Journal of the American Heart Association

ORIGINAL RESEARCH

Combination of Antioxidant Enzyme Overexpression and N-Acetylcysteine Treatment Enhances the Survival of Bone Marrow Mesenchymal Stromal Cells in Ischemic Limb in Mice With Type 2 Diabetes

Qiang Zhu, MD; Hong Hao, PhD; Huifang Xu, MD, PhD; Yosef Fichman, PhD; Yuqi Cui, MD, PhD; Chunlin Yang, PhD; Meifang Wang, MSc; Ron Mittler, PhD; Michael A. Hill, PhD; Peter J. Cowan, PhD; Guangsen Zhang, MD, PhD; Xiaoming He, PhD; Shenghua Zhou, MD, PhD; Zhenguo Liu, MD, PhD

BACKGROUND: Therapy with mesenchymal stem cells remains a promising but challenging approach to critical limb ischemia in diabetes because of the dismal cell survival.

METHODS AND RESULTS: Critical limb ischemia in type 2 diabetes mouse model was used to explore the impact of diabetic limb ischemia on the survival of bone marrow mesenchymal stromal cells (bMSCs). Inhibition of intracellular reactive oxygen species was achieved with concomitant overexpression of superoxide dismutase (SOD)-1 and glutathione peroxidase-1 in the transplanted bMSCs, and extracellular reactive oxygen species was attenuated using SOD-3 overexpression and N-acetylcysteine treatment. In vivo optical fluorescence imaging and laser Doppler perfusion imaging were used to track cell retention and determine blood flow in diabetic ischemic limb, respectively. Survival of the transplanted bMSCs was significantly decreased in diabetic ischemic limb compared with the control. In vitro study indicated that advanced glycation end products, not high glucose, significantly decreased the proliferation of bMSCs and increased their apoptosis associated with increased reactive oxygen species production and selective reduction of SOD-1 and SOD-3. In vivo study demonstrated that concomitant overexpression of SOD-1, SOD-3, and glutathione peroxidase-1, or host treatment with N-acetylcysteine, significantly enhanced in vivo survival of transplanted bMSCs, and improved critical limb ischemia in diabetic mice. Combination of triple antioxidant enzyme overexpression in bMSCs with host N-acetylcysteine treatment further improved bMSC survival with enhanced circulatory and functional recovery from diabetic critical limb ischemia.

CONCLUSIONS: Simultaneous suppression of reactive oxygen species from transplanted bMSCs and host tissue could additively enhance bMSC survival in diabetic ischemic limb with increased therapeutic efficacy in diabetes.

Key Words: cell therapy ■ diabetes ■ ischemic disease ■ mesenchymal stem cells ■ oxidative stress

Diabetes is a major contributing factor to a variety of cardiovascular diseases.[1] Diabetic patients have significantly increased risk for developing severe peripheral artery disease with critical limb ischemia (CLI) and poorly healing chronic ulcers with limited effective treatment options.[2] On the basis of the Inter-Society Consensus for the Management of Peripheral Artery Disease, it is estimated that 25% of patients with

Correspondence to: Zhenguo Liu, MD, PhD, Division of Cardiovascular Medicine, Department of Medicine, Center for Precision Medicine, 1 Hospital Dr CE344, Columbia, MO 65212. E-mail: liuzheng@health.missouri.edu

Supplementary Material for this article is available at https://www.ahajournals.org/doi/suppl/10.1161/JAHA.121.023491

For Sources of Funding and Disclosures, see page 16.

JAHA is available at: www.ahajournals.org/journal/jaha

CLINICAL PERSPECTIVE

What Is New?

- The study demonstrated that intracellular reactive oxygen species from transplanted bone marrow mesenchymal stromal cells (bMSCs) and reactive oxygen species from host tissue equally contributed to the decreased survival of transplanted bMSCs in diabetic ischemic limb.
- Combination of triple antioxidant enzyme overexpression in bMSCs with host N-acetylcysteine treatment significantly improved bMSC survival with enhanced circulatory and functional recovery from diabetic limb ischemia.

What Are the Clinical Implications?

- A combination therapy with simultaneous suppression of reactive oxygen species from transplanted bMSCs and host tissue may provide a novel and effective approach to enhancing the in vivo survival of transplanted bMSCs with increased therapeutic efficacy on the treatment of diabetic limb ischemia.

Nonstandard Abbreviations and Acronyms

AGE	advanced glycation end product
bMSC	bone marrow mesenchymal stromal cell
CLI	critical limb ischemia
FGF-2	basic fibroblast growth factor
Gpx-1	glutathione peroxidase-1
HGF	hepatocyte growth factor
IVIS	in vivo optical fluorescence imaging
PLGF-2	placental growth factor-2
ROS	reactive oxygen species
SOD	superoxide dismutase
WT	wild type

CLI will die within 1 year of diagnosis, and an additional 30% will require limb amputation.[3] This highlights the need for novel and effective therapeutic interventions.

Stem cell therapy remains a viable and attractive option for tissue repair and regeneration.[2] Bone marrow mesenchymal stromal cells (bMSCs) are an ideal source for cell therapy because of the fact that these cells can be easily obtained without ethical concerns, and conveniently expanded ex vivo to clinical scales in a relatively short period of time with minimal loss of potency. Moreover, bMSCs have little (if any) inherent immunogenicity for adverse immune reactions because of their immunosuppressive and/or immunomodulatory properties.[4] However, one of the major challenges for stem cell therapy is the low viability of the implanted cells, with cell loss (and/or death) typically occurring in the first few days after transplantation. Studies in multiple organ systems, including lung, heart, kidney, liver, spleen, and gut, have shown that <1% of systemically administered mesenchymal stromal cells remain present for longer than a week following injection.[5–7] When stem cells are injected directly into the ischemic areas, <10% of the delivered cells are retained or viable in the target areas 4 weeks after delivery (for local delivery via intracoronary or intramyocardial injection).[8]

Many clinical studies have demonstrated that administration of bMSCs (both locally and systematically) in human subjects appeared to be safe, and exhibited promising therapeutic effects for a wide range of disease states, including (but not limited to) myocardial infarction, ischemic and nonischemic cardiomyopathy, ischemic stroke, and spinal cord injury.[9–11] However, some clinical studies have shown limited benefit or no therapeutic value, particularly in diabetic patients.[12–14] It is shown that therapy with $CD34^+$ cells, although beneficial in patients with nonischemic cardiomyopathy with normal glucose metabolism, is ineffective in diabetic patients for unknown reasons.[15] Although the exact cause(s) for the inconsistency in the results of clinical studies is unclear, the outcome of cell therapy could ultimately be associated with the number and function of delivered bMSCs. It is known that reactive oxygen species (ROS) production is increased with increased oxidative stress in diabetes,[16,17] and ROS is closely involved in the fate determination of stem cells.

The present study explored the impact of diabetes on the survival of bMSCs, and the role of intracellular ROS of transplanted bMSCs and extracellular ROS from host tissue on bMSC survival in diabetic ischemic limb. There were 3 objectives: (1) to evaluate the survival of bMSCs in mice with type 2 diabetes using CLI model; (2) to define the role of advanced glycation end products (AGEs) and ROS on bMSC survival in diabetic CLI; and (3) to determine the contributions of ROS from transplanted cells and from the host tissue to cell survival.

METHODS

Animals

The materials, methods, and data that supported the findings of the present study are available from the corresponding author on reasonable request. All animal experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* of US National Institutes of Health. The experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Missouri, Columbia, MO. Male TALLYHO/JngJ mice (16–20 weeks old; stock No. 005314; from the Jackson

Laboratory, ME) were used in creating the CLI model, with age-matched SWR/J mice (stock No. 000689; from the Jackson Laboratory) as nondiabetic control. Wild-type C57BL/6 (WT) mice (4 weeks old; male) and mice with concomitant global overexpression of superoxide dismutase (SOD)-1, SOD-3, and glutathione peroxidase-1 (Gpx-1) (triple-transgenic [TG] mice; 4 weeks old; male) with C57BL/6 background were used for isolation of bMSCs. TG mice were identified and confirmed using genotyping, Western blot analysis, and increased enzyme activities, as described in our previous studies.[18–20] The expression of antioxidant enzymes SOD-1, SOD-3, and Gpx-1 in bMSCs from WT mice and age-matched TG mice was confirmed using Western blot assay, as shown in Figure 3L. Antibodies for SOD-1 (1:1000; Invitrogen; MA1-105), SOD-3 (1:1000; Invitrogen; PA5-93329), and Gpx-1 (1:500; Invitrogen; 702762) were used for immunoblots of the respective proteins. All mice were randomly assigned to each group with the animal experiments performed blindly.

Measurement of Body Weight, Blood Glucose, and Plasma AGEs

Body weight and fasting blood glucose of diabetic and nondiabetic mice (n=20/group) were obtained using a weight scale and blood glucose meter (AimStrip, San Antonio, TX) before femoral artery ligation and day 14 and day 21 after ligation. Plasma AGEs were measured using a mouse AGE ELISA Kit (Biotang Incorporation, Lexington, MA) before ligation and day 21 after ligation.

Cell Culture and Identification

bMSCs were isolated and expanded, as described, with minor modification.[21] The cells were characterized phenotypically using cell surface markers (positive for CD29, Scal-1, CD44, and CD105 and negative for CD34, CD45, CD11b, and CD31) with flow cytometric analysis (Figure S1A) and multilineage differentiation with chemical induction and staining, including adipogenic differentiation using oil red O staining, osteogenic differentiation using alizarin red staining, and chondrogenic differentiation using Alcian blue and nuclear fast red staining (Figure S1B through S1E). The antibodies for flow cytometry analysis, including fluorescein isothiocyanate (FITC) anti-mouse CD29 (102205), Peridinin-Chlorophyll-Protein anti-mouse Sca-1 (108122), FITC anti-mouse CD44 (103005), Allophycocyanin (APC) anti-mouse CD105 (120413), Phycoerythrin anti-mouse CD34 (119308), APC/cyanine7 anti-mouse CD45 (103115), AF 700 anti-mouse CD11b (101222), and PE/cyanine7 anti-mouse CD31 (102418), were from Biolegend. Cell populations were carefully compensated with isotype antibody staining as control. Fluorescence-positive cells were quantitatively evaluated using LSRFortessa X-20 (BD Bioscience, CA), and analyzed using software FlowJo_V10.

Creation of Mouse Diabetic CLI Model and Transplantation of bMSCs

TALLYHO/JngJ diabetic mice and age-matched SWR/J nondiabetic mice were randomly divided into experimental groups, with 8 mice in each group. SWR/J mice are recommended as the "normal" genetically related control for TALLYHO/JngJ mice because the 2 strains share 86.8% of their genotype and 67.1% of their haplotype based on TALLYHO/JngJ single-nucleotide polymorphism alignment with Swiss family strains.[22,23] CLI was accomplished by ligation of the right femoral artery with 6-0 silk sutures after a 3-mm incision with minimal tissue disturbance, as described.[24] Mice with sham operation underwent the same surgical procedure without artery ligation. In brief, the right femoral artery was transected between the sutures. Successful creation of CLI was confirmed using laser Doppler perfusion imaging (Moor Instruments, Devon, UK) that showed no signal of blood perfusion. After 30 minutes, cell injections were made at 3 sites of the downstream area of ligation (right adductor muscle) with a total volume of 100 μL PBS containing 5×10^5 cells labeled with CellTracker DiR (Molecular Probes, Eugene, OR) fluorescence indicator, or same volume of PBS as control using a 29-gauge syringe. For mice with N-acetylcysteine treatment, the animals were treated with N-acetylcysteine (1 mg/mL in the drinking water; Sigma-Aldrich, St. Louis, MO) for 24 hours before creation of CLI and continuously for the rest of the experiment.

Assessment of Blood Perfusion and Function of Ischemic Hind Limb

Laser Doppler perfusion imaging was used to determine blood perfusion recovery of the ischemic limb, as reflected by the ratio of blood perfusion (right or ischemic limb blood perfusion/left or normal limb blood perfusion) before ligation and 30 minutes and days 3, 7, 14, and 21 after ligation under temperature-controlled and air anesthesia (1.5% isoflurane) conditions. Swimming endurance test was performed, as described,[25] to evaluate function recovery of the ischemic limb, as reflected by swimming time for each mouse in a swimming pool before ligation and days 14 and 21 after ligation. The semiquantitative assessment of ambulatory impairment and limitation of ischemic limb (modified clinical standard score) was performed to assess the function of ischemic limb before ligation and days 14 and 21 after ligation, as described.[26,27] The score of ischemic limb recovery was as follows: 0, flexing the toes to resist gentle traction on the tail; 1, plantar flexion; 2, no dragging but no plantar flexion; and 3, dragging of foot, as described.[27]

In Vivo Optical Fluorescent Imaging for Cell Tracking

In vivo optical fluorescence imaging was performed to track and quantitatively monitor the number of viable transplanted bMSCs in the ischemic limb. Fluorescence intensity of DiR-labeled transplanted cells (excitation/emission, 740/790 nm; 0.5-second exposure) was detected using in vivo optical fluorescence imaging (IVIS; Lumina S5; PerkinElmer, Houston, TX). Recipient mice were anesthetized and placed in the imaging chamber. Baseline image was acquired before intramuscular injection of labeled bMSCs. Mice were imaged at days 1, 3, 7, 14, 21, and 28 after cell transplantation. Average signals (photons/s/cm^2/steridian) from the fixed region of interest were assessed using Living Image 4.7.2, as described.[28] In ex vivo experiments, the adductor muscle of the ischemic hind limb was harvested 14 days after CLI operation, and double-immunofluorescence (4′,6-diamidino-2-phenylindole and DiR) imaging of frozen muscle sections (7 μm) were performed using a laser confocal microscope (LeicaSP8-Confocal; Leica, Buffalo Grove, IL) and analyzed using software ImageJ. A linear relationship between fluorescence intensity and cell number was established using in vitro fluorescence assays with different amounts of bMSCs, as described.[29] Briefly, a series of specific amounts of cells were incubated with lipophilic carbocyanine fluorescent dye DiR (2 μmol/L) for 5 minutes at 37 °C, and then for an additional 15 minutes at 4 °C. After washing with PBS for 2 times, the cells were plated into a 24-well plate, and then imaged using PerkinElmer IVIS to determine the linear relationship between fluorescence intensity and cell numbers.

