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Original Article

Beneficial effects of ginsenoside-Rg1 on ischemia-induced angiogenesis in diabetic mice

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Neovascularization and the formation of collateral vessels are often impaired in diabetes mellitus (DM) population compared with non-diabetics. Alterations in vascular endothelial growth factor (VEGF) signaling and endothelial nitric oxide synthase (eNOS) dysfunction have been confirmed to play a crucial role in impaired neovascularization in diabetic mice. Accumulating data have suggested that Rg1, a main component of *Panax ginseng*, has the ability to promote tubulogenesis of human umbilical vein endothelial cells (HUVECs) *in vitro*, and that the mechanism involves increased expression level of VEGF as well as increased eNOS activation. Thus, we speculated that Rg1 might also have therapeutic effects on the impairment of neovascularization in diabetic individuals. The aim of the present study was to investigate whether Rg1 could improve angiogenesis in ischemic hindlimb of diabetic mice *in vivo*. Our data demonstrated that Rg1 treatment resulted in improved angiogenesis in the diabetic ischemic hindlimb, and the potential mechanism might involve increased eNOS activation, upregulated VEGF expression, and inhibited apoptosis. Our results suggest that Rg1 may be used as a novel and useful adjunctive drug for the therapy of peripheral arterial disease in DM.

Keywords ginsenoside-Rg1; hindlimb ischemia; angiogenesis

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Introduction

Peripheral arterial disease (PAD) is common in patients with type 2 diabetes mellitus (DM) and predicts a poor prognosis [1,2]. Neovascularization and the formation of collateral vessels are often impaired in DM population compared with non-diabetics [3–5]. Alterations in vascular endothelial growth factor (VEGF) signaling and eNOS dysfunction have been confirmed to play a crucial role in impaired neovascularization in diabetic mice [4,6].

Ginseng is a highly valued medicinal plant in China, which has gained popularity in the West during the past decade [7,8]. A number of active components of *P. ginseng* have been isolated and characterized, and Rg1 is regarded as the main compound responsible for many pharmaceutical actions of ginseng [9]. Several *in vitro* studies have demonstrated that Rg1 has anti-diabetic activity [10–12]. Recently, accumulating data have suggested that Rg1 also has the ability to promote neovascularization into a polymer scaffold *in vivo* and the proliferation, chemoinvasion, and tubulogenesis of human umbilical vein endothelial cells (HUVECs) *in vitro* [13,14]. Similarly, as a functional ligand of glucocorticoid receptor, Rg1 has been shown to increase phosphorylation and the activities of phosphoinositide 3-kinase (PI3K), protein kinase B (Akt) and eNOS in HUVECs [15]. Moreover, Rg1 is also a potent stimulator of VEGF expression through PI3K/Akt-mediated pathway [16]. These studies indicated that Rg1 may possess anti-hyperglycemic property, as well as potent pro-angiogenic effect. Therefore, Rg1 might be used as a novel therapeutic agent for PAD in diabetic subjects. However, whether Rg1 could increase angiogenesis and blood reperfusion recovery in ischemic limb of streptozotocin (STZ)-DM mice is still unclear.

In the present study, we used a hindlimb ischemia model of STZ-DM mice to determine the effects of Rg1 on angiogenesis. Diabetes impaires endogenous neovascularization of ischemic tissues. Notably, Rg1 promotes angiogenesis and blood reperfusion recovery in both diabetic and non-diabetic control mice. Moreover, Rg1 treatment could also significantly activate eNOS phosphorylation, increase the expression level of VEGF protein, and inhibit cell apoptosis in ischemic tissues, suggesting that Rg1 therapy was effective in restoring ischemic limb reperfusion in a diabetic PAD model.

