Efficacy, safety and influencing factors of intra-calf muscular injection of bone marrow mononuclear cells in the treatment of type 2 diabetes mellitus-induced lower extremity vascular disease

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Abstract. The efficacy, safety and impact of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) associated with the intra-calf muscular injection of bone marrow mononuclear cells (BMMCs) in the treatment of type 2 diabetes mellitus (T2DM)-induced lower extremity vascular disease (LEVD) were evaluated. Patients with T2DM-LEVD were randomly divided into a control group and BMMCs group to assess the efficacy and safety of the treatment; serum VEGF and bFGF levels were detected. The BMMCs group was divided into superior genicular artery (SGA) and inferior genicular artery (IGA) subgroups as well as low-dose and high-dose subgroups for the comparison of efficacy indices. The BMMCs group exhibited significantly improved indices ($P<0.05$) compared with the control group and no fatalities or cancer occurred. There were no significant changes in serum VEGF and bFGF levels ($P>0.05$). The claudication distance in the IGA subgroup was significantly greater that in the SGA subgroup ($P<0.05$); the low-dose subgroup and the high-dose subgroup did not demonstrate any significant differences in each index ($P>0.05$). BMMC treatment for T2DM-LEVD was found to be safe and effective and had no significant impact on serum VEGF and bFGF levels in the short term; However, the degree of LEVD may affect its efficacy.

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Introduction

The risk of peripheral artery disease in patients with type 2 diabetes mellitus (T2DM) is 4-fold higher than that in the non-diabetic population (1). The predominant pathological changes observed in lower extremity vascular disease (LEVD) include thickening of the intimal median layer of the lower limb arteries, development of irregular atherosclerotic plaques in the lumen, stenosis, secondary thrombosis and arterial occlusion; these lesions show a wide range of properties and multi-branch and multi-segment features (2). The stem cell recruiting ability is decreased in patients with DM, as late glycation end-products may inhibit the proliferation and migration of the stem cells (3). Furthermore, reduced neovascularization, collagen matrix formation disorders or infection may cause ischemia or delay the repair of damaged tissues, and thus impede wound healing (4). Currently, medical treatments are ineffective and surgical intervention or vascular bypass surgery is only suitable for certain patients, which causes DM treatment to be challenging and affects patient prognosis.

With the development of renewable medicine, bone marrow mononuclear cell (BMMC) transplantation has been developed as a novel technology useful for the treatment of DM-LEVD (5). BMMCs have self-replication and differentiation potential and may differentiate into vascular endothelial cells and smooth muscle cells at ischemic areas *in vivo* (6). Furthermore, BMMCs secrete pro-angiogenic factors, thus promoting vascular remodeling and improving local blood supply (7). In 2002, Tateishi-Yuyama *et al* (8) used stem cell transplantation to treat low limb ischemic disease for the first time and achieved positive results. Stem cells are a group of relatively primitive cells, considered as 'seeds', and are able to differentiate into a variety of cells in appropriate environments to form new blood vessels, participate in local compensatory revascularization at ischemic sites, and improve and restore blood flow in the lower extremities (9-11). Previous

studies have demonstrated that BMMC therapy effectively promotes ulcer healing and relieves pain in diabetic foot disease (12,13). A meta-analysis showed that stem cell therapy could also prolong walking time and maintain the viability of the affected limb (14). Hirata *et al* (15) reported that when male guinea pigs with DM-induced lower limb ischemia were subjected to BMMC transplantation, lateral branches and new vessels were induced in the ischemic hind limb, but systemic vascular proliferation did not occur. Lee *et al* (16) subcutaneously injected stem cells into the topical skin at the wound edge and ischemic lower limb of diabetic mice and identified that fibroblast growth factor, vascular endothelial growth factor (VEGF), lower limb perfusion and capillary density in the local skin of the mice were significantly higher than those in diabetic mice that were not injected with stem cells, and that the skin healing rate was significantly accelerated. Park *et al* (17) reported that stem cells promoted cell proliferation, cell migration towards the wound area and angiogenesis. Furthermore, Shin and Peterson (18) indicated that after the wound margin in DM mice was transplanted with stem cells, the levels of VEGF and platelet-derived growth factor, which facilitate the repair of skin tissues, were significantly increased, and mobilization of the host's own stem cells surrounding the wound edge was also increased. This suggests that the transplanted stem cells can recruit relevant factors, promote angiogenesis and mobilize the body to produce a series of responses for tissue repair.

