

# Involvement of brain-derived neurotrophic factor in exercise-induced cardioprotection of post-myocardial infarction rats

BI-LEI WANG<sup>1,2\*</sup>, HONG JIN<sup>2\*</sup>, XI-QIONG HAN<sup>2</sup>, YANG XIA<sup>1</sup> and NAI-FENG LIU<sup>2</sup>

<sup>1</sup>Department of Rehabilitation and <sup>2</sup>Department and Institute of Cardiology, Zhongda Hospital, Medical School of Southeast University, Nanjing, Jiangsu 210009, P.R. China

Received April 25, 2018; Accepted August 23, 2018

DOI: 10.3892/ijmm.2018.3841

**Abstract.** Exercise induces a number of benefits, including angiogenesis in post-myocardial infarction (MI); however, the underlying mechanisms have not been fully clarified. Neurotrophic brain-derived neurotrophic factor (BDNF) serves a protective role in certain adult cardiac diseases through its specific receptor, BDNF/NT-3 growth factors receptor (TrkB). The present study explored the mechanisms by which exercise improves cardiac function, with a focus on the involvement of the BDNF/TrkB axis. MI rats were assigned to Sham, sedentary, exercise, exercise with K252a (a TrkB inhibitor), and exercise with NG-nitro-L-arginine methyl ester (L-NAME) groups. The exercise group was subjected to 8 weeks of treadmill running. The results demonstrated that the rats in the exercise group exhibited increased myocardial angiogenesis and improved cardiac function, which was attenuated by K252a. Exercise induced activation of the BDNF/TrkB axis in the ischaemic myocardium and increased serum BDNF levels were abated by exposure to L-NAME. Improvements in angiogenesis and left ventricular function exhibited a positive association, with changes in serum BDNF. In the *in vitro* experiments, human umbilical vein endothelial cells were exposed to shear stress (SS) of 12 dyn/cm<sup>2</sup> to mimic the effects of exercise training on vascular tissue. An increased tube-forming capacity, and a nitric oxide (NO)-dependent prolonged activation of the BDNF/TrkB-full-length axis over 12 h, but not the TrkB-truncated axis, was observed. The SS-related angiogenic response was attenuated by TrkB inhibition. Overall, these results demonstrate that exercise confers

certain aspects of its cardioprotective effects through the activation of the BDNF/TrkB axis in an NO-dependent manner, a process in which fluid-induced SS may serve a crucial role.

## Introduction

Brain-derived neurotrophic factor (BDNF), a major type of neurotrophin, and its receptor BDNF/NT-3 growth factors receptor (TrkB), are known to participate in the regulation of different processes including neuronal development, differentiation, survival and function (1-3). In addition to regulating several nervous system-associated processes, the BDNF/TrkB axis serves a cardioprotective role in certain adult cardiac diseases, a phenomenon that has attracted increasing attention in previous years (4-6). The BDNF/TrkB axis may exert its cardioprotective effects, at least partly, by promoting angiogenesis in the ischaemic myocardium (6). A previous study has demonstrated that BDNF-knockout mice exhibit increased endotheliocyte apoptosis in the coronary arteries and capillary tissue; however, BDNF overexpression in cardiac tissue increases capillary density (6). The exogenous delivery of BDNF to the ischaemic hearts of myocardial infarction (MI) rats improved angiogenesis and cardiac function (7,8). In addition, BDNF/TrkB signalling has been identified to be essential for *in vivo* myocardial performance (9). Loss of TrkB-T in cardiomyocytes caused cardiomyopathy (10). Clinical data also indicated that BDNF serves a beneficial role in cardiovascular homeostasis and/or cardiovascular disease pathogenesis (11-13).

Exercise training has been indicated to induce benefits, including angiogenesis, specifically in ischaemic hearts: Clinical studies have demonstrated that treadmill training yields significant improvements in collateral vessel growth in patients with coronary artery disease (14), and animal experiments have revealed increased microvessel density and improved left ventricular function in post-MI rats following several weeks of moderate exercise (15,16). However, the mechanisms by which these changes occur remain to be elucidated. Previous data have suggested that improvements in endothelial function and structure in response to exercise correlate with shear stress (SS) (17). Exercise training-induced blood flow-mediated elevations in laminar SS have been demonstrated to stimulate

**Correspondence to:** Dr Nai-Feng Liu, Department and Institute of Cardiology, Zhongda Hospital, Medical School of Southeast University, 87 Dingjiaqiao Street, Nanjing, Jiangsu 210009, P.R. China  
E-mail: zdykfk@126.com

\*Contributed equally

**Key words:** exercise training, brain-derived neurotrophic factor, myocardial infarction, shear stress, nitric oxide

growth of collateral vessels in heart tissue (18), and to serve an atheroprotective role in the vasculature (19).

The hypothesis that the BDNF/TrkB axis mediates the cardioprotective effects of exercise in ischaemic hearts derives from studies in which physical training elicited increases in plasma or serum BDNF concentrations in humans (20). In MI rats, exercise was identified to increase cardiac BDNF expression (21). In addition, Prigent-Tessier *et al.* (22) suggested that increases in physiological SS intensity, which are expected with exercise training, have been used to mimic the effects of physical training on the endothelium. In that study, cellular BDNF secretion levels were identified to be proportional to shear stress intensity (22). However, an additional study regarding the effects of SS on the endothelium yielded conflicting results (23). More studies are required to establish the role of the BDNF/TrkB axis in the exercise-induced increase in neoangiogenesis and cardiac function, and additional data are required to establish the effect of exercise on circulating BDNF changes in post-MI status and to associate BDNF levels with exercise-induced improvements in cardiovascular health.

Therefore, the current study aimed to address these issues. For this purpose, prolonged BDNF/TrkB axis activation following exercise training was first determined in post-MI rat hearts. Increased circulating BDNF levels were also identified to be correlated with an exercise-induced increase in neoangiogenesis and cardiac function. Then, the effect of endothelial nitric oxide synthase (eNOS)/nitric oxide (NO) on exercise-associated activation of the BDNF/TrkB axis was assessed by using the post-MI rats and human umbilical vein endothelial cells (HUVECs) subjected to 12 dyn/cm<sup>2</sup> SS and exposed to NG-nitro-L-arginine methyl ester (L-NAME), the NOS inhibitor. Finally, whether the inhibition of the BDNF/TrkB pathway attenuated exercise-responsive neoangiogenesis and improved cardiac function was investigated by using post-MI rats exposed to K252a, the TrkB inhibitor.

## Materials and methods

**Animals.** The present study was approved by the Animal Care and Research Committee of Southeast University, and all experiments were conducted in accordance with the European Convention for the Protection of Vertebrates Used for Experiments and Other Scientific Purposes (European Union Directive 86/609EEC, 1986). A total of 120 male Sprague-Dawley rats (12 weeks old, 220–250 g) were purchased from Shanghai Laboratory Animal Centre (Shanghai, China) and housed in the animal room of the Medical School of Southeast University (Nanjing, China). All rats were randomly divided into the following experimental groups: A sham infarction (Sham) group, a sedentary MI (MIC) group, an MI + exercise (MIE) group, an MI + exercise + L-NAME (MIE+L) group, an MI+exercise+K252a (MIEK) group and an MI + exercise + vehicle (MIEV) group. The MIE+L group included rats subjected to exercise and treatment with L-NAME (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). These rats received L-NAME (100 µg/ml) orally in their drinking water during an 8-week period. Fluid consumption was monitored daily. The dose of L-NAME (25 mg/kg/day) used here was selected based on the results of a previous study (24).

The MIEK group included rats subjected to exercise and daily intraperitoneal injections of a TrkB inhibitor (K252a; Sigma-Aldrich; Merck KGaA), which was administered at a dose of 100 µg/kg/day during the seventh and eighth weeks of the experiment. K252a was prepared in 25% dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA) at a working concentration of 10 µg/ml (25). The MIEV group included rats subjected to exercise and daily intraperitoneal injections of an equivalent volume of DMSO, which was administered during the seventh and eighth weeks of the experiment. The Sham and MIC animals were maintained in separate cages and were allowed *ad libitum* access to chow and water. The room temperature was 20–25°C and the humidity was 40–70%.

**Rat model of MI.** The animals were anaesthetized with intraperitoneal injections of pentobarbital sodium (0.4 mg/10 g). The rats were endotracheally intubated and then ventilated with a rodent ventilator (Harvard Apparatus, Holliston, MA, USA) at 70 breaths per min. Subsequent to exposure of the heart via left thoracotomy, the left anterior descending (LAD) coronary artery was ligated proximally with an 8-0 silk suture. For the rats in the Sham group, the chest was opened but the LAD was left patent. Each animal was allowed to recover for 48 h prior to initiation of exercise training.

