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Original Article

Autologous angiogenic therapy with cultured mesenchymal stromal cells in platelet-rich plasma for critical limb ischemia

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ABSTRACT

Introduction: The prevalence of diabetes mellitus is increasing globally, including in Japan. Patients with diabetes often experience microangiopathy and macroangiopathy, which lead to difficult-to-treat foot ulcers and diabetic gangrene. Conventional cellular therapies have limited safety and are invasive. In this study, we investigated the use of cultured autologous mesenchymal stromal cells derived from the bone marrow and grown in platelet-rich plasma as a potential treatment for diabetic complications.

Methods: A prospective clinical trial was conducted to assess safety as the primary endpoint and efficacy as the secondary endpoint of the aforementioned therapy in five patients with critical limb ischemia, with or without hemodialysis.

Results: Five patients with critical limb ischemia were enrolled between 2016 and 2019, three of whom underwent hemodialysis. Platelet-rich plasma was obtained from 288 ± 39.6 mL of blood/patient, yielding 31.6 ± 1.67 mL of platelet-rich plasma. Bone marrow aspiration yielded 18.4 ± 4.77 mL/patient, and 4.64 ± 1.51 × 10^7 cells were incubated for 16 ± 2.8 days to obtain 3.26 ± 0.33 × 10^7 mesenchymal stromal cells. Although several adverse events were observed, none were directly attributed to cell therapy. Clinical severity, as assessed by both the Fontaine stage and Rutherford category, improved significantly following therapy. This improvement was accompanied by enhancements in the 6-min walking distance, dorsal skin perfusion pressure, ankle transcutaneous partial oxygen pressure, and ankle brachial pressure index.

Conclusion: Autologous angiogenic therapy with cultured mesenchymal stromal cells derived from the bone marrow and grown in platelet-rich plasma is a safe and feasible, and was expected as a potential treatment for critical limb ischemia.

1. Introduction

The number of patients with diabetes is increasing not only in Japan but also worldwide [1,2]. Many of them suffer from complications such as microangiopathy and macroangiopathy, followed by intractable foot ulcers and diabetic gangrene. Many patients experience claudication due to ischemia caused by arteriosclerosis of the lower limb arteries.

In this study, we focused on cultured bone marrow (BM)-derived mesenchymal stromal cells (MSCs) grown in platelet-rich plasma (PRP) because they are safer as fetal bovine serum (FBS) is

Abbreviations: MSC, Mesenchymal stromal cell; PRP, Platelet-rich plasma; FBS, Fetal bovine serum; BM, Bone marrow; MNC, Mononuclear cell; CLI, Critical limb ischemia; HD, Hemodialysis; ASO, Arteriosclerosis obliterans; TAO, Thromboangiitis obliterans; ARB, Angiotensin II receptor blocker; ABPI, Ankle brachial pressure index; SPP, Skin perfusion pressure; $TcPO_2$, Transcutaneous partial oxygen pressure; CABG, Coronary artery bypass grafting; AFS, Amputation-free survival.

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not used in this therapy and are less invasive as the amount of BM required is quite less and longtime general anesthesia [3] is not needed in the clinical approach. In therapeutic angiogenic therapy using BM-derived mononuclear cells (MNCs), a regenerative therapy performed presently, 500 mL of BM is aspirated from the ileum under general anesthesia, separated, purified, and then administered to the patient [3]. The time under general anesthesia, from aspiration of BM to administration of cells, is over 3 h. Administration of CD-34-positive cells for therapeutic angiogenic therapy requires granulocyte-colony stimulating factor (G-CSF), which may induce side-effects, such as interstitial pneumonia and apheresis, with risks, such as bleeding and infection [4]. Here, we have two problems: one is how to obtain a large number of cells required for injection [5,6]. Aspiration of 400–800 mL of BM under general anesthesia is required. However, this approach is not always applicable to all patients. Another method for culturing human MSCs requires FBS. We believe that PRP is safer than FBS. We previously assessed the safety of BM-derived MSCs grown in PRP in an experimental model [7].

