblinded open-labelled pilot study [24–27].

The aim of this study was to assess the safety, feasibility and clinical efficacy of intramuscular application of autologous bone marrow mononuclear cells (MNCs) with plasmid encoding human VEGF165 in diabetic patients with CLI. The idea was that the transfection along with the increase in GH levels would facilitate the sitting process of the marrow cells. Also, the results of this study will be used to estimate the development of angiogenesis caused by combined mononuclear cell and gene therapy.

CLI definitions

CLI is defined as 1) persistent, recurring rest pain requiring analgesia and an ankle systolic pressure of 50 mm Hg and/or toe systolic pressure of 30 mm Hg, and/or 2) ulceration, gangrene, or non-healing wounds in the foot with ankle systolic pressure of 50 mm Hg, or toe systolic pressure of 30 mm Hg. The Fontaine classification stratified patients as class III (rest pain) or class IV (ulceration and/or gangrene) [10].

Material and methods

Cloning and preparation of plasmid DNA (phVEGF165)

For the treatment we used a eukaryotic expression vector encoding the VEGF165 gene [17, 18]. The preparation and purification of the plasmid from cultures of phVEGF165-transformed Escherichia coli were performed with the endotoxin-free column method (Qiagen Mega Kit, Qiagen Inc., Valencia, CA, USA). The purified plasmid was stored in vials and pooled for quality-control analysis. Aliquots of 2,000 μg of phVEGF165 were diluted in sterile saline to the volume of 10 mL. 10mL of a solution containing 2 mg of VEGF165 plasmid gene was added to a mononuclear cell concentrate and incubated for two hours before administration.

Bone marrow collection and preparation

Bone marrow was harvested by 25–30 aspirations from iliac crests under general anaesthesia using a bone marrow collection set (Baxter): no less than four different well-spaced iliac crest puncture sites were carried out on each side. An average volume of 500 mL of bone marrow was collected. ACD formula A was used to prevent clot formation. After collection, the marrow was filtered with 500 μm and 200 μm filters included in the kit. MNCs were separated from the harvested marrow with an albumin-primed blood cell separator (Baxter Fenwall CS 3000 plus) according to the manufacturer's protocol. The final volume of the mononuclear cell concentrate was adjusted to 80 mL, and the final product was filtered with 50-μm-blood product filter (PALL). The CD34+ cell content was estimated by flow-cytometry according to ISHAGE recommendations. The median final number of prepared MNCs was 1.58×10^9 (range from 0.77×10^9 to 3.83×10^9). The median number of collected CD34+ cells was 1.7×10^7 (range from 0.12×10^7 to 4.25×10^7). Finally, we received 120 ml of mononuclear cell solution.

Patient cohort

Inclusion criteria: 1) type 2 DM; 2) CLI, including rest pain and non-healing ischaemic ulcers persisting for

Table I. *Clinical characteristics of patients*
Tabela I. *Charakterystyka kliniczna pacjentów*

No.	Sex	Age	Affected limb	Symptoms	Localisation	Hypertension	Past smoking	Previous history
1	F	68	Right	Rest pain ulcer	Toe	No	Yes	Sympathectomy
2	M	59	Right	Rest pain ulcer	Heel	No	No	Bypass (occluded)
3	M	46	Right	Rest pain ulcer	Toe and heel	No	Yes	Sympathectomy
4	M	58	Left	Rest pain ulcer	Forefoot	Yes	Yes	Bypass (occluded)
5	M	69	Left	Rest pain ulcer	Forefoot	Yes	Yes	Bypass (occluded)
6	F	74	Right	Rest pain ulcer	Forefoot	Yes	Yes	Sympathectomy
7	M	77	Left	Rest pain gangrene	Toe and heel	Yes	Yes	Prostaglandin
8	M	64	Left	Rest pain ulcer	Toe	No	No	Prostaglandin
9	F	48	Right	Rest pain ulcer	Toe	Yes	Yes	Bypass (occluded)
10	M	71	Right	Rest pain gangrene	Forefoot	No	Yes	Sympathectomy
11	F	61	Left	Rest pain ulcer	Toe and heel	Yes	Yes	Bypass (occluded)
12	M	53	Left	Rest pain gangrene	Toe	Yes	Yes	Prostaglandin
13	F	69	Left	Rest pain ulcer	Forefoot	No	No	Prostaglandin
14	F	75	Right	Rest pain ulcer	Forefoot	Yes	No	Prostaglandin
15	M	74	Left	Rest pain ulcer	Toe	No	No	Prostaglandin
16	F	77	Right	Rest pain ulcer	Toe and heel	No	Yes	Prostaglandin
					CLI	Yes	No	

at least 12 weeks; 3) resistance to conventional therapy of CLI for at least four weeks after hospitalisation; 4) ankle-brachial index (ABI) of less than 0.5 in the affected limb; 5) no possibility of surgical or percutaneous revascularisation based on usual practice standards.

Exclusion criteria: 1) severe retinopathy; 2) end-stage renal disease; 3) heart failure and/or angina pectoris New York Heart Association (NYHA) classification III or IV; 4) liver dysfunction (grade B or C in the Child-Pugh classification); 5) malignancy or history of malignancy; 6) and/or inability to stand or walk without help.

Patients were observed for four weeks under conventional drug therapy to confirm that their clinical symptoms and objective parameters did not improve.

