Autologous Bone Marrow Stem Cell Transplantation in Patients with End-Stage Chronical Critical Limb Ischemia and Diabetic Foot

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Summary: A total 37 patients suffering from end stage-IV Fontaine (CLI and diabetic foot) with an ulcerated limb in whom all previous therapeutic strategies failed (e. g. surgical revascularization and endovascular repair) were selected and underwent local transplantation of Autologous Bone Marrow Stem Cells (ABMSCs). The efficacy/safety of this therapy was assessed by using several endpoints such as (a) prevention of amputation, (b) wound healing and (c) degree of angiogenesis. In order to assess the limb ischemia and hypoxia the several tests and measurements were performed pre- and post transplantation at a variety of time intervals. The measurements include: TP-toe pressure measurements (by Periflux 5000 Laser Doppler and Oxymetry system), SPP-skin perfusion pressure, ABI-ankle brachial index, LDP-Laser Doppler baseline and heat perfusion assessment, $TcpO_2$ without and with O_2 provocation inhalation test. In addition, a battery of biochemical and hematological tests of peripheral venous blood samples and bone marrow analysis were performed. Limb salvage was 81% in 30 patients, 7 patients (19%) were amputated for terminal severe ischemia and gangrene progression. In the group of limb salvage patients initial Toe pressure 23.119 (std. error 5.358) increased in 90 days follow-up into 29.888 (std. error 5.99), Toe brachial index increased from 0.1469 (std. 0.0326) to 0.1991(std. 0.401). In LASER doppler and $TcpO_2$, $TcpCO_2$ tissue perfusion examination $TcpO_2$% Increase after O_2 provocation inhalation test was elevated from 162.95 (%) to 229.86% which confirmed a very good tissue vasoreactivity after BMSC transplantation.

Key words: critical limb ischemia – diabetic foot – bon marrow stem cells – transplantation

Transplantace autologních kmenových buněk kostní dřeně u pacientů s chronickou kritickou končetinovou ischemií a diabetickou nohou

Souhrn: Soubor 37 pacientů v konečném stadiu chronické kritické končetinové ischemie st. IV dle Fonataina/CLI, s diabetickou nohou a defektem s vyčerpanou předchozí revaskularizační léčbou (e. g. chirurgickou revaskularizací a intervenční léčbou), byl po předchozím kompletním vyšetření léčen transplantací autologních kmenových buněk kostní dřeně. Efektivita a bezpečnost této léčby byla sledována a) prevencí amputace, b) zhojením defektu, c) stupněm angiogeneze a zlepšením tkáňové perfuze.Ke stanovení stupně ischemie a hypoxie byly provedeny tyto testy před a po provedení transplantace autologních kmenových buněk kostní dřeně. K vyšetření tkáňové perfuze byla provedena měření TP-prstového tlaku, SPP-kožního perfuzního tlaku (přístrojem Periflux 5000 Laser Doppler and Oxymetry System). Byly měřeny ABI- kotníkové tlaky a index, LDP-Laser dopplerometrické vyšetření v bazálním měření a po kožním zahřátí, $TcpO_2$ – transkutánní oxymetrie s inhalačními provokačními testy inhalací O_2. Zároveň byla zpracována kompletní baterie biochemických a laboratorních vyšetření periferní krve a kostní dřeně. Záchrana končetiny byla pozorována u 81 % pacientů (n = 30), končetina byla amputována u 7 pacientů (19 %) pro terminální těžkou ischemii, infekci a progresi gangrény. Ve skupině pacientů se záchranou končetiny iniciální prstový tlak (Toe pressure) 23,119 (std. error 5,358) zvýšil po 90 dnech sledování na 29,888 (std. error 5,99), (Toe brachial index) se zvýšil z 0,1469 (std. 0,0326) na 0,1991 (std. 0,401). V laser-dopplerometrickém vyšetření a $TcpO_2$, $TcpCO_2$ měření tkáňové perfuze došlo k nárůstu $TcpO_2$% z 162,95% na 229,86%, což potvrzuje velmi dobrou vasoreaktivitu po BMSC autologní transplantaci.

