New Therapeutic Approach for Impaired Arteriogenesis in Diabetic Mouse Hindlimb Ischemia

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Background The combined treatment of sustained-release basic fibroblast growth factor (Sr-bFGF) and a 5-hydroxytryptamine_{2A} blocker, sarpogrelate, was evaluated to see whether it reversed the impaired collateral circulation in diabetic (DM) mouse hindlimb ischemia.

Method and Results Diabetic and normal mice with ischemic hindlimb were randomly assigned to 1 of 5 experimental groups (no treatment, sarpogrelate 50 mg·kg⁻¹·day⁻¹, 20 µg or 50 µg Sr-bFGF and a combined treatment of 20 µg Sr-bFGF and sarpogrelate), and treated for 4 weeks. Tissue blood perfusion (TBP), vascular density (angiogenesis) and the number of mature vessels (arteriogenesis) were checked by the use of standard methods. Although angiogenesis was comparable (161±14 vs 154±12 vessels/mm²), the laser Doppler perfusion image index (LDPII) (0.43±0.11 (SD) vs 0.63±0.08, p<0.05) and arteriogenesis (8±3 vs 12±4 vessels/mm², p<0.05) were significantly lower in DM mice than those in normal mice. The dose of Sr-bFGF for the sufficient number of mature vessels (≥45 vessels/mm²) and LDPII (≥0.9) was 20 µg for the normal mice, and 50 µg for the DM mice, which was reduced with the aid of sarpogrelate.

Conclusions A combined therapy of Sr-bFGF and sarpogrelate is effective for neovascularization to reverse the impaired arteriogenesis and TBP in DM mice. (Circ J 2008; 72: 633–640)

Key Words: Diabetic mouse; Growth factors; Hindlimb ischemia; Neovascularization; Serotonin blocker

Peripheral arterial disease (PAD) is one of the critical problems in the cardiovascular field and the prevalence of this disease is now increasing along with the recent rise in the proportion of a geriatric population around the world. Furthermore, PAD with diabetes mellitus (DM) is a major concern as PAD patients with DM have less favorable outcomes compared with those without DM, including a 3- to 4-fold increase in mortality.^{1–4}

Therapeutic angiogenesis with growth factors constitutes a potential alternative approach for the patients with PAD to improve perfusion of ischemic tissues.^{5,6} The establishment of a stable and functional blood vessel network, however, is a complex process that requires several angiogenic factors aimed at stimulating vessel sprouting and remodeling of the pre-existent collateral network.^{7–9} There is strong evidence that endothelial-dependent vasodilatation and collateral development are impaired in DM.^{10–13} Among growth factors, basic fibroblast growth factor (bFGF) is a potent mitogen for growth of both endothelial and vascular smooth muscle cells,^{14,15} and it can also develop functional collaterals in the rabbit ischemic hindlimb model.¹⁶ However,

whether bFGF treatment is effective for the treatment of hindlimb ischemia with DM is not yet established. As bFGF is rapidly degraded in plasma, it is obvious that its delivery system, which is used in therapy, is one of the most important factors to achieve a significant collateral circulation. To overcome this problem, we have developed a sustained-release bFGF incorporated in gelatin hydrogel microspheres for use in various animal models against cardiovascular disease.^{17,18}

The plasma level of 5-hydroxytryptamine (5HT) in patients with DM is elevated.¹⁹ As a result, dilation of collaterals could be compromised by excessive vasoconstrictive reactivity to 5HT, mediated by 5HT_{2A} receptors.²⁰ In contrast, sarpogrelate (a selective 5HT_{2A} blocker) suppresses blood vessel constriction and can improve distal perfusion in an animal model of hindlimb ischemia,^{21,22} and it is also used for patients with chronic PAD.²³

The purpose of the present study was to evaluate: (1) the expression of 5HT_{2A} receptors and the intrinsic pattern of neovascularization in ischemic hindlimb of streptozotocin (STZ) induced DM mice; and (2) the treatment effect of sustained-release bFGF or sarpogrelate alone, or in combination.

