

Original Article

Angiogenic effects of recombinant thymosin β 4 in a mouse hindlimb ischemia model

Jun-Hwan Moon, Jung-Won Lee, Jong-Koo Kang*

College of Veterinary Medicine, Chungbuk National University, Cheongju 362-763, Korea

Recombinant thymosin β 4 (rT β 4) has been reported to migrate and promote vascularization, wound-healing, and hair growth in a mouse hindlimb ischemia model of peripheral vascular disease. C57BL/6 mice (11-weeks-old) were anesthetized and an ischemic model was made by cutting the right aorta femoralis. The ischemic group was intraperitoneally administered with saline (300 μ L/mouse) and the muscular administration group received rT β 4 (150 μ g in 300 μ L of saline) or rT β 4 (150 μ g in 300 μ L saline) to the abdominal cavity at 3-day intervals for 21 days. Myoatrophy of the ischemic group was observed compared to the normal control group. Generation of adjacent vessels was carried out in the rT β 4 administration group compared to the ischemic group. The biopsy results showed significant fibrosis around the muscular undersurface and perimysium in the musculus quadriceps femoris of the ischemic group, whereas partial fibrosis was observed in the perimysium and endomysium in the rT β 4 administration group. Immunostaining indicated that expression levels of hypoxia-inducible factor-1 α (HIF-1 α), vascular endothelial growth factor-1 (VEGF-1), and endothelial nitric oxide synthase (eNOS) in the rT β 4 group were higher than those of the ischemic group. Western blotting showed that expression levels of HIF-1 α , VEGF-1, and eNOS in the rT β 4 group were higher than those of the ischemic group. In conclusion, rT β 4 increases expression levels of HIF-1 α , VEGF-1, and eNOS, resulting in angiogenesis.

Key words: gene recombination thymosin beta4, Peripheral arterial disease (PAD), angiogenesis, Hindlimbschemia, vascular endothelial growth factor (VEGF)

Introduction

Peripheral arterial disease (PAD) is a common disease found in 10~25% of patients over the age of 55 years and

its severity and occurrence are strongly associated with other cardiovascular risk factors that lead to coronary artery disease and stroke [1]. It is associated with diminution in quality of life and increased mortality. However, interventional or surgical procedures are not available in some conditions due to the complexity of the disease. Alternative therapy includes the attempt to develop new collateral vessels using angiogenic growth factors. However, the optimal therapeutic approach has not been developed [2].

Angiogenesis, the formation of new blood microvessels from preexisting ones, in adult organisms is normally limited to the female reproductive system. However, the formation of new blood vessels becomes essential during wound healing and tissue repair and pathological conditions [3].

Recently, activators of the endothelial nitric oxide (eNOS)/nitric oxide (NO) pathway such as vascular endothelial growth factor (VEGF) have been reported to promote angiogenesis or arteriogenesis in critical limb ischemia [4]. The regulation of angiogenesis by hypoxia is an important component of homeostatic control. The major factor which responds to hypoxia is hypoxia-inducible factor-1 α (HIF-1 α). It modulates transcriptional control of several genes such as VEGF and eNOS [5].

Thymosin β 4 (T β 4) is a highly conserved, 43-amino acid acidic peptide that was first isolated from calf thymus tissue. The most prominent physiological function of T β 4 is the regulation of actin polymerization which is necessary for cell motility and organogenesis [6]. Recently, the healing properties of T β 4 have been found in the skin [7, 8] and cornea [9, 10], and more recently it has been shown that T β 4 facilitates cardiac repair after infarction by promoting cell migration and myocyte survival [11, 12]. And T β 4 promotes vasculogenesis and angiogenesis [13, 14] after ischemic injury. Safety, toler-

*Corresponding author: Jong-Koo Kang,

College of Veterinary Medicine, Chungbuk National University, Cheongju 361-763, Korea

Tel: +82-43-261-2607, Fax: +82-43-271-3246, E-mail: jkkang@chungbuk.ac.kr

ability and efficiency of Tβ4 were evaluated in clinical patients with acute myocardial infarction [6] and other diseases [9, 10, 15].

