

Pretreatment of Diabetic Adipose-derived Stem Cells with mitoTEMPO Reverses their Defective Proangiogenic Function in Diabetic Mice with Critical Limb Ischemia

Kun Lian^{1,*}, Qin Wang^{2,*}, Shuai Zhao¹, Maosen Yang¹, Genrui Chen¹, Youhu Chen¹, Congye Li¹, Haokao Gao¹, and Chengxiang Li¹ 

Cell Transplantation
2019, Vol. 28(12) 1652–1663
© The Author(s) 2019
Article reuse guidelines:
sagepub.com/journals-permissions
DOI: 10.1177/0963689719885076
journals.sagepub.com/home/cil


Abstract

Adipose-derived stem cells (ADSCs) have the ability to migrate to injury sites and facilitate tissue repair by promoting angiogenesis. However, the therapeutic effect of ADSCs from patients with diabetes is impaired due to oxidative stress. Given that diabetes is a group of metabolic disorders and mitochondria are a major source of reactive oxygen species (ROS), it is possible that mitochondrial ROS plays an important role in the induction of diabetic ADSC (dADSC) dysfunction. ADSCs isolated from diabetic mice were treated with mitoTEMPO, a mitochondrial ROS scavenger, or TEMPO, a universal ROS scavenger, for three passages. The results showed that pretreatment with mitoTEMPO increased the proliferation, multidifferentiation potential, and the migration and proangiogenic capacities of dADSCs to levels similar to those of ADSCs from control mice, whereas pretreatment with TEMPO showed only minor effects. Mechanistically, mitoTEMPO pretreatment enhanced the mitochondrial antioxidant capacity of dADSCs, and knockdown of superoxide dismutase reduced the restored mitochondrial antioxidant capacity and attenuated the proangiogenic effects induced by mitoTEMPO pretreatment. In addition, mitoTEMPO pretreatment improved the survival of dADSCs in diabetic mice with critical limb ischemia, showing protective effects similar to those of control ADSCs. Pretreatment of dADSCs with mitoTEMPO decreased limb injury and improved angiogenesis in diabetic mice with critical limb ischemia. These findings suggested that short-term pretreatment of dADSCs with a mitochondrial ROS scavenger restored their normal functions, which may be an effective strategy for improving the therapeutic effects of ADSC-based therapies in patients with diabetes.

Keywords

ADSC, ROS, diabetic mice, critical limb ischemia

Introduction

Adipose-derived stem cells (ADSCs) are mesenchymal stem cells that are derived from subcutaneous fat tissues. Since ADSCs are abundant in humans and can be easily isolated, they show promise for biological engineering, especially stem cell-based clinical applications^{1–3}. ADSCs are multipotent stem cells, and isolated ADSCs are capable of differentiating into adipocytes, osteoblasts, and endothelial cells, and thus are candidates for clinical stem cell-based therapies⁴. Owing to their high *in vivo* proliferation efficiency, ADSCs have recently gained popularity over other stem cells for the treatment of peripheral arterial disease (PAD), in which the arteries that carry blood to the limbs become narrowed or clogged due to atherosclerosis^{2,5–8}. ADSCs

¹ Department of Cardiology, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi, China

² Department of Pharmacogenomics, Fourth Military Medical University, Xi'an, Shaanxi, P.R. China

* Both the authors contributed equally to this article.

Submitted: July 2, 2019. Revised: September 1, 2019. Accepted: October 3, 2019.

Corresponding Authors:

Haokao Gao and Chengxiang Li, Department of Cardiology, Xijing Hospital, Fourth Military Medical University, 169 West Changle Road, Xi'an, Shaanxi 710032, P.R. China.

Emails: hk_gao@163.com; lichx1@163.com



have the ability to migrate to injury sites and facilitate tissue repair by promoting angiogenesis, which increases oxygen and the nutrient supply in ischemic areas^{6,9,10}.

Diabetes is a major risk factor for PAD¹¹. The prevalence of PAD in patients with diabetes varies between 20% and 30%, depending on the study population¹². In addition, patients with PAD with diabetes are at higher risk for lower extremity amputation than those without diabetes¹³. Although ADSC-based therapy is a potential strategy for salvaging limbs from amputation due to PAD, it has been reported that the therapeutic effects of diabetic ADSCs (dADSCs) are impaired^{14,15}. Thus, it is of great interest to identify tools to improve the function of dADSCs. Several studies have demonstrated that a major cause of dysfunction in dADSCs is oxidative stress, which impairs their proliferation capacity and angiogenic potential^{6,8,16,17}. It was reported that infection of dADSCs with a lentivirus over-expressing glyoxalase-1, an enzyme that detoxifies methylglyoxal and reduces reactive oxygen species (ROS), reverses their defective proangiogenic function in a diabetic mouse model of critical limb ischemia, which is a type of severe PAD with typical ischemic symptoms and damage⁸. Given that diabetes is a group of metabolic disorders characterized by high blood glucose levels over a prolonged time period and mitochondria are a major source of ROS^{18,19}, it is possible that mitochondrial ROS play an important role in the induction of dysfunction in dADSCs. However, the role of mitochondrial ROS in the dysfunction of dADSCs is currently unknown.

Here, we hypothesized that pretreatment of dADSCs with mitoTEMPO, a mitochondrial ROS scavenger, may improve their function. We found that pretreatment of dADSCs with mitoTEMPO for three passages enhanced their proangiogenic function and improved their protective effects against critical limb ischemia in streptozotocin (STZ)-induced diabetic mice. This finding suggested that a short-term pretreatment of dADSCs with a mitochondrial ROS scavenger restored their proangiogenic capacity both in vitro and in vivo.

Materials and Methods

Animals

The animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health, and the protocol was approved by the Committee on Animal Care of Fourth Military Medical University. Diabetes was induced in 8-week-old C57 mice by a single i.p. injection of STZ (in citrate buffer, pH 4.5; 100 mg/kg body weight) as described previously²⁰. Two weeks after diabetes induction, blood glucose levels were measured with a glucose meter (Life-Scan, Milpitas, CA, USA). Only mice with blood glucose levels >16.7 mM (300 mg/dl) were considered to be diabetic and then subjected to ADSC isolation and induction of critical limb ischemia.

Isolation, Culture, and Characterization of ADSCs

ADSCs were obtained from the subcutaneous adipose tissues in the inguinal area of 10-week-old STZ-induced diabetic and control C57 mice as described previously²¹. Isolated ADSCs were plated at 5×10^5 cells/cm² in DMEM with low glucose (5 mM). To determine the phenotype of the dADSCs, the ADSCs were washed with phosphate-buffered saline (PBS) and incubated with phycoerythrin-conjugated anti-mouse antibodies against CD11b, CD29, CD31, CD44, CD90.1, CD133, and major histocompatibility complex II (MHC-II) for 25 min at 4°C in the dark. The cells were then washed with PBS and collected for flow cytometry analysis (Beckman Coulter, Fullerton, CA, USA). Cultured ADSCs were passaged when they reached 75–80% confluence. The initial confluent culture was designated passage 0. Cultured dADSCs from passage 3 were treated with either a general antioxidant, 4-Hydroxy-TEMPO, formally 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPO, Sigma-Aldrich, St Louis, MO, USA) (1 μM) or a mitochondrially targeted antioxidant, (2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (mitoTEMPO, Sigma) (1 μM) for three passages and then used in experiments. The nADSCs were not treated with TEMPO or mitoTEMPO during the experiments.

Cell Viability Assay

The proliferation of ADSCs was assessed using the cell counting kit-8 assay (CCK-8; Dojindo Laboratories, Shanghai, China) according to the manufacturer's instructions.

Multidifferentiation Potential of ADSCs

ADSCs from passage 5 were incubated in adipogenic or osteogenic medium to evaluate their potential for multidifferentiation as described previously²². Osteoblast formation was evaluated after 3 weeks by assessing calcium accumulation using alizarin red (Sigma-Aldrich). Adipogenic differentiation was assessed using Oil Red-O (Sigma-Aldrich) staining. Endothelial cell differentiation was evaluated by culturing the cells in Endothelial Cell Growth Medium-2 for 2 weeks²³. Then, the endothelial cell phenotype was evaluated by examining the expression of the endothelial cell markers *Pecam-1*, *vWF*, and *Cd105* by RT-qPCR.

