

Limb Salvage Using Intramuscular Injection of Unfractionated Autologous Bone Marrow Mononuclear Cells in Critical Limb Ischemia: A Prospective Pilot Clinical Trial

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Abstract

Objectives: Despite advances in surgical and radiologic vascular techniques, many patients with critical limb ischemia are not eligible for revascularization procedures. Without pharmacologic therapy, the only option left is amputation. Bone marrow-derived progenitor cells represent a good revascularization option. This study sought to evaluate the therapeutic effectiveness of autologous unfractionated bone marrow mononuclear cell injection in revascularization of critical limb ischemia patients.

Materials and Methods: Twenty critical limb ischemia patients were not eligible for open or endo-vascular interventions. Bone marrow mononuclear cells were obtained by aspiration of 300 mL bone marrow, injected intramuscular in affected muscles. Patients were followed-up by walking distance, resting pain, skin condition, and ankle brachial index.

Results: Walking distance improved from a mean of 56 meters to a mean of 132 meters; rest pain improved markedly in 50% of patients, mildly in 5% of patients, and was unaffected in 45% of the patients. Fifty percent of patients showed improved skin condition, while ankle brachial index showed improvement in 40% of patients. No procedure-related complications were encountered.

Conclusions: Autologous bone marrow

mononuclear cell injection provides a safe and effective option for critical limb ischemia patients.

Key words: Ischemia, Endothelial progenitors, Vascular endothelial growth factor receptor, Mononuclear cells, Cell therapy

Introduction

Critical limb ischemia is estimated to develop in 500 to 1000 individuals per million persons in the general population.¹ Despite recent advances in surgical and radiologic vascular techniques, many patients with critical limb ischemia are ineligible for revascularization procedures owing to the anatomic location of the lesion, the extent of the disease, or the extensive comorbidity. Amputation is often the only option left, but is associated with bad prognosis including perioperative mortality in 5% to 20%, a second amputation in 30%; with only 25% to 50% achieving full mobility.² Furthermore, the median cost of managing a patient after amputation is estimated to be almost twice that of successful limb salvage.³

Over the past 2 decades, advances have occurred in understanding the basic mechanisms of blood vessel growth and maturation. Two distinct forms of blood vessel growth have been described: *vasculogenesis* and *angiogenesis*. Vasculogenesis occurs through much of embryonic vascular development where there is differentiation of endothelial cells from pluripotent stem cells. Once endothelial cells have developed, they begin to assemble into primitive vascular network, called the *primary capillary plexus*. Vascularization of several organs, including the endocardium of the heart and the dorsal aorta, occurs by vasculogenesis. In contrast, the brain, the kidneys, and the developing limbs are vascularized by

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angiogenesis, which is defined as the *sprouting of new blood vessels from a pre-existing vascular network*. Angiogenesis is likely the primary mechanism for most new blood vessel growth in the adult, whether it is due to physiologic or pathologic stimuli.⁴

Therapeutic angiogenesis depends upon the phenomenon of angiogenesis to treat disorders of inadequate tissue perfusion. Angiogenesis occurs in stages through which migrated endothelial cells undergo tube formation, remodeling of newly formed vessels into 3-dimensional networks with regression of unnecessary microvessels.⁵

The bone marrow mononuclear cell population contains a heterogeneous population of stem cells that stimulate angiogenesis.⁶ These stem cells are endothelial progenitor cells, mesenchymal stem cells, and hemopoietic stem cells. Mesenchymal stem cells and endothelial progenitor cells are present mainly in the bone marrow, constituting part of the bone marrow side population. They also are present in the cord blood and peripheral blood.

This study was a prospective clinical study conducted to evaluate the safety and therapeutic efficacy of bone marrow mononuclear cells injection in limb salvage in critical limb ischemia cases.

Subjects and Methods

Subjects

Institutional review board approval was obtained before beginning of the study. Protocols conformed to the ethical guidelines of the 1975 Helsinki Declaration. Written, informed consent was obtained from all patients. The study was performed on 20 patients presenting with critical limb ischemia not eligible for open vascular or endovascular interventions or who failed 1 or both of them. Patients were 14 men and 6 women (aged, 43 to 74 years). All patients were evaluated for critical lower limb ischemia; considering rest pain, nonhealing ulcer, and/or gangrene.

Inclusion criteria consisted of critical limb ischemia (presence of 1 or more of the following: ischemic rest pain, ulceration/gangrene, and/or ankle brachial index less than or equal to 0.6), or no possible open surgical or endovascular interventional options.