Basic fibroblast growth factor (bFGF) is a potent pro-angiogenic factor; *in vitro*, bFGF is able to promote the mitosis and chemotaxis of endothelial cells and induce these cells to produce VEGF and other factors (19). VEGF directly and specifically acts on vascular endothelial cells and stimulates the growth of blood vessels; furthermore, it can promote the migration of capillary endothelial cells to form capillary-like microtubes (20), which ultimately form new blood vessels.

To the best of our knowledge, whether the application of BMMCs as a treatment for T2DM-LEVD affects the serum concentrations of VEGF and bFGF, whether this treatment has the same efficacy for different degrees of LEVD and whether different transplantation dosages affect therapeutic efficacy have not been examined. In the present study, the efficacy of intra-calf muscular injection (i-CMI) BMMC therapy for T2DM-LEVD and its impacts on serum VEGF and bFGF levels were investigated. Furthermore, the impacts of transplantation dose and T2DM-LEVD degree on the therapeutic effects were also analyzed to provide a theoretical basis for further clinical applications.

Materials and methods

Subjects. The present study was a randomized, open, parallel-control clinical study. A total of 60 with T2DM-LEVD treated in the First Hospital of Hebei Medical University (Shijiazhuang, China) between January 2010 and January 2014 and who met the inclusion criteria were selected; the patients were subsequently divided into a control group (n=20) and BMMCs group (n=40), according to the random number table method. According to the transplantation dose, the BMMCs group was subdivided into a low-dose subgroup ($<5x10^8$ BMMCs; n=13) and high-dose subgroup ($\geq 5x10^8$ BMMCs; n=24). The BMMCs group was also subdivided into a superior genicular artery (SGA) subgroup (n=16) and inferior genicular artery IGA subgroup (n=21), according to the extent of the LEVD. In the SGA subgroup lesions involved the femoral artery, deep and superficial femoral artery, popliteal artery and inferior genicular artery, and in the IGA subgroup lesions only involved the anterior and posterior tibial artery, peroneal artery, and dorsalis pedis artery, according to the results of color Doppler ultrasound.

Inclusion criteria were as follows: i) Complied with the criteria for T2DM-LEVD issued by the WHO in 1999; ii) did not show improvement after ≥12-week medical circulation improvement or anticoagulation therapy; and iii) not suitable for surgical intervention or vascular bypass surgery. Exclusion criteria were as follows: i) T1DM; ii) associated with diabetic retinopathy (in the proliferation stage) and diabetic nephropathy; iii) associated with iliac artery occlusion; iv) clearly diagnosed with or suspected to have malignant cancer; v) ischemic heart disease or cerebrovascular disease within 1 year; vi) associated with severe heart, liver, kidney or respiratory failure, or in too poor a general condition to tolerate BMMCs transplantation; and vii) participated in another study or observational study at the same time or within 3 months. Exit criteria were as follows: i) Ischemic cardiocerebral disease development during the study; and ii) failure to attend follow-up tests or withdrawal from the study.

The present study was approved by the Ethics Committee of the First Hospital of Hebei Medical University. Prior to the study, all participants and their families were informed of the trial features, purposes and adverse reactions, and informed consent was obtained. When the study ended, 19 patients in the control group completed the study, and 1 patient was withdrawn from the study due to violating the experimental regimen. A total of 37 patients in the BMMCs group completed the study, and 3 patients were withdrawn from the study due to violating the experimental regimen.

All subjects were subjected to comprehensive medical treatment according to their individual conditions during the study, including the control of blood glucose, blood pressure and cholesterol, quitting smoking, improving circulation, anti-platelet aggregation treatment or anti-infection treatment; foot ulcers were periodically dressed and debrided.

