Involvement of endothelial progenitor cells in blood flow recovery through activation of the Wnt/β-catenin signaling pathway and inhibition of high oxidative stress in diabetic hindlimb ischemic rats

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Abstract

Background. Diabetes mellitus (DM) often causes stenosis and occlusion of hindlimb blood vessels, which are also the main cause for hindlimb ischemia in elderly people.

Objectives. To investigate the therapeutic effect of endothelial progenitor cell (EPC) transplantation on diabetic hindlimb ischemia.

Materials and methods. Endothelial progenitor cells were separated, labeled with PKH-26 and transplanted into rat models (10^7 cells/100 g). Dichlorodihydrofluorescein diacetate (DCFH-DA) was used to detect any oxidative stress. Streptozotocin (STZ) was injected to establish a diabetic rat model and hindlimb ischemia model was established via operation. Western blotting was used to detect total β-catenin (T-β-catenin) and non-phospho-β-catenin (NP-β-catenin) levels. The malondialdehyde (MDA), superoxide dismutase (SOD), Wnt3a, Wnt5a and Wnt7a levels were detected using enzyme-linked immunosorbent assay (ELISA). Oxidative stress was measured using DCFH-DA and dihydroethidium (DHE). The endothelial biomarker CD31 was observed to highlight vessels, and PKH-26 to trace migration/adhesion of EPCs.

Results. Endothelial progenitor cells were successfully isolated and identified, and diabetic hindlimb ischemic rat models were created. Tempol remarkably improved blood flow in diabetic hindlimb ischemic rats compared to DM+EPCs rats at 14 days ($p < 0.001$) and 28 days post-operation ($p < 0.001$). High oxidative stress was observed in diabetic hindlimb ischemic rats. Tempol significantly inhibited oxidative stress levels in diabetic hindlimb ischemic rats. Furthermore, Tempol significantly promoted angiogenesis in diabetic hindlimb ischemic rats compared to DM+EPCs rats. The β-catenin inhibitor, XAV (DM+EPCs+Tempol+XAV group), significantly suppressed blood flow recovery and angiogenesis in diabetic hindlimb ischemic rats when compared to the DM+EPCs+Tempol group at 14 days ($p = 0.026$) and 28 days ($p < 0.001$). The XAV remarkably reduced T-β-catenin ($p < 0.001$) and N-β-catenin ($p = 0.030$) levels in Tempol-treated diabetic hindlimb ischemic rats, as compared to the DM+EPCs+Tempol group. The Wnt5a participated in the pathology of diabetic hindlimb ischemia.

Conclusions. There are high oxidative stress levels in both EPCs in high-glucose environments and diabetic hindlimb ischemia, which can lead to limited blood flow recovery. The high oxidative stress caused the inhibition of Wnt/β-catenin signaling pathway, leading to limited blood flow recovery in diabetic hindlimb ischemia. At the same time, Wnt5a participated in the EPC-mediated blood flow recovery.

Key words: diabetic hindlimb ischemia, EPCs, Wnt/β-catenin signaling pathway, oxidative stress

Background

Diabetes mellitus (DM) often causes stenosis and occlusion of hindlimb blood vessels, ischemia and hypoxia, and is also the main cause of hindlimb ischemia in elderly people.[1,2] Currently, the treatment of diabetic hindlimb ischemia includes drug-conservative treatment, interventional surgery, traditional surgery, stem cell transplantation, physical therapy, and appropriate functional exercise, among others.[3] Due to the prolonged course of diabetic hindlimb ischemia and severity of the disease, the first 3 of the abovementioned treatments cannot completely resolve the problems. In the recent years, stem cell transplantation has become an important strategy for the treatment of severe diabetic hindlimb ischemia, whereas other treatment methods have not produced satisfactory clinical outcomes.[4,5]

The treatment with autologous stem cells can bring the light of recovery for patients with severe diabetic hindlimb ischemia.[6] However, there are many bottlenecks in the application of autologous stem cells for treating diabetic hindlimb ischemia. Firstly, even though they exist in limited amounts, the stem cells in peripheral blood or bone marrow need to be collected. Secondly, the treating effect is not stable and might be affected by time, method and number of transplantations. Finally, autologous stem cells can cause some side effects, including those caused by the collection, local infection, anesthesia, induction of tumor formation, and promotion of tumor growth. Presently, there is no treatment strategy that can completely resolve all of the aforenamed problems. However, the treatment with autologous stem cells an important new strategy for improving the condition of patients and promoting the biological function of stem cells.

The cytological basis for neovascularization is the damage of the endothelial cells and endothelial progenitor cells (EPCs) caused by excessive oxidative stress.[7] Endothelial progenitor cells, as the precursor cells of vascular endothelium, demonstrate the abilities of proliferation and differentiation in adulthood. Also, they are capable of developing into primitive vascular structures. Therefore, EPCs have a strong ability to support angiogenesis.[8] Yet, the number of EPCs in peripheral blood of patients with diabetes was lower than that of nondiabetic people. Many factors can lead to the functional damage of EPCs, including oxidative stress, the induction of endothelial nitric oxide synthase (eNOS)/nitric oxide (NO) cell signaling pathway and inflammatory responses.[9] Among them, oxidative stress can mediate many signaling pathways through reactive oxygen species (ROS), such as Wnt/β-catenin, G protein-coupled receptors, Notch, MAPK, JAK-STAT, and NF-κB.[10] At the same time, the proliferation, migration, adhesion, and angiogenesis of EPCs in vitro decreases significantly after the administration of Wnt inhibitors.[11] Therefore, the excessive oxidative stress may regulate the biological function of EPCs by downregulating Wnt/β-catenin signaling pathway.

The Wnt/β-catenin is one of the most important signaling pathways in cells and has a wide range of regulatory effects.[12] It has been shown that this pathway does not only play an important role in the development of embryonic blood vessels, but also regulates the role of EPCs in retinopathy and tumor angiogenesis.[13] However, there are no studies focusing on oxidative stress and the Wnt/β-catenin signaling pathway, or their function effect on EPCs.

Objectives

Therefore, by establishing diabetic hindlimb ischemic rat model and high-glucose environment in vitro, this study aimed to explore whether Wnt/β-catenin signaling is involved in hindlimb ischemia through modulating oxidative stress in vivo and in vitro. This study provides a novel way to treat hindlimb ischemia in diabetic rats employing stem cell transplantation.

Materials and methods

Animals

Two-month-old Sprague Dawley rats (certificate No. 24101115), weighting from 80–120 g, were purchased from Experimental Animal Center of Southwest Medical University (Chongqing, China). The rats were housed at a temperature of 20–25°C with a humidity of 70–80% and 12 h light/12 h dark light cycle. The rats had access to water and food ad libitum.

Ethical statement

All the experiments were approved by the Ethical Committee of Affiliated Hospital of Southwest Medical University (Lanzhou, China; approval No. 201710133). All animal experiments were conducted in accordance with the Declaration of Helsinki.

Isolation and culture of EPCs

The rats were sacrificed by the intraperitoneal injection of pentobarbital at a dosage of 50 mg/kg. In order to isolate the primary cells, the sacrificed rats were soaked in 75% alcohol for 15 min. Then, the femur and tibia of the rats were dissected for isolating EPCs. The bone marrow cavities collected from tibias and femurs were flushed into 5-mL centrifuge tubes using phosphate-buffered saline (PBS) containing 1% heparin. Then, the bone marrow was transferred into a 15-mL centrifuge tube and isolated using the density gradient centrifugation for 20 min at a speed of 2000 rpm. The middle layer, containing the mononuclear cells (MNCs) was transferred into another centrifuge tube and centrifuged for 5 min at 1500 rpm. The supernatants

were removed, and retained bone marrow was incubated in endothelial cell growth medium (EGM)-2 (Lonza Bioscience, Walkersville, USA), supplemented with 10% fetal bovine serum (FBS; Gibco Grand Island, USA) at 37°C and 5% CO_2. The density of cells was adjusted to 1×10^6/mL and cells were seeded onto 6-well or 12-well plates.

Identification of EPCs

In order to identify EPCs, the uptake for Dil-conjugated acetylated low-density lipoprotein (Dil-Ac-LDL) and the binding of fluorescein isothiocyanate-conjugated ulexeuropaeus agglutinin I (FITC-UEA-I) by cells were analyzed. Briefly, the cells were washed twice in PBS and then incubated using 12 μg/mL of Dil-Ac-LDL for 4 h in the dark. Then, the cells were washed thrice using PBS (5 min per wash) and fixed using 4% paraformaldehyde (ZSGB-BIO, Beijing, China) for 20 min. The cells were washed thrice using PBS (5 min per wash) and then incubated using 10 μg/mL of FITC-UEA-1 for 1 h in the dark. Next, the cells were washed twice using PBS (2 washes 5 min each), captured and analyzed for binding of FITC-UEA-1 and the uptake of Dil-Ac-LDL by means of inverted fluorescence microscopy (DMI 6000 B; Leica, Wetzlar, Germany).

PKH-26 labeling and staining of EPCs

The labeling and staining of EPCs was conducted as previously described,[14] with a few modifications. Briefly, the EPCs were cultured, a single cell suspension was generated and 2×10^7 cells were collected per centrifuge tube. Cells were centrifuged for 5 min at 1500 rpm and the supernatants were discarded. The remaining cells were resuspended and incubated with PKH-26 at 25°C for 3 min, and then the reaction was terminated. Finally, the stained EPCs were centrifuged at 25°C and 1500 rpm for 10 min, the cells were resuspended in 0.9% normal saline and transplanted into the tail vein of rats. The PKH-26-labeled frozen sections of tissues were prepared and observed using fluorescence microscopy.