Materials and Methods

Induction of diabetes

The study complied with standards for the Care and Use of Laboratory Animals (Laboratory Animal Center of Nanjing Medical University). Male C57BL/6J mice ($n = 46$, 7 weeks old, 20−25 g) obtained from Laboratory Animal Center of Nanjing Medical University were employed in this study. Diabetes was induced as described previously with minor changes [17]. Briefly, mice were injected intraperitoneally with 50 mg/kg body weight of STZ (Sigma, St Louis, USA) dissolved in citrate buffer (pH 4.5) for 5 days. One week after the fifth injection, diabetes was confirmed by fasting glucose levels equal to or higher than 11.1 mM. Vein blood glucose concentrations were measured using glucose oxidase method (Glucose Analyzer; Roche, Mannheim, Germany). Animal tail vein blood glucose concentrations and fasting weight were measured 1 week after STZ administration as well as both 1 week and 4 weeks after Rg1 treatment.

Rg1 treatment protocol

Ginsenoside-Rg1 (purity >96%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). STZ-DM mice were randomly divided into the following two groups for intraperitoneal treatment: Rg1-treated group (10 mg/kg/day) and saline-treated group (saline, volume 0.1 ml). Nondiabetic mice were randomly divided into saline-treated control group (saline, volume 0.1 ml) and Rg1-treated control group (10 mg/kg/day). Rg1 treatment was started 8 days before surgical induction of unilateral limb ischemia and maintained after operation for 3 weeks.

Mouse model of unilateral hindlimb ischemia

Unilateral hindlimb ischemia was created in all groups as described previously [18]. Briefly, mice were intraperitoneally anesthetized with pentobarbital sodium (50 mg/kg) and the common femoral vein and femoral nerve were dissected free of the artery. Two ligations were performed in the right common femoral artery proximal to the origin of the profunda femoris artery. The common femoral artery was then transected between the ligation sites.

Laser Doppler perfusion imaging

After 3 weeks of operation, laser Doppler perfusion imaging (PeriScan PIM 3; Perimed, Stockholm, Sweden) was performed to assess the superficial blood flow of both feet, as previously described [19]. Relative perfusion data were expressed as the ratio of the ischemic (right) to normal (left) limb blood flow.

Immunofluorescence analysis for CD31 and VEGF expression

Ischemic limb muscles were harvested and cut into frozen tissue sections of 5 μm-thick at 21 days post-surgery. Sections were then blocked with 1% bovine serum albumin in phosphate-buffered saline (PBS). Mouse anti-CD31 antibody (1:100; Abcam, Cambridge, UK) or rabbit anti-VEGF antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, USA) was then applied followed by Alexa Fluor®488 donkey anti-mouse (1:250; Invitrogen, Eugene, USA) or Alexa Fluor®488 goat anti-rabbit (1:1000; Cell Signaling Technology, Danvers, USA) secondary antibody, respectively. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/ml; Sigma). Photographs were taken at 200× magnification. Image-Pro Plus software (Media Cybernetics, Rockville, USA) was used to determine the area of CD31- and DAPI-positive staining. Tissue vascular density was determined as the ratio between CD31-positive areas and DAPI-positive regions [20]. The VEGF fluorescence intensity was semiquantified by the ratio of the fluorescence intensity to per muscle fiber which was calculated using the Image-Pro Plus software [21,22].

VEGF assessment by enzyme-linked immunosorbent assay

Muscle lysates from ischemic adductor and semimembranous muscles at 21 days after operation were prepared and the VEGF contents were determined using VEGF enzyme-linked immunosorbent assay (ELISA) kits (Bio-Swamp, Shanghai, China). Briefly, 0.1 g muscle sample was homogenized in 2 ml of 1× PBS (pH = 7.4), and stored overnight at −20°C. After two freeze–thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 min at 5000 *g*. Samples were then assayed immediately according to the instructions of the manufacturer.

Detection of apoptotic cells in ischemic muscles

At 21 days after ligation, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay was performed to determine apoptotic activity in hindlimb ischemic tissues using *In Situ* Cell Death Detection Kit (Roche) according to the manufacturer's instructions. Apoptotic cells were identified by brown color in their nuclei. A total of 1000 cells were counted at 400× magnification, and the percentage of apoptotic cells per total number of cells was determined [23].

