

Safety and Efficacy of Repeated Bone Marrow Mononuclear Cell Therapy in Patients with Critical Limb Ischemia in a Pilot Randomized Controlled Trial

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Abstract

Background: Critical limb ischemia is a manifestation of peripheral arterial disease characterized by insufficient arterial blood flow for maintaining tissue viability in the lower extremities. Therapeutic angiogenesis is used for peripheral arterial disease patients who are not candidates for surgical revascularization or radiological intervention. There is accumulating evidence for the beneficial impact of autologous bone marrow mononuclear cell transplantation for treatment of critical limb ischemia in humans. This study aims to investigate the safety and efficacy of repeated bone marrow mononuclear cell injections in comparison with a single bone marrow mononuclear cell injection in critical limb ischemia patients.

Methods: Patients with critical limb ischemia (n = 22) were randomized (<http://clinicaltrials.gov/ct2/show/NCT01480414>) to receive either a single (n = 11) or four (n = 11) intramuscular injections of bone marrow mononuclear cells as a cell therapy product.

Results: There were no reported adverse events during the 24-week follow-up period after cell delivery. Efficacy assessment indicated that after cell injections, there was significant improvement in Ankle-Brachial Index, Visual Analog Scale, pain-free walking distance, and Wagner stage as well as reduction in ulcer size. There was no significant difference between the two groups in terms of clinical parameters. However, by the 24th week the pain-free walking distance improved significantly in the group who received four injections of cells.

Conclusion: Favorable clinical outcomes strongly indicate the long-term benefit of bone marrow mononuclear cell transplantation, either as one or several injections, for retrieval from critical limb ischemia. Repeated cell injections have shown increased improvement of pain-free walking distance in patients. These findings warrant further exploration in later-phase clinical trials with repeated injections.

Keywords: Bone marrow, cell therapy, critical limb ischemia, peripheral arterial disease, repeated injections

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Introduction

Critical limb ischemia (CLI) is a manifestation of peripheral arterial disease (PAD) characterized by insufficient arterial blood flow for maintaining tissue viability in the lower extremities.^{1,2} In patients with PAD, intermittent claudication (leg pain after walking) is the most common presenting symptom. However, some patients may be asymptomatic.³⁻⁵ As PAD progresses, leg pain at rest and/or ischemic ulcerations are hallmarks of ischemia.^{6,7} Patients with CLI have few treatment options. The mainstay treatments for PAD include open surgical techniques to remove the blockage, or bypassing it with vein or prosthetic graft, and endovascular treatments to reopen blocked arteries using a variety of catheter-based strategies such as balloon angioplasty, rotablation, and atherectomy, which either crush or remove the blockages.⁸ If these therapies fail or if patients are no longer can-

didates for these treatments, approximately 20% will die and 50%–90% undergo leg amputations either below or above the knee.^{1,9}

Therapeutic angiogenesis has been studied for treatment of patients with PAD who are not candidates for surgical revascularization or radiological intervention. Angiogenesis can be achieved using growth factors¹⁰ and angiogenic genes [11]. Recently, a growing body of reports have described the beneficial effects of autologous bone marrow (BM)- or peripheral blood-derived mononuclear cells (MNCs) for treatment of CLI in humans.¹²⁻²⁵

During the past decade, therapeutic angiogenesis using BM-MNCs has been shown to be an effective approach for treatment of patients who have moderate-to-severe PAD and no alternative treatment.^{7,26} It has been demonstrated that intramuscular (IM) injection of MNCs into critically ischemic legs increases perfusion with significant improvement in ankle-brachial indices (ABI), transcutaneous oxygen pressures, and rest pain.¹² We observed that most outcomes were achieved during 4 weeks after BM-MNC implantation.²⁷ However, it was not determined if repeated transplants of MNCs could improve the symptoms in CLI patients.

With the intent to find a practical and more efficient role for MNCs in CLI damage, the present study has been designed to evaluate the therapeutic potential of single or repeated administration of BM-MNCs in these patients.