**Exercise training protocol.** The training schedule proposed by Leosco *et al.* (15) was used, with specific modifications. Prior to the exercise protocol, all animals underwent a preconditioning training regimen for 3 days comprising 30 min of daily running at a speed of 15 m/min on a rodent treadmill (FT-200; Chengdu Taimeng Technology Co., Ltd., Chengdu, China). A total of 1 week following cardiac surgery, the animals in the MIE, MIE+L, MIEK and MIEV groups began training. In the first week of the running regimen, the animals began with a running speed of 0.3 km/h for 10 min/day. The animals' exercise speed was gradually increased from 0.3 to 1.2 km/h. The animals participated in the exercise regimen 5 times per week throughout the 8-week period, with two days of relaxation allowed per week. At the end of the exercise protocol, certain animals in each group were allowed 3 h of rest prior to undergoing tissue preparation, and the other animals were allowed 48 h of rest prior to cardiac functional evaluations.

**Echocardiographic analysis and heart/body weight measurement.** The rats underwent cardiac functional evaluations, prior to exercise training, at 1 week after cardiac surgery and following the 8-week period of exercise training. Cardiac function was evaluated using transthoracic echocardiography (Visual Sonics Vevo 770; VisualSonics, Inc., Toronto, ON, Canada). When performing echocardiography, isoflurane was used for the induction (4.5%) and maintenance (1.5%) of inhaled anaesthesia. Left parasternal short-axis two-dimensional M-mode images were recorded just below the level of the papillary muscles using a 30-MHz linear transducer. The following haemodynamic parameters were measured: The left ventricular internal diameter at end-systole (LVIDs); the left ventricular internal diameter at end-diastole (LVIDd); the ejection fraction (EF); and left ventricular fractional shortening (LVFS). Each ultrasound examination was performed

by an experienced examiner blinded to the study data. All the values are averages of 5 consecutive cardiac cycles.

Following the ultrasound examination, the animals were anaesthetized with intraperitoneal injections of pentobarbital sodium (0.4 mg/10 g) and the heart was perfused with PBS and rapidly resected. The heart was desiccated with filter paper and then weighed using an electronic analytical balance.

**Tissue preparation.** Prior to exercise training at 1 week after cardiac surgery, venous blood samples were collected from the rats in the Sham, MIC, MIE and MIE+L groups from the angular or caudal veins. The animals were anaesthetized with pentobarbital sodium (0.4 mg/10 g) after 3 h of rest at the end of the exercise protocol. A total of 0.5 ml of venous blood was collected by cardiac puncture, and then the animals were sacrificed. The left ventricles were dissected and then cut in half transversally at the level of the ligation. One section was fixed in 10% formalin and embedded in paraffin prior to use in immunohistochemical assessments at room temperature for 24 h, and the other section was snap-frozen prior to storage at -80°C for subsequent analyses.

**Immunohistochemistry.** BDNF, TrkB and hematopoietic progenitor cell antigen CD34 (CD34) expression levels were detected in serial paraffin-embedded sections of the left ventricle using standard histological and immunostaining procedures. The heart sections were deparaffinized and then rehydrated in a graded alcohol series (xylene I for 20 min, xylene II for 20 min, 100% alcohol I for 5 min, 100% alcohol II for 5 min, 95% alcohol for 5 min, 80% alcohol for 5 min and PBS for 3x3 min). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide. Subsequent to washing with PBS, the sections were subjected to microwave antigen retrieval (800 W; 5 min) in sodium citrate buffer (0.01 mol/l; pH 6.0). Bovine serum albumin (5%; Beyotime Institute of Biotechnology, Haimen, China) was used as blocking agent and the blocking step was performed at 37°C for 30 min. The following antibodies were used for the experiment: A sheep polyclonal antibody to BDNF (cat. no. ab75040; Abcam, Cambridge, UK; 1:200), a rabbit polyclonal antibody to TrkB (cat. no. ab33655; Abcam; 1:50), and a rabbit polyclonal antibody to CD34 (cat. no. pab18289; Abnova, Taipei, Taiwan; 1:200). The sections were incubated with the antibodies overnight at 4°C. The sections were then washed prior to incubation with the appropriate secondary antibodies, including horseradish peroxidase-conjugated anti-goat secondary antibody (cat. no. 81-1620; Thermo Fisher Scientific, Inc., Waltham, MA, USA; 1:800) and anti-rabbit secondary antibody (cat. no. PV-6001; ZsBio, Beijing, China; 1:800), and then colour-developed by DAB (ZsBio) and counterstained with 1 g/l haematoxylin (Sigma-Aldrich; Merck KGaA) at room temperature for 3 min. Light microscopy was used to capture images of the border zone, and 5 high-power fields (HPFs) in each sample were selected at a magnification of x200. The cells were then analysed and counted in a blinded manner.

**Cell culture and shear stress treatment.** HUVECs were purchased from Wuxi Puhe Biotechnological Co., Ltd. (Wuxi, China). The cells were placed in a streamer laminar flow chamber and then exposed to static conditions or high

SS (12 dyn/cm<sup>2</sup>, HSS) for 1, 3, 6 or 12 h. Circulating and non-circulating flow patterns were used to produce the SS. In certain experimental protocols, the cells were treated with the NO synthase inhibitor L-NAME (10<sup>-4</sup> mol/l), the NO donor S-nitroso-N-acetyl-D, L-penicillamine (SNAP; 10<sup>-6</sup> mol/l; Sigma-Aldrich; Merck KGaA), or a TrkB-Fc chimera (1 µg/ml; Sigma-Aldrich; Merck KGaA). The cells and medium were stored at -80°C until BDNF, TrkB and phosphorylated TrkB expression levels were measured.

**Measurement of serum and media BDNF levels.** Serum and media BDNF concentrations were detected using an ELISA kit (cat. no. DBNT00; R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. The samples were analysed in duplicate, and then the mean BDNF concentration for each sample was calculated. No significant cross-reactivity between the BDNF antibodies and any other cytokines was observed. The limit of sensitivity was fixed at <20 pg/ml.

**Western blot analysis.** The heart tissue samples were homogenized, and the HUVECs were isolated and washed twice with cold PBS. Total protein was lysed by radioimmunoprecipitation assay lysis buffer (cat. no. 89900; Thermo Fisher Scientific, Inc.) as described previously (4). The supernatants were collected, and the protein concentrations were determined using a DC Protein Assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein extracts (20 µg) were separated by SDS-PAGE (10% separating gel and 4% stacking gel) and then transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA), which were blocked in TBS containing 5% (w/v) non-fat dry milk at room temperature overnight. BDNF, TrkB and phosphorylated TrkB (p-TrkB) expression levels were detected with the following primary antibodies: An anti-BDNF antibody (cat. no. ab75040; Abcam; 1:200), an anti-TrkB antibody (cat. no. ab33655; Abcam; 1:200), and an anti-TrkB (phospho Y515) antibody (cat. no. ab109684; Abcam; 1:200). The membranes were incubated with these primary antibodies overnight at 4°C and then visualized using an anti-rabbit IgG secondary antibody (cat. no. A27036; Thermo Fisher Scientific, Inc.; 1:10,000) or anti-goat IgG secondary antibody (cat. no. 81-1620; Thermo Fisher Scientific, Inc.; 1:5,000) for 1 h at room temperature and an EZ-ECL system (ChemiDoc XRS+ System, Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal protein loading was confirmed by measuring total actin expression levels (cat. no. ab8226; 1:2,000; Abcam), and the data pertaining to target protein expression levels were analysed using ImageJ 4.0.1 software (National Institutes of Health, Bethesda, MD, USA).

**Reverse transcription quantitative polymerase chain reaction (RT-qPCR).** Total RNA was isolated from HUVECs and purified with TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The isolated total RNA (3 µg) was converted into cDNA in a 20 µl reaction mixture using the RevertAid First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA) according to the manufacturer's protocol. qPCR was performed on a StepOnePlus PCR System (Bio-Rad

Laboratories, Inc.) using Fast SYBR Green PCR Master Mix (Toyobo Life Sciences, Osaka, Japan) according to the manufacturer's protocol. The  $2^{-\Delta\Delta C_q}$  method was used for quantification (26). Pre-denaturation process was at 95°C for 5 min, denaturation process was at 95°C for 15 sec, annealing process was at 60°C for 20 sec, and stretching process was at 72°C for 40 sec. Melting curve analysis of the PCR products was performed to ensure the specificity of the amplification. The expression levels of the target genes were normalized to those of GAPDH, which served as an endogenous control. Primers specific for BDNF, TrkB and GAPDH were constructed by Genescript Co., Ltd. (Nanjing, China). The sequences of the primers were as follows: BDNF forward, 5-AACATAAGG ACGCGACTT-3; BDNF reverse, 5-TGCAGTCTTTTATC TGCCG-3; TrkB forward, 5-GATCTTCACCTACGGCAA GC-3; TrkB reverse, 5-TCGTCAAGTTCTGAAGGAGT-3; GAPDH forward: 5-AACTTTGGCATTGTGGAAGG-3; and GAPDH reverse: 5-TGTGAGGGAGATGCTCAGTG-3.

**Tube formation.** HUVECs ( $1.5 \times 10^4$ ) exposed to SS or static conditions for 6 h were suspended in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) with 5% FBS (Gibco; Thermo Fisher Scientific, Inc.) and then seeded in a 96-well plate precoated with Matrigel® (BD Biosciences, Franklin Lakes, NJ, USA). The precoating step was performed at 0°C and the plate was placed in the cell incubator at 37°C for 1 h. The cells were incubated for 24 h at 37°C, and then the capillary-like tube structures that had formed from the HUVECs were observed by a phase-contrast microscope (x100). The number of tubes per low-power field was calculated and compared using ImageJ 4.0.1 software.