We hypothesized that recombinant Tβ4 (rTβ4) may be involved in the mechanisms of angiogenic pathways and formation of collateral vessel after PAD, resulting in the promotion of angiogenesis. Our study reveals the effects of rTβ4 on peripheral angiogenesis in a mouse hindlimb ischemia model and rTβ4 may contribute to the advancement of alternative therapeutic medicine in this field.

Materials and Methods

Animals and reagents

Specific pathogen-free 11-week-old male C57BL/6 mice were purchased from a commercial animal breeder (Koatech Inc., Pyeongtaek, Korea) and acclimated for 1 week before initiation of the study. During the acclimation and experimental periods, the mice were housed in polycarbonate cages in an animal room with controlled temperature ($22 \pm 3^\circ\text{C}$) and humidity ($50 \pm 20\%$) and a 12-hr light/dark cycle. The animals were provided commercial pellets (Purina, Seoul, Korea) and tap water ad libitum. All procedures were conducted in compliance to the Standard Operating Procedures of Laboratory Animals and approved by Laboratory Animal Care and Use Committee of the Laboratory Animal Research Center, Chungbuk National University. rTβ4 was obtained from Biototech Co., Ltd. (Ochang, Korea). Sterile saline was obtained from Choongwae Pharma Co., Ltd. (Seoul, Korea) and used as the vehicle.

Hindlimb ischemia operation

Mice were anesthetized with intramuscular injection of Zoletil (30 mg/kg) and xylazine (8 mg/kg). Aseptic surgery was performed to ligate the right common femoral artery. In brief, an incision was made in right groin of mice. Blunt dissection was performed and the right common femoral artery was identified by its pale pink color and pulsatile nature. The common femoral vein and femoral nerve were dissected free of the artery. Two ligations were performed in the common femoral artery proximal to the origin of the profundafemoris artery using 7-0 nylon sutures. The common femoral artery was then transected between the ligation sites. Immediate blanching was noted in the distal right hind limb after ligation. The incision was sutured in a single layer using the same suture material as for ligation. Mice were noted to be limping and dragging the right hind limb after recovery from anesthesia [16].

Recombinant thymosin β4 treatment

Sixteen operated mice received rTβ4 immediately after ligation and the remaining 6 animals received saline

alone. Treatment was given intramuscularly (IM) or intraperitoneally (IP) with rTβ4 (150 μg in 300 μL saline/mouse, $n=8$). Injections were given every 3 days during 3 weeks [11, 17] (Fig. 1). Dosages were based on previous studies on Tβ4 biodistribution [18].

Gross examination

Right hindlimbs were removed, weighed, and fixed for histological sectioning. The quadriceps femoris muscles were excised and fixed in 4% paraformaldehyde solution. Some muscle samples were routinely processed, paraffin-embedded, cut into 4-μm sections, and stained with Masson's trichrome.

Immunohistochemical analysis

Remaining samples were used for immunohistochemical study. CD31, HIF-1α, VEGF-1 and eNOS immunoreactivities were identified using monoclonal anti-CD31 antibody at 1:100 (M0823) (Dako Biotechnology, Carpinteria, CA, USA), monoclonal anti-HIF-1α antibody at 1:100 (sc-53546) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal anti-VEGF-1 antibody at 1:100 (sc-53462) (Santa Cruz Biotechnology) and monoclonal anti-eNOS antibody at 1:100 (sc-654) (Santa Cruz Biotechnology). The reaction was visualized with ABC reagent (Santa Cruz Biotechnology). Briefly, the sections were incubated in 3% hydrogen peroxide to block endogenous peroxidase activity. The slides were treated with antigen retrieval solution (0.1% trypsin) at 37°C for 20 min. To prevent nonspecific antibody binding, sections were preincubated for 30 min in PBS containing 10% goat serum. The sections were incubated with the primary antibodies directed against CD31, HIF-1α, VEGF-1 or eNOS at appropriate dilutions 2 hr at 37°C and then rinsed for 15 min with PBS followed by incubation with secondary antibody (rabbit monoclonal to mouse IgG, ABC kit) for 1 hr at 37°C . Again the sections were rinsed with PBS and incubated with diaminobenzidine and counterstained with hematoxylin. Negative control slides were prepared by substituting PBS for CD31, HIF-1α, VEGF-1 and eNOS staining.