General information. Patient age, disease duration, smoking and alcohol consumption were recorded. Additionally, height and body weight were measured to calculate body mass index (BMI) using the following formula: BMI=weight (kg)/height^2 (m^2).

Preoperative preparation. On the day of surgery [week 0 (W0)], venous blood samples were taken from the subjects (fasted for 12 h overnight) from the median cubital vein to detect levels of glycosylated hemoglobin (HbA1c; DCA2000+HbA1c analyzer and HbA1c kit; Bayer AG, Leverkusen, Germany), fasting plasma glucose (FPG), triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), liver function and kidney function (LX20 automatic biochemical analyzer; Beckman Coulter, Inc., Brea, CA, USA). The 5 ml of venous blood was naturally solidified at room temperature

Table I. Comparison of baseline indicators between the control and BMMCs groups.

Group	Cases	Sex (M/F)	Age (years)	Disease duration (years)	Smoking history (Y/N)	Alcohol consumption history (Y/N)	BMI (kg/m^2)
Control	19	10/9	56.32±5.57	14.79±3.10	9/10	8/11	24.61±2.98
BMMCs	37	19/18	56.81±4.76	16.38±3.47	21/16	20/17	25.80±2.89

Data are presented as the mean ± standard deviation. BMMCs, bone marrow mononuclear cells; BMI, body mass index.

and then centrifuged (256 x g, 4˚C, 10 min) to collect the serum, which was subsequently stored at -80˚C until the detection of VEGF and bFGF for all specimens simultaneously. Additionally, subjective (resting pain, limb coldness score and numbness) and objective indicators [intermittent claudication distance, lower limb skin temperature, transcutaneous oxygen pressure ($TcPO_2$) and resting ankle-brachial pressure index (ABI)] were evaluated. Blood glucose was determined using the glucose oxidase method (21). TC, TG and LDL-C were detected using an enzyme assay (22) and HbA1c was determined by the immune agglutination method (23). VEGF and bFGF were detected using ELISA kits (cat. no. 107751GR-H; Shanghai ExCell Bio Co., Shanghai, China). Intra- and inter-batch coefficients of variation of the kits were both <10%. Detections were performed by experienced professional staff in strict accordance with the kit instructions.

Preparation and transplantation of autologous BMMCs. Under strict aseptic conditions, 150-200 ml autologous bone marrow was sampled from the iliac crest of the subject after local anesthesia, which was then prepared into a 50-ml BMMC suspension in the hospital for future use. After intravenous anesthetization, the control group was injected with saline (50 ml, multi-point intramuscular injection), whereas the BMMCs group was intramuscularly injected with the BMMC suspension (50 ml) at 1.5 cm apart in a grid-like pattern, and patients with severe foot lesions were injected at 1 cm apart in a grid-like pattern. The number of BMMCs was counted using the trypan blue staining method (24). The peri-ulcer area was intensively injected; each injection volume was 1 ml. Following injection, the injection site was dressed with aseptic dressing and kept warm; the dressing was removed 3 days later.

Postoperative follow-up. Fasting venous blood was sampled from the median cubital vein at W12 and W24 to detect HbA1c, FPG, TG, TC, LDL-C, liver function and kidney function. A total of 5 ml venous blood was naturally solidified at room temperature and centrifuged (256 x g, 4˚C, 10 min) to collect the serum. The serum was then stored at -80˚C until the detection of VEGF and bFGF, when the same batch of specimens had been completely collected. Additionally, efficacy indices (subjective indicators and objective indicators) and safety were comprehensively assessed.

Efficacy assessment. Subjective indicators included resting pain score, limb coldness score and numbness score. The assessment was divided into 10 levels, where a higher score indicated a more severe degree. Objective indicators included intermittent claudication distance, lower extremity skin temperature (measured using a Piccolo multifunction infrared temperature instrument; Eurotherm SRL Inc., Guanzate, Italy), $TcPO_2$ (TCM400; Radiometer Medical ApS, Brønshøj, Denmark) and resting ABI (determined using an ES 1,000 SPM Doppler blood flow detector; Hadeco, Inc., Kawasaki, Japan). The above detections were performed by experienced technicians.

