Diazoxide accelerates wound healing by improving EPC function

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1. ABSTRACT

Endothelial cell dysfunction is the primary cause of microvascular complications in diabetes. Diazoxide enables beta cells to rest by reversibly suppressing glucose-induced insulin secretion by opening ATP-sensitive K^+ channels in the beta cells. This study investigated the role of diazoxide in wound healing in mice with streptozotocin (STZ)-induced diabetes and explored the possible mechanisms of its effect. Compared to the controls, mice with STZ-induced diabetes exhibited significantly impaired wound healing. Diazoxide treatment (30 mg/kg/d, intragastrically) for 28 days accelerated wound closure and stimulated angiogenesis in the diabetic mice. Circulating endothelial progenitor cells (EPCs) increased significantly in the diazoxide-treated diabetic mice. The adhesion, migration, and tube formation abilities of bone marrow (BM)-EPCs were impaired by diabetes, and these impairments were improved by diazoxide treatment. The expression of both p53 and TSP-1 increased in diabetic mice compared to that in the controls, and these increases were inhibited significantly by diazoxide treatment. In vitro, diazoxide treatment improved the impaired BM-EPC function and diminished the increased expression of p53 and TSP-1 in cultured BM-EPCs caused by high glucose levels. We conclude that diazoxide improved BM-EPC function in mice with STZ-induced diabetes, possibly via a p53- and TSP-1-dependent pathway.

2. INTRODUCTION

Diabetes mellitus (DM) is a serious, complex metabolic disorder and a growing health threat that affects approximately 4% of the population worldwide and its incidence is expected to increase to 5.4.% by 2025 (1, 2). Microvascular and macrovascular diseases are among the principal complications of DM, and are the major causes of death among diabetic patients (3-5). Chronic hyperglycemia invokes the onset of microvascular complications, such as diabetic foot ulcers, which increases the risk of amputation.

The activity of endothelial cells is the basis of endothelial integrity and function. Endothelial cell dysfunction initiates vascular diseases and is the primary cause of impaired wound healing (6, 7). Endothelial progenitor cells (EPCs) are a population of rare cells that
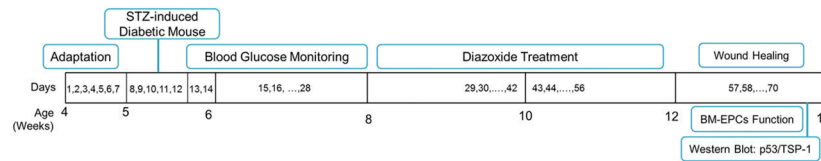
Figure 1. Illustration of the experimental protocols. Streptozotocin (STZ, 60 mg/kg/d × 5 d, *i.p.*) was administered to induce diabetes in C57BL/6 mice. Blood glucose was monitored, and diazoxide (30 mg/kg/d, *i.g.*) was given for 28 consecutive days. Wound healing, BM-EPC function and proteins were then measured.

circulate in the blood and possess the ability to differentiate into endothelial cells, i.e., the cells that compose the lining of blood vessels (8, 9). The process by which blood vessels are formed de novo from EPCs is known as vasculogenesis. In diabetes, the number of EPCs is decreased and their functions are impaired (10, 11).

Thrombospondin-1 (TSP-1) is a subunit of a disulfide-linked homotrimeric adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions, and is a natural inhibitor of neovascularization in healthy tissues (12). Down-regulation of TSP-1 positively modulates endothelial cell adhesion, motility, and growth. In DM and cardiovascular diseases, the transcription of TSP-1 mRNA is augmented in EPCs (13, 14). The p53 protein is crucial in multicellular organisms and functions as a tumor suppressor. The activation of p53 contributes to mitochondrial damage and dysfunction in diabetic platelets (15), and p53 can inhibit vasculogenesis via activation of TSP-1 in fibroblasts (16, 17).

Diazoxide is an ATP-dependent potassium channel activator and causes the local relaxation of smooth muscle by increasing membrane permeability to potassium ions. This change in potassium permeability switches off voltage-gated calcium ion channels, preventing calcium flux across the sarcolemma and activation of the contractile apparatus. In pancreatic beta cells, diazoxide inhibits the secretion of insulin via its action on potassium channels. Six months of treatment with low-dose diazoxide (100 mg at bedtime) for type 1 diabetes, and three months of treatment with diazoxide (5-7.5. mg/kg/d) for pediatric type 1 diabetes, have beneficial effects on the preservation of beta cell function and metabolic control (18, 19). However, little is known about the actions of diazoxide on EPCs and angiogenesis in diabetic conditions. Thus, in the present work, we hypothesized that diazoxide would accelerate wound healing by improving EPC function in mice with STZ-induced diabetes.

3. MATERIALS AND METHODS

3.1. Animals

Male C57BL/6 mice (4 weeks of age, 18-22 g) were purchased from Sino-British SIPPR/BK Lab Animal Ltd. (Shanghai, China). Mice were housed in controlled conditions (temperature: 23 ± 2°C; humidity: 70%;

lighting: 8:00-20:00) and received standard mouse chow and tap water *ad libitum*. All animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Bethesda, MD, USA).