Previous clinical studies have reported that the implantation of BM-derived MNCs or MSCs results in improved symptoms and healing of ulcers in patients with critical limb ischemia (CLI) up to stage IV of Fontaine's classification [5,6,8]. However, a meta-analysis revealed no advantages of stem cell therapy in the primary outcome measures of amputation, survival, and amputation-free survival (AFS) in patients with CLI [9]. The issues mentioned remain unresolved. We believe that one of the most important endpoints of therapeutic angiogenesis in patient with CLI is the AFS rate. In this prospective clinical study, we evaluated safety as the primary endpoint and efficacy as the secondary endpoint of intramuscular injection of cultured autologous BM-MSCs grown in their own platelet-rich plasma (PRP) in patients with CLI.

Guidelines for identifying severity and providing appropriate treatment have been updated over the years. Treatment decisions are based on the guidelines recommended by the Transatlantic Intersociety Consensus II (TASC II) established in 2007 [10]. Options that are currently available include risk modification techniques, exercise, pain and ulcer management, and revascularization interventions performed via endovascular or bypass surgical approaches [11,12]. Without proper treatment, patients diagnosed with CLI face a risk of major amputation. The high limb amputation rate (30% annually) after undergoing the best therapy available and poor mortality (25% annually) are common issues for patients with CLI [10]. Recent reports have shown that mortality rate of patients with CLI was 20% within six months after diagnosis and over 50% at five years. Whereas non-revascularized patients, patients with so-called no-option CLI, have been reported to show one-year mortality rate of 20%–40% [13,14]. Advances in surgical and medical interventions have improved post-treatment AFS [15]. However, the one-year mortality rate for non-revascularized CLI cases remains 49%, with infection being the most common cause, followed by cardiovascular disease [16]. In the RENDEZVOUS study reported from Japan, endovascular Treatment (EVT) for lesions below the ankle did not improve AFS [17]. In other words, despite advances in treatment, a certain number of no-option patients whose AFS is still poor remains. This scenario encouraged us to establish a new approach against CLI.

2. Methods

2.1. Study design

This study was designed to prospectively evaluate the safety, feasibility, and efficacy of autologous BM-derived MSCs grown in PRP in five patients with CLI with or without hemodialysis (HD).

2.2. Criteria for enrollment

The ethics committee of the National Center for Global Health and Medicine Hospital (NCGM; NCGM-G-001596-07) and the Evaluation Committee on Regenerative Medicine of Tokyo Medical and Dental University (RM27-003) have approved this study, and informed consent was obtained from all patients.

Eligible subjects fulfilled the following inclusion criteria: (a) presented with arteriosclerosis obliterans (ASO) or thromboangiitis obliterans (TAO), (b) presented with CLI with a Fontaine category of 3–4, (c) presented with CLI with a Rutherford category of 4–6, (d) failure of or no indication for transluminal angioplasty/stenting and bypass surgery, (e) men or women aged 20–80 years, and (f) provided written informed consent. The exclusion criteria were as follows: (a) malignancy or history of malignancy within the past 5 years and (b) diabetic retinopathy (new Fukuda Classification: B II-B V). Subjects aged 81 years were allowed to enroll if the case enrollment committee confirmed them appropriate to participate in this trial.

2.3. Preparation of PRP

PRP was prepared following the procedures previously described by Hamada et al. with minor modifications [18]. Peripheral venous blood (200 mL) was drawn from each patient into bags (KBS-200CA8L; Kawasumi) containing an acid citrate dextrose solution anticoagulant. Aliquots (50 mL blood; Falcon™ 50 mL High Clarity PP Centrifuge Tube, Conical Bottom; Corning Inc.) were centrifuged for 10 min at 200×*g* at room temperature (15 °C-30 °C). Yellow plasma (buffy coat with platelets) was separated from the other components and centrifuged again for 20 min at 1500×*g* at room temperature. The upper platelet-poor plasma layer was then separated and discarded. The remaining PRP layer was then divided into 500 μL aliquots (Nalgene™ General Long-Term Storage Cryogenic Tubes; Thermo Fisher Scientific) and stored at −30 °C until use. Each aliquot was defrosted at room temperature for 1 h. Thereafter, it was centrifuged for 10 min at 2500×*g* (Falcon™ 15 mL High Clarity PP Centrifuge Tube, Conical Bottom; Corning Inc.) at room temperature before being added to the medium.