Methods

Experimental Animals

Six-week-old male C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). The Kyoto University Animal Experiment Committee approved the experiment protocol. Animals were cared for in compliance with the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, Commission on Life Sciences, National

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Table 1 Primer Sequences Used for Real Time-PCR Receptors Expression

Primer	Sequence
<i>5HT_{2A} receptors</i>	
Forward primer	TAGCCGCTTCAACTCCAGAACC
Reverse primer	AAGACCTTCGAATCATCCTGTAGCC
<i>GAPDH</i>	
Forward primer	AAATGGTGAAGGTCGGTGTG
Reverse primer	TGAAGGGGTCGTTGATGG

5HT, 5-hydroxytryptamine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Research Council. To generate a diabetic model, 6-week-old male C57BL/6 mice were randomized to receive an intraperitoneal injection of STZ (150 mg·kg⁻¹·dose⁻¹) dissolved in 1 mol/L sodium citrate buffer, pH 4.5 or vehicle. Two weeks after receiving the injection, the serum glucose level was measured by using an Accucheck II Advantage Glucometer (Roche Diagnostics, Basel, Switzerland). The mice with a serum glucose level less than 200 mg/dl received an additional half-dose of STZ and finally mice with a serum glucose level less than 200 mg/dl were excluded from the study.²⁴

Mouse Ischemic Hindlimb Model With Various Treatment Profiles

Hindlimb ischemia was created in the 8-week-old male wild-type and DM mice. Briefly, after the mice were anesthetized by an intraperitoneal sodium pentobarbital (60 mg/kg) injection, the right groin area was shaved and prepared with povidone-iodine. The entire right saphenous artery and vein, right external iliac artery and vein, and deep femoral and circumflex arteries and veins were ligated, cut, and excised to obtain a mouse model of severe hindlimb ischemia as described previously.¹⁷

After surgery, the mice with hindlimb ischemia were randomly assigned to 1 of 5 experimental groups and treated for 4 weeks: group N, no treatment; group S, treated with a supplemented diet containing sarpogrelate (50 mg·kg⁻¹·day⁻¹); group F20, single intramuscular injection of a sustained-release form of bFGF microspheres (20 µg); group F50, single intramuscular injection of a sustained-release form of bFGF microspheres (50 µg); group F20+S, combined treatment of a sustained-release bFGF (20 µg) and sarpogrelate.

An intraperitoneal injection of pentobarbital (60 mg/kg), subjected to the measurements described below, was provided and then the mice were killed with an overdose of pentobarbital so that a histological examination could take place.

Expression and Quantification of the 5HT_{2A} Receptor by Real Time (RT)-PCR

For the RT-PCR analysis, wild-type and DM mice were treated as mentioned above and killed on days 3 and 7 after the induction of ischemia.

The ischemic (right thigh muscles) and the non-ischemic (left thigh muscles) tissues were excised and snap-frozen in liquid nitrogen. Total muscle RNA was extracted by using an RNeasy[®] fibrous tissue mini RNA isolation kit (Qiagen, Mainz, Germany), according to the manufacturer's recommendations. Briefly, total RNA was eluted from the RNeasy[®] mini columns with 30 µl of RNase-free water and centrifugation with 10,000 rpm for 1 min. Quantitative RT-PCR was performed by using a QuantiTect[®] SYBR Green RT-PCR kit (Qiagen) in a 50 µl reaction volume. The final reac-

Fig 1. Expression of 5-hydroxytryptamine (5HT)_{2A} receptors in non-ischemic (open bars) and ischemic (closed bars) tissues of the thigh muscle in normal and diabetic (DM) mice on days 3 and 7 after the induction of hindlimb ischemia. *p<0.05 vs non-ischemic tissue; †p<0.05 vs normals.

tion contained a 1x QuantiTect[®] SYBR Green RT-PCR Master Mix and a 0.5 µl QuantiTect[®] RT Mix. Primers used for the PCR are listed in Table 1. The final concentration of the forward and reverse primer was 500 nmol. The thermal cycle parameters for reverse transcription and amplification were as follows: 50°C, 30 min (Hold); again 95°C, 15 min (Hold); 94°C, 15 s (Melt); 60°C, 30 s (Anneal); 72°C, 1 min (Extend); 40 cycles for each. Compensation between the PCR plate wells was conducted by using the Well Factor Plate Method using a Well Factor Solution (Bio-Rad, Hercules, CA, USA), according to the iCycler iQ[®] manual. The mRNA value was measured with the threshold of 3,700 for 5HT_{2A} receptors and 1,130 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by using the iCycler iQ[®] (Bio-Rad) Real-Time PCR Detection System, Software Version 3.0A. A standard curve was depicted by plotting the mean C_T values, which was measured by the total RNA (0.2–200 ng). The slope and y-intercept were then calculated. Each amount of mRNA was calculated according to the formula:

$$\text{mRNA amount (ng as total RNA)} = 10^{(C_T - b)/m}$$

where b is the y-intercept of the standard curve line and m is the slope of the standard curve line.