Western blot analysis of eNOS and phospho-eNOS

Gastrocnemius tissue samples were prepared at 21 days after operation and western blotting was performed as previously described [24]. Proteins were separated by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and

transferred to nitrocellulose membranes (Bio-Rad, Hercules, USA). After blocking with 5% skimmed milk, membranes were then incubated with primary antibodies including rabbit anti-eNOS (1:1000; Sigma), rabbit anti-phospho-eNOS (1:100; Santa Cruz Biotechnology), rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:4000; Cell Signaling Technology) at 4°C overnight, respectively. The membranes were then incubated with peroxidase-labeled secondary antibodies (1:2000; Cell Signaling Technology) at room temperature for 1 h. Results were expressed as density values normalized to GAPDH.

Statistical analysis

Statistical analysis was conducted using SPSS 13.0 (SPSS Inc., Chicago, IL). Data were expressed as mean ± SEM. Differences were determined using two-tailed Student's *t* test or analysis of variance, with a Newman–Keuls test to determine *post hoc* differences. The comparative incidence of toe necrosis was evaluated by χ^2 test. Differences were considered significant at a two-tailed *P* value of <0.05.

Results

Effects of Rg1 treatment on blood glucose and body weight

As shown in **Fig. 1(A)** , there was no significant difference of baseline fasting glucose levels between the saline-treated diabetic group and Rg1-treated diabetic group. Likewise, when detected both 1 week and 4 weeks after Rg1 treatment, the fasting glucose levels were still similar between the two groups. To further explore the time course effect of Rg1 treatment on glucose level, after 1 week of Rg1 or saline treatment, STZ-DM mice were fasted for 6 h (from 8 a.m. to 2 p.m.), and then received Rg1 (10 mg/kg) or saline(0.1 ml) intraperitoneal injection. **Figure 1(B)** shows that the blood glucose levels were lower at the time point of 2 h in Rg1-treated diabetic mice than that in saline-treated diabetic group. Interestingly, Rg1 administration for 4 weeks corrected the STZ-induced body weight loss [**Fig. 1(C)**].