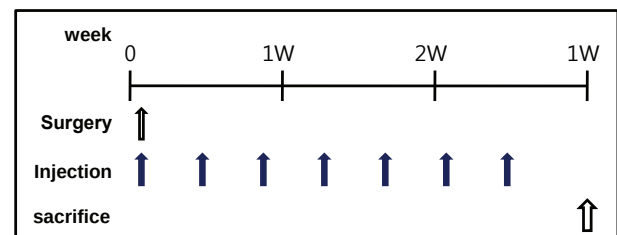


Fig. 1. Experimental design. After surgery (↑) for induction of hindlimb ischemia, recombinant thymosin β4 or vehicle (↑) was injected intraperitoneally or intramuscularly on every 3 days during 3 weeks. At the end of the experiment, all rats were sacrificed (↑).

Western blot analysis

Whole muscle cell lysates were fractionated by SDS-PAGE and transferred onto membranes (Millipore Corp., Billerica, MA, USA). Monoclonal antibodies against HIF-1 α (Santa Cruz Biotechnology), VEGF-1 (Santa Cruz Biotechnology) and eNOS (Santa Cruz Biotechnology) were diluted at 1:500 and β -actin (Fermentas Biotechnology, Burlington, Ont, Canada) was diluted at 1:2,000. Each antibody was used in conjunction with a horse radish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology).

Statistical analysis

The differences between the groups were examined using a standard one-way analysis of variance. If statistical significance differences were evident, the data were analyzed using the multiple comparison procedures of Duncan's or Dunnett's multiple range comparison. SPSS for Windows 12.0 K software package was used.

Results

Gross findings

Mice were noted to be limping and dragging the right hindlimb after surgery. To evaluate the ischemic injury and distribution of collateral vessels in the right hindlimb muscular tissue, right hindlimb without skin was observed by stereoscopic microscope at the end of the experiment. The ischemia control group exhibited marked ischemia-induced muscular atrophy compared to the normal control (Figs. 2A and 2B). However, there were no significant differences between the treatment groups with

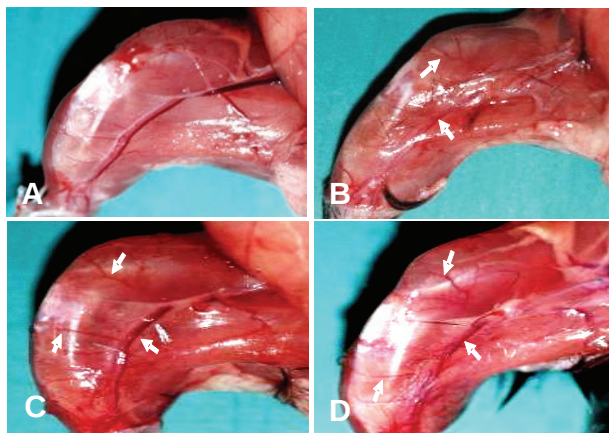


Fig. 2. Gross findings of the hindlimbs. In comparison with normal features of muscular tissue in normal control group (A), the ischemia control (B) showed ischemia-induced muscular atrophy. Treatment groups with recombinant thymosin β 4 (rT β 4) (C and D) induced more number of collateral vessels in comparison to the ischemia control group. A: Normal control, B: Ischemia control, C: Ischemia + rT β 4 (150 μ g, IM), D: Ischemia + rT β 4 (150 μ g, IP).

rT β 4 (IM and IP) and the ischemia control group in the ratio of right to left hindlimb (Fig. 3).