Histological Analysis

Adductor muscles of the ischemic hind limb were harvested, weighed, and prepared for histological fluorescent staining and hematoxylin and eosin staining. Detection of in situ formation of O_2^- was performed using the fluorescent dye dihydroethidium, as described.[30] Briefly, the muscle frozen sections were fixed with paraformaldehyde at room temperature for 10 minutes, and incubated with dihydroethidium (1:1000; Invitrogen; D1168) at room temperature for 15 minutes. After washing (×3) with PBS, the tissue preparations were incubated with 4′,6-diamidino-2-phenylindole at room temperature for 10 minutes, and then examined with a laser confocal microscope and analyzed with software ImageJ. Multiple sections across the length of muscle were obtained from each sample, and 2 sections were randomly selected for each sample for each of the histological classifications (dihydroethidium fluorescence, CD31$^+$ capillary density, and hematoxylin and eosin staining, see below). From each section, 3 independent fields were examined with a laser confocal microscope or an inverted light microscope. The average value from the 6 fields (2 sections with 3 fields each) was calculated and used for data analysis for each animal.

CD31 immunofluorescent staining was performed to evaluate the capillary density in the ischemic limb. Frozen muscle sections (7 μm) were fixed with paraformaldehyde for 10 minutes, blocked with 2% BSA for 30 minutes, and incubated with AF 594 anti-mouse CD31 immunofluorescent antibody (1:400; Biolegend; 102432) at 4 °C overnight. After washing (×3) with PBS, the preparations were incubated with 4′,6-diamidino-2-phenylindole. CD31-positive capillary structure was pictured using a laser confocal microscope, and the capillary density was quantified using software ImageJ. The morphology and structure of the adductor muscle in the ischemic limb were evaluated using frozen section hematoxylin and eosin staining kit (Thermo Scientific, Waltham, MA). Muscle fiber areas were quantified using software ImageJ for all animals.

Flow Cytometric Analysis of Cell Apoptosis and Intracellular ROS

bMSCs were incubated in high D-glucose medium (30 mmol/L), high L-glucose medium (24.5 mmol/L plus 5.5 mmol/L high D-glucose medium for high osmolarity control), and low D-glucose medium (5.5 mmol/L) under hypoxic condition (5% O_2, 5% CO_2, and 90% N_2) at 37 °C for 24 hours. The cells were then harvested and prepared for flow cytometric analysis for cell apoptosis (FITC Apoptosis Detection Kit; BD Pharmingen, San Jose, CA) and intracellular ROS (CM-H2DCFDA; Invitrogen, Waltham, MA), as described.[19] AGEs and their receptors have been implicated in pathogenesis of diabetic complications, including cardiovascular complications, and associated with worse prognosis.[31,32] Therefore, we used AGE-BSA (prepared by reaction between BSA and glycolaldehyde; Millipore, Billerica, MA) to treat bMSCs to investigate the effects of AGEs on bMSCs at the concentration of 400 μg/mL that was determined through preliminary experiments using WT-bMSCs and previous studies.[33,34] There were 5 groups: (1) WT+BSA, (2) TG+BSA, (3) WT+AGE-BSA, (4) TG+AGE-BSA, and (5) WT+AGE-BSA+N-acetylcysteine (2 mmol/L). Cells were cultured with 5% O_2 at 37 °C for 24 hours, and then harvested for flow cytometric analysis for early apoptosis (annexin V FITC positive), late apoptosis (annexin V FITC and propidium iodide double positive), and intracellular ROS (ROS Detection Reagents-FITC; Invitrogen), as described.[19] Fluorescence-positive cells were quantitatively evaluated using LSRFortessa X-20 (BD Bioscience, CA), and analyzed using software FlowJo_V10. For all fluorescence-activated cell sorting analysis, at least 5×10^4 cells were counted with a total of at least 1×10^6 cells for each sample.

Cell Proliferation Assay

Cell proliferation was evaluated using EdU Cell Proliferation Kit (Invitrogen; AF488 for Imaging), as described.[35] Briefly, bMSCs were cultured in medium with AGE-BSA or BSA (400 μg/mL) with 5% O_2 at 37 °C for 24 hours, and then moved to the medium with 1× 5-ethynyl-2'-deoxyuridine (EdU) solution and incubated for 6 hours. After washing (×3), cells were fixed and permeabilized, and then mixed with Click-iT reaction cocktail for imaging. Proliferative cells were examined using a fluorescence microscope and quantified using software ImageJ.

Tube Formation and Cell Migration Assay

Tube formation assay was performed using Matrigel basement membrane matrix (Corning, Tewksbury, MA) in 96-well plates (50 μL/well), as described.[35] Briefly, bMSCs were incubated in medium with BSA (control) or AGE-BSA (400 μg/mL) for 24 hours with 1% O_2, then seeded onto prepared Matrigel with a cell density of 3×10^4 cells/well and cultured for 4 hours. Tube formation was examined and quantified by calculating the lengths of tubes with software ImageJ. Cell migration assay was performed using transwell culture chambers (24-well, 8-μm pore size; Corning, NY). After incubating in medium with BSA (control) or AGE-BSA (400 μg/mL) for 24 hours with 1% O_2, the cells were suspended in serum-free medium, and plated (5×10^4/well) in the upper compartment of the chamber with medium containing 10% fetal bovine serum in the lower chamber as an inducer for migration. After incubation at 37 °C for 12 hours, cells were fixed with ice-cold acetone for 10 minutes and stained with crystal violet for 15 minutes. The upper surface of the chamber was wiped with a cotton swab, and the cells on the downside were imaged from 5 random microscopic fields and quantified using software ImageJ.

Measurement of Angiogenetic Growth Factors and Western Blot Analysis

To determine the effect of high glucose and AGEs on the paracrine function of bMSCs, the conditioned media of bMSCs were collected after 24 hours of culture with high glucose or AGEs for measurement of angiogenetic growth factors, including vascular endothelial growth factor (VEGF), epidermal growth factor, basic fibroblast growth factor (FGF-2), hepatocyte growth factor (HGF), and placental growth factor-2 (PLGF-2), using ELISA assay by Eve Technologies Corporation (Calgary, Canada). Cells were collected and prepared for Western blot analysis for protein expression, as described.[35] Protein lysates were loaded and separated on 10% SDS-PAGE gels, and then transferred onto 0.45 μm nitrocellulose membrane. The preparations were blocked with 5% milk in 1× tris-buffered saline and Tween 20, and incubated with primary antibodies at 4 °C overnight, followed by incubation with corresponding second antibodies at room temperature for 1 hour. The primary antibodies were as follows: SOD-1 (1:1000; Invitrogen; MA1-105), SOD-3 (1:1000; Invitrogen; PA5-93329), and Gpx-1 (1:500; Invitrogen; 702762). The protein bands were detected using electrochemiluminescence reagent with Odyssey Imaging System (Li-Cor Biosciences, Lincoln, NE), and analyzed using software ImageJ.

Hydrogen Peroxide Measurement

Hydrogen peroxide (H_2O_2) is considered a key ROS implicated in cell signaling and diabetic pathology.[36,37] Thus, H_2O_2 production was measured using quantitative peroxide assay kit (Lipid Compatible; Thermo Scientific, Waltham, MA), as per manufacturer's instructions. The conditional media from bMSCs and mouse plasma were collected and prepared for peroxide measurements. For each sample, 10 μL of methanol was mixed with 90 μL conditional media or plasma in microcentrifuge tubes. After incubation for 30 minutes at room temperature, an aliquot of 900 μL reaction reagent was added into each sample in microcentrifuge tube for a second incubation of 30 minutes. After centrifuging at 12 000*g* for 10 minutes, 300 μL of supernatant was transferred to a 48-well plate for analysis using a microplate reader at the wavelength of 595 nm (Biotek, VT).

Statistical Analysis

All the data were expressed as means±SDs and analyzed with GraphPad Prism 8.0 software (San Diego, CA). A 2-tailed unpaired Student *t* test was used for comparisons of 2 groups of data with normal distribution and equal variance, and a 2-tailed unpaired Student *t* test with Welch correction was used for 2 groups of data with normal distribution and unequal variance. A Mann-Whitney test was used for the analysis of 2 groups of data with abnormal distributions. ANOVA with Bonferroni post hoc test or Tukey test was used for data analysis of multiple groups with normal distributions and equal variance, and Kruskal-Wallis test with Dunn post hoc test was used for comparisons of multiple groups with normal distributions and unequal variance or abnormal distributions. Correlation data were evaluated with polynomial regression analysis. $P<0.05$ was considered statistically significant for the comparisons.

RESULTS

Survival of bMSCs Was Significantly Decreased in Diabetic Mice With Decreased Blood Flow Recovery From Limb Ischemia

First, we tested the hypothesis that a mouse model of type 2 diabetes would be associated with decreased in vivo survival of bMSCs, thus contributing to their

decreased therapeutic efficacy in diabetes. Because >90% of diabetic patients have type 2 diabetes,[38] male TALLYHO/JngJ mice (16–20 weeks old; from Jackson Laboratory) were used in the present study. The TALLYHO/JngJ mouse is a polygenic mouse model of spontaneous type 2 diabetes that exhibits phenotypic characteristics similar to human type 2 diabetes with the plasma glucose levels of 200 to 400 mg/dL.[22] Age-matched male nondiabetic SWR/J mice were used as controls.

Body weight, blood glucose level, and plasma AGE level were significantly increased in diabetic TALLYHO/JngJ mice compared with control mice (Figure 1A through 1C), confirming the suitability of the mouse model. To determine if the therapeutic potential of bMSCs on limb ischemia was impaired in diabetic mice, bMSCs were prepared from male WT C57BL/6 mice. The cells were identified with immunophenotype markers using flow cytometric analysis (positive for CD29, Sca-1, CD44, and CD105 and negative for CD34, CD45, CD11b, and CD31; Figure S1A) and multilineage differentiation using chemical induction and staining (adipogenic differentiation with oil red O staining, osteogenic differentiation with alizarin red staining, and chondrogenic differentiation with Alcian blue and nuclear fast red staining; Figure S1B through S1E). CLI was created by ligation of right femoral artery with minimal tissue disturbance, as described,[24] and confirmed by laser Doppler perfusion imaging (FLPI-2; Moor Instruments, Devon, UK) that showed no signal for blood flow perfusion. After 30 minutes, a total of 5×10^5 cells labeled with CellTracker DiR (Molecular Probes, Eugene, OR) fluorescence indicator in 100 μL or the same volume of PBS were injected into 3 sites of ischemic right limb adductor muscle. IVIS (Lumina S5; PerkinElmer, Houston, TX) was performed to quantitatively monitor viable transplanted bMSCs. A linear relationship between viable cell number and fluorescence intensity was established to quantify the bMSCs in vivo with an r^2 of 0.990 (polynomial regression analysis; Figure S2A and S2B). IVIS imaging showed that the survival of bMSCs was significantly decreased in the ischemic limb in diabetic mice compared with nondiabetic controls (from 32.90% to 7.03% at week 1, and from 8.28% to undetectable level at week 2 following cell delivery; Figure 1D and 1F). Decreased bMSC number was confirmed by ex vivo histological examination (Figure 1E and 1G).

Blood flow recovery, as calculated by the ratio of ischemic limb/nonligated limb blood perfusion, was significantly decreased in diabetic mice compared with nondiabetic controls (Figure 2A and 2B). Functional recovery of the ischemic limb, as assessed using a swimming endurance test and a semiquantitative assessment of ambulatory impairment and limitation of the ischemic limb (modified clinical standard score), was also significantly decreased in diabetic mice compared with nondiabetic mice, as expected (Figure 2C and 2D). Histological analysis showed that the capillary density and muscle fiber area were significantly decreased in diabetic ischemic limbs compared with nondiabetic controls (Figure 2E through 2H). Treatment with bMSCs significantly improved the recovery of ischemic limb blood flow in diabetic ischemic limbs. However, the recovery of blood flow was significantly decreased compared with nondiabetic mice with bMSC treatment.

Overexpression of Triple Antioxidant Enzymes Significantly Improved the Viability and Function of bMSCs in the Presence of AGEs

We then investigated the mechanism for decreased survival of bMSCs in diabetic mice. We have shown that high glucose had no effect on the proliferation and apoptosis of bMSCs.[39] AGEs and their receptors have been implicated in the pathogenesis of diabetes, and are significantly associated with cardiovascular complications and worse prognosis.[31,32] Thus, we investigated the effects of AGE-BSA on bMSCs. We observed that high glucose and high osmolarity with L-glucose did not significantly change the proliferation and apoptosis of bMSCs (Figure S3A through S3E). Culture of bMSCs with AGE-BSA (100–400 μg/mL with nonglycated BSA as control) significantly increased intracellular ROS production and apoptosis dose dependently (Figure 3A through 3C). AGE-BSA treatment (400 μg/mL) decreased proliferation and increased apoptosis, as determined with EdU Cell Proliferation and FITC Apoptosis Detection Kits, respectively (Figures 3 and 4). AGE-BSA treatment also significantly decreased their capability of migration in hypoxic condition (Figure 4). In addition, intracellular ROS were suppressed with N-acetylcysteine, whereas AGE-induced inhibition of cell proliferation and the increase in cell apoptosis were significantly prevented (Figures 3 and 4), confirming that ROS production was critically involved in AGE-induced reduction in survival of bMSCs.