Scratch and Cell Migration Assays

ADSCs at 90% confluence were used for the scratch assay as described previously⁸, and a standard, 3 mm scratch was made, bisecting the ADSC cultures. Photographs were taken under an inverted microscope (Olympus Microscopes, Tokyo, Japan). The cell migration assay was performed using Transwell chambers (24-well, 8-mm pore size; Corning, NY, USA). DMEM containing 10% serum was used as an attractant and was placed in the lower chamber. ADSCs (10^5) in DMEM containing 0.5% serum were added to the

upper chamber of the insert and incubated at 37°C for 24 h to allow the cells to migrate toward the underside of the insert filter. After incubation, ADSCs that did not migrate through the pores were gently removed with a cotton swab. Cells on the lower side of the filter were fixed with 4% paraformaldehyde and stained with 1% crystal violet in 2% methanol.

Proangiogenic Analysis of ADSCs

The capacity of the ADSCs to stimulate angiogenesis was assessed in a tube formation assay using Matrigel (BD Biosciences, San Jose, CA, USA) as previously described⁸. In brief, conditioned medium was collected from confluent cultures of normal ADSCs, dADSCs, and mitoTEMPO-treated dADSCs after 48 h of hypoxia (1% O₂). Then, the levels of vascular endothelial growth factor A (VEGFA), hepatocyte growth factor (HGF), and fibroblast growth factor 2 (FGF2) were measured using enzyme-linked immunosorbent assays (R&D Systems, MN, USA). Next, human umbilical vein endothelial cells (HUVECs) were seeded on Matrigel (Corning) and incubated in ADSC-conditioned medium for 16 h. Images were taken using an inverted phase contrast microscope. The expression levels of the proangiogenic genes *Hif-1a*, *Vegfa*, and *Sdf-1a* were detected using RT-qPCR.

Establishment of a Critical Limb Ischemia Model in Diabetic Mice

STZ-induced diabetic mice were anesthetized with 3% pentobarbital sodium (50 mg/kg), and limb ischemia was induced as described previously²¹. Briefly, the left femoral artery and its branches were ligated. Sham-operated mice received an incision without artery ligation. After arterial ligation, the ischemic hind limb was intramuscularly injected with either PBS or cultured ADSCs (5×10^6 cells in 100 μ l).

Bioluminescence Imaging of ADSCs In Vivo

The survival of engrafted ADSCs in diabetic mice with critical limb ischemia was tracked by bioluminescence as described previously²¹. The engrafted ADSCs were infected with a lentivirus carrying a luciferase gene. Then, the mice were anesthetized and intraperitoneally injected with D-luciferin (150 mg/kg). Using an IVIS, images were acquired at 3-min intervals until the peak signal was observed.

Confocal Imaging

An inverted confocal microscope (Zeiss LSM 800) equipped with a 40 \times , 1.3 NA oil-immersion objective was used for imaging. To detect mitochondrial ROS in isolated ADSCs, the cells were loaded with mitoSOX (5 μ M) for 20 min and then washed. To detect mitoSOX fluorescence, the excitation and emission wavelengths were 488 nm and 540–625 nm, respectively. To detect intracellular ROS in isolated ADSCs, the cells were loaded with DCFH (5 μ M) for 10

min and then washed. To detect DCF fluorescence, the cells were exposed to the excitation and emission wavelengths (488 nm and >500 nm, respectively) generated using a low-intensity laser to minimize the photochemical reaction of DCF. Paraffin sections of the left gastrocnemius muscle were obtained and sequentially analyzed by immunohistochemical staining using a rat monoclonal anti-CD31 antibody (1:50, ab7388; Abcam, Cambridge, USA) to visualize the CD31-positive cells. All experiments were performed at room temperature.

Western Blotting

Protein expression was measured by Western blotting as described previously²⁴. The immunoblots were probed with anti-superoxide dismutase 2 (SOD2), anti-catalase, anti-glutathione peroxidase (GPx), anti-VEFG, or anti-GAPDH antibodies overnight at 4°C and then incubated with the corresponding secondary antibodies at room temperature for 1 h. The blots were visualized with ECL-plus reagent.

Statistical Analysis

All values are presented as the mean \pm SEM. Data were compared by one-way ANOVA or two-way ANOVA, followed by an unpaired *t*-test, as appropriate. The data distributions were analyzed for normality by the Kolmogorov–Smirnov normality test. Bonferroni's correction for multiple comparisons was also used. Differences were considered significant at *p*-values less than 0.05.

Results

Pretreatment of dADSCs with mitoTEMPO Scavenged Mitochondrial ROS and Improved Multidifferentiation Potential

Flow cytometry analysis showed that the isolated ADSCs were positive for the stem cell surface antigens CD29, CD44, and CD90.1, with little contamination by inflammatory, hematopoietic, or immune cells as indicated by the low expression levels of CD11b, CD31, CD133, and MHC-II (Fig. S1). Compared with ADSCs from normal control mice (nADSCs), dADSCs displayed higher ROS levels in both the intracellular space and mitochondria as assessed by DCF and mitoSOX fluorescence, respectively (Fig. 1A). Compared with nADSCs, dADSCs showed impaired cell viability (Fig. 1B) and decreased multidifferentiation potential, including adipogenic and osteogenic potential (Fig. 1C, D), suggesting that diabetes impaired the differentiation potential of ADSCs.

To test whether mitochondrial ROS plays an important role in the induction of dADSCs dysfunction, cultured dADSCs were treated with either TEMPO (1 μ M), which scavenges intracellular ROS, or mitoTEMPO (1 μ M), which scavenges mitochondrial ROS, for three passages (passages 3–5). As shown in Fig. 1A, pretreatment with TEMPO and