**Statistical analysis.** SPSS 21.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. The data in the present study conformed to Gaussian distribution. The data are expressed as the mean  $\pm$  standard error of the mean and are representative of at least 3 independent experiments. Differences among different groups were assessed using one-way analysis of variance followed by a Least Significant Difference post-hoc test. The associations between serum BDNF levels and LV function and CD34<sup>+</sup> cell counts were analysed using Pearson's correlation coefficient analysis.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**BDNF and TrkB protein expression increases in the ischaemic heart zone (IHZ) following exercise training.** BDNF and TrkB expression levels in MI heart tissues were measured by immunostaining. MI rats subjected to exercise exhibited markedly increased BDNF protein expression in the IHZ compared with sedentary MI rats. The exercise-induced increase in BDNF protein expression was inhibited by L-NAME (Fig. 1A). Representative original images indicating BDNF staining are presented in Fig. 1B. TrkB protein expression in the ischaemic heart tissues was increased compared with that in Sham heart tissues; however, no significant difference in TrkB protein expression was identified among the MIC, MIE and MIE+L groups (Fig. 1C). Representative original images demonstrating TrkB staining are presented in Fig. 1D.

**BDNF/TrkB axis is activated in the IHZ following exercise training.** BDNF expression in the IHZ by was examined by western blot analysis. As demonstrated in Fig. 2A, BDNF protein expression in the ischaemic heart tissues was decreased compared with that in the Sham heart tissues. Exercise training increased BDNF protein expression, which was inhibited by L-NAME. The MIEK rats indicated increased BDNF expression compared with the MIE and MIEV rats.

The expression and phosphorylation levels of the two types of TrkB [the full-length (FL) and truncated (T) isoforms] were examined by western blot analysis. As demonstrated in Fig. 2B-a, the MIE, MIEV and MIEK rats exhibited increased TrkB-FL expression compared with the MIC rats. No significant differences among the Sham, MIE, MIE+L, MIEV and MIEK groups were observed (Fig. 2B-a). As indicated in Fig. 2B-b, TrkB-T protein expression levels in MI hearts were increased compared with those in Sham hearts; no significant difference in TrkB-T expression was observed among the MIC, MIE, MIE+L and MIEV groups. The MIEK rats exhibited an increased TrkB-T protein content compared with the other groups (Fig. 2B-b).

It was also identified that TrkB-FL and TrkB-T phosphorylation levels in the IHZs of the groups subjected to exercise, with the exception of the MIEK group, were increased compared with those in the IHZs of the other groups (Fig. 2C and D). The phosphorylation levels of the two receptors in the MIE+L-NAME and MIEK groups were decreased compared with those in the MIE and MIEV groups (Fig. 2C and D).

**Angiogenic responses to exercise training are attenuated by the TrkB inhibitor in post-MI rat hearts.** Subsequent to the assessment of the effects of exercise on BDNF/TrkB axis activation in post-MI rats, CD34 expression was quantified by immunohistochemistry. CD34 is an antibody identified specifically in vascular endothelial cells, and its expression may be used to evaluate angiogenesis in ischaemic myocardial tissue (27). The surviving myocardium was stained with standard immunohistochemical reagents, and then the numbers of CD34<sup>+</sup> microvascular endothelial cells were counted. As demonstrated in Fig. 3B, the numbers of CD34<sup>+</sup> microvascular cells were increased in the MI exercise groups compared with those in the MIC group, indicating that exercise promoted angiogenesis in the surviving myocardium. Notably, the number of CD34<sup>+</sup> microvascular cells was decreased in the MIEK group compared with those in the MIE and MIEV groups; however, the number of CD34<sup>+</sup> microvascular cells in the MIEK group was increased compared with that in the MIC group. Representative original images demonstrating CD34<sup>+</sup> staining are presented in Fig. 3A. These results suggest a possible explanation of why cardiac function was decreased in the MIEK group and additionally demonstrate the necessity of BDNF/TrkB axis activation in exercise-induced angiogenesis following MI.

**Effects of exercise training and K252a on cardiac function and the echocardiographic data.** As summarized in Table I, training decreased body weight in the MI rats subjected to exercise compared with the sedentary rats. In addition, heart weight and the heart weight: Body weight ratio were increased in the trained rats compared with those in the



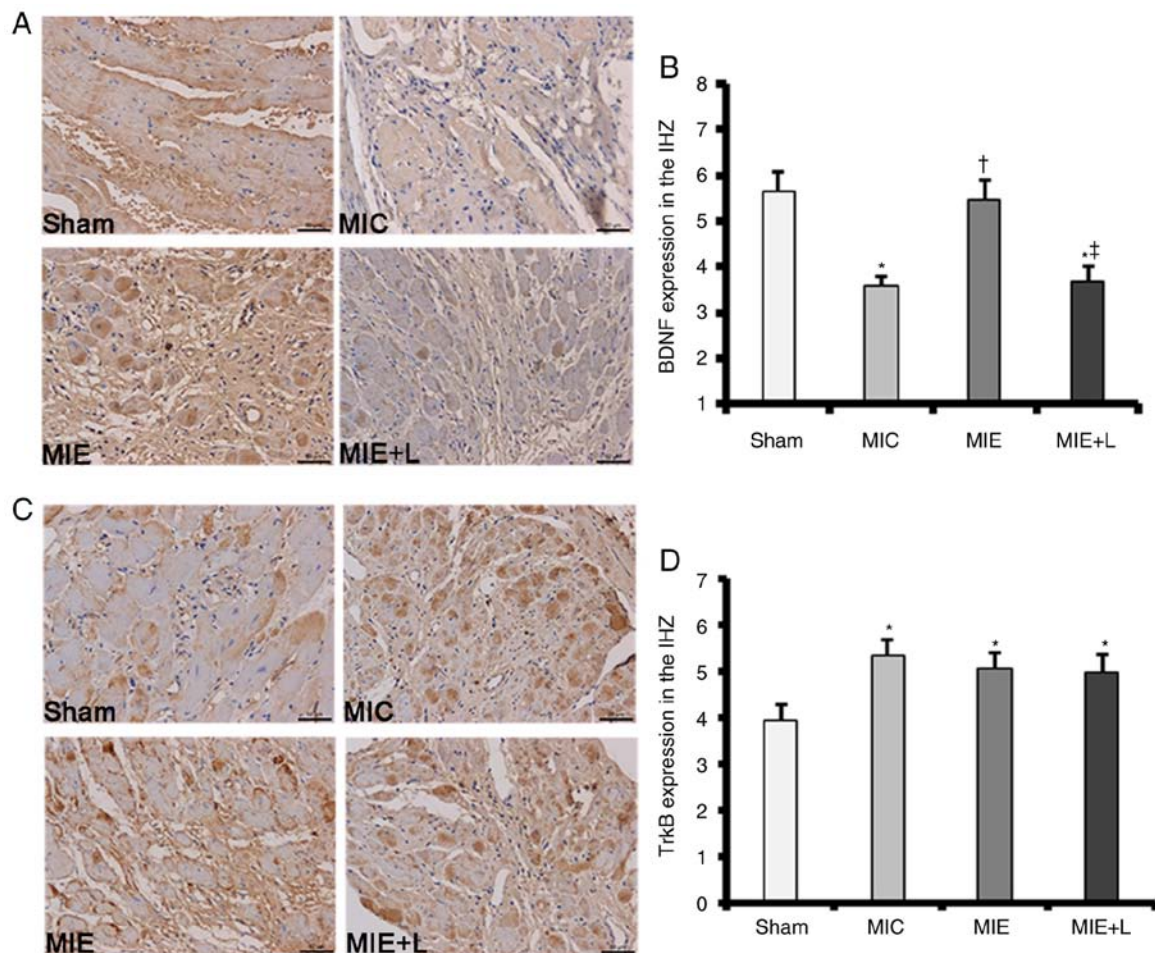


Figure 1. Exercise training upregulates BDNF and TrkB protein expression in the IHZ in the myocardium, and L-NAME inhibits this effect. (A) Representative images of BDNF immunostaining (DAB) in the IHZ of each group. (B) Quantitative results of BDNF immunostaining in the IHZ of each group. \* $P < 0.01$  vs. Sham; † $P < 0.01$  vs. MIC and ‡ $P < 0.01$  MIE+L vs. MIE. (C) Representative images of TrkB immunostaining (DAB) in the IHZ of each group. (D) Quantitative results of TrkB immunostaining in the IHZ of each group \* $P < 0.01$  vs. Sham. Scale bar =  $50 \mu\text{m}$ . The values are presented as the mean  $\pm$  standard error of the mean ( $n=6$ ). BDNF, brain-derived neurotrophic factor; TrkB, BDNF/NT-3 growth factors receptor; IHZ, ischaemic heart zone; L-NAME, NG-nitro-L-arginine methyl ester; MI, myocardial infarction; MIC, sedentary MI group; MIE, MI + exercise group; MIE+L, MI + exercise + L-NAME group.

sedentary rats. Heart weight and the heart weight: body weight ratio measurements indicated modest but non-significant reductions in the MIEK group compared with those in the MIE and MIEV groups. At 9 weeks after surgery, the EF was evidently improved in the MIE and MIEV groups; however, the EF remained sub-optimal in the MIEK group compared with those in the other exercise groups. Training favourably affected LV geometry, as the rats in the training groups exhibited decreased LVIDd and LVIDs diameters compared with those in the MIC group. Nevertheless, the MIEK group demonstrated larger LV diastolic and systolic diameters compared with the MIE and MIEV groups. LVFS was increased in the exercise groups compared with that in the MIC group; however, the MIEK group indicated less LVFS compared with the MIE and MIEV groups. These data indicate that the effects of exercise on cardiac function were antagonized by K252a and suggest that the BDNF/TrkB axis served a role in mediating the cardioprotective effects of exercise (Table I).