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Materials and Methods

Patients

This study enrolled 22 patients with CLI whose symptoms included rest pain, non-healing ischemic ulcers, or both. Patients were not candidates for surgical revascularization and endovascular interventions due to lack of good run off and the presence of a patent artery for reperfusion in the limb, as diagnosed by an independent vascular surgeon. None of the patients had responded to standard conventional therapy for a total of 8 weeks and all were recommended to undergo major amputation. All patients read and signed a consent form before participating in the study.

The exclusion criteria were poorly controlled diabetes mellitus (presence of retinopathy or HgA1c >8%), malignancy in the last 5 years and a history of radiation or chemotherapy that affected the BM, heart failure (EF <30%), renal impairment (creatinine >2.5 mg%), previous organ transplantation, current serious infectious diseases, acute myocardial infarction, cerebrovascular accidents within 2 months, and life expectancy less than 12 months. Our group of patients had end stage clinical limb ischemia that was not candidate for revascularization or angioplastic surgery or other possible treatment. With this narrow inclusion criteria and rarity of this disease (Buerger's disease), we could not estimate a perfect sample size, but based on similar studies of peripheral blood derived mono-nuclear cells and whole bone marrow cells with sample sizes of 27, 29, 28, 16, 14, and 15 cases, we decided to enroll 22 patients (11 in each group) in our study.

The protocol of the study was approved by the Ethics Committees of Tehran University of Medical Sciences and Royan Institute. The trial was registered at NIH Clinical Trials with the identifier, NCT 01480414. Patients were randomized into two groups: 11 patients treated with one IM injection of BM-MNC (Group A) and 11 patients who received four IM injections (Group B) by block randomization method (using a block size of four). The person who directed the patients in the study, those who performed the procedures, and the person who performed the follow up and outcome assessments all wore masks.

Interventions

Approximately 150 mL of autologous BM was aspirated under spinal anesthesia from the iliac crest using a Jamshidi needle. The samples were collected into plastic bags that contained anticoagulant citrate phosphate dextrose adenine (CPDA) one day before the BM-MNC implantation. MNCs were purified by centrifugation under good manufacturing practice conditions by Ficoll-Hypaque (Lymphodex, Inno Train, H9L6114, Kronberg, Germany), which has a density separation within 90–120 minutes. After separation, the cells were washed twice with normal saline and then counted and assessed for viability using trypan blue. The cells were suspended in 50 mL of normal saline (which included 2% of the patient's own serum) and maintained at 4°C before the procedure.

The cells were injected IM into the calf and interosseous foot muscles of the ischemic leg, under spinal anesthesia, the day after BM aspiration. We implanted approximately 1 mL of BM-MNC suspension into each injection site using a 26-gauge injection needle (40 sites, 1.5 cm deep) with 3×3-cm grid intersects as injection site markers.

Each aspiration only had enough cells to prepare BM-MNC for two injections. Thus, before the third injection in group B, we had

to aspirate another sample of autologous BM from the iliac crest for the third and fourth injections. In group B, the injection was repeated every 3 weeks until the fourth injection. Then, the samples were frozen and stored.

Cell freezing protocol

The cellular freezing stage was very important and had to be performed quickly and accurately, so that the frozen cells could save their viability and ability to regrow. BM-MNCs were transferred into CryoMACS freezing bags (Miltenyi Biotec, Germany) and washed twice with normal saline and 2% human serum albumin (20% Octalbin, Switzerland). Then, supernatants were discarded and the cell pellets resuspended in a freshly prepared cold freezing media which contained 40% normal saline, 40% human serum albumin, and 20% DMSO (Sigma-Aldrich, USA). The cell suspensions were cryopreserved by a computer-assisted controlled-rate freezer (Planer, Kryo560, UK) in a slow manner from 4°C to -150°C over 90 minutes. At the end of the freezing program, the bags were stored at -196°C in a liquid nitrogen tank (MVE 1800 Series, USA) until transplantation.