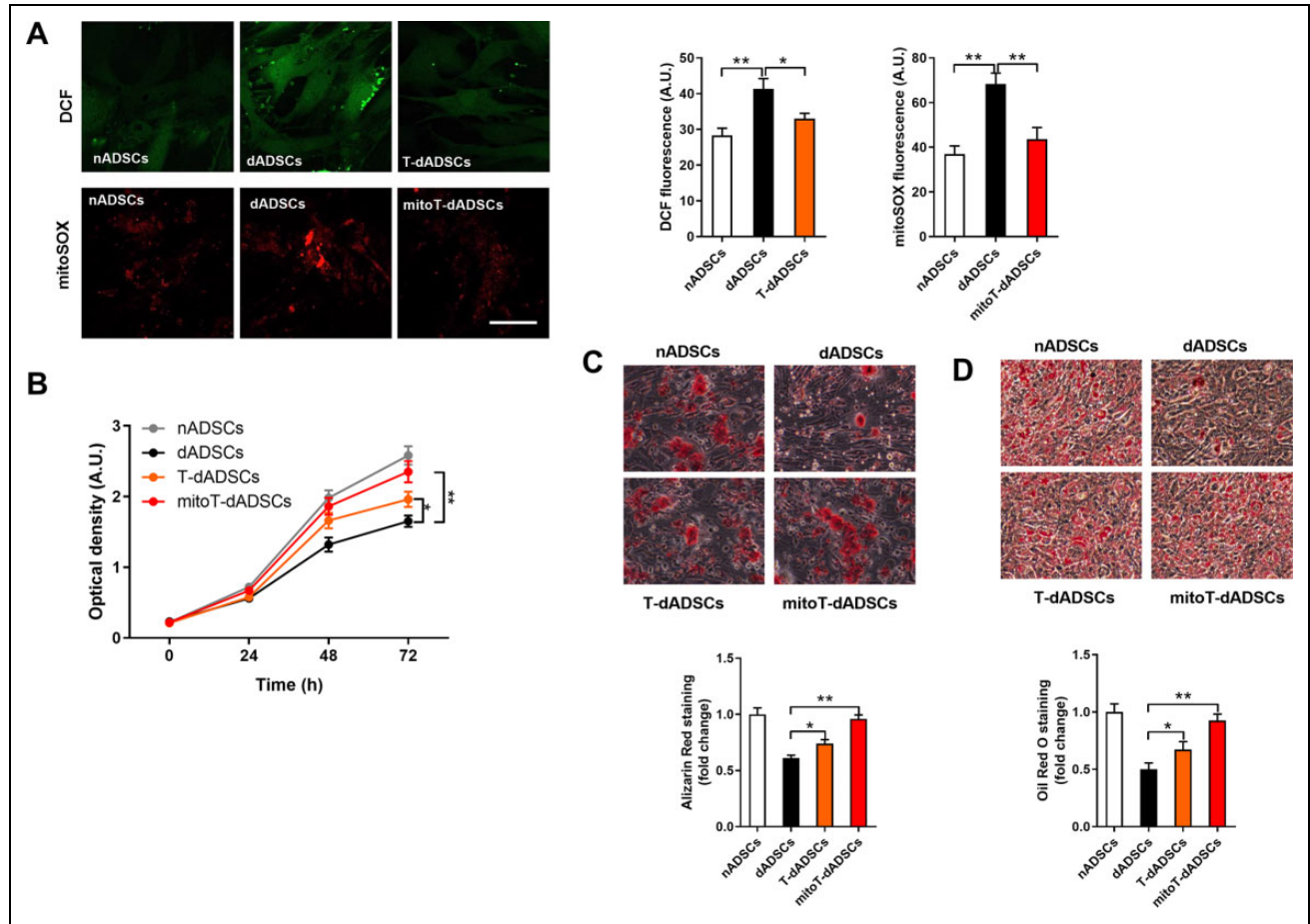


Figure 1. Pretreatment of diabetic ADSCs with mitoTEMPO improved cell viability and multidifferentiation capability in vitro.

A. TEMPO and mitoTEMPO pretreatment scavenged intracellular and mitochondrial ROS in diabetic ADSCs, respectively. Intracellular ROS was monitored by assessing DCF fluorescence, and mitochondrial ROS was monitored by assessing mitoSOX fluorescence. Abbreviations: nADSCs, normal ADSCs from nondiabetic mice; dADSCs, diabetic ADSCs; T-dADSCs, TEMPO-pretreated dADSCs; mitoT-dADSCs, mitoTEMPO-pretreated dADSCs. Scale bar, 50 μ m. B. Proliferation curve of dADSCs pretreated with either TEMPO or mitoTEMPO. C. Pretreatment with TEMPO and mitoTEMPO increased the osteogenic capability of dADSCs. D. Pretreatment with TEMPO and mitoTEMPO increased the adipogenic capability of dADSCs. $n = 6$. * $p < 0.05$; ** $p < 0.01$.

mitoTEMPO decreased intracellular and mitochondrial ROS levels, respectively. Importantly, incubation of dADSCs with TEMPO or mitoTEMPO for three passages improved cell function, as evidenced by the increased cell viability and multidifferentiation potential (Fig. 1B–D). In particular, mitoTEMPO-pretreated dADSCs (mitoT-dADSCs) displayed viability and multidifferentiation potential similar to that of nADSCs (Fig. 1B–D), suggesting that mitoTEMPO pretreatment restored the function of dADSCs in vitro.

Pretreatment of dADSCs with mitoTEMPO Improved Migration Capacity

As the migration of stem cells from the transplant site to the injured area is essential for successful stem cell-based therapy, we detected whether mitoTEMPO could increase the migration capacity of dADSCs by using scratch and Transwell assays. In the scratch assay, the mitoT-dADSCs showed

faster migration than the dADSCs (Fig. 2A). Similarly, the Transwell assay results showed that more mitoT-dADSCs migrated through the Transwell membrane than dADSCs (Fig. 2B). In contrast, TMEPO pretreatment had little effect on the cell migration of dADSCs (Fig. 2A, B). These results reinforced the notion that mitoTEMPO pretreatment restored the function of dADSCs in vitro.

Pretreatment of dADSCs with mitoTEMPO Enhanced Proangiogenic Capacity

Angiogenesis, which involves the migration, growth, and differentiation of endothelial cells, has been suggested as a major mechanism underlying stem cell-promoted tissue repair^{25,26}. Pretreatment with mitoTEMPO promoted the differentiation of dADSCs into endothelial cells as evidenced by the higher expression levels of the endothelial cell-specific genes *Pecam1*, *vWF*, and *Cd105* (Fig. 3A).

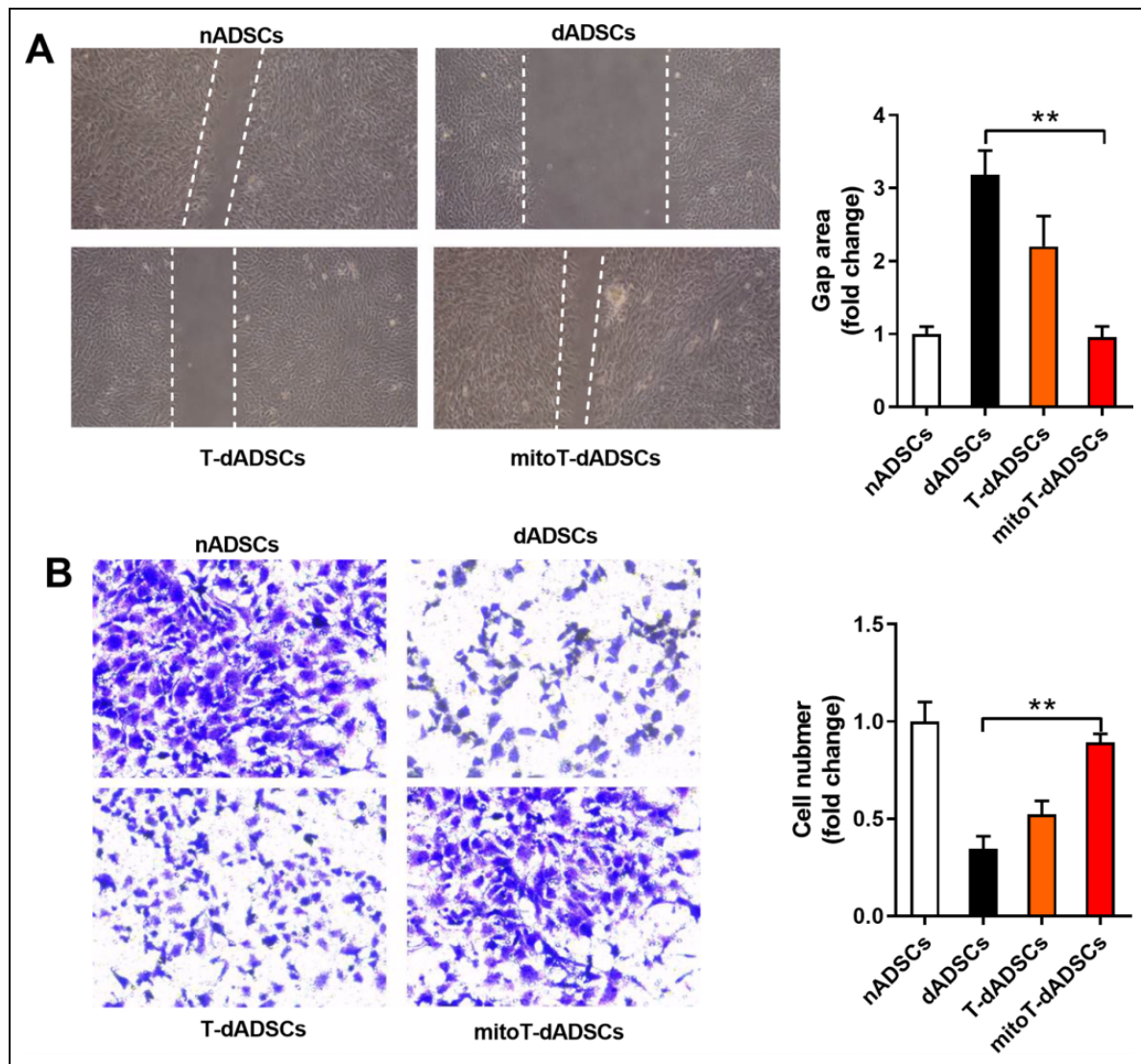


Figure 2. Pretreatment of diabetic ADSCs with mitoTEMPO improved their migration capacity in vitro.

