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**Abstract**

Buerger’s disease or Thromboangiitis obliterans is an orphan vascular disease that most commonly affects nerves, small or medium-sized vessels in the upper and lower extremities, and is characterized by a non-atherosclerotic, segmental, inflammatory disorder. The etiology and the pathogenesis of the disease have not been fully elucidated. Although various interventions have been adopted recently, there is still no effective treatment for the prevention of the progression of the disease. This report presents three clinical cases that show the efficacies of autologous adipose tissue-derived mesenchymal stem cell (AdMSC) treatment in Buerger’s disease. Three male patients diagnosed with Buerger’s disease were between 46 and 55 years and had a smoking history. AdMSCs (5X10^6 cells/kg body weight) were injected intramuscularly into at least 38 points of the ischemic legion of the lower limb at one time. The patients were checked for safety and efficacy at one, three, and six months after AdMSC injection. No severe adverse events and no adverse drug events were observed in physical examination, vital signs, and laboratory tests for all three patients. Ulcers in the affected legs of the patients were healed completely after the treatment. Visual Analogue Scale scores and all the criteria (activities, emotional, pain, social, symptoms and total) of the King’s College Hospital’s Vascular Quality of Life Questionnaire (VascuQOL) of all the patients were improved from baseline to six months follow-up. Digital Infrared Thermal Imaging showed the gradual alleviation of lesions in the leg. Angiogenesis in the affected limbs was identified by CT-Angiography after AdMSC injection. The present cases show the improvement in patients with Buerger’s disease with the observation of angiogenesis after intramuscular injection of autologous AdMSCs. This suggests that autologous AdMSC can be an effective alternative treatment for Buerger’s disease.

**Keywords**

Buerger’s disease, autologous adipose tissue-derived mesenchymal stem cell, intramuscular injection
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Introduction

Buerger’s disease or Thromboangiitis obliterans is an orphan vascular disease that most commonly affects small/medium-sized vessels and is characterized by a non-atherosclerotic, occlusive, thrombotic, segmental, inflammatory disorder in the upper and lower extremities\(^1\). The etiology is unknown, and the pathogenesis of the disease has not been fully elucidated. Typical Buerger’s disease patients are mostly men (<45 years) with a history of smoking, and exhibit various symptoms, such as progressive claudication, ischemic ulcers and rest pain, which are thought be due to peripheral vascular disorders. In severe cases, lesions in the affected limbs are exacerbated and lead to tissue death, and eventually amputation.

Currently, the cessation of smoking is known to be the most effective treatment, however, the management of smoking is considered difficult and cessation has been known to be insufficient for severe ischemic states in patients with Buerger’s disease\(^2\)-\(^6\). There have been various treatments based on the symptoms and pathophysiology of Buerger’s disease, including medications for pain-relief, vascular inflammation, vasodilatation or antiplatelet effect, and medical (surgical) intervention, such as sympathectomy, endovascular angioplasty and bypass surgery\(^7\). However, these are only palliative for symptoms and there is no treatment or interventions reported to be effective for the prevention of the progression of the disease. Recently, mesenchymal stem cells (MSCs) from various sources emerge as a promising alternative for Buerger’s disease treatment\(^8\)-\(^10\). Angiogenesis is thought to be due to peripheral vascular disorders. In severe cases, lesions in the affected limbs are exacerbated and lead to tissue death, and eventually amputation.

We previously studied that the safety and the efficacy of adipose-derived mesenchymal stem cells (AdMSC) on the treatment of Buerger’s disease\(^1\). Here, we report three patients diagnosed with Buerger’s disease where treatment with AdMSCs brought about angiogenesis in the affected limbs.

Treatment with AdMSCs

Administration of autologous AdMSCs for Buerger’s disease was approved by the Korean Ministry of Food and Drug Safety with Investigational New Drug Application for Emergency Use (Approval Nos. 20170072755, 20170072828, 20170072872). The protocol for administration of AdMSCs was conducted in compliance with the Helsinki declaration and approved by the Institutional Review Board of Bethesda Hospital, Yangsan, Korea (Approval No. 2016-6). Written informed consent to take part in the treatment was acquired from all patients before the initiation of treatment.

