Original Article

Nerve Growth Factor Promotes Angiogenesis and Skeletal Muscle Fiber Remodeling in a Murine Model of Hindlimb Ischemia

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Abstract

Background: Therapeutic angiogenesis has been shown to promote blood vessel growth and improve tissue perfusion. Nerve growth factor (NGF) has been reported to play an important role in both physiological and pathological angiogenesis. This study aimed to investigate the effects of NGF on angiogenesis and skeletal muscle fiber remodeling in a murine model of hindlimb ischemia and study the relationship between NGF and vascular endothelial growth factor (VEGF) in angiogenesis.

Methods: Twenty-four mice were randomly allocated to normal control group ($n = 6$), blank control group ($n = 6$), VEGF gene transfection group ($n = 6$), and NGF gene transfection group ($n = 6$). The model of left hindlimb ischemia model was established by ligating the femoral artery. $VEGF_{165}$ plasmid (125 μg) and NGF plasmid (125 μg) was injected into the ischemic gastrocnemius of mice from VEGF group and NGF group, respectively. Left hindlimb function and ischemic damage were assessed with terminal points at 21th day postischemia induction. The gastrocnemius of four groups was tested by hematoxylin-eosin staining, proliferating cell nuclear antigen and CD34 immunohistochemistry staining, and myosin ATPase staining. NGF and VEGF protein expression was detected by enzyme-linked immunosorbent assay.

Results: On the 21th day after surgery, the functional assessment score and skeletal muscle atrophy degree of VEGF group and NGF group were significantly lower than those of normal control group and blank control group. The endothelial cell proliferation index and the capillary density of VEGF group and NGF group were significantly increased compared with normal control group and blank control group ($P < 0.05$). The NGF and VEGF protein expression of NGF group showed a significant rise when compared with blank control group ($P < 0.05$). Similarly, the VEGF protein expression of VEGF group was significantly higher than that of blank control group ($P < 0.05$), but there was no significant difference of the NGF protein expression between VEGF group and blank control group ($P > 0.05$). The type I skeletal muscle fiber proportion in gastrocnemius of NGF group and VEGF group was significantly higher than that of blank control group ($P < 0.05$).

Conclusions: NGF transfection can promote NGF and VEGF protein expression which not only can induce angiogenesis but also induce type I muscle fiber expression in ischemic limbs.

Key words: Angiogenesis; Limb Ischemia; Muscle Fiber Type; Nerve Growth Factor; Vascular Endothelial Growth Factor

Introduction

Peripheral arterial disease (PAD) is a major cause of morbidity and mortality in China, and the two major manifestations of PADs are intermittent claudication and critical limb ischemia. The importance of angiogenesis in the development of collateral circulation to compensate for inadequate tissue perfusion is now well established,

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but the clinical effect of angiogenic treatment with growth factor gene or stem cell in patients with PAD was not very satisfactory. Some studies[1-3] demonstrated neural factors might also play a vital role in angiogenesis in the treatment of PAD. However, the sole endpoint of these studies has been angiogenesis, while neglecting the effect on remodeling of skeletal muscle fibers, a responding tissue of ischemia. This study aimed to observe the effect of nerve growth factor (NGF) on angiogenesis and remodeling of sleketal muscle fibers in ischemic limbs and explore the relationship between NGF and vascular endothelial growth factor (VEGF) in angiogenesis.

Methods

Experimental animals

Six-week-old female Institute of Cancer Research (ICR) mice were obtained from the Vital River Laboratories (Beijing, China). Mice were maintained in an air-conditioned room with a 12:12 h light/dark cycle where the temperature was 22 ± 2°C and relative humidity was 60 ± 10%. Water and food were available *ad asbitsium*. All protocols and procedures involving animals were approved by the Peking Union Medical College Animal Care and Use Committee. All animals received care in accordance with the Principles of Laboratory Animal Care (2006) formulated by the Chinese Ministry of Science and Technology. Twenty-four ICR mice were randomly assigned to normal control group ($n = 6$), blank control group ($n = 6$), VEGF treatment group ($n = 6$), and NGF treatment group ($n = 6$) according to random number table.