A. Pretreatment of dADSCs with mitoTEMPO increased their migration as assessed by a scratch assay. Abbreviations: nADSCs, normal ADSCs from nondiabetic mice; dADSCs, diabetic ADSCs; T-dADSCs, TEMPO-pretreated dADSCs; and mitoT-dADSCs, mitoTEMPO-pretreated dADSCs. B. Pretreatment of dADSCs with mitoTEMPO increased their migration as assessed by a Transwell assay. $n = 6$. ** $p < 0.01$.

Pretreatment with mitoTEMPO also enhanced the angiogenic potential of endothelial cells (Fig. 3B, C). The expression levels of the proangiogenic genes *Hif-1a*, *Vegfa*, and *Sdf-1a* were higher in mitoT-dADSCs than in dADSCs under hypoxia (Fig. 3B). In addition, mitoTEMPO pretreatment increased the levels of VEGFA, HGF, and FGF2 in the conditioned medium of dADSCs (Fig. 3C).

A tube formation assay was performed with HUVECs. The HUVECs were incubated with conditioned medium from nADSCs, dADSCs, or mitoT-dADSCs. Compared with tube formation in the presence of conditioned medium from dADSCs, conditioned medium from mitoT-dADSCs induced greater tube formation, which was comparable to that formed in medium from nADSCs

(Fig. 3D). These results suggest that mitoTEMPO pretreatment effectively restored the proangiogenic potential of dADSCs in vitro.

Enhancement of Mitochondrial Antioxidant Capacity Contributed to the Proangiogenic Effects of mitoTEMPO Pretreatment on dADSCs

The mitochondrial redox balance is not only dependent on ROS generation, but also antioxidant capacity, which determines ROS scavenging ability²⁷. The major antioxidant enzymes in mitochondria are SOD2, catalase, and GPx. The levels of these antioxidant enzymes were lower in dADSCs than in nADSCs (Fig. 4A). Pretreatment with mitoTEMPO

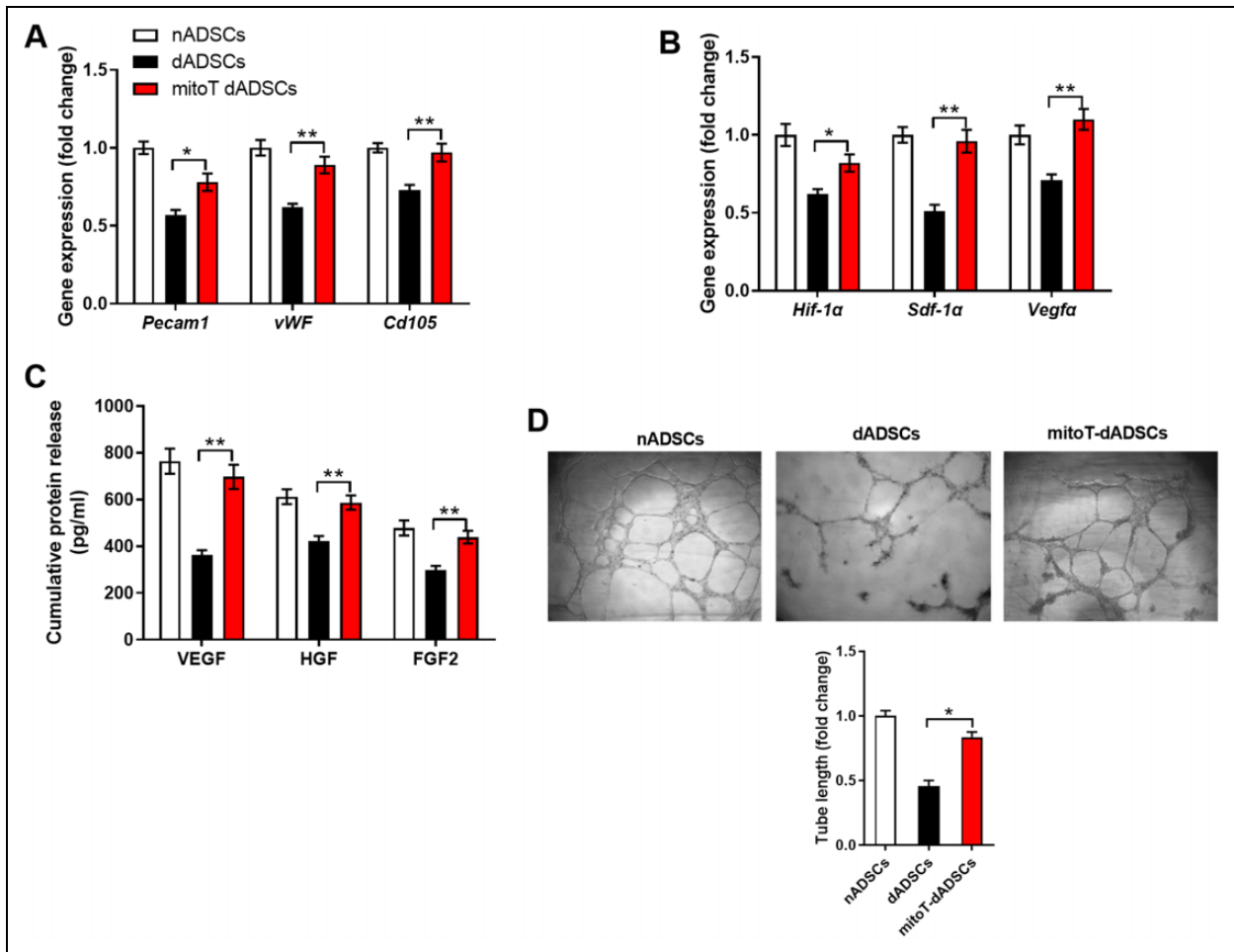


Figure 3. Pretreatment of diabetic ADSCs with mitoTEMPO increased their proangiogenic capacity.

A. The expression levels of proangiogenic genes in ADSCs under hypoxic conditions. Abbreviations: nADSCs, normal ADSCs from nondiabetic mice; dADSCs, diabetic ADSCs; and mitoT-dADSCs, mitoTEMPO-pretreated dADSCs. B. Pretreatment with mitoTEMPO increased the levels of proangiogenic proteins in the conditioned medium of dADSCs. C. Conditioned medium from mitoTEMPO-pretreated dADSCs enhanced tube formation by HUVECs. $n = 6$. * $p < 0.05$; ** $p < 0.01$.

increased the mitochondrial antioxidant capacity of dADSCs, as evidenced by increased levels of these antioxidant enzymes (Fig. 4A). To test whether the enhanced antioxidant capacity is involved in the proangiogenic effect of mitoTEMPO pretreatment, SOD2 expression in dADSCs was silenced by siRNA (Fig. 4B). SOD2 knock-down had little effect on cell viability in untreated dADSCs, but decreased cell viability in mitoTEMPO-pretreated dADSCs (Fig. 4C). SOD2 knockdown decreased the expression levels of the proangiogenic genes *Hif-1α*, *Vegfa*, and *Sdf-1α* in mitoT-dADSCs under hypoxia, but showed little effect on the expression of these genes in dADSCs (Fig. 4D). In addition, conditioned medium from SOD2-knockdown mitoT-dADSCs showed impaired proangiogenic effects on HUVECs compared with the negative control (Fig. 4E). These results suggested that enhancement of mitochondrial antioxidant capacity contributed to the proangiogenic effects of mitoTEMPO pretreatment on dADSCs.