A delicate balance between ROS formation and clearance is apparent in biological systems. Antioxidant enzymes, including SODs and Gpx-1, are vital components of the physiological systems that remove excessive ROS.[40] Western blotting analysis showed that AGE-BSA treatment (400 μg/mL) significantly decreased protein levels of SOD-1 and SOD-3 in bMSCs (Figure 3I and 3J). To determine if decreased levels of SOD-1 and SOD-3 contributed to AGE-induced increase of ROS production and apoptosis in bMSCs, as well as subsequent inhibition of cell function and viability, experiments were repeated with bMSCs from mice with concomitant overexpression of SOD-1, SOD-3, and Gpx-1 (TG-bMSCs). The reason

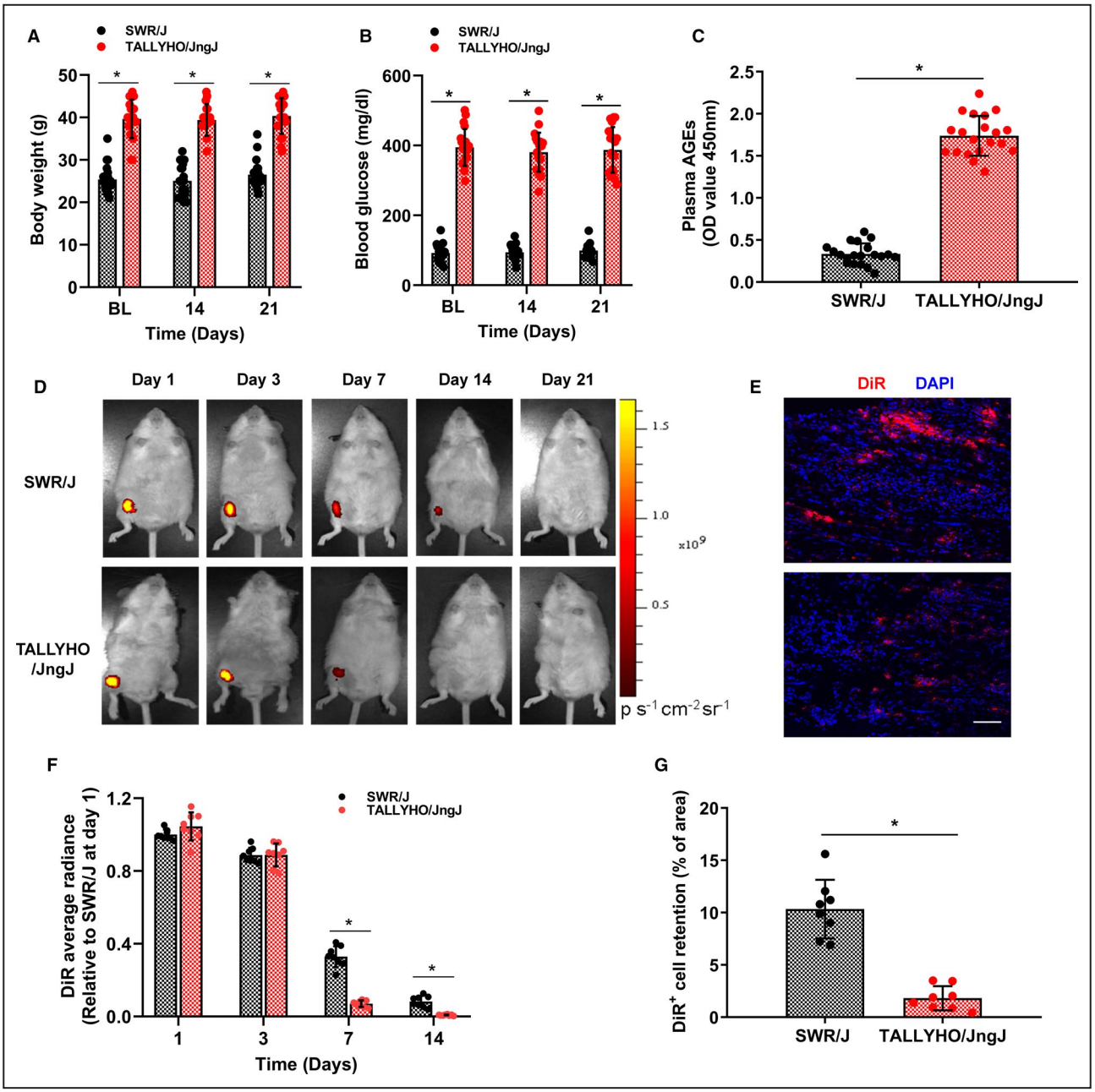


Figure 1. Survival of bone marrow mesenchymal stromal cells (bMSCs) was significantly decreased in the ischemic limb of mice with type 2 diabetes.

Body weight, blood glucose level, and plasma advanced glycation end product level were significantly increased in diabetic TALLYHO/JngJ mice than the control mice (**A** through **C**; **P*<0.01; a repeated-measures ANOVA for **A** and **B**; Student 2-tailed *t* test for **C**; n=20/group). Critical limb ischemia was created by ligation of right femoral artery. After 30 minutes, a total of 5×10^5 bMSCs labeled with CellTracker DiR fluorescence indicator in 100 μL or same volume PBS were injected into 3 sites of ischemic limb adductor muscle. In vivo optical fluorescence imaging study showed that the survival of bMSCs was significantly decreased in the ischemic limb in diabetic mice compared with the nondiabetic controls (from 32.90% to 7.03% at week 1, and from 8.28% to undetectable level at week 2 after cell delivery; **P*<0.05; a repeated-measures ANOVA; **D** and **F**). Decreased cell number of bMSCs in diabetic ischemic limb was confirmed with ex vivo histological examinations of DiR^+ fluorescence 14 days after cell transplantation (Student 2-tailed *t* test; n=8/group, **P*<0.01 for TALLYHO/JngJ vs SWR/J mice; **E** and **G**). Bar=50 μm. All data were shown as means±SD. BL indicates before ligation; and DAPI, 4′,6-diamidino-2-phenylindole.

for using a triple antioxidant enzyme overexpression approach was that SOD overexpression alone can be paradoxically associated with an increased level of H_2O_2 with increased oxidative stress, both in vitro and in vivo.[41] As expected, TG-bMSCs effectively resisted AGE-induced ROS, inhibition of cell proliferation and

migration, and increase in apoptosis (Figures 3 and 4), confirming that decreased expressions of SOD-1 and SOD-3 were important to AGE-induced ROS production in bMSCs.

Angiogenic growth factors from bMSCs are considered an important mechanism in their therapeutic benefits.[42] Thus, we investigated the effect of AGEs on the production of various angiogenesis-related

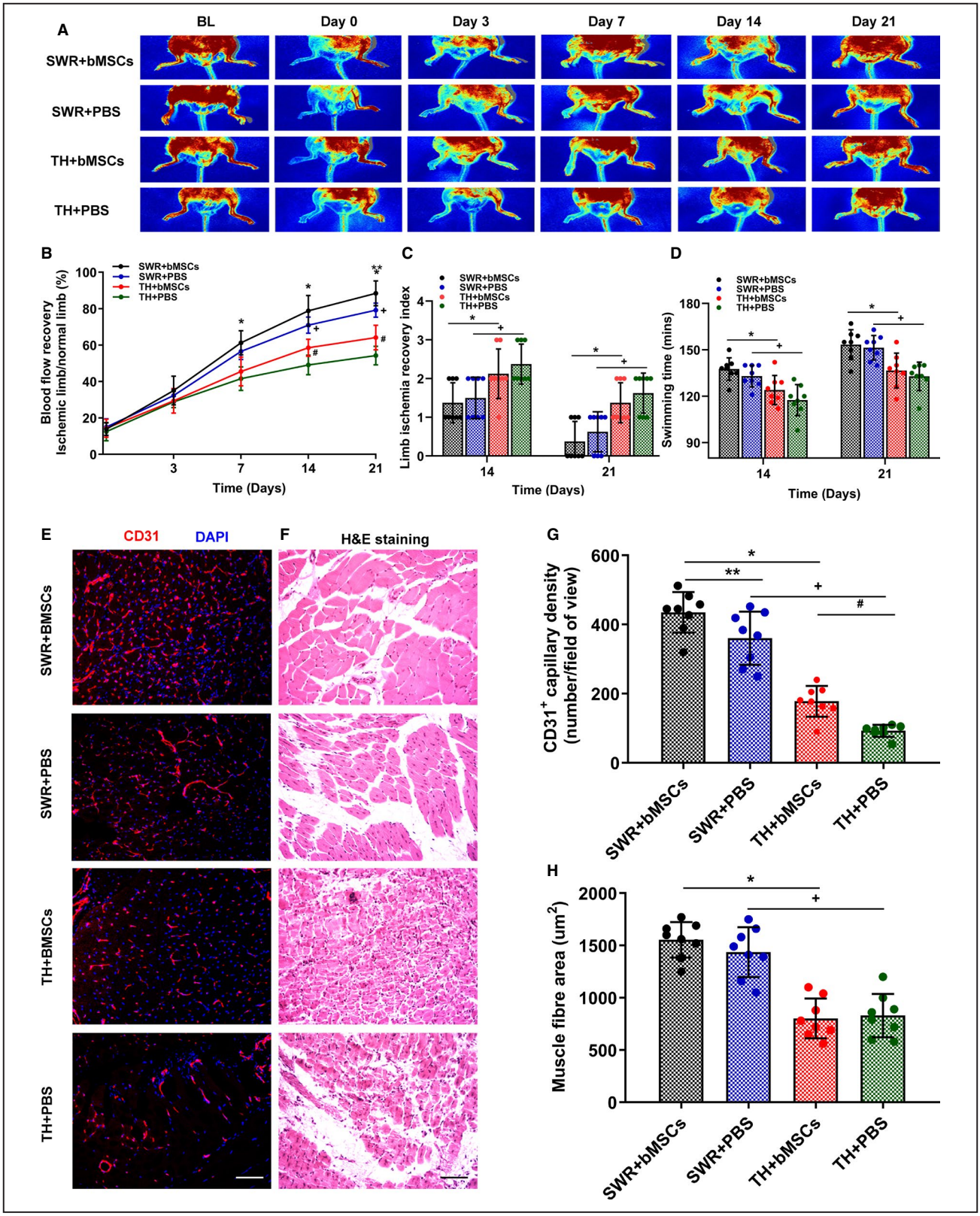


Figure 2. Decreased survival of bone marrow mesenchymal stromal cells (bMSCs) was associated with decreased blood flow recovery from limb ischemia in mice with type 2 diabetes.
Blood flow recovery, as reflected by the ratio of ischemic limb blood perfusion/normal limb blood perfusion, was significantly decreased in diabetic mice compared with nondiabetic controls (*P<0.01 for nondiabetic SWR/J mice [SWR]+bMSCs vs diabetic TALLYHO/JngJ mice [TH]+bMSCs; #P<0.05 for TH+bMSCs vs TH+PBS; +P<0.01 for SWR+PBS vs TH+PBS; **P<0.05 for SWR+bMSCs vs SWR+PBS; 2-way repeated-measures ANOVA; n=8/group; **A** and **B**). Function recovery of the ischemic limb, as assessed using the semiquantitative assessment of ambulatory impairment and limitation of ischemic limb (modified clinical standard score; **C**) and swimming endurance test (**D**), was also significantly decreased in diabetic mice compared with nondiabetic mice, as expected (*P<0.05 for SWR+bMSCs vs TH+bMSCs; +P<0.05 for SWR+PBS vs TH+PBS; 2-way repeated-measures ANOVA; n=8/group; **C** and **D**). Histological analysis of the adductor muscle from ischemic hind limb 21 days after ligation with CD31 immunofluorescent staining demonstrated that the capillary density was significantly reduced in diabetic ischemic limb compared with nondiabetic ischemic limb (*P<0.01 for SWR+bMSCs vs TH+bMSCs; #P<0.05 for TH+bMSCs vs TH+PBS; +P<0.01 for SWR+PBS vs TH+PBS; **P<0.05 for SWR+bMSCs vs SWR+PBS; 1-way ANOVA; n=8/group; **E** and **G**). Hematoxylin and eosin (H&E) staining revealed that muscle fiber area was significantly decreased with increased inflammatory infiltration in diabetic ischemic limb compared with nondiabetic ischemic limb (*P<0.01 for SWR+bMSCs vs TH+bMSCs; +P<0.01 for SWR+PBS vs TH+PBS; 1-way ANOVA; n=8/group; **F** and **H**). Bar=50 μm. All data were shown as means±SD. BL indicates before ligation; and DAPI, 4′,6-diamidino-2-phenylindole.

growth factors, including VEGF, epidermal growth factor, FGF-2, HGF, and PLGF-2, from bMSCs and the role of ROS in mediating the effect of AGEs on growth factor production. ELISA revealed that AGE-BSA treatment substantially decreased the levels of VEGF, FGF-2, HGF, and PLGF-2, but not epidermal growth factor, under hypoxic conditions. Concomitant overexpression of SOD-1, SOD-3, and Gpx-1 or N-acetylcysteine treatment significantly prevented AGE-induced reduction in the production of VEGF, FGF-2, HGF, and PLGF-2 from bMSCs (Figures 4E through 4I), suggesting that decreased production/release of these growth factors from bMSCs by AGEs was ROS dependent.

Concomitant Overexpression of SOD-1, SOD-3, and Gpx-1 Significantly Enhanced In Vivo Survival of bMSCs in Diabetic Ischemic Limb

To determine if inhibition of ROS production in bMSCs could enhance the survival of bMSCs in diabetic ischemic limb, bMSCs with or without concomitant overexpression of SOD-1, SOD-3, and Gpx-1 were injected into the ischemic limb in diabetic mice. Because it was AGEs, not high glucose per se, that contribute to decreased survival of bMSCs, we measured plasma AGE level in diabetic mice. As expected, diabetic mice had significantly higher levels of plasma AGEs compared with nondiabetic mice (Figure 1C). IVIS analysis showed that concomitant overexpression of SOD-1, SOD-3, and Gpx-1 significantly improved the survival of bMSCs in diabetic ischemic limb by ≈5 times over the control (from 7.63% to 33.52%; Figure 5C and 5D) 1 week after implantation. Increased levels of viable bMSCs in the ischemic tissue compared with control cells from WT mouse were confirmed by ex vivo histological analysis (Figure 5E and 5F). Treatment with bMSCs overexpressing the triple antioxidant enzymes also improved their therapeutic efficacy on ischemic limb in diabetic mice with enhanced recovery of blood flow and function, as reflected with regional blood perfusion, swimming endurance test, and limb ischemia recovery index, compared with control cells (Figure 6A through 6D). Improved circulation and function recovery of diabetic ischemic limb was associated with increased capillary density and preserved muscle mass and decreased inflammatory infiltration over the control, as demonstrated with histological analysis (Figure 6E through 6H). However, the in vivo survival of these cells was still less optimal, with most cells (>80%) disappearing within 2 weeks of delivery into the ischemic limb of diabetic mice.