Finally, the 5HT_{2A} receptor mRNA expressions were normalized to GAPDH mRNA.

Monitoring of Hindlimb Blood Perfusion

Hindlimb blood perfusion was scanned by using a laser Doppler perfusion image (LDPI) analyzer (Moor Instruments, Devon, UK) on the first day of treatment and then every 7 days until the end of the study period. To eliminate the influence of the surgical procedure, the average blood perfusion of the bilateral feet was evaluated. To minimize influential variables including ambient light and temperature, perfusion was expressed as the ratio of the blood perfusion in the right (ischemic) limb to that in the left (non-ischemic) limb of the same mouse, that is, in terms of the LDPI index (LDPII).^{17,25} Mice with a LDPII less than 0.2 on the first day of the experiment were defined as having hindlimb ischemia.

Fig 2. Left panels: Time-course of ischemic/non-ischemic blood perfusion ratio in normal and diabetic (DM) mice with various treatments. Right panels: ischemic/non-ischemic blood perfusion ratio in normal and DM mice at 4 weeks after various treatments. Group N, no treatment; group S, treatment with sarpogrelate; group F20, treatment with sustained-release 20 μ g basic fibroblast growth factor (bFGF); group F50, treatment with sustained-release 50 μ g bFGF; group F20+S, combined treatment with sustained-release 20 μ g bFGF and sarpogrelate. *p<0.05 vs group N; †p<0.05 vs group S; ‡p<0.05 vs group F20. LDPII, laser Doppler perfusion image index; wk, week.

Immunohistological Analysis

Four weeks after the initiation of treatment, the mice were euthanized and perfusion-fixed with 4% paraformaldehyde. The ischemic calf muscles were embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) and frozen at -80°C . Cryostat sections (5- μm thick) of the tissues were stained with rabbit polyclonal anti-human von Willebrand factor antibody (vWF; Dako Japan, Kyoto, Japan) or mouse monoclonal anti-human smooth muscle actin (SMA; Sigma-Aldrich Japan K.K., Tokyo, Japan) antibody. As the negative control, rabbit normal immunoglobulin fraction (Dako Japan, Kyoto, Japan) was used to show antibody specificity. From each mouse, 8 random fields on 2 different sections (approximately 3 mm apart) were photographed with a digital camera (Olympus, Tokyo, Japan). The numbers of vWF (endothelial marker)-positive, SMA (vascular smooth muscle marker)-positive vessels and muscle fibers were counted manually in a blind fashion. Vascular density (angiogenesis) was determined by the counted number of vWF-positive vessels and mature vessel density (arteriogenesis) was determined by the counted number of SMA positive vessels.¹⁷

Statistical Analysis

Results are expressed as the mean $\pm$ SD. Differences among groups were determined by using one-way analysis of variance followed by multiple comparisons using the Bonferroni/Dunn's test. All statistical analyses were performed by using StatviewTM software (Abacus, MI, USA). A p value <0.05 was considered to be significant.

Results

Survival of Mice

One hundred of 112 mice survived 4 weeks after surgery. Thus, each treatment group of the normal and DM mice consisted of 10 mice.

Blood Sugar Level and Body Weight Profile

The serum blood sugar level in DM mice was 400 ± 109 mg/dl, which was significantly higher than 146 ± 22 mg/dl in normal mice (p<0.05). In contrast, the body weight of DM mice was significantly lower than that of normal mice (23 ± 1 g vs 25 ± 1 g, p<0.05).

5HT_{2A} Receptor Expression

The expression of 5HT_{2A} receptors in the ischemic hindlimb was significantly higher than that in the non-ischemic hindlimb in both normal and DM mice at days 3 and 7 after surgery. The expression in the non-ischemic and the ischemic hindlimb in the DM mice were significantly higher than that in the non-ischemic and the ischemic hindlimb in normal mice, respectively (Fig 1).

