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Safety and Effect of Adipose Tissue-Derived Stem Cell Implantation in Patients With Critical Limb Ischemia

– A Pilot Study –

Han Cheol Lee, MD, PhD; Sung Gyu An, MD; Hye Won Lee, MD; Jin-Sup Park, MD; Kwang Soo Cha, MD, PhD; Taek Jong Hong, MD, PhD; Jong Ha Park, PhD; Sun Young Lee, BSc; Sang-Pil Kim, MD; Yeong Dae Kim, MD, PhD; Sung Woon Chung, MD, PhD; Yong Chan Bae, MD, PhD; Yong Beom Shin, MD, PhD; Jeung Il Kim, MD; Jin Sup Jung, MD, PhD

Background: Treatment of critical limb ischemia (CLI) by bypass operation or percutaneous vascular intervention is occasionally difficult. The safety and efficacy of multiple intramuscular adipose tissue-derived mesenchymal stem cells (ATMSC) injections in CLI patients was determined in the study.

Methods and Results: The study included 15 male CLI patients with ischemic resting pain in 1 limb with/without non-healing ulcers and necrotic foot. ATMSC were isolated from adipose tissue of thromboangiitis obliterans (TAO) patients (B-ATMSC), diabetes patients (D-ATMSC), and healthy donors (control ATMSC). In a colony-forming unit assay, the stromal vascular fraction of TAO and diabetic patients yielded lesser colonies than that of healthy donors. D-ATMSC showed lower proliferation abilitythan B-ATMSC and control ATMSC, but they showed similar angiogenic factor expression with control ATMSC and B-ATMSC. Multiple intramuscular ATMSC injections cause no complications during the follow-up period (mean follow-up time: 6 months). Clinical improvement occurred in 66.7% of patients. Five patients required minor amputation during follow-up, and all amputation sites healed completely. At 6 months, significant improvement was noted on pain rating scales and in claudication walking distance. Digital subtraction angiography before and 6 months after ATMSC implantation showed formation of numerous vascular collateral networks across affected arteries.

Conclusions: Multiple intramuscular ATMSC injections might be a safe alternative to achieve therapeutic angiogenesis in patients with CLI who are refractory to other treatment modalities.

Key Words: Adipose tissue; Angiogenesis; Cell therapy; Critical limb ischemia; Mesenchymal stem cell

Critical limb ischemia (CLI) is difficult to manage using current treatment modalities. Percutaneous transluminal angioplasty (PTA) is only 60–70% successful in treating CLI. Between 30% and 40% of CLI patients are not successfully revascularized.[1] Particularly, the rate of restenosis is higher in lesions in infrapopliteal arteries than that in lesions in the superficial femoral artery. Performing PTA and a bypass operation in patients with thromboangiitis obliterans (TAO) is difficult because of the segmental nature of this disease, the inflammatory process, and the involvement of small distal vessels. Long-term patency rates in patients with TAO are low.[2]

Editorial p????

Cell therapies using bone marrow mononuclear cells (BM-MNC) and peripheral blood mononuclear cells (PBMNC) have effective outcomes in patients with peripheral artery disease and TAO.[3–6] Mesenchymal stem cells (MSC) are multipotent and differentiate into osteoblasts, chondrocytes, endothelial cells, and vascular smooth muscle cells.[7,8] Recently, MSC transplantation was found to induce neovascularization in a hindlimb ischemia model.[9] The adipose tissue is abundant in the human

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Division of Cardiology, Department of Internal Medicine (H.C.L., S.G.A., H.W.L., J.-S.P., K.S.C., T.J.H., J.H.P.), Department of Physiology, College of Medicine (S.Y.L., J.S.J.), Department of Thoracic & Cardiovascular Surgery, College of Medicine (S.-P.K., Y.D.K., S.W.C.), Department of Plastic Surgery, College of Medicine (Y.C.B.), Department of Rehabitation, College of Medicine (Y.B.S.), Department of Orthopedic Surgery, College of Medicine (J.I.K.), Pusan National University, Medical Research Institute, Pusan Medical University Hospital, Busan, Korea

Mailing address: Jin Sup Jung, MD, PhD, Departments of Physiology, College of Medicine, Pusan National University, Beomeo-ri, Mulgeum-eup, Yangsan-si, Gyeongsangnam-do, 626-870, Korea. E-mail: jsjung@pusan.ac.kr

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Table 1. Patients' Characteristics

Case	Age/ gender	Diagnosis	Ischemic site/status	Risk factors	Previous treatment for critical limb ischemia
1	33/M	TAO	Left toe/resting pain (II-4)	Smoking	Minor amputation, sympathectomy
2	52/M	TAO	Left toe/non-healing ulcer (III-5)	Smoking, quit, hyperlipidemia	Femoral to femoral artery bypass operation, major amputation
3	24/M	TAO	Left toe/Non-healing ulcer (III-5)	Smoking, quit	Minor amputation
4	46/M	TAO	Right toe/Necrosis (III-6)	Smoking, quit	Minor amputation
5	36/M	TAO	Right toe/resting pain (II-4)	Smoking, quit	Femoral to popliteal artery bypass operation
6	42/M	TAO	Left foot/Necrosis (III-6)	Smoking, quit	Superficial femoral artery PTA, bypass operation
7	64/M	Diabetic foot	Left foot/non-healing ulcer (III-5)	DM, HT	
8	55/M	TAO	Right toe/non-healing ulcer (III-5)	Smoking	
9	55/M	TAO	Right toe/non-healing ulcer (III-5)	Smoking	Femoral to popliteal artery bypass operation
10	69/M	Diabetic foot	Right foot/Necrosis (III-6)	DM, HT	
11	60/M	TAO	Left foot/non-healing ulcer (III-5)	Smoking, HT	Axillary to femoral artery bypass operation
12	46/M	TAO	Left toe/non-healing ulcer (III-5)	Smoking	Minor amputation
13	73/M	TAO	Left foot/resting pain (II-4)	None	
14	39/M	TAO	Left toe/non-healing ulcer (III-5)	Smoking, DM, HT	Minor amputation
15	73/M	Diabetic foot	Left toe/non-healing ulcer (III-5)	HT, DM	PTA for superficial femoral artery.

TAO, thromboangiitis obliterans; PTA, percutaneous trnasliminal angioplasty; DM, diabetus mellitus; HT, hypertension.

body and is consistently replenished. Therefore, this tissue is an ideal source of MSC. It has been shown that adipose tissue-derived MSC (ATMSC) have characteristics similar to those of bone marrow stromal cells (BMSC).[10,11] ATMSC differentiate into endothelial cells[12,13] and have a proangiogenic effect in the hindlimb ischemia model.[12–14] Our previous study showed that ATMSC can be differentiated into endothelial cell[15–17] and that the implantation of in vitro cultured ATMSC in murime hindlimb ischemia model was effective for causing proangiogenic action.[15] However, therapeutic efficacy of culture-expanded ATMSC transplantation in CLI patients have not been reported.

The present study was designed to implant ATMSC in patients with CLI, which resulted from TAO and diabetic foot, and to evaluate the safety and efficacy of the implantation procedure.