Establishment of hindlimb ischemia model and gene transfection

The mice of blank control group, VEGF group, and NGF group underwent surgically induced left hindlimb ischemia, using methods described previously.[4] Briefly, mice were anesthetized with intraperitoneal injection of pentobarbital sodium (35 mg/kg, Sigma, USA). A skin incision paralleling the inguinal ligament was made to allow proper isolation, ligation, and excision of the femoral artery from its origin just above the inguinal ligament to its bifurcation at the origin of the saphenous and popliteal arteries. The branches, inferior epigastric, lateral circumflex, and superficial epigastric arteries were also isolated and ligated with 8-0 prolene. The incision was closed using 6-0 prolene. All animals were closely monitored during the postoperative period.

Seven days after surgery, the mice of blank control group, VEGF group, and NGF group were anesthetized using pentobarbital sodium as previous described, and then intramuscular injection was administered by injecting evenly across the belly of the gastrocnemius muscle using a 27-gauge needle as follows: Blank control group (125 μl 1% poloxamer/50 mmol NaCl), VEGF group (125 μg $VEGF_{165}$ plasmid [Invitrogen, USA] in 125 μl 1% poloxamer/50 mmol NaCl), and NGF group (125 μg NGF plasmid [Invitrogen, USA] in 125 μl 1% poloxamer/50 mmol NaCl). Each injection was performed smoothly over at least 15 s, and the needle was left in place for at least 10 s afterward to prevent back leakage.

Assessment of hindlimb function and ischemic damage

At postoperative 21 days (14 days after gene transfection), function and ischemic damage assessment for the left hindlimbs of all mice were made according to the following scoring criteria: 4 = any amputation; 3 = dragging of foot, severe discoloration, or subcutaneous tissue or necrosis; 2 = not dragging but no plantar, moderate discoloration; 1 = plantar flexion, mild discoloration; and 0 = flexing the toes to resist gentle traction on the tail.[5]

Tissue harvest and preparation

The mice were anesthetized after hindlimb function assessment as previous described and then the mice were euthanized by cervical vertebrae dislocation following excision of the ischemic and contralateral nonischemic gastrocnemius muscles. The gastrocnemius muscle was divided into three parts; the distal portion was snap-placed in 30% sucrose-phosphate-buffered saline solution and mounted in cross-section in optimal cutting temperature. Cryostat sections (5 μm) were prepared on microscope slides (Fisher Scientific Inc., Leics, UK) for histological analysis. The middle part was snap-frozen in liquid nitrogen for protein extraction, and the remaining part was stored at 4% paraformaldehyde.

Analysis of capillary density and endothelial cells proliferation

Endothelial cells were identified using immunohistochemistry (IHC) staining for CD34 (1:200 dilution, Serotec, USA). Capillary density was measured by counting 5 random high-power (magnification ×200) fields or a minimum of 200 fibers from each (ischemic and nonischemic) limb on an inverted light microscope and expressed by the number of CD34-positive cells per mm^2. Area was measured with an NIH image analysis system.

Endothelial cells proliferation in skeletal muscle was detected with IHC staining of proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology Inc., CA, USA). The proliferation index was expressed by the percentage of the number of positive nuclei divided by the total number of counted nuclei. The count was performed on 5 randomly selected fields (magnification ×200), with at least 200 nuclei per sample. A single reader who was blinded to the sample type performed all the analysis.

Measurement of vascular endothelial growth factor protein and nerve growth factor protein

Available ELISA for VEGF and NGF was performed on homogenized muscle samples as the manufacturer's instructions (R & D Systems Inc., MN, USA). Briefly, approximately 100 mg of each muscle was homogenized in 1.5 ml NP-40 lysis buffer containing protease inhibitors. Muscle homogenates were centrifuged at 13000 r/min.