Diabetic hindlimb ischemic rat model

Sprague Dawley weighing 80–120 g were used to generate the diabetic hindlimb ischemic model, as previously described.[15] Briefly, the diabetes was generated by the intraperitoneal injection of streptozotocin (STZ) at a dosage of 50 mg/kg. At 3, 5, 7, 14, 28, 42, and 56 days post-STZ administration, plasma glucose concentrations were examined. After the treatment of STZ, the rats were fed with high-fat food for 2 months, and then the experiments were carried out. The rats with plasma glucose concentration >16.65 mmol/L over 4 readings were considered diabetic rats. Eight weeks after the diabetes induction, the rats were anaesthetized by the intraperitoneal injection of pentobarbital (50 mg/kg), and the left femoral artery, distal portion and all lateral branches were dissected and ligated with 7-0 sutures. The treatment for blood vessels in diabetic hindlimb ischemic model was carried out according to a previous report.[16]

Trial grouping

The rats in this study were divided into 2 groups, namely the diabetes group and the control group. The control group consisted of rats injected with normal saline instead of STZ. After laser Doppler monitoring was conducted, the rats were divided into a healthy group (H group, n = 6) and a diabetic hindlimb ischemic rat model group (DM group, n = 6). Some rats were transplanted with EPCs, which were further divided into DM+NS (normal saline) group (n = 6), DM+EPCs group (n = 6), DM+EPCs+Tempol group (n = 6), and DM+EPCs+Tempol+XAV group (n = 6). In the process of EPC transplantation, the EPC single cell suspension was mixed well to avoid cell aggregates and vascular obstruction.

ROS examination

The production of ROS was examined using dichlorodihydrofluorescein diacetate (DCFH-DA) and dihydroethidium (DHE) staining. In brief, the frozen ischemic gastrocnemius muscles of the left hindlimb sections or EPCs were incubated with DCFH-DA (5 μmol/L) or DHE (2 μmol/L) for 30 min at 37°C in dark. Then, the sections were washed 3 times with PBS (5 min per wash). The fluorescent images of DCFH-DA/DHE-stained tissues/cells were observed using fluorescence microscopy (IX53; Olympus Corp., Tokyo, Japan). At least 5 visual fields of 1 section were analyzed regarding the fluorescence intensity using Image-Pro Plus software (v. 6.0; Media Cybernetics, Inc., Bethesda, USA).

Oxidative stress evaluation

In order to evaluate oxidative stress levels, the production of malondialdehyde (MDA) and superoxide dismutase (SOD) in the serum of rats was measured, as previously described.[17] The MDA activity was measured using the MDA assay kit (Cat. No. S0131S; Beyotime, Shanghai, China), following the manufacturer's instructions. The SOD activity was evaluated with the SOD detection kit (Cat. No. S0101M; Beyotime), according to the manufacturer's protocol. The absorbance was captured with the spectrophotometer at the wavelength of 450 nm.

Immunofluorescence and microvascular density evaluation

Briefly, the gastrocnemius muscle of the left hindlimb was fixed with 4% paraformaldehyde at 4°C for 24 h and frozen sections (5 μm) were made. Then, the sections were treated with 0.5% Triton X-100 for 10 min and 0.1% trypsin for 20 min, and blocked with 10% goat serum for 15 min.

The sections were incubated with mouse anti-rat CD31 antibody (1:200) at 4°C overnight. The CD31 is an important cellular immunological biomarker for the vascular endothelial cells.[18] The next day, sections were incubated with FITC-labeled goat anti-mouse immunoglobulin G (IgG) (1:200) for 60 min in dark. In this study, 1 green fluorescence point or cluster was used to represent 1 blood vessel. Five visual fields were selected, green fluorescent spots were photographed (×200 magnification), the microvascular density (MVD) was analyzed, and the mean was generated.

Wnt3a/Wnt5a/Wnt7a levels determined using ELISA

On the 28th day post-operation, the rats were sacrificed to obtain blood. Blood was centrifuged at 2000 rpm for 10 min to obtain serum for the detection of Wnt3a, Wnt5a and Wnt7a. The Wnt3a, Wnt5a and Wnt7a levels were determined using the Wnt3a Detection ELISA kit (Cat. No. 153260; US Biological, Swampscott, USA), Wnt5a Detection ELISA kit (Cat. No. 153265; US Biological) and Wnt7a Detection ELISA kit (Cat. No. ABIN6374423; Rockland Immunochemicals, Inc., Aachen, Germany), respectively, according to the manufacturers' protocols.

Western blotting assay

The total proteins in the gastrocnemius muscles of the left hindlimb tissues were extracted, and the concentration was determined using BCA protein assay kit (Cat. No. P0012S; Beyotime Biotech. Co. Ltd., Wuhan, China). The obtained proteins were separated on a 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to polyvinylidene fluoride (PVDF) membranes. Then, the membranes were blocked with 5% non-fat milk and incubated with rabbit anti-rat total β-catenin (T-β-catenin) monoclonal antibody (1:1000, Cat. 8480; Cell Signaling Technology, Beverly, USA), rabbit anti-rat non-phospho-β-catenin (NP-β-catenin) polyclonal antibody (1:1000, Cat. No. 19807; Cell Signaling Technology) and rabbit anti-rat GAPDH monoclonal antibody (Cat. No. 5174; Cell Signaling Technology) at 4°C overnight, followed by the incubation with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (1:500, Cat. No. A0208; Beyotime) at room temperature for 2 h. Western blotting bands were imaged using an enhanced chemiluminescence (ECL) kit (Amersham Biosciences, Piscataway, USA) and analyzed using Quantity One software (Bio-Rad Laboratories, Inc., Hercules, USA).

Statistical analyses

Data were defined as mean ± standard deviation (SD) and analyzed using IBM SPSS software v. 20.0 (IBM Corp., Armonk, USA). The assumption of the normality and homogeneity of the variances required for the comparison of means were analyzed using the Kolmogorov–Smirnov test and Levene's test, respectively. The differences between the 2 groups were compared using Student's t-test. The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. When parameters of the same animals were measured over consecutive days, a repeated measures ANOVA was used. A value of $p < 0.05$ was considered statistically significant.

Results

Identification and labeling of EPCs

Endothelial progenitor cells were identified using double staining and labeled with PKH-26. After 3 days of culture, the EPCs were adherent cells, mainly consisting of round and a few oval shapes (Fig. 1A). After 7 days of continuous culture, the amount of cells increased, with obvious oval or spindle-shaped cells, demonstrating the colony-like growth under the microscope (Fig. 1A). After 14 days of culture, the pebble-like cells appeared with vortex-like growth (Fig. 1A). After 7 days of culture, EPCs were stained with Dil-Ac-LDL and FITC-UEA-1 double fluorescence and observed under confocal microscope. The red fluorescence-stained cells were considered Dil-Ac-LDL-positive and yellow fluorescence cells were considered FITC-UEA-1-positive (Fig. 1B). Cells with both red and yellow fluorescence were considered EPCs. The PKH-26-labeled EPCs could also migrate to ischemic sites (Fig. 1C).

Generation of diabetes model and diabetic hindlimb ischemic rat model

After STZ injection, fasting plasma glucose (FPG) was measured on the 3rd, 5th, 7th, 14th, 28th, and 56th day. The average blood glucose level of the experimental group was measured at 16.7 mmol/L, which was significantly higher than in the control group (Fig. 2A; all $p < 0.001$). According to the laser Doppler monitoring, the blood flow of the H group and the DM group were significantly changed before and 0 days after the operation (Fig. 2B). These results showed that the left hindlimb of the 2 groups demonstrated an obvious ischemia (Fig. 2B). According to the repeated measures ANOVA, there were significant differences in L/R blood flow ratio pre-operation, and 0, 7, 14, and 28 days post-treatment, in both the H group (ANOVA, $F = 89.011$, degrees of freedom (df) = 4, $p < 0.001$) and the DM group (ANOVA, $F = 128.794$, df = 4, $p < 0.001$). Therefore, the blood flow ratio was changed along with the treatment time. The Tukey's post hoc test showed that the L/R blood flow ratio of the DM group was significantly lower compared to that of the H group 14 days ($F = 68.903$, df = 10, $p = 0.003$) and 28 days ($F = 6.699$, df = 10, $p < 0.001$) after the operation (Fig. 2B).