Exclusion criteria consisted of renal failure or dialysis, active overwhelming infection in the

affected limb that threatens the patient's life, proliferative retinopathy, bone marrow diseases.

Consent: Detailed explanation of the procedure, its outcome, possible complications, and expected improvement were included in the consent form and signed by all patients before management.

Materials and Methods

1. Bone marrow aspiration: Under deep sedation and strict aseptic conditions, 300 mL bone marrow was aspirated from multiple sites from both iliac crests in heparinized syringes.
2. Mononuclear cell separation was done using Ficoll-Hypaque density gradient centrifugation. Mononuclear cells were incubated overnight in a density of 1 million cells/mL in X-vivo medium with 2% heat-inactivated autologous serum. After 24 hours, cells were washed 3 times in sterile saline, filtered, and suspended in sterile saline.
3. Cell count, immunophenotyping, and viability testing were done.
4. The cells were suspended in 30 to 50 mL sterile saline.
5. Stem cell injection:
 - Sedation using 10 mg diazepam was injected intravenously.
 - Under local anesthesia and complete aseptic conditions, a 26-gauge needle was used to inject the mononuclear cell concentrate into 40 to 50 sites in the muscle of the ischemic limb (Figure 1).
 - One mL mononuclear cell concentrate was injected in each site, 1.5 cm depth.
6. Follow-up: all patients were followed-up monthly for 3 months by:
 - Ischemic pain: Assessed by rest pain score, visual analogue scale (VAS)⁷ graded from grade 0 (pain free) to grade 10 (maximum pain).
 - Improvement of walking distance in ambulant patients.
 - Signs of wound healing (decrease ulcer size, start granulation tissue).
 - Appearance of a line of separation for the gangrenous part of the foot, intraoperative bleeding during debridement, the appearance of granulation tissue, and healing.

- Improved ankle brachial index.
- Duplex ultrasound was done at the end of follow-up to detect the degree of improvement of perfusion of the limb.

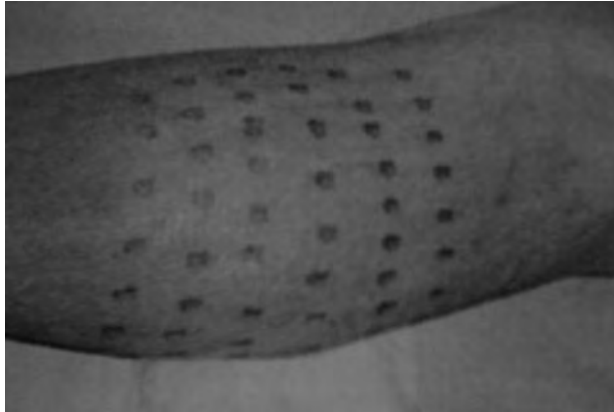


Figure 1. Marking the sites of bone marrow mononuclear cells injection.

Results

This study included 20 patients with critical limb ischemia (epidemiologic data are listed in Table 1). Characteristics of the injected cell population are described in Table 2 and Figure 2. During follow-up, 4 patients were lost owing to other reasons (3 from myocardial infarction and 1 from acute renal failure). Five patients underwent amputation owing to poor improvement. Patients were followed-up for 3 months using the following criteria.

Table 1. Initial data of the study population.

Age distribution		
Age	Number of patients	Percentage (%)
Under 40 years	0	0
41-60 years	10	50
61-70 years	8	40
Above 70 years	2	10
Sex distribution		
Sex	Number of patients	Percentage (%)
Male	14	70
Female	6	30
Presenting symptoms		
Complaint	Number	Percentage (%)
Rest pain only	8	40
Rest pain + ulceration	4	20
Rest pain + gangrene	8	40
Comorbidities		
Associated comorbidity	Number	Percentage (%)
Diabetes mellitus	13	65
Hypertension	10	50
Ischemic heart disease	11	55
Stroke	4	20
Duplex study		
Level of occlusion	Number	Percentage (%)
Infrapopliteal	12	60
Femoropopliteal	6	30
Aortoiliac disease	2	10