Klíčová slova: kritická končetinová ischemie – diabetická noha – kmenové buňky kostní dřeně – transplantace

Introduction

The local method of autologous bone marrow stem cells transplantation consists of harvesting bone marrow from the iliac crest, its separation using cell separator to generate ABMSCs (CD 34+) and their subsequent multiple injections into a limb suffering from chronic and critical ischemia. The aim of this therapy is to induce angiogenesis and vasculogenesis which would correct ischemia and hypoxia, improve tissue perfusion and metabolism, and thus prevent limb amputation. In general, such reparative process could be divided into the **initial phase** characterized by the release of inflammatory mediators and neurotransmitters followed by **phase of formation** of primitive vascular tubes composed of the endothelial cell precursors which are later on adhered to and stabilized by pericyte cells. Process of angiogenesis (by sprouting as well as nonsprouting) takes usually between 4–8 weeks depending on degree of the initial tissue ischemia and hypoxia.

Methodology and techniques

A total of 37 patients was recruited over the period of 8 month for this study.

Patient inclusion criteria: (a) Patients suffering from chronic and critical limb ischemia according to the TASC classification Rutherford 4–6, Fontaine IV, (b) with failed basic conservative and revascularization treatment (surgical or endovascular), (c) with age over 18 years, (d) with signed informed consent, (e) without acute CLI that would require early limb amputation.

Patient exclusion criteria: (a) Patients with estimated survival less than 6 months, (b) with known bone marrow diseases (e. g. lymphoma, leukemia, myelodysplastic syndrome, metastasis in the bone marrow), (c) with the final stage of renal failure and dialysed, (d) with acute stage of severe limb ischemia with severe inflammatory process affecting patient's life which require limb amputation to avert deterioration in clinical condition and death.

Separation of ABMSCs and their application: Concentrate of autologous stem cells (CD 34 positive) was separated from the bone marrow aspirate using the cell separator from Harvest Technologies. Bone marrow is collected from both iliac bone crests according to standard Yamshidi puncture aspiration methodology. During this procedure the patient is under analgosedation using propophol iv bolus and physiological signs including blood pressure (BP), pulse, ECG, pO_2 saturation are continuously monitored and adjusted according to needs. In brief, 240 ml of the bone marrow is aspirated into an aspiration set pre filed with ACD-A anticoagulant (30 ml) by hematologist and processed using the cell separator from Harvest Technologies. This equipment is easy to operate and has several advantages compared to other cell separators on the market. The methodology is (a) simple, easy, fast, and safe using gradient density centrifugation to separate all blood elements including white cells,

CD-34+(BMA) × Age

Nonparametric Correlations (CD34+, Platelets, Age)

Correlations

		Age	CD34+ BMA (10.9/l)	CD34+ BMC (10.9/l)
Spearman's rho				
Age	Correlation Coefficient		–,363*	–,262
	Sig. (2-tailed)		**,027**	,118
	N		37	37
CD34+ BMA (10.9/l)	Correlation Coefficient	–,363*		,829**
	Sig. (2-tailed)	**,027**		**,000**
	N	37		37
CD34+ BMC (10.9/l)	Correlation Coefficient	–,262	,829**	
	Sig. (2-tailed)	,118	**,000**	
	N	37	37	
Concentration CD34+	Correlation Coefficient	,071	–,301	,181
	Sig. (2-tailed)	,682	,074	,290
	N	36	36	36
Platelets BMA	Correlation Coefficient	–,120	–,101	–,040
	Sig. (2-tailed)	,485	,557	,816
	N	36	36	36
Platelets BMC	Correlation Coefficient	–,135	,042	,151
	Sig. (2-tailed)	,433	,809	,378
	N	36	36	36
Concentration Plateles	Correlation Coefficient	–,072	,383*	,449**
	Sig. (2-tailed)	,677	**,021**	**,006**
	N	36	36	36