Combination of Triple Antioxidant Enzyme Overexpression in bMSCs and Host Treatment With N-Acetylcysteine Additively Increased the Survival of bMSCs in Diabetic Ischemic Limb

Local microenvironment plays a critical role in the survival and function of transplanted bMSCs. Excessive ROS production significantly contributes to cell death shortly after bMSC implantation. Although concomitant overexpression of SOD-1, SOD-3, and Gpx-1 significantly improved the survival of bMSCs in the ischemic limb in diabetic mice, most cells were no longer present at the target site 2 weeks after their delivery. Tissue ROS production and oxidative stress level are known to be significantly increased in diabetic conditons.[16,17] Thus, we hypothesized that increased tissue ROS production was an important determinant for bMSC survival in diabetic ischemic limb.

To test this hypothesis, we measured the ROS levels in the ischemic limb in both diabetic mice and nondiabetic mice. Indeed, tissue ROS level in diabetic mice was significantly increased compared with the nondiabetic mice (Figure 5A and 5B). To determine the role of increased tissue ROS in the in vivo survival of bMSCs, diabetic mice were treated with N-acetylcysteine for 24 hours before creation of limb ischemia and continuously for the rest of the experiment to block ROS

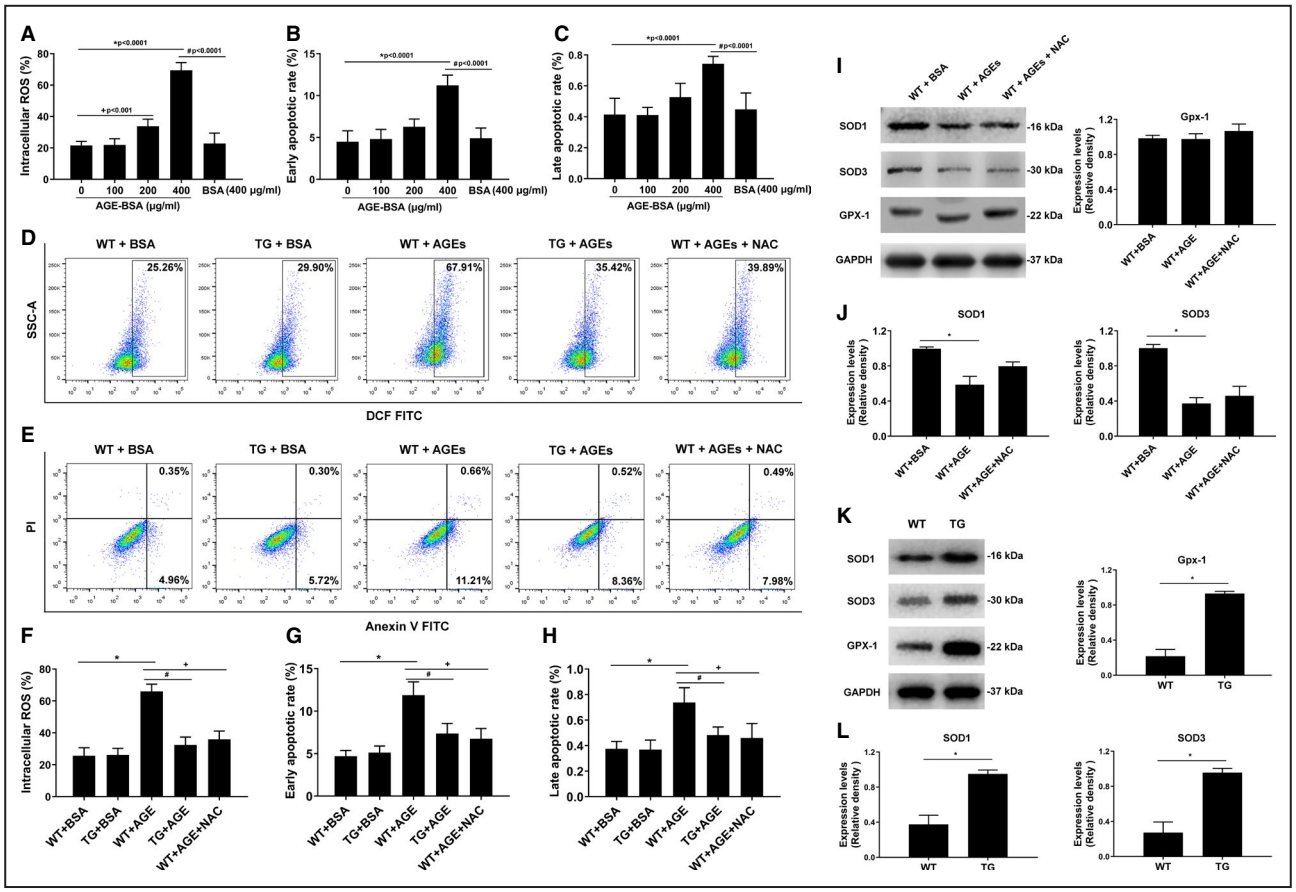


Figure 3. Advanced glycation end products (AGEs) impaired the viability of bone marrow mesenchymal stromal cells (bMSCs) through increased reactive oxygen species (ROS) production attributable to selective reduction of superoxide dismutase (SOD)-1 and SOD-3 expression.

A through **C**, Culture of bMSCs with AGE-BSA (with concentration from 100 to 400 μg/mL with BSA as control) significantly increased intracellular ROS (**A**) and early (**B**) and late apoptosis (**C**) in a dose-dependent manner. n=6/group. **P*<0.0001 for 0 vs 400 μg/mL AGE-BSA; #*P*<0.0001 for 400 μg/mL BSA vs 400 μg/mL AGE-BSA; +*P*<0.001 for 0 vs 200 μg/mL AGE-BSA. **D** through **H**, The representative images of flow cytometric analysis (**D** and **E**) and quantification of intracellular ROS (**F**) and early (**G**) and late apoptosis (**H**). AGE-BSA treatment (400 μg/mL with BSA as control) significantly increased intracellular ROS, and apoptosis of bMSCs. Inhibition of ROS production with N-acetylcysteine (NAC) significantly prevented AGE-induced increase in intracellular ROS, and apoptosis of bMSCs (n=6/group; **P*<0.01 for WT+AGE vs WT+BSA; +*P*<0.01 for WT+AGE+NAC vs WT+AGE). **I** through **L**, The representative images (**I** and **K**) and quantification (**J** and **L**) of Western blot analysis. AGE-BSA treatment (400 μg/mL) significantly decreased the protein levels of SOD-1 and SOD-3 in bMSCs (n=3; **P*<0.05 for WT+AGE vs WT+BSA; **J**). The expression levels of SOD-1, SOD-3, and glutathione peroxidase-1 (Gpx-1) were significantly increased in bMSCs from triple-transgenic (TG) mice compared with bMSCs from wild-type (WT) mice (n=3; **P*<0.05 for TG vs WT; **L**). To determine if decreased levels of SOD-1 and SOD-3 contributed to AGE-induced increase of ROS production in bMSCs and subsequent increase in apoptosis, experiments were repeated with bMSCs from mice with concomitant overexpression of SOD-1, SOD-3, and Gpx-1 (TG-bMSCs). Indeed, AGE-induced ROS and apoptosis were significantly decreased in TG-bMSCs compared with WT-bMSCs (n=6/group; #*P*<0.05 for TG+AGE vs WT+AGE). Statistical differences were determined with 1-way ANOVA with Bonferroni post hoc test or Kruskal-Wallis test with Dunn post hoc test. All data were shown as means±SD. FITC indicates fluorescein isothiocyanate; DCF, dichlorofluorescein; PI, propidium iodide; SSC-A, side scatter area.

production. One main reason to use N-acetylcysteine is that N-acetylcysteine is a US Food and Drug Administration–approved drug with a long safety record, and could be readily used for clinical studies with human subjects. N-acetylcysteine is able to enter cells easily, and increases intracellular glutathione level. The sulfhydryl group within N-acetylcysteine molecule could also directly scavenge ROS, modulate oxidation-reduction state both extracellularly and intracellularly, regulate cytokine synthesis (anti-inflammatory effect), and inhibit some important inflammation-related signaling pathways (including nuclear factor-κB), leading to reduced ROS production. As expected, N-acetylcysteine significantly decreased tissue ROS levels (Figure 5A and 5B), and effectively improved the survival of bMSCs in the diabetic ischemic limb by almost 5-fold (from 7.63% to 35.66%; Figure 5C and 5D) compared with that in mice not receiving N-acetylcysteine treatment 1 week after implantation. Increased survival of bMSCs was associated with significant improvement of blood flow

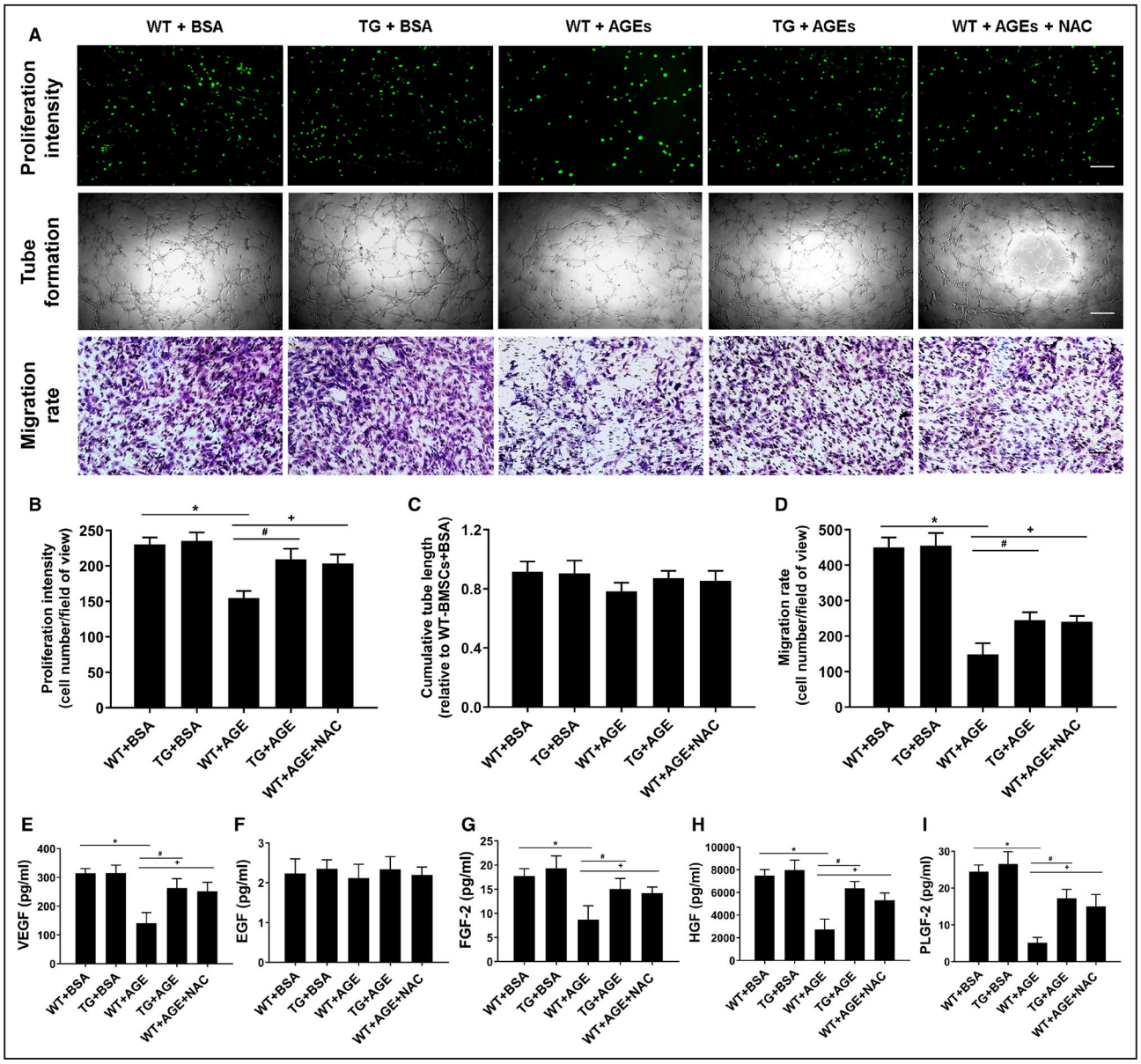


Figure 4. Inhibition of reactive oxygen species (ROS) production significantly prevented the reduction of bone marrow mesenchymal stromal cell (bMSC) function by advanced glycation end products (AGEs).
AGE-BSA treatment (400 μg/mL with BSA as control) significantly decreased the function of bMSCs, including proliferation (**A** and **B**) and migration (**A** and **D**) of bMSCs in vitro (n=6/group; **P*<0.05 for wild type [WT]+AGE vs WT+BSA). ELISA assay revealed that AGE-BSA treatment substantially decreased the levels of vascular endothelial growth factor (VEGF) (**E**), basic fibroblast growth factor (FGF-2) (**G**), hepatocyte growth factor (HGF) (**H**), and placental growth factor-2 (PLGF-2) (**I**), but not epidermal growth factor (EGF) (**F**), in hypoxia condition (5% O_2 and 5% CO_2 at 37 °C; n=3/group; **P*<0.05 for WT+AGE vs WT+BSA). Inhibition of ROS production with either concomitant overexpression of superoxide dismutase (SOD)-1, SOD-3, and glutathione peroxidase-1 or N-acetylcysteine (NAC) treatment significantly prevented AGE-induced reduction of bMSC function with preserved cell proliferation, migration, and production of VEGF, FGF-2, HGF, and PLGF-2 from bMSCs (n=3/group; $^{\#}P$<0.05 for TG+AGE vs WT+AGE; ^{+}P<0.05 for WT+AGE+NAC vs WT+AGE; **A** through **I**). Bar=100 μm. Statistical differences were determined with 1-way ANOVA with Bonferroni post hoc test or Kruskal-Wallis test with Dunn post hoc test. All data were shown as means±SD.

and recovery of function of the diabetic ischemic limb (Figure 6A through 6D), and increased capillary density and preserved muscle mass (Figure 6E through 6H).