Methods

Patients

Fifteen patients with CLI were enrolled in the study. Among these, 12 patients had TAO and 3 patients had diabetic foot. Inclusion criteria included a 6-month history of CLI (atherosclerosis obliterans, diabetic foot, or TAO); age: between 20 and 80 years; Rutherford's class: II-4, III-5, or III-6; resting pain or ischemic ulcer/necrosis; and unsuitability for percutaneous vascular intervention or a bypass operation. All patients provided written informed consent. Patients who had a history of cancer atrial fibrillation or primary hematologic disease, osteomyelitis, infectious disease, acute febrile illness, chronic glomerulonephritis, or chronic obstructive pulmonary disease and proliferative retinopathy or entrapment syndrome; patients who consumed alcohol and abused drugs; and patients receiving immunosuppressive drugs, corticosteroids, and cytotoxic drugs were excluded from the study. Primary endpoints were estimated with the absence of major adverse events and collateral vessel formation on digital subtraction angiography (DSA). Secondary endpoints were estimated with maximal walking distance, cludication distance, temperature change on thermography, ankle-brachial index (ABI) and Wong-Baker FACES pain rating scores.

Isolation and Culture of ATMSC

Human adipose tissues were obtained by simple liposuction from the abdominal subcutaneous fats of 3 abdominoplasty patients with no underlying diseases, 12 patients with TAO, and 3 patients with diabetic foot with informed consent. Subcutaneous adipose tissues were digested with collagenase I (1 mg/ml) under gentle agitation for 60 min at 37°C. The digested tissues were centrifuged and the pellet (stromal vascular fraction [SVF]) was resuspended in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA)–based media containing 0.2 mmol/L ascorbic acid and 10% fetal bovine serum (FBS) obtained from bovine spongiform encephalopathy free herd. The cell fraction was cultured overnight at 37°C/5% CO_2 in DMEM-based media containing 0.2 mmol/L ascorbic acid and 10% FBS. After 24 h, the cell medium was changed to Keratinocyte-SFM (Invitrogen)-based media containing 0.2 mmol/L ascorbic acid, 0.09 mmol/L calcium, 5 ng/ml rEGF, and 5% FBS. The cells were subculture-expanded in the same media until passage 3. Characteristics of ATMSC including surface marker expression and angiogenic factor secretion were examined in our previous studies.[11,15,16] FBS contaminant from cultured MSC were completely removed by several washing with PBS and was verified through the test of albumin concentration below the measurement limit using a bovine albumin enzyme-linked immunosorbent assay quantitiation kit (Bethyl Laboratories, Montgomery, TX, USA). The Korea Food and Drug Administration permitted the FBS-eliminated MSC for clinical study. Aliquots of the ATMSC are then tested for cell viability and fungal, bacterial, endotoxin, and mycoplasma contamination as demanded by the Code of Federal Regulations, Title 21 (21CFR) before further use. The procedure for ATMSC preparation was performed at the GMP laboratory of RNL Bio (Seoul, Korea) under GMP conditions. In vitro hypoxia experiments were performed with a hypoxic incubator (APM-30D, ASTEC, Japan) that continuously infuses a calibrated gas mixture (95% N2, 5% CO_2). Experiments were performed at oxygen concentrations of 21% and 1%.

Determination of Colony-Forming Unit (CFU) in SVF

Isolated cells after collagenase digestion (SVF) were collected and cell viability was determined by trypan blue assay. Identi-

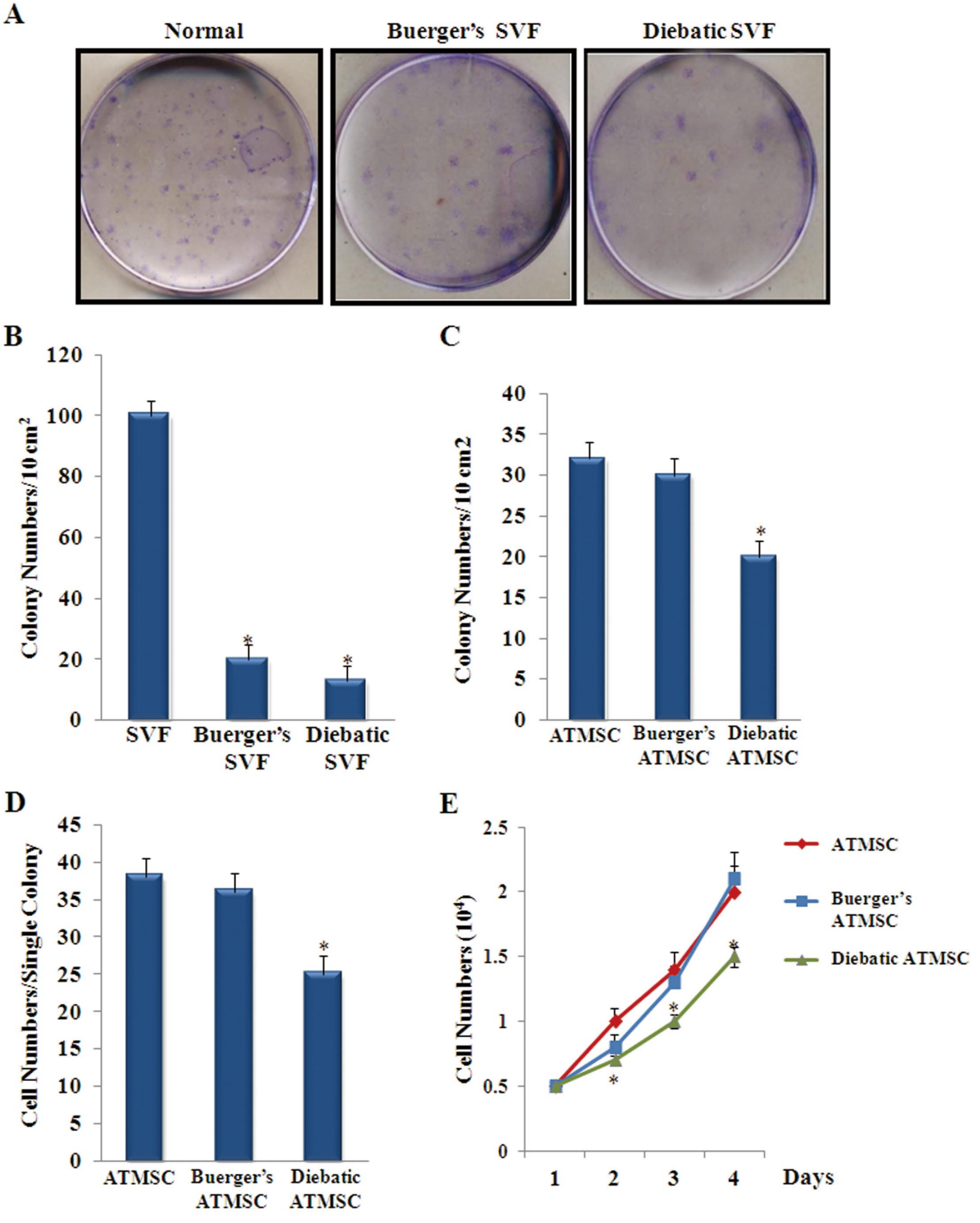

Figure 1. Colony-forming unit (CFU) assay of stromal vascular fractions (SVF) and proliferation of adipose tissue-derived mesenchymal stem cells (ATMSC) isolated from Buerger's and diabetic patients. (**A**,**B**) Colony number was counted after plating of 10^5 viable SVF cells. (**C**,**D**) The CFU assay was performed by plating 60 cells onto a 100 mm culture dish. The colonies and cell numbers were counted 7 days after plating, and the numbers of cells in each colony were counted. (**E**) ATMSC proliferation was determined by direct cell counting with a hemocytometer 1–4 days after plating. Data represent mean±SEM. *P<0.05, compared with normal ATMSC (n=3).