3.2. Experimental protocols

Mice were separated randomly into the following three groups: control, streptozotocin (STZ)-induced diabetes, and diazoxide-treated STZ-induced diabetic mice. After one week of adaptation, mice were injected intraperitoneally daily for 5 days with 60 mg/kg STZ (Amresco, Solon, OH, USA) dissolved in 0.1. mM sodium citrate buffer (pH 4.5.) to induce diabetes. Control mice were administered an equal volume of vehicle. Glucose levels in whole blood obtained from the tail vein were monitored using a blood glucose monitoring system (Maochang, Taipei, China). On day 28, mice with blood glucose levels greater than 250 mg/dL were defined as STZ-induced diabetic mice and treated with diazoxide (30 mg/kg/d, intragastrically) or vehicle (0.5.% sodium carboxymethyl cellulose for an additional 28 days. Control mice received vehicle. On day 56, the mice were used for wound healing experiments or anesthetized for harvesting of the bone marrow (BM) to isolate BM-EPCs (Figure 1).

3.3. Assessments of wound healing and angiogenesis

After the induction of anesthesia with ketamine (100 mg/kg, intraperitoneally), the hair on the dorsum was removed and the area swabbed with betadine and 75% ethanol three times. A 6-mm circular wound was created by punch biopsy. The wounded area was covered with a bioclusive transparent dressing (Johnson & Johnson, Arlington, TX, USA) and its size measured every 2 days until day 12. The wound area was digitized and measurements calculated with Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

Angiogenesis evaluations were conducted with CD31 immunochemistry and hematoxylin (VWR Scientific, Radnor, PA, USA) staining. Briefly, the skin at the wounded area was collected on days 3, 6, and 9 after the punch biopsies. Samples were then fixed and embedded in paraffin. After deparaffinization and rehydration, the slides were placed in Tris-buffered saline (pH 7.5.) for 5 min followed by blocking of endogenous

peroxidases. After serum blocking for 30 min (Vector Laboratories, Burlingame, CA, USA), the samples were incubated with an anti-CD31 antibody (10 μg/mL, 1:50; BD Biosciences, San Jose, CA, USA) for 60 min at room temperature and subsequently further incubated with a biotinylated secondary antibody (anti-mouse IgG, Vectastain Elite ABC kit, Vector Laboratories) for 30 min and Vectastain Elite ABC Reagent (Vector Laboratories) for 30 min, and Nova Red (Vector Laboratories) for 15 min. The slides were then counterstained with hematoxylin for 10 sec followed by differentiation in 1% aqueous glacial acetic acid and rinsing in running tap water. Capillaries were recognized as CD31-positive tubular structures. The capillary density in the wound healing area was quantified on two slides from each mouse. For each slide, five high-power fields (200×) were examined. The results were summed and averaged as the capillaries per high-power field.

3.4. Determination of circulating EPCs

Approximately, 0.7 mL of blood was harvested from an anesthetized mouse and maintained in cool heparin anticoagulant tubes. After mixing with phosphate-buffered saline (1:1), 1 mL of gradient centrifugation liquid 1083 (Sigma-Aldrich, St. Louis, MO, USA) was added gently to the samples, which were then centrifuged at 1,400 g for 25 min. The cell layer was extracted and incubated with 1X erythrocyte lysing solution for 5 min. After washing and suspension, FITC-CD34 (eBioscience, San Diego, CA, USA) and PE-Flk-1 (BD Biosciences) antibodies were added to the samples for flow cytometry. $CD34^+Flk-1^+$ cells were recognized as circulating EPCs.

3.5. Evaluation of BM-EPC functions

BM-EPCs from C57BL/6 mice were isolated, cultured, and characterized as described previously [20]. The adhesion, migration, and tube formation abilities of BM-EPCs were determined to evaluate the functions of the cells. Briefly, seven days after isolating the BM-EPCs, the cells were harvested using 0.1.25% trypsin. After suspension, the cells (2×10^4/mL) were seeded into a 96-well plate. After 6 h of incubation, the cells were stained with Hoechst 33258 (1:1000, Sigma-Aldrich). The adhered cells in five randomly selected fields from each well were counted (200×).

A modified Boyden chamber assay was adopted to assess the migration abilities of the EPCs. Cells (1×10^5/mL) were suspended in serum-free endothelial growth medium and seeded on the upper chamber with a polycarbonate membrane (8 μm pores). Vascular endothelial growth factor (50 ng/mL) was added to the cell-free medium in the lower chamber of a 24-well Transwell plate (Corning, Lowell, MA, USA). After incubation for 24 h, the cells were fixed to the washed membrane and stained with Hoechst 33258 (10 μg/mL). The cells that migrated were counted in five randomly selected microscopic fields (200×) per sample.

The Matrigel tube formation assay was adopted to determine the angiogenic capacities of EPCs. Briefly, BM-EPCs (1×10^5/mL) were seeded in 96-well plates pre-coated with growth factor-induced Matrigel (BD Biosciences) (50 μL/well) and incubated for 6 h at 37°C. Neovascularization was evaluated by counting the numbers of tubes with a computer-assisted microscope (Leica Microsystems Inc., Wetzlar, Germany). Images of the tube morphologies were collected from five randomly selected microscopic fields (200×) per sample.

3.6. Cell apoptosis/proliferation assay and western blot analyses

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Beyotime Institute of Biotechnology, Hanmen, China) was used to evaluate EPC viability. Cell proliferation was determined using the CCK-8 assay (Dojindo Molecular Technology, Kumamoto, Japan). Flow cytometry (BD PharMigen, San Diego, CA, USA) was used to identify apoptotic cells as Annexin V-positive-propidium iodide negative. All experiments were performed in accordance with the manufacturer's instructions.