In the distribution of collateral vessels, treatment groups with rT β 4 induced more number of collateral vessel in comparison to the ischemia control group (Figs. 2B, 2C and 2D).

Histopathological findings

To investigate fibrotic changes in the hindlimb, Masson's trichrome stain was used. The ischemia control exhibited fibrous connective tissue components around the endomysium and intramuscular area caused by muscular dystrophy compared to the normal control (Figs. 4A and 4B). However, treatment groups with rT β 4 (IM and IP) showed diminished fibrosis in both the perimysium and endomysium area (Figs. 4C and 4D).

Immunohistochemistry

To assess the formation of microvessels, CD31 immu-

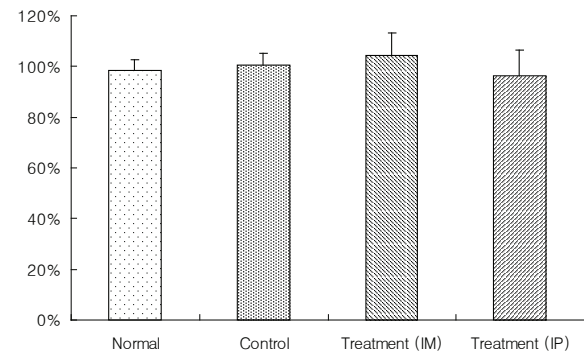


Fig. 3. Ratio of right to left hindlimb. There were no significant differences between the treatment groups with recombinant thymosin β 4 (rT β 4) and the ischemia control. Normal: normal control, Control: ischemia control, Treatment (IM): Ischemia + rT β 4 (150 μ g, IM), Treatment (IP): Ischemia + rT β 4 (150 μ g, IP).

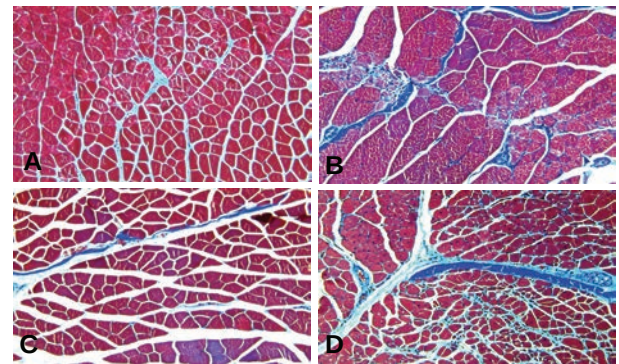


Fig. 4. Masson's trichrome staining of the muscular tissues. A fibrosis was observed in the ischemia control (B). Treatment groups with recombinant thymosin β 4 (rT β 4) (C and D) were showed diminished fibrosis compared to the ischemia control. A: Normal control, B: Ischemia control, C: Ischemia + rT β 4 (150 μ g, IM), D: Ischemia + rT β 4 (150 μ g, IP), $\times 200$.

nostaining was used. CD31 in the ischemic control group was observed to be similarly expressed compared with the normal control group. While the treatment group compared with the ischemia control group as a whole showed a high expression of CD31. These results suggest that the expression of CD31 is considered to be one to stimulate microvascular formation. As a result, rT β 4 treatment group compared with the ischemia control group showed high CD31 expression patterns (Fig. 5).

Also, the expression of HIF-1 α exhibited similarly in the ischemic control group compared to the normal control group, while the expression of HIF-1 α increased in the treatment group compared to the ischemic control group (Fig. 6). In the immunohistochemical staining for VEGF-1 and eNOS, similar patterns were observed as the HIF-1 α (Figs. 7 and 8).

