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CLINICAL PRACTICE ARTICLE

Treatment of Buerger's disease (Thromboangiitis obliterans) with autologous adipose tissue-derived mesenchymal stem cell: Report of three cases [version 1; peer review: 2 approved with reservations]

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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 (5×10^6 cells/kg body weight) were injected intramuscularly into at least 38 points of the ischemic leg 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.

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Approval Status

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2. Kenji Yanishi , Kyoto Prefectural University of Medicine, Kyoto, Japan		
Any reports and responses or comments on the article can be found at the end of the article.		

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 extremities¹⁻³. 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 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.

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^{2,4-6}. There have been various treatments based on the symptoms and pathophysiology of Buerger's disease, including medications for pain-relief, vascular inflammation, vasodilation or antiplatelet effect, and medical (surgical) intervention, such as sympathectomy, endovascular angioplasty and bypass surgery². 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^{1,7}. Angiogenesis is considered as a crucial condition to preserve the affected limbs and prohibit the aggravation of the disease, and previous studies report that MSCs has an angiogenic nature⁷.

We previously studied that the safety and the efficacy of adipose-derived mesenchymal stem cells (AdMSC) on the treatment of Buerger's disease⁸. 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-5⁹ 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 protocol⁸ 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 I (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% CO₂, 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 high level of >92% and >99%, respectively. Negative markers of CD31, CD34, and CD45 were expressed 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 protocols⁸, AdMSCs were prepared at a concentration of 1X10⁷ cells/0.5 ml saline/syringe before IM injection. Finally adjusted AdMSCs were administered at the dose of 5X10⁶ 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 reports⁸. 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 (Vascu-QoL), consisting of 25 questions grouped into 5 domains (activity, emotional, pain, symptoms, social), for the disease-specific quality of life assessment^{10,11}; 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.

Patient (Age, years)	Rutherford scale	Rest pain before AdMSC treatment	Ulcers at baseline	Symptom onset (years ago)	Smoking duration (years)	Previous amputation history	Previous treatment	Previous medication (continued to last F/u)	Claudication	Pain area	Other symptoms
001 (48)	III-5	Yes	No	18	6	Lt. 1,2,3,4 th toe partial amputation; Rt 3 rd toe total amputation	Angioplasty 4 times; Allogenic stem cell treatment: 8yrs ago	Aspirin (100mg, qd), clopidogrel (75mg, qd), beraprost (0.06mg, tid), Vytorin (10mg, qd.), Mypol (as codeine phosphate 20mg, qd)	30 m	Lt thigh, both calves, both feet, both hands Lt leg is more severe	Allodynia
002 (55)	III-5	Yes	Rt big toe, Rt 2 nd toe	10	34	Both big toe partial amputation; Rt 2 nd toe partial amputation	Hyperbaric oxygen therapy	Sarpogrelate (100mg, tid), ginkgo leaf extract (80mg, bid), aceclofenac (100mg, bid), pregabalin (75mg, bid), ciprofloxacin (250mg, bid)	300 m	Both calves, both feet, both hands	None
003 (45)	III-5	Yes	No	19	25	Rt 4,5 th toe total amputation	Bypass graft, angioplasty	Cilostazol (200mg, qd), aspirin (100mg, qd), warfarin (5mg, qd), oxycodone (10mg, tid)	50 m	Both calves, Rt foot	Allodynia, Raynaud's symptom

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), Vytarin (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. 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.

Figure 2. DITI images of patient 001 showing the improvement of the left leg at baseline (A), six months (B), one year (C) after 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 legs 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 lumbodysnia 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 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).

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**).

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 smoking¹. There are no standard diagnostic criteria and no treatment guidelines or protocols for Buerger's disease². 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 disease⁷.

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 VasuQoL indicated treatment satisfaction without any adverse events. VasuQoL is vascular disease-specific, and is a reliable and validated assessment¹⁰⁻¹¹. Along with our previous findings demonstrating the safety and functional improvement in the patients with Buerger's disease⁸, the present cases suggest that AdMSCs are involved in modulating inflammation and pain.

Figure 6. 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.

Figure 7. 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).

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 protocols¹². 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^{13–14}. Previous studies report that AdMSCs secrete soluble angiogenetic factors, such as vascular endothelial growth factor (VEGF), fibroblast grow factor-2 (FGF-2), interleukin-6 (IL-6)^{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

angiogenesis^{13,14,16–18}. The angiogenetic potential is essential for recovering damaged tissues¹⁴. 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^{13,14}.

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 angiogenetic 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.