*Serum BDNF levels increase in response to chronic exercise training and are correlated with angiogenesis and cardiac*

*function.* No significant differences in serum BDNF concentrations were observed among the Sham, MIC, MIE and MIE+L groups at the beginning of the exercise programme (Fig. 4A-a). However, the BDNF concentrations were increased in the MIE group ( $236.3 \pm 35.7 \text{ pg/ml}$ ) compared with those in the MIC ( $186.5 \pm 19.1 \text{ pg/ml}$ ) and MIE+L groups ( $180.4 \pm 20.9 \text{ pg/ml}$ ) at the end of exercise programme (Fig. 4A-b). Simultaneously, a significant decrease in serum BDNF was observed in the rats of the MIC and MIE+L groups compared with that in the Sham rats ( $223.9 \pm 30.1 \text{ pg/ml}$ ; Fig. 4A-b).

The associations between serum BDNF concentrations and LV function *in vivo* and the numbers of CD34<sup>+</sup> endothelial cells in the IHZ were also examined; LV function was determined by the EF value. A positive association was identified between serum BDNF levels and the CD34<sup>+</sup> cell counts ( $r=0.542$ ,  $P=0.006$ ; Fig. 4B) and the EF ( $r=0.502$ ,  $P=0.013$ ; Fig. 4C). Therefore, increased levels of serum BDNF were associated with cardiac function and angiogenesis. In addition, the association between the variation of BDNF serum levels and the EF prior and subsequent to exercise is demonstrated in Fig. 4D. Improvement of the EF exhibited a positive association with changes in serum BDNF.

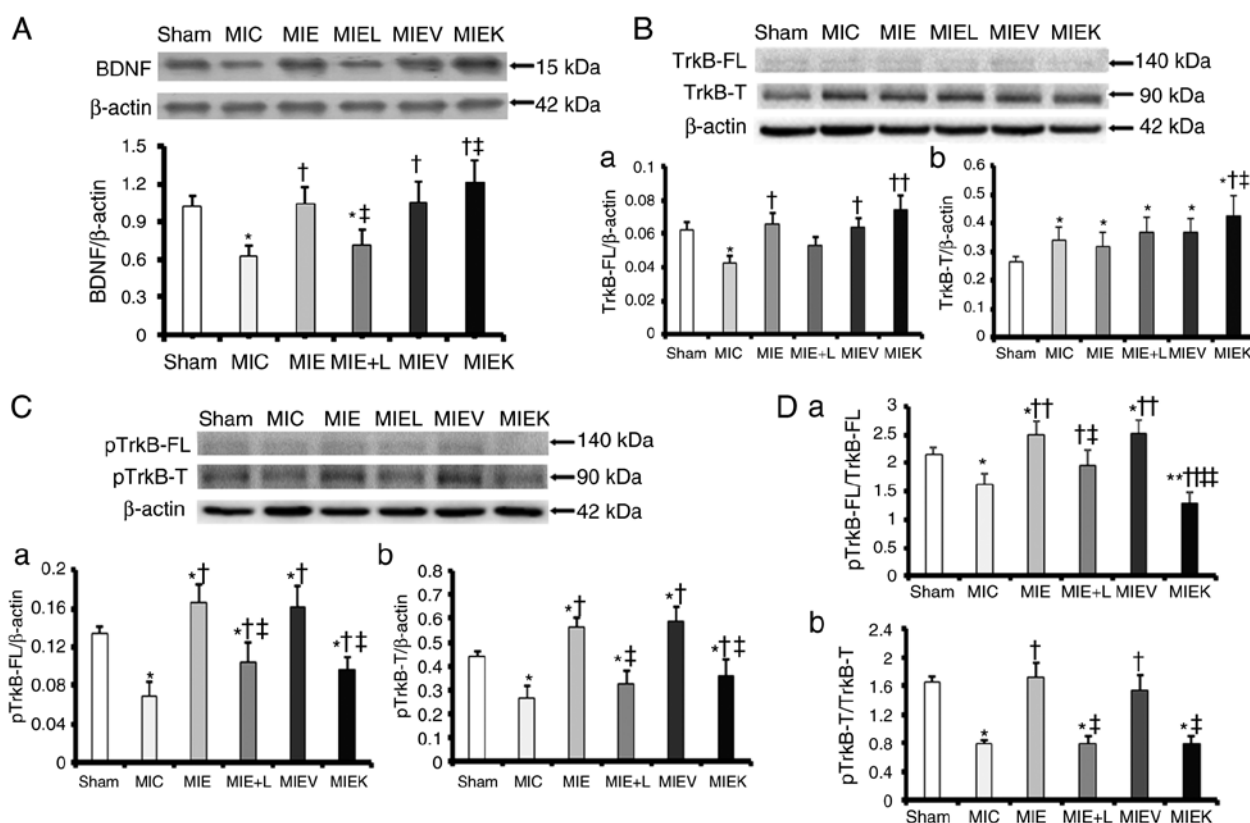


Figure 2. Exercise training activates the BDNF/TrkB axis in the IHZ of the myocardium, which is attenuated by L-NAME and the TrkB inhibitor. (A) Representative western blot analysis images for BDNF and the protein level analysis. \*P<0.05 vs. Sham, †P<0.05 vs. MIC and ‡P<0.05 vs. MIE. (B) Representative western blot analysis images for TrkB-FL and TrkB-T: (a) TrkB-FL protein level analysis. \*P<0.05 vs. Sham, †P<0.05 vs. MIC and ‡P<0.01 MIEK vs. MIC; (b) TrkB-T protein level analysis. \*P<0.05 vs. Sham, †P<0.05 vs. MIC and ‡P<0.05 vs. MIE. (C) Representative western blot analysis images for phosphorylated TrkB-FL and TrkB-T: (a) Phosphorylated TrkB-FL protein level analysis. \*P<0.01 vs. Sham, †P<0.01 vs. MIC, ‡P<0.01 vs. MIE; (b) phosphorylated TrkB-T protein level analysis. \*P<0.01 vs. Sham, †P<0.01 vs. MIC and ‡P<0.01 vs. MIE. (D) The ratio of (a) pTrkB-FL to total TrkB-FL (\*P<0.05 MIC, MIE, MIEV vs. Sham, \*\*P<0.01 MIEK vs. Sham, †P<0.05 MIE+L vs. MIC, ‡P<0.01 MIE, MIEV, MIEK vs. MIC; §P<0.05 MIE+L vs. MIE, ¶P<0.01 MIEK vs. MIE) and the ratio of (b) phosphorylated TrkB-T to total TrkB-T (\*P<0.01 vs. Sham, †P<0.01 vs. MIC and ‡P<0.01 vs. MIE). The values are presented as the mean  $\pm$  standard error of the mean (n=6). BDNF, brain-derived neurotrophic factor; TrkB, BDNF/NT-3 growth factors receptor; p, phosphorylated; MI, myocardial infarction; L-NAME, NG-nitro-L-arginine methyl ester; MIC, sedentary MI group; MIE, MI + exercise group; MIE+L, MI + exercise + L-NAME group; MIEK, MI+exercise+K252a group; MIEV, MI + exercise + vehicle group.