The surface lipid layer was removed, and the supernatant was saved at −80°C for ELISA and protein assay. Protein concentration was measured using a 1:500 dilution of the supernatant in the microtiter plate by micro-BCA protein assay (Pierce, Rockford, IL, USA). Supernatant dilutions of 1:6 were used for the VEGF and NGF ELISA assays.

Analysis of muscle fiber type

Muscle fiber type analysis was performed using the myosin ATPase method optimized at pH 9.4 after acid (pH 4.6) or alkaline (pH 10.3) preincubation. The main fibers were classified as type I and type II. The fiber type percentages were calculated from approximately 50 fibers in the muscle sections (magnification ×400).

Statistical analysis

Data are presented as mean ± standard deviation (SD) and analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Statistical significant was determined using the one-way analysis of variance followed by least-significant difference *post-hoc* analysis for comparison among multiple groups. $P < 0.05$ was considered statistically significant.

Results

Assessment of hindlimb function and ischemic damage

On the 21th day postoperatively, mice from normal control group had no symptoms relating hindlimb ischemia while mice from the blank control group, VEGF group and NGF group had various ischemia on affected legs. The ambulatory impairment and ischemic tissue damage score of blank control group was significantly higher than those of normal control group, VEGF group and NGF group [$P < 0.05$, Table 1].

Hematoxylin-eosin staining

Muscle tissue of mice from normal control group had no indications of atrophy [Figure 1a]. On the 21th day postoperatively, the gastrocnemius muscle atrophy of VEGF group and NGF group was significantly less serious than blank control group [Figure 1b-1d].

Immunohistochemical staining

CD34

IHC staining results on the 21th day postoperatively indicated significantly larger numbers of CD34-positive endothelial cells in blank control group [Figure 2b], VEGF group [Figure 2c], and NGF group [Figure 2d] than normal control group [Figure 2a]. The capillary density of VEGF group (0.36 ± 0.03) and NGF group (0.34 ± 0.05) was significantly increased compared with normal control group (0.11 ± 0.03) and blank control group (0.22 ± 0.04) [$P < 0.05$, Figure 2e].

Table 1: Ambulatory impairment and ischemic tissue damage score on the 21th day postoperatively (mean ± SD)

Index	Normal control group ($n = 6$)	Blank control group ($n = 6$)	VEGF group ($n = 6$)	NGF group ($n = 6$)
Score	0	2.17 ± 0.75*	1.17 ± 0.41*,†	1.00 ± 0.63*,†

*Compared with normal control group: $P<0.05$; †Compared with blank control group: $P<0.05$. SD: Standard deviation; VEGF: Vascular endothelial growth factor; NGF: Nerve growth factor.

Proliferating cell nuclear antigen

IHC staining results on the 21th day postoperatively indicated the number of PCNA-positive endothelial cells were significantly larger in blank control group [Figure 3b], VEGF group [Figure 3c], and NGF group [Figure 3d] than normal control group [Figure 3a]. Moreover, the endothelial cell proliferation index was significantly higher in VEGF group (0.31 ± 0.06) and NGF group (0.39 ± 0.19) than blank control group (0.14 ± 0.03) [$P < 0.05$, Figure 3e].

Expression of nerve growth factor and vascular endothelial growth factor protein

On the 21th day postoperatively, the VEGF protein expression were significantly increased in blank control group (22.27 ± 3.95 pg/ml), VEGF group (50.71 ± 5.45 pg/ml), and NGF group (46.29 ± 5.54 pg/ml) compared with normal control group (10.71 ± 2.69 pg/ml) [$P < 0.05$, Figure 4a]. Besides, VEGF protein expression was higher in VEGF group and NGF group than blank control group [$P < 0.05$, Figure 4a]. Similarly, the NGF protein expression was significantly increased in blank control group (13.42 ± 3.76 pg/ml), VEGF group (16.67 ± 3.14 pg/ml), and NGF group (35.26 ± 4.55 pg/ml) compared with normal control group (6.43 ± 2.64 pg/ml) [$P < 0.05$, Figure 4b], among which there was a significant difference between NGF group and blank control group [$P < 0.05$, Figure 4b], while the NGF protein expression in VEGF group was comparable to blank control group [$P > 0.05$, Figure 4b].