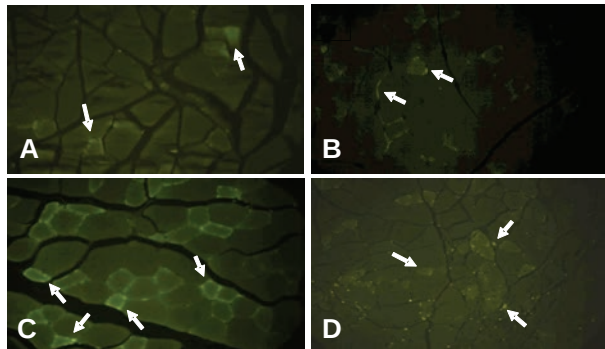


Fig. 5. CD31 immunohistochemistry of the muscular tissues. Capillaries were stained positive for CD31 (FITC). Treatments with recombinant thymosin β 4 (rT β 4) (C and D) increased the expression of CD31 compared with the ischemia control (B). A: Normal control, B: Ischemia control, C: Ischemia + rT β 4 (150 μ g, IM), D: Ischemia + rT β 4 (150 μ g, IP), $\times 200$.

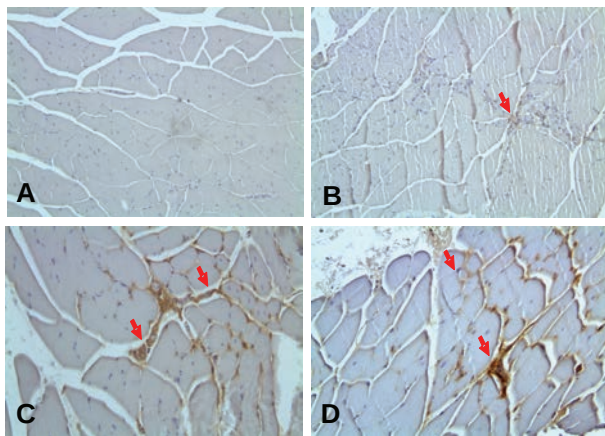


Fig. 6. Hypoxia-inducible factor-1 α (HIF-1 α) immunohistochemistry of the muscular tissues. Treatment groups with recombinant thymosin β 4 (rT β 4) (C and D) increased the expression of HIF-1 α compared with the ischemia control (B). A: Normal control, B: Ischemia control, C: Ischemia + rT β 4 (150 μ g, IM), D: Ischemia + rT β 4 (150 μ g, IP), $\times 200$.

Western blot

The expressions of HIF-1 α , VEGF-1 and eNOS were enhanced in the treatment groups with rT β 4 (IM and IP) by Western blot analysis. The expression of HIF-1 α , VEGF-1 and eNOS was up-regulated in treatment groups with rT β 4 as compared with the ischemia control (Fig. 9).

Discussion

The effects of rT β 4 was tested on angiogenesis in a mouse hindlimb ischemia model. During 3 weeks, rT β 4 was administered every 3 days. To assess the recovery of

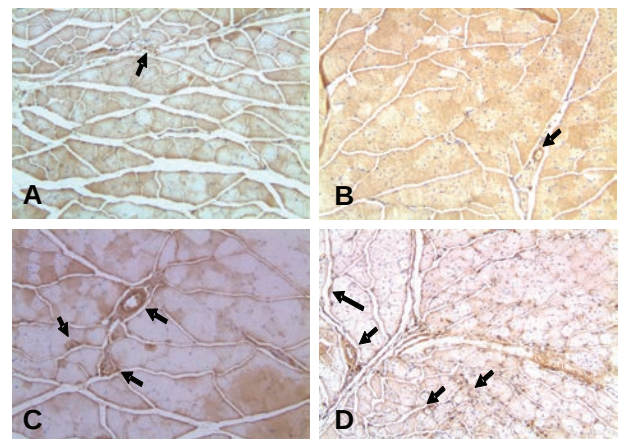


Fig. 7. Vascular endothelial growth factor-1 (VEGF-1) immunohistochemistry of the muscular tissues. Treatment groups with recombinant thymosin β 4 (rT β 4) (C and D) increased the expression of VEGF-1 compared with the ischemia control (B). A: Normal control, B: Ischemia control, C: Ischemia + rT β 4 (150 μ g, IM), D: Ischemia + rT β 4 (150 μ g, IP), $\times 200$.