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Kenji Yanishi

Department of Cardiology, Kyoto Prefectural University of Medicine, Kyoto, Japan

Thank you very much for the opportunity of paper review. Cell therapy using autologous adipose tissue-derived mesenchymal stem cells (AdMSCs) is expected as a new therapy for patients with no-option chronic limb-threatening ischemia (CLTI). In addition, it is very interesting that this manuscript shows that cell therapy using AdMSCs tended to be effective. However, I have some concerns below follows to accept these results.

1. The number of patients in this study is small. So, it is difficult to judge the efficacy of this cell therapy in patients with CLTI based on these results.
2. Thermography imaging technics may be effective for screening of severe ischemic limb, but this finding is insufficient as a clinical endpoint compared with skin perfusion pressure or transcutaneous oxygen tension.
3. It is interesting to use CT-angiography to evaluate the peripheral angiogenesis after this cell therapy. However, the protocol of the CT-angiography is unclear in three cases. Did authors undergo CT-angiography under uniform conditions before and after this cell therapy? In addition, it is unclear where is the CT value cut-off set. Authors should describe the protocol of CT-angiography.

Is the background of the cases' history and progression described in sufficient detail?

Yes

Are enough details provided of any physical examination and diagnostic tests, treatment given and outcomes?

Partly

Is sufficient discussion included of the importance of the findings and their relevance to future understanding of disease processes, diagnosis or treatment?

Partly

Is the conclusion balanced and justified on the basis of the findings?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Therapeutic angiogenesis, Chronic limb threatening ischemia

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 30 Jun 2021

Jeong Chan Ra, R Bio Co. Ltd, Seoul, South Korea

First of all, I am very sorry for the too late reply to your kind comments, and Thank you for your careful reading and considerate comments on our manuscripts.
I hope our late reply hasn't caused any inconvenience to you

1. The number of patients in this study is small. So, it is difficult to judge the efficacy of this cell therapy in patients with CLTI based on these results.

I would like to mention that this clinical study is not written as explorative research nor designed clinical trials, but a case report reporting the treatment result of 3 emergency cases with the approval of the Korean Ministry of Food and Drug Safety for Emergency Use. The condition for the approval of the KMFDS for Emergency Use is restrictive. In these cases, the number of enrolled patients was small, because they were to be satisfied with several conditions, such as the term after diagnosed with TAO (over 6 months) and showing the recurrence or no improvement after previous treatments. The present study is a case report on the improvement status of the patients with TAO, a rare disease having difficulty recruiting enough patients for collecting clinical data.

2. Thermography imaging technics may be effective for screening of severe ischemic limb, but this finding is insufficient as a clinical endpoint compared with skin perfusion pressure or transcutaneous oxygen tension.

Yes, ABI(ankle-brachial index) or TBI(toe brachial index) have been used in many CLTI studies. However, the ABI, or ankle pressure may not predict a walking distance or reflect the status of the disease (PMID: 12099140). In the present study, the patients were not applied ABI or TBI for the difficulty of the adequate comparison; the patients with partial amputation of the big toe or with complete amputation of the 4th and 5th toes could not be considered for measuring TP (toe pressure). The hospital to which the enrolled patients were referred had not equipped TcPO2 (Transcutaneous oximetry) and the measuring TcPO2 was not considered.

Of course, there may be lots of difficulties to apply DITI (Digital Infrared Thermal Imaging) as an assessment standard for clinical status. DITI instrument is set up by the relative

temperature, not absolute temperature, and, even in the identical individual, the coloring information from DITI usually reflects intrinsic & extrinsic variables, which may influence the blood circulation, such as coffee, nicotine, seasonal effect, use of vasodilator, or patients' exercise status. However, DITI has the advantage to detect and comparing abnormal thermal distribution by measuring thermal symmetry (PMID: 3418388). DITI easily allows to assessing thermal difference and thermal change by comparing affected and unaffected limbs, or by systemic thermal distribution before and after administration, so allows determining improvement or aggravation of the patients' status. Thus, though DITI may not be unsuitable for measuring the quantitative comparison of the thermal change, it can be used for evaluating the patients' status, progressing to whether improvement or worsen by the comparison of the change in the legions before and after administration of AdMSC.

3. It is interesting to use CT-angiography to evaluate peripheral angiogenesis after this cell therapy. However, the protocol of the CT-angiography is unclear in three cases. Did authors undergo CT-angiography under uniform conditions before and after this cell therapy? In addition, it is unclear where is the CT value cut-off set. The authors should describe the protocol of CT-angiography.

DSA (digital subtraction angiography) would be an effective tool for comparing or predicting the prognosis of TAO patients. However, DSA is known for an invasive procedure and might burden patients who already suffering from angiopathy. CT-angiography was determined to be adapted for the patients in the present case report for alleviating the burden of the patients and with reference that CT-angiography could show the adjacent results to DSA data (PMID: 21658691).