Pretreatment of dADSCs with mitoTEMPO Improved their Survival in Diabetic Mice with Critical Limb Ischemia

To test whether mitoTEMPO pretreatment could enhance the function of dADSCs in vivo, a model of critical limb ischemia was induced in diabetic mice. After the operation, the ischemic hind limb was intramuscularly injected with PBS, nADSCs, dADSCs, or mitoT-dADSCs (5×10^6 cells in 100 μ L). Then, the survival of the engrafted ADSCs in the diabetic mice was evaluated by bioluminescence imaging. As shown in Fig. 5, the intensity of the bioluminescence signal did not differ significantly among the groups at 3 days after cell transplantation. However, in the subsequent days, the signal intensities in the dADSCs group were lower than those in the nADSCs and mitoT-dADSCs groups. In particular, no bioluminescence signal was detected in the dADSCs group at 28 days after cell transplantation, while bioluminescence signals were still detectable in mice treated

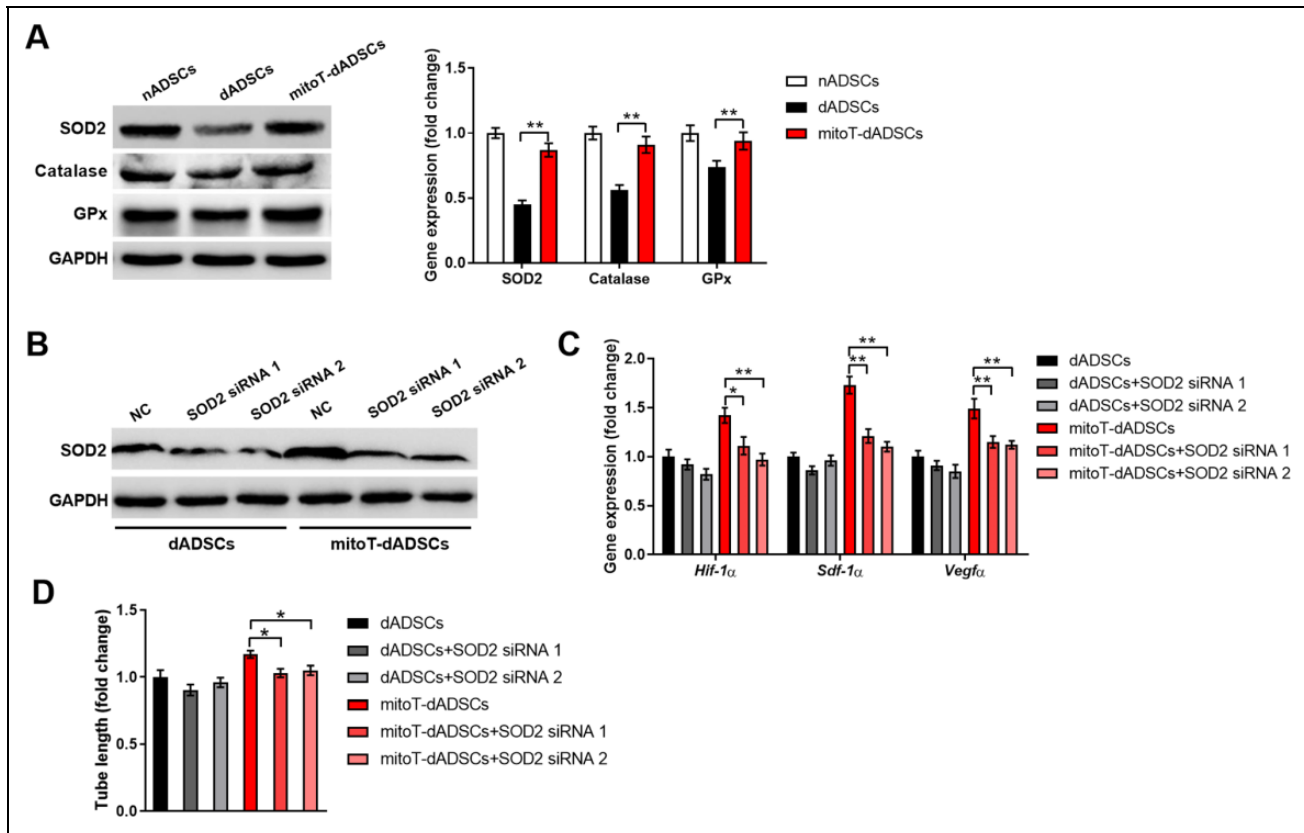


Figure 4. Pretreatment of diabetic ADSCs with mitoTEMPO improved proangiogenic capacity by enhancing mitochondrial antioxidant capacity.

The expression levels of the major mitochondrial antioxidant enzymes (superoxide dismutase 2 [SOD2], catalase, and glutathione peroxidase [GPx]) in normal ADSCs from nondiabetic mice (nADSCs), diabetic ADSCs (dADSCs), and mitoTEMPO-pretreated dADSCs (mitoT-dADSCs). B SOD2 expression in dADSCs was silenced by specific SOD2 siRNAs. C. Knockdown of SOD2 decreased cell viability in mitoTEMPO-pretreated dADSCs. D. Knockdown of SOD2 attenuated the proangiogenic effects of mitoTEMPO as evidenced by the expression levels of proangiogenic genes in dADSCs under hypoxia. E. Knockdown of SOD2 attenuated the proangiogenic effects of mitoTEMPO pretreatment as evidenced by the assessment of HUVEC tube formation in conditioned medium. $n = 6$. * $p < 0.05$; ** $p < 0.01$.

with either nADSCs or mitoT-dADSCs, suggesting that mitoTEMPO pretreatment improved the survival of dADSCs in diabetic mice with critical limb ischemia.

Pretreatment of dADSCs with mitoTEMPO improved their Proangiogenic Effects in Diabetic Mice with Critical Limb Ischemia

To further validate the restored angiogenic capacity of dADSCs induced by mitoTEMPO pretreatment in vivo, ischemic injury and angiogenesis were assessed in diabetic mice with critical limb ischemia. Nearly all mice in the PBS group experienced limb loss or necrosis at 28 days post operation, whereas less limb necrosis and loss and higher salvage rates were observed in mice in the nADSCs and mitoT-dADSCs groups than in the dADSCs group (Fig. 6A). We further detected the proangiogenic capacity of mitoT-dADSCs by Western blotting and histological analysis of microvessel densities (Fig. 6B, C). The levels of

VEGF, a key promoter of angiogenesis, were higher in the nADSCs and mitoT-dADSCs groups than that in the dADSCs group (Fig. 6B), suggesting that mitoTEMPO pretreatment restored the proangiogenic effects of dADSCs in vivo. This was further reinforced by the histological CD31 staining results, which showed more microvessels in the nADSCs and mitoT-dADSCs groups than in the dADSCs group (Fig. 6C).

Discussion

Diabetes impairs the proangiogenic function of ADSCs, limiting their clinical application for the treatment of ischemic diseases. Here, we found that short-term pretreatment of dADSCs with mitoTEMPO restored their biological function by reducing mitochondrial ROS accumulation and enhancing mitochondrial antioxidant capacity. Pretreatment of dADSCs with mitoTEMPO improved their viability and their differentiation, migration, and proangiogenic capacities to levels similar to those of nondiabetic ADSCs. Moreover,