Subjects

The study was performed on 16 patients presenting type 2 DM and CLI not eligible for open vascular or endovascular interventions, or who failed one or both of them. The patients were nine men and seven women aged from 48 to 78 years (mean age 60.83). The duration of diabetes ranged from 8.5 years to 21 years. All patients needed constant insulin administration every day with an average dose of 0.63 U/kg (range from 0.38 to 0.91 U/kg per day). Three patients used both insulin and an oral hypoglycaemic drug (metformin) for optimal glucose control. The average haemoglobin A1c level was 8.1% (range from 5.9–11.2%) All patients were evaluated for DM and CLI; including rest pain, non-healing ulcer, and/or gangrene (Table I). The protocol of this study was approved by the Commission of Bioethics at the Wrocław Medical University (Approval no. KB-926/2003) and written informed consents were obtained from all the patients before enrollment in the study.

Administration of therapy

The patients received conventional care for their ulcers. To remove extensive callus and necrotic tissue, wound debridement was performed. After wound dressing, pressure relief was provided. Broad-spectrum antibiotics were prescribed if ulcers showed clinical signs of infection. Adjustments to the treatment were performed when indicated on the basis of microbiologic cultures and sensitivity testing. The patients received an intramuscular injection of MNCs and phVEGF165. After marrow aspiration and two hours of incubation with phVEGF 165, the concentrate was injected intramuscularly into the ischaemic lower limb below knee level. The injection sites to calf muscles were based on our previous study and data from literature [13, 24]. The volume of each injection was 1.5 mL (approximately 80 injections to calf muscles — each 2 cm deep per session). The injections were given no later than three hours after bone marrow harvesting because our intention was to eliminate the possible loss of stem

cells caused by extracorporeal storage of bone marrow cells, and to avoid prolonged exposure of plasmid to enzymatic degradation by nucleases from monocytes present in the mononuclear cell solution. In RT-PCR stem cell tests, similar levels of VEGF165 mRNA were detected before and after incubation with plasmid. These results prove a low rate of stem cell transfection by naked VEGF plasmid. However, based on our own data and data from the literature, we expected the occurrence of the transfection of connective tissue cells at the place of injection of VEGF plasmid [23, 24, 28]. The expected effect would be an increase in the production of VEGF by cells in ischaemic tissue, which would significantly improve the settlement of the injected stem cells and their transformation into angioblasty cells.

Study protocol

Cardiac, haematological, infectious, renal, hepatic, metabolic, and clinical parameters were measured both before and after cell and gene application in order to monitor the effect of MNCs and phVEGF165, as well as possible side effects of the combined stem cell and VEGF165 plasmid therapy. During a 12-week follow-up period, all patients received a constant fixed dose of insulin on a daily basis. Heart rate, blood pressure, body temperature, haemoglobin, thrombocytes, leukocytes, C-reactive protein, plasma glucose level, creatinine, urea, uric acid, gamma GT, alkaline phosphatase, and alanine aminotransferase were measured at the beginning and then one week, four weeks, and three months after the cells and gene transplantation. Before the administration of the gene, venous blood was drawn from the upper limb in order to evaluate VEGF165 concentration. This material was then obtained seven, 28 and 90 days following plasmid injection. Serum was centrifuged and frozen. VEGF165 concentration was then evaluated by means of the ELISA method using a kit from R&D Systems according to the manufacturer's instructions.

Resting ABI were calculated as the ratio of the lowest pressure from either the posterior or anterior tibial artery divided by the highest brachial systolic pressure, which were obtained one week before and both one and three months after completing the injections of MNCs with phVEGF 165.

Multislice helical computed tomography (MSHCT) performed using a GE Medical Systems LightSpeed 16-slice device was used to evaluate the arterial supply of the lower limb in our patients. The first examination was carried out one week before the administration of stem cells and VEGF gene. The follow-up examination was performed three months after the initial test. CT angiography was performed after intravenous administration of 150 ml (ca 2 mL/kg b.w.) non-ionic low-osmolar monomer contrast medium (Iomeron 350 mg I/mL) injected by a power-injector with a flow-rate set at 4 mL/sec. The scanning was initiated automatically at the peak concentration of contrast medium in the distal part of abdominal aorta (about 25–30 seconds after the onset of contrast administration) and the examination included only the arterial phase. The field of view ranged from the aortic bifurcation down to the level of the feet. The following parameters were used in acquisition: 3.00 mm slice with overlapping sections of 2.0 mm, pitch of 0.9, tube voltage of 120 kVp, tube current of 170 mAs. Axial scans and basic sagittal and coronal reconstructions were initially reviewed and then additionally two- and three-dimensional reconstructions were performed. Each artery and collateral was analysed individually by two researchers in various reconstructions. We used: multiplanar reconstruction (MPR), curved planar reformation (CPR), and maximum intensity projection (MIP). MPR is the method that proved particularly useful in the assessment of thrombotic material, atherosclerotic plaques and CPR additionally in the case of tortuous collaterals. The MIP technique proved substantially useful after calcium-subtraction, but in order to avoid the potential reconstructing-related mistakes we also evaluated MIPs prior to calcium-subtraction. The calcium-subtraction and the 'clipping tool' functions significantly facilitated the evaluation of arterial vessels localised close to bones, i.e. the popliteal artery between the femoral condyles. A radiologist who was unaware of the treatment status of the patients interpreted the CT angiograms. The evaluation was based on measurements of:

— the number of vessels;
— the length of arterial vessels;
— the width of the flow channel of each arterial vessel;
— the presence of calcifications in walls and thrombotic material in the lumen of the vessels.