2.4. Culture of BM-MSC

The BM (10–20 mL) was aspirated from the iliac bones of the patient under local anesthesia using disposable Illinois BM needles (DIN1515X; CareFusion). Equal volumes of phosphate-buffered saline (PBS; Thermo Fisher Scientific, Japan) were added to the BM aspirate and overlaid onto 10 mL of Ficoll (Ficoll™-Paque Premium; Cytiva, UK). After centrifugation for 20 min at 500×*g* (Falcon™ 50 mL High Clarity PP Centrifuge Tube, Conical Bottom; Corning Inc.), the buffy coat was separated. The cells were then washed twice with PBS and suspended in 15 mL α-MEM (Gibco, Life Technologies) containing 20 μg/mL of gentamicin (MSD KK, Japan) and 2 IU/mL of heparin-supplemented PRP. More than 3×10^7 cells were disseminated in the T75 flask (Falcon™ 75 cm^2 Rectangular Canted Neck Cell Culture Flask with tissue culture-treated surface; Corning Inc.) with media containing 10% of their own PRP and incubated at 37 °C in 5% CO_2. The medium was changed four days later to remove floating cells. When the cells became subconfluent seven days later, they were detached using TrypLE Select Enzyme (Gibco, Life Technologies), suspended in 35 mL medium, and placed in a T175 flask (Falcon™ 175 cm^2 Rectangular Straight Neck Cell Culture Flask with tissue culture-treated surface; Corning Inc.). After approximately 11 d, the cells were passaged and counted. The shipping standard value of the cell number in our protocol was 1×10^7 to

1×10^8. Some cells were used for sterility, endotoxin, and mycoplasma tests at passage 2.

The cultured cells were confirmed to be MSCs by applying FACS analysis and Chromosomal analysis through G-band test after culturing the BM of three healthy volunteers in the same manner as described in a previous report [7]. In this study, the cultured cells that were spindle-shaped and exhibited adhesiveness were determined to be MSCs.

2.5. Sterility test

Ten milliliters of each culture supernatant were sent to BML, Inc. Japan, to culture.

2.6. Endotoxin test

Ten milliliters of each culture supernatant were sent to BML, Inc. Japan, to measure the endotoxin concentration.

2.7. Mycoplasma test

Ten milliliters of each culture medium containing approximately 1×10^5 cells were sent to BML, Inc. Japan, to detect Mycoplasma DNA through real time PCR.

2.8. BM-MSC transplantation

Cell transplantation was performed under general anesthesia. After sanitization with povidone iodine, all shipped cells dissolved in 25 mL physiological saline were intramuscularly administered at 50 evenly distributed sites (0.5 mL/site) on the leg with more severe ischemia in each patient. The points and muscle tissue-depths of all administration points from the knee joint to the ankle joint were selected under ultrasound guidance.

2.9. Endpoints

The primary endpoint was safety at 12 months after cell therapy. Safety was evaluated on the basis of adverse events, the severity of which were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (version 4.0).

The secondary endpoint was efficacy, which included the following parameters: (a) amputation-free 12 months after cell therapy; (b) rate of improvement from CLI to non-CLI stage assessed according to the Fontaine stage and Rutherford category six months after cell therapy; (c) death due to CLI 12 months after cell therapy; (d) all-cause death 12 months after cell therapy; (e) changes in ulcer size 12 months after cell therapy, pain-free walking distance in 6-min walking test six months after cell therapy, and physiological tests including ankle brachial pressure index (ABPI) (BP203RPEIII; Omron Colin, San Antonio, TX, USA) six months after cell therapy, skin perfusion pressure (SPP) (PAD4000; Kaneka, Tokyo, Japan) six months after cell therapy, and transcutaneous partial oxygen pressure ($TcPO_2$) (PO-850; Sumitomo Electric System Solutions, Tokyo, Japan)six months after cell therapy.

2.10. Data management and statistical analysis

The data were managed at an independent data center for the NCGM. Following the data input, data cleaning and logic checks were performed to guarantee data quality.

Categorical and continuous data are presented as numbers (percentage) and means (±SD), respectively. Amputation-free and all-cause survival rates were calculated using the Kaplan–Meier method. All analyses were performed using the SAS software (version 9.4; SAS Institute, Cary, NC, USA).

3. Results

3.1. Patients

Five patients with CLI were enrolled between April 2016 and June 2019. All patients were men; four had ASO and diabetes mellitus, one had TAO, and three received HD. The cause of renal failure was diabetic kidney disease in three patients (Table 1).