Safety assessment. Chest computed tomography, liver-, gallbladder-, pancreas-, spleen-, kidney- and bladder-color ultrasound, liver function, kidney function and fundus examinations were performed to investigate the post-transplantation complications and comorbidities. The above detections were performed by experienced technicians.

Statistical analysis. All data were processed using SPSS 19.0 statistical software (IBM SPSS, Armonk, NY, USA). The measurement data were expressed as mean ± standard deviation. Results were subjected to tests of normality and homogeneity of variance. Intergroup averages were compared using Student's t-test, whereas multi-group averages were compared using one-way analysis of variance with Student-Newman-Keuls post hoc test. Countable data were compared using the χ^2 test or non-parametric test. $P<0.05$ was considered to indicated a statistically significant difference.

Results

Comparison of baseline indicators in the control and BMMCs groups. Sex, age, disease duration, smoking history, alcoholic consumption history and BMI were not significantly different between the control and BMMCs groups ($P>0.05$; Table I).

Comparison of blood pressure, blood sugar and blood lipid in the control and BMMCs groups. The values of systolic blood pressure (SBP), diastolic blood pressure (DBP), HbA1c, FPG, TG, TC and LDL-C between the control and BMMCs groups were not significantly different before treatment at WO ($P>0.05$) and no significant changes were detected after treatment ($P>0.05$) with the exception of FPG. At W24, FPG in the BMMCs group was significantly reduced compared with that in the control group ($P<0.05$). The remaining indicators between the two groups exhibited no significant differences at the same time points ($P>0.05$; Table II).

Table II. Comparison of blood pressure, blood sugar, blood lipid, VEGF and bFGF between the control group and the BMMCs group before and after treatment.

Group	Time point	Cases	FPG (mmol/l)	HbA1c (%)	SBP (mmHg)	DBP (mmHg)	TG (mmol/l)	TC (mmol/l)	LDL-C (mmol/l)	VEGF (pg/ml)	bFGF (pg/ml)
Control	W0	19	7.36±0.60	7.03±0.57	134.05±6.68	71.21±7.44	1.95±0.52	5.17±0.46	3.61±0.58	322.05±108.25	557.26±72.15
	W12	19	7.47±0.49	6.96±0.58	132.21±4.44	70.74±3.89	1.98±0.47	5.18±0.36	3.69±0.57	303.95±96.70	529.89±90.75
	W24	19	7.73±0.72	6.98±0.44	134.68±4.56	72.63±3.77	1.91±0.36	5.03±0.51	3.46±0.53	281.37±84.62	524.58±91.98
BMMCs	W0	37	7.30±0.63	6.91±0.67	134.62±5.62	72.92±6.64	1.86±0.44	5.19±0.40	3.70±0.56	358.11±109.29	523.27±94.75
	W12	37	7.35±0.42	6.79±0.50	133.32±4.76	72.97±5.56	1.86±0.29	5.04±0.39	3.52±0.42	324.70±101.58	503.41±95.38
	W24	37	7.30±0.74[a]	7.00±0.39	133.89±4.69	73.68±4.75	1.86±0.42	5.08±0.47	3.51±0.49	316.03±89.63	508.54±99.30

Data are presented as the mean ± standard deviation. [a]P<0.05 vs. the control group at the same time point. W, week; BMMCs, bone marrow mononuclear cells; FPG, fasting plasma glucose; HbA1c, glycosylated hemoglobin; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglyceride; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; VEGF, serum vascular endothelial growth factor; bFGF, basic fibroblast growth factor.

Table III. Comparison of efficacy indicators between the control and the BMMCs groups before and after treatment.