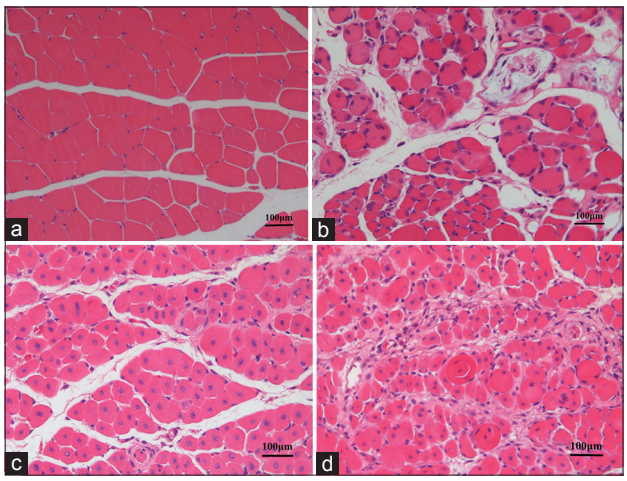

Figure 1: Hematoxylin and Eosin staining (×200). On the 21th day postoperatively, the gastrocnemius muscle from blank control group (b), vascular endothelial growth factor group (c) and nerve growth factor group (d) all manifested muscle atrophy, broadened intermuscular space and myocyte proliferation to some extend compared with normal control group (a), among which muscle atrophy in blank control group was more significant than vascular endothelial growth factor group and nerve growth factor group.

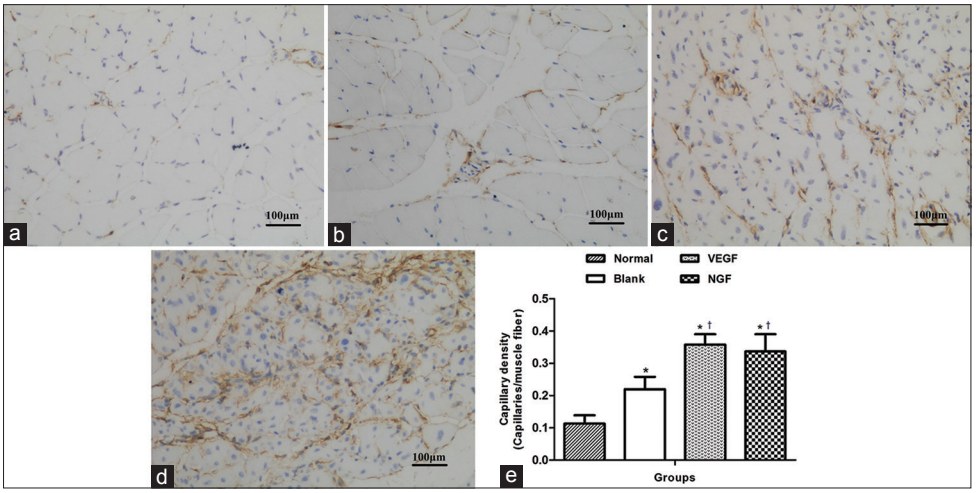

Figure 2: CD34 immunohistochemical staining (×200). On the 21th day postoperatively, compared with the normal control group (a), the number of CD34-positive endothelial cells was significantly increased in blank control group (b), vascular endothelial growth factor (VEGF) group (c) and nerve growth factor (NGF) group (d). The capillary density was significantly higher in VEGF group and NGF group than blank control group (e) (Compared with normal control group: *$P < 0.05$; Compared with blank control group: †$P < 0.05$).