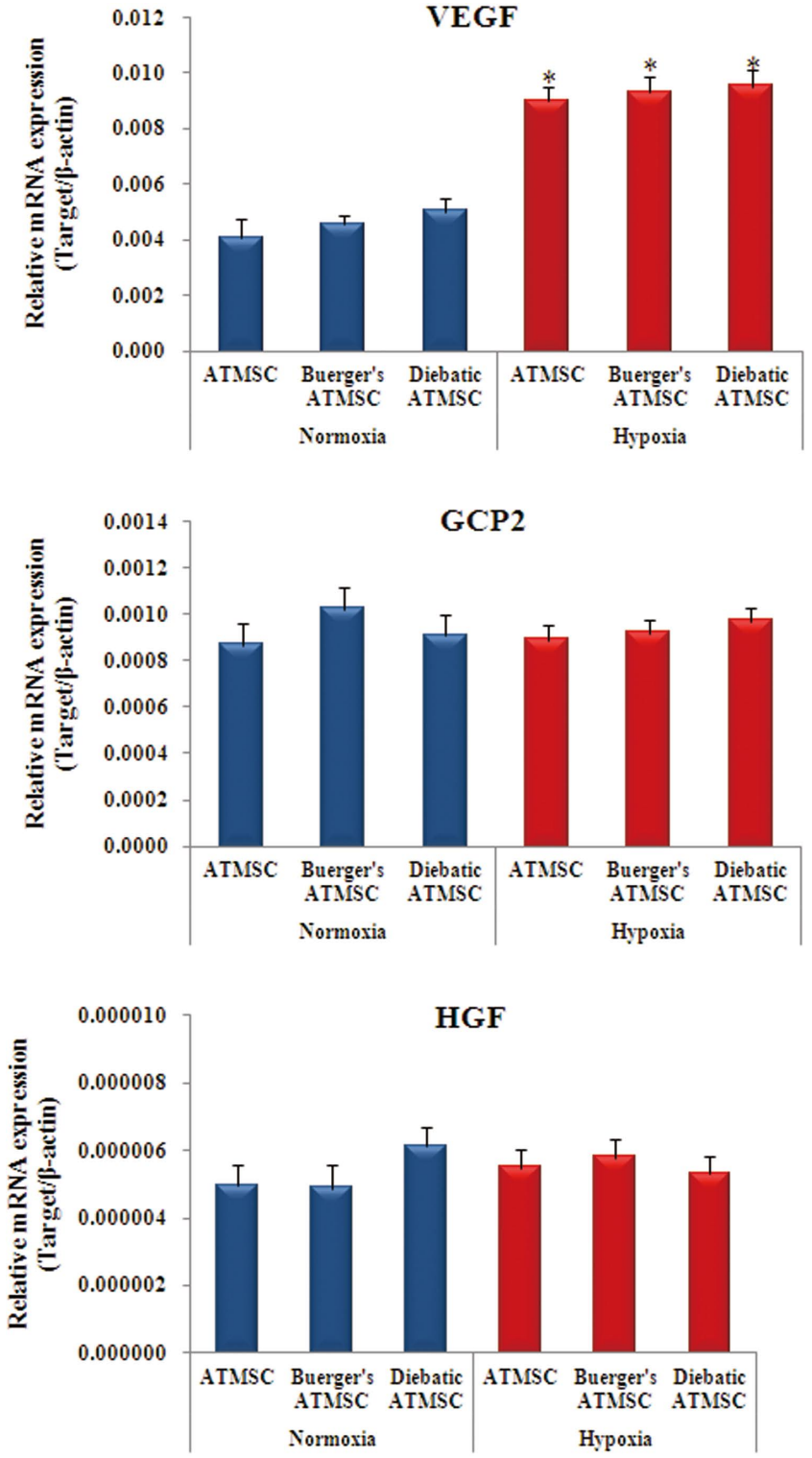

Figure 2. Expression of angiogenic factors in adipose tissue-derived mesenchymal stem cells (ATMSC) isolated from Buerger's and diabetic patients. Angiogenic factors expression was determined by real-time polymerase chain reaction analysis. Data represent mean±SEM of 3 different experiments. *P<0.05 compared with normoxic condition.

cal number of SVF cells (1×10^5 cells) was plated on a 100 mm plate, and the number of colony was counted 10 days after plating.

Evaluation of Proliferation of Culture-Expanded ATMSC

Passage 3 ATMSC were plated on a 6-well plate at a density of 1×10^4 cells/well. At the indicated days, the cells were trypsinized, stained with 0.4% trypan blue (Sigma, St Louis, MO, USA) and cell number was counted. For CFU assay, 60 ATMSC were transferred to a 100 mm plate, and the number of colonies and cells in each colony were then counted.

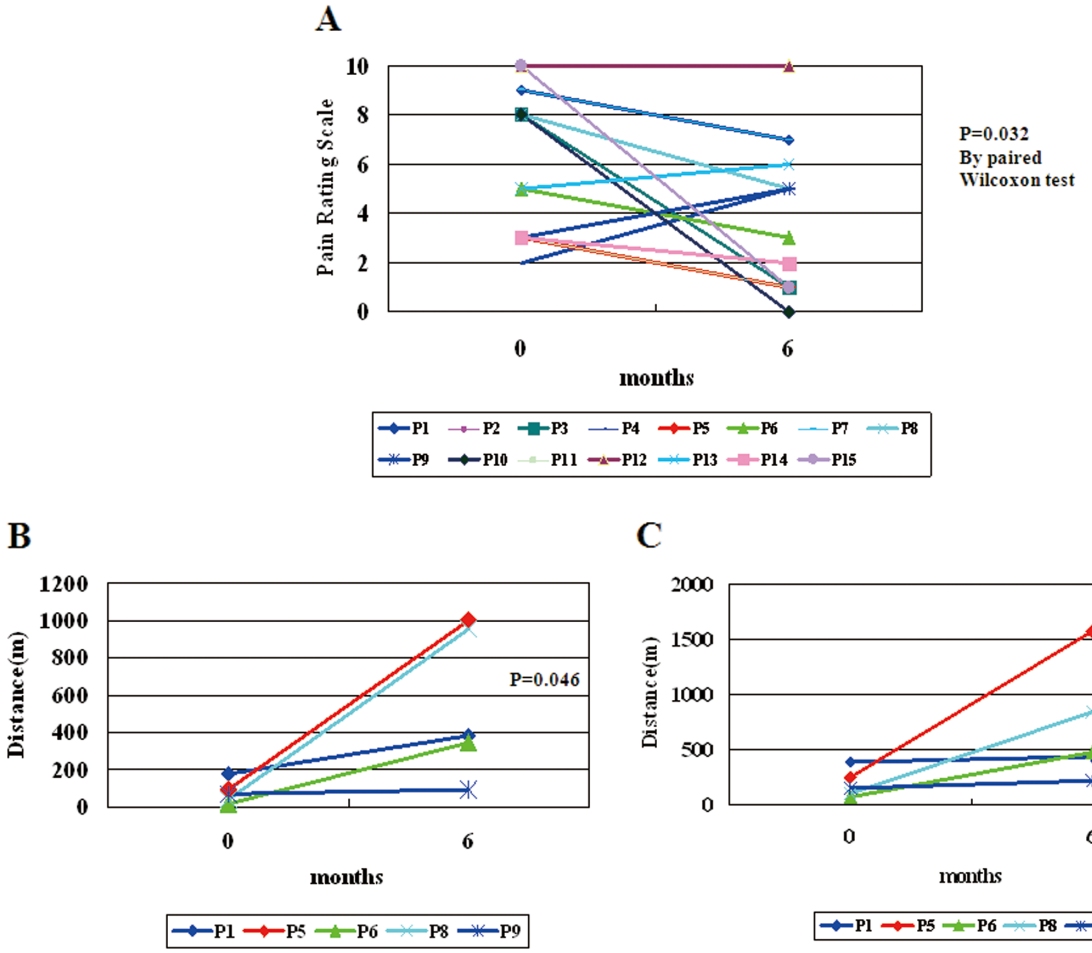

Figure 3. The Wong-Baker FACES Pain Rating Scale is checked at baseline and 6 months after adipose tissue-derived mesenchymal stem cells implantation and is improved (**A**). Claudication walking distance (**B**) and the maximal walking distance (**C**) are improved at baseline and 6 months after ATMSC implantation in 5 patients.