Group	Time point	Cases	Resting pain score	Limb coldness score	Numbness score	Intermittent claudication distance (m)	Lower extremity skin temperature (˚C)	$TcPO_2$ (mmHg)	ABI
Control	W0	19	6.00±1.11	5.21±0.92	4.16±0.90	185±41.15	29.9±1.48	21.16±5.10	0.39±0.12
	W12	19	5.74±1.10	4.89±0.88	4.00±0.82	184±39.50	29.7±1.16	21.32±4.19	0.40±0.09
	W24	19	5.95±1.18	5.11±0.94	4.05±0.78	188±43.32	29.2±1.27	19.58±4.60	0.35±0.11
BMMCs	W0	37	5.97±1.26	5.35±1.59	4.16±0.73	195±44.01	29.8±1.15	23.24±5.38	0.37±0.10
	W12	37	4.78±0.95[a,b]	3.73±1.02[a,b]	2.57±0.77[a,b]	224±44.28[b,c]	30.3±1.17[d]	24.22±5.12[d]	0.41±0.09
	W24	37	3.84±0.90[a,b]	2.76±0.98[a,b]	2.00±0.85[a,b]	323±57.07[a,b]	32.6±1.01[a,b]	32.84±6.15[a,b]	0.57±0.08[a,b]

Data are presented as the mean ± standard deviation. [a]P<0.01 vs. the same group at W0; [b]P<0.01 vs. the control group at the same time point; [c]P<0.05 vs. the same group at W0; [d]P<0.05 vs. the control group at the same time point. W, week; BMMCs, bone marrow mononuclear cells; $TcPO_2$, transcutaneous oxygen pressure; ABI, ankle-brachial pressure index.

Table IV. Comparison of baseline indicators in low- and high-dose subgroups.

Variable	Low-dose subgroup	High-dose subgroup	P-value
Sex (male/female)	5/8	14/10	>0.05
Age (years)	56.54±4.48	56.96±4.99	>0.05
Disease duration (years)	15.77±4.34	16.38±3.49	>0.05
Smoking history (n)	5	16	>0.05
Alcohol consumption history (n)	7	13	>0.05
Body mass index (kg/m^2)	25.96±3.08	25.72±2.85	>0.05

Comparison of efficacy in the control and BMMCs groups. After treatment, the subjective and objective indicators in the control group showed no significant changes compared with those prior to treatment (P>0.05). At W12, all subjective indicators in the BMMCs group were significantly reduced (P<0.01) and the intermittent claudication distance was significantly increased (P<0.05) compared with those at W0; at W24, all objective indicators were significantly improved compared with those at W0 and W12 (P<0.01). At W12, the subjective indicators, intermittent claudication distance (P<0.01) and $TcPO_2$ (P<0.05) were significantly improved in the BMMCs group compared with the control group. At W24, the subjective and objective indicators were all significantly improved in the BMMCs group compared with the control group (P<0.01; Table III).

Safety assessment in the control and BMMCs groups. During the study, the liver and renal functions in the BMMCs group showed no signs of abnormalities and no fatalities, cancer or proliferative retinopathy occurred. No significant differences in VEGF and bFGF between the two groups were exhibited before treatment at W0 (or after treatment at W12 or W24 (P>0.05). Moreover, comparison of VEGF and bFGF between the two groups at the same time point indicated no statistically significant differences (P>0.05; Table II).

Comparison of baseline indicators in low- and high-dose subgroups. The low-dose subgroup included 5 males and 8 females (age, 56.54±4.48 years; disease duration, 15.77±4.34 years). A total of 5 cases had smoking history and 7 cases had a history of alcohol consumption. The BMI was 25.96±3.08 kg/m^2 and the transplantation dose was 3.59±0.94x10^8 cells. The high-dose subgroup included 14 males and 10 females (age, 56.96±4.99 years; disease duration, 16.38±3.49 years). A total of 16 cases had smoking history and 13 cases had a history of alcohol consumption. The BMI was 25.72±2.85 kg/m^2 and transplantation dose was 7.21±1.35x10^8 cells. The sex, age, disease duration, smoking history, alcohol consumption history and BMI between the two subgroups were not significant different (P>0.05; Table IV).

Table V. Comparison of blood pressure, blood sugar and blood lipids between the low- and high-dose subgroups before and after treatment.