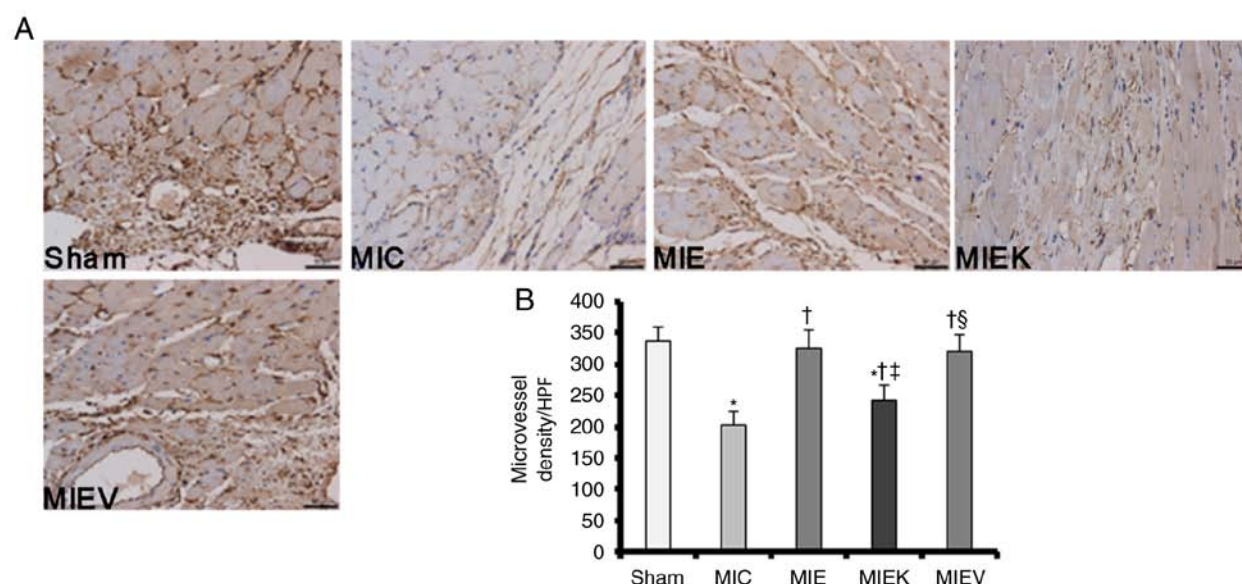


Figure 3. Angiogenic responses to exercise training were attenuated by the TrkB inhibitor in post-MI rat hearts. (A) Representative images of CD34-stained myocardium in each group. Scale bar=50  $\mu$ m. (B) Quantitative results of CD34-positive cells per field. \*P<0.01 vs. Sham, †P<0.01 vs. MIC, ‡P<0.01 vs. MIE and §P<0.01 vs. MIEK. The values are presented as the mean  $\pm$  standard error of the mean (n=8). TrkB, brain-derived neurotrophic factor/NT-3 growth factors receptor; CD34, hematopoietic progenitor cell antigen CD34; MI, myocardial infarction; MIC, sedentary MI group; MIE, MI + exercise group; MIEK, MI+exercise+K252a group; MIEV, MI + exercise + vehicle group.

Table I. Physiological, haemodynamic and echocardiographic data for the Sham and MI rats prior and subsequent to the exercise training protocol.

Measurements	Groups				
	Sham	MIC	MIE	MIEK	MIEV
Physiological data					
Body wt, kg	0.389±0.022	0.360±0.019 <sup>a</sup>	0.317±0.023 <sup>b</sup>	0.335±0.021 <sup>b,c</sup>	0.321±0.025 <sup>b</sup>
Heart wt, g	1.003±0.053	1.998±0.178 <sup>a</sup>	2.287±0.106 <sup>b</sup>	2.199±0.109 <sup>b</sup>	2.278±0.137 <sup>b</sup>
Heart wt/body wt ratio	2.59±0.13	5.53±0.53 <sup>a</sup>	7.17±0.29 <sup>b</sup>	6.89±0.33 <sup>b</sup>	7.11±0.39 <sup>b</sup>
Haemodynamic and echocardiographic data					
EF, %					
1 week	71.68±5.19	32.97±3.22 <sup>a</sup>	33.76±3.21 <sup>a</sup>	35.4±2.77 <sup>a</sup>	34.12±2.93 <sup>a</sup>
9 weeks	75.45±5.63	36.96±8.05 <sup>a</sup>	56.89±9.21 <sup>b</sup>	45.4±8.54 <sup>b,c</sup>	55.72±8.90 <sup>b</sup>
LVID (mm)					
Diastolic					
1 week	7.27±0.51	11.29±0.57 <sup>a</sup>	11.23±0.61 <sup>a</sup>	11.20±0.63 <sup>a</sup>	11.30±0.69 <sup>a</sup>
9 weeks	7.41±0.22	11.69±0.63 <sup>a</sup>	9.06±0.31 <sup>b</sup>	11.09±0.55 <sup>a,c</sup>	9.34±0.34 <sup>b</sup>
Systolic					
1 week	3.77±0.19	10.07±0.69 <sup>a</sup>	10.04±0.53 <sup>a</sup>	10.01±0.55 <sup>a</sup>	10.11±0.61 <sup>a</sup>
9 weeks	3.82±0.20	9.95±0.60 <sup>a</sup>	6.54±0.40 <sup>b</sup>	8.88±0.40 <sup>b,c</sup>	6.98±0.30 <sup>b</sup>
LVFS, %					
1 week	48.19±4.94	10.84±1.98 <sup>a</sup>	10.69±1.97 <sup>a</sup>	10.75±1.41 <sup>a</sup>	10.67±1.79 <sup>a</sup>
9 weeks	48.45±4.40	14.88±2.37 <sup>a</sup>	27.81±2.92 <sup>b</sup>	19.93±2.13 <sup>b,c</sup>	25.27±3.17 <sup>b</sup>

Wt, weight; EF, ejection fraction; LVID, left ventricular internal diameter; LVFS, left ventricular fractional shortening; MI, myocardial infarction; MIC, sedentary MI group; MIE, MI + exercise group; MIEK, MI+exercise+K252a group; MIEV, MI + exercise + vehicle group. <sup>a</sup>P<0.05 vs. Sham, <sup>b</sup>P<0.05 vs. Sham and MIC and <sup>c</sup>P<0.05 vs. MIE and MIEV.

SS of 12 dyn/cm<sup>2</sup> increases BDNF/TrkB-FL protein levels and sustains TrkB-FL activation in HUVECs. To gain insight into the mechanisms by which physical training induces angiogenesis and to explain the upregulated BDNF/TrkB protein expression observed in the animal experiments, the effects of SS on the expression of BDNF and the two types of TrkB were examined. HUVECs were placed in a laminar flow chamber and exposed to high SS at 12 dyn/cm<sup>2</sup> for 1, 3, 6 or 12 h. The western blot analysis results, which are demonstrated in Fig. 5A, revealed that BDNF protein expression levels were persistently upregulated by exposure to SS of 12 dyn/cm<sup>2</sup>. Specifically, BDNF protein expression levels were increased markedly after 1 h of SS exposure (147±17% of the static control), peaked after 3 h of SS exposure (347±41%) prior to partially normalizing after 6 h of SS exposure (265±33%), and remained elevated after 12 h of SS exposure (163±21%). Consistent with the data indicating that SS elicited sustained increases in cellular BDNF secretion, additional data suggested that BDNF levels in the medium exposed to SS for 12 h were 379±27% (407.65±27.31 pg/ml) increased compared with those in the medium exposed to static conditions (Fig. 5B).

Western blot analysis also revealed that TrkB-FL protein expression levels were upregulated by SS. Specifically, as indicated in Fig. 5C, TrkB-FL protein expression levels increased after 1 h of SS exposure (211±32% of the static control), peaked after 3 h of SS exposure (388±27%) prior to

partial normalization after 6 h of SS exposure (146±20%), and decreased to baseline after 12 h of SS exposure (114±17%). As demonstrated in Fig. 5E, phosphorylated TrkB-FL protein expression levels increased more markedly compared with TrkB-FL protein expression levels and remained elevated after 12 h of SS exposure (558±65% after 1 h; 1,509±193% after 3 h; 1,216±107% after 6 h; and 739±79% after 12 h).

Notably, it was identified that TrkB-T protein expression and phosphorylation levels were not modified by SS at 12 dyn cm<sup>2</sup> (Fig. 5D and F).

#### eNOS/NO mediates SS-induced BDNF/TrkB regulation.

To assess whether eNOS/NO participates in the regulation of BDNF/TrkB expression, HUVECs were treated with the eNOS inhibitor L-NAME (10<sup>-4</sup> mol/l). Fig. 6A and B indicate that SS-induced increases in BDNF/TrkB-FL expression were completely abolished by L-NAME. To additionally assess whether NO contributes to upregulation of BDNF/TrkB expression, HUVECs were then exposed to SNAP, an NO donor, under static conditions (10<sup>-6</sup> mol/l). SNAP treatment increased BDNF protein expression levels after 1 h (227±26%) but did not have an effect on BDNF protein expression at subsequent time points (Fig. 6C). However, TrkB-FL protein levels were similar between unstimulated HUVECs and HUVECs exposed to SNAP (Fig. 6D). Therefore, SS regulates BDNF/TrkB expression in