Two patients with Rutherford category 4 (Fontaine stage 3) and three patients with Rutherford category 5–6 (Fontaine stage 4) underwent cell therapy (Table 2). Cell therapy was performed in the left leg in three patients and in the right leg in two patients. Three patients had ulcerative lesions ranging in size from 17 to 71 mm. The 6-min walking distance ranged from 0 to 418 m. Four patients with ASO had diabetes mellitus.

3.2. PRP and cell culture

The blood (288 ± 39.6 mL) for PRP was collected from each patient, and 31.6 ± 1.67 mL PRP was obtained from each sample, as shown in Table 2.

The BM (18.4 ± 4.77 mL) was aspirated from each patient. After centrifugation and wash, $4.64 \pm 1.51 \times 10^7$ cells were disseminated on the flask and incubated for 16 ± 2.8 d to obtain $3.26 \pm 0.33 \times 10^7$ MSCs.

Sterility, Endotoxin, and Mycoplasma tests at shipping reported all negative results.

Table 1
Baseline characteristics.

Characteristic	Value
Age (year)	67.4 ± 14.5
Male/female (*n*)	5/0
ASO/TAO (*n*)	4/1
Underlying disease, *n* (%)	
Diabetic kidney disease	4 (80.0)
Hemodialysis	3 (60.0)
Comorbidity, *n* (%)	
Ischemic heart disease	4 (80.0)
Stroke	1 (20.0)
Hypertension	5 (100)
Diabetes	4 (80.0)
Dyslipidemia	5 (100)
Smoking habit, *n* (%)	
No	0 (0)
Ex	5 (100)
Laboratory variables	
Aspartate transaminase (U/l)	24.4 ± 17.6
Alanine transaminase (U/l)	42.8 ± 44.7
γ-glutamyl transpeptidase (U/l)	46.2 ± 54.2
Blood urea nitrogen (mg/dl)	29.2 ± 15.0
Creatinine (mg/dl)	3.95 ± 2.54
Blood sugar (mg/dl)	129 ± 34.6
C-reactive protein (mg/dl)	0.474 ± 0.530
White blood cell ($\times 10^3/\mu l$)	5.82 ± 1.65
Hemoglobin (g/dl)	12.0 ± 3.33
Platelet ($\times 10^4/\mu l$)	21.3 ± 4.31
Medication, *n* (%)	
Aspirin	5 (100)
Clopidogrel	1 (20.0)
Serotonin 5HT2 antagonist	0 (0)
Cilostazol	5 (100)
Prostanoid	0 (0)
Statin	5 (100)
ARB	3 (60.0)

Table 2
Information about CLI, blood and cell.

Parameters	Case 1	Case 2	Case 3	Case 4	Case 5	Value
	Rt	Lt	Lt	Lt	Rt	
Clinical severity						
Fontaine stage	4	4	3	4	3	
Rutherford category	6	5	4	5	4	
Visual analog scale	9	6	4	8	9	
Ulcer size (mm)	71	24	0	17	0	
6 min' walking distance (m)						
Absolute claudication distance	0	77	418	180	358	207 ± 179
Diabetes	Yes	Yes	Yes	Yes	No	
PRP and cell						
Blood for PRP						
Total blood (ml)	220	290	310	300	320	288 ± 39.6
Serum (ml)	156	160	140	165	140	152 ± 11.6
PRP (ml)	30	30	32	34	32	31.6 ± 1.67
Cell product						
Bone marrow (ml)	10	20	20	20	22	18.4 ± 4.77
Initial cell (x10^7 counts)	2.9	6.2	6.2	4.3	3.6	4.64 ± 1.51
Cell at scale up (x10^6 counts)	0.6	5.1	9.7	6	4.1	5.10 ± 3.29
Product MSC (x10^7 counts)	3.4	2.8	3.2	3.7	3.2	3.26 ± 0.33
Culture period (days)	18	18	18	12	14	16 ± 2.8
Culture period from the scale up (days)	9	7	8	4	6	6.8 ± 1.9
Product quality						
Sterility test at shipping	Negative	Negative	Negative	Negative	Negative	
Endotoxin test at shipping (EU/ml)	<0.001	<0.001	<0.001	<0.001	<0.001	
Mycoplasma test at shipping	Negative	Negative	Negative	Negative	Negative	

3.3. Safety evaluation

Mean operation time to administer cells under general anesthesia was 10.8 min (8, 9, 11, 13, and 13 min). No perioperative complications were observed.