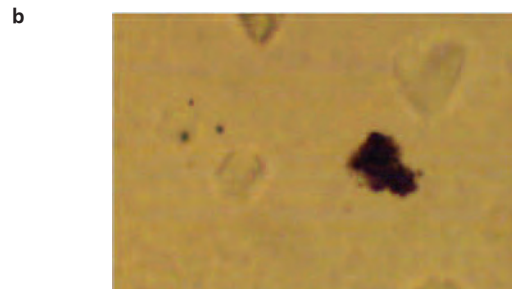
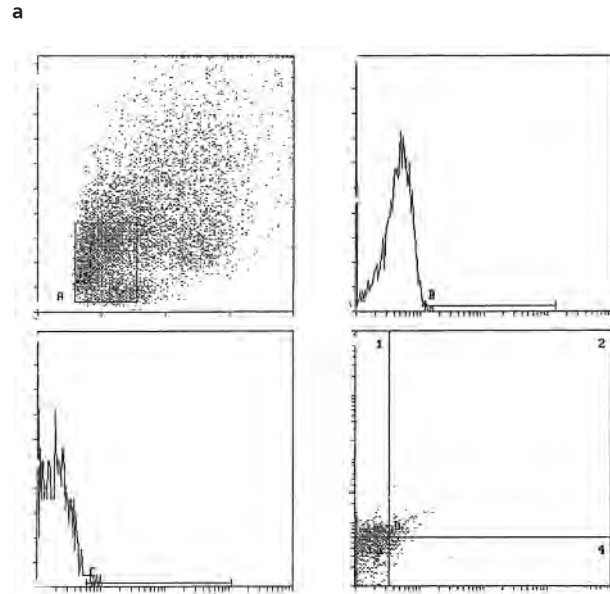


Figure 2. Characterization of the cell population:

- a. Immunophenotyping pattern: scatter diagram, CD133 positivity, CD34 positivity, and double positive population.
b. Factor VIII expression in cytospin preparation of the cell population (immunohistochemistry).

Rest pain

Rest pain was evaluated by a VAS at 2 weeks, and at 1, 2, and 3 months. In the first 2 weeks after therapy, there was detectable improvement in only 2 patients: 1 with good improvement (score 0-3), and the other with fair improvement (4-6). This percentage increased to 60% after 3 months (Table 3).

Table 2. Characteristics of the injected cell population.

Total number	Viability	CD34 Positivity	CD133 Positivity	CD34+/133+
37-68 × 10 ⁶ /mL	92-98	2% to 3%	0.7% to 1.3%	0.5% to 1.0%
(Total: 1.11 × 10 ⁹)				

Walking distance

On presentation, 14 of the 20 patients were nonambulant either because of old age or amputation of the other limb. Thus, walking was evaluated only in the 6 ambulant patients. Before

stem cell injection, the mean walking distance was about 56 ± 14 meters, and it increased to 132 ± 33 meters after 3 months.

Table 3. Rest pain score over follow-up.

Pain score	After 2 weeks	After 1 month	After 2 months	After 3 months
Good improvement (0-3 score) (%)	5	20	40	50
Fair improvement (4-6 score) (%)	5	30	15	5
Poor improvement (7-8 score) (%)	5	10	10	5
Excluded patients (owing to mortality) (%)	0	5	15	20

Signs of wound healing

Four of the 20 patients showed ischemic ulcers at presentation. At the end of the follow-up, 1 patient had complete healing, 1 had partial healing (decrease in the circumference and depth of the ulcer), while the other 2 showed no improvement regarding ulcer healing.

Gangrene

On presentation, gangrene was observed in 8 patients (40%), 3 with gangrenous toes, 4 with a gangrenous forefoot, and 1 with a gangrenous patch over the heel. Improvement occurred only in 3 patients, while the remaining 5 patients included 3 mortalities and 2 major amputations. Three successful patients showed improvement in the form of line of demarcation, fair bleeding during debridement, good granulation tissue, and complete healing of the stump.

Duplex study

A Duplex study showed infrapopliteal disease in 12 patients (60%), femoropopliteal disease in 6 patients (30%), and aortoiliac disease in 2 patients (10%). Follow-up Duplex study was done monthly on all patients. There was an improvement in ankle brachial index in 8 cases (40%). Ankle brachial index ranged from 0.3 to 1.01, with a mean of 0.49 before injection, and reached a mean of 0.65 ± 0.08 after 3 months of injection.