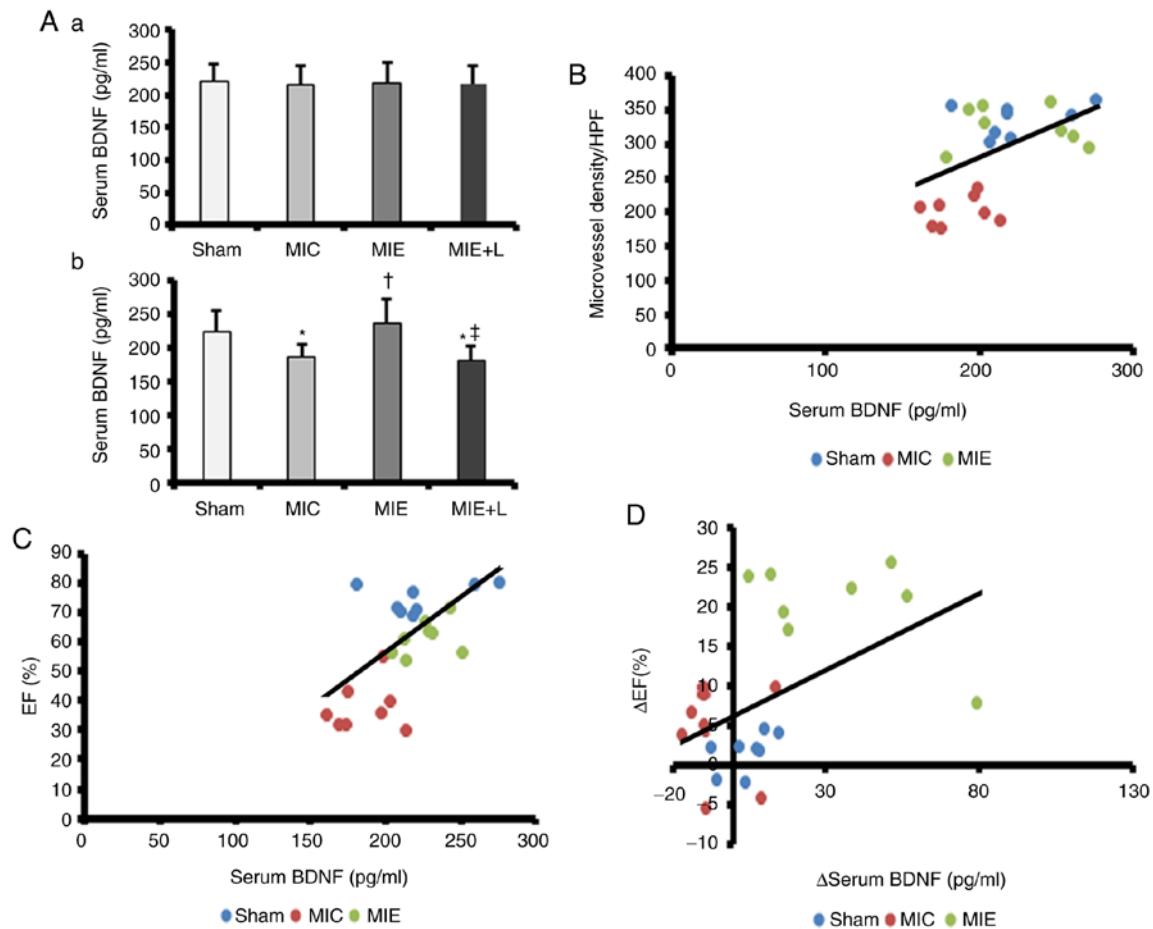


Figure 4. Serum BDNF level is associated with myocardium angiogenesis and left ventricle function. (A) ELISA measurements of the serum BDNF concentration at the (a) beginning and the (b) end of the exercise programme. \* $P < 0.05$  vs. Sham and † $P < 0.05$  vs. MIC. (B) The serum BDNF concentration was positively correlated with myocardial microvessel density ( $r = 0.542$ ,  $P = 0.006$ ). (C) The serum BDNF concentration was positively correlated with the EF ( $r = 0.631$ ,  $P = 0.001$ ). (D) A positive correlation was identified between improved serum BDNF levels and the EF ( $r = 0.502$ ,  $P = 0.013$ ). The values are presented as the mean  $\pm$  standard error of the mean ( $n = 8$ ). MI, myocardial infarction; L-NAME, NG-nitro-L-arginine methyl ester; MIC, sedentary MI group; MIE, MI + exercise group; MIE+L, MI + exercise + L-NAME group; BDNF, brain-derived neurotrophic factor; EF, ejection fraction; HPF, high-powered fields.

an NO-dependent manner in HUVECs, and NO release is sufficient to increase BDNF expression.

*SS-induced TrkB-FL upregulation is dependent on increases in BDNF levels in media.* To determine whether the aforementioned increases in BDNF/TrkB-FL protein levels reflected a transcriptional regulatory event, BDNF/TrkB-FL mRNA expression levels were assessed by RT-qPCR in HUVECs exposed to high SS (12 dyn/cm<sup>2</sup>). Prolonged increases in BDNF mRNA expression in HUVECs exposed to high SS for 1, 3, 6 or 12 h ( $190 \pm 15$ ,  $430 \pm 31$ ,  $525 \pm 53$  and  $641 \pm 52\%$  of the static control, respectively) were observed (Fig. 7A). Prolonged increases in TrkB-FL mRNA expression in HUVECs exposed to high SS for 1, 3, 6 or 12 h ( $258 \pm 17$ ,  $387 \pm 32$ ,  $498 \pm 43$  and  $697 \pm 55\%$  of the static control, respectively) were observed (Fig. 7B). To determine whether these SS-mediated increases in TrkB-FL expression were dependent on changes in BDNF levels in the media, HUVECs were treated with non-circulating fluid and BDNF/TrkB-FL mRNA expression levels were examined after 1, 3, 6 and 12 h. As demonstrated in Fig. 7C, BDNF mRNA expression levels increased continuously from 1–12 h post-treatment ( $251 \pm 15$ ,  $348 \pm 17$ ,  $525 \pm 21$  and  $693 \pm 25\%$  of the static control, respectively), while TrkB-FL mRNA expression

levels decreased steadily from 1–12 h post-treatment ( $35.3 \pm 4$ ,  $16.2 \pm 2$ ,  $11.0 \pm 1$  and  $6.98 \pm 0.7\%$  of static control, respectively) (Fig. 7D).

*BDNF/TrkB activation is associated with increases in tube-forming capacity.* To investigate whether the BDNF/TrkB pathway participated in high SS-induced capillary-like tube formation, a tube formation assay was performed, the results of which were examined microscopically. As indicated in Fig. 8A and B, HUVECs exposed to high SS formed increased numbers of tubes compared with control HUVECs cultured under static conditions at 12 h post-SS initiation. SS-induced tube formation was completely inhibited by the eNOS inhibitor L-NAME. To determine whether the BDNF/TrkB pathway mediated high SS-induced improvements in tube-forming capacity in HUVECs, a chimeric TrkB-Fc ( $1 \mu\text{g/ml}$ ) was used to neutralize BDNF secretion under fluid conditions. It was identified that tube formation was impaired in the chimera-treated group compared with that in the SS-treated group; however, the number of tubes that formed in the chimera-treated group was increased compared with that in the control group. These data suggested that SS confers a proportion of its tube-forming effects through the activation of the BDNF/TrkB axis.