The measurement of length was made twice for each vessel and the median length was calculated. We compared the results of both initial and follow-up examinations. An increase in either the number of vessels, vessel length or vessel width was considered an improvement.

Pain was evaluated using the Visual Analogue Scale (VAS) one week before treatment and 12 weeks after the administration of autologous bone marrow MNCs and VEGF plasmid.

Statistical analysis

Paired chi square and Wilcoxon tests were used to compare continuous variables before and after therapy, and to evaluate the differences between the clusters of

Table II. *Safety analysis after administration of VEGF plasmid and stem cells*
Tabela II. *Analiza bezpieczeństwa terapii z użyciem plazmidu VEGF oraz komórek macierzystych*

Clinical findings after administration of phVEGF/stem cells	Number of patients (total = 16)
Deaths	0
Major complications: shock, peripheral embolisation, necrosis and pseudoaneurysm	0
Fever	2
Oedema	2
New cancer	0

Table III. *Results of laboratory tests*
Tabela III. *Wyniki badań laboratoryjnych*

Lab. parameters	Before administration (mean ± SD)	Seven days after administration (mean ± SD)	28 days after administration (mean ± SD)	90 days after administration (mean ± SD)
Haemoglobin [g/dL]	13 ± 2.6	13 ± 1.4	12 ± 2.1	12 ± 1.5
Thrombocytes [× 1,000/μL]	242 ± 50	220 ± 25	238 ± 46	245 ± 31
Leukocytes [× 1,000/ μL]	8 ± 2	8 ± 3	9 ± 2	8 ± 2
C-reactive protein [mg/L]	12 ± 6	18 ± 6	13 ± 3	12 ± 3
Alanine aminotransferase [U/L]	25 ± 15	28 ± 16	26 ± 10	27 ± 13
Creatinine [μmol/L]	107 ± 20	99 ± 24	101 ± 25	102 ± 15

measurements taken at individual time points. $P < 0.05$ was considered significant.

Results

Clinical follow-up

The series of intramuscular injections of MNCs with VEGF165 gene were well tolerated by most patients. Only two of them reported intense pain during the injections. No major complications were noted. Two patients reported lower limb tenderness at the injection sites for up to 24 hours following injections. Mild and transient limb oedema occurred in two patients. Fever was observed in two patients (Table II). However, neither leukocytosis increase and other reactions, nor any side effects, were observed in the patients in the course of our study. There were no significant changes in laboratory parameters during this study. No patient died or was hospitalised (for any other reason than follow-up) in the course of our 90-day observation period (Table III).

All patients were followed up for at least three months. Limb amputation due to advanced CLI with extensive foot ulceration or necrosis and severe wound infection was performed in four patients. All of them underwent below-knee amputation. The amputations were performed between the 10th and 12th week following MNCs and gene administration. In these four patients, the results were not satisfactory. In another 12 patients, therapy caused rest pain recession and total healing of chronic foot ulcerations occurred up to 12 weeks after the administration of MNCs with VEGF gene. In the course of the healing process, we performed the surgical debridement of necrotic tissue. In these patients, the combined mononuclear cell/gene therapy was successful and their lower limbs were saved from amputation (Fig. 1–4).

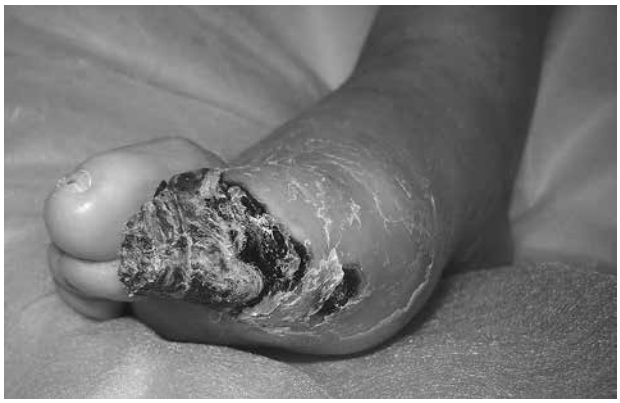

Figure 1. *Patient No. 1 before treatment*
Rycina 1. *Pacjent nr 1 przed leczeniem*

Change in VEGF serum levels

During our 90-day observation period, fluctuations in cytokine VEGF levels occurred in the study subjects. Mean VEGF serum levels increased from 257 ± 80