Defreezing protocol of cells

The frozen cells were quickly thawed at 37°C in a water bath. The same volume of pre-warmed (22°C–37°C) RPMI supplemented by 1% L-glutamine (Invitrogen, USA) and 10% fetal bovine serum (FBS, PAA, Austria) was used. After centrifuging at 300 g for 10 minutes, the cell pellets were rinsed twice with normal saline and 2% HSA. Then, cell viability was determined with trypan blue. The desired volumes of normal saline for transplantation were adjusted (21, 22).

Flow cytometry analysis

Fluorescence-activated cell sorting (FACS) analysis for the expression of specific cell-surface markers by BM-MNC was performed for all samples using a flow cytometer device (FACS Calibur; Becton Dickinson, San Jose, CA, USA). The number of cells was adjusted to 1×10^5 – 2×10^5 cells/mL. Fc receptor blocking reagents were used for blocking non-specific antibody bindings (Miltenyi Biotec, Bergish Galdbach, Germany) according to the manufacturer's instructions. Then, the cells were stained for 30 minutes at 4°C with fluorochrome-labeled monoclonal antibodies (Supplementary Table 1). The controls were appropriately diluted isotype-matched antibodies (Supplementary Table 1). Data from 10000 events was stored. The list mode files were analyzed with WinMDI (version 2.9).

Clinical assessment

On the first admission before cell implantation, complete laboratory tests and serological profiles were obtained for all patients. The tests included complete blood count with differential, liver function test, renal function test, fasting blood glucose, HbA1C, urine analysis, erythrocyte sedimentation rate, C-reactive protein, lipid profile, and troponin T.

All patients underwent a standard vascular examination that included measurement of ABI determined with duplex ultrasonography for both the *dorsalis pedis* and the posterior tibial arteries (ABI is the systolic pressure at the ankle, divided by the systolic pressure at the arm). According to international standards, an increase of at least 0.1 is recognized as a significant improvement and a value >0.9 is regarded as normal.²⁸ To assess the pain-free

Table 1. Patients' baseline characteristics.

	Single injection (Group A)	Repeated injection (Group B)	P-value
Age (y)	53±8	49±7	.25
Sex (male)	11(100%)	11(100%)	
Disease (TAO)	9(81%)	8(72%)	.33
Previous treatment			
Bypass graft	4(36%)	3(27%)	.39
Sympathectomy	3(27%)	5(45%)	.14
Risk Factors			
Smoking	9(81%)	8(72%)	.33
Diabetes	2(18%)	3(27%)	.33
Hypertension	3(27%)	5(45%)	.14
Wagner Stage			
Stage I	2(18%)	2(18%)	
Stage II	2(18%)	1(9%)	
Stage III	3(27%)	1(9%)	
Stage IV	3(27%)	7(63%)	
Pain Relief Medication	6(54%)	10(90%)	
ABI-Baseline	0.52±0.29	0.48±0.20	.33
PFWD-Baseline	59±58	17±30	.12
VAS-Baseline	5.73±2.19	7.18±1.73	.60
Wagner-Baseline	1.27 ± 1.27	3.3 ± 1.25	.07

The data is presented as mean ± SD. TAO: Thromboangiitis obliterans

walking distance (PFWD), the patients underwent a validated progressive treadmill protocol with a reduced initial speed (1.6 km/h) in which they continued walking until they felt the claudication pain or another clinical indication for stopping the test (expressed in meters).

To quantify and evaluate the grade of resting pain, a Visual Analog Scale (VAS) was used as the standard. The patients assessed the severity of their resting pain by depicting a length from 0 to 10 cm, where 0 cm meant "pain free" or "no pain", and 10 cm meant "maximum excruciating pain". The limb condition of patients was assessed by the Rutherford classification for ischemic limbs.⁹ Ischemic ulcers were evaluated with the Wagner classification.²⁸ Angiographic assessment was performed with digital subtraction angiography for all patients before transplantation to exclude any option for open or endovascular revascularization. Clinical data was obtained 1 day before transplantation and at 4, 12, and 24 weeks after transplantation. Most patients did not consent to follow-up arteriographies.