Adverse events during the 12-month follow-up period after cell therapy are listed in Table 3. Death (case 2; 152 days after cell therapy) due to the physical and mental weakness after coronary artery bypass grafting (CABG) (62 days after cell therapy) owing to the progression of angina pectoris; and below-knee limb amputation (case 4; 185 days after cell therapy) to avoid general infection, colon diverticulitis, and pneumonia were the serious adverse events requiring in-hospital treatment. However, none of these events were thought to be directly related to cell therapy, as approved by the ethics committee of the NCGM Hospital and the Evaluation Committee on Regenerative Medicine of Tokyo Medical and Dental University.

3.4. Efficacy evaluation

The 1-year AFS and all-cause survival rates were 60.0% and 80.0%, respectively (Fig. 1). The improvement rate from CLI stage to non-CLI stage 1 year after cell therapy was 60.0% (Fig. 2; three of five patients). The category of clinical severity on both Fontaine stage and Rutherford category significantly improved following cell therapy.

All three ulcers showed a reduction in size after cell therapy, and the largest ulcer (maximum diameter, 71 mm) completely healed at 12 months (Fig. 3). One of the three patients with ulcer had a major amputation at 12 months, and another died 6 months after cell therapy. These were not thought to be directly related to cell therapy. Case 1 with diabetes mellitus who needed HD suffered from MRSA bacteremia and osteomyelitis in metatarsal bone. Case 2 died after CABG, case 4 underwent major amputation, and their ulcers showed shrinkage in size.

Table 3
Adverse events during 12 months' follow-up period after cell transplantation.

Adverse event	Number of events
Serious adverse event	
Cardiovascular	
Angina	1 (Case 2)
CABG (Coronary artery bypass grafting)	1 (Case 2)
Death	1 (Case 2)
Gastrointestinal	
Colon diverticulitis	1
Infectious	
Pneumonia	1
Peripheral arterial	
Limb amputation	1 (Case 4)
Nonserious adverse event	
Blood	
Thrombocytopenia	1

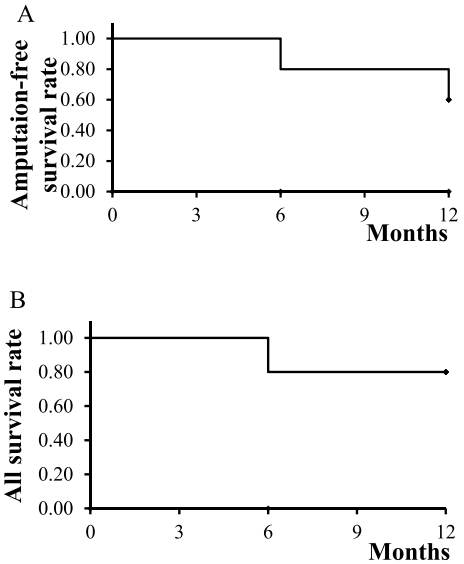


Fig. 1. Amputation-free and all-cause survival rates. (A): Amputation-free survival at one year was 60.0%. (B): All-cause survival rate was 80.0%.

Fontaine classification

	Pre-treatment	1 month	2 months	6 months	12 months
Case 1	IV	IV	IV	IV	II
Case 2	IV	IV	IV		
Case 3	III	III	II	II	II
Case 4	IV	IV	IV	IV	
Case 5	III	III	II	II	I

CLI
Non-CLI

Rutherford classification

	Pre-treatment	1 month	2 months	6 months	12 months
Case 1	6	6	6	5	2
Case 2	5	5	5		
Case 3	4	4	3	3	3
Case 4	5	5	5	5	
Case 5	4	4	3	2	2

CLI
Non-CLI

Fig. 2. Changes in clinical severity. Fontaine stage and Rutherford category. The improvement rate from CLI stage to non-CLI stage at one year after cell transplantation was 60.0%.

Change in clinical severity was partially accompanied by a change in 6-min walking distance, dorsal SPP and ankle $TcPO_2$. The 6-min walking distance was increased in three patients at six months, but not in the remaining two patients, dorsal SPP and ankle $TcPO_2$ was found to be increased in three patients and was not evaluated in two patients. These changes were not statistically significant due to the absence of data (Fig. 3).