Proteins were extracted as described previously [21, 22]. Specifically, TSP-1, a protein secreted by BM-EPCs, was obtained by condensing the cell media. We used the bicinchoninic acid assay (Thermo Scientific, Rockford, IL, USA) to determine the concentration of protein in the condensed media. Protein samples of approximately 30 μg were run on 10% SDS-PAGE. The proteins were then electrotransferred to nitrocellulose membranes. The unbound sites in the membrane were blocked with 5% bovine serum albumin/phosphate-buffered saline Tween-20 for 1 h at 37°C. After washing, the blots were incubated for 2 h at 25°C with primary antibodies for p53 or TSP-1 (Cell Signaling Technology, Beverly, MA, USA) and subsequently with an IRDye 800CW-conjugated goat anti-rabbit secondary antibody (1:5,000; Li-Cor Bioscience, Lincoln, NE, USA) for 30 min at 25°C in the dark. Infrared fluorescence images were obtained using an Odyssey infrared imaging system (Li-Cor Bioscience, Lincoln, NE, USA). The bands were quantified with Quantity One software (Bio-Rad, Hercules, CA, USA).

3.7. Statistical analyses

Data are expressed as the means ± SEM. The results were analyzed with one-way ANOVA followed by Newman-Keuls multiple comparison tests using GraphPad Prism v5.0. software. Two-sided P values less than 0.0.5 were considered statistically significant.

4. RESULTS

4.1. Body weight and blood glucose changes in diabetic mice

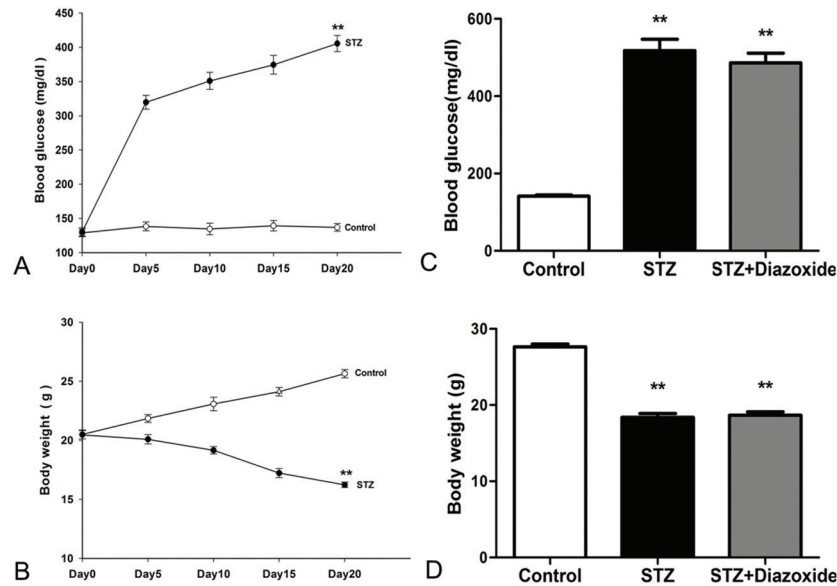Blood glucose increased significantly, and body weight decreased significantly, in the

Figure 2. Establishment of STZ-induced diabetic mice. Blood glucose (A) was significantly increased, and body weight (B) was decreased in the STZ-induced diabetic mice. The mice with blood glucose levels greater than 250 mg/dl were defined as diabetic. Twenty-eight days of diazoxide treatment did not alter the blood glucose levels (C) or body weights (D) of the STZ-induced diabetic mice. The data are expressed as the means ± the SEMs (**$P < 0.01$ vs control, n=11-13 per group).
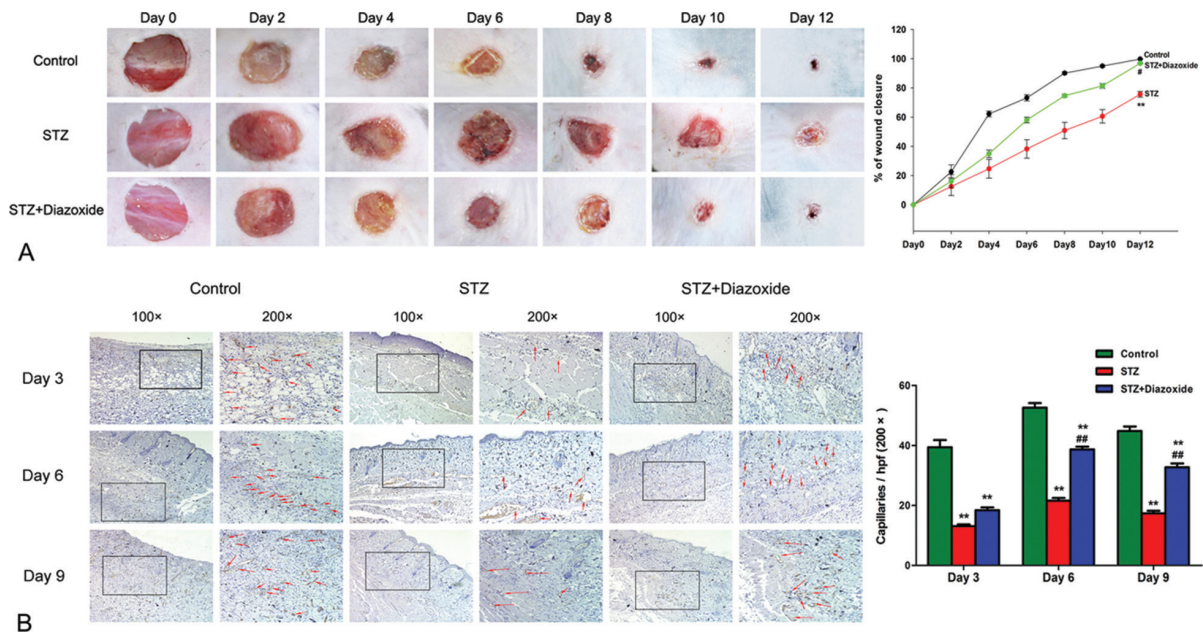