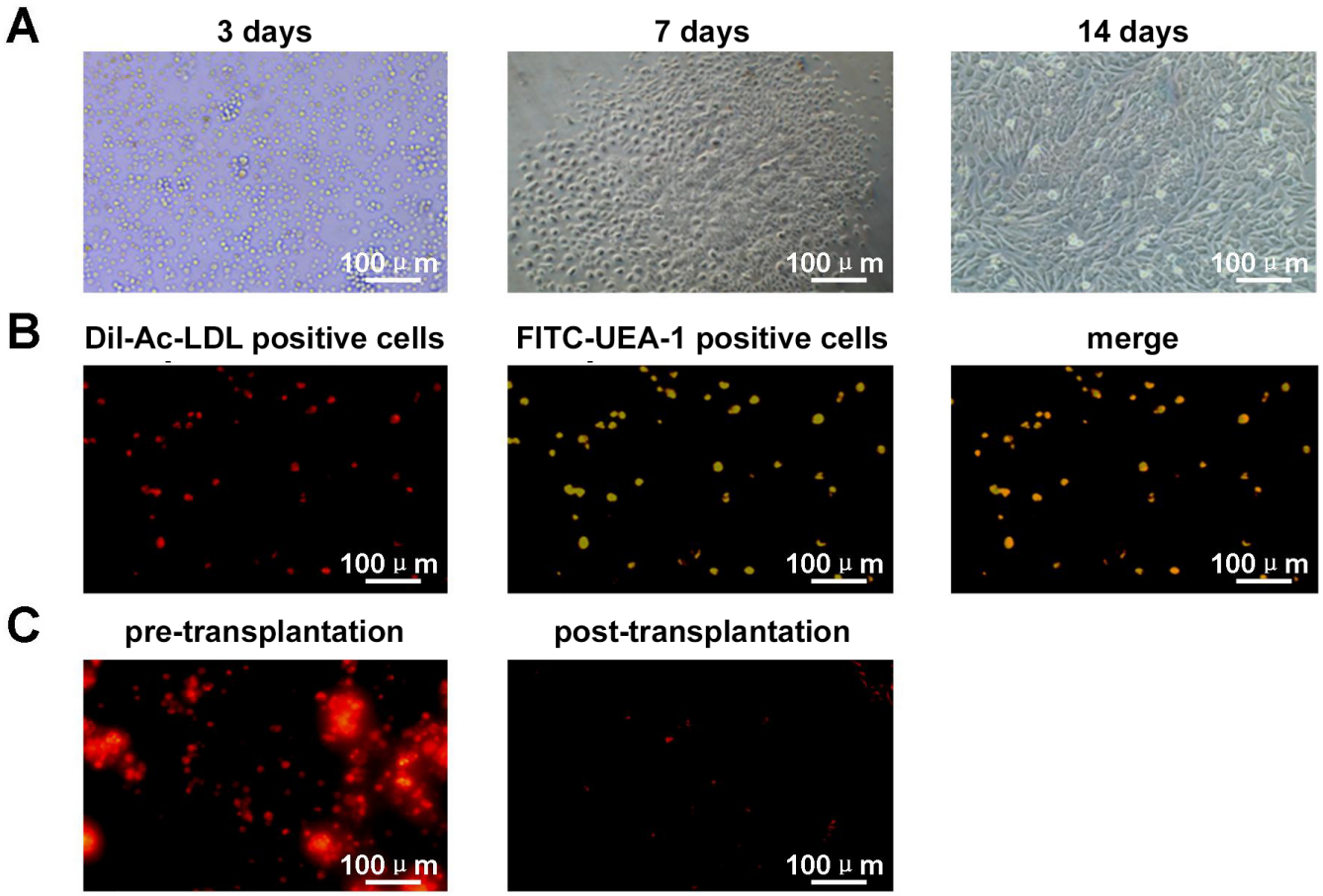

Fig. 1. Identification and label for the endothelial progenitor cells (EPCs). A. EPC morphological changes at 3, 7 and 14 days, ×200 magnification; B. Detection of EPCs by staining with DiI-conjugated acetylated low-density lipoprotein (DiI-Ac-LDL) and fluorescein isothiocyanate-conjugated ulexeuropaeus agglutinin I (FITC-UEA-I). The images of DiI-Ac-LDL-positive cells and FITC-UEA-I-positive cells were merged, ×200 magnification; C. Pre-transplantation and post-transplantation EPCs marked with PKH-26. The post-transplantation EPCs migrated to the gastrocnemius muscle, ×200 magnification

Oxidative stress inhibitor Tempol improved blood flow in hindlimb of diabetic hindlimb ischemic rats

According to the repeated measures ANOVA, there were significant differences for L/R blood flow ratio at the pre-operation time point, in DM+NS (ANOVA, $F = 124.482$, $df = 4$, $p < 0.001$), DM+EPCs (ANOVA, $F = 399.254$, $df = 4$, $p < 0.001$) and DM+EPCs+Tempol (ANOVA, $F = 114.956$, $df = 4$, $p < 0.001$) groups. Thus, the effects of Tempol on L/R blood flow ratio changed with the treatment times. The results showed that there was no significant difference in blood flow of hindlimbs between the DM+NS group and the DM+EPCs group, 0, 7, 14, and 28 days after the operation (Fig. 3A,B; all $df = 10$, $p > 0.05$). The results also demonstrated that the recovery of blood flow in diabetic hindlimb ischemic rats was poor after the transplantation of EPCs. However, following Tempol administration, the recovery of blood flow in diabetic hindlimb ischemic rats was significantly improved compared with that of the DM+EPCs group at 14 days (Tukey's post hoc test, $F = 69.283$, $df = 10$, $p = 0.013$) and 28 days (Tukey's post hoc test, $F = 106.872$, $df = 10$, $p < 0.001$) post-operation (Fig. 3A,B).

High oxidative stress observed in hindlimbs of diabetic hindlimb ischemic rats

The MDA levels in the DM group were significantly higher compared to that in the H group, both for pre-operation (Student's t-test, $t = -15.123$, $df = 10$, $p < 0.001$) and post-operation (Student's t-test, $t = -9.852$, $df = 10$, $p < 0.001$) (Fig. 4A). Furthermore, there was a significant difference between the MDA levels among the H group (pre- and post-operation) and DM group (pre- and post-operation) (Fig. 4A; Tukey's post hoc test (ANOVA), $F = 81.708$, $df = 3$, $p < 0.001$). However, there were no significant differences for the MDA levels between the pre-operation group and the post-operation group, for both the H group ($p = 0.708$) and the DM group (Fig. 4A; $p = 0.191$). There was a significant difference in the MDA levels among H+NS, DM+NS and DM+EPCs groups (Fig. 4B; Tukey's post hoc test (ANOVA), $F = 55.342$, $df = 2$, $p < 0.001$). However, there were no differences in the MDA levels between the DM+NS group and the DM+EPCs group (Fig. 4B; $p = 0.900$). Moreover, there were significant differences in SOD levels among H+NS, DM+NS and DM+EPCs groups (Fig. 4C; Tukey's post hoc

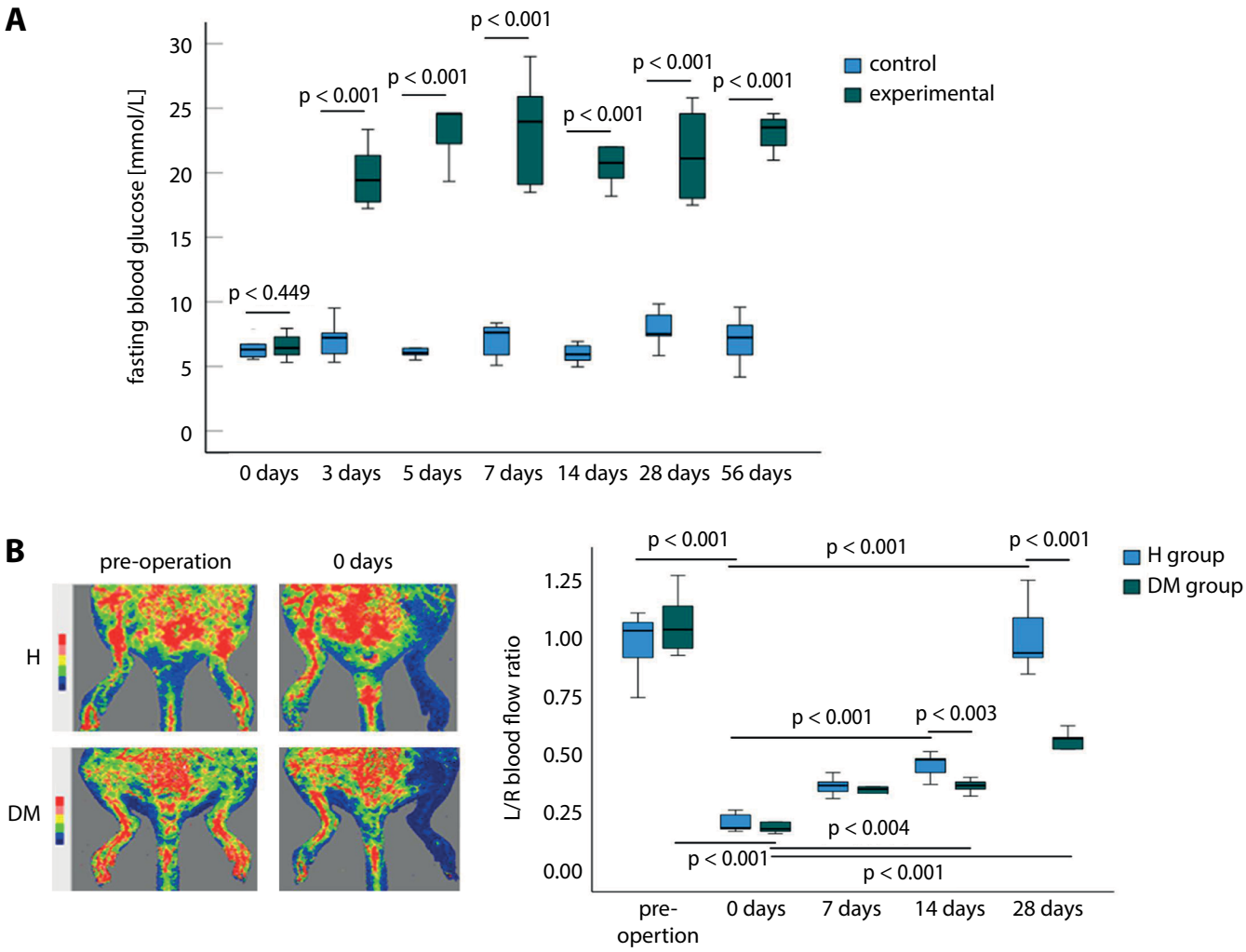

Fig. 2. Establishment of the diabetes rat models and diabetic hindlimb ischemic rat models. A. The fasting blood glucose changes in the rats of the control and experimental group; B. Laser Doppler perfusion imaging (LDPI) in the healthy (H) group and diabetes mellitus (DM) group, preoperative (Pre-) and postoperative (0, 7, 14 and 28 days) showed changes in blood flow (n = 6). The L/R blood flow ratio statistical line chart was drawn. The Tukey's post hoc test (analysis of variance (ANOVA)) and Student's t-test were conducted to analyze the data. The statistical differences (p-values) between the 2 labeled groups (horizontal lines) are depicted

test (ANOVA), $F = 22.933$, $df = 2$, $p < 0.001$). The SOD levels were significantly lower in DM+NS (Fig. 4C; $p < 0.001$) and DM+EPCs groups (Fig. 4C; $p < 0.001$) compared to that in the H+NS group. However, there were no differences between the DM+NS and DM+EPCs groups (Fig. 4C; $p = 0.903$).