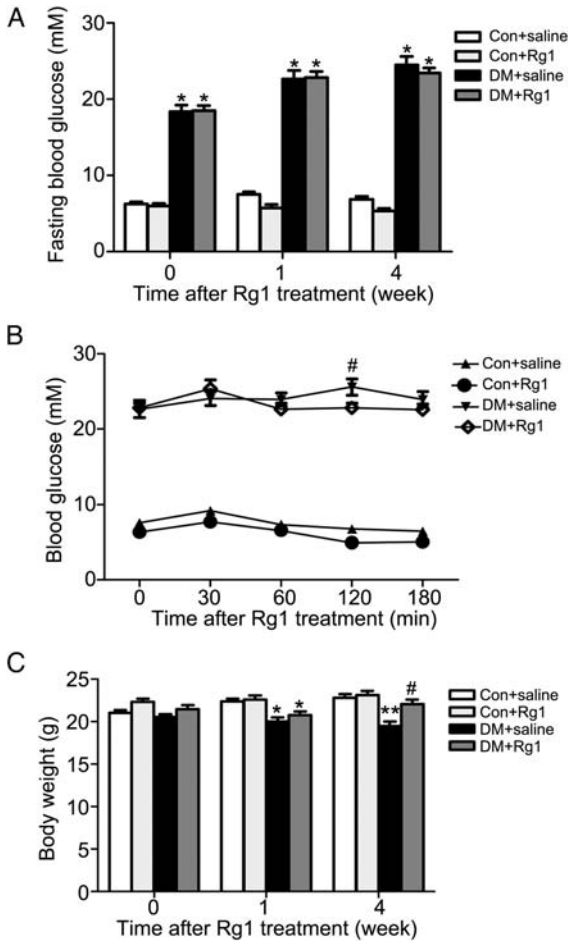

Figure 1 Effects of Rg1 treatment on blood glucose and body weight (A) Fasting blood glucose levels before and after Rg1 administration at a dose of 10 mg/kg/day. No significant difference was detected between saline-treated diabetic group and Rg1-treated diabetic group. (B) Time course effect of Rg1 on glucose levels of STZ-DM mice. Blood glucose levels were lower at the time point of 2 h in Rg1-treated diabetic mice than that in saline-treated diabetic group. (**C**) Effects of Rg1 on body weight changes. Rg1 administration for 4 weeks corrected the STZ-induced body weight loss. $n = 10-13$ per group, $^{*}P < 0.05$ versus control + saline group, $^{**}P < 0.01$ versus control + saline group, $^{\#}P < 0.05$ versus DM + saline group.

Rg1 treatment improves vascular recovery in a diabetic mouse model of hindlimb ischemia

We observed that toe necrosis occurred in 5 of 13 saline-treated diabetic mice (38.5%), 1 of 12 Rg1-treated diabetic mice (8.3%), none of 10 Rg1-treated non-diabetic mice, and 1 of 11 non-diabetic mice (9.1%) ($P < 0.05$; control + Rg1 versus DM + saline). The blood perfusion at the day 0 (preligation) in all groups were similar [**Supplementary Fig. S1(A,B)**]. When compared in saline-treated groups, restoration of foot perfusion was significantly lower in diabetic mice than controls. After Rg1 treatment, the ischemic foot perfusion was improved in both diabetic and non-diabetic control mice, as documented by laser Doppler [**Fig. 2(A,B)**]. Moreover, Rg1 treatment restored foot perfusion in diabetic mice to level similar to that in saline-treated non-diabetic controls.

Rg1 treatment increases vascular density in the ischemic tissues

The vascular density at the day 0 (preligation) in all groups was similar [**Supplementary Fig. S1(C,D)**]. **Figure 2(C)** shows quantitative immunohistochemical measurement of vascular density (CD31/DAPI ratio), confirming the beneficial effect of Rg1 on angiogenesis in STZ-DM mice. Capillary number ratio was decreased in diabetic mice when compared in saline-treated groups. Rg1 treatment increased vascular density in the ischemic tissues of both