Figure 3. Diazoxide accelerated wound healing and stimulated angiogenesis in the STZ-induced diabetic mice (A) Six-millimeter-diameter wounds were created via punch biopsy, and the closure of the wound area was measured every 2 days until day 12. Diazoxide accelerated wound closure in the STZ-induced diabetic mice compared with the controls. (B) Typical photographs (200×) of CD31^{+} staining of the wounded areas on days 3, 6 and 9 and quantification of the CD31^{+} capillaries. The data are expressed as the means ± the SEMs (**$P < 0.01$ vs Control; #$P < 0.05$, ##$P < 0.01$ vs STZ; n=5-10 per group).

STZ-induced diabetic mice compared to that in the controls ($P < 0.01$; Figures 2A and 2B). Treatment with diazoxide for 28 days did not alter either blood glucose levels or the body weights of the mice with STZ-induced diabetes (Figures 2C and 2D).

4.2. Diazoxide accelerated wound closure and angiogenesis in diabetic mice

To assess the effects of diazoxide on wound healing in mice with STZ-induced diabetes, the wound closure percentage was calculated every other day until
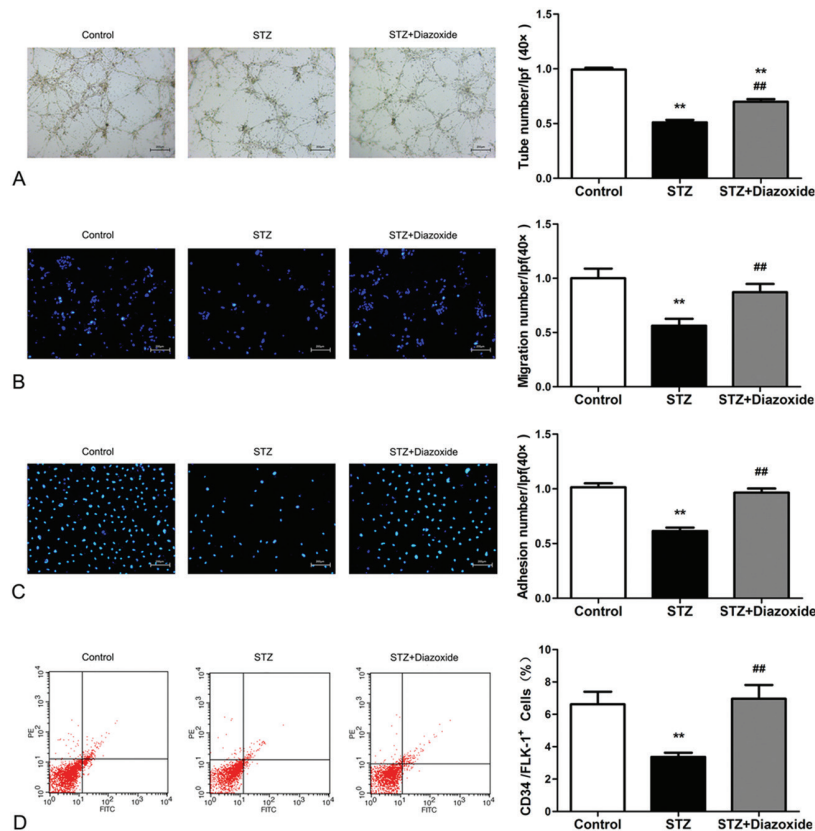
Figure 4. Diazoxide enhanced BM-EPC function in the STZ-induced diabetic mice. Diazoxide improved the tube formation (A), migration (B) and adhesion (C) abilities of the BM-EPCs and increased circulating EPC numbers (D) in the STZ-induced diabetic mice. Scale bar: 200 μm. The data are expressed as the means ± the SEMs (**$P < 0.01$ vs Control; ##$P < 0.01$ vs STZ group, n=5-6 per group).
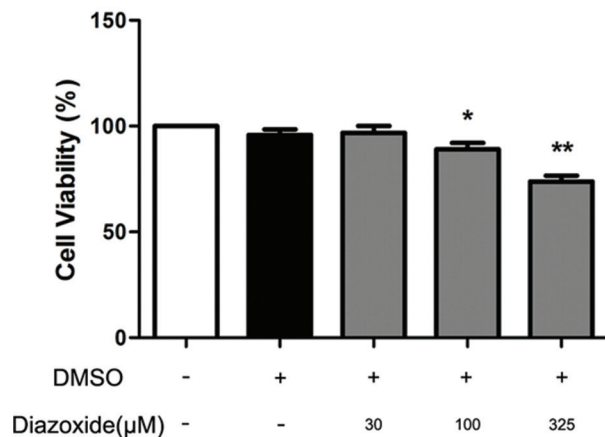

Figure 5. Optimization of the diazoxide concentration with MTT cell viability tests. Diazoxide at 30 μM did not affect cell viability. However, diazoxide concentrations of 100 and 325 μM significantly decreased BM-EPC viability. The data are expressed as the means ± the SEMs (*$P < 0.05$, **$P < 0.01$ vs Control, n=6 per group).

day 12 as (1-(average wound area on day X/average wound area on day 0)) × 100. Wound healing was significantly slowed in diabetic mice compared to that in the controls ($P < 0.01$). Diazoxide treatment significantly accelerated wound closure in the diabetic mice (Figure 3A).