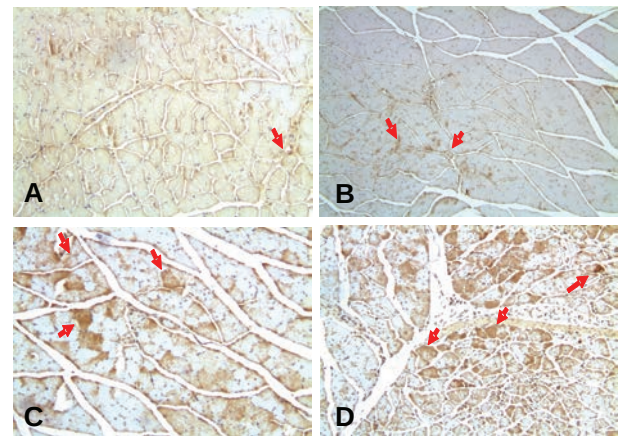


Fig. 8. Endothelial nitric oxide synthase (eNOS) immunohistochemistry of the muscular tissues. Treatment groups with recombinant thymosin β 4 (rT β 4) (C and D) increased the expression of eNOS compared with the ischemia control (B). A: Normal control, B: Ischemia control, C: Ischemia + rT β 4 (150 μ g, IM), D: Ischemia + rT β 4 (150 μ g, IP), $\times 200$.

ischemic tissue damage and the expression of angiogenic growth factors in an ischemic hindlimb, histopathological analysis and Western blotting were performed. Data was presented the alleviation of ischemic tissue damage and the overexpression of angiogenic growth factors.

Ischemia can cause ischemic damage to hindlimb muscle and eventually lead to tissue necrosis [2]. In gross findings, the ischemia controls displayed muscular atrophy. Previous studies showed muscular atrophy by ischemia compared to the normal control, leading to decreased ratio of left to right leg volume [19, 20]. However, there were no significant differences between the treatment groups with rTβ4 (IM and IP) and the ischemia control in the present study. According to the study by Hoefer *et al* (2001), there was no difference between treatment groups and ischemia control when the treatment was performed 3 weeks after femoral ligation. Additional experiments to evaluate the ratio of ischemic to non-ischemic hindlimb by date are needed. To investigate fibrotic changes in the ischemic hindlimb, Masson's trichrome staining was used. Microscopic analysis revealed the ischemia control exhibited fibrous connective tissue components around endomysium and intramuscular area caused by muscular dystrophy. Treatment groups with rTβ4 (IM and IP) showed diminished fibrosis in both the perimysium and endomysium area. These data indicated that rTβ4 reduced the ischemic injury and prevented the muscular dystrophy.

Ischemia-induced hypoxia stimulates the release of HIF-1α. It modulates transcriptional control of VEGF-

1 and eNOS. NO, produced by eNOS, is an important regulator of angiogenesis and can induce angiogenesis *in vivo* [21]. rTβ4 induced angiogenesis via eNOS using thermal therapy in mice with hindlimb ischemia [17]. To explain the mechanism of rTβ4, the expression of angiogenic growth factors was evaluated by Western blotting. Data revealed that the expression of HIF-1α, VEGF-1 and eNOS enhanced in the treatment groups with rTβ4 (IM and IP) compared to the ischemia control. Also, the histological analysis showed that the expression of HIF-1α, VEGF-1 and eNOS increased in the treatment groups with rTβ4 (IM and IP) compared to the ischemia control in ischemic hindlimb muscle. These results suggest that rTβ4 promotes the expression of angiogenic growth factors related to the NO/eNOS pathway.

Angiogenesis is the postnatal process whereby an existing microvascular network is expanded [3]. In angiogenesis, collateral vessels were observed [22]. Although we could not perform an angiography, we speculated the formation of blood microvessels by CD31 immunostaining. CD31 immunostaining was widely used in detecting new blood microvessels and expression of CD31 increased depending on number of new blood microvessels [23]. The treatment groups with rTβ4 (IM and IP) induced the over-expression of CD31 compared to the ischemia control. This study found an increase in the expression of angiogenic growth factors after administration of rTβ4 in a mouse hindlimb ischemia model. Therefore, it is assumed that rTβ4 was involved in the angiogenesis and formation of collateral vessels.