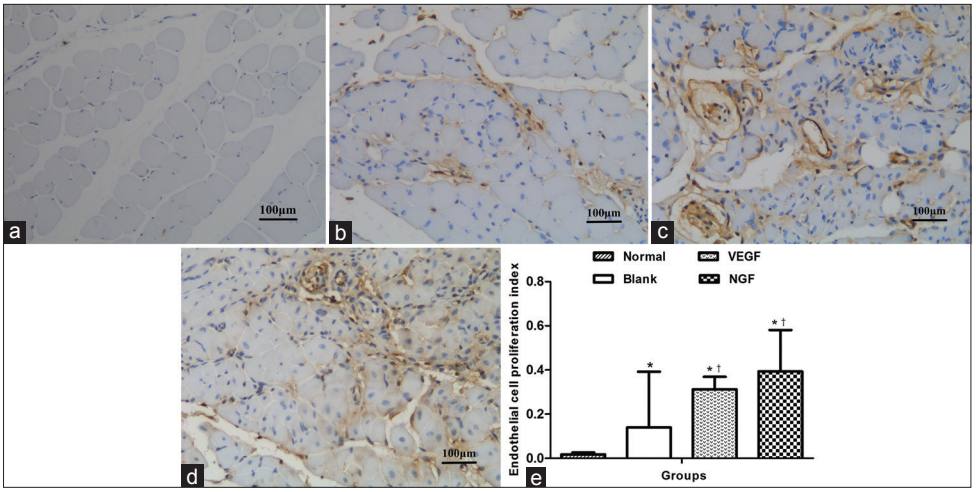

Figure 3: Proliferating cell nuclear antigen immunohistochemical staining (×200). On the 21th day postoperatively, the number of proliferating cell nuclear antigen-positive endothelial cells was significantly larger in blank control group (b), vascular endothelial growth factor (VEGF) group (c) and nerve growth factor (NGF) group (d) than normal control group (a). Moreover, the endothelial cell proliferation index was significantly higher in VEFG group and NGF group than blank control group (e) (Compared with normal control group: *$P < 0.05$; Compared with blank control group: †$P < 0.05$).

Changes in skeletal muscle fiber type

On the 21th day postoperatively, the type I muscle fibers percentage of gastrocnemius muscle was significantly lower in blank control group [20.55 ± 4.42, Figure 5b] than normal control group [40.52 ± 5.09, Figure 5a]. However, the type I muscle fibers percentage was significantly increased in VEGF group [51.50 ± 6.84, Figure 5c] and NGF group [54.25 ± 7.64, Figure 5d] compared with blank control group after gene transfection [$P < 0.05$, Figure 5e].

Discussion

Several studies have revealed interaction relationship between blood vessels and nerve fibers in their development and regeneration.[6,7] Several growth factors have been identified as important mediators that direct angiogenic sprouting and regulate axon terminal arborization. VEGF was originally described as a key angiogenic factor; it is now well established that VEGF also plays a therapeutic role in the nervous system.[8,9] NGF was the most important neurotrophin which can promote peripheral nerve development and regeneration, and it also has the effect that promote angiogenesis.[1,2] Renault *et al*.[10] found in a mouse model study that angiogenesis induced by ischemia and expression of VEGF-A, angiogenin-1, and NGF-3 were significantly reduced in ischemic muscle after denervation, which implied a potential effect of neural factors on angiogenesis in PAD. However, the relationship between VEGF and NGF in angiogenesis of ischemic hindlimbs has not been fully elucidated.

In the present study, we found the hindlimb function recovery was significantly faster in hindlimb ischemic