Discussion

Therapeutic angiogenesis using stem cells, autologous progenitor cells, growth factors such as basic fibroblast

growth factor, and transcription factors such as hypoxia-inducible factor- α that induce synthesis of angiogenic cytokines have been used in critical limb ischemia patients who lack options for endovascular or surgical revascularization.⁸

Bone marrow cells are composed of extensive complex cell fractions containing many kinds of undifferentiated stem cells and differentiated cells. Implantation of autologous bone marrow cells is demonstrated to be an effective and feasible technique of inducing therapeutic angiogenesis in both clinical and experimental studies. However, the angiogenic potency might differ among cell fractions of bone marrow cells, and which of these play a key role remains unclear.⁹ The most important cell populations involved in angiogenesis are CD133+ve cells,¹⁰ CD117+ve cells,¹¹ CD34+ve cells,¹² in addition to the mesenchymal stem cells.¹³

Endothelial progenitor population comprise a heterogenous population of cells such as CD34-/CD133+/VEGFR2+, CD34+/CD133+/VEGFR2+, in addition to the mature endothelial cells. The CD34-/CD133+/VEGFR2+ fraction is the precursor of the CD34+/CD133+ population and shows more-potent vasoregenerative capacities.^{14, 15}

Endothelial progenitors are reported to be mobilized by several agents such as chemotherapeutic agents,¹⁶ maximum tolerated dose, metronomic chemotherapy,¹⁷ and erythropoietin.¹⁸ These agents can be used therapeutically either to enhance angiogenesis in ischemic cases or to reduce angiogenesis in cases of malignancies. It is crucial that we understand the angiogenic potency induced by different cell fractions, because selecting the most effective cell fraction for implantation could improve this treatment.⁹

Injection of unfractionated bone marrow mononuclear cells has been reported to promote neovascularization of ischemic tissues effectively.¹⁹ This angiogenic effect may be related to their ability to induce vascular and muscle regeneration by direct de novo vascular and muscle differentiation or paracrine mechanisms through vascular endothelial growth factor secretion.²⁰

This study was a prospective clinical trial to evaluate the safety and efficacy of bone marrow-derived mononuclear cell injection in cases of critical limb ischemia. The current study enrolled 20 critical limb ischemia patients who were no-option cases.

The route of administration of stem cells was by intramuscular injection. According to the level of occlusion, we injected it in the calf muscles in the infrapopliteal disease and in the calf muscles and quadriceps for femoropopliteal disease. Zhou and associates documented 3 routes of stem cell administration in critical limb ischemia: intramuscular, intra-arterial, or a combination of both.²¹ With intra-arterial administration of mononuclear cells, stem cells reach the region of maximum ischemia by blood flow.²² While traveling in the circulation, nutrient and oxygen supply are preserved and provide a favorable environment for survival and engraftment, but cell uptake from the circulation may be limited. In this type of delivery, homing requires migration of endothelial progenitor cells out of the vessels into the surrounding tissue, which make ischemic tissue targeted less efficiently.²³

Most studies on cell therapy for critical limb ischemia used whole mononuclear cell fraction. At this moment, it is unclear whether administration of more-selected cell populations or ex-vivo culture toward an endothelial phenotype would be more effective.

In this study, we used bone marrow mononuclear cells. Iba and associates compared the angiogenic effects of the same numbers of bone marrow mononuclear cells and peripheral blood mononuclear cells in a rat model, and showed the superior angiogenic effect of mononuclear cells.²⁴ However, Tateno and associates showed that there was no significant difference in stimulation of neovascularization after infusion of peripheral blood mononuclear cells and bone marrow mononuclear cells.²⁵

Infused cell dose plays a pivotal role the effectiveness of therapy. In this study, the total cell number of mononuclear cells was 1.11×10^9 . In other studies using bone marrow mononuclear cells, total cell number ranged from $0.1-101 \times 10^9$.^{17, 26, 27} The fraction of CD34+ cells in the isolated mononuclear cells population varies from 0.6% in the study by Kajiguchi and associates²⁸ to 2.4% in the therapeutic angiogenesis using cell therapy study,²⁹ while it ranged from 2% to 3% in this study. CD133+ve cell population ranged from 0.7% to 1.3% in this study; endothelial progenitor cells—which are CD34+CD133+—ranged from 0.5% to 1.0%. This fraction was not evaluated in other studies, while it plays an important role in angiogenesis.

We evaluated the improvement in limb perfusion by reducing ischemic pain (assessed by a VAS), improved walking distance, signs of wound healing, appearance of a line of separation for gangrenous parts, and improved ankle brachial index. These indicators are more objective than improvement of pain, cold sensation, and numbness used by Chung and associates.³⁰

Improvement of rest pain was reported in 55% of patients, Chung and associates reported 88.2% improvement,³⁰ while Tateishi and associates reported 50% regression of rest pain.⁷ Failure of response was encountered in 25% of cases, as opposed to 9.1% of patients in the study conducted by Chung and associates in 2007.³⁰ This may be due to the choice of patients who were old with severe comorbidities.