Tempol inhibited high oxidative stress in diabetic hindlimb ischemic rats

According to the DCFH-DA staining results, there were plenty of DCFH-DA-positive cells in the gastrocnemius tissues in both DM+NS and DM+EPCs rats, and no DCFH-DA-positive cells in the H+NS rats (Fig. 4D). However, the Tempol administration remarkably reduced the oxidative stress (DCFH-DA staining) compared to both the DM+NS and DM+EPCs groups (Fig. 4D). The DHE staining also indicated that Tempol administration significantly suppressed oxidative stress, compared to both the DM+NS and DM+EPCs groups (Fig. 4D).

Tempol promoted angiogenesis in gastrocnemius of diabetic hindlimb ischemic rats

Compared with the DM+NS group, there was no obvious difference in the number of new microvessels in the DM+EPCs group (Fig. 5A). Furthermore, there were no EPCs in the DM+NS group migrating to the gastrocnemius, indicating that the PKH-26-labeled EPCs (red) demonstrated no false positive value and had a higher specificity in this experiment. Compared with the DM+EPCs group, the oxidative stress was inhibited and new microvessels were generated in the DM+EPCs+Tempol group (Fig. 5A,B; Tukey's post hoc test (ANOVA), $F = 183.797$, $df = 2$, $p < 0.001$). Meanwhile, the number of microvessels migrating to the ischemic gastrocnemius muscle was also increased in diabetic hindlimb ischemic rats.

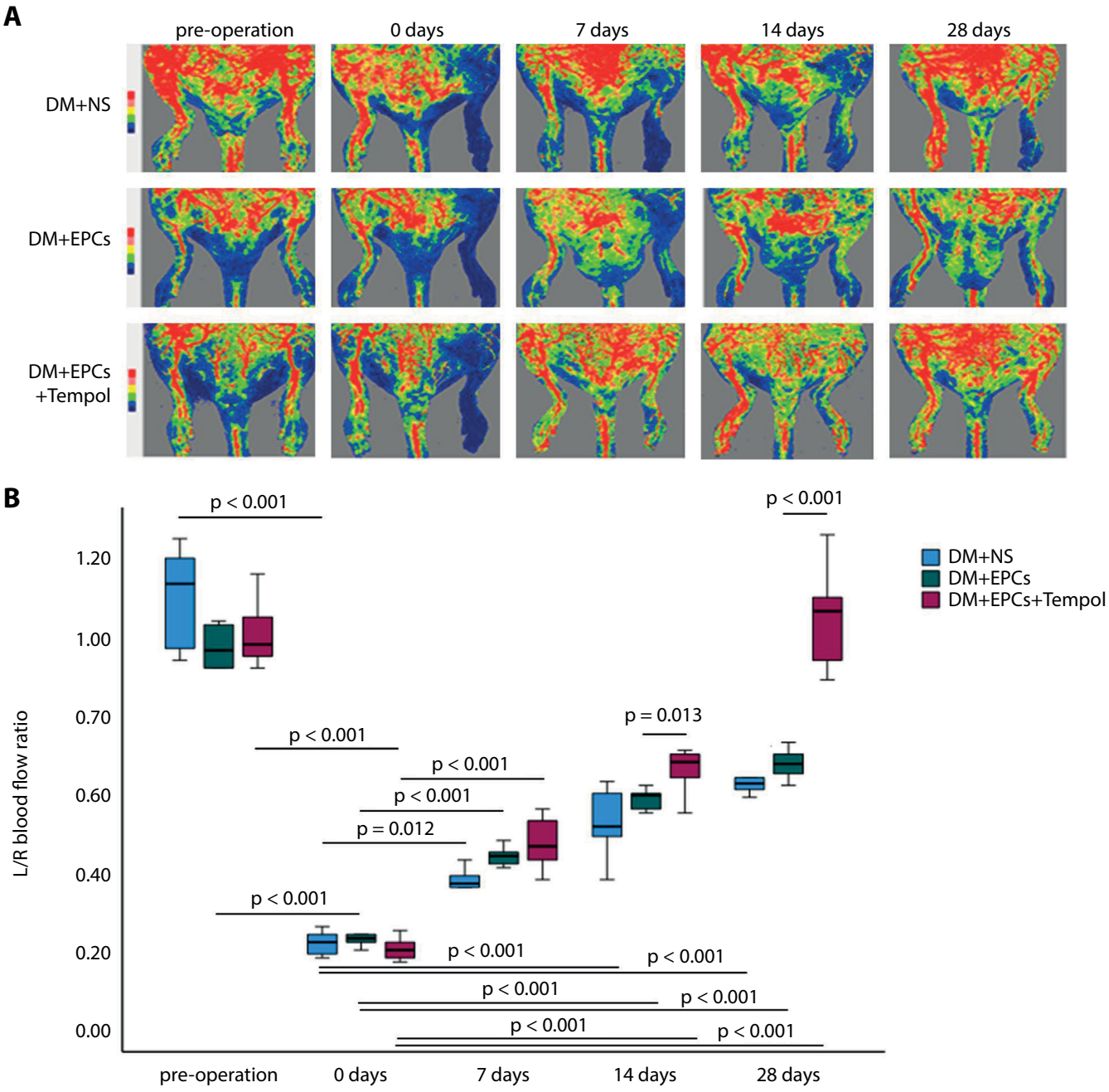

Fig. 3. Effects of oxidative stress inhibitor Tempol on the blood flow in the hindlimbs of diabetic hindlimb ischemic rats. A. Blood flow changes of rats in DM+NS, DM+EPCs and DM+EPCs+Tempol groups at pre-operation and 0, 7, 14, and 28 days post-operation, determined with laser Doppler monitoring; B. The continuous flow ratio statistical analysis for DM+NS, DM+EPCs and DM+EPCs+Tempol groups. The Tukey's post hoc test (analysis of variance (ANOVA)) and Student's t-test were conducted to analyze the data. The statistical differences (p-values) between the 2 labeled groups (horizontal lines) are depicted

DM – diabetes mellitus; NS – normal saline; EPCs – endothelial progenitor cells.

β-catenin inhibitor XAV suppressed blood flow recovery in diabetic hindlimb ischemic rats

The Wnt/β-catenin signaling pathway inhibitor, XAV, significantly decreased the L/R blood flow ratio in the DM+EPCs+Tempol+XAV group compared to that in the DM+EPCs+Tempol group at 14 days (Tukey's post hoc test, $F = 83.56$, $df = 10$, $p = 0.026$) and 28 days post-treatment (Tukey's post hoc test, $F = 118.34$, $df = 10$, $p < 0.001$) (Fig. 6A,B). In the DM+EPCs+Tempol+XAV group, there were significant differences in L/R blood flow ratio at different time points (pre-operation 0, 7, 14, and 28 days post operation) (Fig. 6A,B; Tukey's post hoc test (ANOVA), $F = 47.978$, $df = 4$, $p < 0.001$). In order to exclude the effect of DMSO on the L/R blood flow ratio, the L/R blood flow ratio was also examined in dimethyl sulfoxide (DMSO)-treated group. However, there was no significant difference between the DM+EPCs+DMSO group and the DM+EPCs group (Fig. 6A,B; all $p > 0.05$),