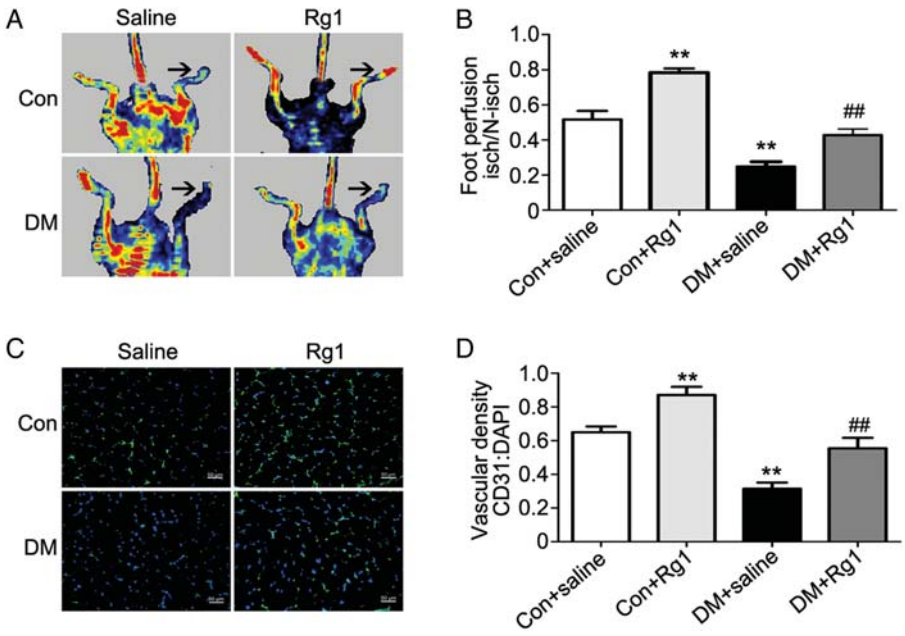

Figure 2 Rg1 treatment improves vascular recovery and angiogenesis in ischemic hind limbs of both diabetic and non-diabetic mice (A) Representative scans of blood flow in ischemic limbs measured by laser Doppler imaging analysis at day 21 following ligation of the right femoral artery. Arrows show ischemic hind limbs. (B) Quantification of the limb perfusion as a ratio of blood flow reperfusion in ischemic and non-ischemic feet ($n = 7$). (C) CD31 (endothelial stain; green), and DAPI (nuclear stain; blue) staining of ischemic gastrocnemius tissues on day 21 after operation. (D) Quantification of number of CD31-stained vessels of gastrocnemius muscle sections ($n = 10$). $^{**}P < 0.01$ versus control + saline group, $^{\#\#}P < 0.01$ versus DM + saline group.

diabetic and non-diabetic control mice. Vascular density in ischemic leg was increased by 1.1-fold in Rg1-treated diabetic mice compared with untreated diabetic animals [**Fig. 2(D)**].

Rg1 treatment decreases cell apoptosis *in vivo*

To determine whether improved limb reperfusion by Rg1 treatment was associated with increased cell survival in the ischemic tissues, the cell apoptosis was assessed by TUNEL assay. The percentage of TUNEL-positive cells markedly increased in the ischemic hindlimb of diabetic mice when compared in saline-treated mice. Rg1 treatment decreased cells apoptosis in the ischemic tissues of both diabetic and non-diabetic control groups [**Fig. 3(A,B)**].

Rg1 treatment enhances the expression of VEGF in ischemic tissue

To determine if Rg1 treatment increased VEGF levels, tissues were isolated to perform VEGF immunofluorescence analysis and VEGF ELISA. VEGF staining [**Fig. 3(C)**] shows that the expression of VEGF was decreased in ischemic tissue of diabetic mice when compared in saline-treated groups. However, Rg1 treatment enhanced the expression of VEGF protein in diabetic mice to the level similar to saline-treated control group. The fluorescence intensity of Rg1-treated diabetic group was 4.4-fold than that of untreated diabetic group [**Fig. 3(D)**]. Data from ELISA indicated VEGF levels were increased significantly after Rg1 treatment (control + saline versus control + Rg1, 32.5 ± 4.5 versus 44.2 ± 3.3 pg/ml; DM + saline versus DM + Rg1, 14.3 ± 2.0 versus 31.2 ± 3.4 pg/ml) [**Fig. 3(E)**].

Rg1 treatment rescues eNOS signaling

We examined whether Rg1 treatment altered eNOS protein expression or phosphorylation in ischemic gastrocnemius tissues. **Figure 4(A,B)** illustrates that the amount of total eNOS expression in ischemic tissues was similar among four groups. However, Rg1 treatment increased S1177-phosphorylated eNOS protein in ischemic limbs of both diabetic and non-diabetic control mice [**Fig. 4(A,C)**].

Discussion

The data of *in vivo* studies and clinical trials have revealed that the root of *P. ginseng* and the root of *Panax quinquefolius* have hypoglycemic effects [25,26]. Studies at the cellular level have indicated that Rg1, the main active component of ginseng, plays its anti-diabetic role via promoting glucose-stimulated insulin secretion, inhibiting hepatic glucose production, and inducing glucose transporter type 4 expression [10–12]. However, our time course analysis showed that although blood glucose levels were lower at the time point of 2 h in Rg1-treated diabetic mice