As regards ankle brachial index, 40% of cases showed improvement, 60% showed no improvement. However, 15% of patients who showed no improvement in ankle brachial index showed improved rest pain and walking distance and reduced use of analgesics. This may be attributed to the formation of minute collaterals were insufficient to improve ankle brachial index. No procedure-related complications were encountered in this study.

In conclusion, cellular therapy is well-tolerated and offers rising hope for patients with peripheral arterial disease. Administration of autologous bone marrow mononuclear cells injection is easy, inexpensive, and safe, with a definite ameliorating effect on limb ischemia. However, specification of the target cell population, route of administration, and dose-escalation need larger case-controlled studies.

Limitations

This study was not placebo-controlled. The subjects were heterogeneous in terms of the underlying disease, age distribution, and coronary risk factors. Patients were all in terminal comorbid states, which made long-term follow-up unpractical. Therefore, it was difficult to assess precisely the long-term efficacy of cell therapy in the present study. In future studies, case-control studies should be set using patients receiving conventional therapy as a control. Evaluation of the efficacy of treatment should be done using clinical evaluation, Doppler study, and transcutaneous oxygen saturation in postischemic tissues.

References

- Dormandy J, Heeck L, Vig S. Predicting which patients will develop chronic critical leg ischemia. *Semin Vasc Surg.* 1999;12(2):138-141.
- Norgren L, Hiatt WR, Dormandy JA, et al. Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II). *Eur J Vasc Endovasc Surg.* 2007;33(suppl 1):S1-S75.
- Hirsch AT, Haskal ZJ, Hertzner NR, et al. ACC/AHA 2005 guidelines for the management of patients with peripheral arterial disease (lower extremity, renal, mesenteric, and abdominal aortic): executive summary a collaborative report from the American Association for Vascular Surgery/Society for Vascular Surgery, Society for Cardiovascular Angiography and Interventions, Society for Vascular Medicine and Biology, Society of Interventional Radiology, and the ACC/AHA Task Force on Practice Guidelines (Writing Committee to Develop Guidelines for the Management of Patients With Peripheral Arterial Disease) endorsed by the American Association of Cardiovascular and Pulmonary Rehabilitation; National Heart, Lung, and Blood Institute; Society for Vascular Nursing; TransAtlantic Inter-Society Consensus; and Vascular Disease Foundation. *J Am Coll Cardiol.* 2006;47(6):1239-1312.
- Kocher AA, Schuster MD, Szabolcs MJ, et al. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med.* 2001;7(4):430-436.
- Choksy SA, Chan P. Therapeutic angiogenesis. *Br J Surg.* 2006;93(3):261-263.
- Finney MR, Greco NJ, Haynesworth SE, et al. Direct comparison of umbilical cord blood versus bone marrow-derived endothelial precursor cells in mediating neovascularization in response to vascular ischemia. *Biol Blood Marrow Transplant.* 2006;12(5):585-593.
- Tateishi-Yuyama E, Matsubara H, Murohara T, et al. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. *Lancet.* 2002;360(9331):427-435.
- Slovut DP, Sullivan TM. Critical limb ischemia: medical and surgical management. *Vasc Med.* 2008;13(3):281-291.
- Li TS, Hamano K, Nishida M, et al. CD117+ stem cells play a key role in therapeutic angiogenesis induced by bone marrow cell implantation. *Am J Physiol Heart Circ Physiol.* 2003;285(3):H931-H937.
- Tang J, Wang J, Yang J, Kong X. Adenovirus-mediated stromal cell-derived factor-1alpha gene transfer induces cardiac preservation after infarction via angiogenesis of CD133+ stem cells and anti-apoptosis. *Interact Cardiovasc Thorac Surg.* 2008;7(5):767-770.
- Beohar N, Rapp J, Pandya S, Losordo DW. Rebuilding the damaged heart: the potential of cytokines and growth factors in the treatment of ischemic heart disease. *J Am Coll Cardiol.* 2010;56(16):1287-1297.