The three cases presented herein were male patients with smoking history (currently non-smoking) and aged between 46 and 55 years. All patients showed the corkscrew appearance or luminal obstruction of the medium-sized or smaller arteries by angiogram, and were diagnosed Buerger’s disease at least six months before the start of AdMSC treatment (Table 1). These patients were suggested for treatment with AdMSCs (between April 2017 and April 2018) as they were classified as Rutherford class III-5\(^5\) by clinical description, experienced ischemic rest pain and ulcers, and showed the recurrence or no improvement after previous treatments. The baseline characteristics including previous treatment and medications are shown in Table 1.

The isolation and characterization of the autologous AdMSCs were performed using a previously established culture protocol\(^1\) under good manufacturing practice conditions in the Stem Cell Research Institute of R Bio (Seoul, Republic of Korea). Briefly, abdominal subcutaneous adipose tissue was obtained through liposuction three weeks before administration, and digested with collagenase \(1\) (Gibco/Life Technologies, Grand Island, NY, USA). After centrifugation, the pellet was resuspended in DMEM (Invitrogen, Carlsbad, CA, USA)-based media containing 0.2 mM ascorbic acid and 10% fetal bovine serum (FBS; JR Scientific, Woodland, CA, USA). The cell fraction was cultured overnight at 37°C/5% CO\(_2\), and cell adhesion was checked after 24 h. Cells were maintained for 4 to 5 days until confluent (passage 0). When the cells reached 90% confluency, they were subcultured to expand in keratinocyte SFM-based media (Invitrogen, USA) containing 0.2 mM ascorbic acid, 0.09 mM calcium, 5 ng/ml rEGF, and 5% FBS until passage 3. Before transporting the cells for administration, aliquots of the AdMSCs were tested for cell viability, fungal, bacterial, endotoxin, and mycoplasma contamination and immunophenotype for MSCs. Cell viability evaluated by trypan blue exclusion was >91%, and no evidence of bacterial, fungal and mycoplasma contamination was observed. The AdMSCs showed a homogenous population of cells with high positive marker expression levels of CD73 and CD90 at a very low level of <0.08%.

Since most patients showed allodynia, intramuscular (IM) injections were carried out under spinal anesthesia. According to our previously reported protocols\(^6\), AdMSCs were prepared at a concentration of 1X10\(^7\) cells/0.5 ml saline/syringe before IM injection. Finally adjusted AdMSCs were administered at the dose of 5X10\(^6\) cells/kg (based on body weight of the patient) were injected into multiple sites (at least 38 points) of the ischemic zone of the lower extremities (the feet of the three patients) at one time. Before the IM injection, the ischemic legions on the affected limbs were identified by Digital Infrared Thermal Imaging (DITI). To assess safety and efficacy, all the patients were followed up at one, three, and six months after the IM injection.

Safety was assessed during follow-up by looking at vital signs, physical examination, laboratory tests, adverse events, and serious adverse events as described in our previous reports\(^7\). For the evaluation of the efficacy, the following assessment were performed at every follow-up: Visual Analogue Scale (VAS) for rest pain, designated as 1 (best) to 10 (worst); King’s College Hospital’s Vascular Quality of Life Questionnaire (VascuQoL), consisting of 25 questions grouped into 5 domains (activity, emotional, pain, symptoms, social), for the disease-specific quality of life assessment\(^8\); assess ulcer size and wound healing; assess the risk of additional amputation; DITI for the identification of ischemic legions before and after the
Table 1. Baseline characteristics of patients.