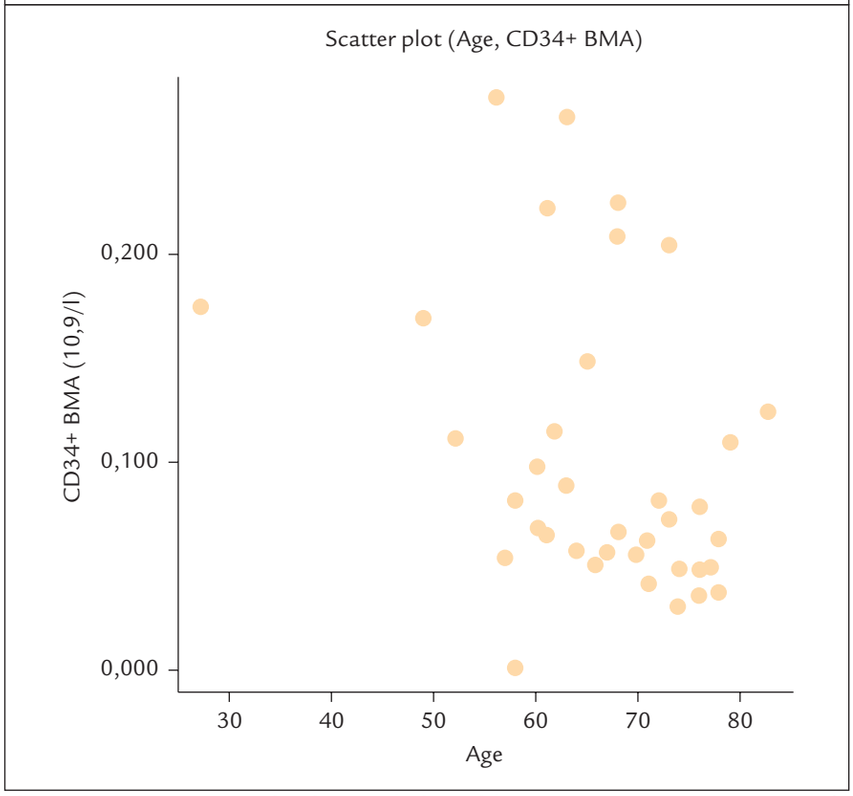

Fig. 1. CD34+ concentration in correlation with age. BMA – Bone Marrow Aspirate, BMC – Bone Marrow Concentrate.

Ratio Lympho/Neutrophiles BMC

Test Statistics[b,c]

			Ratio Lympho/Neu BMC
Chi-Square			9,739
df			3
Asymp. Sig.			**,021**
Monte Carlo	Sig.		**,013a**
Sig.	99% Confidence	Lower Bound	,010
	Interval	Upper Bound	,016

a. Bassed on 10000 sampled tables with starting seed 743671174.
b. Kruskal Wallis Test
c. Grouping Variable: Healing

Npar Tests

Kruskal-Wallis Test

Ranks

	Healing	N	Mean Rank
Ratio Lympho/ /Neu BMC	healed	10	24,60
	healing	16	16,38
	non healing	2	25,50
	amputation	7	10,14
	total	35	