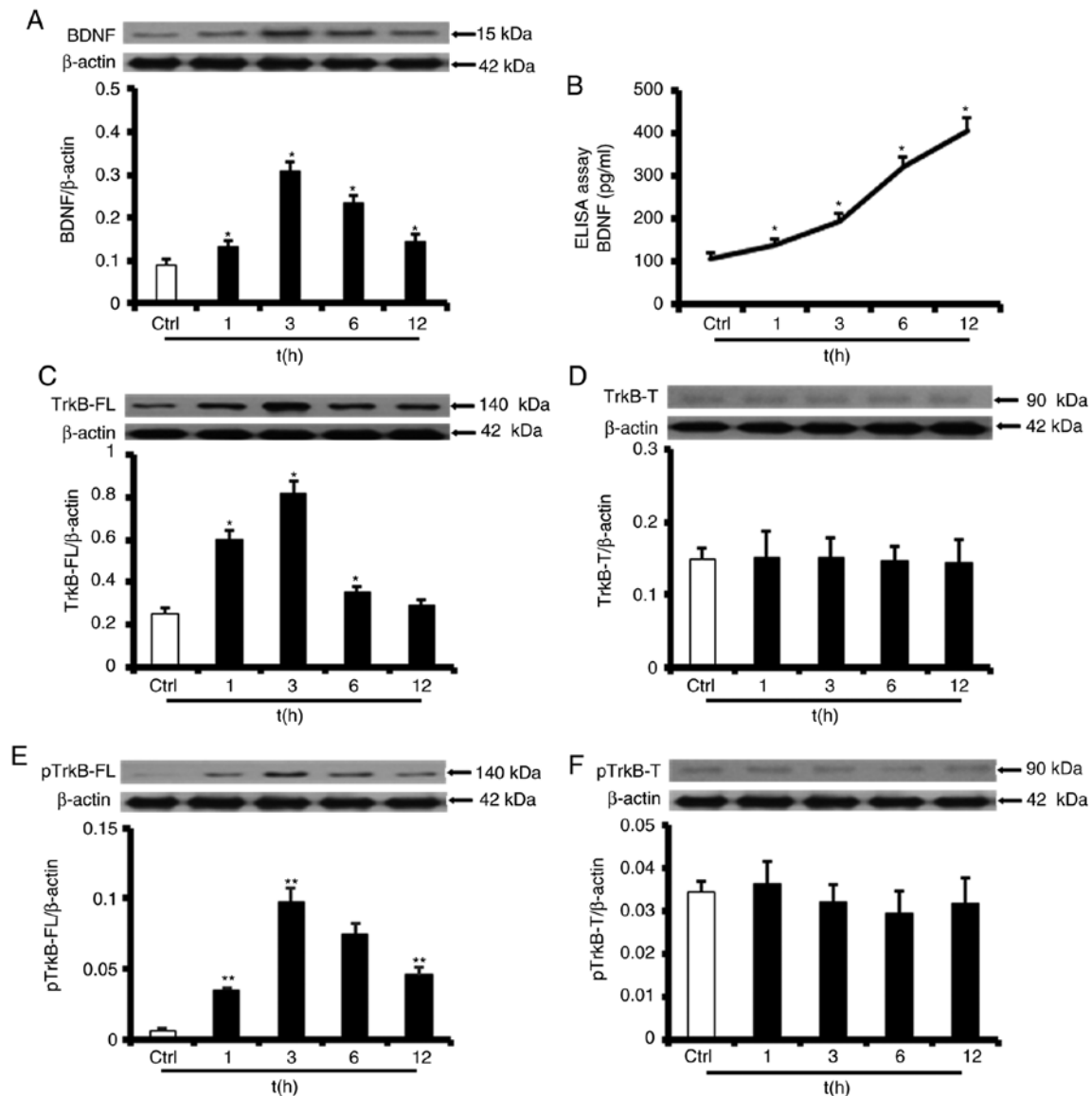


Figure 5. SS of 12 dyn/cm<sup>2</sup> increased BDNF/TrkB-FL protein levels and sustained TrkB-FL activation in HUVECs. (A) Representative western blot analysis images for BDNF and the protein level analysis in HUVECs exposed to SS (\* $P$ <0.01 vs. Ctrl). (B) ELISA measurement of BDNF levels in the culture medium following exposure of HUVECs for 1, 3, 6 and 12 h of SS (\* $P$ <0.01 vs. Ctrl). (C) Representative western blot analysis images for TrkB-FL and the protein level analysis in HUVECs exposed to SS (\* $P$ <0.01 vs. Ctrl). (D) TrkB-T protein expression and phosphorylation levels were not modified by SS at 12 dyn/cm<sup>2</sup>. (E) Representative western blot analysis images for phosphorylated TrkB-FL and the protein level analysis in HUVECs exposed to SS (\*\* $P$ <0.001 vs. Ctrl). (F) TrkB-T phosphorylation levels were not modified by SS at 12 dyn/cm<sup>2</sup>. The values are presented as the mean  $\pm$  standard error of the mean (n=6). SS, shear stress; BDNF, brain-derived neurotrophic factor; TrkB, BDNF/NT-3 growth factors receptor; FL, full-length; T, truncated; p, phosphorylated; ctrl, control; HUVECs, human umbilical vein endothelial cells; t, time.

## Discussion

The present study provided evidence associating the BDNF/TrkB axis with exercise-induced improvements in cardiovascular health. The data indicated that circulating and cardiac BDNF protein levels were increased within 8 weeks in rats subjected to exercise training, and that increases in BDNF expression coincided with improved vascularization and cardiac function. The decreases in capillary density and cardiac function noted in the trained rats treated with the known TrkB inhibitor K252a supported this result.

One hypothesis explaining the association between the BDNF/TrkB axis and improved cardiovascular function in response to exercise implicates alterations in TrkB expression

and phosphorylation in the IHZ. There are 2 major isoforms of TrkB that exist, including a full-length functional isoform (140 KDa) and a truncated non-functional isoform (90 KDa). Increased TrkB-T expression levels in the nervous system have been associated with neurologic disorders, and TrkB-T production is induced in response to brain injury (28). BDNF/TrkB signalling was identified to modulate heart contraction force and long-term heart tissue homeostasis (10). TrkB-FL was demonstrated to exist in cardiomyocytes isolated from healthy and failing rodent hearts, and TrkB-T expression levels were markedly increased in failing hearts compared with those in healthy hearts (9). In the present study, it was also identified that TrkB-T protein expression levels were increased in MI rat hearts, indicating a response to ischaemic injury. Notably, exer-

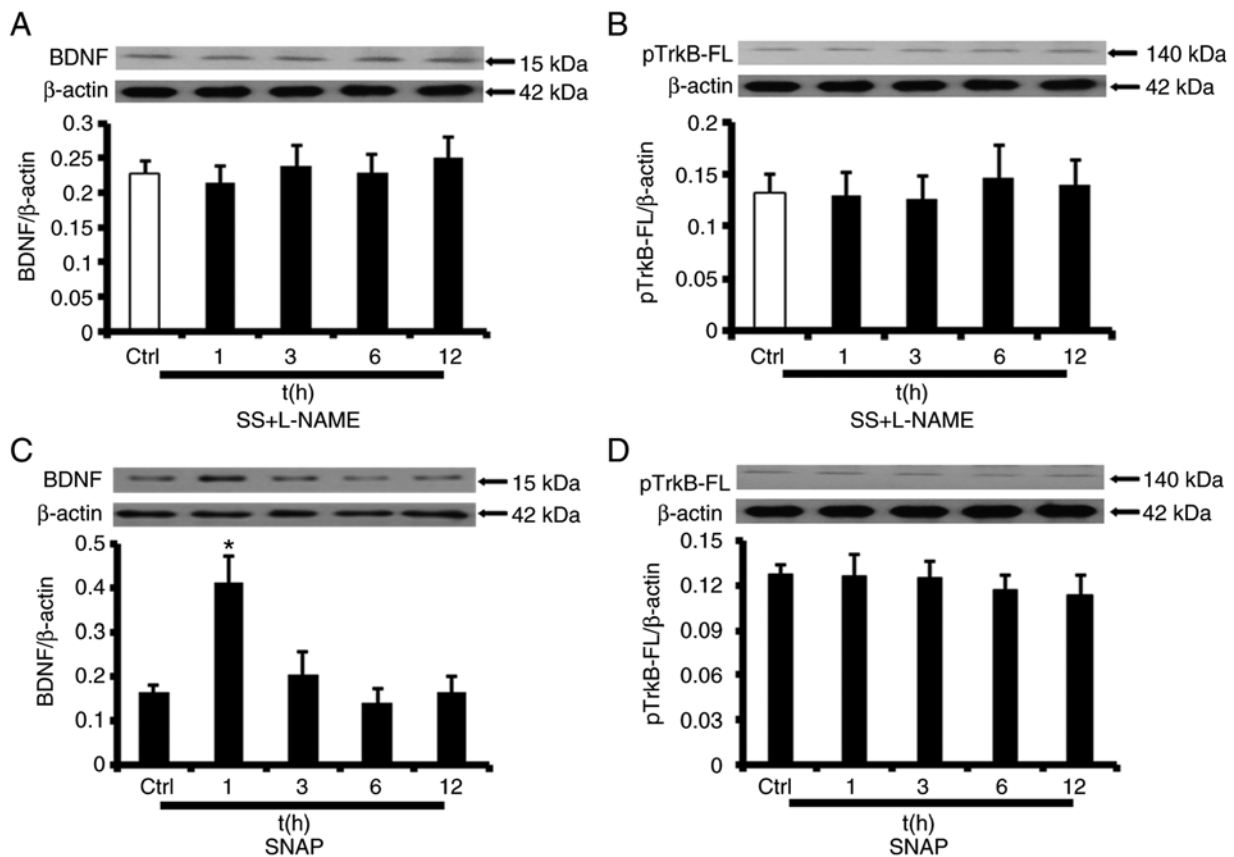


Figure 6. NO-dependent activation of the BDNF/TrkB axis. Representative western blot analysis images and protein level analysis for BDNF and phosphorylated TrkB-FL in HUVECs. (A and B) Cultured HUVECs were incubated with L-NAME and exposed to SS at 12 dyn/cm<sup>2</sup> for 1, 3, 6 or 12 h. (A) Representative western blot analysis images for BDNF and the protein level analysis. (B) Representative western blot analysis images for phosphorylated TrkB-FL and the protein level analysis. (C and D) Static HUVECs incubated with the NO donor SNAP. (C) Representative western blot analysis images for BDNF and the protein level analysis (\**P*<0.01 vs. Ctrl). (D) Western blot analysis images for phosphorylated TrkB-FL and the protein level analysis. The values are presented as the mean ± standard error of the mean (n=6). BDNF, brain-derived neurotrophic factor; TrkB, BDNF/NT-3 growth factors receptor; FL, full-length; p, phosphorylated; ctrl, control; NO, nitric oxide; HUVECs, human umbilical vein endothelial cells; L-NAME, NG-nitro-L-arginine methyl ester; SS, shear stress; t, time.

cise training induced a modest but non-significant decrease in TrkB-T protein expression. The marked decrease in TrkB-T protein expression that occurred in response to training may be attributed to improvements in myocardial damage due to exercise training. However, TrkB-T phosphorylation levels increased markedly in the MIE group. These novel data regarding exercise-induced increases in TrkB-T phosphorylation also support the previous hypothesis that exercise training has compensatory effects and contributes to post-MI enhancement of cardiac performance via TrkB-T activation (9). This hypothesis requires additional investigation.