<table>
<thead>
<tr>
<th>Patient (Age, years)</th>
<th>Rutherford scale</th>
<th>Rest pain before AdMSC treatment</th>
<th>Ulcers at baseline</th>
<th>Symptom onset (years ago)</th>
<th>Smoking duration (years)</th>
<th>Previous amputation history</th>
<th>Previous treatment (continued to last F/u)</th>
<th>Claudication</th>
<th>Pain area</th>
<th>Other symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>001 (48)</td>
<td>III-5</td>
<td>Yes</td>
<td>No</td>
<td>18</td>
<td>6</td>
<td>Lt. 1, 2, 3, 4th toe partial amputation; Rt 3rd toe total amputation</td>
<td>Angioplasty 4 times; Allogenic stem cell treatment: 8yrs ago</td>
<td>Aspirin (100mg, qd), clopidogrel (75mg, qd), beraprost (0.06mg, tid), Vytorin (10mg, qd, ), Mypol (as codeine phosphate 20mg, qd)</td>
<td>30 m</td>
<td>Lt thigh, both calves, both feet, both hands</td>
</tr>
<tr>
<td>002 (55)</td>
<td>III-5</td>
<td>Yes</td>
<td>Rt big toe, Rt 2nd toe</td>
<td>10</td>
<td>34</td>
<td>Both big toe partial amputation; Rt 2nd toe partial amputation</td>
<td>Hyperbaric oxygen therapy</td>
<td>Sarpogrelate (100mg, tid), ginkgo leaf extract (80mg, bid), aceclofenac (100mg, bid), pregabalin (75mg, bid), ciprofloxacin (250mg, bid)</td>
<td>300 m</td>
<td>Both calves, both feet, both hands</td>
</tr>
<tr>
<td>003 (45)</td>
<td>III-5</td>
<td>Yes</td>
<td>No</td>
<td>19</td>
<td>25</td>
<td>Rt 4,5th toe total amputation</td>
<td>Bypass graft, angioplasty</td>
<td>Clostazol (200mg, qd), aspirin (100mg, qd), warfarin (5mg, qd), oxycodone (10mg, tid)</td>
<td>50 m</td>
<td>Both calves, Rt foot</td>
</tr>
</tbody>
</table>
injection of AdMSC. Computed Tomography (CT)-Angiography was carried out for the evaluation of angiogenesis at baseline and the last follow-up.

**Case 1 (patient 001)**

A man, aged 48 years, whose onset of Buerger’s disease symptoms had begun 18 years ago, was admitted to the hospital and identified for treatment with AdMSCs. The patient had a smoking history from age of 24 to 30, and had stopped smoking after the diagnosis of Buerger’s disease. At the time of visiting the hospital for treatment with AdMSCs, the patient had partial amputation in the left 1, 2, 3, 4th toes and total amputation in the right 3rd toe, with no ulcers. The patient already had a history of angioplasty four times and received allogenic stem cell therapy, but the effect of this treatment disappeared two months after the treatment. The patients showed severe pain, allodynia, rest pain, claudication (30 m), and pains in the left thigh, both calves, both feet, and both hands. The patient was on the following medication, which was maintained during treatment with AdMSCs: Aspirin (100mg, qd), clopidogrel (75mg, qd), beraprost (0.06mg, tid), Vytorin (10mg, qd), Mypol (as codeine phosphate 20mg, qd) (summarized in Table 1, with other characteristics). The analgesics were decreased in dose (Mypol 20mg per three days) during the follow-up period as symptoms improved.

No severe adverse events and no adverse drug events were observed during follow-up after treatment with AdMSCs regarding physical examination (including ulcer size check, capillary refill test), vital signs (temperature, pulse, and blood pressure respiration), and laboratory tests (hematology, biochemistry, urinalysis). Vas and VascuQoL scores showed improvement one month after the treatment (Figure 1A, B). No additional ulcers were observed and no amputations were required. Rest pain and allodynia disappeared, and quality of sleep was improved as night pain disappeared during the follow-up period. Claudication was also improved from 30 m at baseline to 100 m at the final follow-up. DITI images in Figure 2 showed gradual alleviation in the affected lower limbs after three months from the treatment, and also improvement in the non-affected opposite limb six months after AdMSC injection. The persistence of this alleviation effect was identified by DITI images in the additional visiting

![Figure 1](image-url). Changes in Visual Analogue Scale (A) and the King’s College Hospital’s Vascular Quality of Life Questionnaire (VascuQoL) scores (B-D) of the individual patients from baseline to last follow-up (six months) after the injection of AdMSCs.
the hospital one year after AdMSC injection. Angiogenesis in the affected limbs was identified by CT-Angiography after AdMSC injection. The formation of collateral arteries in the lesions was newly observed in the non-injected right leg (Figure 3).