Hindlimb Blood Perfusion

The time-course of LDPII in normal and DM mice are depicted in Fig 2; Left panels. In normal mice, the LDPII in groups S, F20, F50 and F20+S was significantly higher than that in the no-treatment group at 4 weeks after initiation of treatment (0.63 ± 0.08 , 0.72 ± 0.11 , 0.85 ± 0.11 , 0.88 ± 0.11 and

Fig 3. Comparison of the laser Doppler perfusion image index (LDPII) between normal (open bars) and diabetic (closed bars) mice at 4 weeks after various treatments. Group N, no treatment; group S, treatment with sarpogrelate; group F20, treatment with sustained-release 20 μ g basic fibroblast growth factor (bFGF); group F50, treatment with sustained-release 50 μ g bFGF; group F20+S, combined treatment with sustained-release 20 μ g bFGF and sarpogrelate; NS, not significant. * p <0.05 vs normal mice.

Fig 4. Representative photomicrographs of the ischemic calf muscles double stained with the anti-human von Willebrand factor antibody (vWF) and the anti-human smooth muscle actin (SMA) antibody at 4 weeks after surgery. Original magnification, $\times 200$; scale bars indicate 100 μ m. Left panel: normal mice; right panel: diabetic (DM) mice. Left column: vWF positive vessels; middle column: SMA positive vessels; right column: double-stained vessels. Group N, no treatment; group S, treatment with sarpogrelate; group F20, treatment with sustained-release 20 μ g basic fibroblast growth factor (bFGF); group F50, treatment with sustained-release 50 μ g bFGF; group F20+S, combined treatment with sustained-release 20 μ g bFGF and sarpogrelate.

0.94 $\pm$ 0.04 for groups N, S, F20, F50, and F20+S, respectively; p <0.05). The LDPII in groups F20, F50 and F20+S was significantly higher than that in group S. No statistically significant difference in the LDPII was found in the F20, F50 and F20+S groups (Fig 2; Upper right panel). In DM mice, the LDPII in the S, F20, F50 and F20+S groups was significantly higher (p <0.05) than that in the no-treatment group at 4 weeks after initiation of treatment (0.43 $\pm$ 0.11, 0.66 $\pm$ 0.10, 0.68 $\pm$ 0.06, 0.89 $\pm$ 0.11 and 0.94 $\pm$ 0.06 for groups N, S, F20, F50 and F20+S, respectively). The LDPII in groups F50 and F20+S was significantly higher than that in

S and F20 groups. No statistically significant difference in terms of the LDPII was found between either the S and F20 groups or the between the F50 and F20+S groups (Fig 2; Lower right panel).

The LDPII in the no-treatment group in normal mice was significantly higher (p <0.05) than that in the no-treatment group in DM mice. The LDPII in normal mice with F20 treatment was also significantly higher (p <0.05) than that in DM mice with the same treatment. There was no statistically significant difference in the LDPII between normal and DM mice with S, F50 and F20+S treatments (Fig 3).

Fig 5. Comparison of the vascular density in the ischemic calf muscles between normal (open bars) and diabetic (closed bars) mice at 4 weeks after surgery. Group N, no treatment; group S, treatment with sarpogrelate; group F20, treatment with sustained-release 20 μ g basic fibroblast growth factor (bFGF); group F50, treatment with sustained-release 50 μ g bFGF; group F20+S, combined treatment with sustained-release 20 μ g bFGF and sarpogrelate; NS, not significant. * p <0.05 vs group N; † p <0.05 vs group S.

Fig 6. Comparison of the vascular/muscle fiber ratio in the ischemic calf muscles between normal (open bars) and diabetic (closed bars) mice at 4 weeks after surgery. Group N, no treatment; group S, treatment with sarpogrelate; group F20, treatment with sustained-release 20 μ g basic fibroblast growth factor (bFGF); group F50, treatment with sustained-release 50 μ g bFGF; group F20+S, combined treatment with sustained-release 20 μ g bFGF and sarpogrelate; NS, not significant. * p <0.05 vs group N; † p <0.05 vs group S.

The percent increase in the LDPII for DM mice treated with sarpogrelate was a mean 54%, which is higher than the 14% mean in normal mice.