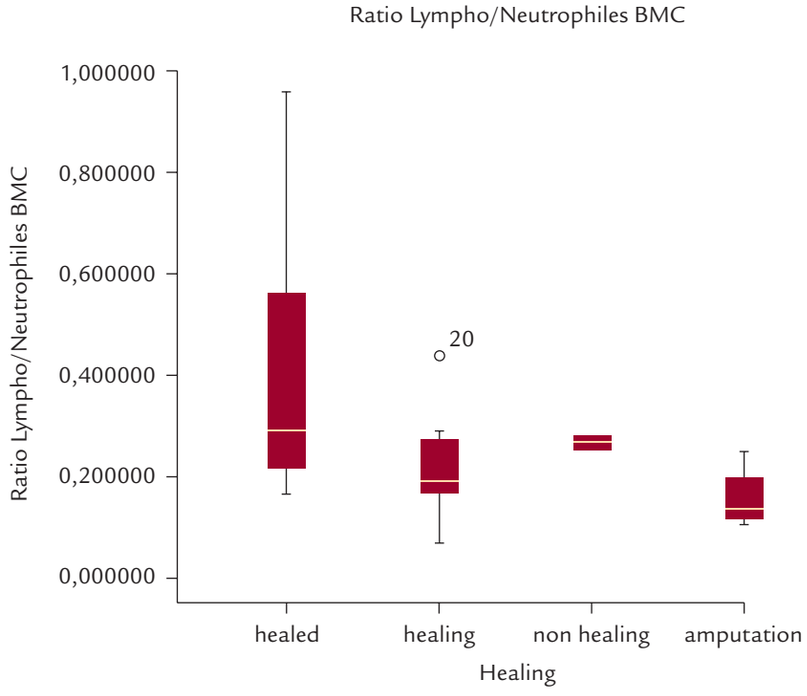

Fig. 2. Bone marrow lymphopenia and neutrophilia in Lympho/Neu ratio in amputation group of patients. BMC – Bone Marrow Concentrate.

platelets, red cells from collected bone marrow in centrifuge containers into a sample with 5% hematocrit (containing ABMSCs and platelets) within 15 min. Thereafter 40 ml of sample is aspirated into a syringe and immediately applied by multiple injections in the ischemic limb. Whole procedure should take less than one hour.

Monitoring quality of life and clinical effects of ABMSCs transplantation: Bone marrow was examined before and after separation of ABMSCs to assess number of CD 34+ progenitors as well as number of other cellular components including platelets. Prior- (0 days-baseline) and post transplantation (1, 7, 14, 30, 60, and 120, 180 days) all patients underwent following examinations: Laser Doppler Flowmetry – assessment of blood flow; measurements of transcutaneous oxygen ($TcpO_2$) and CO_2 ($TcpCO_2$) levels with and without provocation test (with O_2 inhalation). Complete battery of tests such as CBC and differential, fibrinogen and CPR was established. In addition, ABI-Ankle-Brachial Index, TP-Toe Pressure, SPP-Skin Perfusion Pressure, Rutherford and Fontaine Cathegory, analgetics consumption (requirements), claudication interval in non-amputated patients was monitored. These values were collected and then correlated with the degree of initial ischemia/hypoxia as well as with changes in ischemia/hypoxia developed within 90/120/180 postoperative days.

Results

A) After failed or impossible surgical and/or interventional revascularisation and after unsuccessful maximum conservative therapy, 37 patients mean age 66.32 (Min = 27 y; Max = 83 y) with impending major amputation due to severe critical limb ischemia had autologous BMSC transplanted into ischemic leg. Significant (0.027 before and 0.000 after concentration) correlation between Age and CD34+ counts

Ratio: Platelets/CD+34+ (BMA)

			Ratio Platelets/CD34+ BMA
N			35
Median			50777,77776
Chi-Square			8,200[a]
df			3
Asymp. Sig.			**,042**
Monte Carlo Sig.	Sig.		**,032[b]**
	99% Confidence Interval	Lower Bound	,027
		Upper Bound	,036

a. 5 cells (62,5%) have expected frequencies less than 5. The minimum expected cell frequency is 1,0

b. Bassed on 10000 sampled tables with starting seed 92208573.

c. Grouping Variable: Healing

Npar Tests

Kruskal-Wallis Test

Ranks

	Healing	N	Mean Rank
Ratio Platelets/ /CD34+ BMA	healed	10	18,60
	healing	16	14,81
	non healing	2	12,50
	amputation	7	26,00
	total	35	