Subgroup	Time point (weeks)	Cases (n)	FPG (mmol/l)	HbA1c (%)	SBP (mmHg)	DBP (mmHg)	TG (mmol/l)	TC (mmol/l)	LDL-C (mmol/l)
Low-dose	W0	13	7.20±0.66	6.85±0.58	133.38±6.85	72.77±5.45	2.03±0.47	5.23±0.32	3.85±0.38
	W24	13	7.11±0.75	6.97±0.42	134.31±3.25	74.00±3.83	1.87±0.29	5.11±0.53	3.57±0.51
High-dose	W0	24	7.35±0.63	6.94±0.72	135.29±4.86	73.00±7.32	1.77±0.39	5.17±0.44	3.62±0.63
	W24	24	7.40±0.73	7.01±0.38	133.67±5.36	73.50±5.25	1.86±0.48	5.06±0.44	3.48±0.49

Data are presented as the mean ± standard deviation. FPG, fasting plasma glucose; HbA1c, glycosylated hemoglobin; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglyceride; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol.

Table VI. Comparison of subjective and objective indicators between the low- and high-dose subgroups before and after treatment.

Subgroup	Time point (weeks)	Cases (n)	Resting pain score	Limb coldness score	Numbness score	Intermittent claudication distance (m)	Lower extremity skin temperature (˚C)	$TcPO_2$ (mmHg)	ABI
Low-dose	W0	13	5.69±1.60	5.23±1.88	4.38±0.65	209.77±39.77	29.66±1.15	22.63±6.05	0.38±0.10
	W24	13	3.85±0.90[a]	2.46±1.13[a]	2.00±0.91[a]	335.62±58.75[a]	32.45±0.99[a]	33.54±7.10[a]	0.60±0.07[a]
High-dose	W0	24	6.13±1.04	5.42±1.44	4.04±0.75	187.67±45.03	29.90±1.17	23.58±5.09	0.37±0.10
	W24	24	3.83±0.92[a]	2.92±0.89[a]	2.00±0.83[a]	316.88±56.28[a]	32.72±1.02[a]	32.46±5.70[a]	0.58±0.09[a]

Data are presented as the mean ± standard deviation. [a]P<0.01 vs. same subgroup at W0. W, week; $TcPO_2$, transcutaneous oxygen pressure; ABI, ankle-brachial pressure index.

Table VII. Comparison of baseline indicators in SGA and IGA subgroups

Variable	SGA subgroup	IGA subgroup	P-value
Sex (male/female)	8/8	11/10	>0.05
Age (years)	57.69±4.99	56.14±4.59	>0.05
Disease duration (years)	17.38±3.26	15.24±3.92	>0.05
Smoking history (n)	9	12	>0.05
Alcohol consumption history (n)	9	11	>0.05
Body mass index (kg/m^2)	25.80±2.72	25.80±3.08	>0.05

SGA, superior genicular artery; IGA, inferior genicular artery.

Comparison of blood pressure, blood sugar and blood lipids in low- and high-dose subgroups. The values of SBP, DBP, HbA1c, FPG, TG, TC, and LDL-C between the low- and high-dose BMMC subgroups were not significantly different before (W0) and after (W24) treatment (P>0.05). Furthermore, there was no significant difference in each indicator between the two subgroups at the same time point (P>0.05; Table V).

Comparison of efficacy indicators in low- and high-dose subgroups. No significant differences in any of the indicators were detected before (W0) the treatment between the low- and high-dose BMMCs subgroups (P>0.05). After treatment (W24), the subjective and objective indicators in the two subgroups were all significantly improved compared with those in the same subgroup at W0 (P<0.01). However, no significant difference in each indicator between the two subgroups at the same time point was observed (P>0.05; Table VI).