Statistical analysis

All cellular and clinical data were presented as mean ± standard deviation (SD). All P-values were two-tailed and $P < 0.05$ was considered statistically significant. We used a general linear model with repeated-measure for analyzing the data change from baseline to weeks 4, 12, and 24. The Bonferroni-Holm correction was applied to control for type I error. The Bonferroni correction was used for the ABI, subjective pain index (VAS), PFWD and Wagner stage. All cellular and clinical statistical analyses were conducted by SPSS (version 20; SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics

The patients' demographic and clinical data are shown in Table 1. A total of 14 patients had severe pain at rest and difficulty ambulating and sleeping upon admission. Sixteen patients consumed pain relief medications due to their ischemia-related resting pain. The angiographic assessment showed infra-popliteal artery involvement in 18 (81%) patients. According to the Wagner classification,²⁸ one (5%) patient had no ulcer, 4 (18%) patients were Wagner stage I (superficial ulcer involving the full skin thickness but not underlying tissues), 3 (13%) were Wagner stage II (deep ulcer, which penetrates down into ligaments and muscles, without bone involvement or abscess formation), 4 (18%) were Wagner stage III (deep ulcer with cellulitis, abscess, or osteomyelitis) and 10 (45%) patients were Wagner stage IV (localized gangrene).

Cell analysis

The data of BM harvesting volume, cell viability and total MNCs is summarized in Table 2. The mean harvesting volume was 155 mL for Group A and 157 mL for Group B. For each patient, approximately 9×10^8 viable cells in normal saline, supplemented with 2% patient serum, were injected. BM-MNCs expressed angiogenic markers CD34, CD133, VEGFR, VWF, and TIE2. The analysis of BM-MNCs in both groups revealed no significant difference in the expression of angiogenesis markers.

Clinical outcomes

Evaluation of clinical parameters indicated that ABI, VAS, and PFWD improved significantly in both groups compared with

Table 2. Results of BM cell preparation.

Patient #	BM volume (mL)	Viability (%)	Total viable MNC×10 ⁸
Single injection (Group A)			
1	149	98	7.2
5	151	100	11.0
6	152	96	4.0
8	163	94	15.9
9	155	100	5.8
12	156	100	8.6
13	159	98	17.4
15	152	100	10.8
18	151	100	3.6
20	162	98	4.1
22	155	96	6.2
Mean ±SD	155.0±4.6	98.3±1.9	8.6±4.7
Repeated injection (Group B)			
2	162	97	7.5
3	157	94	10.1
4	156	94	6.8
7	148	97	11.6
10	169	88	9.41
11	165	93	4.4
14	158	99	11.5
16	153	100	11.8
17	150	96	11.1
19	153	100	13.1
21	156	100	4.5
Mean ± SD	157.0 ± 6.3	96.2 ± 3.7	9.3 ± 3.0



Figure 1. Limb salvage after BM-MNC implantation in two patients after repeated injections. Patient no.2 was a 49-year-old male diagnosed with TAO who presented with a non-healing ulcer that led to the amputation of his second finger. Patient no.19 was a 46-year-old male diagnosed with TAO with a non-healing ulcer that resulted in the amputation of his first finger due to gangrene. The pictures were taken five months after the first implantation. TAO: thromboangiitis obliterans.

baseline during 24 weeks of follow-up. Mean ulcer size decreased significantly from 9.81 ± 5.37 cm² before treatment (baseline) to 3.50 ± 4.06 cm² at week 24 ($P=0.00001$, Figure 1). Improvement of ulcers was defined as regression in both ulcer size and necrotic regions which was observed in 19 (90%) out of 21 patients (one patient had no ulcer before the treatment or during follow-up).