In conclusion, the results indicate that rTβ4 exerts angiogenic properties through increased angiogenic growth factor expression and that rTβ4 is likely to be developed as an alternative therapeutic medicine in peripheral arterial disease.

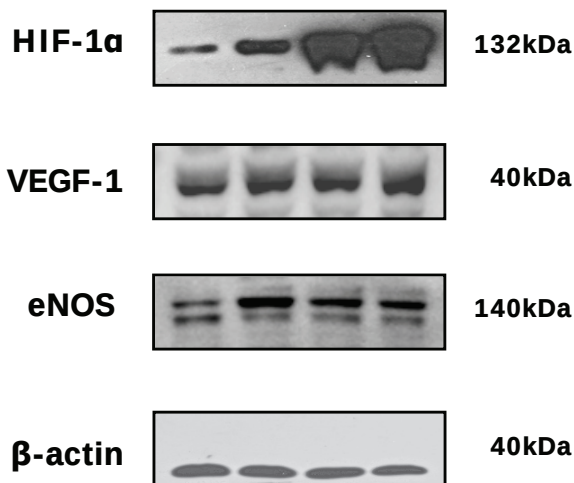


Fig. 9. Hypoxia-inducible factor-1α (HIF-1α), Vascular endothelial growth factor-1 (VEGF-1) and Endothelial nitric oxide synthase (eNOS) by Western blot in the muscular tissues. Treatment groups with recombinant thymosin β4 (rTβ4) increased the expression of HIF-1α, VEGF-1 and eNOS compared with the ischemia control. Normal: normal control, Control: ischemia control, Treatment (IM): Ischemia + rTβ4 (150 μg, IM), Treatment (IP): Ischemia + rTβ4 (150 μg, IP).

Acknowledgements

This work was supported by Chungbuk National University Grant in 2011.

ORCID

Jong-Koo Kang, <http://orcid.org/0000-0002-8921-7454>

References

1. Baumgartner I, Schainfeld R, Graziani L. Management of peripheral vascular disease. *Annu Rev Med* 2005;56:249-272.
2. He Y, Luo Y, Tang S, Rajantie I, Salven P, Heil M, Zhang R, Luo D, Li X, Chi H, Yu J, Carmeliet P, Schaper W, Sinusas AJ, Sessa WC, Alitalo K, Min W. Critical function of Bmx/Etk in ischemia-mediated arteriogenesis