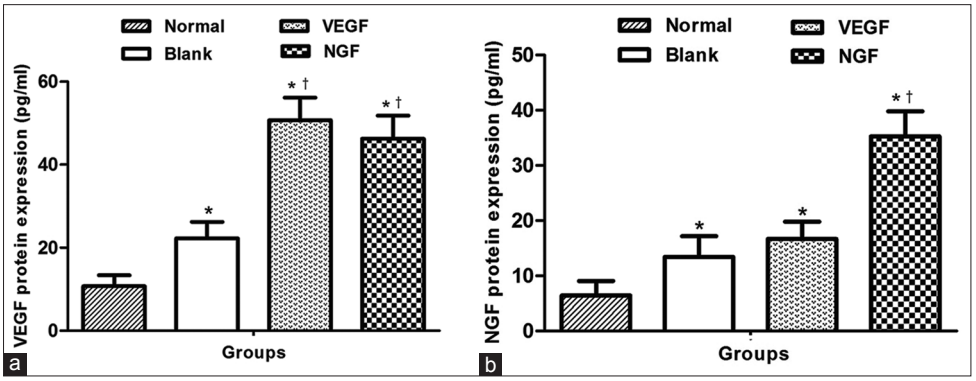

Figure 4: On the 21th day postoperatively, the vascular endothelial growth factor (VEGF) protein expression was significantly increased in VEGF group and nerve growth factor (NGF) group compared with blank control group (a) (Compared with normal control group: $^{*}P < 0.05$; Compared with blank control group: $^{\dagger}P < 0.05$). The NGF protein expression in NGF group was significantly higher than blank control group, but there was no significant difference between the VEGF group and blank control group (b) (Compared with normal control group: $^{*}P < 0.05$; Compared with blank control group: $^{\dagger}P < 0.05$).

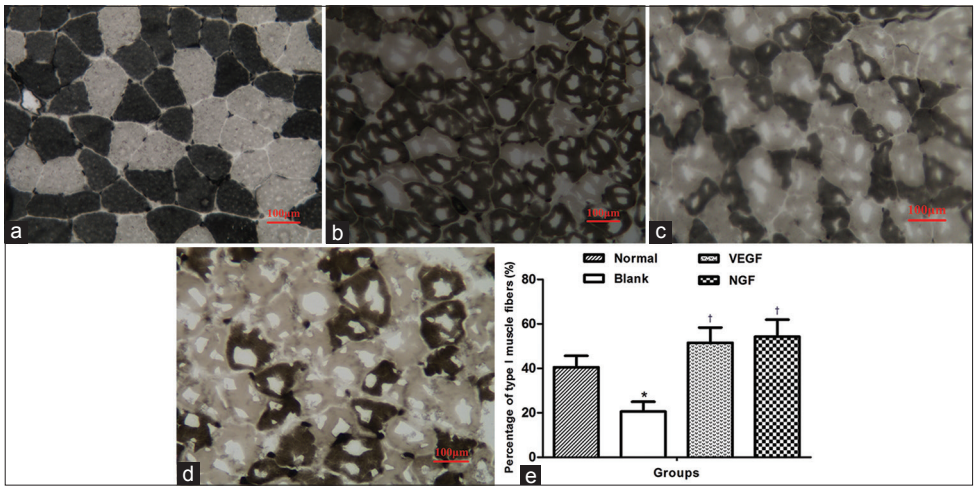

Figure 5: Myosin ATPase staining (white: type I; black: type II; ×400). On the 21th day postoperatively, there was lower percentage of type I muscle fibers in blank control group (b) than normal control group (a). There was significant higher percentage of type I muscle fibers in vascular endothelial growth factor (VEGF) group (c) and nerve growth factor (NGF) group (d) than blank control group (e) (Compared with normal control group: $^{*}P < 0.05$; Compared with blank control group: $^{\dagger}P < 0.05$).

mice transfected with NGF and VEGF. Histological assessment found significantly milder muscle atrophy, proliferated endothelial cells, and more angiogenesis after NGF and VEGF gene transfection, suggesting angiogenic function of NGF and VEGF. There are a few signaling pathways may be mediated the angiogenesis which induced by NGF, including promoting the proliferation, migration, and differentiation of endothelial progenitor cells,[11,12] activating Src-PI3K-Akt pathway which lead to the activation of protein kinase G and mitogen-activated protein kinase and then promote the proliferation of endothelial cells,[13] activating the PI3K and ERK pathways which can promote the proliferation and migration of endothelial cells by inducing matrix metalloproteinase-2 and nitric oxide expression. NGF can also directly increase VEGF expression in endothelial cells.[1,14] We found that VEGF protein expression was significantly increased in gastrocnemius transfected with NGF gene or VEGF gene. NGF protein expression was up-regulated after NGF gene transfection but there was no significant difference between the VEGF group and blank control group. The results indicated that NGF gene transfection may induce the VEGF expression in ischemic muscle which mechanism need further research.