- Madeddu P, Emanueli C, Pelosi E, et al. Transplantation of low dose CD34+KDR+ cells promotes vascular and muscular regeneration in ischemic limbs. *FASEB J.* 2004;18(14):1737-1739.
- Crisostomo PR, Wang Y, Markel TA, Wang M, Lahm T, Meldrum DR. Human mesenchymal stem cells stimulated by TNF-alpha, LPS, or hypoxia produce growth factors by an NF kappa B- but not JNK-dependent mechanism. *Am J Physiol Cell Physiol.* 2008;294(3):C675-C682.
- Salven P, Mustjoki S, Alitalo R, Alitalo K, Rafii S. VEGFR-3 and CD133 identify a population of CD34+ lymphatic/vascular endothelial precursor cells. *Blood.* 2003;101(1):168-172.
- Friedrich EB, Walenta K, Scharlau J, Nickenig G, Werner N. CD34-/CD133+/VEGFR-2+ endothelial progenitor cell subpopulation with potent vasoregenerative capacities. *Circ Res.* 2006;98(3):e20-e25.
- Furuya Y, Okazaki Y, Kaji K, Sato S, Takehara K, Kuwana M. Mobilization of endothelial progenitor cells by intravenous cyclophosphamide in patients with systemic sclerosis. *Rheumatology (Oxford).* 2010;49(12):2375-2380.
- Bertolini F, Paul S, Mancuso P, et al. Maximum tolerable dose and low-dose metronomic chemotherapy have opposite effects on the mobilization and viability of circulating endothelial progenitor cells. *Cancer Res.* 2003;63(15):4342-4326.
- Heeschen C, Aicher A, Lehmann R, et al. Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. *Blood.* 2003;102(4):1340-1346. Epub 2003. Erratum in: *Blood.* 2004;103(12):4388.
- Moriya J, Minamino T, Tateno K, et al. Long-term outcome of therapeutic neovascularization using peripheral blood mononuclear cells for limb ischemia. *Circ Cardiovasc Interv.* 2009;2(3):245-254.
- Lian Q, Zhang Y, Zhang J, et al. Functional mesenchymal stem cells derived from human induced pluripotent stem cells attenuate limb ischemia in mice. *Circulation.* 2010;121(9):1113-1123.
- Zhou B, Poon MC, Pu WT, Han ZC. Therapeutic neovascularization for peripheral arterial diseases: advances and perspectives. *Histol Histopathol.* 2007;22(6):677-686.
- Bartsch T, Falke T, Brehm M, et al. Intra-arterial and intramuscular transplantation of adult, autologous bone marrow stem cells. Novel treatment for therapy-refractory peripheral arterial occlusive disease [in German]. *Dtsch Med Wochenschr.* 2006;131(3):79-83. Erratum in: *Dtsch Med Wochenschr.* 2006;131(8):402.
- Dimmeler S, Zeiher AM, Schneider MD. Unchain my heart: the scientific foundations of cardiac repair. *J Clin Invest.* 2005;115(3):572-583.
- Iba O, Matsubara H, Nozawa Y, et al. Angiogenesis by implantation of peripheral blood mononuclear cells and platelets into ischemic limbs. *Circulation.* 2002;106(15):2019-2025.
- Tateno K, Minamino T, Toko H, et al. Critical roles of muscle-secreted angiogenic factors in therapeutic neovascularization. *Circ Res.* 2006;98(9):1194-1202.
- Higashi Y, Kimura M, Hara K, et al. Autologous bone-marrow mononuclear cell implantation improves endothelium-dependent vasodilation in patients with limb ischemia. *Circulation.* 2004;109(10):1215-1218.
- Durdu S, Akar AR, Arat M, Sancak T, Eren NT, Ozyurda U. Autologous bone-marrow mononuclear cell implantation for patients with Rutherford grade II-III thromboangiitis obliterans. *J Vasc Surg.* 2006;44(4):732-739.
- Kajiguchi M, Kondo T, Izawa H, et al. Safety and efficacy of autologous progenitor cell transplantation for therapeutic angiogenesis in patients with critical limb ischemia. *Circ J.* 2007;71(2):196-201.
- Miyamoto K, Nishigami K, Nagaya N, et al. Unblinded pilot study of autologous transplantation of bone marrow mononuclear cells in patients with thromboangiitis obliterans. *Circulation.* 2006;114(24):2679-2684.
- Gu Y, Zhang J, Qi L. Comparative study on autologous implantation between bone marrow stem cells and peripheral blood stem cells for treatment of lower limb ischemia [in Chinese]. *Zhongguo Xue Fu Chong Jian Wai Ke Za Zhi.* 2007;21(7):675-678.