Increased survival of bMSCs in diabetic ischemic limb with N-acetylcysteine treatment was short-lived, however, with 85% of injected cells no longer present within 2 weeks of injection (Figure 5C and 5D). We, thus, tested the hypothesis that a combination of inhibiting intracellular ROS production in bMSCs with concomitant overexpression of SOD-1, SOD-3, and Gpx-1 and extracellular ROS production with N-acetylcysteine treatment could further enhance the survival of bMSCs

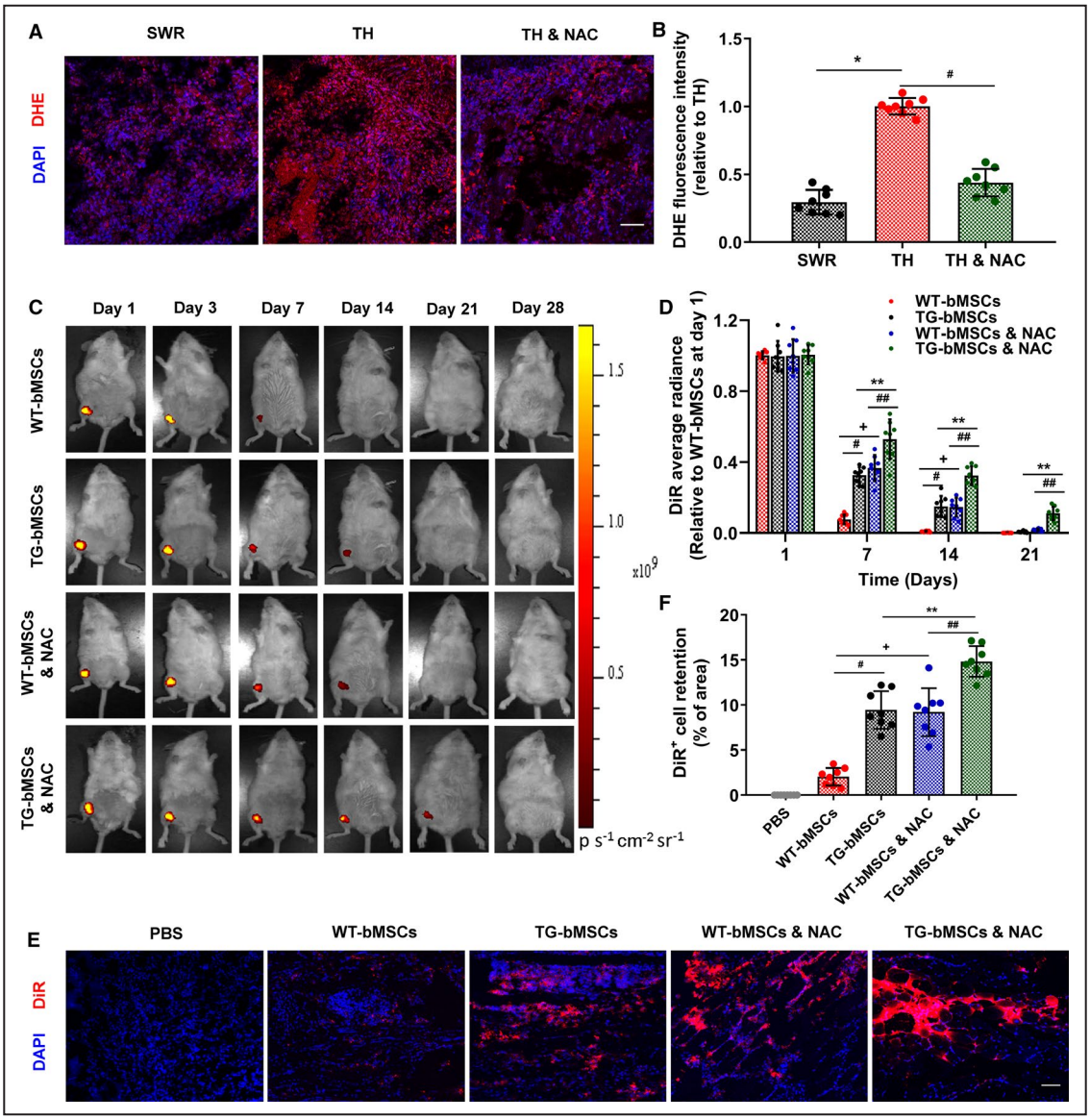


Figure 5. Combination of triple antioxidant enzyme overexpression in bone marrow mesenchymal stromal cells (bMSCs) and host treatment with N-acetylcysteine (NAC) additively enhanced the survival of bMSCs in diabetic ischemic limb.

Dihydroethidium (DHE) staining (from the adductor muscle of ischemic hind limb 21 days after ligation) demonstrated that reactive oxygen species (ROS) production in diabetic ischemic limb was significantly increased compared with the nondiabetic ischemic limb (n=8/group; $^{*}P<0.01$ for TALLYHO/JngJ mice [TH] vs SWR/J mice [SWR]; **A** and **B**). NAC treatment substantially decreased tissue ROS level in the ischemic limb in diabetic mice (n=8/group; $^{\#}P<0.01$ for TH and NAC vs TH). In vivo optical fluorescent imaging analysis showed that concomitant overexpression of superoxide dismutase (SOD)-1, SOD-3, and glutathione peroxidase-1 (Gpx-1) significantly improved the survival of bMSCs (TG-bMSCs) in diabetic ischemic limb by almost 5 times over the control (from 7.63% to 33.52%; $^{\#}P<0.01$ for TG-bMSCs vs wild-type [WT] bMSCs) 1 week after implantation. The survival of bMSCs was substantially increased to >50% in the ischemic limb in diabetic mice with combination of inhibiting intracellular ROS production in bMSCs with concomitant overexpression of SOD-1 and Gpx-1 (TG-bMSCs) and extracellular ROS production with NAC treatment and SOD-3, compared with survival rate of about 30% to 35% for either triple enzyme overexpression or NAC treatment alone at week 1 after cell delivery, and >30% and 10% for the cells with combined treatment at weeks 2 and 3, respectively (n=8/group; $^{+}P<0.05$ for WT-bMSCs vs WT-bMSCs and NAC; $^{**}P<0.05$ for TG-bMSCs and NAC vs TG-bMSCs; $^{\#\#}P<0.05$ for TG-bMSCs and NAC vs WT-bMSCs and NAC; **C** and **D**). Increased cell numbers in TG-bMSCs and TG-bMSCs and NAC group were confirmed with ex vivo histological analysis of DiR^{+} fluorescence 14 days after transplantation (n=8/group; $^{\#}P<0.01$ for TG-bMSCs vs WT-bMSCs; $^{+}P<0.05$ for WT-bMSCs vs WT-bMSCs and NAC; $^{**}P<0.01$ for TG-bMSCs and NAC vs TG-bMSCs; $^{\#\#}P<0.01$ for TG-bMSCs and NAC vs WT-bMSCs and NAC; **E** and **F**). Bar=50 μm. Statistical differences were determined by 2-way repeated-measures ANOVA followed by Tukey test (**D**) and 1-way ANOVA with Bonferroni post hoc test (**B** and **F**). All data were shown as means±SD. DAPI indicates 4′,6-diamidino-2-phenylindole.

in diabetic ischemic limb. Mice were therefore transplanted with TG-bMSCs after surgical CLI and treated with N-acetylcysteine for 24 hours before ligation and continuously for the rest of the experiment. IVIS analysis showed that the survival of bMSCs was substantially increased to >50% in the ischemic limb of diabetic mice with combination therapy compared with survival rate of about 30% to 35% for either triple enzyme overexpression or N-acetylcysteine treatment alone at week 1 after cell delivery, and >30% and 10% for the cells with combined treatment at weeks 2 and 3, respectively (Figure 5C and 5D). As expected, improved survival of bMSCs with combination treatment was associated with enhanced recovery of ischemic limb blood flow and function (Figure 6A through 6D) with preserved capillary and muscle mass (Figure 6E through 6H). These data demonstrated that intracellular ROS in transplanted bMSCs and extracellular ROS in the target tissue are equally important to the survival of bMSCs in diabetic ischemic limb, and their subsequent therapeutic efficacy on diabetic CLI.

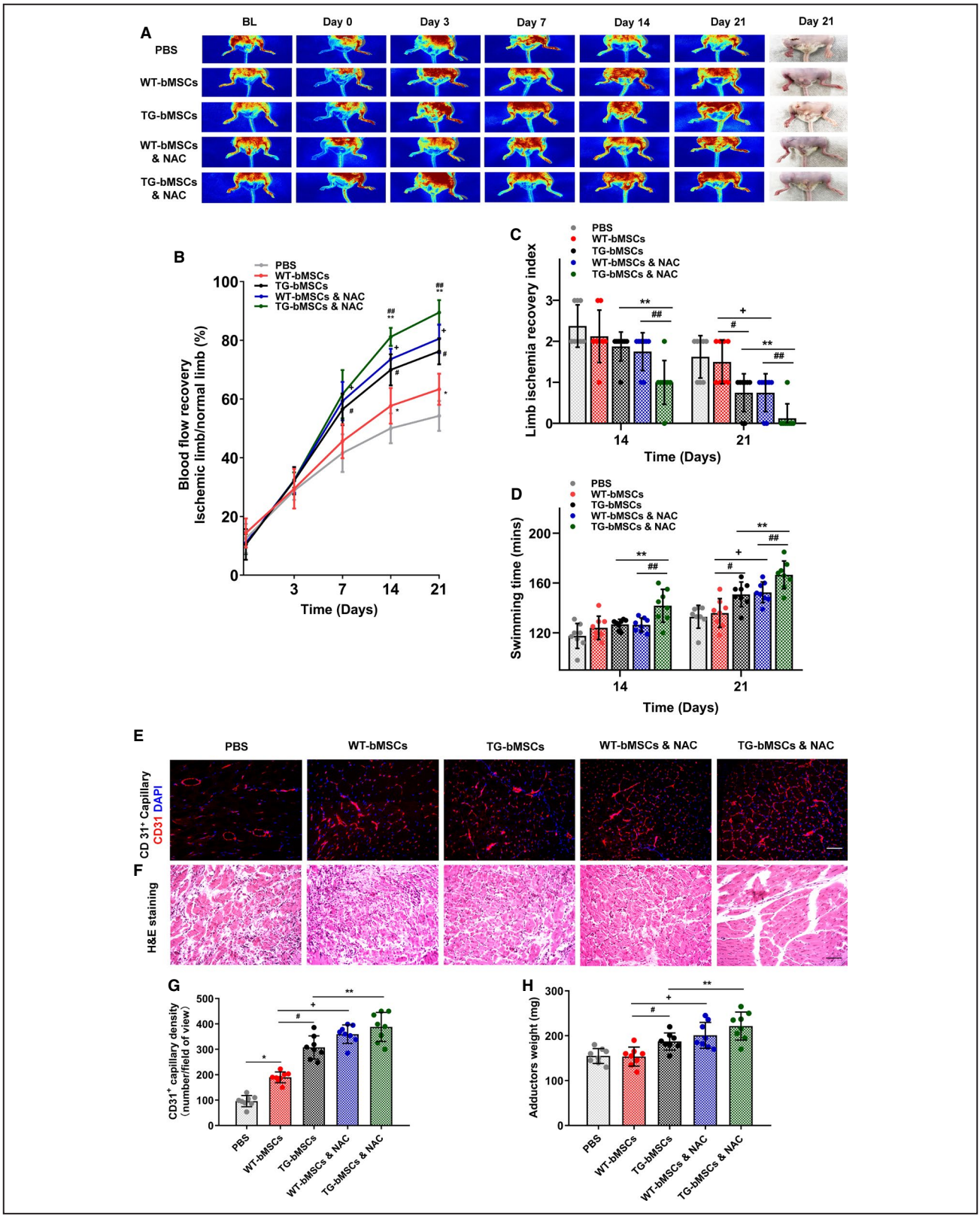
DISCUSSION

It is challenging to treat CLI in diabetic patients, and stem cell therapy is not as effective in this population for largely undefined reasons. However, there is no doubt that the efficacy of stem cell therapy is ultimately associated with the number and function of cells in the target areas. In the present study, we demonstrated that the viability and function of bMSCs were significantly improved by overexpression of triple antioxidant enzymes or N-acetylcysteine treatment in the presence of increased AGEs. Transplantation of bMSCs with concomitant overexpression of SOD-1, SOD-3, and Gpx-1 or treatment with N-acetylcysteine significantly increased the survival of bMSCs in diabetic ischemic limb with enhanced therapeutic efficacy on CLI in diabetic mice. The combined strategy of inhibiting intracellular ROS production in transplanted bMSCs with concomitant overexpression of SOD-1, SOD-3, and Gpx-1 and local tissue ROS production with host N-acetylcysteine treatment further enhanced the survival of bMSCs in the ischemic limb of diabetic mice with improved recovery of blood flow and function as well as preserved vascular and muscle structure.

Considerable effort has been made to explore effective cell therapies for diabetes[43,44] and its complications, including diabetic cardiomyopathy,[45] diabetic polyneuropathy,[46] diabetic cystopathy,[47] and diabetic limb ischemia.[48] Nevertheless, the functional survival of transplanted stem cells remains the major problem attributable to inflammation, insufficient blood perfusion, tissue dysfunction, and other causes.[49] Given the complicated disease conditions, cell therapy has become increasingly integrated with new technologies and materials to improve the functional survival of transplanted cells in many proof-of-concept and preclinical investigations. In this study, we combined cell transplantation with host treatment to leverage the additive or complementary effects on the cells. As severe ischemia and excessive ROS production significantly decrease the survival of bMSCs in diabetic CLI (Figure 5), we used bMSCs overexpressing 3 antioxidant enzymes together with host N-acetylcysteine treatment to reduce ROS and oxidative stress and alleviate diabetic limb ischemia.

To appropriately model type 2 diabetes in human subjects, we used a relatively new polygenic mouse model, TALLYHO/JngJ mouse, which is characterized by spontaneous type 2 diabetes occurring as the mice age. More commonly used mice with type 2 diabetes include the *db/db* and *ob/ob* strains. However, these mice are obese and deficient in leptin signaling (leptin receptor deficient in *db/db* mice and leptin production deficient in *ob/ob* mice), and usually have high plasma glucose levels (>600 mg/dL).[50] Leptin signaling is important for energy homeostasis and obesity, and is known to influence ventricular and vascular remodeling.[51] Clinically, leptin deficiency (either receptor or protein) in human subjects is rare.[50] Leptin levels are either normal or, in most cases, elevated (up to 10 times normal) in patients with type 2 diabetes.[50] No differences in the expression and distribution of the leptin receptor are identified in obese or lean diabetic patients. Interestingly, the mutation responsible for leptin receptor defect in obese *db/db* mice is not present in any obese human subject, indicating that leptin resistance observed in obese humans is not attributable to a defect in the leptin receptor.[51] TALLYHO/JngJ mouse is a non–insulin-dependent polygenic mouse model for type 2 diabetes with plasma glucose levels of 200 to 400 mg/dL. These mice exhibit phenotypic characteristics similar to human type 2 diabetes with elevated plasma leptin level (about 3 times of normal).[22] Thus, TALLYHO/JngJ mouse model was used as the type 2 diabetes model for the present study.