Real-Time Polymerase Chain Reaction

The primer sequences used in the experiment were as follows: *β*-actin, 5'-CTGGTGCCT GGG GCG-3', 5'-AGCCTCGCCTT-TGCCGA-3'; VEGF, 5'-CGAAACCATGAACTTTCTGC-3', 5'-CCTCAGTGGGCACACACTCC-3'; HGF, 5'-CCTATG-CAGAGGGACAAAGG-3', 5'-TGCTATTGAAGGGGAAC-CAG-3'; GCP2, 5'-GTCCTTCGGGCTCCTTGT-3', 5'-AA-CTTGCTTCCCGTTCTTCA-3'. Real-time quantitation was done as described in a previous study.[18]

Preparation of ATMSC for Intramuscular Injection and Evaluation of Clinical Outcomes After Cell Transplantation

This study was approved by the IRB committee of Pusan National University Hospital (PNUH IRB-2008074) and the Korean Food and Drug Association (KFDA-1273). We intramuscularly injected doses of ATMSC with guidelines based on a previous mouse animal model.[14] We placed 0.5 cc ATMSC in each syringe, which included 5×10^6 ATMSC. Total 3×10^8 ATMSC were injected 60 points into the lower extremities of 12 patients. Multiple intramuscular injections were given under spinal anesthesia to reduce pain.

The Wong-Baker FACES pain rating scale (from 0 to 10), ABI, claudication walking distance, and maximal walking distance (determined using a treadmill test) were evaluated, and thermography and DSA were performed before cell implantation and at 6 month after cell transplantation. Adverse effects, major amputation, minor amputation, ulcer healing, and clinical improvement were estimated in the hospital 7 days after the injection was given as well as at 1, 3, and 6 months after injection according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE; Version 4). Clinical improvement was defined as more than 2 steps of the Wong-Baker FACES pain rating score. Primary endpoints included the absence of major adverse effects, degree of collateral vessel formation on DSA, and the Wong-Baker FACES pain rating score. Secondary endpoints consisted of the claudication walking distance, maximal walking distance, ABI, thermography findings, major amputation, minor amputation, and ulcer healing. Three cardiologists rated the grade of collateral vessel formation as +1, +2, and +3 between baseline DSA and DSA performed at 6 months. Three rehabilitation doctors estimated the improvement on thermography as +1, +2, and +3.

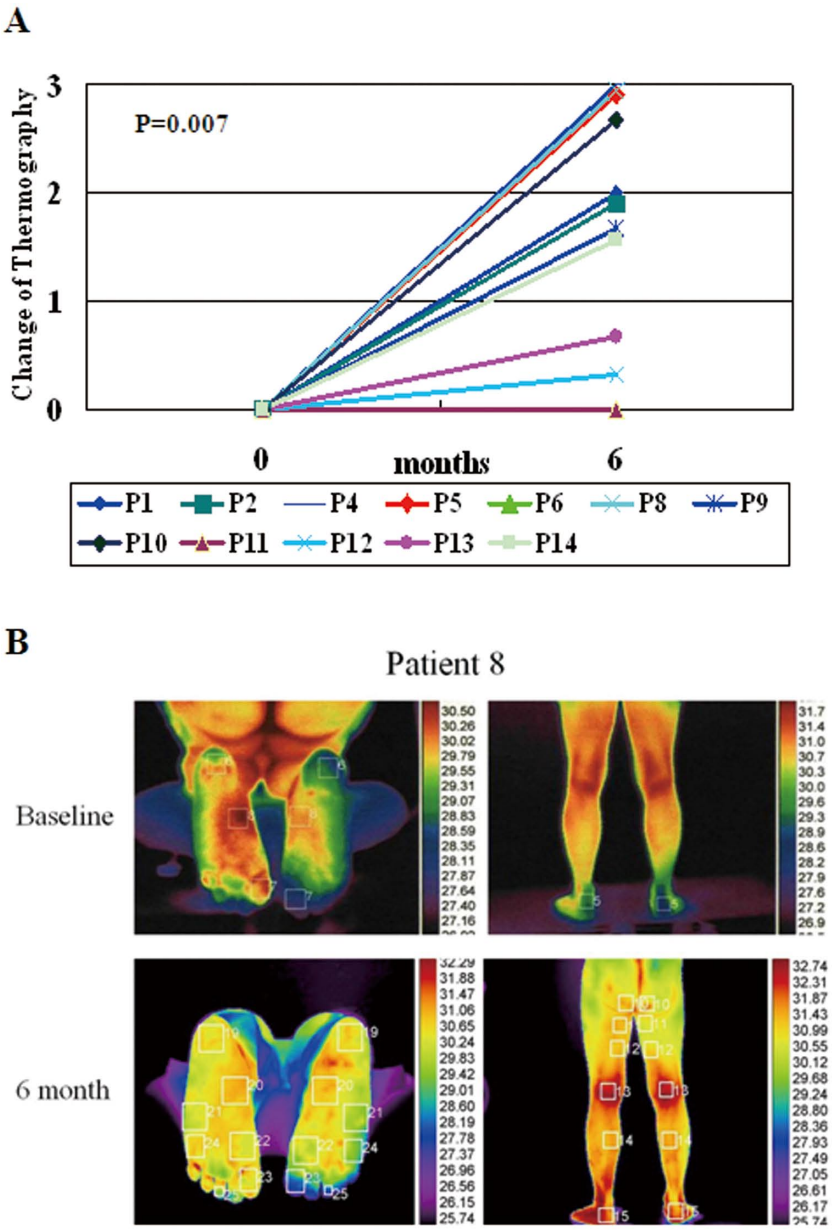

Figure 4. Change of thermography. (**A**) The findings of thermography and ranked the degree of improvement as +1, +2, and +3 according to the temperature color change. Statistically significant improvement was observed in the temperature color change after the adipose tissue-derived mesenchymal stem cells (ATMSC) implantation. (**B**) Patient 8: a 55-year-old man with thromboangiitis obliterans was implanted with ATMSC on the right leg. Improvement of the temperature color change in ATMSC transplanted right leg and foot was observed after 6 months.

Statistical Analysis

Comparisons between groups in the in vitro study were analyzed using the Student's t-test. The paired Wilcoxon test was applied for the Wong-Baker pain rating scores, ABI, claudication walking distance, maximal walking distance, collateral grades on DSA, and improvement on thermography between baseline and 6 months. Probability values of $P<0.05$ were considered statistically significant.

Results

Baseline Characteristics of Patients

Fifteen male patients were included in this study, which was conducted between January 2009 and March 2010. Among these, 12 patients had TAO and 3 patients had diabetic foot. The mean age of the enrolled patients was 51.1±14.9 years. All TAO patients, except 1, were smokers. Eighty percent of the patients (12/15) had non-healing ulcer or necrotic foot (Rutherford class III-5, III-6), and 66.7% (10/15) had previously undergone minor or major amputation and/or a bypass operation. Patient characteristics are summarized in **Table 1**.