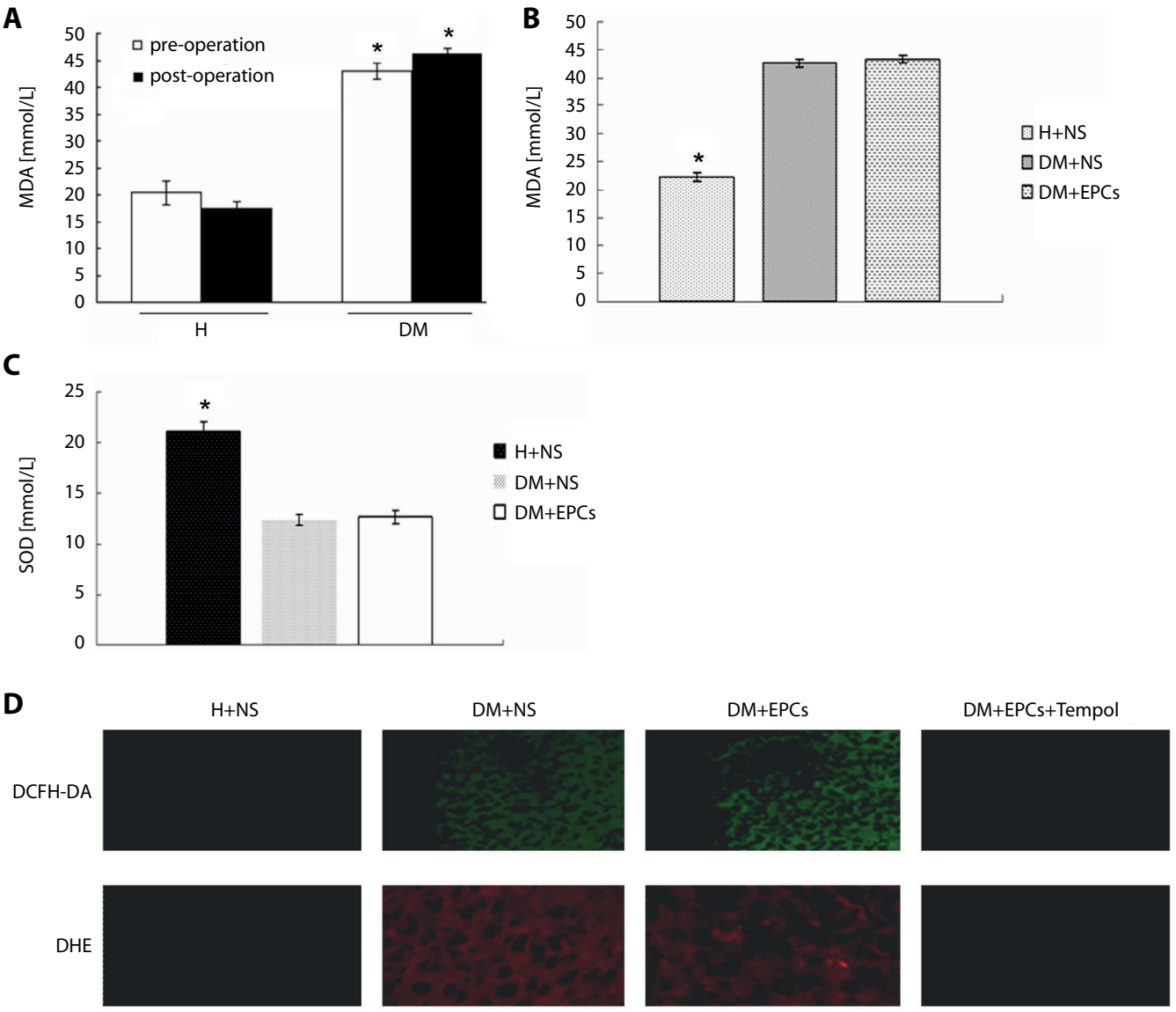

Fig. 4. Determination of high oxidative stress in the hindlimbs of diabetic hindlimb ischemic rats. A. Serum malondialdehyde (MDA) levels in the healthy (H) group and diabetes mellitus (DM) group at pre-operation and post-operation (* $p < 0.05$ for comparison of pre- and post-operation MDA level in the H group); B. Serum MDA levels in the endothelial progenitor cell (EPC)-transplanted diabetic rats (* $p < 0.05$ for comparison of pre- and post-operation MDA level in the DM+EPCs group); C. Effect of the transplantation of EPCs on serum superoxide dismutase (SOD) levels of DM rats (* $p < 0.05$ for comparison of pre- and post-operation MDA level in the DM+EPCs group); D. Determination of the reactive oxygen species (ROS) levels in ischemic gastrocnemius muscle of rats using fluorescent microscope. Green fluorescent dichlorodihydrofluorescein diacetate (DCFH-DA) reflected the ROS levels in gastrocnemius muscle of rats in each group. Red fluorescent dihydroethidium (DHE) reflected the ROS levels in the gastrocnemius muscle of rats in each group (×200 magnification). The Tukey's post hoc test (analysis of variance (ANOVA)) and Student's t-test were conducted to analyze the data. The statistical differences (p-values) between the 2 labeled groups (horizontal lines) have been depicted

NS – normal saline.

which suggests that the XVA buffer DMSO (as a solvent and as a negative control) did not affect blood flow recovery. Moreover, repeated measures ANOVA showed that there were significant differences in the L/R blood flow ratio between different time points (before the operation and 0, 7, 14, and 28 days following the operation) in the DM+EPCs (ANOVA, $F = 60.546$, $df = 4$, $p < 0.001$), DM+EPCs+DMSO (ANOVA, $F = 67.757$, $df = 4$, $p < 0.001$) and DE+EPCs+Tempol (ANOVA, $F = 56.550$, $df = 4$, $p < 0.001$) groups. In summary, L/R blood flow ratio in various groups changed at different treatment time points.

XAV suppressed angiogenesis in gastrocnemius muscle of diabetic hindlimb ischemic rats following Tempol administration

Our results demonstrated that XAV administration suppresses microvessel migration to ischemic gastrocnemius muscle in diabetic hindlimb ischemic rats after the administration of Tempol (Fig. 7A). The ANOVA analysis showed that there were obvious differences for angiogenesis (value of EPCs/MVD) in gastrocnemius muscle among

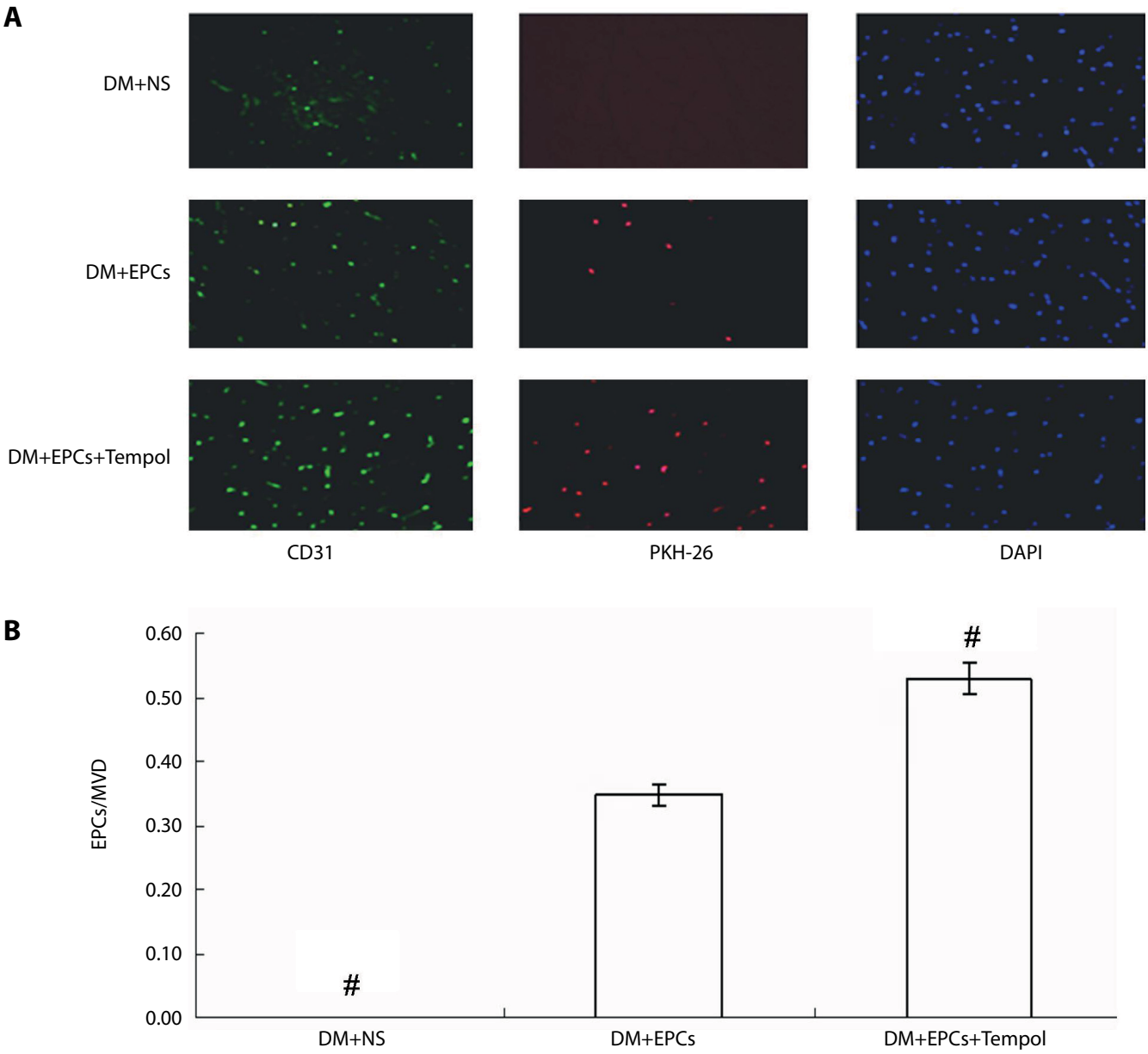

Fig. 5. Effects of Tempol on angiogenesis in the gastrocnemius muscle of diabetic hindlimb ischemic rats. A. Counting the numbers of microvessels in frozen section of gastrocnemius muscles of rats by staining with PKH-26. The 4′,6-diamidino-2-phenylindole (DAPI) (blue fluorescence) represents the nuclei. The CD31 (green fluorescence) represents the new microvessels. The PKH-26-labeled red fluorescence or clusters represent the migration of endothelial progenitor cells (EPCs) to the ischemic sites (×200 magnification); B. Statistical analysis for the ratio of number of labeled EPCs migrated to ischemic site to the number of new microvessels. The Tukey's post hoc test (analysis of variance (ANOVA)) was conducted to analyze the data.
* $p < 0.05$ for comparison of DM+NS group or DM+EPCs+Tempol group with the DM+EPCs group.