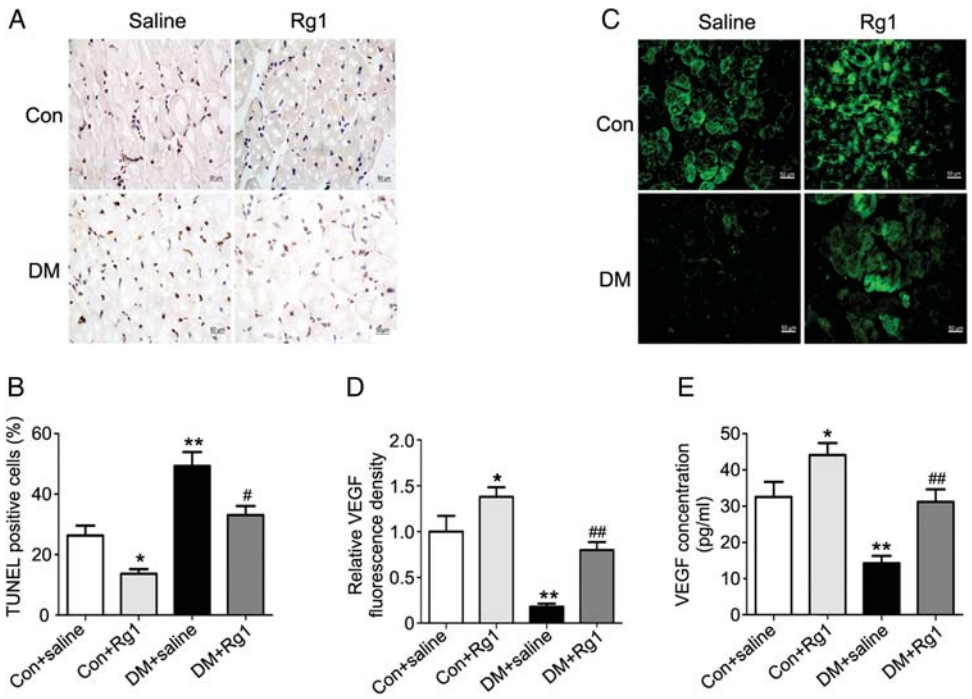

Figure 3 Rg1 treatment decreases cell apoptosis and increases VEGF expression in ischemic limb (A) Representative images of TUNEL-positive nuclei staining (brown) in ischemic limb. (B) Rg1 treatment decreased cells apoptosis in ischemic limbs of both diabetic and non-diabetic control mice ($n = 9$). (C) Representative images of VEGF expression in skeletal myocytes of ischemic limbs (immunofluorescent staining). (D) Semiquantitative analysis of VEGF expression of ischemic limbs by immunofluorescence analysis ($n = 10$). (E) Quantitative analysis of VEGF expression of ischemic limbs by ELISA ($n = 8$). $^{*}P < 0.05$ versus control + saline group, $^{**}P < 0.01$ versus control + saline group, $^{\#}P < 0.05$ versus DM + saline group, $^{\#\#}P < 0.01$ versus DM + saline group.

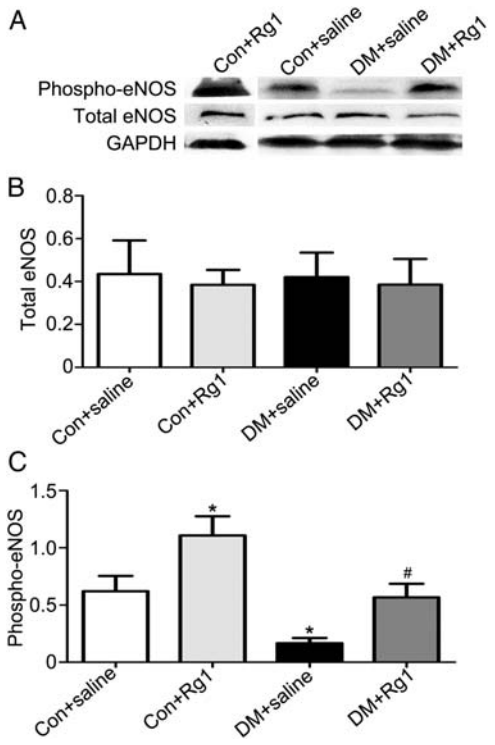

Figure 4 Analysis of eNOS expression in the calf muscles of mice (A) Western blot analysis of total eNOS and phospho-eNOS Ser1177 expression. GAPDH was used as an internal control. (B) Densitometry for total eNOS expression normalized to GAPDH. (C) Densitometry for phospho-eNOS expression normalized to GAPDH. $n = 6$ per group, $^{*}P < 0.05$ versus control + saline group, $^{\#}P < 0.05$ versus DM + saline group.