Characterization of Transplanted ATMSC Isolated From Patients With TAO and Diabetic Foot

To determine whether characteristics of ATMSC were altered in CLI patients, we compared density of stem cells in adipose tissues, proliferation, and differentiation potential of culture expanded ATMSC with those of normal individuals. The number of CFU reflects density of ATMSC in SVF that are isolated from fresh adipose tissue.[19,20] CFU analysis showed that patients with TAO and diabetic foot showed significantly lower CFU values than those of control cells (**Figures 1A**,**1B**).

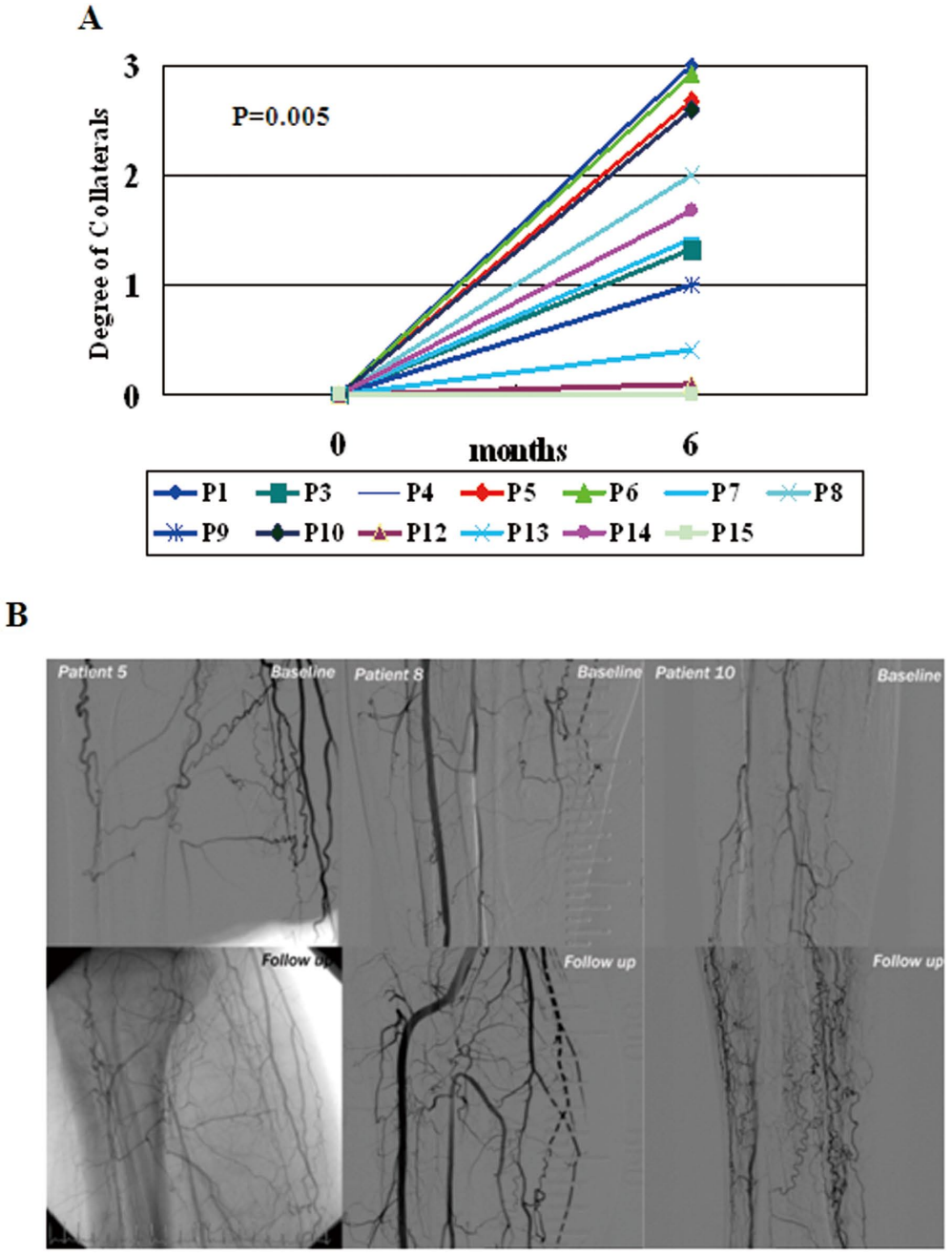

Figure 5. Change of collateral vessel formation on digital subtraction angiography (DSA). (**A**) The degree of collateral vessel formation were ranked as +1, +2, and +3 according to the number of collaterals. (**B**) Collateral vessel formation was strikingly increased in the knee, upper tibia, and lower tibia at 6 months after adipose tissue-derived mesenchymal stem cells (ATMSC) implantation. Patient 5: a 36-year-old man with TAO showed increased multiple corkscrew collaterals around the areas of occlusion at 6 months after ATMSC implantation (follow up). Patient 8: a 55-year-old man with TAO showed a number of collaterals and visible peroneal artery at 6 months after ATMSC implantation (follow up). Patient 10: a 69-year-old man with diabetic foot numerous collaterals are connected to anterior and posterior tibial artery (follow up).

CFU analysis of cultured ATMSC (passage number 3) showed normal proliferation potential of ATMSC obtained from TAO patients (B-ATMSC), in contrast with decreased proliferation of ATMSC obtained from diabetic foot patients (D-ATMSC) (**Figures 1C–E**).

The proangiogenic action of ATMSC resulted from the paracrine action of angiogenic factors that are secreted from transplanted cells.[12–16] Therefore, we compared the expression of major angiogenic factors in control, B-ATMSC, and D-ATMSC at normoxic or hypoxic condition. Real-time polymerase chain reaction anlaysis revealed no significant differences in HGF, VEGF, and GCP-2 expression among the

Table 2. Clinical Outcome on 6 Months After Implantation of ATMSC

Case	Age/ gender	Diagnosis	Ischemic site/status	Clinical symptom, wound healing
1	33/M	TAO	Left toe/resting pain (II-4)	Well improved
2	52/M	TAO	Left toe/non-healing ulcer (III-5)	Ulcer healing, well improved
3	24/M	TAO	Left toe/non-healing ulcer (III-5)	Minor amputation, well improved
4	46/M	TAO	Right toe/necrosis (III-6)	Minor amputation, no change
5	36/M	TAO	Right toe/resting pain (II-4)	Well improved
6	42/M	TAO	Left foot/necrosis (III-6)	Minor amputation, well improved
7	64/M	Diabetic foot	Left foot/non-healing ulcer (III-5)	Ulcer healing, well improved
8	55/M	TAO	Right toe/non-healing ulcer (III-5)	Ulcer healing, well improved
9	55/M	TAO	Right toe/non-healing ulcer (III-5)	Ulcer healing, no change
10	69/M	Diabetic foot	Right foot/necrosis (III-6)	Minor amputation, well improved
11	60/M	TAO	Left foot/non-healing ulcer (III-5)	Ulcer healing, well improved
12	46/M	TAO	Left toe/non-healing ulcer (III-5)	No change
13	73/M	TAO	Left foot/resting pain (II-4)	No change
14	39/M	TAO	Left toe/non-healing ulcer (III-5)	Minor amputation, well improved
15	73/M	Diabetic foot	Left toe/non-healing ulcer (II-5)	Ulcer healing, well improved

#Well improved ≥2 steps, mild improved ≥1 step in the Wong-baker score.
ATMSC, adipose tissue-derived mesenchymal stem cells; TAO, thromboangiitis obliterans.

groups (Figure 2). Culture of ATMSC under 1% oxygen for 3 days significantly increased VEGF expression without affecting HGF and GCP-2 expression (Figure 2).