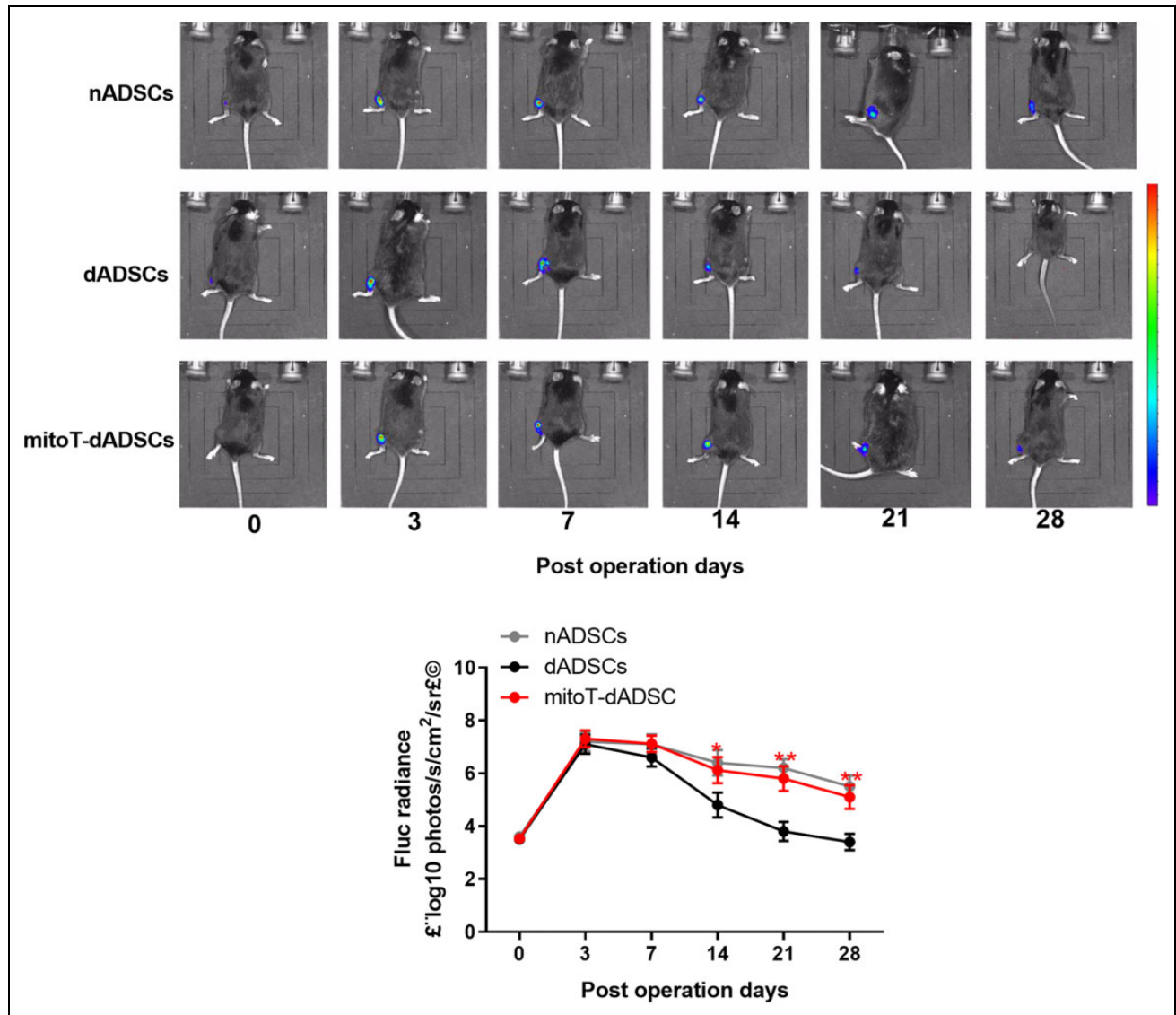


Figure 5. Pretreatment of diabetic ADSCs with mitoTEMPO increased survival in diabetic mice with critical limb ischemia. Longitudinal bioluminescence imaging was used to track ADSC^{Fluc} survival in diabetic mice with critical limb ischemia in vivo. Typical images are shown on the left and a quantitative analysis of the Fluc optical signals is shown on the right. $n = 6$. * $p < 0.05$; ** $p < 0.01$ vs. the dADSCs group. Abbreviations: nADSCs, normal ADSCs from nondiabetic mice; dADSCs, diabetic ADSCs; and mitoT-dADSCs, mitoTEMPO-pretreated dADSCs.

mitoTEMPO pretreatment restored the protective effects of dADSCs, as mitoTEMPO-pretreated dADSCs could reduce limb ischemic injury and enhance angiogenesis in diabetic mice with critical limb ischemia. This study has provided a solution to the impaired angiogenic capacity of transplanted dADSCs and the application of dADSC-based therapy for patients with diabetes.

Diabetes is a lifestyle disease that currently affects 8.3% of the world's adult population, and the prevalence is increasing at an alarming rate, making it one of the most common non-communicable diseases in the current era²⁸. It is not only a metabolic disorder characterized by hyperglycemia, insulin resistance, and pancreatic beta cell

dysfunction, but also a complex syndrome accompanied by systemic inflammation, oxidative stress, nitrate stress, and the formation of advanced glycation end products^{29–31}. dADSCs exhibit impairments in viability, differentiation, and other functions^{16,32,33}. The mechanism underlying diabetes-induced ADSC dysfunction remains unknown. However, it has been suggested that oxidative stress plays a causal role in insulin resistance and can be induced by hyperglycemia, inflammation, and advanced glycation end products^{31,34}. In addition, increasing evidence has shown that oxidative stress contributes to the development of various pathological processes³⁵. Thus, the elimination of ROS might be a critical factor in restoring of the effectiveness of

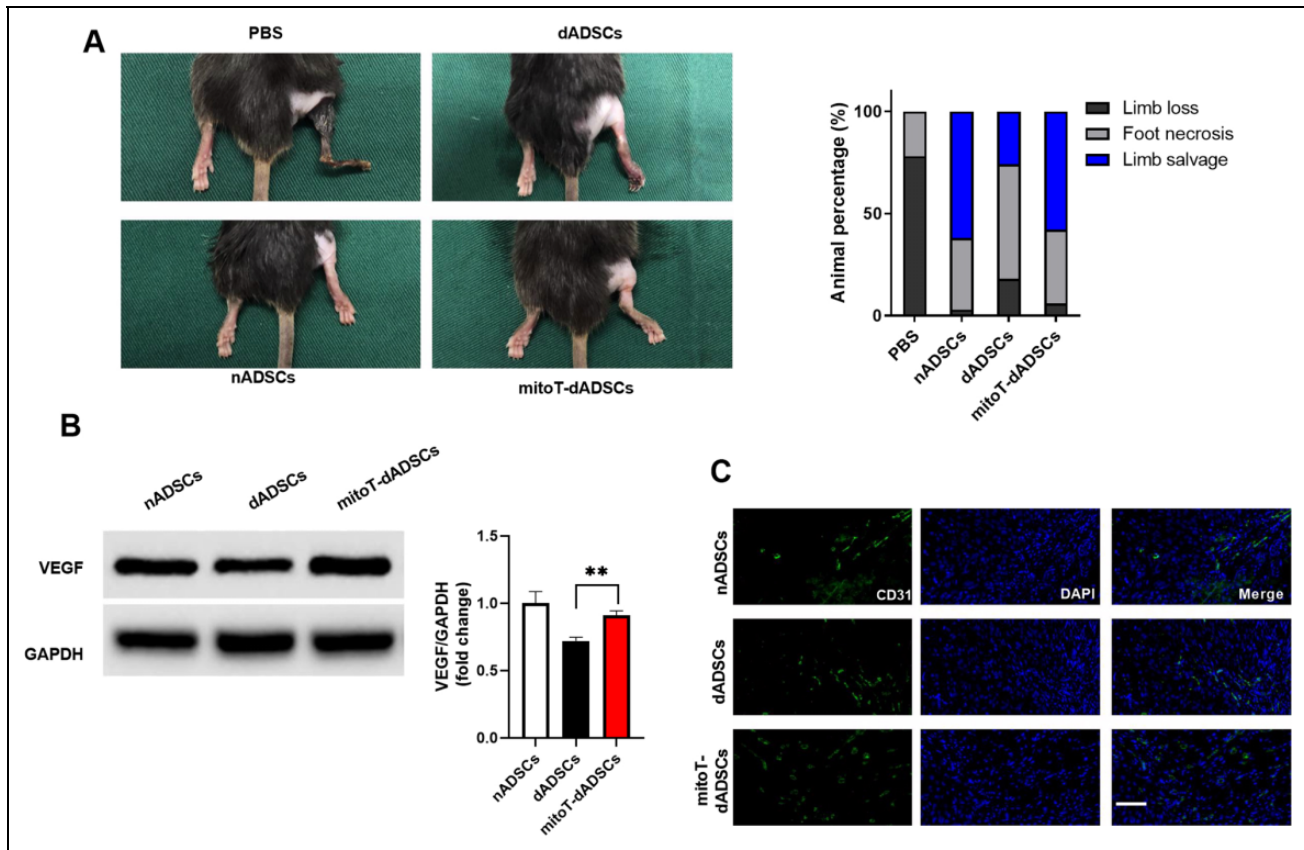


Figure 6. Pretreatment of diabetic ADSCs with mitoTEMPO improved their proangiogenic capacity in diabetic mice with critical limb ischemia.