In addition to the comparison of the thermal distribution, the improvement status of the patients after the treatment was checked by the findings of the newly formed collateral arteries. The neogenesis of collateral arteries was macroscopically identified using the anatomical landmarks in the images which were taken in the same condition (the same regions, the same radio-intensity, and non-manipulated contrasts) and the same protocol before and after administration. The present emergency case estimated the efficacy of the administration of AdMSC by the findings of the newly formed collateral arteries after the treatment, not by its quantitative comparisons with the statistical meaning, as in the efficacy test of the clinical trials or research articles, which could be assessed by the Cut-off set or the Hounsfield unit (HU) scale. Again, I would like to comment that the present case study reported the improvement status of 3 TAO cases based on the clinical observations from the point of view of a clinician for emergency patients. Of course, further studies should be needed with an intensive evaluation of the protocol assessment on larger scales.

Competing Interests: No competing interests were disclosed.

Reviewer Report 15 May 2020

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Pawan K. Gupta

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Comments:

1. Once the MSCs are being isolated from the adipose tissue, the following details can be added:
 - a. The passage number or doubling time of the MSCs.
 - b. Time period of storage of the cells and the stability time of these cryopreserved cells.
 - c. Any *in vitro* studies potency assay done for this indication for use of Adipose tissue-derived MSCs.
 - d. Was Karyotyping analysis done as it is not mentioned in the text?
 - e. Please mention the values of HLA DR during the release of the cells, if done.
2. All the patients enrolled in the study were labeled as Rutherford III - 5. As per the definition Rutherford III - 5 is defined as "Minor tissue loss—nonhealing ulcer, focal gangrene with diffuse pedal ischemia". But patient numbers 001 and 003 do not have ulcers at baseline. Please reconsider the grade of these patients.
3. Was ABPI, ankle pressure or TcPO2 measured in these patients as they are very important indirect findings of increased oxygenation of tissues.
4. Case number 002, the hands were also involved. Whether any injections of MSCs were given in the upper limbs also.
5. In the discussion section more can be discussed on the advantage of CT angiography on MRA or DSA as CT angiography was done in the three patients.
6. No new data is coming out from this small study of three patients as it is now a well-known fact that stem cells improved angiogenesis in these patients.

Is the background of the cases' history and progression described in sufficient detail?

Yes

Are enough details provided of any physical examination and diagnostic tests, treatment given and outcomes?

Yes

Is sufficient discussion included of the importance of the findings and their relevance to future understanding of disease processes, diagnosis or treatment?

Partly

Is the conclusion balanced and justified on the basis of the findings?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Hematology, Stem cells and Regenerative Medicine.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 19 Jun 2020

Jeong Chan Ra, R Bio Co. Ltd, Seoul, South Korea

First of all, I'm very sorry for the late reply and Thank you for your careful reading and kind comments on our manuscripts.

1. Once the MSCs are being isolated from the adipose tissue, the following details can be added:

- a. The passage number or doubling time of the MSCs.**
- b. Time period of storage of the cells and the stability time of these cryopreserved cells.**
- c. Any in vitro studies potency assay done for this indication for use of Adipose tissue-derived MSCs.**
- d. Was Karyotyping analysis done as it is not mentioned in the text?**
- e. Please mention the values of HLA DR during the release of the cells, if done.**

Because the present study was carried out for Emergency use, not for the formal clinical trials, it was not needed to include the analysis of karyotyping and HLA DR. So, the karyotyping analysis and HLA-DR analysis was not performed. Also, for emergency use, the fresh AdMSC was injected within hours after testing as written in the text and the passage number was described in the text. The AdMSC in the present study was routinely manufactured by previously established culture protocol under good manufacturing practice conditions (<https://pubmed.ncbi.nlm.nih.gov/24449146/> , <https://pubmed.ncbi.nlm.nih.gov/28713639/>)

There have been considerable reports on the in vitro angiogenetic potency of adipose tissue-derived mesenchymal stem cells. The angiogenetic property of AdMSC has been well-known and has been suggested as one of the underlying cure mechanisms for various ischemic diseases in many previous studies including the present study, which is basically a clinical case report.

The present study was dealt with the emergency use of AdMSC under the approval of related government ministry, and there was no need to carry out additional in vitro potency assay of AdMSC.

2. All the patients enrolled in the study were labeled as Rutherford III - 5. As per the definition Rutherford III - 5 is defined as "Minor tissue loss—nonhealing ulcer, focal

gangrene with diffuse pedal ischemia". But patient numbers 001 and 003 do not have ulcers at baseline. Please reconsider the grade of these patients.