4. Discussion

We demonstrated that angiogenic therapy with cultured autologous BM-derived MSCs grown in PRP can be prospectively evaluated for safety and feasibility, and can be a potential treatment for CLI. To the best of our knowledge, this is the first report to evaluate the safety and relative efficacy of angiogenic therapy prospectively with cultured autologous BM-derived MSCs grown in PRP in patients with CLI.

Several therapeutic options include genetic medicine, cell transplantation with G-CSF [4,19], and aspiration of 500 mL BM under general anesthesia [3], and there may be potential or physical risks. In our procedure, autologous 10–20 mL MSCs were incubated with autologous PRP and mean operation time under general anesthesia was 10.8 min, which may be safer and less invasive.

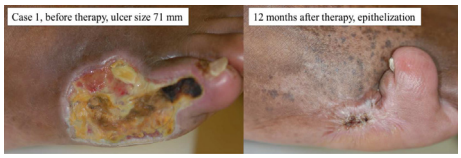
In an experimental study, the number of MSCs cultured with PRP was greater than that cultured with 10% FBS [7]. A similar situation was observed in the present study. The number of MSCs cultured with PRP increased 6.4-times (from $5.10 \pm 3.3 \times 10^6$ to $3.26 \pm 0.33 \times 10^7$) in seven days (Table 2), that cultured with 10% FBS increased 4.5-times in the same time [20]. When comparing patients and healthy subjects, it took 4 d for 5.4×10^6 MSCs from healthy subjects to attain the shipping criteria of 1×10^7 MSCs [7], but 6.8 d for 5.1×10^6 MSCs from patients (Table 2). In other words, it took more time to culture MSCs obtained from patients with PRP than those collected from healthy subjects did. Furthermore, qPCR was used to examine the production of several microRNAs in young and old MSCs. Consequently, a difference was observed in the amount of microRNA produced by the cells of different ages (unpublished data). We hypothesized that the presence of disease and age may be related to the ability to culture MSCs.

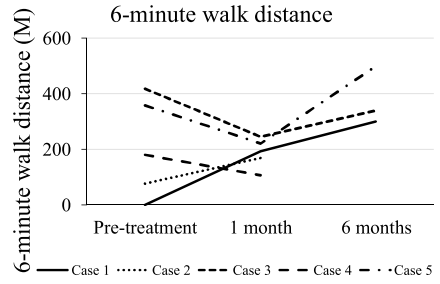
Furthermore, we believe that the quality of PRP may affect the quality of MSCs. No significant differences were noted in the amount of PRP between cases; however, without a qualitative study, it is difficult to discuss its effect on MSC quality. A qualitative evaluation of PRP was not conducted in this study; however, it may be explained by the differences in microRNA. This is a speculation and should be further explored in future studies.

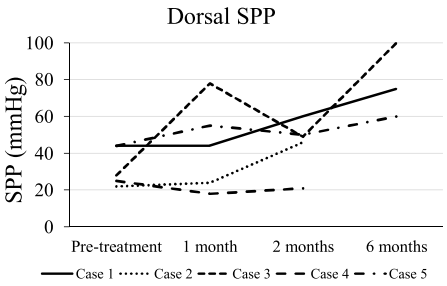
Since the number of cells required for transplantation was achieved in all cases, PRP can be considered an adequate substitute for FBS in terms of proliferation of MSCs.

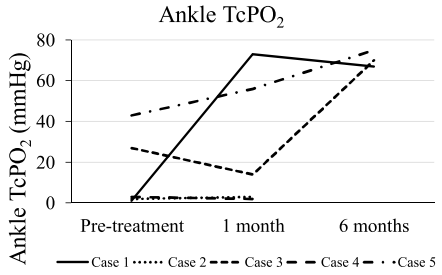
Substantial improvements in clinical severity of three patients who underwent the treatment were evaluated according to both Fontaine stage and Rutherford category one year after the cell therapy. In addition to the improvements in clinical severity, dorsal SPP and ankle $TcPO_2$ increased in all three patients. Although these changes seem to be caused by cell therapy, we also observed the following discrepancies. Fig. 3 showed several discrepancies between the measurements. In case 3, the discrepancy between SPP and 6-min walking distance may be due to the other factors that may influence 6-min walking distance, such as aerobic capacity, endurance, and foot joints conditions as well as improved blood flow to the feet. The discrepancy between ABPI and 6-min walking distance may be due to the fact that ABPI below 50 mmHg is not measurable by the oscillometric method used in this study. Therefore, even if the ABPI was not measured, the patient may have some ankle blood pressure and is able to walk to some extent.