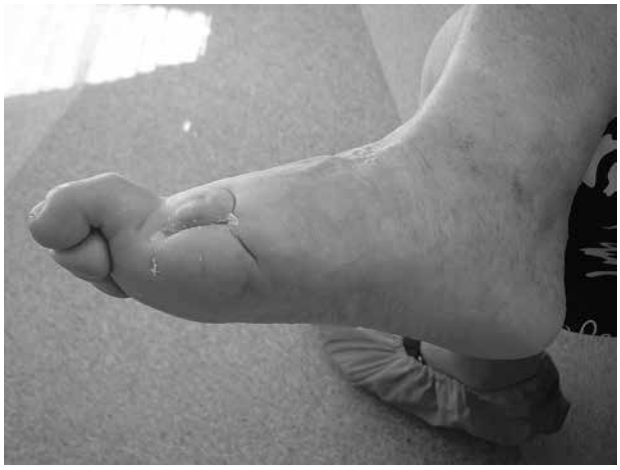

Figure 2. *Patient No. 1 after treatment*
Rycina 2. *Pacjent nr 1 po leczeniu*

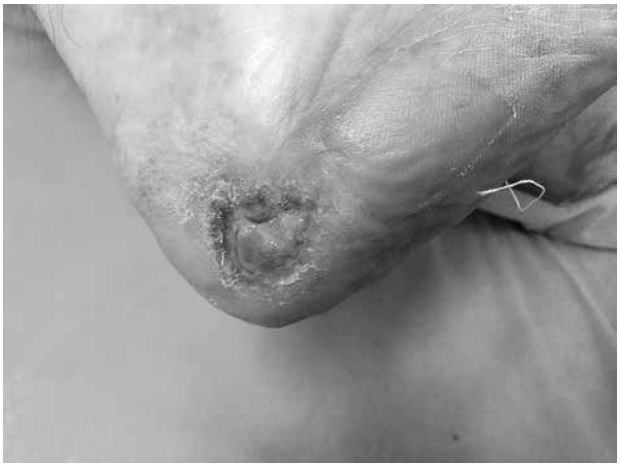

Figure 3. *Patient No. 2 before treatment*
Rycina 3. *Pacjent nr 2 przed leczeniem*

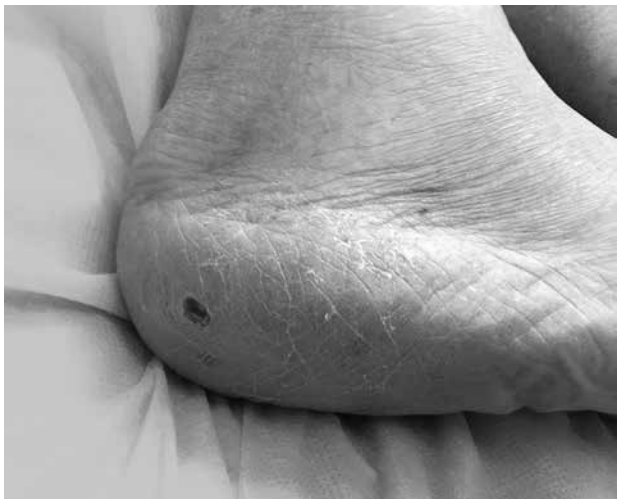

Figure 4. *Patient No. 2 after treatment*
Rycina 4. *Pacjent nr 2 po leczeniu*

pg/L to 391 ± 82 pg/L (P > 0.05) two weeks after the stem cell and gene therapy. It was the highest mean serum level recorded during this study. However, the changes of VEGF levels were highly variable. The observed levels did not significantly differ between patients with healed ulceration and those with an amputated limb (Table IV).

Plasma glucose levels

After 90 days of treatment, the mean fasting plasma glucose level significantly decreased from 8.00 ± 0.75 mmol/L at baseline to 6.14 ± 0.67 mmol/L ($p < 0.001$) (Table V).

ABI results

The mean ABI increased significantly from 0.26 ± 0.22 to 0.42 ± 0.19 ($p < 0.001$) four weeks after the adminis-

Table IV. *Plasma level of VEGF*
Tabela IV. *Stężenie VEGF w osoczu*

Time of measurement	Before administration (mean ± SD)	Seven days after administration (mean ± SD)	14 days after administration (mean ± SD)	28 days after administration (mean ± SD)	90 days after administration (mean ± SD)
Level of VEGF	257 ± 80	342 ± 85	391 ± 82	288 ± 78	259 ± 59

Table V. *Plasma glucose level*
Tabela V. *Stężenie glukozy w osoczu*

Time of measurement	Before administration (mean ± SD)	Seven days after administration (mean ± SD)	28 days after administration (mean ± SD)	90 days after administration (mean ± SD)
Glucose (mmol/l)	8.00 ± 0.75	7.54 ± 0.83	6.49 ± 0.71	6.14 ± 0.67

Table VI. *Ankle–brachial index results*
Tabela VI. *Ocena współczynnika kostka–ramię*

Time of measurement	One week before (mean ± SD)	One month after (mean ± SD)	Three months after (mean ± SD)
Ankle-brachial index (10 patients)	0.26 ± 0.22	0.42 ± 0.19	0.49 ± 0.30

tration of stem cells and gene. At the end of the study (after three months), the index increased significantly to 0.49 ± 0.30 (Table VI). The increase in the ABI was observed in 12 patients with completely healed ulceration. Unfortunately, four patients did not have a chance to complete the ABI examination due to amputation.

CT angiography results

Generally, CT angiography documented the typical findings in advanced atherosclerotic changes in lower limb arteries in all 16 cases enrolled to this study. These findings included segmental occlusive disease involving primarily the distal superficial femoral artery and/or the popliteal artery (Fig. 5–7).