Comparison of baseline indicators in SGA and IGA subgroups. The SGA subgroup included 8 males and 8 females (age, 57.69±4.99 years; disease duration, 17.38±3.26 years). There were 9 cases with smoking history and 9 cases with a history of alcohol consumption. The BMI was 25.80±2.72 kg/m^2 and the transplantation dose was $6.14±2.25x10^8$ cells. The IGA subgroup included 11 males and 10 females (age, 56.14±4.59 years; disease duration, 15.24±3.92 years). A total of 12 cases had smoking history and 11 cases had a history of alcohol consumption. The BMI was 25.80±3.08 kg/m^2 and transplantation dose was $5.79±2.07x10^8$ cells. Sex, age, disease duration, smoking history, alcohol consumption history, BMI and transplantation dose between the two groups were not significantly different (P>0.05; Table VII).

Comparison of blood pressure, blood sugar and blood lipids in the SGA and IGA subgroups. The values of SBP, HbA1c, FPG, TG, TC and LDL-C between the two subgroups were not significantly different before treatment at W0 (P>0.05);

Table VIII. Comparison of blood pressure, blood sugar and blood lipids between the SGA and IGA subgroups before and after treatment.

Subgroup	Time point	Cases	FPG (mmol/l)	HbA1c (%)	SBP (mmHg)	DBP (mmHg)	TG (mmol/l)	TC (mmol/l)	LDL-C (mmol/l)
SGA	W0	16	7.42±0.65	6.85±0.70	134.50±4.72	72.25±3.49	1.76±0.41	5.22±0.44	3.81±0.68
	W24	16	7.41±0.73	6.97±0.38	134.75±5.36	69.75±6.53	1.88±0.51	5.15±0.46	3.66±0.48
IGA	W0	21	7.20±0.62	6.96±0.67	134.71±6.33	75.33±5.77[a]	1.94±0.45	5.16±0.37	3.61±0.44
	W24	21	7.22±0.76	7.01±0.40	133.24±4.12	74.76±5.35	1.85±0.34	5.02±0.47	3.39±0.49

Data are presented as the mean ± standard deviation. [a]P<0.01 vs. the SGA subgroup at the same time point. W, week; SGA, superior genicular artery; IGA, inferior genicular artery; FPG, fasting plasma glucose; HbA1c, glycosylated hemoglobin; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglyceride; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol.

Table IX. Comparison of subjective and objective indicators between the SGA and IGA subgroups before and after treatment.

Subgroup	Time point	Cases	Resting pain score	Limb coldness score	Numbness score	Intermittent claudication distance (m)	Lower extremity skin temperature (°C)	$TcPO_2$ (mmHg)	ABI
SGA	W0	16	5.56±1.15	2.88±0.81	4.31±0.70	184.69±48.24	29.94±1.01	22.19±5.56	0.39±0.10
	W24	16	3.88±0.89[a]	5.25±1.39[a]	2.19±0.75[a]	299.06±57.44[a]	32.58±1.07[a]	33.75±6.48[a]	0.58±0.08[a]
IGA	W0	21	6.29±1.27	5.43±1.75	4.05±0.74	203.62±39.74	29.72±1.26	24.05±5.23	0.36±0.10
	W24	21	3.81±0.93[a]	2.67±1.11[a]	1.86±0.91[a]	342.05±50.48[a,b]	32.67±0.98[a]	32.14±6.00[a]	0.60±0.09[a]

Data are presented as the mean ± standard deviation. [a]P<0.01 vs. the same group at W0. [b]P<0.05 vs. SGA subgroup at the same time point. W, week; SGA, superior genicular artery; IGA, inferior genicular artery; $TcPO_2$, transcutaneous oxygen pressure; ABI, ankle-brachial pressure index.

however, DBP in the SGA subgroup was significantly lower compared with that in the IGA subgroup before treatment at W0 (P<0.01). At W24, the values of SBP, DBP, HbA1c, FPG, TG, TC, and LDL-C were not significantly differently from the values before treatment (P>0.05) and there was no significant difference in each indicator between the two subgroups at the same time point (P>0.05; Table VIII).