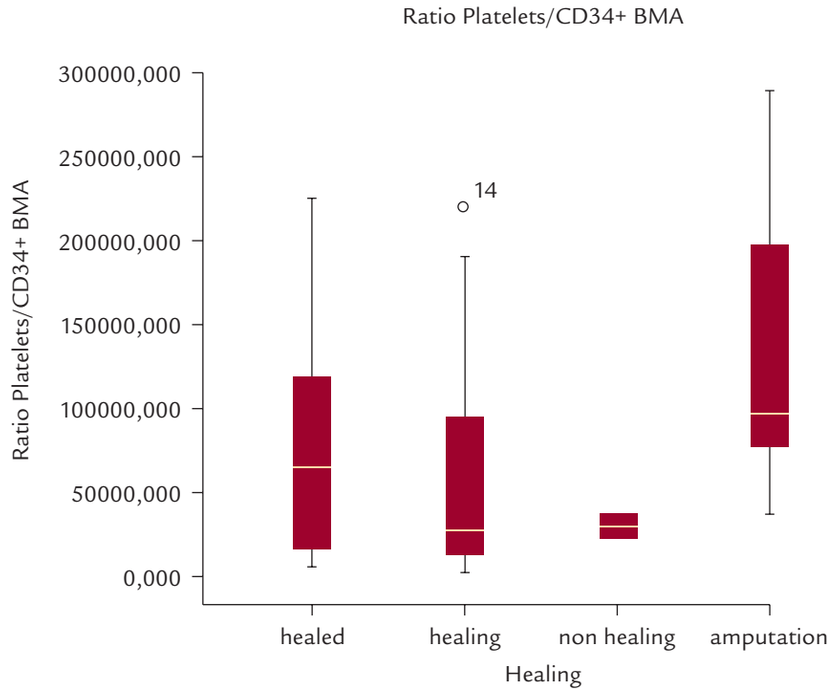

Fig. 3. Significant higher levels of Plateles/CD34+ ratio in amputation group. BMA – Bone Marrow Aspirate.

was observed, that corresponds to the stem cells aging (Fig. 1).

Mean concentration of CD34+ (5.17 × times) and Platelets (3.9 × times) after Harvest technologies separation show strong impact of gradient densit centrifugation for stem cells harvesting and concentrate prepare.

B) **Limb salvage was 81% in 30 patients, 7 patients (19%) were amputated** for terminal severe ischemia and gangrene progression. In the group of limb salvage patients initial Toe pressure 23.119 (std. error 5.358) increased in 90 days follow-up into 29.888 (std. error 5.99), **Toe brachial index** increased from 0.1469 (std. 0.0326) to 0.1991 (std. 0.401). In LASER doppler and $TcpO_2$, $TcpCO_2$ tissue perfusion examination with Periflux system (Perimed, Sweeden) **$TcpO_2$% Increase** after O_2 provocation inhalation test was elevated from 162.95 (%) to 229.86% which confirmed a very good tissue vasoreactivity after BMSC transplantation.

$TcpCO_2$% of Decrease in catabolism confirms the level of diminishing tissue catabolic status after O_2 inhalation provocation test. In the limb salvage group the initial level of 0.022% was diminished to –3.50% which confirmes better tissue metabolism after cell therapy and better vasoreactivity.

C) In the bone marrow significant elevation of Neutrophiles and Platelets with sever lymphopenia was confirmed by bone marrow cytoflowmetry analysis (Fig. 2).

Significant elevation of Neutrophiles and Platelets in bone marrow in the group of amputated patients may signalised VEGF, SDF-1 growth factor collection in neutrophiles and PDGF-BB in platelets, that might be reason of unstabile angiogenesis with unstabile primary endothelial progenitor cell tubules and worse pericytes cell stabilisation of primitive capilary network.