The role of diazoxide in neovascularization was evaluated in the wounded skin of mice. Compared to the controls, the number of CD31^{+} capillaries in the diabetic mice was reduced significantly on days 3, 6, and 9 ($P < 0.01$; Figure 3B). Diazoxide treatment significantly augmented the capillary densities in diabetic mice on days 6 and 9 (Figure 3B).

4.3. Diazoxide improved BM-EPC function in diabetic mice

The adhesion, migration, and tube formation capacities of BM-EPCs were measured to assess cell functions. The functions of BM-EPCs from diabetic mice reduced significantly compared to that in controls. Treatment with diazoxide significantly improved the capacity of BM-EPCs for tube formation ($P < 0.01$; Figure 4A), migration ($P < 0.01$; Figure 4B), and adhesion ($P < 0.01$; Figure 4C) in the diabetic mice.

The numbers of CD34^{+} Flk-1^{+} cells in peripheral blood were measured to determine whether diazoxide stimulated BM-EPCs. The number of circulating EPCs in diabetic mice decreased significantly compared to that in the controls. Diazoxide treatment increased the number of circulating EPCs ($P < 0.01$; Figure 4D).
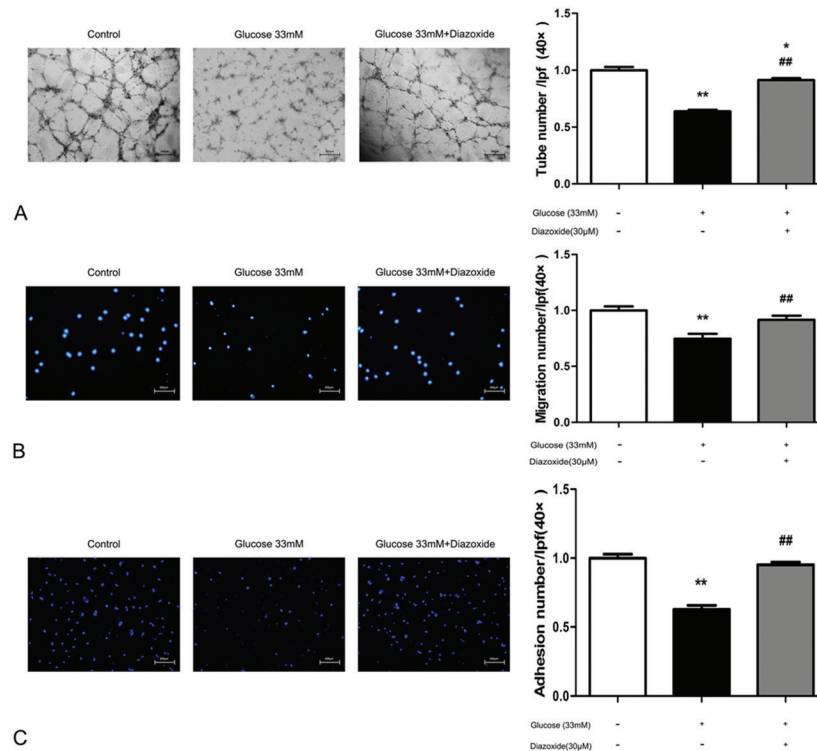
Figure 6. Diazoxide restored cultured BM-EPC functions that were impaired by high glucose levels *in vitro*. Diazoxide significantly restored the high glucose-impaired tube formation (A), migration (B) and adhesion (C) abilities of the cultured BM-EPCs *in vitro*. Scale bar: 200 μm. The data are expressed as the means ± the SEMs (*$P < 0.05$, **$P < 0.01$ *vs* Control; ##$P < 0.01$ *vs* High glucose, n=6 per group).

4.4. Diazoxide alleviated the glucose-induced dysfunction of BM-EPCs *in vitro*

To investigate whether the effects of diazoxide on BM-EPCs in STZ-induced diabetic mice were directly related to high glucose levels, 33 mM glucose was used to induce cell damage *in vitro*. This high concentration of glucose significantly decreased the adhesion, migration, and tube formation capacities of cultured BM-EPCs compared with the controls.

To optimize the concentration of diazoxide, the MTT assay was used to assess the viability of BM-EPCs following treatment with diazoxide alone. Thirty micromolar diazoxide did not affect cell viability (Figure 5). Moreover, this concentration of diazoxide decreased the damage to BM-EPC functions induced by 33 mM glucose ($P < 0.01$) when compared with untreated cells (Figure 6). Additionally, as illustrated in Figure 7, 33 mM glucose significantly increased BM-EPC apoptosis and inhibited BM-EPC proliferation, effects that were significantly prevented by diazoxide.