**Case 2 (patient 002)**
A man, aged 55 years, was diagnosed with Buerger’s disease 10 years ago. The patient had 34 years of smoking history from the age of 20 to 54 and stopped smoking one year ago prior to treatment with AdMSCs. Both hands and legs were affected and amputations had been performed partially in both big toes and right 2nd toe. Ulcers were observed in the right big and 2nd toes. The patient had a history of hyperbaric oxygen therapy and no angioplasty. After the partial amputation of affected toes, the following medication was taken by the patient, and was continued during the follow-up period after AdMSC treatment: sarpogrelate (100mg, tid), ginkgo leaf extract (80mg, bid), aceclofenac (100mg, bid), pregabalin (75mg, bid), and ciprofloxacin (250mg, bid). There were pains in both calves, feet, and hands, and stiffness in the feet and lumbodynia after long-distance walking, but no signs in the thigh.

No severe adverse events and no adverse drug events were observed during follow-up after treatment with AdMSCs regarding physical examination (including ulcer size check, capillary refill test), vital signs (temperature, pulse, and blood pressure respiration), laboratory tests (hematology, biochemistry, urinalysis). Vas and VascuQoL scores showed improvement one month after the treatment (Figure 1A, C). Claudication was improved from 300 m at baseline to 600 m at the final follow-up. The ulcers on the right big and 2nd toes at baseline showed bone exposure and after six months exhibited a complete healed state with no additional ulcers observed and amputations required (Figure 4). This complete healed state was identified in the reinspection one year after AdMSC injection. Rest pain had disappeared, and most symptoms were improved, and all medications were stopped one month after treatment with AdMSCs. DITI images showed alleviation in the affected right limb one month after treatment and also showed improvement in the non-affected opposite limb three months after treatment. The improved state was maintained six months after treatment. Angiogenesis in the right limb was identified by CT-Angiography and the formation of new collateral arteries was observed in the right leg after AdMSC injection (Figure 5).

**Case 3 (patient 003)**
A man, aged 45 years, whose onset of Buerger’s disease symptoms had begun 19 years ago, was admitted to the hospital. The patient had 25 years of smoking history from the age of 20 to 45 and stopped smoking after angioplasty 8 months ago prior to the treatment with AdMSCs. Both legs were affected and amputations had been carried out on the right 4 and 5th toes. There were no ulcers at the time of treatment with AdMSCs. The patient had bypass graft on the right leg 5 years previous to treatment. The patient reported pain in both calves and right foot, allodynia, rest pain, claudication (50 m.), Raynaud’s symptom in both hands, and slow capillary filling in both fingers and toes. The patient was on the following medication which was maintained during treatment with AdMSCs: Cilostazol (200mgm qd), aspirin (100mg, qd), warfarin (5mg, qd), oxycodone (10mg, tid).

No severe adverse events and no adverse drug events were observed during follow-up after treatment with AdMSCs regarding physical examination (including ulcer size check, capillary refill test), vital signs (temperature, pulse, and blood pressure respiration), laboratory tests (hematology, biochemistry, urinalysis) during the study. Vas and VascuQoL scores showed improvement one month after treatment (Figure 1A, D). Rest pain, allodynia, and Raynaud’s symptoms disappeared and quality of sleep was improved as night pain disappeared during the follow-up period. Most of the symptoms were improved and the analgesics have decreased the dose (oxycodone 5 mg, tid) during the follow-up period as pains alleviated. Claudication was also improved from 50 m at baseline to 300 m at final follow-up. DITI images showed the gradual alleviation process in the affected lower limb three months after treatment, and also showed improvement in the non-affected opposite limb at the final follow-up (Figure 6). Angiogenesis in the affected left limb was identified by CT-Angiography after AdMSC injection. Newly formed collateral
Figure 3. CT-angiography images of the left leg of patient 001 at baseline (A, C) and at six months (B, D) after injection of AdMSCs showed the increased numbers of collateral arteries (arrows). The arrowheads (B) indicated the newly formed collateral arteries in the right leg that was not injected.