After the end of the therapy, the formation of new collateral vessels was observed in all cases without amputation (Fig. 8). The comparison of CT angiograms

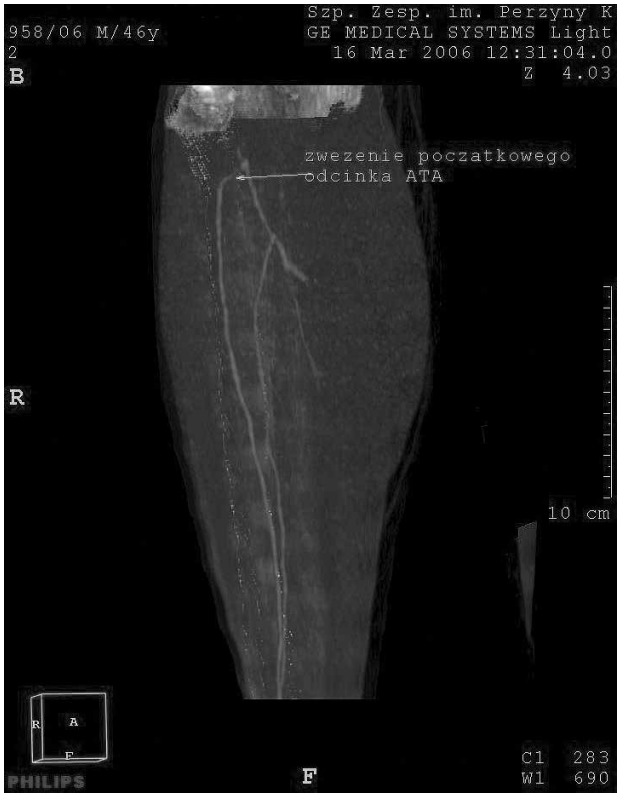

Figure 6. *Patient No. 3 before treatment, CT angiography*
Rycina 6. *Pacjent nr 3 przed leczeniem, tomografia komputerowa z opcją naczyniową*

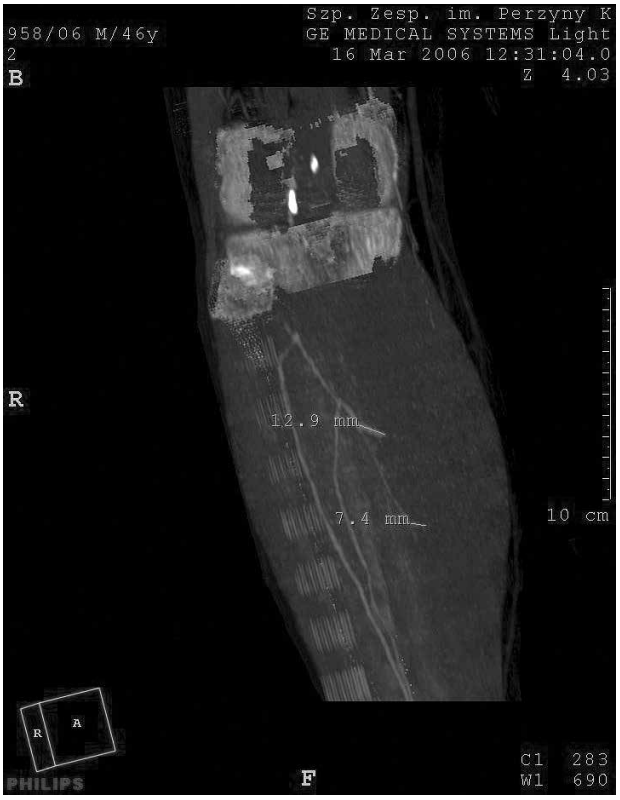

Figure 5. *Patient No. 3 before treatment, CT angiography*
Rycina 5. *Pacjent nr 3 przed leczeniem, tomografia komputerowa z opcją naczyniową*

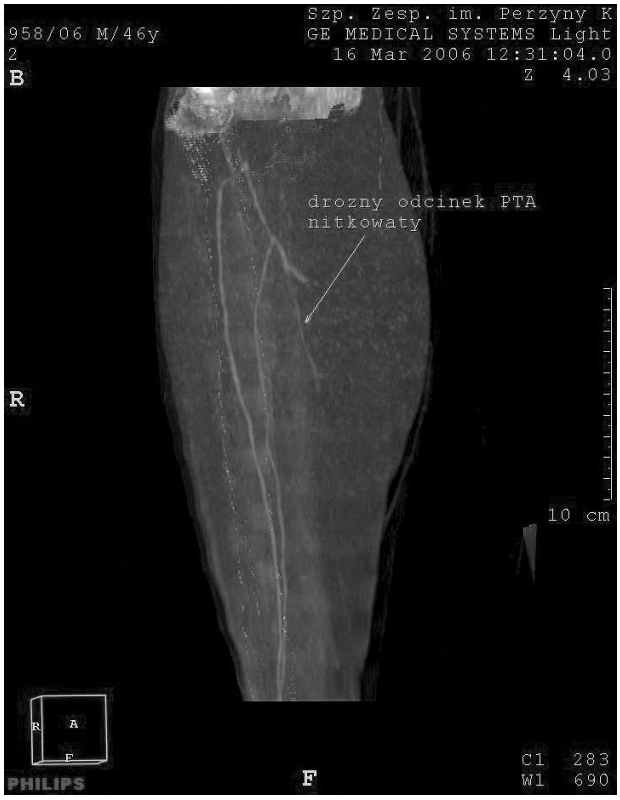

Figure 7. *Patient No. 3 before treatment, CT angiography*
Rycina 7. *Pacjent nr 3 przed leczeniem, tomografia komputerowa z opcją naczyniową*