Ulcer size (mm)					
	Pre treatment	1 month	2 months	6 months	12 months
Case 1	71	60	60	7	0
Case 2	24	10	8		
Case 4	17	10	12	12	









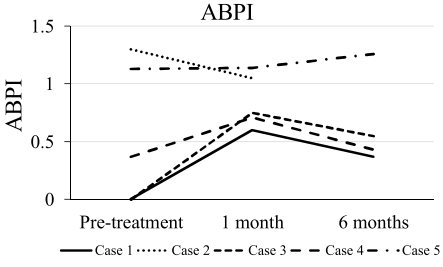


Fig. 3. Changes in ulcer size, 6-min walking distance, dorsal SPP, ankle SPP, and ABPI after cell transplantation. All three ulcers showed reduction in size after cell therapy. Six months after transplantation, absolute claudication distance improved from 206.6 m at baseline to 380.0 m, dorsal SPP increased from 32.6 mmHg to 78.3 mmHg, ankle $TcPO_2$ improved from 15.2 mmHg to 70.7 mmHg, And ABPI changed from 0.56 to 0.65. Abbreviations: SPP, skin perfusion pressure; $TcPO_2$, transcutaneous partial oxygen pressure; ABPI, ankle brachial pressure index.

It is difficult to obtain results that lead to absolute conclusions from individual measurement, and it is important to implement multiple established measurement to assess blood flow. However, since discrepancies between measurements may occur, it is more realistically useful to consider AFS rate as the primary efficacy endpoint.

In this study, one of the five patients died within one year of undergoing cell therapy; CABG was performed for angina pectoris, but the postoperative course was poor (Table 3). Among the patients with PAD, 45.7% died over five years, with the highest number of deaths caused by cardiac or major vascular disease [21]. Early diagnosis and therapy for comorbidities, especially cardiovascular diseases, are crucial to improve AFS rate in patients with CLI.

Angiogenic cell therapy using BM and peripheral blood MNCs in patients with CLI has shown poor clinical outcomes due to the inclusion of patients with diabetes mellitus and renal dysfunction [5,8]. In case 1, BM was harvested the same day after HD; however, the number of cultured MSCs did not meet the shipping criteria. For the second time, the BM was collected the next day after HD, and good culture results were obtained. These results suggested that the pathology of patients, especially in HD cases, may affect MSCs, which may consequently affect the efficacy and safety of cell therapy. In this study, our cell therapy, which included three patients on HD, induced a change in the clinical severity of one patient with CLI (Fig. 2).

Whether the presence of diabetes mellitus or HD affects the efficacy of this cell therapy has not been clearly evaluated. The HD group had a poor prognosis; of the three patients on HD, case 2 died and case 4 was amputated, but was free of CLI. Unlike peripheral blood MNCs, our therapy may allow the possibility of treating patients on HD.

Regarding the mechanism underlying the improvement in ischemic legs, both this study and our experimental study refer to angiogenesis. Currently, with the support of Grant-in-Aid for Scientific Research (grant number: 20K09133), we are investigating angiogenesis-related signal transduction in human MSCs culture. Unpublished, but several specific extracellular vesicles have been speculated to induce angiogenesis in our recent experimental models. Additional experimental studies are required to determine whether the administered MSCs change into vessels and to determine the mediators involved.

The small sample size and single-arm design were the major limitations of this study. However, this prospective interventional study provided encouraging findings. Health of the patients with CLI, with or without HD, improved following angiogenic therapy with cultured autologous BM-derived MSCs grown in their own PRP. The AFS rate was 60.0% and CLI-free ratio was 60.0% one year after cell therapy.

5. Conclusions

Our autologous angiogenic therapy, which uses cultured BM-derived MSCs grown in PRP, is safe and feasible, and can be a potential treatment for CLI. Long-term observations and large-scale clinical studies are required to confirm the potential benefits of this cell therapy.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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