ROS and oxidative stress are critically involved in modulation of stem cell growth, migration, differentiation, apoptosis, and senescence.[52,53] Although low levels of ROS are necessary for normal physiological function, including the intracellular signals to trigger angiogenesis,[54,55] high concentrations of ROS can induce senescence and apoptosis of stem/progenitor cells and are associated with defective neovascularization.[56,57] N-acetylcysteine as an effective antioxidant has been used to inhibit ischemia-induced ROS formation and promote ischemic limb recovery in human subjects and mice.[58,59] Inhibition of ROS production with codelivery of N-acetylcysteine has been shown to significantly increase mesenchymal stromal cell/human umbilical vein endothelial cell retention after transplantation in



nondiabetic ischemic limb.[60] Furthermore, bMSCs with SOD overexpression show enhanced survival in irradiated mice,[61] and improve their therapeutic effect on myocardium infarction[62] and brain trauma.[63] Oxidative stress has also been proposed to play an important role in the development of diabetic complications, including vascular dysfunction in both types of diabetes, particularly in type 2 diabetes.[64] In the present study,

Figure 6. Combination of triple antioxidant enzyme overexpression in bone marrow mesenchymal stromal cells (bMSCs) and host treatment with N-acetylcysteine (NAC) effectively improved blood flow and function of the diabetic ischemic limb. **A**, The representative images of laser Doppler perfusion imaging for blood perfusion before and after critical limb ischemia for diabetic mice with different treatments. **B**, Quantitative analysis of limb blood flow using the ratio of blood flow perfusion (right or ischemic limb blood perfusion/left or normal limb blood perfusion). Limb function recovery was evaluated using the semiquantitative assessment of ambulatory impairment and limitation of ischemic limb (modified clinical standard score [**C**] and swimming endurance test [**D**]). Treatment with bMSCs with concomitant triple antioxidant enzyme overexpression or combination treatment improved their therapeutic efficacy on ischemic limb in diabetic mice with enhanced recovery of blood flow and function (n=8/group; $^{\#}P<0.05$ for TG-bMSCs vs wild-type [WT] bMSCs; $^{+}P<0.05$ for WT-bMSCs vs WT-bMSCs and NAC; $^{**}P<0.05$ for TG-bMSCs and NAC vs TG-bMSCs; $^{\#\#}P<0.05$ for TG-bMSCs and NAC vs WT-bMSCs and NAC; **B** through **D**). Improved circulation and function recovery of diabetic ischemic limb was associated with increased capillary density, preserved muscular mass, and decreased inflammatory infiltration (n=8/group; $^{\#}P<0.05$ for TG-bMSCs vs WT-bMSCs; $^{+}P<0.05$ for WT-bMSCs vs WT-bMSCs and NAC; $^{**}P<0.05$ for TG-bMSCs and NAC vs TG-bMSCs; **E** through **H**). $^{*}P<0.05$ for WT-bMSCs vs PBS. Bar=50 μm. The significant differences were determined with 2-way repeated-measures ANOVA followed by Tukey test (**B**–**D**) and 1-way ANOVA with Bonferroni post hoc test (**G** and **H**). All data were shown as means±SD. BL indicates before ligation; DAPI, 4′,6-diamidino-2-phenylindole; and H&E, hematoxylin and eosin.

we verified that tissue ROS levels in diabetic ischemic limb were significantly increased compared with nondiabetic ischemic limb. Both concomitant overexpression of SOD-1, SOD-3, and Gpx-1 of bMSCs and host N-acetylcysteine treatment significantly reduced ROS production and enhanced therapeutic efficacy with increased capillary density, preserved muscular structure, and decreased inflammatory infiltration in diabetic ischemic limb. In addition, the results from the in vitro experiments demonstrated that AGEs significantly decreased the proliferation of bMSCs and increased their apoptosis associated with increased ROS production attributable to decreased expression of SOD-1 and SOD-3. Inhibition of ROS production with either N-acetylcysteine treatment or overexpression of SOD-1, SOD-3, and Gpx-1 significantly prevented AGE-induced inhibition of bMSC proliferation and increase in apoptosis. Angiogenic growth factors from bMSCs are considered one of the important mechanisms for their therapeutic benefits. AGE treatment substantially decreased production/release of angiogenic growth factors (ie, VEGF, FGF-2, HGF, and PLGF-2). Concomitant overexpression of SOD-1, SOD-3, and Gpx-1 and N-acetylcysteine treatment significantly prevented AGE-induced reduction in the production of these angiogenic growth factors from bMSCs. These results suggested that an enhanced therapeutic efficacy on diabetic limb ischemia could be attributed to the increased viability and function of bMSCs.

One of the important findings in the present study was that either manipulation of bMSCs with overexpression of antioxidant enzymes causing reduced endogenous ROS production or suppression of ROS production in the host microenvironment in diabetic limb ischemia could significantly improve the survival of bMSCs to a similar extent in the critically ischemic limb and enhance the recovery of blood flow and function. However, the protective effect on bMSCs for either approach was short-lived and far less than desirable, with 80% of injected cells no longer present within 2 weeks after delivery. More important, the combined overexpression of antioxidant enzymes in the transplanted bMSCs and treatment of the host with N-acetylcysteine substantially enhanced the survival of bMSCs in critically ischemic limb in diabetic mice with significant improvement on their therapeutic efficacy on limb ischemia. The additive effect of this combined approach on the survival of bMSCs strongly suggests that ROS production from transplanted bMSCs and from host tissue equally contributes to decreased bMSC survival in diabetes. The combination therapy described in our study could provide a novel and effective treatment option for CLI in diabetic patients. However, clinical studies are required to confirm the findings of the present study on the effect of combination treatment on bMSC survival and therapeutic efficacy in patients with diabetic CLI. Further studies are also needed to define the mechanism underlying the interactions between intracellular ROS generated by the transplanted bMSCs and ROS (both intracellular and extracellular) of the host tissue, on the survival of bMSCs in diabetic ischemic limb, and the additive therapeutic effect of host N-acetylcysteine treatment and concomitant overexpression of SOD-1 and SOD-3 in transplanted cells for diabetic ischemic conditions.

The mechanisms for the poor in vivo survival of transplanted bMSCs have not been well defined. Clearly, the fate of transplanted stem cells in tissues could be determined by a complex and multifactorial mechanism, including (but not limited to) types and sources of cells, mode of delivery, locations, host microenvironment, oxygenation level, mechanical stress, metabolic status, and disease conditions.[4,65] The amount of viable transplanted cells in the target tissue could be the combined outcome of cell loss attributable to washing out, apoptosis, necrosis, and proliferation. In the present study, we observed that the number of viable cells remained stable for the first 72 hours after delivery in the ischemic limb muscle in both diabetic and nondiabetic mice, suggesting that cell loss was not attributable to initial washing out. The data from the in vitro experiments suggested that

increased apoptosis and decreased proliferation might play an important role in decreased number of viable bMSCs in diabetic ischemic limb because of increased level of AGEs (at least partially). However, further studies are needed to confirm the potential contributions of increased apoptosis and decreased proliferation to the poor survival of transplanted bMSCs in diabetic ischemic limb. Although high glucose level had no significant impact on intracellular ROS production and apoptosis and proliferation of bMSCs in vitro, it could be certainly important and interesting mechanistically to determine if optimal control of glucose levels with insulin could enhance the survival of bMSCs in diabetic ischemic limb.

ROS formation is known to be increased with increased oxidative stress in diabetes.[16,17] Hydrogen peroxide is considered a key ROS implicated in cell signaling and diabetic pathology, including diabetic cardiovascular complications.[36,37] In the present study, we observed that AGE treatment moderately increased the level of H_2O_2 in the conditioned media from bMSCs. N-acetylcysteine treatment or overexpression of SOD-1, SOD-3, and Gpx-1 effectively blocked AGE-induced increase of H_2O_2 in the conditioned media (Figure S4A). The level of H_2O_2 in the plasma from diabetic mice was significantly increased as expected. Interestingly, systematic N-acetylcysteine treatment only moderately, yet significantly, decreased the plasma H_2O_2 level in diabetic mice, whereas N-acetylcysteine treatment effectively attenuated excessive ROS production in diabetic ischemic limb (Figure S4B). Further studies are needed to determine the role of H_2O_2 in increased ROS production and decreased bMSC survival in diabetic ischemic limb.

In conclusion, our study has demonstrated that a combination of bMSCs subjected to concomitant triple antioxidant enzyme overexpression and treatment of recipients with N-acetylcysteine additively enhanced the survival of bMSCs, and improved their therapeutic efficacy in diabetic CLI. The combined treatment approach also effectively preserved the viability and function of bMSCs against AGEs, a known stimulant for ROS production. These data suggest that intracellular ROS from bMSCs and host tissue equally contribute to decreased bMSC survival in diabetic ischemic limb. The combination therapy may provide a novel and effective approach to the treatment of diabetic CLI.

ARTICLE INFORMATION

Received August 3, 2021; accepted August 19, 2021.

Affiliations

Center for Precision Medicine and Division of Cardiovascular Medicine, Department of Medicine, University of Missouri School of Medicine, Columbia, MO (Q.Z., H.H., H.X., Y.C., C.Y., M.W., Z.L.); Department of Cardiology (Q.Z., S.Z.) and Institute of Molecular Hematopathy (G.Z.), Second Xiangya Hospital, Central South University, Changsha City, Hunan Province, China; College of Agriculture, Food and Natural Resources (Y.F., R.M.) and Dalton Cardiovascular Research Center (M.A.H.), University of Missouri, Columbia, MO (Y.F., R.M.); Department of Surgery, University of Missouri School of Medicine, Christopher S. Bond Life Sciences Center, University of Missouri, Columbia, MO; Dalton Cardiovascular Research Center, University of Missouri, Columbia, MO (M.A.H.); Department of Medicine, University of Melbourne, Australia (P.J.C.); Immunology Research Centre, St. Vincent's Hospital, Melbourne, Australia (P.J.C.); Fischell Department of Bioengineering, University of Maryland, College Park, MD (X.H.).

Sources of Funding

This work was supported by US National Institutes of Health (NIH) grants to Dr Liu (NIH HL124122 and ES026200).

Disclosures

None.

Supplementary Material

Figures S1–S4

REFERENCES

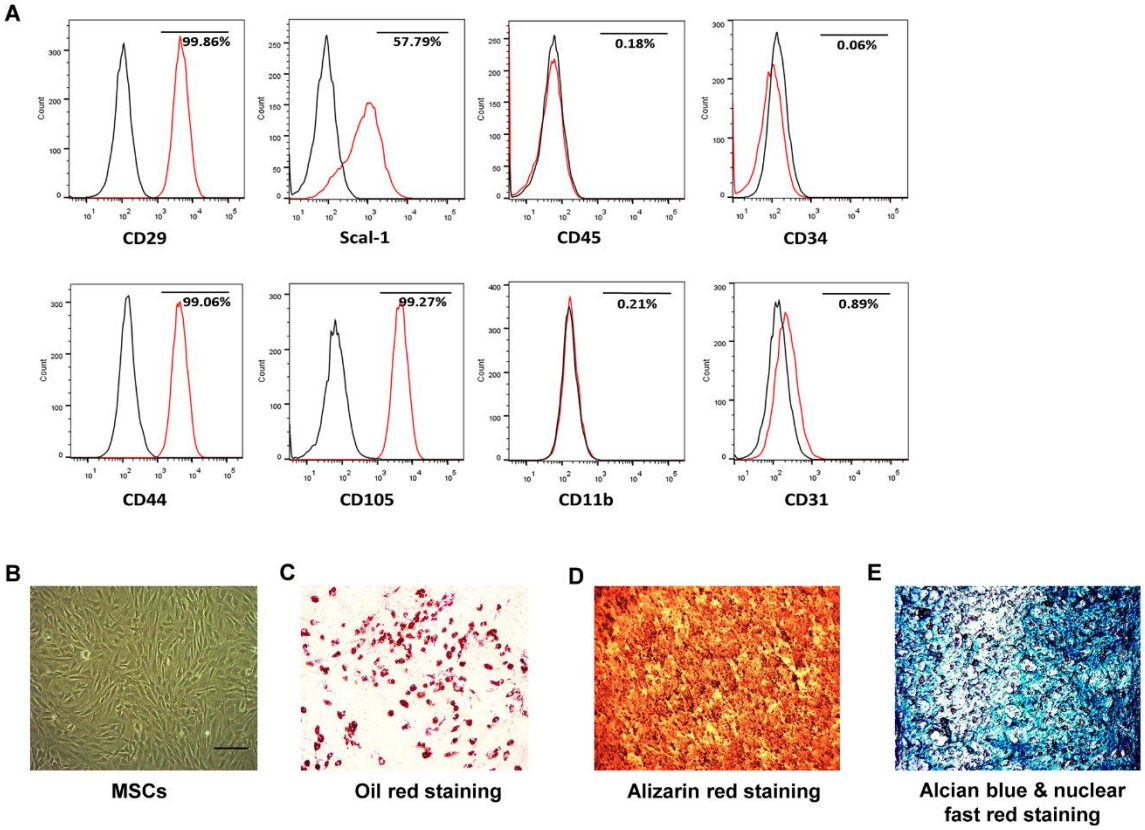
1. Strain WD, Paldánius PM. Diabetes, cardiovascular disease and the microcirculation. *Cardiovasc Diabetol*. 2018;17:57. doi: 10.1186/s12933-018-0703-2
2. Qadura M, Terenzi DC, Verma S, Al-Omran M, Hess DA. Concise review: cell therapy for critical limb ischemia: an integrated review of preclinical and clinical studies. *Stem Cells*. 2018;36:161–171. doi: 10.1002/stem.2751
3. Jaff MR, White CJ, Hiatt WR, Fowkes GR, Dormandy J, Razavi M, Reekers J, Norgren L. An update on methods for revascularization and expansion of the TASC lesion classification to include below-the-knee arteries: a supplement to the inter-society consensus for the management of peripheral arterial disease (TASC II). *J Endovasc Ther*. 2015;22:663–677. doi: 10.1177/1526602815592206
4. Golpanian S, Wolf A, Hatzistergos KE, Hare JM. Rebuilding the damaged heart: mesenchymal stem cells, cell-based therapy, and engineered heart tissue. *Physiol Rev*. 2016;96:1127–1168. doi: 10.1152/physrev.00019.2015
5. Kallmeyer K, André-Lévigne D, Baquié M, Krause K-H, Pepper MS, Pittet-Cuénod B, Modarressi A. Fate of systemically and locally administered adipose-derived mesenchymal stromal cells and their effect on wound healing. *Stem Cells Transl Med*. 2020;9:131–144. doi: 10.1002/sctm.19-0091
6. Kean TJ, Lin P, Caplan AI, Dennis JE. MSCs: delivery routes and engraftment, cell-targeting strategies, and immune modulation. *Stem Cells Int*. 2013;2013:732–742. doi: 10.1155/2013/732742
7. Nystedt J, Anderson H, Tikkanen J, Pietilä M, Hirvonen T, Takalo R, Heiskanen A, Satomaa T, Natunen S, Lehtonen S, et al. Cell surface structures influence lung clearance rate of systemically infused mesenchymal stromal cells. *Stem Cells*. 2013;31:317–326. doi: 10.1002/stem.1271
8. Abdelwahid E, Kalvelyte A, Stulpinas A, de Carvalho KAT, Guarita-Souza LC, Foldes G. Stem cell death and survival in heart regeneration and repair. *Apoptosis*. 2016;21:252–268. doi: 10.1007/s10495-015-1203-4
9. Cofano F, Boido M, Monticelli M, Zenga F, Ducati A, Vercelli A, Garbossa D. Mesenchymal stem cells for spinal cord injury: current options, limitations, and future of cell therapy. *Int J Mol Sci*. 2019;20:2698. doi: 10.3390/ijms20112698
10. Menasché P. Cell therapy trials for heart regeneration—lessons learned and future directions. *Nat Rev Cardiol*. 2018;15:659–671. doi: 10.1038/s41569-018-0013-0
11. Tang YH, Ma YY, Zhang ZJ, Wang YT, Yang GY. Opportunities and challenges: stem cell-based therapy for the treatment of ischemic stroke. *CNS Neurosci Ther*. 2015;21:337–347. doi: 10.1111/cns.12386
12. Halban PA. Cell therapy for type 2 diabetes: is it desirable and can we get it? *Diabetes Obes Metab*. 2008;10(suppl 4):205–211. doi: 10.1111/j.1463-1326.2008.00957.x
13. Liew A, O'Brien T. The potential of cell-based therapy for diabetes and diabetes-related vascular complications. *Curr Diab Rep*. 2014;14:469. doi: 10.1007/s11892-013-0469-6