Complete healing of the ulcers was noted in 7 (33%) patients (2 from group A and 5 from group B).

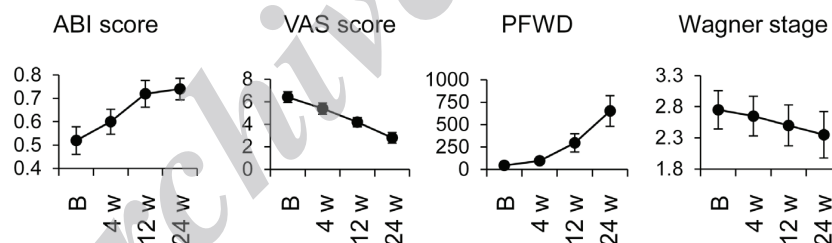
As seen in Table 3 and Figure 2, single and repeated injection groups showed significant improvements in all patients as well as each group at different times (4, 12, and 24 weeks of follow-up).

Additionally, comparison between single and repeated injec-

Table 3. Clinical parameters in two groups.

Test	Single injection (Group A)	P-value	Repeated injection (Group B)	P-value
ABI				
Baseline	0.53 ± 0.31		0.49 ± 0.21	
Wk 4	0.62 ± 0.24	.17	0.59 ± 0.24	.02
Wk 12	0.74 ± 0.21	.02	0.69 ± 0.29	.01
Wk 24	0.77 ± 0.17	.01	0.71 ± 0.23	.002
VAS				
Baseline	5.80 ± 2.3		7.00 ± 1.83	
Wk 4	5.20 ± 2.44	.17	5.60 ± 2.06	.003
Wk 12	4.20 ± 2.04	.01	4.20 ± 1.75	<.0001
Wk 24	2.90 ± 1.85	.001	2.70 ± 2.45	.001
PFWD				
Baseline	65.00 ± 57.90		18.90 ± 31.30	
Wk 4	109.00 ± 102.90	.1	76.10 ± 66.90	.01
Wk 12	181.00 ± 167.50	.03	422.80 ± 609.40	.08
Wk 24	298.00 ± 204.20	.003	1045.00 ± 926.80	.01
Wagner				
Baseline	2.20 ± 1.30		3.30 ± 1.25	
Wk 4	1.90 ± 1.20	.27	3.40 ± 1.26	.34
Wk 12	1.70 ± 0.95	.13	3.30 ± 1.49	.9
Wk 24	1.40 ± 1.26	.07	3.30 ± 1.49	.9
Ulcer Size				
Baseline	9.32 ± 5.59		10.32 ± 5.36	
Wk 24	4.00 ± 4.07	.001	3.00 ± 4.17	.004

A) All patients



B) One inj. —▲— Four inj. —■—

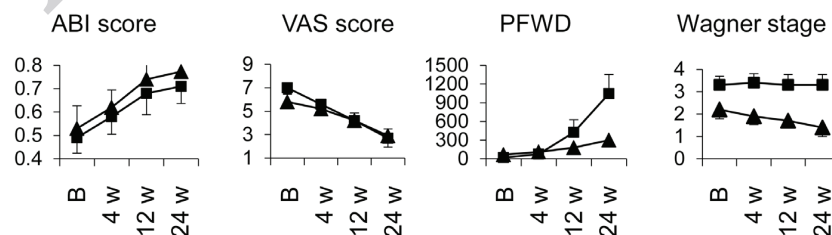


Figure 2. Changes in clinical parameters after treatment. Clinical parameters in all patients (A) and in single and repeated injections (B). ABI, VAS, and PFWD significantly improved at weeks 4, 12, and 24 compared to baseline levels (at least $P < .05$). Wagner stage showed improvement just after 24 weeks. There was no significant difference in the clinical parameters between two groups within week 4, 12 and 24. However, there was a significant difference in PFWD at week 24 between the two groups ($P = .001$). Improvement in VAS was achieved in 20 (91%) patients and PFWD in 16 (73%). Improvement in ABI was observed in 18 (82%) patients at 24 weeks follow up. ABI: Ankle-Brachial Index, B: baseline, PFWD: pain-free walking distance, VAS: Visual Analog Scale, w: week.