Serious limb ischemia can finally lead to limb ulcer, necrosis, and even amputation. Previous studies mainly focused on how to recover or improve blood perfusion in ischemic limbs but rarely studied histological structure change of muscle, the target organ of ischemia. The contractive characteristics of mammal skeletal muscles are determined by myosin heavy chain (MHC) subtypes. MHC is also called myosin ATPase for its ATPase activity.[15] Different subtypes of MHC have distinct ATPase characteristics and subsequently determined various physiological features of skeletal muscle fibers. Skeletal muscle fibers can be divided into slow-twitch muscle fibers (type I) and fast-twitch muscle fibers (type II) according to MHC subtypes. Slow-twitch muscle fibers mainly express MHC-I subtype, so they have stronger aerobic capacity but slower contraction rate, which leads to longer duration of contraction and higher resistance to fatigue.

On the contrary, fast-twitch muscle fibers mainly express MHC-II subtype, so they have stronger anaerobic capacity and faster contraction with higher local tension but shorter duration of contraction and easy fatigue.[16,17] Muscle fiber types can be altered by mutual conversion of MHC subtypes which mainly happens when sports load, blood perfusion, innervation, or energy metabolism was changed.[18-21] For PAD patients whose muscles are in long-term ischemic and oxygen-starved status, the mitochondrial enzymes may adaptively alter functions.

Normal gastrocnemius is fast-contraction muscle which mainly made up of type II muscle fibers. In the present study, the type I muscle fibers percentage in ischemic gastrocnemius of blank control group was markedly reduced compared with normal control group, and the corresponding percentage of type II muscle fibers in blank control group was increased which suggesting a remodeling process from type I to type II muscle fibers. McGuigan *et al*.[22] performed myosin ATPase staining on gastrocnemius of PAD patients and also found similar muscle fiber remodeling. This transformation seems adaptively to provide energy for shorter-distance walking thus leading to reduction in walking performance. Our further study found the percentage of type I fibers was significantly increased after NGF or VEGF gene transfection than that of the blank control group. It means that the remodeling process from type I fibers to type II fibers was possibly inhibited by NGF and VEGF, which may improve the aerobic capacity of gastrocnemius. The mechanism of skeletal muscle fiber remodeling is considered to be related to the energy metabolic pathway transformation.[23,24] Transcription activating factor proliferator-activated receptor-γ coactivator-1α (PGC-1α) and nuclear receptors peroxisome proliferator-activated receptor-β/δ (PPAR-β/δ) and estrogen-related receptor-γ (ERR-γ) are important factors in modulating cellular energy metabolism. In skeletal muscle, up-regulated expression of PGC-1α, PPAR-β/δ, and ERR-γ can increase the percentage of type I fibers.[25-27] Moreover, Matsakas *et al*.[28] found ERR-γ-dependent remodeling of myofibers may promote angiogenesis and restoration of blood perfusion in ischemic skeletal muscle. In the present study, the type I fibers percentage was increased in ischemic muscle transfected with NGF and VEGF genes, possibly mediated by more angiogenesis, blood perfusion recovery, and the amount and activity of oxidase restore in mitochondria. Furthermore, ischemic nerve injury can be partly relieved by blood perfusion recovery, which also will cause the muscle fibers transformation.[29,30]

In conclusion, this study demonstrates that NGF transfection can promote NGF and VEGF protein expression which not only can induce angiogenesis but also induce type I muscle fiber expression in ischemic limbs.

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Conflicts of interest

There are no conflicts of interest.

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