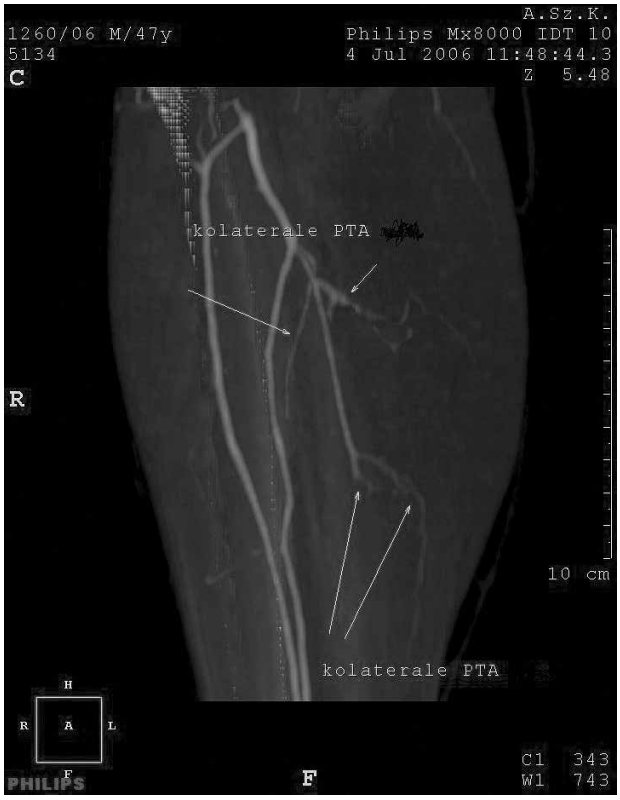

Figure 8. *Patient No. 3 after treatment, CT angiography*

Rycina 8. *Pacjent nr 3 po leczeniu, tomografia komputerowa z opcją naczyniową*

demonstrated an increase in the number (from initially two to four in the follow-up), length and width of collaterals arising from posterior tibial artery (PTA). Moreover, in 12 patients who saved their legs, in follow-up angio-CTs, the lumen of PTA and tibio-fibular trunk appeared wider and more regular compared to the first examination (Fig. 9). The number of collaterals arising from the superficial femoral artery did not change, but CT angiograms showed qualitative evidence of improved distal flow after the stem cell and gene therapy (Fig. 10).

Rest pain (VAS)

Pain was measured using the VAS and decreased significantly from 6.3 ± 1.4 before treatment to 1.2 ± 1.1 after three months ($p < 0.002$). At the end of the study, decrease in pain severity was observed in 12 patients with completely healed ulceration.

Discussion

The natural history of CLI has been well documented to have an inexorable downhill course [6–11]. Therefore, amputation is often recommended as the solution of choice in these patients [12, 13]. This is the first study known to us describing the application of a combined

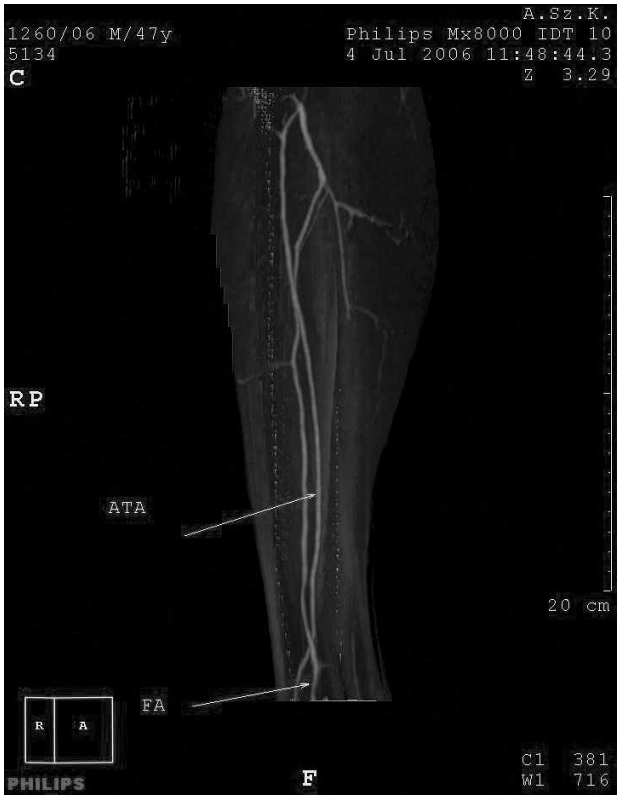

Figure 9. *Patient No. 3 after treatment, CT angiography*

Rycina 9. *Pacjent nr 3 po leczeniu, tomografia komputerowa z opcją naczyniową*

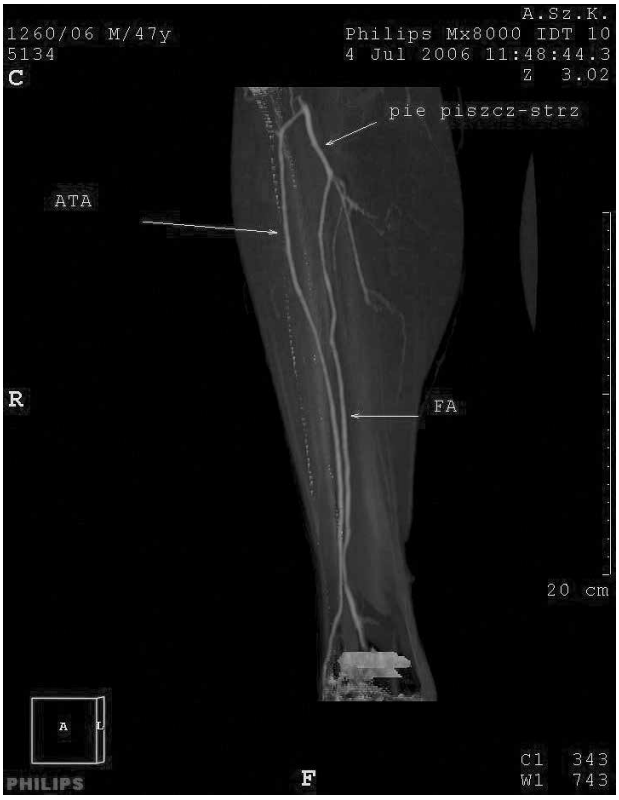

Figure 10. *Patient No. 3 after treatment, CT angiography*

Rycina 10. *Pacjent nr 3 po leczeniu, tomografia komputerowa z opcją naczyniową*

therapy using the simultaneous administration of MNCs and gene in patients with CLI due to diabetes. To the best of our knowledge, earlier trials described only treatment either with MNCs, or VEGF plasmid [21–23, 25, 27].