Table 4. Differences in clinical outcomes between two groups.

	Change From Baseline		Difference (95% CI)	P-value
	Single injection (Group A)	Repeated injection (Group B)		
ABI				
4 wk	0.05 ± 0.18	0.09 ± 0.10	-0.03(-0.17 to 0.09)	.58
12 wk	0.17 ± 0.26	0.18 ± 0.18	-0.01(-0.22 to 0.19)	.87
24 wk	0.24 ± 0.24	0.21 ± 0.16	0.02(-0.17 to 0.21)	.79
VAS				
4 wk	-0.18 ± 1.8	-1.45 ± 1.03	1.27(-0.05 to 2.59)	.06
12 wk	-1.09 ± 2.34	-2.54 ± 1.03	1.45(-0.16 to 3.06)	.07
24 wk	-2.9 ± 1.97	-4.6 ± 2.67	1.7(-0.5 to 3.91)	.12
PFWD				
4 wk	40.90 ± 73.02	56.50 ± 54.6	-15.6(-75 to 43.82)	.59
12 wk	105.45 ± 144.6	373.50 ± 589.2	-268.04(-651.08 to 114.99)	.15
24 wk	233.00 ± 177.95	1026.11 ± 934.9	-793.1(-1515 to -70.5)	.001
Wagner Stage				
4 wk	-0.18 ± 0.87	0.1 ± 0.31	-0.28 (-0.89 to 0.33)	.33
12 wk	-.036 ± 0.31	0.0 ± 0.47	-0.36 (-1.09 to 0.37)	.31
24 wk	-.08 ± 1.22	0.0 ± 0.47	-0.80 (-1.71 to 0.11)	.08
Ulcer Size				
24 wk	5.31 ± 3.96	-7.31 ± 6.41	2.00 (-2.74 to 6.74)	.38

tions demonstrated no significant differences between the groups in terms of monitored parameters (Table 4, Figure 2B). However, the PFWD values demonstrated a significant ($P = 0.001$) difference between the group which received repeated injections compared with single injection group at the 24th week [95% CI 793.1 m (70.5–1515)].

One patient from each group did not respond to implantation of BM-MNCs. Both underwent surgery for below-knee amputation (Table 5). Minor amputations were performed in 4 patients due to their previously localized gangrene (finger amputation in week 8, 2 fingers amputation in week 15, one finger amputation in week 21 and 2 finger amputation in week 26). In these patients, improvement in their ulcers was observed during follow-up.

Following the BM harvesting and MNC implantation, we observed no adverse events such as injection site infection or localized reaction in the patients. In addition, thromboembolic complications such as coronary or cerebrovascular events were not observed during the 24-week follow-up of 21 patients. Only one patient had myocardial infarction in the 23rd week of follow-up.

Discussion

Therapeutic angiogenesis has been studied for treatment of patients with PAD who are not candidates for surgical revascularization or other radiological interventions. Therapeutic angiogenesis using BM-MNCs has become a promising treatment for patients with moderate-to-severe PAD who have no other treatment options.^{7,12,26}