Clinical Results

The Wong-Baker FACES pain rating scores after 6 months of ATMSC transplantation were significantly improved compared to the baseline pain rating scores (P=0.032). The pain rating scores in 3 patients worsened compared to the baseline pain rating scores (Figure 3A).

The claudication walking distance and the maximal walking distance determined using the treadmill test were determined for only 5 patients. The remaining patients could not participate in the treadmill test because some patients had amputated toes, feet, lower legs, or painful ulcer lesions. For the 5 patients tested, the claudication walking distances improved significantly (P=0.046) (Figure 3B). Maximal walking distances also improved, but this improvement was not statistically significant (P=0.075) (Figure 3C). Two patients showed a >10-fold improvement in claudication walking distance and maximal walking distance (baseline claudication distance/maximal walking distance of patient 5: 90 m/249 m and 6-month claudication distance/maximal walking distance: 1,000 m/1,570 m; baseline claudication distance/maximal walking distance of patient 8: 38 m/102 m and 6-month claudication distance/maximal walking distance: 959 m/1,918 m).

No change in ABI was noted because this value primarily depends on the pressure of large arteries (mean baseline ABI: 0.73±0.3; 6-month mean ABI: 0.70±0.28, P=0.21), and ATMSC form numerous small collateral arteries.

Thermography

We analyzed the findings of thermography and ranked the degree of improvement as +1, +2, and +3 according to the temperature color change, as assessed by 3 rehabilitation doctors. The data showed that significant improvement was observed in the temperature color change after the ATMSC implantation (P=0.007) (Figures 4A,4B).

DSA

We analyzed the findings of DSA and ranked the degree of collateral vessel formation as +1, +2, and +3 according to the number of collaterals, as assessed by 3 cardiologists. Improvement of more than 1 grade in collateral vessel formation estimated by 6-month DSA was observed in 2 of the 3 patients with diabetic foot (66.7%) and in 8 of the 10 patients with TAO (80%) (Figures 5A,5B).

Clinical Outcomes

Clinical outcomes at 6 months after ADSC transplantation were summarized in Table 2. Eleven patients were responsive to ATMSC treatment. Patients 4, 9, 12, and 13 were not responsive to ATMSC treatment. We performed minor amputations in 5 patients following ATMSC implantation, and wounds after amputation clearly healed in these 5 patients. Of patients with non-healing ulcers, 66.7% experienced ulcer healing (6/9). In the cases with an initially necrotic foot, no observable tissue regeneration occurred (Figure 6; patients 4, 6, and 10). Overall, 66.7% of patients showed clinical improvement (10/15) (Table 2). There was clinical improvement in 3 of 3 patients with a diabetic foot (100%) and in 7 of 12 patients with TAO (58.3%).

Adverse Events

No adverse events classified as greater than NCI CTCAE (version 4) grade 2 occurred. One patient experienced mild fever, 1 patient had flu-like symptoms, 2 patients complained of pain at the injection site, and 1 patient had a headache.

Discussion

Cell therapy using BM-MNC and PBMNC as a new modality of treatment for peripheral arterial disease (PAD) has been reported to be effective in treating these diseases, including atherosclerotic disease and TAO (Buerger's disease).[3–6,21] An initial clinical trial was conducted in 25 patients with ischemia of 1 leg and 22 patients with ischemia of both legs (therapeutic angiogenesis by cell transplantation [TACT study]).[6] At 4 weeks, ABI, transcutaneous oxygen pressure (TcO_2), resting pain, and pain-free walking time were significantly improved in legs injected with BM-MNC compared to those injected with saline. To establish the long-term safety and clinical

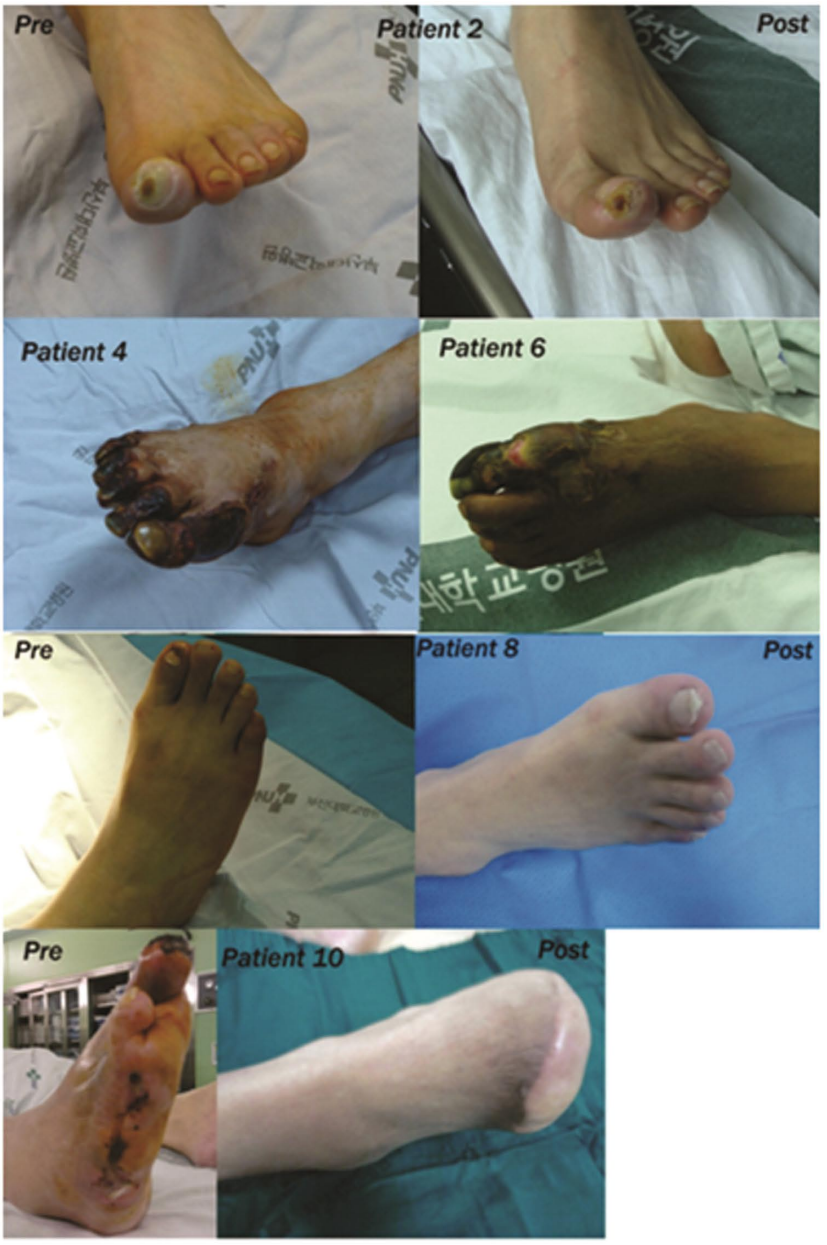

Figure 6. Wound healing after adipose tissue-derived mesenchymal stem cells (ATMSC) implantation. Patient 2: a 52-year-old man with thromboangiitis obliterans (TAO) presented with non-healing ulcer on toe was much improved at 6 months after ATMSC implantation. Patient 8: a 55-year-old man with TAO presented with small non-healing ulcer on toe was healed completely at 6 months after ATMSC implantation. Patient 10: a 69-year-old man with diabetic foot showed clear healing of ischemic necrosis on foot was clearly healed after amputation at 6 months after ATMSC implantation. Patients 4, 6: initially advanced necrotic foot, no observable tissue regeneration occurred.