MVD – microvascular density.

DM+EPCs, DM+EPCs+DMSO, DM+EPCs+Tempol, and DM+EPCs+Tempol+XAV groups (Fig. 7A,B; Tukey's post hoc test (ANOVA), $F = 16.921$, $df = 3$, $p < 0.001$). In a similar manner, Tempol treatment remarkably enhanced angiogenesis in gastrocnemius muscle of Tempol-administered diabetic hindlimb ischemic rats (DM+EPCs+Tempol group) compared to the DM+EPCs group (Fig. 7A,B; $p < 0.001$). Meanwhile, the angiogenesis in XAV and Tempol-administered diabetic hindlimb ischemic rats (DM+EPCs+Tempol+XAV group) was significantly reduced compared to that in the DM+EPCs+Tempol group (Fig. 7A,B; $p < 0.001$).

XAV reduced β-catenin expression in Tempol-treated diabetic hindlimb ischemic rats

The T-β-catenin and NP-β-catenin expressions were determined using western blotting assay (Fig. 8A). The results showed that there were significant differences in T-β-catenin expression (Fig. 8B; Tukey's post hoc test (ANOVA), $F = 70.149$, $df = 3$, $p < 0.001$) and NP-β-catenin expression (Fig. 8C; Tukey's post hoc test (ANOVA), $F = 9.562$, $df = 3$, $p = 0.005$) among DM+EPCs, DM+EPCs+DMSO, DM+EPCs+Tempol, and

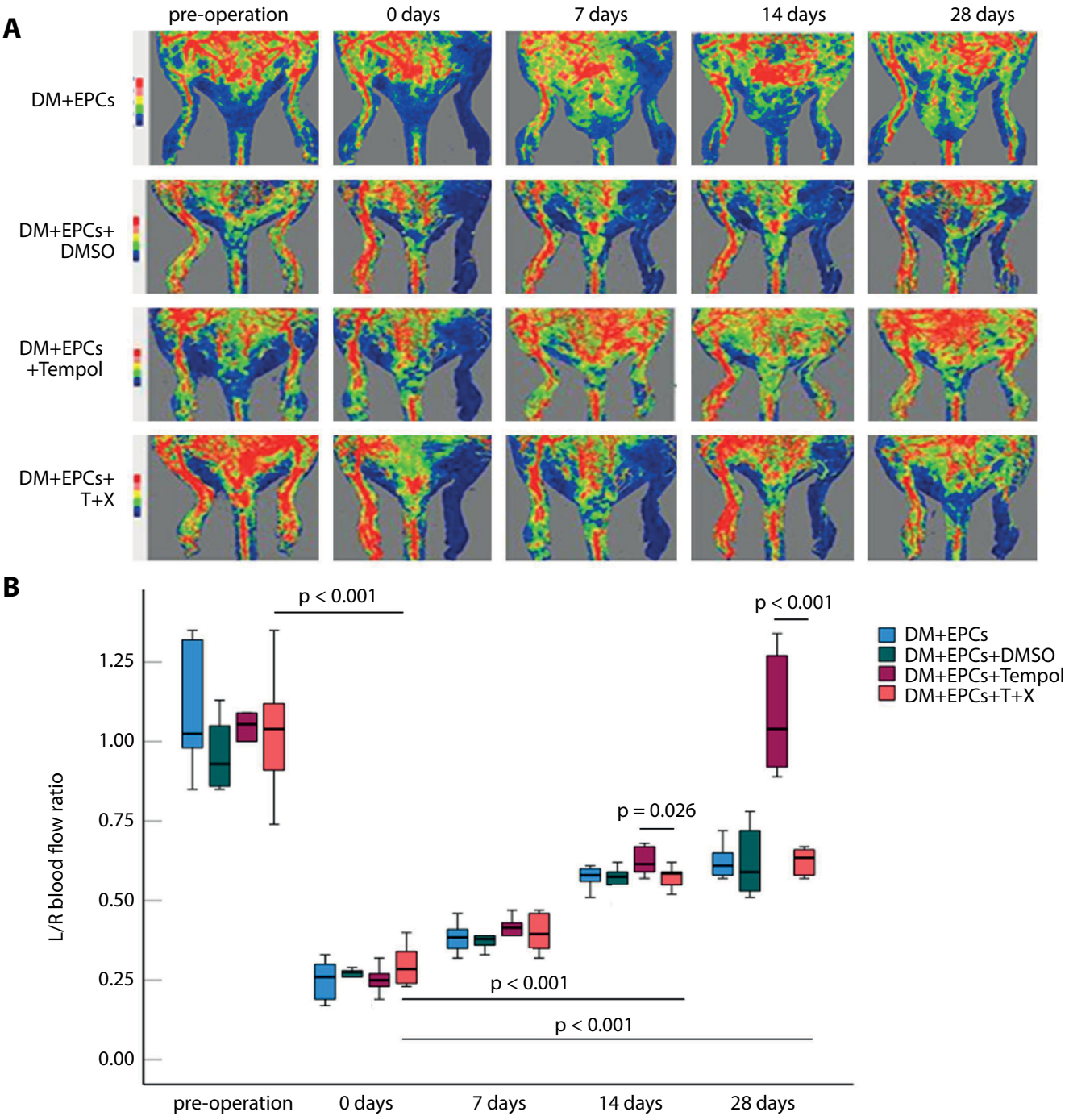

Fig. 6. The effect of inhibition of Wnt/β-catenin signaling pathway on the recovery of blood flow. A. The blood changes for the rats in DM+EPCs, DM+EPCs+DMSO, DM+EPCs+Tempol, and DM+EPCs+Tempol+XAV groups at pre-operation and 0, 7, 14, and 28 days post-operation were determined using laser Doppler monitoring; B. The statistical analysis of the continuous L/R blood flow ratio for each group. The Tukey's post hoc test (analysis of variance (ANOVA)) and Student's t-test were conducted to analyze the data. The statistical differences (p-values) between the 2 labeled groups (horizontal lines) have been depicted

DM – diabetes mellitus; EPCs – endothelial progenitor cells; DMSO – dimethylsulfoxide; T+X – tempol+XAV.

DM+EPCs+Tempol+XAV groups. The Tempol treatment significantly increased T-β-catenin expression (Fig. 8B; $p < 0.001$) and NP-β-catenin expression (Fig. 8C; $p = 0.008$) in EPC-transplanted diabetic hindlimb ischemic rats (DM+EPCs+Tempol), compared to those in the DM+EPCs group. However, the XAV administration decreased T-β-catenin expression (Fig. 8B; $p < 0.001$) and NP-β-catenin expression (Fig. 8C; $p = 0.030$) in Tempol administered and EPC-transplanted diabetic hindlimb ischemic rats (DM+EPCs+Tempol+XAV group), compared to those in the DM+EPCs+Tempol group.

Wnt5a participated in the pathology of diabetic hindlimb ischemia

The results showed that the Wnt3a level (Fig. 9A; Tukey's post hoc test (ANOVA), $F = 11.210$, $df = 3$, $p < 0.001$) was