Comparison of subjective and objective indicators in SGA and IGA subgroups. Before treatment at W0, none of the indicators were significantly different between the two subgroups (P>0.05). At W24, the subjective and objective indicators in the two subgroups were significantly improved compared with those before treatment at W0 (P<0.01). Additionally, the intermittent claudication distance in the IGA subgroup was significantly increased compared with that in the SGA subgroup (P<0.05; Table IX).

Safety evaluation. No significant abnormalities were found in the liver function and renal function in the control and BMMCs groups, and no mortality, cancer or proliferative retinopathy occurred.

Discussion

The present study was designed to investigate whether i-CMI BMMCs therapy is safe and effective for treating T2DM-LEVD, whether it affects the serum concentrations of VEGF and bFGF, and whether different degrees of LEVD and transplantation doses affect its therapeutic efficacy.

The present study demonstrated that subjective symptoms (resting pain, limb coldness score and numbness) and objective indicators (intermittent claudication distance, lower limb skin temperature, $TcPO_2$ and resting ABI) in patients in the BMMCs group were significantly improved compared with those in the control group. Additionally, no fatalities or cancer occurred during the study, suggesting that i-CMI autologous BMMCs were effective and safe for treating T2DM-LEVD, which is consistent with the results of previous studies (25-27). The present study further showed that patients in the BMMCs group exhibited improvements in subjective symptoms 3 months after surgery, which was earlier than the improvements observed in the objective indicators (6 months after surgery).

Previous studies have indicated that VEGF could promote angiogenesis in DM, whose upregulation has been closely associated with diabetic nephropathy and diabetic retinopathy (28-31). Hirata *et al* (15) transplanted BMMCs into guinea pigs with DM-LEVD and the formation of collateral blood vessels and neovessels in the transplantation group significantly increased. Furthermore, the study indicated that the plasma VEGF level did not affect vascular proliferation throughout the body.

The present study further revealed that 6 months after transplantation, serum VEGF and bFGF were not significantly altered. Additionally, no cases of proliferative retinopathy were reported, suggesting that the transplantation of BMMCs only promotes angiogenesis at the transplantation site, but does not affect the serum concentrations of VEGF and bFGF over the short-term or promote the occurrence and development of diabetic retinopathy and diabetic nephropathy, indicating the safety of BMMC therapy for T2DM-LEVD. However, further investigation is required into whether the local concentrations of VEGF and bFGF at the injection site were changed.

Retrospective analysis indicated that the efficacies in the low- and high-dose subgroups were not significantly different, suggesting that at a dose range of $1\text{-}10\text{x}10^8$ BMMCs, the transplantation dose does not impact the transplantation effects.

The gold diagnostic standard of T2DM-LEVD is digital subtraction angiography, which is an invasive and expensive examination, and thus this method is currently not suitable for the routine examination of T2DM-LEVD. Color Doppler ultrasound can rapidly, easily and accurately detect blood flow changes in lower limb arteries (32). Results from this type of imaging are consistent with those of computed tomography angiography, and thus it is also useful for application in the diagnosis of T2DM-LEVD (33). A previous study showed that the incidence of inferior genicular arterial lesions was higher than that of superior genicular arterial lesions in patients with DM. These lesions were also more severe and the proportion of anterior and posterior tibial arterial occlusion was high (34). In the present study, subjects from the BMMCs group were subdivided into SGA and IGA according to the results of color Doppler ultrasound. Retrospective analysis revealed that the intermittent claudication distance in the IGA subgroup was significantly increased when compared with that in the SGA group, indicating that compared with T2DM-LEVD patients with SGA involvement, the effects in patients with simple IGA involvement were superior. The remaining indicators between the two subgroups were not significantly different, which may be related to the short observation time and small sample size.

In summary, i-CMI BMMCs therapy was safe and effective for use in the treatment of T2DM-LEVD, showed no significant short-term effect on serum VEGF and bFGF and provided improved results in patients with IGA involvement when compared with patients with SGA involvement. A transplantation dose of $1\text{-}10\text{x}10^8$ BMMCs did not affect the transplantation effects. However, the present results were obtained from a single center and small sample size over a relatively short observation time; therefore, further multi-center, large-sample and long-term clinical studies are required.

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