Vascular Density

Representative photomicrographs of histological sections in the normal and DM mice are shown in Fig 4. In normal mice, the vascular density in the groups F20, F50, and F20+S was significantly higher than that in the other 2 groups (154 ± 12 , 166 ± 13 , 326 ± 39 , 352 ± 27 and 390 ± 64 vessels/mm² for groups N, S, F20, F50 and F20+S, respectively; p <0.05). There was no statistically significant difference in the vascular density between groups N and S. No statistically significant difference in the vascular density was found among the groups F20, F50, and F20+S (Fig 5). In DM mice, the vascular density in the groups F20, F50 and F20+S was significantly higher than that in the other 2 groups (161 ± 14 , 170 ± 18 , 336 ± 31 , 348 ± 46 and 381 ± 58 vessels/mm² for groups N, S, F20, F50 and F20+S, respectively; p <0.05). There was no statistically significant difference in the vascular density between the groups N and S. No statistically significant difference in the vascular density was found among groups F20, F50, and F20+S (Fig 5).

The vascular density in the no-treatment group in normal mice was comparable with that in the no-treatment group in DM mice. There was no statistically significant difference

in the vascular density between normal and DM mice with each treatment (Fig 5). The percentage increase in the vascular density with sarpogrelate treatment in DM mice was 6% in mean, which is comparable with 8% in mean in normal mice.

Vascular/Muscle Fiber Ratio

The vascular/muscle fiber ratios in normal and DM mice are shown in Fig 6. In normal mice, the vascular/muscle fiber ratio was 0.62 ± 0.05 , 0.65 ± 0.05 , 1.42 ± 0.13 , 1.56 ± 0.12 and 1.63 ± 0.10 vessels/muscle fiber for groups N, S, F20, F50 and F20+S, respectively. In DM mice, it was 0.52 ± 0.05 , 0.61 ± 0.08 , 1.31 ± 0.08 , 1.42 ± 0.06 and 1.51 ± 0.15 vessels/muscle fiber for groups N, S, F20, F50 and F20+S, respectively. All data in the vascular/muscle fiber ratio were directionally similar to those in the vascular density.

Mature Vessel Density

Representative photomicrographs of histological sections in normal and DM mice are shown in Fig 4. In normal mice, the mature vessel density in groups S, F20, F50 and F20+S was significantly higher than that in the no-treatment group (12 ± 4 , 21 ± 3 , 45 ± 15 , 45 ± 8 and 51 ± 14 vessels/mm² for groups N, S, F20, F50 and F20+S, respectively; p <0.05). The mature vessel density in groups F20, F50 and F20+S was also significantly higher than that in group S, but no

Fig 7. Comparison of the mature vessel density in the ischemic calf muscles between normal (open bars) and diabetic (closed bars) mice at 4 weeks after surgery. Group N, no treatment; group S, treatment with sarpogrelate; group F20, treatment with sustained-release 20 μ g basic fibroblast growth factor (bFGF); group F50, treatment with sustained-release 50 μ g bFGF; group F20+S, combined treatment with sustained-release 20 μ g bFGF and sarpogrelate; NS, not significant. * p <0.05 vs normal mice; † p <0.05 vs group N; ‡ p <0.05 vs group S; § p <0.05 vs group F20.

statistically significant difference was found among groups F20, F50 and F20+S (Fig 7). In DM mice, the mature vessel density in groups S, F20, F50 and F20+S was significantly higher than that in the no-treatment group (8 ± 3 , 24 ± 4 , 31 ± 5 , 47 ± 6 and 48 ± 5 vessels/mm² for groups N, S, F20, F50 and F20+S, respectively; p <0.05). The mature vessel density in groups F20, F50 and F20+S was significantly higher than that in group S. The mature vessel density in groups F50 and F20+S was also significantly higher than that in group F20. No statistically significant difference in the mature vessel density was found between groups F50 and F20+S (Fig 7).

The mature vessel density in the no-treatment group in normal mice was significantly higher (p <0.05) than that in the no-treatment group in DM mice. The mature vessel density in normal mice with F20 treatment was significantly higher (p <0.05) than that in DM mice receiving the same treatment (Fig 7). The percentage increase in the mature vessel density with sarpogrelate treatment in DM mice was 200% in mean, which was higher than 75% in mean in normal mice.

Discussion

The new findings in this study are as follows: (1) sarpogrelate treatment for hindlimb ischemia was more effective in DM mice compared with normal mice; (2) the treatment with sustained-release bFGF was also effective in DM mice, which needed a higher dose of bFGF or a combined treatment of bFGF and sarpogrelate.