outcomes of intramuscular BM-MNC implantation in ischemic limbs, this research group conducted a 3-year follow-up study. In contrast to the previous TACT study, ABI and TcO_2 did not change significantly; however, a significant improvement in leg pain, ulcer size, and pain-free walking distance was maintained for at least 2 years after therapy.[22] Idei et al compared the therapeutic efficacy of BM-MNC implantation in 25 PAD patients and 26 TAO patients.[23] Four-year amputation-free rates after BM-MNC implantation were 48% in PAD patients and 95% in TAO patients, and these values were 0% in control PAD patients and 6% in control TAO patients. Kim et al transplanted human leukocyte antigen-matched human umbilical cord blood-derived MSC into 4 men with TAO and observed disappearance of ischemic resting pain from affected extremities between 5h and 14 days and healing of necrotic skin lesions within 120 days.[24] However, in this study, the number of cases was small and limited functional evaluation was performed. To the best of our knowledge, this is the first report of ATMSC therapy for treating CLI in humans. The number of transplanted cells (3×10^8) used in our study was approximately 0.01 of the number of transplanted cells used in MNC studies. We have shown that autologous ATMSC implantation effectively increases blood flow, as assessed by new collateral vessel formation on DSA or by substantial improvement of the Wong-Baker FACES pain rating score, claudication walking distance, and thermography results. Clinical improvement of more than 2 steps was noted in 66.7% of patients. Only 5 patients could be examined using the treadmill test because many patients experienced major/minor amputation and/or a bypass operation or they had painful foot ulcers before enrolling in our study. In our study, the 6-month amputation-free rate was 66.7%, which is comparable to the

rates noted in previous studies. However, our results cannot be directly compared with results of other studies because differences in CLI severity and the underlying CLI causes in recruited patients can affect the clinical outcomes of cell implantation therapy.[23]

Underlying mechanisms of the beneficial effects of ADSC transplantation in PAD patients can infer from the previous animal studies. Our previous study[15,16] and other studies[12–14] demonstrated that the proangiogenic action of adipose tissue-derived cells in hindlimb ischemia model of nude mice is mostly resulted from ATMSC-derived paracrine factors including angiogenic and antiapoptotic factors, although small fraction of transplanted cells are differentiated into endothelial cells.

While most MNC transplantation studies have reported favorable results in patients with CLI,[25,26] Miyamoto et al reported that after BM-MNC implantation, 50% of 8 TAO patients experienced adverse effects during follow-up, including 1 case of sudden death at 20 months in a 30-year-old patient with no previous cardiac history (the cause was unknown), 1 patient with arteriovenous fistula formation at 7 months, and 2 patients with worsened symptoms.[26] In this study, no patients who underwent ATMSC implantation showed severe adverse effects during follow-up periods.

Our data shows that SVF in TAO patients and diabetic patients yield lower CFU values than normal individuals. However, culture-expanded B-ATMSC (P3) showed normal proliferation and differentiation potential. These results indicate that the adipose tissue of TAO patients contains lower MSC levels than that of normal individuals, but ATMSC exhibit normal function. However, in contrast with B-ATMSC, D-ATMSC exhibit altered proliferation potential. Hyperglycemia-induced impairment of stem cell functions have been reported in endothelial progenitor cells, muscle stem cells, and MSC.[27–29] Our previous study showed that exposure to high glucose and the presence of diabetes in mice impairs ATMSC functions.[30] In a rat diabetic model, MSC decreased osteogenic potential,[31] and exposure to high-glucose medium inhibited osteogenic differentiation and increased adipogenic differentiation in MG-63 osteosarcoma cells[32] and MSC.[33] Idei et al showed that the number of endothelial progenitor cells (EPC) and cell migration response to VEGF were significantly lower in atherosclerotic PAD patients compared to TAO patients, but the number and migration of EPC were similar between the TAO group and control group.[23] These results indicated that the functions of stem cells, including ATMSC, are compromised in diabetic patients. Our study showed that despite impaired functions of D-ATMSC, their transplantation resulted in a significant functional improvement of ischemic symptoms in 3 diabetic patients. These finding can be explained by the expression levels of angiogenic paracrine factors in D-ATMSC similar to those of normal ATMSC and the increase of VEGF expression under hypoxic condition. Selective increase of VEGF expression under hypoxic condition has been reported in the previous studies.[14,34] However, we cannot compare the statistical difference of the efficacy of ATMSC transplantation between TAO and diabetic foot groups directly, because our study is a phase I pilot study for the determination of safety and effects of ATMSC transplantation in CLI and the number of patients in this study is limited. Therefore, we cannot exclude the possibility that the use of immunocompetent allogeneic cells exhibiting normal functions might provide better therapeutic efficacy for CLI treatment in diabetic patients.

Jeong et al reported that growing tumors were observed in 30% of hearts in the MI model, and in 46% of hindlimbs in the diabetic neuropathy model during the follow-up at 4 to 8 weeks after human bone marrow-derived MSC transplantation.[35] However, human ATMSC maintains genomic stability in long term culture.[36,37] A recent study showed that intravenous infusion of ATMSC which were prepared with the same method in our study does not form any tumor in nude mice and human.[38] Although we have to analyze the safety issue of stem cells transplantation carefully, the possibility that early passage of ATMSC (3rd passage in our study) forms tumors during long term observation is low.

Our data indicate that multiple intramuscular ATMSC injections are a safe alternative to achieve therapeutic angiogenesis in CLI patients who are refractory to other treatment modalities.

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References

1. Dormandy JA, Rutherford RB. Management of peripheral arterial disease (PAD): TASC Working Group: TransAtlantic Inter-Society Consensus (TASC). *Eur J Vasc Endovasc Surg* 2000; **31**(1Pt2)**:** S1–S296.
2. Sasajima T, Kubo Y, Inaba M, Goh K, Azuma N, et al. Role of infrainguinal bypass in Buerger's disease: An eighteen-year experience. *Eur J Vasc Endovasc Surg* 1997; **2:** 186–192.
3. Esato K, Hamano K, Li TS, Furutani A, Seyama A, Takenaka H, et al. Neovascularization induced by autologous bone marrow cell implantation in peripheral arterial disease. *Cell Transplant* 2002; **8:** 747–752.
4. Higashi Y, Kimura M, Hara K, Noma K, Jitsuiki D, Nakagawa K, et al. Autologous bone-marrow mononuclear cell implantation improves endothelium-dependent vasodilation in patients with limb ischemia. *Circulation* 2004; **10:** 1215–1218.
5. Saigawa T, Kato K, Ozawa T, Toba K, Makiyama Y, Minagawa S, et al. Clinical application of bone marrow implantation inpatients with arteriosclerosis obliterans, and the association between efficacy and the number of implanted bone marrow cells. *Circ J* 2004; **12:** 1189–1193.
6. Tateishi-Yuyama E, Matsubara H, Murohara T, Ikeda U, Shintani S, Masaki H, et al; Therapeutic Angiogenesis using Cell Transplantation (TACT) Study Investigators. 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:** 427–435.
7. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; **284:** 143–147.
8. Reyes M, Dudek A, Jahagirdar B, Koodie L, Marker PH, Verfaillie CM, et al. Origin of endothelial progenitors in human postnatal bone marrow. *J Clin Invest* 2002; **3:** 337–346.
9. Al-Khaldi AH, Al-Sabti J, Galipeau J, Lachapelle K. Therapeutic angiogenesis using autologous bonemarrow stromal cells: Improved blood flow in a chronic limb ischemia model. *Ann Thorac Surg* 2003; **1:** 204–209.
10. Kim SJ, Cho HH, Kim YJ, Seo SY, Kim HN, Leo JB, et al. Human adipose stromal cells expanded in human serum promote engraftment of human peripheral blood hematopoietic stem cells in NOD/SCID mice. *Biochem Biophys Res Commun* 2005; **1:** 25–31.
11. Lee RH, Kim B, Choi I, Kim H, Choi HS, Suh K, et al. Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue. *Cell Physiol Biochem* 2004; **14:** 311–324.
12. Miranville A, Heeschen C, Sengenès C, Curat CA, Busse R, Bouloumié A, et al. Improvement of postnatal neovascularization by human adipose tissue-derived stem cells. *Circulation* 2004; **3:** 349–355.
13. Planat-Benard V, Silvestre JS, Cousin B, André M, Nibbelink M, Tamarat R, et al. Plasticity of human adipose lineage cells toward endothelial cells: Physiological and therapeutic perspectives. *Circulation* 2004; **5:** 656–663.
14. Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove CJ, Bovenkerk JE, et al. Secretion of angiogenic and antiapoptotic fac-