14. Creusot RJ, Battaglia M, Roncarolo MG, Fathman CG. Concise review: cell-based therapies and other non-traditional approaches for type 1 diabetes. *Stem Cells*. 2016;34:809–819. doi: 10.1002/stem.2290
15. Vrtovec B, Sever M, Jensterle M, Poglajen G, Janez A, Kravos N, Zemljic G, Cukjati M, Cernelc P, Haddad F, et al. Efficacy of CD34+ stem cell therapy in nonischemic dilated cardiomyopathy is absent in patients with diabetes but preserved in patients with insulin resistance. *Stem Cells Transl Med*. 2016;5:632–638.
16. Howangyin KY, Silvestre JS. Diabetes mellitus and ischemic diseases: molecular mechanisms of vascular repair dysfunction. *Arterioscler Thromb Vasc Biol*. 2014;34:1126–1135. doi: 10.1161/ATVBAHA.114.303090
17. Forrester SJ, Kikuchi DS, Hernandes MS, Xu Q, Griendling KK. Reactive oxygen species in metabolic and inflammatory signaling. *Circ Res*. 2018;122:877–902. doi: 10.1161/CIRCRESAHA.117.311401
18. Cui Y, Narasimhulu CA, Liu L, Li X, Xiao Y, Zhang J, Xie X, Hao H, Liu JZ, He G, et al. Oxidized low-density lipoprotein alters endothelial progenitor cell populations. *Front Biosci (Landmark Ed)*. 2015;20:975–988. doi: 10.2741/4351
19. Liu L, Cui Y, Li X, Que X, Xiao Y, Yang C, Zhang J, Xie X, Cowan PJ, Tian J, et al. Concomitant overexpression of triple antioxidant enzymes selectively increases circulating endothelial progenitor cells in mice with limb ischaemia. *J Cell Mol Med*. 2019;23:4019–4029. doi: 10.1111/jcmm.14287
20. Mysore TB, Shinkel TA, Collins J, Salvaris EJ, Fisicaro N, Murray-Segal LJ, Johnson LE, Lepore DA, Walters SN, Stokes R, et al. Overexpression of glutathione peroxidase with two isoforms of superoxide dismutase protects mouse islets from oxidative injury and improves islet graft function. *Diabetes*. 2005;54:2109–2116. doi: 10.2337/diabetes.54.7.2109
21. Kern S, Eichler H, Stoeve J, Klüter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells*. 2006;24:1294–1301. doi: 10.1634/stemcells.2005-0342
22. Kim JH, Sen S, Avery CS, Simpson E, Chandler P, Nishina PM, Churchill GA, Naggert JK. Genetic analysis of a new mouse model for non-insulin-dependent diabetes. *Genomics*. 2001;74:273–286. doi: 10.1006/geno.2001.6569
23. Bhatti JS, Thamarai K, Kandimalla R, Manczak M, Yin X, Kumar S, Vijayan M, Reddy PH. Mitochondria-targeted small peptide, SS31 ameliorates diabetes induced mitochondrial dynamics in male TallyHO/JngJ mice. *Mol Neurobiol*. 2021;58:795–808. doi: 10.1007/s12035-020-02142-7
24. Haberzettl P, Conklin DJ, Abplanalp WT, Bhatnagar A, O'Toole TE. Inhalation of fine particulate matter impairs endothelial progenitor cell function via pulmonary oxidative stress. *Arterioscler Thromb Vasc Biol*. 2018;38:131–142. doi: 10.1161/ATVBAHA.117.309971
25. Luttun A, Tjwa M, Moons L, Wu Y, Angelillo-Scherrer A, Liao F, Nagy JA, Hooper A, Priller J, De Klerck B, et al. Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat Med*. 2002;8:831–840. doi: 10.1038/nm731
26. Rutherford RB, Baker JD, Ernst C, Johnston KW, Porter JM, Ahn S, Jones DN. Recommended standards for reports dealing with lower extremity ischemia: revised version. *J Vasc Surg*. 1997;26:517–538. doi: 10.1016/S0741-5214(97)70045-4
27. Ke X, Liu C, Wang Y, Ma J, Mao X, Li Q. Netrin-1 promotes mesenchymal stem cell revascularization of limb ischaemia. *Diab Vasc Dis Res*. 2016;13:145–156. doi: 10.1177/1479164115611594
28. Cao F, Lin S, Xie X, Ray P, Patel M, Zhang X, Drukker M, Dylla SJ, Connolly AJ, Chen X, et al. In vivo visualization of embryonic stem cell survival, proliferation, and migration after cardiac delivery. *Circulation*. 2006;113:1005–1014. doi: 10.1161/CIRCULATIONAHA.105.588954
29. Fan W, Cheng K, Qin X, Narsinh KH, Wang S, Hu S, Wang Y, Chen Y, Wu JC, Xiong L, et al. 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;31:203–214. doi: 10.1002/stem.1265
30. Luo JD, Wang YY, Fu WL, Wu J, Chen AF. Gene therapy of endothelial nitric oxide synthase and manganese superoxide dismutase restores delayed wound healing in type 1 diabetic mice. *Circulation*. 2004;110:2484–2493. doi: 10.1161/01.CIR.0000137969.87365.05
31. Meerwaldt R, Links T, Zeebregts C, Tio R, Hillebrands JL, Smit A. The clinical relevance of assessing advanced glycation endproducts accumulation in diabetes. *Cardiovasc Diabetol*. 2008;7:29. doi: 10.1186/1475-2840-7-29
32. Pu LJ, Lu L, Xu XW, Zhang RY, Zhang QI, Zhang JS, Hu J, Yang ZK, Ding FH, Chen QJ, et al. Value of serum glycated albumin and high-sensitivity C-reactive protein levels in the prediction of presence of coronary artery disease in patients with type 2 diabetes. *Cardiovasc Diabetol*. 2006;5:27. doi: 10.1186/1475-2840-5-27
33. Weinberg E, Maymon T, Weinreb M. AGEs induce caspase-mediated apoptosis of rat BMSCs via TNFalpha production and oxidative stress. *J Mol Endocrinol*. 2014;52:67–76.
34. Kim S, Kwon J. COMP-Ang1 inhibits apoptosis as well as improves the attenuated osteogenic differentiation of mesenchymal stem cells induced by advanced glycation end products. *Biochim Biophys Acta*. 2013;1830:4928–4934. doi: 10.1016/j.bbagen.2013.06.035
35. Xia X, Zhang L, Chi J, Li H, Liu X, Hu T, Li R, Guo Y, Zhang X, Wang H, et al. *Helicobacter pylori* infection impairs endothelial function through an exosome-mediated mechanism. *J Am Heart Assoc*. 2020;9:e014120. doi: 10.1161/JAHA.119.014120
36. Byon CH, Heath JM, Chen Y. Redox signaling in cardiovascular pathophysiology: a focus on hydrogen peroxide and vascular smooth muscle cells. *Redox Biol*. 2016;9:244–253. doi: 10.1016/j.redox.2016.08.015
37. Yan J, Jiang J, He L, Chen L. Mitochondrial superoxide/hydrogen peroxide: an emerging therapeutic target for metabolic diseases. *Free Radic Biol Med*. 2020;152:33–42. doi: 10.1016/j.freeradbiomed.2020.02.029
38. Fan W, Song Y, Inzucchi SE, Sperling L, Cannon CP, Arnold SV, Kosiborod M, Wong ND. Composite cardiovascular risk factor target achievement and its predictors in US adults with diabetes: the Diabetes Collaborative Registry. *Diabetes Obes Metab*. 2019;21:1121–1127. doi: 10.1111/dom.13625
39. Liu Z, Lei M, Jiang Y, Hao H, Chu L, Xu J, Luo M, Verfaillie CM, Zweier JL, Liu Z. High glucose attenuates VEGF expression in rat multipotent adult progenitor cells in association with inhibition of JAK2/STAT3 signalling. *J Cell Mol Med*. 2009;13:3427–3436. doi: 10.1111/j.1582-4934.2008.00502.x
40. Reczek CR, Chandel NS. Ros-dependent signal transduction. *Curr Opin Cell Biol*. 2015;33:8–13. doi: 10.1016/j.ceb.2014.09.010
41. Gardner R, Salvador A, Moradas-Ferreira P. Why does sod overexpression sometimes enhance, sometimes decrease, hydrogen peroxide production? A minimalist explanation. *Free Radic Biol Med*. 2002;32:1351–1357. doi: 10.1016/S0891-5849(02)00861-4
42. Bronckaers A, Hilkens P, Martens W, Gervois P, Ratajczak J, Struys T, Lambrichts I. Mesenchymal stem/stromal cells as a pharmacological and therapeutic approach to accelerate angiogenesis. *Pharmacol Ther*. 2014;143:181–196. doi: 10.1016/j.pharmthera.2014.02.013
43. Carlsson P-O, Schwarcz E, Korsgren O, Le Blanc K. Preserved β-cell function in type 1 diabetes by mesenchymal stromal cells. *Diabetes*. 2015;64:587–592. doi: 10.2337/db14-0656
44. Sen S, Domingues CC, Rouphael C, Chou C, Kim C, Yadava N. Genetic modification of human mesenchymal stem cells helps to reduce adiposity and improve glucose tolerance in an obese diabetic mouse model. *Stem Cell Res Ther*. 2015;6:242. doi: 10.1186/s13287-015-0224-9
45. ShamsEldeen AM, Ashour H, Shoukry HS, Fadel M, Kamar SS, Aabdelbaset M, Rashed LA, Ammar HI. Combined treatment with systemic resveratrol and resveratrol preconditioned mesenchymal stem cells, maximizes antifibrotic action in diabetic cardiomyopathy. *J Cell Physiol*. 2019;234:10942–10963. doi: 10.1002/jcp.27947
46. An X, Liao G, Chen Y, Luo AI, Liu J, Yuan Y, Li L, Yang L, Wang H, Liu F, et al. Intervention for early diabetic nephropathy by mesenchymal stem cells in a preclinical nonhuman primate model. *Stem Cell Res Ther*. 2019;10:363. doi: 10.1186/s13287-019-1401-z
47. WenBo W, Fei Z, YiHeng D, Wei W, TingMang Y, WenHao Z, QianRu L, HaiTao L. Human umbilical cord mesenchymal stem cells overexpressing nerve growth factor ameliorate diabetic cystopathy in rats. *Neurochem Res*. 2017;42:3537–3547. doi: 10.1007/s11064-017-2401-y
48. Soria-Juan B, Escacena N, Capilla-González V, Aguilera Y, Llanos L, Tejedo JR, Bedoya FJ, Juan V, De la Cuesta A, Ruiz-Salmerón R, et al. Cost-effective, safe, and personalized cell therapy for critical limb ischemia in type 2 diabetes mellitus. *Front Immunol*. 2019;10:1151. doi: 10.3389/fimmu.2019.01151
49. Volarevic V, Arsenijevic N, Lukic ML, Stojkovic M. Concise review: mesenchymal stem cell treatment of the complications of diabetes mellitus. *Stem Cells*. 2011;29:5–10. doi: 10.1002/stem.556
50. Wang B, Chandrasekera PC, Pippin JJ. Leptin- and leptin receptor-deficient rodent models: relevance for human type 2 diabetes. *Curr Diabetes Rev*. 2014;10:131–145.
51. Cui H, López M, Rahmouni K. The cellular and molecular bases of leptin and ghrelin resistance in obesity. *Nat Rev Endocrinol*. 2017;13:338–351.