In this study, BM-MNC implantation, either once or four times, showed no adverse impact on the patients. During 24 weeks, PFWD improved in 16 (73%) patients and ABI by more than 0.1 in 18 (82%) patients. A decrease of VAS and pain at rest was achieved in 20 (90%) patients. Regression in ulcer size was observed in 19 (90%) out of 21 patients; however, one patient did

not have any evidence of ulcer. In 7 patients, the ulcers healed completely. There was no significant difference in the clinical parameters between the 4th, 12th, and 24th weeks of follow-up in the single and repeated injection groups. However, PFWD was notably better in the group that received repeated injections compared to the single injection group in the 24th week, which suggested that repeated injections of BM-MNC could be more effective than a single injection in CLI patients. In the present study, 17 (78%) patients had thromboangiitis obliterans (TAO) and 5 were diagnosed with diabetes mellitus. TAO is a non-atherosclerotic inflammatory disease that affects small and medium sized arteries and veins of the upper and lower extremities.²⁹ This disease occurs worldwide with the highest rate in Middle Eastern populations, including Iran. We have not observed any improvement in differences between patients who had TAO and diabetes mellitus. In this study, there were 22 male patients.

In 2 patients, the complications did not improve – rather, they became worse with decreased ABI and increased rest pain. Both underwent major amputations.

To date, the mechanisms by which BM-MNC may induce angiogenesis have not yet been completely elucidated. This may be attributed to an indirect paracrine impact from stem cell-mediated angiogenic factors, such as vascular endothelial growth factor, or a direct contribution of hematopoietic stem cells (HSC)-derived EPCs to new vessel formation.³⁰ Angiogenesis can result in the growth of new capillaries and pre-existing collateral vessels.³¹⁻³³ The CD34+ fraction in BM-MNCs reportedly synthesizes not only angiogenic growth factors like VEGF and basic fibroblast growth factor (bFGF), but also angiopoietin I, which plays a vital role in the maturation and maintenance of the vascular system, resulting in blood vessels.^{34,35}

According to several reports, there is a correlation between clinical efficacy and the total number of implanted MNCs²⁶ and CD34+ cells.¹⁴ However, in the current study, we observed no cor-

Table 5. Clinical and demographic characteristics of two patients who did not respond to MNC implantation.

	Patient No.9	Patient No.10
Treatment Group	Single injection (Group A)	Repeated injection (Group B)
Age (y)	47	52
Gender	Male	Male
Diagnosis	TAO	TAO
Time Of Amputation	4 mo after transplantation	6 mo after transplantation
Smoking during treatment	Yes	No
Diabetes	No	No
ABI		
Baseline	0.40	0.28
Wk 4	0.16	0.25
Last follow-up	0.15	0.25
VAS		
Baseline	5	4
Wk 4	9	2
Last follow-up	9	4
Wagner stage of ulcer		
Baseline	3	4
Wk 4	4	4
Last follow-up	4	4

relation between the number of implanted cells and the clinical outcome, which is in line with other studies.³⁶

In summary, these results confirm that the IM injection of BM-MNCs, either once or several times, is a safe and efficient treatment for patients with CLI. However, more research is needed to develop a clearer understanding of the causal processes underlying such undesired outcomes after MNC therapy.

Conflict of interest: none.

Author contributions

BM designed the study, screened patients for enrollment in study, performed the bone marrow cell aspiration, performed the cell transplantation, followed up the patients after cell transplantation, monitored the clinical evaluation, data collection and edited the article.

MRZ participate in designing the study, enrollment of patients in study and edited the article.

EA participate in designing the study, followed up the patients after cell transplantation, performed image analysis, data collection and statistical analysis and prepared the primary draft of the article.

SEH performed cell separation for transplantation.

HM performed the bone marrow aspiration, performed cell transplantation, followed up the patients after cell transplantation, monitored the clinical evaluation and motor function assessment.

LA followed up the patients after cell transplantation.

HB designed the study, and was the principal editor of the article.

NA designed the study, oversaw data acquisition and analysis, editor of the article.

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Supplement Table 1. Flow cytometry analysis.