tors by human adipose stromal cells. *Circulation* 2004; **10:** 1292–1298.
15. Moon MH, Kim SY, Kim YJ, Kim SJ, Lee JB, Bae YC, et al. Human adipose tissue-derived mesenchymal stem cells improve postnatal neovascularization in a mouse model of hindlimb ischemia. *Cell Physiol Biochem* 2006; **17:** 279–290.
16. Cho HH, Kim YJ, Kim JT, Song JS, Shin KK, Bae YC, et al. The role of chemokines in proangiogenic action induced by human adipose tissue-derived mesenchymal stem cells in the murine model of hindlimb ischemia. *Cell Physiol Biochem* 2009; **24:** 511–518.
17. Jeon ES, Moon HJ, Lee MJ, Song HY, Kim YM, Bae YC, et al. Sphingosylphosphorylcholine induces differentiation of human mesenchymal stem cells into smooth-muscle-like cells through a TGF-beta-dependent mechanism. *J Cell Sci* 2006; **119:** 4994–5005.
18. Cho HH, Park HT, Kim YJ, Bae YC, Suh KT, Jung JS. Induction of osteogenic differentiation of human mesenchymal stem cells by histone deacetylase inhibitors. *J Cell Biochem* 2005; **96:** 533–542.
19. Mitchell JB, McIntosh K, Zvonic S, Garrett S, Floyd ZE, Kloster A, et al. Immunophenotype of human adipose-derived cells: Temporal changes in stromal-associated and stem cell–associated markers. *Stem Cells* 2006; **24:** 376–385.
20. Casteilla L, Planat-Benard V, Laharrague P, Cousin B. Adipose-derived stromal cells: Their identity and uses in clinical trials, an update. *World J Stem Cells* 2011; **3:** 25–33.
21. Behan MW, Holm NR, Curzen NP, Erglis A, Stables RH, de Belder AJ, et al. Simple or complex stenting for bifurcation coronary lesions: A patient-level pooled-analysis of the nordic bifurcation study and the british bifurcation coronary study. *Circ Cardiovasc Interv* 2011; **4:** 57–64.
22. Matoba S, Tatsumi T, Murohara T, Imaizumi T, Katsuda Y, Ito M, et al. TCT Follow-up Study Investigators. Long-term clinical outcome after intramuscular implantation of bone marrow mononuclear cells (Therapeutic Angiogenesis by Cell Transplantation [TACT] trial) in patients with chronic limb ischemia. *Am Heart J* 2008; **5:** 1010–1018.
23. Idei N, Soga J, Hata T, Fujii Y, Fujimura N, Mikami S, et al. Autologous bone-marrow mononuclear cell implantation reduces long-term major amputation risk in patients with critical limb ischemia: A comparison of atherosclerotic peripheral arterial disease and buerger disease. *Circ Cardiovasc Interv* 2011; **4:** 15–25.
24. Kim SW, Han H, Chae GT, Lee SH, Bo S, Yoon JH, et al. Successful stem cell therapy using umbilical cord blood-derived multipotent stem cells for Buerger's disease and ischemic limb disease animal model. *Stem Cells* 2006; **6:** 1620–1626.
25. Kajiguchi M, Kondo T, Izawa H, Kobayashi M, Yamamoto K, Shintani S, et al. Safety and efficacy of autologous progenitor cell transplantation for therapeutic angiogenesis in patients with critical limb ischemia. *Circ J* 2007; **71:** 196–201.
26. Miyamoto K, Nishigami K, Nagaya N, Akutsu K, Chiku M, Kamei M, et al. Unblinded pilot study of autologous transplantation of bone marrow mononuclear cells in patients with thromboangiitis obliterans. *Circulation* 2006; **114:** 2679–2684.
27. Aguiari P, Leo S, Zavan B, Vindigni V, Rimessi A, Bianchi K, et al. High glucose induces adipogenic differentiation of muscle-derived stem cells. *Proc Natl Acad Sci USA* 2008; **105:** 1226–1231.
28. Kränkel N, Adams V, Linke A, Gielen S, Erbs S, Lenk K, et al. Hyperglycemia reduces survival and impairs function of circulating blood-derived progenitor cells. *Arterioscler Thromb Vasc Biol* 2005; **5:** 698–703.
29. Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res* 2001; **89:** E1–E7.
30. Kim HK, Kim YJ, Kim JT, Kwon CH, Kim YK, Bae YC, et al. Alterations in the proangiogenic functions of adipose tissue-derived stromal cells isolated from diabetic rats. *Stem Cells Dev* 2008; **17:** 669–680.
31. Stolzing A, Sellers D, Llewelyn O, Scutt A. Diabetes induced changes in rat mesenchymal stem cells. *Cells Tissues Organs* 2010; **191:** 453–465.
32. Wang W, Zhang X, Zheng J, Yang J. High glucose stimulates adipogenic and inhibits osteogenic differentiation in MG-63 cells through cAMP/protein kinase A/extracellular signal-regulated kinase pathway. *Mol Cell Biochem* 2010; **338:** 115–122.
33. Cramer C, Freisinger E, Jones RK, Slakey DP, Dupin CL, Newsome ER, et al. Persistent high glucose concentrations alter the regenerative potential of mesenchymal stem cells. *Stem Cells Dev* 2010; **19:** 1875–1884.
34. Thangarajah H, Vial IN, Chang E, El-Ftesi S, Januszyk M, Chang EI, et al. IFATS collection: Adipose stromal cells adopt a proangiogenic phenotype under the influence of hypoxia. *Stem Cells* 2009; **27:** 266–274.
35. Jeong JO, Han JW, Kim JM, Cho HJ, Park CW, Lee NH, et al. Malignant tumor formation after transplantation of short-term cultured bone marrow mesenchymal stem cells in experimental myocardial infarction and diabetic neuropathy. *Circulation Research* 2011; **108:** 1340–1347.
36. Grimes BR, Steiner CM, Merfeld-Clauss S, Traktuev DO, Smith D, Reese A, et al. Interphase FISH demonstrates that human adipose stromal cells maintain a high level of genomic stability in long-term culture. *Stem Cells Dev* 2009; **18:** 717–724.
37. Meza-Zepeda LA, Noer A, Dahl JA, Micci F, Myklebost O, Collas P, et al. High-resolution analysis of genetic stability of human adipose tissue stem cells cultured to senescence. *J Cell Mol Med* 2008; **12:** 553–563.
38. Ra JC, Shin IS, Kim SH, Kang SK, Kang BC, Lee HY, et al. Safety of intravenous infusion of human adipose tissue-derived mesenchymal stem cells in animals and humans. *Stem Cells Dev* 2011; **20:** 1297–1308.