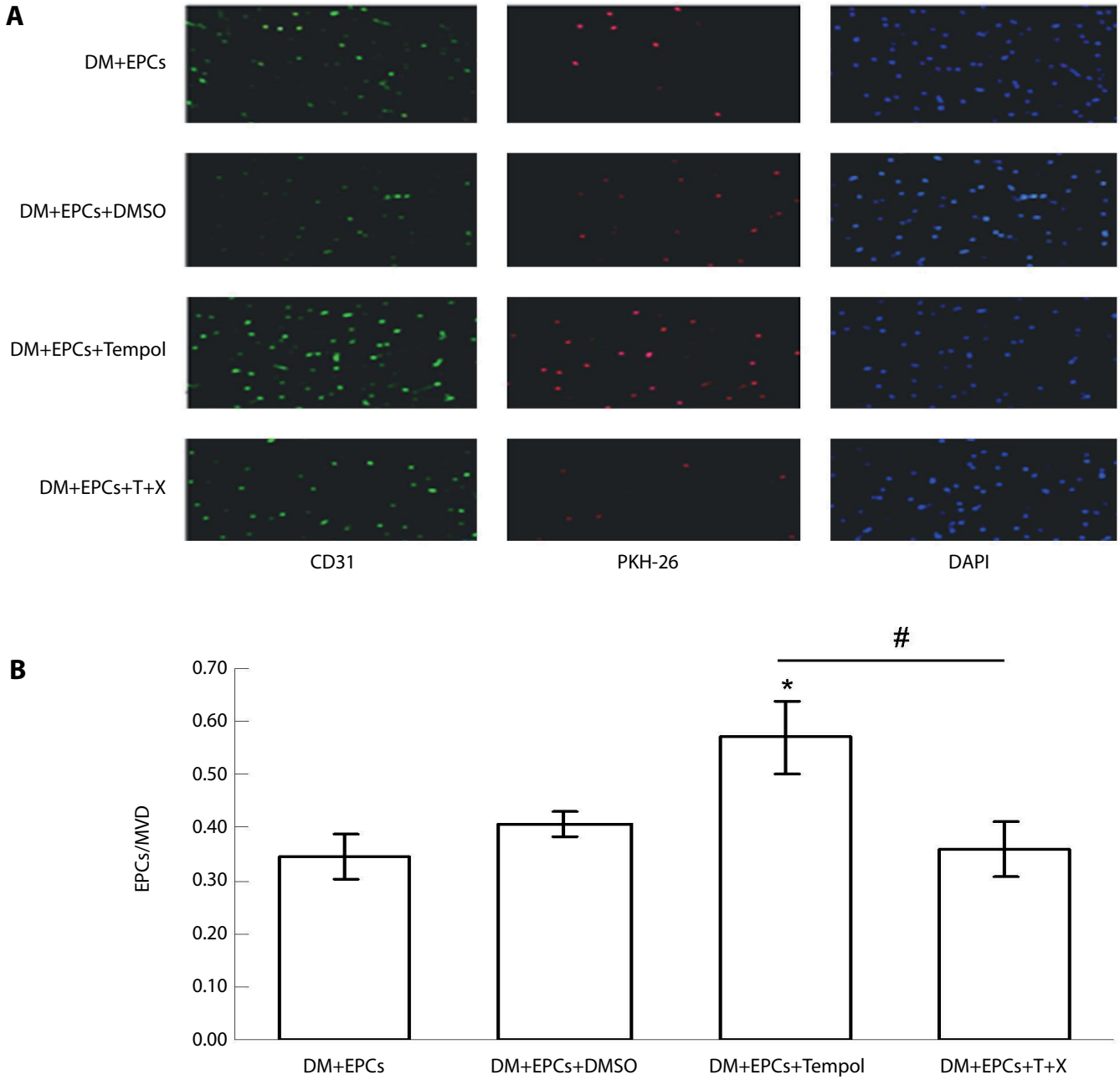

Fig. 7. Effects of Tempol and/or XAV on angiogenesis in gastrocnemius muscles of diabetic hindlimb ischemic rats. A. Counting the numbers of microvessels in the frozen section of gastrocnemius muscles of rat and the numbers of PKH-26-labeled endothelial progenitor cells (EPCs). The 4′,6-diamidino-2-phenylindole (DAPI) (blue fluorescence) represents the nuclei. The CD31 (green fluorescence) represents the new microvessels. The PKH-26-labeled red fluorescence or clusters represent the migration of EPCs to ischemic sites (×200, magnification); B. Statistical analysis for the ratio of number of labeled EPCs migrated to ischemic site to the number of new microvessels. The Tukey's post hoc test (analysis of variance (ANOVA)) was conducted to analyze the data. *$p < 0.05$ represented EPCs/MVD in DM+PECs+DMSO group compared to the DM+PECs+Tempol group. #$p < 0.05$ represented EPCs/MVD in DM+PECs+Tempol group compared to the DM+PECs+Tempol+XAV group.

DM – diabetes mellitus; DMSO – dimethylsulfoxide; MVD – microvascular density; T+X – tempol+XAV.

significantly increased, whereas Wnt5a (Fig. 9B; Tukey's post hoc test (ANOVA), $F = 18.293$, $df = 3$, $p < 0.001$) and Wnt7a (Fig. 9C; Tukey's post hoc test (ANOVA), $F = 13.149$, $df = 3$, $p < 0.001$) levels were remarkably decreased in diabetic hindlimb ischemic rats compared to those of healthy rats. Furthermore, the administration of Tempol (DM+EPCs+Tempol group) significantly enhanced Wnt5a levels compared to those in the DM+EPCs group (Fig. 9B; $p < 0.001$).

Discussion

Diabetic hindlimb ischemia presents with vascular inflammation, lipid deposition and atherosclerosis, which can lead to vascular stenosis and occlusion, followed by ischemia and hypoxia, ulceration, infection, and necrosis of the corresponding blood supply site.[19] In particular, atherosclerosis and acute arterial thrombosis are

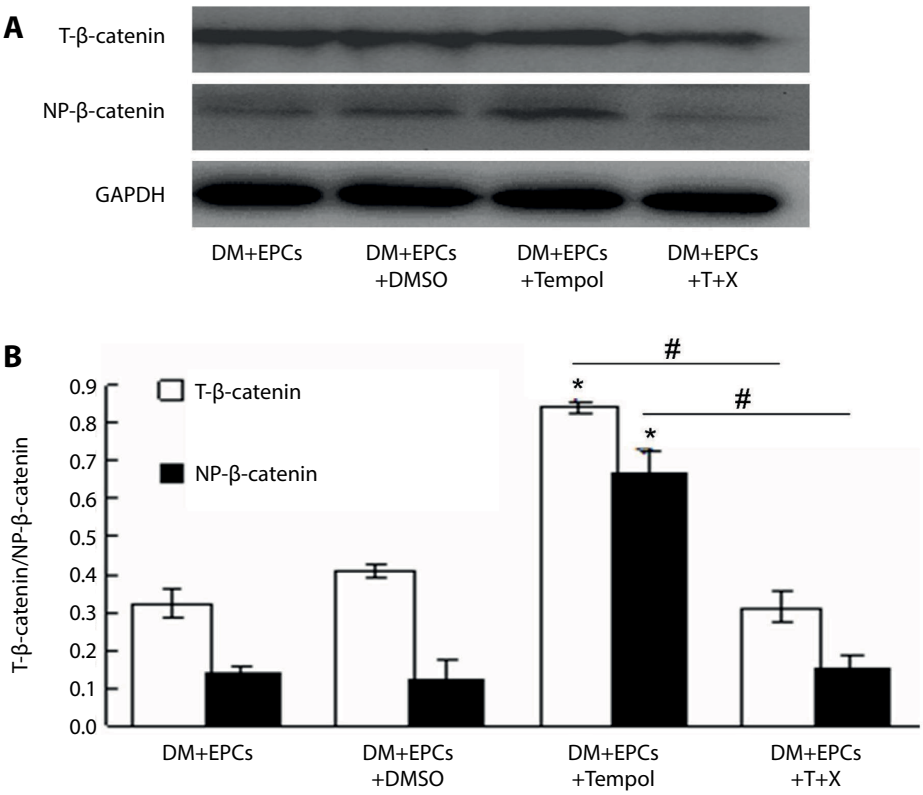

Fig. 8. Effects of Tempol and/or XAV on total β-catenin (T-β-catenin) and non-phospho-β-catenin (NP-β-catenin) expression in gastrocnemius muscles of diabetic hindlimb ischemic rats. A. Western blotting assay for the T-β-catenin and NP-β-catenin expression; B. Statistical analyses for the relative expression of T-β-catenin and β-catenin. Statistical analyses for the relative expression of NP-β-catenin. The Tukey's post hoc test (analysis of variance (ANOVA)) was conducted to analyze the data. * $p < 0.05$ for DM+EPCs+Tempol group compared to the DM+PECs group, for both T-β-catenin group and NP-β-catenin group. # $p < 0.05$ for DM+EPCs+Tempol group compared to the DM+PECs+Tempol+XAV group.

GAPDH – glyceraldehyde-3-phosphate dehydrogenase; DM – diabetes mellitus; EPCs – endothelial progenitor cells; DMSO – dimethylsulfoxide; T+X – tempol+XAV for both T-β-catenin and NP-β-catenin.

the primary causes of amputation in the elderly. Diabetic hindlimb ischemia is not only an ischemic disease, but is also often associated with neurotrophic disorders. However, diabetic hindlimb ischemia leads to vascular damage and ischemic disease, accompanied by an impairment of collateral angiogenesis. Diabetic hindlimb ischemia further aggravates ischemia, and any ulcer and necrotic lesions present are more difficult to repair. In this way, a vicious circle will be formed. If timely intervention and treatment are not carried out, the extent and scope of the lesions will be significantly increased.

There are many other factors affecting diabetic hindlimb ischemia, including hypertension, smoking, hyperlipidemia, overweight or obesity, lack of exercise, and poor mental health. In addition, local hemodynamic changes and structural changes of the arterial wall are important factors, creating a complex presentation.[20] Many of the abovementioned factors lead to diabetic limb ischemia and poor blood flow recovery. The same performance can be observed in this experiment. Through the establishment of a diabetic single limb ischemia model, the relative value of blood flow in the left femoral artery and its branches were observed 14 days and 28 days after the operation. The relative blood flow at the same time point in the diabetic group was relatively small, and laser Doppler imaging was found to create better comparisons, indicating that the blood flow recovery effect of diabetic rats after hindlimb ischemia was worse than that of healthy rats. Diabetic angiopathy is a slow and continuous pathological process.[10] After STZ destroys the B cells of islets of Langerhans, hyperglycemia emerges, indicating that the hindlimb model cannot be established immediately. Therefore, the experiment continued to feed high-fat food for 2 months, and then the follow-up experiment was carried out.

Asahara et al. were the first to isolate EPCs from the bone marrow.[21] The cell surface antigens were $CD34^+$, KDR^+, CD133, and VEGFR-2.[22,23] In recent years, it has been found that the mobilization of bone marrow-derived EPCs can repair damaged blood vessels. Therefore, EPCs play an important role in the regeneration of various vascular lesions.[24] Furthermore, EPCs play key roles in generating blood vessels in utero and during post-partum development. Because of the relatively large number of MNCs in the bone marrow of Sprague Dawley rats, simplicity of the method and low costs, bone marrow-derived EPCs were used in this experiment. Cells cultured for 7 days were infused with Dil labeled with acLDL and UEA-1 labeled with FITC. The double-positive cells of UEA-1 and acLDL were considered undifferentiated EPCs.