- and angiogenesis. *J Clin Invest* 2006;116:2344-2355.
3. Griffioen AW, Molema G. Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. *Pharmacol Rev* 2000;52:237-268.
 4. Lekas M, Lekas P, Latter DA, Kutryk MB, Stewart DJ. Growth Factor Induced Therapeutic Neovascularization For Ischemic Vascular Disease: Time for a Re-evaluation? *Curr Opin Cardiol* 2006;21:376-384.
 5. Milkiewicz M, Ispanovic E, Doyle JL, Haas TL. Regulators of angiogenesis and strategies for their therapeutic manipulation. *Int J Biochem Cell Biol* 2006;38:333-357.
 6. Crockford D. Development of thymosin beta4 for treatment of patients with ischemic heart disease. *Ann N Y Acad Sci* 2007;1112:385-395.
 7. Philp D, Goldstein AL, Kleinman HK. Thymosin beta4 promotes angiogenesis, wound healing, and hair follicle development. *Mech Ageing Dev* 2004;125:113-115.
 8. Philp D, Nguyen M, Scheremeta B, St-Surin S, Villa AM, Orgel A, Kleinman HK, Elkin M. Thymosin beta4 increases hair growth by activation of hair follicle stem cells. *FASEB J* 2004;18:385-387.
 9. Fine JD. Epidermolysis bullosa: a genetic disease of altered cell adhesion and wound healing, and the possible clinical utility of topically applied thymosin beta 4. *Ann N Y Acad Sci* 2007;1112:396-406.
 10. Guarnera G, DE Rosa A, Camerini R. Thymosin beta-4 and venous ulcers: clinical remarks on a european prospective, randomized study on safety, tolerability, and enhancement on healing. *Ann N Y Acad Sci* 2007;1112:407-412.
 11. Bock-Marquette I, Saxena A, White MD, Dimaio JM, Srivastava D. Thymosin beta4 activates integrin-linked kinase and promotes cardiac cell migration, survival and cardiac repair. *Nature* 2004;432:466-472.
 12. Cava sin MA. Therapeutic potential of thymosin-beta4 and its derivative N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) in cardiac healing after infarction. *Am J Cardiovasc Drugs* 2006;6:305-311.
 13. Smart N, Risebro CA, Melville AA, Moses K, Schwartz RJ, Chien KR, Riley PR. Thymosin beta4 induces adult epicardial progenitor mobilization and neovascularization. *Nature* 2007;445:177-182.
 14. Smart N, Risebro CA, Melville AA, Moses K, Schwartz RJ, Chien KR, Riley PR. Thymosin beta-4 is essential for coronary vessel development and promotes neovascularization via adult epicardium. *Ann N Y Acad Sci* 2007;1112:171-188.
 15. Smart N, Rossdeutsch A, Riley PR. Thymosin beta4 and angiogenesis: modes of action and therapeutic potential. *Angiogenesis* 2007;10:229-241.
 16. Hiroshi N, Ngan FH, Mark DR, John PC. Murine model of hindlimb ischemia. *J Vis Exp* 2009;23:1-3.
 17. Zhang J, Zhang ZG, Morris D, Li Y, Roberts C, Elias SB, Chopp M. Neurological functional recovery after thymosin beta4 treatment in mice with experimental auto encephalomyelitis. *Neuroscience* 2009;164:1887-1893.
 18. Mora CA, Baumann CA, Paino JE, Goldstein AL, Badamchian M. Biodistribution of synthetic thymosin beta 4 in the serum, urine, and major organs of mice. *Int J Immunopharmacol* 1997;19:1-8.
 19. Kakinuma Y, Furihata M, Akiyama T, Arikawa M, Handa T, Katare RG, Sato T. Donepezil, an acetylcholinesterase inhibitor against Alzheimer's dementia, promotes angiogenesis in an ischemic hindlimb model. *J Mol Cell Cardiol* 2010;48:680-693.
 20. Akasaki Y, Miyata M, Eto H, Shirasawa T, Hamada N, Ikeda Y, Biro S, Otsuji Y, Tei C. Repeated thermal therapy up-regulates endothelial nitric oxide synthase and augments angiogenesis in a mouse model of hindlimb ischemia. *Circ J* 2006;70:463-470.
 21. Ziche M, Morbidelli L, Masini E, Amerini S, Granger HJ, Maggi CA, Geppetti P, Ledda F. Nitric oxide mediates angiogenesis in vivo and endothelial cell growth and migration in vitro promoted by substance P. *J Clin Invest* 1994;94:2036-2044.
 22. Kumar D, Branch BG, Pattillo CB, Hood J, Thoma S, Simpson S, Illum S, Arora N, Chidlow JH Jr, Langston W, Teng X, Lefer DJ, Patel RP, Kevil CG. Chronic sodium nitrite therapy augments ischemia-induced angiogenesis and arteriogenesis. *Proc Natl Acad Sci* 2008;105:7540-7545.
 23. Li J, Wei Y, Liu K, Yuan C, Tang Y, Quan Q, Chen P, Wang W, Hu H, Yang L. Synergistic effects of FGF-2 and PDGF-BB on angiogenesis and muscle regeneration in rabbit hindlimb ischemia model. *Microvasc Res* 2010;80:10-17.
 24. Hoefer IE, Royen NV, Buschmann IR, Piek JJ, Schaper W. Time course of arteriogenesis following femoral artery occlusion in the rabbit. *Cardiovasc Res* 2001;49:609-617.