4.5. Diazoxide inhibited the expression of p53 and TSP-1 in BM-EPCs

The expression of p53 in BM-EPCs and levels of TSP-1 in the supernatants from BM-EPCs of the STZ-induced diabetic mice, were determined. Both p53 and TSP-1 levels increased in cells from diabetic mice compared to that in controls. Diazoxide treatment significantly inhibited p53 expression ($P < 0.01$; Figure 8A) and TSP-1 secretion ($P < 0.01$; Figure 8B) in the diabetic mice compared with the untreated mice, respectively. Similarly, high glucose concentrations increased the expression of p53 and TSP-1 by BM-EPCs *in vitro*, while diazoxide treatment prevented these changes (Figure 8C and 8D). Furthermore, treatment with pifithrin-α (a specific inhibitor of p53) significantly decreased the damage to BM-EPC functions caused by high glucose concentrations ($P < 0.01$) (Figure 9). Moreover, the inhibition of p53 by pifithrin-α downregulated the enhanced expression of TSP-1 induced by high glucose in the BM-EPCs that were cultured *in vitro* (Figure 10).

5. DISCUSSION

In the present study, we made the following observations: 1) diazoxide accelerated wound healing and stimulated angiogenesis in mice with STZ-induced diabetes; 2) diazoxide improved BM-EPC functions (including adhesion, migration, and tube formation) that were damaged owing to high glucose concentrations both *in vivo* and *in vitro*; and 3) the protective effects of diazoxide in BM-EPCs were possibly related to inhibition of the expression of p53 and TSP-1.
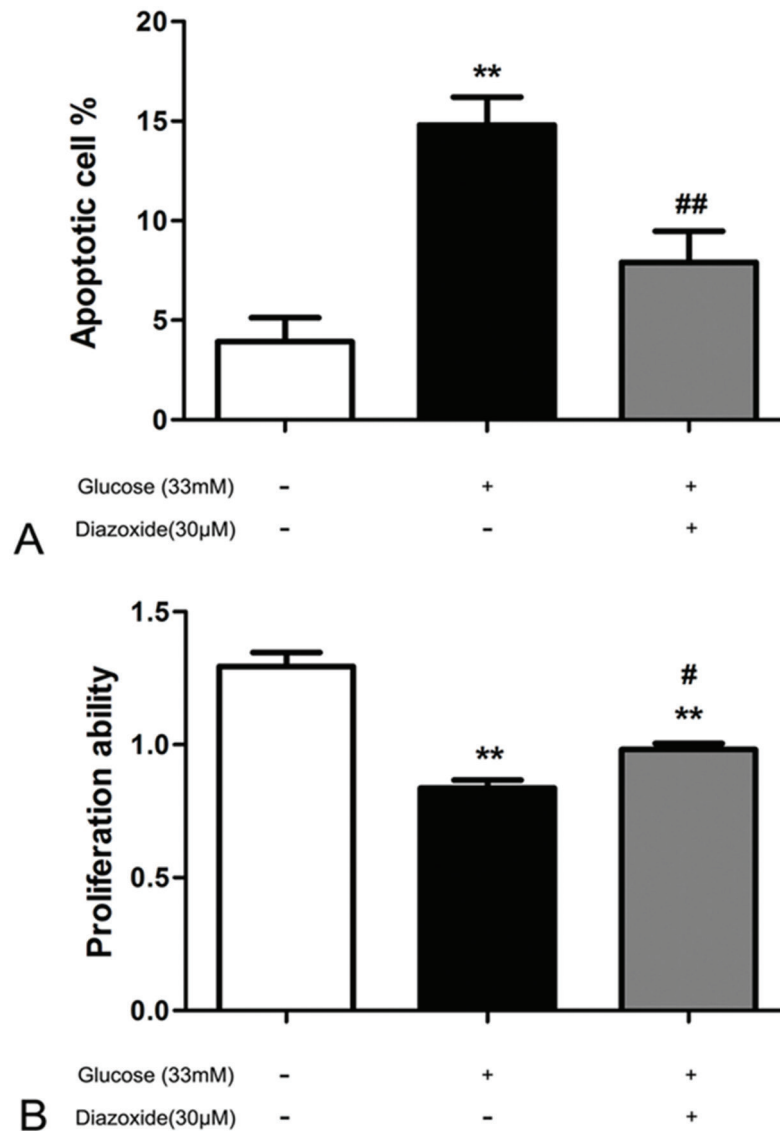
Figure 7. Diazoxide inhibited cell apoptosis and stimulated cell proliferation in the high glucose-treated BM-EPCs. (A) Diazoxide decreased the proportion of apoptotic cells in the BM-EPCs treated with high glucose by 46.6%. (B) Diazoxide increased cell proliferation in the BM-EPCs treated with high glucose. The data are expressed as the means ± the SEMs (**$P < 0.01$ *vs* Control; #$P < 0.05$, ##$P < 0.01$ *vs* high glucose, n=6 per group).

The opening of K_{ATP} channels appears to be beneficial in subjects with DM (23-26). In mouse pancreatic beta cells, diazoxide can counteract the inhibitory action of glucose on K^+ channels and enables beta cells to rest by reversibly suppressing glucose-induced insulin secretion by opening K_{ATP} channels in the beta cells (23, 27). These findings suggest that the beneficial role of diazoxide might be insulin-dependent. However, research has also revealed that long-term low-dose diazoxide treatment improves glycemic control without influencing beta cell function in subjects with newly diagnosed type 1 diabetes (18). This suggests that the protective effect of diazoxide in diabetes involves insulin-independent mechanisms.