Platelets (1) Day 0 Peripheral Blood

Test Statistics[b,c]

			Platelets 1
Chi-Square			6,379
df			3
Asymp. Sig.			,095
Monte Carlo Sig.	Sig.		,079a
	99% Confidence Interval	Lower Bound	,072
		Upper Bound	,086

a. Bassed on 10000 sampled tables with starting seed 1993510611.
b. Kruskal Wallis Test
c. Grouping Variable: Healing

Npar Tests

Kruskal-Wallis Test

Ranks

	Healing	N	Mean Rank
Platelets 1	healed	10	15,65
	healing	17	16,29
	non healing	2	21,00
	amputation	7	27,21
	total	36	

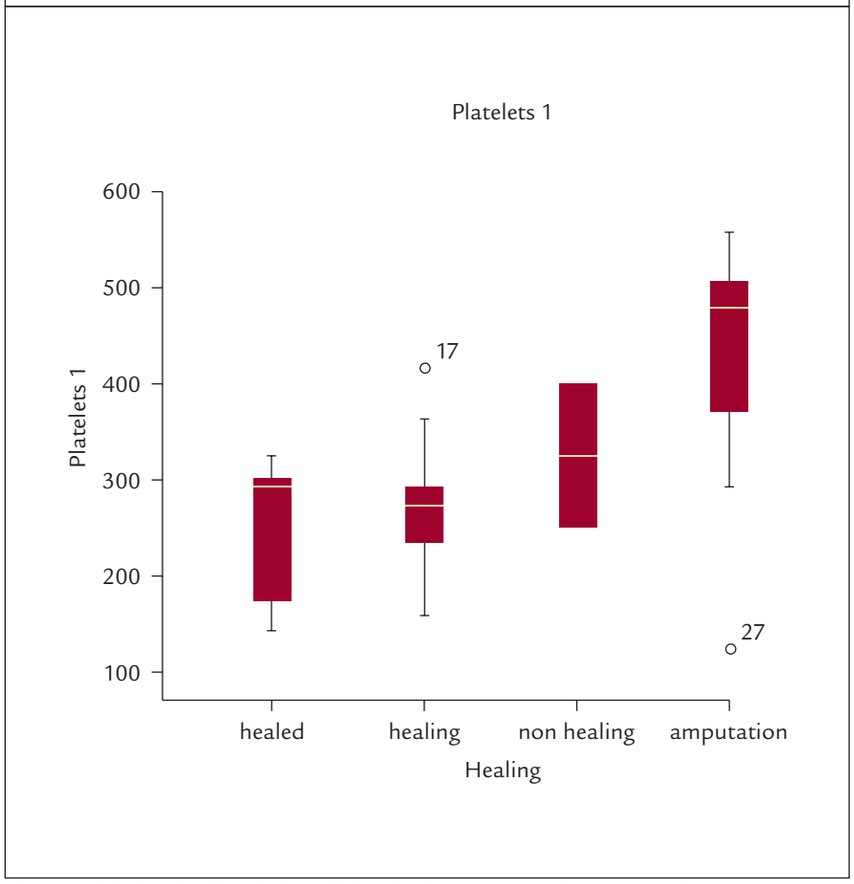

Fig. 4. High levels of peripheral blood plateles count in amputation group.

D) **In the amputated group** of patients significant higher levels of fibrinogen and platelets in the peripheral blood count, might correlate with peripheral vascular trombophilia and early thrombosis in unstable neovascularised capillaries, that in combination with bone marrow lymphopenia which correspondes with T-cell imunodeficiency and infection in the diabetic foot ulcers, leades into poor leg prognosis and finaly amputation (Fig. 4).

Conclusions

Local autologous BMAC administration for CLI patients with diabetic foot seems to be very promising technique for end-stage limb salvage. In our group of patients we were able to safe 80% of legs with significant improvement in the toe pressure, tissue perfusion and better metabolism of criticaly ill legs. Severe infection of diabetic foot ulcers might be in correlation with bone marrow lymphopenia. Nonhealing diabetic foots might be also in conjunction with growth factors diminishing, which are necessary for angiogenesis and are poolled in bone marrow neutrophiles and platelets, with consequent unstability of capillary angiogenesis.

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