Our results are very promising. We achieved a significant long-term (over 90 days) clinical improvement in patients with CLI initially qualifying for amputation. As a result of the treatment, in 12 out of 16 patients surgery proved unnecessary and limb salvage was achieved. We observed the healing of ulcerations in the ischaemic limbs and rest pain regression in these patients. Unfortunately, such spectacular effects were not observed in all patients following the combined mononuclear cell and gene treatment. In four patients with advanced CLI symptoms, amputations were necessary, but amputation levels were lowered below knee level, despite having been set above the knee before the combined therapy. This is also considered to be a positive effect of the treatment. In these cases, the need for amputation was due to severe ulceration of the lower limb. It seems that the combined mononuclear cell and gene therapy should be introduced much earlier in such cases. From the perspective of patients' safety, no significant adverse effects were observed in any patients. Aside from some minor discomfort at the injection sites and peripheral oedema in two cases with fever in two cases, no other side effects were observed and – specifically – on long-term follow-up there was no evidence of any systemic effects of VEGF in terms of the development of retinopathy or new tumour growth.

The analysis of serum tests shows high initial VEGF165 concentration in the study subjects. It was statistically higher in all patients compared to healthy controls. The levels of VEGF also showed high variability from patient to patient. This situation seems to be due to much increased production of VEGF protein by the critical ischaemic muscles in the affected limb [28, 29]. During our 90-day observation period, fluctuations in cytokine levels occurred in the study subjects. Our results of VEGF level in serum demonstrate a significant increase in VEGF up to four weeks after gene therapy. The prolonged high level of VEGF in our trials seems to be related to an increased production of cytokine by the transfected muscle cells at the injection sites, and we hope that we have successfully transfected the stem cells [13, 15, 18, 24, 28]. It seems to depend on the high production of VEGF by the transfected cells in the ischaemic tissues [29]. The analysis of the clinical outcomes obtained in these patients, as well as the haemodynamic and imaging data, should be cautiously interpreted as very promising. Our results also provided preliminary evidence of the potential efficiency of the mononuclear cell and gene therapy in the treatment of CLI due to diabetes. Especially, the ABI increased significantly in 12 patients with successfully healed ulcerations ($p < 0.05$). Improved blood supply was achieved due to an increase in the number, length and width of collaterals arising from PTA, widening of PTA and tibio-fibular trunk that also became more regular (despite persistent stenosis or occlusion of the superficial femoral artery and/or the popliteal artery). To the best of our knowledge, such an improvement of the ABI is difficult to achieve with pharmacological treatment in patients with CLI due to diabetes [30]. Unfortunately, at the same time no improvement was documented in the amputated limbs of four patients. It seems that in these cases the damage to microcirculation in the affected limbs was too advanced to be improved by angiogenesis induced by stem cells from MNCs and VEGF165 plasmid. CT angiography showed increased flow in calf arterial vessels in all cases with successfully healed ulcerations. Also angiograms showed the formation of new collateral vessels in all 12 surviving limbs. Improvement of rest pain was reported in 12 (75%) patients with successfully healed wounds in the lower limb. The failed results in four cases (25%) can probably be explained by advanced critical ischaemia symptoms with irreversible damage to microcirculation. In these cases, therapeutic angiogenesis was too weak a signal of the restoration of the peripheral vascular bed in critical ischaemic muscles. It seems that the mononuclear cell and gene therapy should be introduced earlier in such cases [30, 31].

Conclusions

Our study has several limitations: it was not randomised, placebo-controlled, or double-blind. However, we evaluated the improvement in limb perfusion by reducing ischaemic pain, signs of wound healing, improved ABI and formation of new vessels after cell and gene transplantation. We found that the combined administration of intramuscular MNCs and VEGF gene was safe and effective in 75% of our patients with lower limb ischaemic necrosis due to DM.

In summary, from our observations we conclude that our method is a very promising form of therapeutic angiogenesis.

References

1. Weck M, Slesaczeck T, Rietzsch H et al. Noninvasive management of the diabetic foot with critical limb ischemia: current options and future perspectives. Ther Adv Endocrinol Metab 2011; 2: 247–55.
2. Prompers L, Huijberts M, Apelqvist J et al. High prevalence of ischaemia, infection and serious comorbidity in patients with diabetic foot disease in Europe. Baseline results from the Eurodiale study. Diabetologia 2007; 50: 18–25.
3. Wang L, Zhao S, Mao H et al. Autologous bone marrow stem cell transplantation for the treatment of type 2 diabetes mellitus. Chin Med J (Engl) 2011; 124: 3622–8.