Neovascularization is categorized into angiogenesis, arteriogenesis and vasculogenesis. In the present study, we focused on the 2 former components. Angiogenesis represents the process of endothelial cell replication and migration leading to formation of capillary networks, while arteriogenesis is the maturation and enlargement of size of the pre-existing small vessels through vascular remodeling^{26,27}. Some investigators have demonstrated that arteriogenesis is defective and angiogenesis is enhanced or unaffected in DM^{11,12,28–30}. The present study showed that arteriogenesis (capillary spouting) was unaffected, although arteriogenesis and blood flow were impaired in DM mice. The aim was to restore blood flow as a result of enhanced arteriogenesis, which supports the concept that the clinical impact of collateral arteries is based on their ability to proliferate

into large conductance arteries (arteriogenesis), which can very efficiently restore blood flow to the ischemic territories^{31,32}.

Arteriogenesis plays a crucial role in collateral growth^{16,31}. Therapeutic angiogenesis with various growth factors has been extensively investigated over the last several years. Among angiogenic growth factors, bFGF is known to be one of the most potent stimulators of arteriogenesis^{27,33,34}. The process of arteriogenesis involves a variety of vessel wall cells including endothelial cells, smooth muscle cells and fibroblasts, and bFGF has broad potential to act as a growth factor for these cells³³. Additionally, bFGF does not only promote proliferation of endothelial cells but it also induces the development of a medial layer and adventitia, which could sustain and support the endothelium from the outside³⁵. To achieve the optimal collateral circulation, a choice of growth factors and their delivery system are the most important factors. Because the half-life of bFGF in its free form is very short, we used controlled and sustained-release bFGF incorporated in gelatin hydrogel microspheres^{17,18,22}.

In the present study, beneficial effects of sarpogrelate on ischemic hindlimb perfusion was exaggerated in the DM mice, presumably due to blockade of severe constriction of native collateral vessels as a result of increased expression of 5HT_{2A} receptors. Our findings on the enhanced expression of 5HT_{2A} receptors in DM mice were in agreement with those previously reported by Janiak et al²¹. It is tempting to speculate on the mechanism of augmented arteriogenesis with sarpogrelate treatment. The increased collateral blood flow mediated by sarpogrelate, a blockade of the 5HT_{2A} receptor, possibly augments shear stress at the site of pre-existent collateral vessels. Arteriogenesis is potentiated by the amplification of endogenous endothelial signaling mediated by changes in shear stress^{36–38}. Our findings are in agreement with earlier reports, which demonstrated that a serotonin blocker effectively improved the distal perfusion in hindlimb ischemia in the fatty Zucker rat²¹. While sarpogrelate has been reported to be effective for rabbits with hindlimb ischemia²² and anginal patients^{39,40}, more favorable effects of sarpogrelate are expected to be observed in patients with PAD and effort angina complicated by DM.

In this current study, we utilized GAPDH to normalize the 5HT_{2A} receptor expression based on results from an earlier study²¹. However, in a diabetic condition, GAPDH

expression might be suppressed by reactive oxygen species production.⁴¹ Therefore, this means that the use of other genes such as β -actin or 18s rRNA might be better for mRNA normalization.^{42,43} During this study, a conventional fluorescence light microscope was used, which was, to some extent, unable to distinguish endothelial and smooth muscle cell layers conspicuously but did confirm the overlapping or collocated cells. In order to distinguish double labeling in the case of 2 distinct collocated cells from a single one in the same tissue section, a confocal microscope instead of conventional microscope is required.^{44,45}

Collectively, the results of the present study showed that angiogenesis is preserved but regional blood flow and arteriogenesis are impaired in hindlimb ischemia of STZ-induced DM mice. Although 20 μ g bFGF was sufficient to induce therapeutic angiogenesis in hindlimb ischemia of normal mice, a higher dose of bFGF (50 μ g) was required for the same effect in DM mice. Furthermore, a combined treatment with sustained-release 20 μ g bFGF and sarpogrelate showed the same outcome as the treatment with 50 μ g bFGF. Thus, in DM mice, an intrinsic response to the stimuli for arteriogenesis was impaired, but the treatment effect of sustained-release bFGF was well preserved. These results might be applicable in the clinical setting in patients with PAD complicated by DM. Further investigations are certainly needed to fully define the mechanisms of the detrimental effect of DM on vascular disease using new and more sophisticated approaches.

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