Figure 4. The healing process of a toe ulcer in patient 002. The patient's right second and big toe showed ulcers and exposed bone on the second toe at baseline (A, B). At six months of AdMSC treatment (C, D), the big toe ulcer was completely healed and the second toe is shown just after the crust was removed during healing. The ulcer remained completely healed at one year after AdMSC treatment (E, F).

Figure 5. CT-angiography images of the right leg of patient 002 at baseline (A, C) and at six months (B, D) after the injection of AdMSCs showed increased numbers of collateral arteries (arrows). Arteries in the injected lesions was observed in the non-injected right leg at the final follow-up (Figure 7).

Discussion
Etiology, e.g. genetics, and pathophysiology of Buerger’s disease still remain uncertain, with the exception of its high correlation with smoking. There are no standard diagnostic criteria and no treatment guidelines or protocols for Buerger’s disease. Treatment and assessment of its efficacy remain debatable for these reasons. However, recent studies and clinical trials have indicated that the restoration of angiogenesis is the key for alleviation of symptoms and the fundamental therapy for Buerger’s disease.

The present cases reported the improvement of patients diagnosed with Buerger’s disease after the administration of AdMSCs. Ulcers present in some of the patients on affected limbs were completely healed, a major symptom of Buerger’s disease, and rest pain and claudication were alleviated. In addition, assessment of the patients using VAS scale and VascuQoL indicated treatment satisfaction without any adverse events. VascuQoL is vascular disease-specific, and is a reliable and validated assessment. Along with our previous findings demonstrating the safety and functional improvement in the patients with Buerger’s disease, the present cases suggest that AdMSCs are involved in modulating inflammation and pain.
Angiogenesis in the ischemic limb in the present cases were identified by non-invasive CT-angiography after the injection of AdMSC, and corresponded with the results of the previous study using the AdMSC provided by our previous established protocols\textsuperscript{12}. Moreover, angiogenesis was also found in the counterpart limb, which had not been injected. These findings demonstrate that focally injected AdMSCs could conduct systemic angiogenetic characteristics in patients with Buerger’s disease. The outcomes of the present clinical cases are considered as a result of the synergy between angiogenetic properties and anti-inflammatory/immunomodulatory action of AdMSC. These various functions of the MSCs are known to be exerted by paracrine actions with the release of extracellular vesicles, exosomes\textsuperscript{13-14}. Previous studies report that AdMSCs secret soluble angiogenetic factors, such as vascular endothelial growth factor (VEGF), fibroblast grow factor-2 (FGF-2), interleukin-6 (IL-6)\textsuperscript{13-15}. AdMSCs have been reported to show various advantages over MSCs from other sources, including a less invasive sampling procedure, higher cell numbers from tissue harvested, higher capacity for proliferation and higher capacity of

\begin{figure}
\centering
\includegraphics[width=\textwidth]{image1.png}
\caption{DITI images of patient 003 showing the improvement of the right leg at baseline (A), three months (B), six months (C) after injection of AdMSCs.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{image2.png}
\caption{CT-angiography images of the right leg of patient 003 at baseline (A, C) and six months (B, D) after the injection of AdMSCs showing thicker and more abundant arteries (arrows and arrowheads).}
\end{figure}
angiogenesis. The angiogenic potential is essential for recovering damaged tissues. Newly formed blood vessels are extremely helpful for the migration of versatile stem cells to affected lesions and for the transportation of various factors, which is vital for the regeneration of tissues and tissue function.

Taken together, these cases would suggest that AdMSCs have potential advantages for regenerative medicine, and especially AdMSCs may be promising alternatives in orphan disease or emergency cases, such as Buerger’s disease. However, the restricted numbers of patients presented here and the short period of follow-up limit the assessment of the long lasting angiogenic potential of AdMSCs. Nonetheless, the present clinical cases show improvement and safety of IM injection of AdMSCs in patients with Buerger’s disease, leading to an alleviation of symptoms and observation of angiogenesis in the affected limbs. Further studies are needed for continuous follow-up to optimize the treatment protocol. A precise assessment of the efficacy of AdMSCs in larger clinical trials will also be needed.

Data availability
All data underlying the results are available as part of the article and no additional source data are required.

Consent
Written informed consent for publication of the patients’ clinical details and associated images was obtained from each patient.

References

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