4. Kügler CF, Rudofsky G. The challenges of treating peripheral arterial disease. Vasc Med 2003; 8: 109–114.
5. Eckardt A, Kraus O, Küstner E et al. Interdisciplinary treatment of diabetic foot syndrome. Orthopade 2003; 32: 190–198.
6. Loomans CJ, de Koning EJ, Staal FJ et al. Endothelial progenitor cell dysfunction: a novel concept in the pathogenesis of vascular complications of type 1 diabetes. Diabetes 2004; 53: 195–199.
7. Tepper OM, Galiano RD, Capla JM et al. Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. Circulation 2002; 106: 2781–2786.
8. Lechner A, Habener JF. Stem/progenitor cells derived from adult tissues: potential for the treatment of diabetes mellitus. Am J Physiol Endocrinol Metab 2003; 284: E259–266.
9. Eskelinen E, Eskelinen A, Albäck A et al. Major amputation incidence decreases both in non-diabetic and in diabetic patients in Helsinki. Scand J Surg 2006; 95: 185–189.
10. Norgren L, Hiatt WR, Dormandy JA et al. Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II). Eur J Vasc Endovasc Surg. 2007; 33 (Suppl. 1): S1–75.
11. Schatteman GC, Hanlon HD, Jiao C et al. Blood-derived angioblasts accelerate blood-flow restoration in diabetic mice. J Clin Invest 2000; 106: 571–578.
12. Takahashi T, Kalka C, Masuda H et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 1999; 5: 434–438.
13. Al-Khaldi A, Al-Sabti H, Galipeau J et al. Therapeutic angiogenesis using autologous bone marrow stromal cells: improved blood flow in a chronic limb ischemia model. Ann Thorac Surg 2003; 75: 204–209.
14. Iwaguro H, Yamaguchi J, Kalka C et al. Endothelial progenitor cell vascular endothelial growth factor gene transfer for vascular regeneration. Circulation 2002; 105: 732–738.
15. Tateishi-Yuyama E, Matsubara H, Murohara T et al. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. Lancet 2002; 360: 427–435.
16. Kurpisz M, Czepczyński R, Grygielska B et al. Bone marrow stem cell imaging after intracoronary administration. Int J Cardiol 2007; 121: 194–195.
17. Shyu KG, Chang H, Wang BW et al. Intramuscular vascular endothelial growth factor gene therapy in patients with chronic critical leg ischemia. Am J Med 2003; 114: 85–92.
18. Kusumanto YH, van Weel V, Mulder NH et al. Treatment with intramuscular vascular endothelial growth factor gene compared with placebo for patients with diabetes mellitus and critical limb ischemia: a double-blind randomized trial. Hum Gene Ther 2006; 17: 683–691.
19. Hedman M, Hartikainen J, Syvänne M et al. Safety and feasibility of catheter-based local intracoronary vascular endothelial growth factor gene transfer in the prevention of postangioplasty and in-stent restenosis and in the treatment of chronic myocardial ischemia: phase II results of the Kuopio Angiogenesis Trial (KAT). Circulation 2003; 107: 2677–2683.
20. Isner JM, Pieczek A, Schainfeld R et al. Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb. Lancet 1996; 348: 370–374.
21. Isner JM. Arterial gene transfer of naked DNA for therapeutic angiogenesis: early clinical results. Adv Drug Deliv Rev 1998; 30: 185–197.
22. Miyamoto K, Nishigami K, Nagaya N et al. Unblinded pilot study of autologous transplantation of bone marrow mononuclear cells in patients with thromboangiitis obliterans. Circulation 2006; 114: 2679–2684.
23. Isner JM, Baumgartner I, Rauh G et al. Treatment of thromboangiitis obliterans (Buerger's disease) by intramuscular gene transfer of vascular endothelial growth factor: preliminary clinical results. J Vasc Surg 1998; 28: 964–973.
24. Skóra J, Sadakierska-Chudy A, Pupka A et al. Application of VEGF165 plasmid in treatment of critical lower limb ischemia. Pol Merkur Lekarski 2006; 20: 655–659.
25. Stewart DJ, Hilton JD, Arnold JM et al. Angiogenic gene therapy in patients with nonrevascularizable ischemic heart disease: a phase 2 randomized, controlled trial of AdVEGF(121) (AdVEGF121) versus maximum medical treatment. Gene Ther 2006; 13: 1503–1511.
26. Kalka C, Masuda H, Takahashi T et al. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci U S A 2000; 97: 3422–3427.
27. Yamamoto K, Kondo T, Suzuki S et al. Molecular evaluation of endothelial progenitor cells in patients with ischemic limbs: therapeutic effect by stem cell transplantation. Arterioscler Thromb Vasc Biol 2004; 24: 192–196.
28. Sadakierska - Chudy A, Baczyńska D, Skóra J et al. Transfection efficiency and cytotoxicity of transfection reagents in human umbilical vein endothelial cells. Adv Clin Exp Med 2008; 17: 625–634.
29. Choksy S, Pockley AG, Wajeh YE et al. VEGF and VEGF receptor expression in human chronic critical limb ischaemia. Eur J Vasc Endovasc Surg 2004; 28: 660–669.
30. Rutherford RB, Becker GJ. Standards for evaluating and reporting the results of surgical and percutaneous therapy for peripheral arterial disease. Radiology 1991; 181: 277–281.
31. Takeshita S, Isshiki T, Mori H et al. Use of synchrotron radiation microangiography to assess development of small collateral arteries in a rat model of hindlimb ischemia. Circulation 1997; 95: 805–808.