Patients	CD45	CD34	CD3	CD19	CD14	CD16	CD133	CD90	CD31	CD33	CD44	CD38	CD29	CD73	CD105	VEGFR
Group A(1 Injection)																
1	10.58	0.06	10.86	0.4	1.82	1.42	1.55	5.55	13.7	10.79	64.33	9.86	62.2	0.21	7.3	11.28
5	68.32	0.05	35.65	6.55	2.77	5.87	1.06	5.87	79.32	68.43	76.3	48.5	45.54	0.32	32.92	41.3
6	20.93	0.32	8.95	2.43	0.76	0.05	0.98	67.39	54.66	45.65	34.78	12.04	27.8	4.91	9.02	8.14
8	11.22	0.09	61.54	0.45	45.45	0.98	0.34	34.88	10.22	22.43	87.66	76.92	93.43	7.92	45.27	83.27
9	34.54	0.54	14.2	9.93	3.99	3.87	0.45	23.13	88.11	0.54	89.1	52.47	76.01	0.28	1.69	79.6
12	2.35	0.73	12.75	2.54	0.34	27.66	0.07	16.72	34.62	3.91	78.55	81.56	65.3	0.59	13.71	38.81
13	45.43	0.65	39.87	0.34	18.23	26	0.88	44.34	36.3	73.23	63.33	32.05	91.84	0.16	24.67	5.02
15	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
18	12.87	0.03	2.34	0.96	1.88	2.66	0.04	76.6	2.32	4.7	94.39	57.93	52.23	17.48	4.03	67.36
20	13.08	0.19	11.03	18.22	3.43	22.98	0.31	3.05	6.56	32.54	84.28	0.23	86.29	5.36	83.67	27.05
22	12.89	0.23	9.85	10.33	0.07	0.04	0.08	25.65	56.21	1.32	79.06	7.06	39.62	0.74	51.45	26.37
Mean±SD	23.22 ± 20.32	0.29 ± 0.26	20.70 ± 18.70	5.21 ± 5.98	7.87 ± 14.22	9.15 ± 11.50	0.57 ± 0.51	30.31 ± 25.70	38.20 ± 30.66	26.35 ± 27.69	75.17 ± 17.38	37.86 ± 29.84	64.02 ± 22.78	3.79 ± 5.55	27.37 ± 26.32	38.82 ± 29.05
Group B (repeated Injections)																
2	31.38	0.36	58.75	7.65	0.2	5.36	0.54	18.71	64.74	7.34	99.4	79.02	61.58	6.26	42.66	40.66
3	76.13	0.05	55.34	10.82	47.79	0.02	0.06	33.21	53.95	66.31	93.07	52.94	92.54	4.44	8	64.32
4	65.69	0.71	47.18	17.38	1.6	0.74	0.88	46.5	75.25	68.26	88.5	58.92	89.08	8.46	23.46	26.04
7	37.46	0.75	41.91	5.39	17.98	0.06	0.25	3.25	49.76	3.67	58.62	50.92	74.77	1.46	28.28	63.34
10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
11	32.32	0.05	20.48	4.17	1.74	0.08	0.17	47.42	10.64	0.08	50.02	8.36	39.07	1.95	10.42	19.97
14	1.48	0.03	7.29	0.96	15.3	23.18	0.06	66.71	5.2	9.34	90.5	37.63	42.74	0.63	42.08	5.11
16	56.73	0.76	12.2	2.96	56.53	0.84	0.21	78.32	84.16	77.11	79.57	84.01	91.37	4.22	12.83	80.24
17	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
19	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
21	10.64	0.03	8.81	0.94	3.97	0.84	1.2	2.17	2.56	3.38	90.02	6.66	51.1	0.13	1.72	3.65
Mean±SD	38.97 ± 25.97	0.34 ± 0.35	31.49 ± 21.58	6.28 ± 5.59	18.13 ± 22.12	3.89 ± 7.99	0.42 ± 0.41	37.03 ± 28.00	43.28 ± 32.69	29.43 ± 34.30	81.21 ± 17.62	47.30 ± 28.77	67.78 ± 22.16	3.44 ± 2.92	21.18 ± 15.54	37.91 ± 28.92
P-value	.16	.71	.27	.7	.25	.29	.5	.6	.73	.83	.47	.5	.73	.87	.56	.94