A. Representative images of ischemic limbs from different groups of mice at days 28 after ligation and ADSC therapy are shown on the left. The percentages of ischemic outcomes are shown on the right. B. The expression of CD31 and VEGF in the gastrocnemius muscle of ischemic limbs in diabetic mice with critical limb ischemia. C. Histological CD31 staining in the gastrocnemius muscle of ischemic limbs in diabetic mice with critical limb ischemia. Scale bar, 100 μ m. $n = 6$. * $p < 0.05$; ** $p < 0.01$.

dADSC-based therapy. In fact, several studies have demonstrated that oxidative stress is a cause of dysfunction in dADSCs that impairs their proliferation capacity and angiogenic potential^{6,8,16,17}. These studies suggest that oxidative stress plays a causal role in the induction of dysfunction in dADSCs, and rebalancing the redox status in dADSCs may be a potential strategy for improving the function of dADSCs.

Given that diabetes is a group of metabolic disorders and mitochondria are a major source of ROS^{18,19}, it is possible that mitochondrial ROS plays an important role in the induction of dysfunction in dADSCs. However, the role of mitochondrial ROS in dADSC dysfunction is unknown. Recent studies have shown strong support for the direct involvement of mitochondria in the regulation of stem cell function^{36–38}. Here, we provided a solution for restoration of the impaired proangiogenic capacity of dADSCs. Cultured dADSCs were pretreated with either TEMPO or mitoTEMPO for three passages, and the results showed that mitoTEMPO pretreatment restored the function of dADSCs, whereas TEMPO pretreatment had only minor effects, suggesting that

mitochondrial ROS plays a more important role in the induction of dysfunction of dADSCs. These findings are consistent with several studies showing that scavenging mitochondrial ROS improves stem cell function^{37,39}. Although a previous study showed that overexpression of glyoxalase-1 induced by lentivirus infection in dADSCs reverses its defective proangiogenic function in a diabetic mouse model of critical limb ischemia⁸, our study showed that scavenging mitochondrial ROS reestablished the function of dADSCs in vitro. It appears that mitoTEMPO pretreatment restored the function of dADSCs by remodeling, whereas overexpression of glyoxalase-1 enhanced the function of ADSCs by increasing their survival under oxidative stress. Short-term treatment of dADSCs with mitoTEMPO improved their viability and differentiation, migration, and proangiogenic capacities. The mitoTEMPO-pretreated dADSCs showed characteristics similar to those of non-diabetic ADSCs.

Stem cells are characterized by two key properties, self-renewal (i.e., the ability to proliferate without lineage commitment) and pluripotency (i.e., the ability to differentiate

into more than one cell type)⁴⁰. dADSCs showed impaired capacities for both self-renewal and pluripotency. However, mitoTEMPO pretreatment for three passages restored the function of dADSCs, suggesting that dADSCs could be remodeled in vitro and that mitoTEMPO pretreatment changed dADSCs to normal ADSCs. These results provided a solution for the restoration of dADSC function in vitro. However, as there are multiple risk factors in diabetes, such as inflammation, hyperglycemia, and oxidative stress, the restoration of dADSC function may not be sufficient to improve the therapeutic effects of transplanted ADSCs. Therefore, additional strategies to enhance the function of dADSCs in the diabetic environment should be pursued.

It should be noted that in the clinic, patients with diabetes often go through a long course before the development of limb vascular disease. The long course of hyperglycemia and other environments in diabetes remodel ADSCs and impair their function in patients with diabetes. Here, we only isolated ADSCs from diabetic mice 2 weeks after STZ injection. This cannot totally mimic the true situation in clinical patients. Further studies are warranted to explore the potential application of mitochondrial ROS scavenger in remodeling of diabetic ADSC function in clinic.

Conclusions

This study provided direct evidence that pretreatment of dADSCs with mitoTEMPO for three passages can effectively restore their impaired cell viability and migration, differentiation, and proangiogenic capacities both in vitro and in vivo, leading to a better prognosis for diabetic ischemic diseases. These findings suggest that short-term pretreatment of dADSCs with a mitochondrial ROS scavenger may be an effective strategy for improving the therapeutic effects of ADSC-based therapy in patients with diabetes.

Acknowledgments

The authors thank Dr. Yan Fei (Fourth Military Medical University) for advice on the STZ-induced diabetic mouse model.

Author Contributions

LK and WQ performed the statistical analyses, evaluated the results and drafted the paper. HK, CC and LK participated in the conception and design of the study. GR, YH and CY contributed to laboratory measurement and data assurance. ZS and MS contributed to analyzing data.

Availability of Data and Material

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Ethical Approval

All procedures in this study were approved by the Committee on Animal Care of Fourth Military Medical University.

Statement of Human and Animal Rights

All the guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health, and the protocol was approved by the Committee on Animal Care of Fourth Military Medical University.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.


Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Program for the National Natural Science Foundation of China-Youth Projects (No. 81500272, 81600356, 81702733), the Science and Technology Development Research Project of Shaanxi Province-Key Problems of Science and Technology in Social Development (No. 2016SF-034), and the National Natural Science Foundation of China (No. 81670229, 81570210).

ORCID iD

Chengxiang Li  <https://orcid.org/0000-0001-8297-1481>

Supplemental Material

Supplemental material for this article is available online.

References

- Mizuno H, Tobita M, Uysal AC. Concise review: adipose-derived stem cells as a novel tool for future regenerative medicine. *Stem Cells*. 2012;30(5):804–810.
- Gimble JM, Bunnell BA, Guilak F. Human adipose-derived cells: an update on the transition to clinical translation. *Regen Med*. 2012;7(2):225–235.
- Fan W, Sun D, Liu J, Liang D, Wang Y, Narsinh KH, Li Y, Qin X, Liang J, Tian J, Cao F. Adipose stromal cells amplify angiogenic signaling via the VEGF/mTOR/Akt pathway in a murine hindlimb ischemia model: a 3D multimodality imaging study. *PLoS One*. 2012;7(9):e45621.
- Taha MF, Hedayati V. Isolation, identification and multipotential differentiation of mouse adipose tissue-derived stem cells. *Tissue Cell*. 2010;42(4):211–216.
- Bateman ME, Strong AL, Gimble JM, Bunnell BA. Concise review: using fat to fight disease: a systematic review of non-homologous adipose-derived stromal/stem cell therapies. *Stem Cells*. 2018;36(9):1311–1328.
- Zhao L, Johnson T, Liu D. Therapeutic angiogenesis of adipose-derived stem cells for ischemic diseases. *Stem Cell Res Ther*. 2017;8(1):125.
- Writing Group M, Writing Committee M, Accf/Aha Task Force M. 2011 ACCF/AHA Focused Update of the Guideline for the Management of patients with peripheral artery disease (Updating the 2005 Guideline): a report of the American