Diabetic angiopathy is closely related to the decrease of EPCs that can be mobilized and migrate to the ischemic site, as well as the dysfunction of vascular repair, neovascularization, angiogenesis, and arterial angiogenesis caused by this functional impairment. An excessive oxidative stress is an important mechanism of diabetic angiopathy.[10] In recent years, it has been found that the high-glucose environment affects the mitochondrial electron transport chain, continuously producing large amounts of ROS,[25,26] causing oxidative stress damage, and affecting the physiological growth and survival of EPCs. Finally,

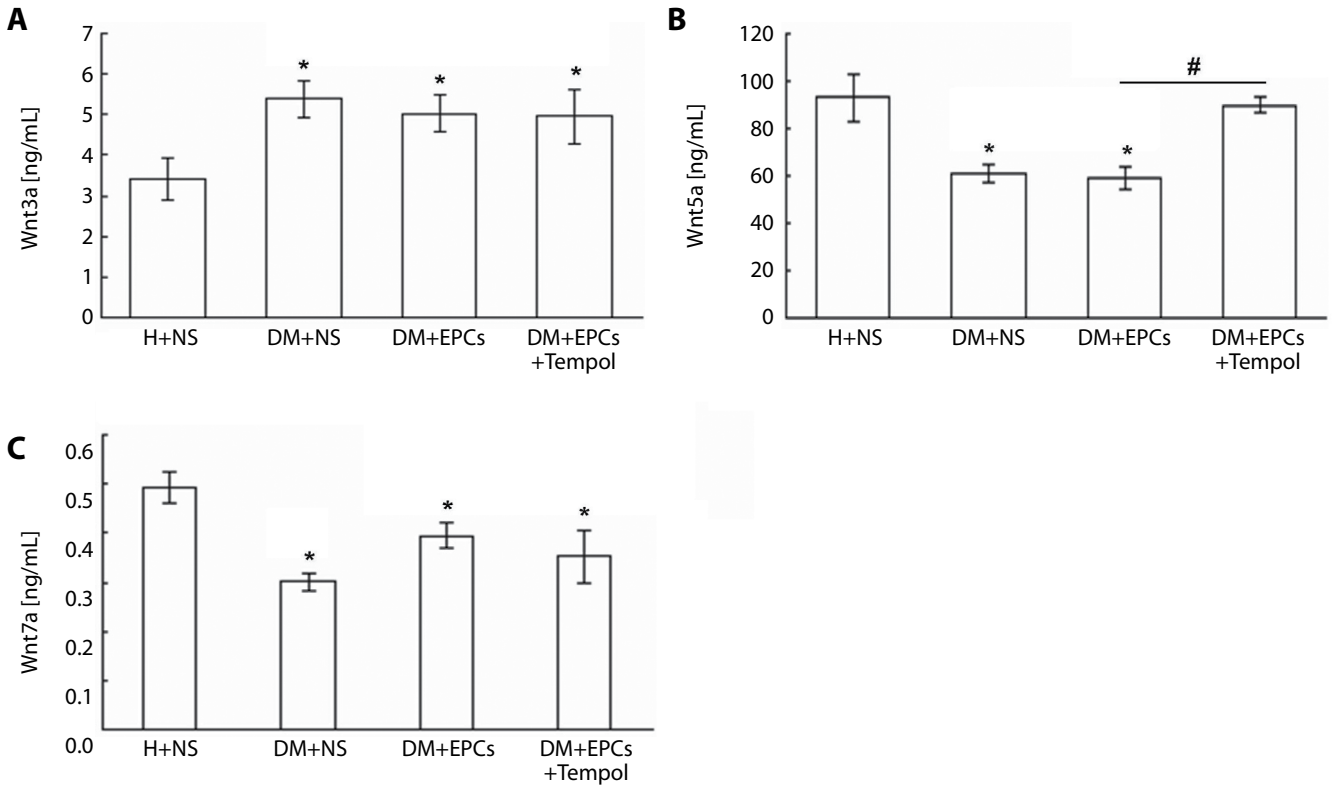

Fig. 9. Serum levels of Wnt3a, Wnt5a and Wnt7a in diabetic hindlimb ischemic rats of the H+NS, DM+NS, DM+EPCs, and DM+EPCs+Tempol groups. A. Levels of serum Wnt3a; B. Levels of serum Wnt5a; C. Levels of serum Wnt7a. The Tukey's post hoc test (analysis of variance (ANOVA)) was conducted to analyze the data. * $p < 0.05$ for DM+NS, or DM+EPCs, or DM+EPCs+Tempol group compared to the H+NS group. # $p < 0.05$ for DM+EPCs+Tempol group compared to the DM+PECs group.

H – healthy; DM – diabetes mellitus; NS – normal saline; EPCs – endothelial progenitor cells.

this causes abnormal metabolism and function of cells, or even apoptosis. In this study, there was no significant change in the amount of MDA in the diabetic serum before or after the operation, which was 2 times higher than that in the normal group. The serum SOD of healthy rats was significantly higher than that of diabetic rats. There were no significant changes in the MDA serum levels of diabetic and healthy rats before and after the operation. Moreover, there was no significant change in MDA and SOD in the serum of diabetic rats when they were injected with EPCs. Therefore, the effects of the operation and transplantation of EPCs on oxidative stress level in diabetic rats can be excluded. In addition, the rats were divided into 4 groups, including H+NS, DM+NS, DM+EPCs, and DM+EPCs+Tempol. The levels of ROS were measured by DCFH-DA and DHE fluorescence. The results showed that the relative fluorescence value in gastrocnemius muscle of diabetic rats was higher than that of normal rats. The relative fluorescence values of the DM+EPCs+Tempol and DM+EPCs groups were significantly lower than those of the DM+EPCs group. At the same time, EPCs were divided into 3 groups, including the blank group, high-glucose (HG) group and normal glucose (N) concentration group. After 120 h of culture, the level of ROS in EPCs was detected with flow cytometry. The results showed that ROS was also produced by EPCs under normal glucose concentration, but the amount was significantly lower than in the HG group. The abovementioned in vitro and in vivo experiments showed that high glucose increases the ROS products and the degree of oxidative stress. Compared with the normal rats, the level of oxidative stress in diabetic rats was increased, both locally and systemically, which was not related to cell transplantation and operation.

The level of ROS in vivo affects the number of transplanted cells migrating to the ischemic gastrocnemius muscle, the degree of neovascularization and the recovery of blood flow. In previous studies in vitro, it has been found that oxidative stress induced by high-glucose environment can damage the proliferation and tube-forming ability of EPCs[27] and increase the apoptosis.[28] The ability for migration and adhesion of stem cells transplanted intravenously is of extreme importance. It is possible that the transplantation of stem cells can promote the migration of self-mobilized EPCs to the ischemic site. Therefore, EPCs labeled with PKH-26 can be used to trace the migration of EPCs to the ischemic site. The rats included in our study were divided into 3 groups, namely the DM+NS group, DM+EPCs group and DM+EPCs+Tempol group. This experimental result first confirmed that oxidative

stress is the main factor affecting the therapeutic effect of EPC. In the following experiment, we would further explore Wnt/β-catenin signaling pathway-associated mechanism. The laser Doppler perfusion imaging (LDPI) was used to continuously monitor the blood flow recovery of ischemic hindlimbs, and immunofluorescence was used to detect the neovascularization of the ischemic gastrocnemius muscle and transplanted EPCs. The results showed that the MVD of the DM+EPCs+Tempol rats was higher than that of the DM+EPCs rats, and the relative number of PKH-26-labeled EPCs migrating to ischemic sites was higher, together with the ratio of labeled cells to MVD. The increase in the oxidative stress level in diabetes mellitus leads to the limitation of blood flow recovery in hindlimbs after ischemia. It may be that the oxidative stress level has been increased for a long time, resulting in a decreased number of EPCs adhering to the ischemic site, which in turn decreased the number of new blood vessels. However, this can be reversed by oxidative inhibitors which promote blood flow recovery. However, in this study, the blood flow of the nonsurgical hindlimb (the right hindlimb) was also detected using LDPI and it might have led to the false positive findings, which is a limitation of our study.

The β-catenin is a cytoskeleton protein and a crucial component of the Wnt/β-catenin signaling pathway.[29] The stability of β-catenin and its position in cells is regulated by a series of positive and negative regulatory factors. The abnormal regulation can lead to the inhibition or abnormal activation of Wnt signaling, resulting in a series of abnormalities in gene expression, cell adhesion and development, which are closely related to the cancer occurrence and metastases.[30,31] Our results indicated that Tempol treatment significantly increased T-β-catenin and NP-β-catenin expression in the EPC-transplanted diabetic hindlimb ischemic rats (DM+EPCs+Tempol) compared to those in the DM+EPCs group. However, XAV administration decreased T-β-catenin and NP-β-catenin expression in Tempol administration and in the EPC-transplanted diabetic hindlimb ischemic rats (DM+EPCs+Tempol+XAV) compared to the DM+EPCs+Tempol group. Furthermore, the administration of Tempol (DM+EPCs+Tempol group) significantly enhanced Wnt5a levels compared to those in the DM+EPCs group. All of these results suggest that the limited effect of blood flow recovery in diabetic hindlimb ischemia may be related to the regulation of Wnt/β-catenin signaling pathway.

Limitations

The most obvious limitation of this study is that the sample size for the STZ-induced diabetic rat model and hindlimb ischemia model was small. In future investigations, we aim to involve more animals for clarifying the associated findings.

Conclusions

High oxidative stress levels were noted in both EPCs in high-glucose environment and diabetic hindlimb ischemia, which can lead to limited blood flow recovery post-treatment. The high oxidative stress caused the inhibition of Wnt/β-catenin signaling, leading to limited blood flow recovery in the diabetic hindlimb ischemia. Meanwhile, Wnt5a participated in the EPC-mediated blood flow recovery. The findings of this study would benefit the basic study of a high-glucose environment in animal models of induced diabetic hindlimb ischemia. Moreover, the study might provide insight for the further clinical treatment of diabetic hindlimb ischemia.

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