than that in saline-treated diabetic group, there was no significant change compared with 0 h in Rg1-treated diabetic group. Moreover, when detected both one week and four weeks after Rg1 treatment, the fasting glucose levels were still similar between saline-treated diabetic mice and Rg1-treated diabetic mice. Our present data are consistent with Yang et al's [27] study, which also found no prominent effect of Rg1 on lowering fasting blood glucose level in KK-Ay mice. Thus, it is hasty to conclude that Rg1 possesses anti-diabetic effect in STZ-DM mice. Further studies investigating the exact effects of Rg1 on blood glucose in STZ-DM mice are required.

Neovascularization and the formation of collateral vessels are often impaired in DM. VEGF, an endothelial cell-specific mitogen triggered by hypoxia, has been proved to be a critical growth factor in therapeutic angiogenesis [28–31]. Reduced expression of VEGF in diabetic mice impairs endogenous neovascularization in ischemic tissues, while application of adenoviral-VEGF gene transfer can overcome the impaired neovascularization [4]. It can be inferred that the agent with activity to increase VEGF expression may treat the impairment of neovascularization in diabetic individuals. Previous studies have showed that Rg1 is a potent stimulator of VEGF expression and can induce angiogenesis [16,32]. Consistent with these results, our study also showed that Rg1 treatment significantly improved blood perfusion, and increased capillary density

and VEGF expression in ischemic tissues in both diabetic and non-diabetic mice.

Our present study also demonstrated that Rg1 improved the activity of eNOS in ischemic hind limbs. Previous study found that activation of eNOS is impaired in diabetic bone marrow, resulting in the deficiency of endothelial progenitor cells mobilization [33]. In addition, observations indicated that eNOS-derived NO is requisite for acute adaptation to severe ischemia to prevent tissue necrosis and for sustained arteriogenesis [34]. Leung *et al.* [15] have shown that Rg1 can increase phosphorylation and the activities of PI3K, Akt, and eNOS, leading to increased NO production in HUVECs. These studies, taken together with our data, show a link between diabetes, decreased activation of eNOS, and impaired vascularization.

Many studies have revealed that the fundamental cellular process of apoptosis contributes to myocyte loss in different forms of ischemic injury [23,31,35]. The ischemia occurring in diabetic condition dramatically increases endothelial cell death [36]. Thus, uncontrolled apoptosis may prevent the attempt to build up functional collateralization. Consistently, we found that apoptotic cells were prominent in STZ-DM mice. Our data manifested that Rg1 administration might have the capability of reducing apoptosis in both diabetic and non-diabetic mice. Previous study found that Rg1 can promote functional neovascularization through PI3K/Akt pathway, which has been proved to play an important role in the regulation of cell apoptosis [16,37]. Further studies are needed to investigate whether the activation of PI3K/Akt pathway is involved in the decreased cell apoptosis in ischemic tissue in Rg1-treated diabetic mice.

The limitations of the present study mainly contain the following two points. First, although STZ-induced diabetic mice is appropriate to explore the effects of Rg1 treatment on PAD, mice maintained on a high-fat diet may better model the clinical conditions for PAD. Second, we just chose only one dose of Rg1, without further dose-related studies. The exact effects of different treatment doses of Rg1 on ischemia-induced angiogenesis in STZ-DM mice remain unknown. Further studies are needed to answer these questions.

In summary, Rg1 treatment had beneficial effect on ischemia-induced angiogenesis in diabetic PAD model. Moreover, Rg1 treatment could also significantly activate eNOS phosphorylation, increase the expression of VEGF protein, and inhibit cell apoptosis in ischemic tissues. Our results suggest that Rg1 may be used as a novel useful adjunctive drug for the therapy of PAD in DM.

Supplementary Data

Supplementary data are available at *ABBS* online.

Funding

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