- College of Cardiology Foundation/American Heart Association Task Force on practice guidelines. *Circulation*. 2011; 124(18):2020–2045.
8. Peng Z, Yang X, Qin J, Ye K, Wang X, Shi H, Jiang M, Liu X, Lu X. Glyoxalase-1 overexpression reverses defective proangiogenic function of diabetic adipose-derived stem cells in streptozotocin-induced diabetic mice model of critical limb ischemia. *Stem Cells Transl Med*. 2017;6(1):261–271.
 9. Uccelli A, Moretta L, Pistoia V. Mesenchymal stem cells in health and disease. *Nat Rev Immunol*. 2008;8(9):726–736.
 10. Maxson S, Lopez EA, Yoo D, Danilkovitch-Miagkova A, Leroux MA. Concise review: role of mesenchymal stem cells in wound repair. *Stem Cells Transl Med*. 2012;1(2):142–149.
 11. Tavintharan S, Ning C, Su Chi L, Tay W, Shankar A, Shyong Tai E, Wong TY. Prevalence and risk factors for peripheral artery disease in an Asian population with diabetes mellitus. *Diab Vasc Dis Res*. 2009;6(2):80–86.
 12. Marso SP, Hiatt WR. Peripheral arterial disease in patients with diabetes. *J Am Coll Cardiol*. 2006;47(5):921–929.
 13. Yang SL, Zhu LY, Han R, Sun LL, Li JX, Dou JT. Pathophysiology of peripheral arterial disease in diabetes mellitus. *J Diabetes*. 2017;9(2):133–140.
 14. Saki N, Jalalifar MA, Soleimani M, Hajizamani S, Rahim F. Adverse effect of high glucose concentration on stem cell therapy. *Int J Hematol Oncol Stem Cell Res*. 2013;7(3):34–40.
 15. Ferrer-Lorente R, Bejar MT, Tous M, Vilahur G, Badimon L. Systems biology approach to identify alterations in the stem cell reservoir of subcutaneous adipose tissue in a rat model of diabetes: effects on differentiation potential and function. *Diabetologia*. 2014;57(1):246–256.
 16. Li TS, Furutani A, Takahashi M, Ohshima M, Qin SL, Kobayashi T, Ito H, Hamano K. Impaired potency of bone marrow mononuclear cells for inducing therapeutic angiogenesis in obese diabetic rats. *Am J Physiol Heart Circ Physiol*. 2006; 290(4):H1362–1369.
 17. Li X, Xie X, Lian W, Shi R, Han S, Zhang H, Lu L, Li M. Exosomes from adipose-derived stem cells overexpressing Nrf2 accelerate cutaneous wound healing by promoting vascularization in a diabetic foot ulcer rat model. *Exp Mol Med*. 2018;50(4):29.
 18. Escribano-Lopez I, Diaz-Morales N, Iannantuoni F, Lopez-Domenech S, de Maranon AM, Abad-Jimenez Z, Banuls C, Rovira-Llopis S, Herance JR, Rocha M, Victor VM. The mitochondrial antioxidant SS-31 increases SIRT1 levels and ameliorates inflammation, oxidative stress and leukocyte-endothelium interactions in type 2 diabetes. *Sci Rep*. 2018; 8(1):15862.
 19. Rueggsegger GN, Creo AL, Cortes TM, Dasari S, Nair KS. Altered mitochondrial function in insulin-deficient and insulin-resistant states. *J Clin Invest*. 2018;128(9):3671–3681.
 20. Huang YW, Zhu QQ, Yang XY, Xu HH, Sun B, Wang XJ, Sheng J. Wound healing can be improved by (-)-epigallocatechin gallate through targeting Notch in streptozotocin-induced diabetic mice. *FASEB J*. 2019;33(1):953–964.
 21. Fan W, Cheng K, Qin X, Narsinh KH, Wang S, Hu S, Wang Y, Chen Y, Wu JC, Xiong L, Cao F. mTORC1 and mTORC2 play different roles in the functional survival of transplanted adipose-derived stromal cells in hind limb ischemic mice via regulating inflammation in vivo. *Stem Cells*. 2013;31(1):203–214.
 22. Qin JB, Li KA, Li XX, Xie QS, Lin JY, Ye KC, Jiang ME, Zhang GX, Lu XW. Long-term MRI tracking of dual-labeled adipose-derived stem cells homing into mouse carotid artery injury. *Int J Nanomedicine*. 2012;7:5191–5203.
 23. Policha A, Zhang P, Chang L, Lamb K, Tulenko T, DiMuzio P. Endothelial differentiation of diabetic adipose-derived stem cells. *J Surg Res*. 2014;192(2):656–663.
 24. Guo Y, Wang Z, Qin X, Xu J, Hou Z, Yang H, Mao X, Xing W, Li X, Zhang X, Gao F. Enhancing fatty acid utilization ameliorates mitochondrial fragmentation and cardiac dysfunction via rebalancing optic atrophy 1 processing in the failing heart. *Cardiovasc Res*. 2018;114(7):979–991.
 25. Liu X, Li Q, Niu X, Hu B, Chen S, Song W, Ding J, Zhang C, Wang Y. Exosomes secreted from human-induced pluripotent stem cell-derived mesenchymal stem cells prevent osteonecrosis of the femoral head by promoting angiogenesis. *Int J Biol Sci*. 2017;13(2):232–244.
 26. Hao L, Zou Z, Tian H, Zhang Y, Zhou H, Liu L. Stem cell-based therapies for ischemic stroke. *Biomed Res Int*. 2014; 2014:468748.
 27. Maciejczyk M, Zebrowska E, Zalewska A, Chabowski A. Redox balance, antioxidant defense, and oxidative damage in the hypothalamus and cerebral cortex of rats with high fat diet-induced insulin resistance. *Oxid Med Cell Longev*. 2018;2018:6940515.
 28. Hameed I, Masoodi SR, Mir SA, Nabi M, Ghazanfar K, Ganai BA. Type 2 diabetes mellitus: from a metabolic disorder to an inflammatory condition. *World J Diabetes*. 2015;6(4): 598–612.
 29. Karam BS, Chavez-Moreno A, Koh W, Akar JG, Akar FG. Oxidative stress and inflammation as central mediators of atrial fibrillation in obesity and diabetes. *Cardiovasc Diabetol*. 2017; 16(1):120.
 30. Rochette L, Zeller M, Cottin Y, Vergely C. Diabetes, oxidative stress and therapeutic strategies. *Biochim Biophys Acta*. 2014; 1840(9):2709–2729.
 31. Nowotny K, Jung T, Hohn A, Weber D, Grune T. Advanced glycation end products and oxidative stress in type 2 diabetes mellitus. *Biomolecules*. 2015;5(1):194–222.
 32. Rennert RC, Sorkin M, Januszyk M, Duscher D, Kosaraju R, Chung MT, Lennon J, Radiya-Dixit A, Raghvendra S, Maan ZN, Hu MS, et al. Diabetes impairs the angiogenic potential of adipose-derived stem cells by selectively depleting cellular subpopulations. *Stem Cell Res Ther*. 2014;5(3):79.
 33. Hascall V, Wang A. Hyperglycemia diverts dividing stem cells to pathological adipogenesis. *Stem Cell Res Ther*. 2014;5(6):128.
 34. Houstis N, Rosen ED, Lander ES. Reactive oxygen species have a causal role in multiple forms of insulin resistance. *Nature*. 2006;440(7086):944–948.
 35. Bashan N, Kovsan J, Kachko I, Ovadia H, Rudich A. Positive and negative regulation of insulin signaling by reactive oxygen and nitrogen species. *Physiol Rev*. 2009;89(1):27–71.
 36. Teslaa T, Teitell MA. Pluripotent stem cell energy metabolism: an update. *EMBO J*. 2015;34(2):138–153.

37. Khacho M, Slack RS. Mitochondrial and reactive oxygen species signaling coordinate stem cell fate decisions and life long maintenance [published online ahead of print July 31, 2017]. *Antioxid Redox Signal*. 2017.
38. Li Q, Gao Z, Chen Y, Guan MX. The role of mitochondria in osteogenic, adipogenic and chondrogenic differentiation of mesenchymal stem cells. *Protein Cell*. 2017;8(6): 439–445.
39. Yang F, Yan G, Li Y, Han Z, Zhang L, Chen S, Feng C, Huang Q, Ding F, Yu Y, Bi C, et al. Astragalus polysaccharide attenuated iron overload-induced dysfunction of mesenchymal stem cells via suppressing mitochondrial ROS. *Cell Physiol Biochem*. 2016;39(4):1369–1379.
40. Zhang H, Wang ZZ. Mechanisms that mediate stem cell self-renewal and differentiation. *J Cell Biochem*. 2008;103(3): 709–718.