Rutherford classification deals with both chronic and acute limb ischemia. The enrolled patients in the present study were not acute cases but chronic cases with no improvement after previous treatment in other hospitals for over 6 months. According to Rutherford classification, the patients with only pain could be grouped into the grade 0-2 (category 0-4), and the patients starting to show the wounds could be grouped into grade 3 (category 5, 6). The patients 001 & 003 had already toe amputation and had shown the continuous aggravating process at the time of the recruitment for the present study. Considering that Buerger's disease is a progressive and worsening disease rather than healing, it could be assumed that the patients 001 & 003 had the amputations as a result of the progression of low ankle/toe pressure and worsening of toe circulation. Even if the toe amputation was carried out due to necrosis, it could not mean that ABI and AP/TP showed improvement after the treatment. Thus, it is difficult to say that the patients diagnosed with gangrene and ulcer at first diagnosis which requires the amputation had improved in AP/TP and in the Rutherford classification after the amputation. In other words, considering that the amputation is a common solution for non-healing ulcers in the therapeutic strategy in the Buerger's disease, it could be said that the patients with previous amputations and severe ischemic rest pain at the time of enrollment are relevant to Rutherford III-5 (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4232437/table/TB00869-2/>).

As referred to Hardman et al. (Semin Intervent Radiol. 2014; 31(4): 378-388. Overview of Classification Systems in Peripheral Artery Disease), Rutherford did not include the strict temporal division in the definition. So, it can say that there is no clear temporal distinction on the occurrence of the ulcer at the time of diagnosis.

3. Was ABPI, ankle pressure or TcPO2 measured in these patients as they are very important indirect findings of increased oxygenation of tissues.

Ankle (ABI) or toe (TBI) brachial indices were not applied as ABI cannot predict walking distance and has difficulty accurately describing the disease state (Vasa Zeitschrift für Gefasskrankheiten. 2002;31(2):107-10. Leder et al. Exercise capacity and Doppler pressure measurements in symptomatic peripheral arterial obstructive disease). Moreover, these were not considered as efficacy measures because of measurement difficulties owing to wounds or the lack of toes in some patients. TcPO2 was not measured because we do not have the device.

4. Case number 002, the hands were also involved. Whether any injections of MSCs were given in the upper limbs also.

As an extension of our previous report (Cell Med. 2017;9(3):87-102, Ra et al., Prospective, Nonrandomized, no Placebo-Controlled, Phase I/II Clinical Trial Assessing the Safety and Efficacy of Intramuscular Injection of Autologous Adipose Tissue-Derived Mesenchymal Stem Cells in Patients With Severe Buerger's Disease), the present study was carried out under the approval of Korean Ministry of Food and Drug Safety with Investigational New Drug Application for emergency Use. As required by the KMFDS, the administration of AdMSC injection was carried out once in the affected lower limb, not the upper limb, the

same as the previous report.

5. In the discussion section more can be discussed on the advantage of CT angiography on MRA or DSA as CT angiography was done in the three patients.

Although angiography is considered to be the most accurate method for precise identification of the vascular condition, this method is invasive, difficult to perform, and cumbersome and was determined to be inappropriate for the comparison before and after stem cell administration. However, as the previous patient status check was performed using angiography, it was possible to identify the existing disease state. Compared to conventional angiography, CT-angiography has a disadvantage with regard to accuracy and locating small vessels but provided good results of 99% sensitivity, 98% specificity, and 98% accuracy in a comparative study in patients with critical limb ischemia (Clinical radiology. 2011;66(10):945-52, Fotiadis et al., 64-section CT angiography in patients with critical limb ischemia and severe claudication: comparison with digital subtractive angiography), and thus was deemed suitable for noninvasive examination in the present study. Nevertheless, as conventional CT angiography constitutes a 3D reconstruction of the cephalic to a caudal axis, it is difficult to compare the axial image in the case of a 90° vertical axis on the vertical axis of the human body, such as the foot, indicating the need to improve the imaging and reconstruction methods

6. No new data is coming out from this small study of three patients as it is now a well-known fact that stem cells improved angiogenesis in these patients.

Buerger's disease is a rare disease to have difficulty recruiting enough patients for collecting clinical data which could be analyzed for research. The patients in the present report visited the hospital for the treatment of the aggravated condition and enrolled in the case study. We tried the single focal injection of autologous AdMSC to the patients with almost incurable symptoms and identified the systemic angiogenesis in the improvement status of the patients. The present study is a clinical practice article consisting of 3 clinical case reports according to the submission guideline of F100research, rather than a novel research article for investigation. We hope that our study would be promising opportunities to adapt to AdMSC therapy for the treatment of rare diseases

Competing Interests: No competing interests were disclosed.

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