52. Qin Y, Lu M, Gong X. Dihydrorhodamine 123 is superior to 2,7-dichlorodihydrofluorescein diacetate and dihydrorhodamine 6G in detecting intracellular hydrogen peroxide in tumor cells. *Cell Biol Int*. 2008;32:224–228. doi: 10.1016/j.cellbi.2007.08.028
53. Humtsoe JO, Bowling RA Jr, Feng S, Wary KK. Murine lipid phosphate phosphohydrolase-3 acts as a cell-associated integrin ligand. *Biochem Biophys Res Commun*. 2005;335:906–919. doi: 10.1016/j.bbrc.2005.07.157
54. Hoefer IE, van Royen N, Rectenwald JE, Deindl E, Hua J, Jost M, Grundmann S, Voskuil M, Ozaki CK, Piek JJ, et al. Arteriogenesis proceeds via ICAM-1/Mac-1-mediated mechanisms. *Circ Res*. 2004;94:1179–1185. doi: 10.1161/01.RES.0000126922.18222.F0
55. Ushio-Fukai M, Alexander RW, Akers M, Griendling KK. p38 Mitogen-activated protein kinase is a critical component of the redox-sensitive signaling pathways activated by angiotensin II: role in vascular smooth muscle cell hypertrophy. *J Biol Chem*. 1998;273:15022–15029. doi: 10.1074/jbc.273.24.15022
56. Taylor BM, Fleming WE, Benjamin CW, Wu Y, Mathews WR, Sun FF. The mechanism of cytoprotective action of lazaroids I: inhibition of reactive oxygen species formation and lethal cell injury during periods of energy depletion. *J Pharmacol Exp Ther*. 1996;276:1224–1231.
57. Ushio-Fukai M, Urao N. Novel role of NADPH oxidase in angiogenesis and stem/progenitor cell function. *Antioxid Redox Signal*. 2009;11:2517–2533. doi: 10.1089/ars.2009.2582
58. de Medeiros WA, da Silva LA, Dall'Igna DM, Michels M, Manfredini A, Dos Santos Cardoso J, Constantino L, Scaini G, Vuolo F, Streck EL, et al. N-acetylcysteine effects on a murine model of chronic critical limb ischemia. *Biochim Biophys Acta Mol Basis Dis*. 2018;1864:454–463. doi: 10.1016/j.bbadis.2017.10.027
59. Martinez de Lizarrondo S, Gakuba C, Herbig BA, Repessé Y, Ali C, Denis CV, Lenting PJ, Touzé E, Diamond SL, Vivien D, et al. Potent thrombolytic effect of N-acetylcysteine on arterial thrombi. *Circulation*. 2017;136:646–660.
60. Huang CC, Pan WY, Tseng MT, Lin KJ, Yang YP, Tsai HW, Hwang SM, Chang Y, Wei HJ, Sung HW. Enhancement of cell adhesion, retention, and survival of HUVEC/cbMSC aggregates that are transplanted in ischemic tissues by concurrent delivery of an antioxidant for therapeutic angiogenesis. *Biomaterials*. 2016;74:53–63. doi: 10.1016/j.biomaterials.2015.09.043
61. Abdel-Mageed AS, Senagore AJ, Pietryga DW, Connors RH, Giambernardi TA, Hay RV, Deng W. Intravenous administration of mesenchymal stem cells genetically modified with extracellular superoxide dismutase improves survival in irradiated mice. *Blood*. 2009;113:1201–1203. doi: 10.1182/blood-2008-07-170936
62. Pan Q, Qin X, Ma S, Wang H, Cheng K, Song X, Gao H, Wang Q, Tao R, Wang Y, et al. Myocardial protective effect of extracellular superoxide dismutase gene modified bone marrow mesenchymal stromal cells on infarcted mice hearts. *Theranostics*. 2014;4:475–486. doi: 10.7150/thno.7729
63. Shi X, Bai Y, Zhang G, Liu Y, Xiao H, Liu X, Zhang W. Effects of overexpression of SOD2 in bone marrow-derived mesenchymal stem cells on traumatic brain injury. *Cell Tissue Res*. 2018;372:67–75. doi: 10.1007/s00441-017-2716-7
64. Akoumianakis I, Antoniades C. Impaired vascular redox signaling in the vascular complications of obesity and diabetes mellitus. *Antioxid Redox Signal*. 2019;30:333–353. doi: 10.1089/ars.2017.7421
65. Jameel MN, Li Q, Mansoor A, Qiang X, Sarver A, Wang X, Swingen C, Zhang J. Long-term functional improvement and gene expression changes after bone marrow-derived multipotent progenitor cell transplantation in myocardial infarction. *Am J Physiol Heart Circ Physiol*. 2010;298:H1348–H1356. doi: 10.1152/ajpheart.01100.2009

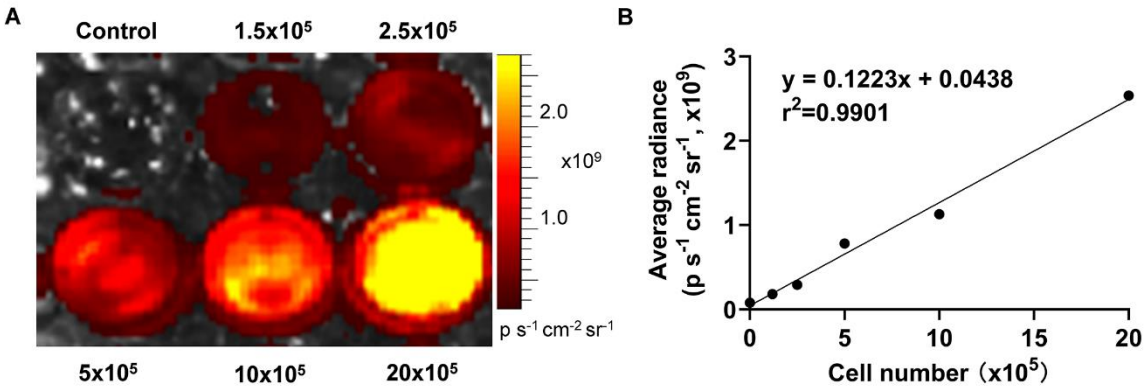
Supplemental Material

Figure S1. Identification and characterization of bMSCs.



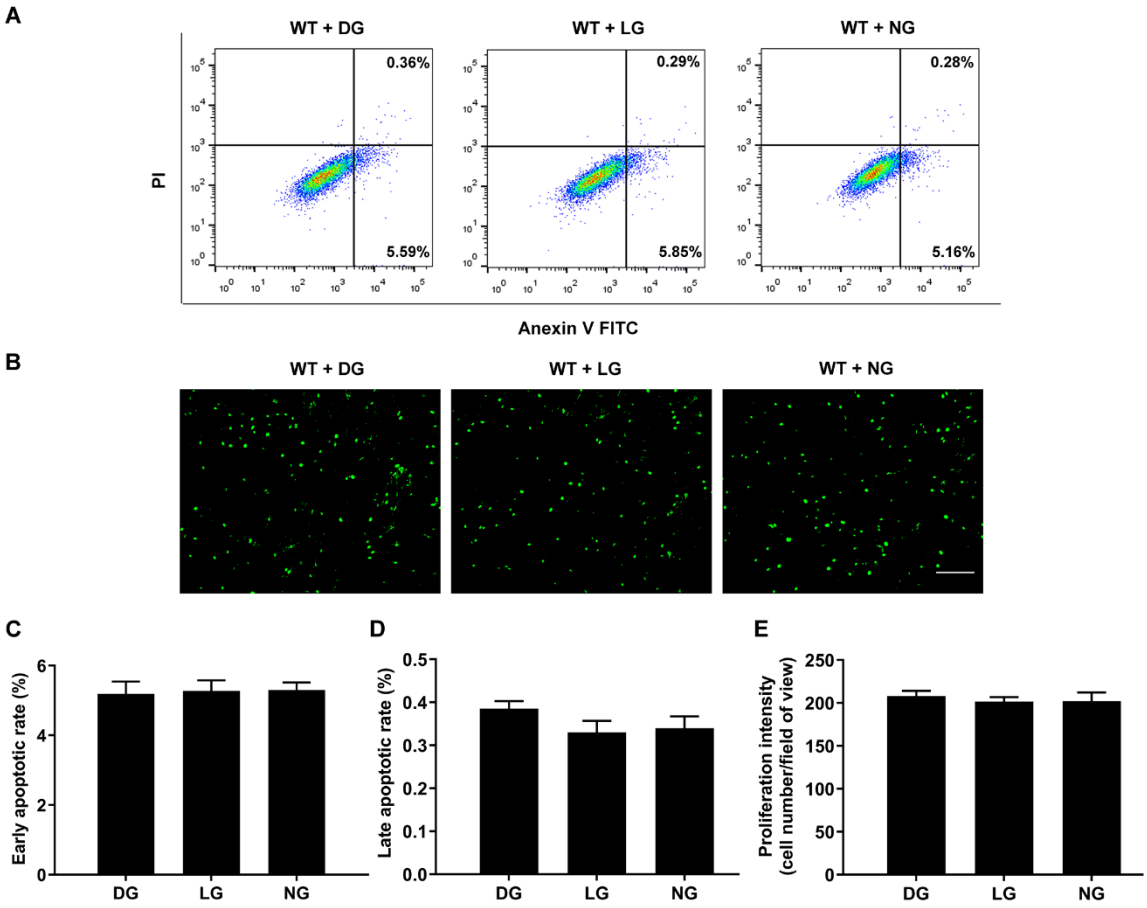
The isolated bMSCs were identified with their morphology (**B**), immunophenotype makers (positive for CD29, Scal-1, CD44, CD105 and negative for CD34, CD45, CD11b, CD31) using flow cytometric analysis (**A**), and multilineage differentiation using chemical induction and staining (**C-E**). Multilineage differentiation capacities of bMSCs included adipogenic differentiation using oil red-O staining (**C**), osteogenic differentiation using alizarin red staining (**D**), chondrogenic differentiation using alcian blue and nuclear fast red staining (**E**). n=3, scale bar, 100 μm.

Figure S2. A linear relationship between fluorescence intensity and cell number was established using in vitro fluorescence assays with different amounts of bMSCs.



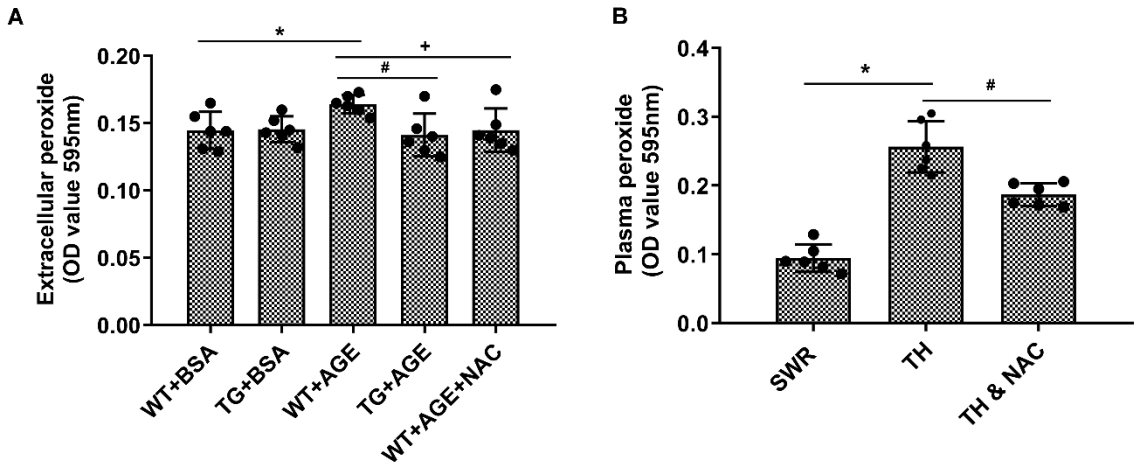
A series of specific amounts of cells were incubated with lipophilic carbocyanine fluorescent dyes DiR (2 uM/L), and imaged using PerkinElmer *In Vivo* Imaging System (IVIS) to determine the linear relationship between fluorescence intensity and cell numbers. Fluorescent imaging of different amount DiR-labeled bMSCs was quantified based on their optical radiance intensity as shown on colored scale bars with photons second^{-1} cm^{-2} steradian^{-1} (p s^{-1} cm^{-2} sr^{-1}, **A**). A linear relationship between cell number and fluorescence signal intensity was obtained in vitro with the r^2 of 0.990 (polynomial regression analysis, **B**).

Figure S3. Effect of high glucose on bMSCs.



Experiments were conducted to determine if high glucose could have a significant effect on apoptosis and proliferation of bMSCs in vitro. There was no significant difference in cell apoptosis and proliferation when bMSCs were incubated in media with D-glucose (DG) of 30 mmol/l, L-glucose (LG) of 24.5 mmol/l plus 5.5 mmol/l DG (for high osmolarity control), and D-glucose of 5.5 mmol/l (NG) under hypoxic condition (5% O2, 5% CO2, and 90% N2) at 37°C for 24 hours. (**A-B**) Representative images of flow cytometric analysis for early and late apoptotic cells (**A**), and EdU-AF488 assay for proliferative cells (**B**). Quantitative analysis on early, late apoptosis and proliferation of bMSCs were shown in panel **C-E**, respectively. n=4/group. Data were shown as means ± SD.

Figure S4. Levels of H_2O_2 in the conditioned media from bMSCs and plasma from TH and SWR mice.



AGE-BSA treatment (400 μg/ml with BSA as control) moderately, and yet significantly, increased the level of H_2O_2 in the conditioned media from bMSCs. NAC treatment or overexpression of SOD-1, SOD-3 and Gpx-1 effectively blocked AGE-induced increase of H_2O_2 in the conditioned media (n=6/group, *$p<0.05$ for WT+AGE versus WT+BSA; #$p<0.05$ for TG+AGE versus WT+AGE; +$p<0.05$ for WT+AGE+NAC versus WT+AGE; A). The level of H_2O_2 in the plasma from diabetic TH mice were significantly increased as expected. Interestingly, systematic NAC treatment only moderately, yet significantly, decreased the plasma H_2O_2 level in TH mice (n=6/group, *$p<0.01$ for TH versus SWR; #$p<0.05$ for TH & NAC versus TH; B). WT, bMSCs from C57 BL/6 mice; TG, bMSCs from TG mice; SWR, SWR/J mice; TH, TALLYHO/JngJ mice; NAC, N-acetylcysteine; All data were shown as means ± SD. Statistical differences were determined with one-way ANOVA with Bonferroni's post hoc test.