DM is a group of metabolic diseases that are characterized by mechanisms involved in the pathogenesis of vascular complications, including excess cardiovascular diseases and impaired wound healing abilities (33). Accumulating evidence has demonstrated that endothelial cell functions play an important role in diabetes (28-32). BM-EPCs have been identified as circulating precursors of adult neo-vasculogenesis and vascular homeostasis. These cells reside in the bone marrow and are mobilized to the peripheral blood following many stimuli (34-36). BM-EPCs play a vital role in re-endothelialization in DM (10, 11, 37-42). Decreased numbers of circulating EPCs have been found in diabetic conditions, and these reductions are related to the worsening of peripheral vascular complications (10).
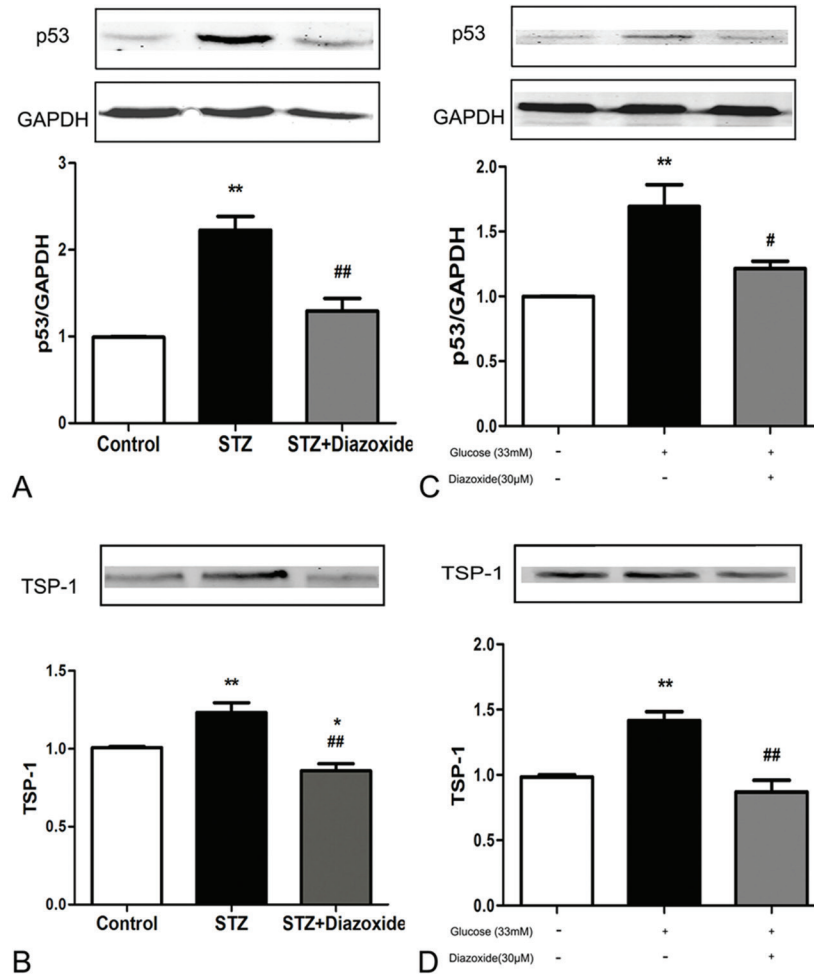
Figure 8. Diazoxide decreased the expressions of p53 and TSP-1 both *in vivo* and *in vitro*. Diazoxide significantly inhibited p53 expression (A) and reduced TSP-1 secretion (B) by the BM-EPCs from the STZ-induced diabetic mice. Diazoxide significantly inhibited high glucose-augmented p53 expression (C) and TSP-1 secretion (D) in the cultured BM-EPCs. The data are expressed as the means ± the SEMs (*$P < 0.05$, **$P < 0.01$ *vs* Control; #$P < 0.05$, ##$P < 0.01$ *vs* STZ or High glucose, n=6 per group).

Conversely, increases in circulating EPCs in patients with type 2 DM can restore endothelium-dependent vasomotion (39). Furthermore, Huang *et al.* demonstrated that diazoxide inhibited aortic endothelial cell apoptosis in diabetic rats *via* the activation of extracellular signal-regulated kinases (43). In the present work, we found that diazoxide accelerated wound healing, stimulated angiogenesis, and improved EPC function both *in vivo* and *in vitro*. These findings indicate that the beneficial effects of diazoxide on wound healing and angiogenesis in diabetes could be at least partially attributed to improved EPC function.

Tumor protein p53, also known as p53, is crucial for normal function in multicellular organisms. p53 regulates the cell cycle and functions as a tumor suppressor. The loss of p53 can lead to an increase in vascular endothelial growth factor levels, which subsequently promotes angiogenesis (44). Tumor suppressor p53 has been also demonstrated to mediate the accelerated onset of EPC senescence in diabetes (45). TSP-1 is a naturally occurring inhibitor of angiogenesis that causes normal endothelial cells to become unresponsive to a wide variety of inducers (46, 47). TSP-1 can limit vessel density in normal tissues and curtail tumor growth (48). Dameron *et al.* demonstrated that p53 stimulated the endogenous TSP-1 gene and positively regulated TSP-1 promoter sequences. This indicated that p53 could inhibit angiogenesis via the regulation of TSP-1 synthesis (17). Importantly, TSP-1 in BM-EPCs may mediate diabetes-induced delays in re-endothelialization following arterial injury (49). The work by Hong *et al.* suggested that the elevated expression of TSP-1 contributed to severe damage in diabetic rats (50).

Diazoxide is a potassium channel activator that protects cells from hypoxia by suppressing p53
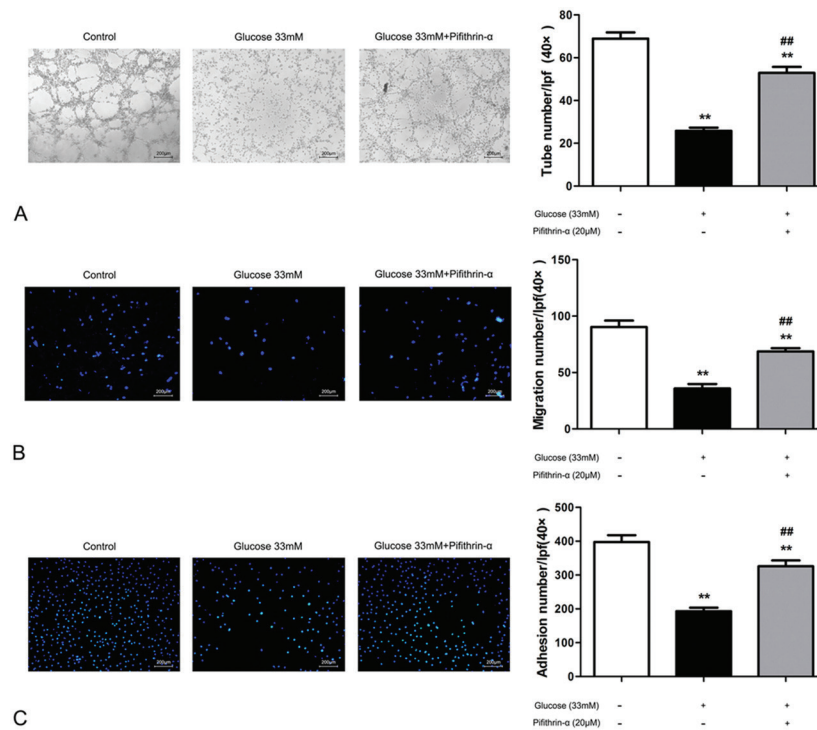
Figure 9. The P53 inhibitor pifithrin-α restored the BM-EPC function that was impaired by the high glucose *in vitro*. Pifithrin-α significantly restored the high glucose-impairments of tube formation (A), migration (B) and adhesion (C) abilities of the cultured BM-EPCs *in vitro*. Scale bar: 200 μm. The data are expressed as the means ± the SEMs (**P < 0.01 *vs* Control; ##P < 0.01 *vs* high glucose, n=6 per group).
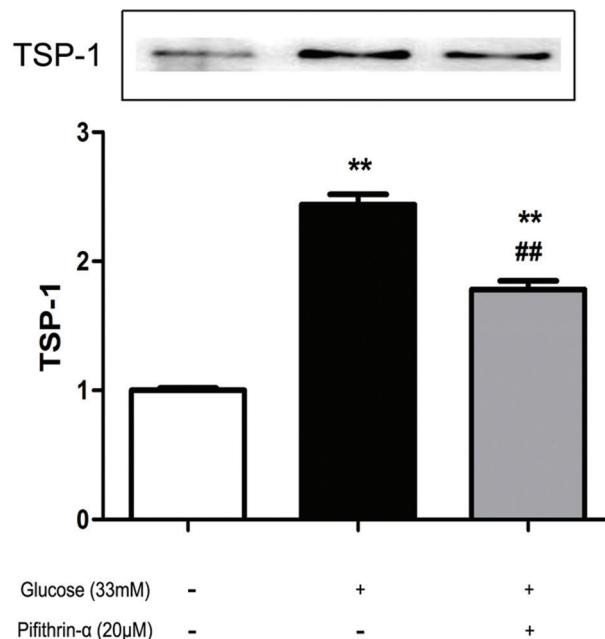

Figure 10. The P53 inhibitor pifithrin-α downregulated the TSP-1 expression that was enhanced by high glucose in the BM-EPCs that were cultured *in vitro*. The data are expressed as the means ± the SEMs (**P < 0.01 *vs* control; ##P < 0.01 *vs* high glucose, n=6 per group).

expression in hippocampal CA1 neurons in a manner dependent on the activation of ATP-sensitive potassium channels (51). Moreover, diazoxide treatment can protect cultured rat myocardium microvascular endothelial cells from apoptosis related to p53 (52). Our work revealed that the expression of p53 and TSP-1 was elevated in BM-EPCs from mice with STZ-induced diabetes. In addition, BM-EPCs isolated from control mice and cultured in high-glucose conditions exhibited increases in p53 and TSP-1 expression that could be inhibited by diazoxide. Thus, diazoxide was effective under both *in vivo* and *in vitro* conditions. These findings indicate that the beneficial effects of diazoxide on improving EPC function in diabetes might be related to the inhibition of p53 and TSP-1 expression.

Overall, the results of the current study demonstrate that diazoxide accelerated wound healing by improving the functions of BM-EPCs in STZ-induced diabetic mice. The mechanism of this effect appears to involve the p53/TSP-1 pathway, although further work is needed to confirm this conclusion. Importantly, our findings provide new evidence regarding the potential clinical use of diazoxide for the treatment of diabetic complications.

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6. ACKNOWLEDGEMENT

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