To the best of our knowledge, the western blot analysis results of the present study represent the first evidence demonstrating that exercise training upregulates TrkB-FL protein expression and phosphorylation levels in the IHZs of MI rats. Previous studies have identified that the TrkB-FL receptor was expressed primarily in young cardiac microvascular endothelial cells (CMECs) (7). BDNF may induce young CMECs to migrate via BDNF-TrkB-FL-phosphoinositide 3-kinase/protein kinase B pathway activation (7). Therefore, we hypothesized that exercise-induced TrkB-FL upregulation and phosphorylation may be the primary factors driving angiogenesis following exercise training. The rats in the exercise group that received K252a exhibited decreased phos-

phorylation levels of TrkB and decreased capillary density and cardiac function, which additionally confirmed the role of the activated BDNF/TrkB axis in exercise-induced cardioprotection.

The animal studies also exhibited elevated peripheral BDNF levels following exercise, and the serum BDNF concentration was positively correlated with myocardial angiogenesis and cardiac function. However, the increased serum BDNF observed subsequent to physical exercise in patients following MI events, and the association between its variation and an improved cardiac state, have not been fully investigated. The data from the present study indicate that circulating BDNF may be a potential biomarker for assessing the effect of exercise rehabilitation in patients following MI events; however, additional clinical studies are required for verification.

At present, how exercise training activates the BDNF/TrkB axis remains unknown. The cardiovascular benefits of physical training are known to depend heavily on exercise-induced uniaxial laminar flow and shear stress, which is considered one of the central signalling mechanisms underlying improvements in vascular function (29-31). The average haemodynamic SS level in human veins under resting conditions is ~6 dyn/cm<sup>2</sup> (32,33). Laminar shear stress levels >10 dyn/cm<sup>2</sup> have been demonstrated to trigger

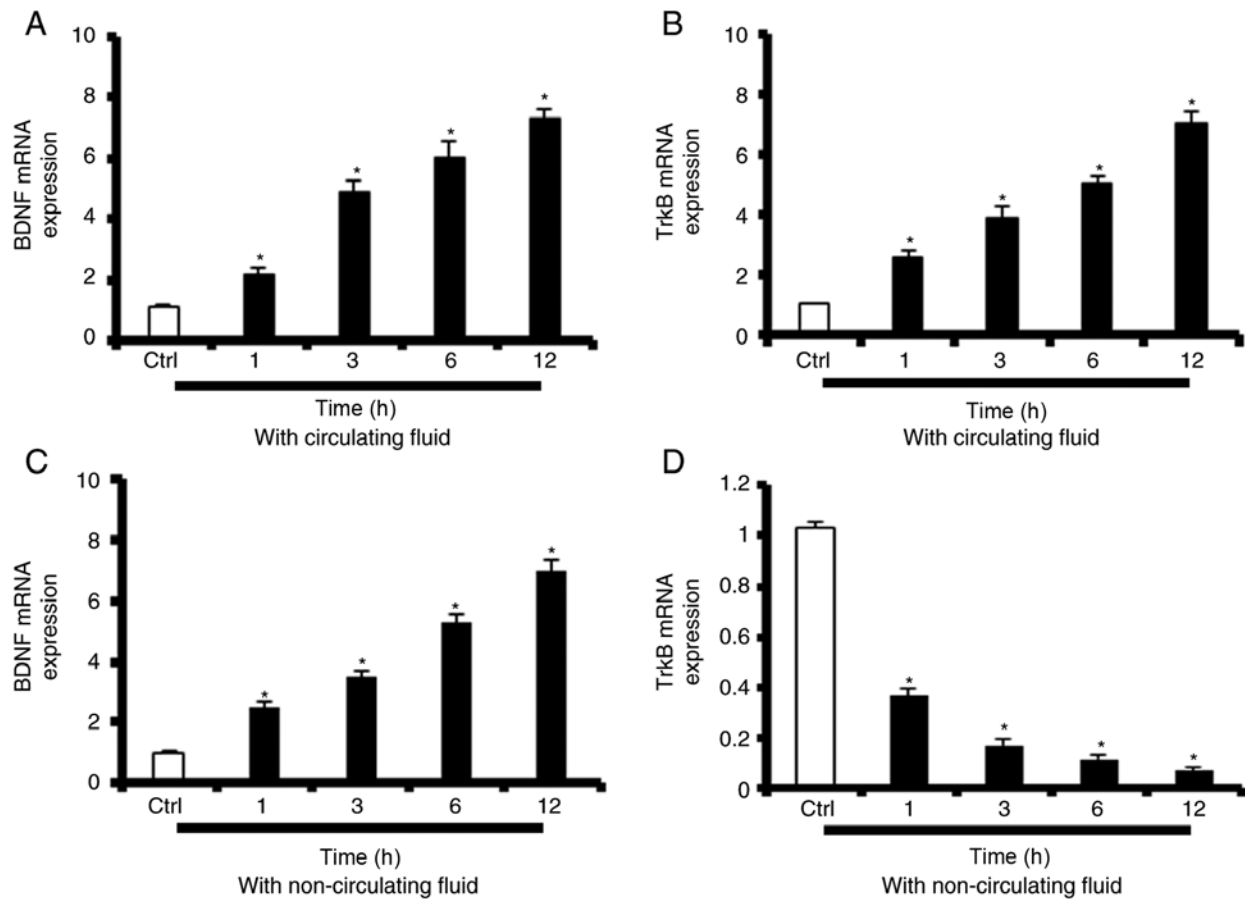


Figure 7. SS-induced TrkB-FL upregulation is dependent on increases in BDNF levels in the media. (A and B) mRNA expression levels of BDNF and TrkB-FL in HUVECs exposed to SS for 1, 3, 6 or 12 h. (A) BDNF mRNA levels. \* $P < 0.001$  vs. Ctrl. (B) TrkB-FL mRNA levels. \* $P < 0.001$  vs. Ctrl. (C and D) mRNA expression levels of BDNF and TrkB-FL in HUVECs exposed to non-circulating fluid SS for 1, 3, 6 or 12 h. (C) BDNF mRNA levels. \* $P < 0.001$  vs. Ctrl. (D) TrkB-FL mRNA levels. \* $P < 0.001$  vs. Ctrl. The values are presented as the mean  $\pm$  standard error of the mean ( $n = 6$ ). BDNF, brain-derived neurotrophic factor; TrkB, BDNF/NT-3 growth factors receptor; FL, full-length; ctrl, control; HUVECs, human umbilical vein endothelial cells; SS, shear stress; t, time.

endothelial cell sprouting (34,35). Exercise increases the heart rate, resulting in increases in blood flow and vascular shear stress; therefore, a circulatory system that produces SS (12 dyn/cm<sup>2</sup>) similar to the *in vivo* conditions elicited by exercise was developed to mimic the effects of physical training on the BDNF/TrkB axis in the vasculature. It was identified that SS-induced increases in tube-formation capacity were substantially inhibited by chimeric TrkB-Fc treatment. Similar to the animal experimental results, this result additionally indicated that the BDNF/TrkB axis is involved in exercise-induced angiogenesis.

Dynamic observations of BDNF secretion under SS in HUVECs were also conducted. Exposure of HUVECs to SS at 12 dyn/cm<sup>2</sup> was associated with a significant and persistent increase in BDNF protein expression. These data contradict the results described by Nakahashi *et al* (23), who noted that BDNF levels were decreased in the medium of HUVECs exposed to SS (24 dyn/cm<sup>2</sup>) for 24 h (23). However, Prigent-Tessier *et al* (22) identified that BDNF secretion levels were proportional to shear stress intensity in cultured endothelial cells exposed to SS at 14 dyn/cm<sup>2</sup> for 24 h. These contradictory results suggest that the responses of BDNF expression to SS are likely to be cell- and context-specific.

Notably, increased SS increased the expression and phosphorylation of TrkB-FL but not TrkB-T in HUVECs.

Concomitantly, the variation trend of TrkB-FL phosphorylation was similar to that for BDNF expression. These data also support the results of the animal experiments, which demonstrated that TrkB-FL may be the primary receptor mediating the angiogenic response to exercise, while TrkB-T primarily mediates exercise-induced increases in cardiac contraction force.

Exogenous BDNF (70 ng/ml) has been suggested to induce only a transient increase in TrkB receptor levels in cultured neurons and CMECs (7,36). Therefore, we hypothesized that the sustained increases in TrkB expression that occurred under flow conditions are ligand-dependent. This hypothesis was verified using a laminar flow chamber containing non-circulating fluid, in which only a small amount of BDNF was present. Time-dependent increases in BDNF mRNA expression and decreases in TrkB-FL mRNA expression, were observed, confirming the previous hypothesis. It was also identified that SS induced sustained TrkB-FL phosphorylation, whereas exogenous BDNF application triggered only a transient increase in TrkB phosphorylation (37). The increased BDNF concentration that manifested under flow conditions was decreased compared with the dose of exogenous BDNF administered; however, the increase resulted in more significant and longer TrkB receptor activation (12 h). The cellular mechanisms responsible for this phenomenon are

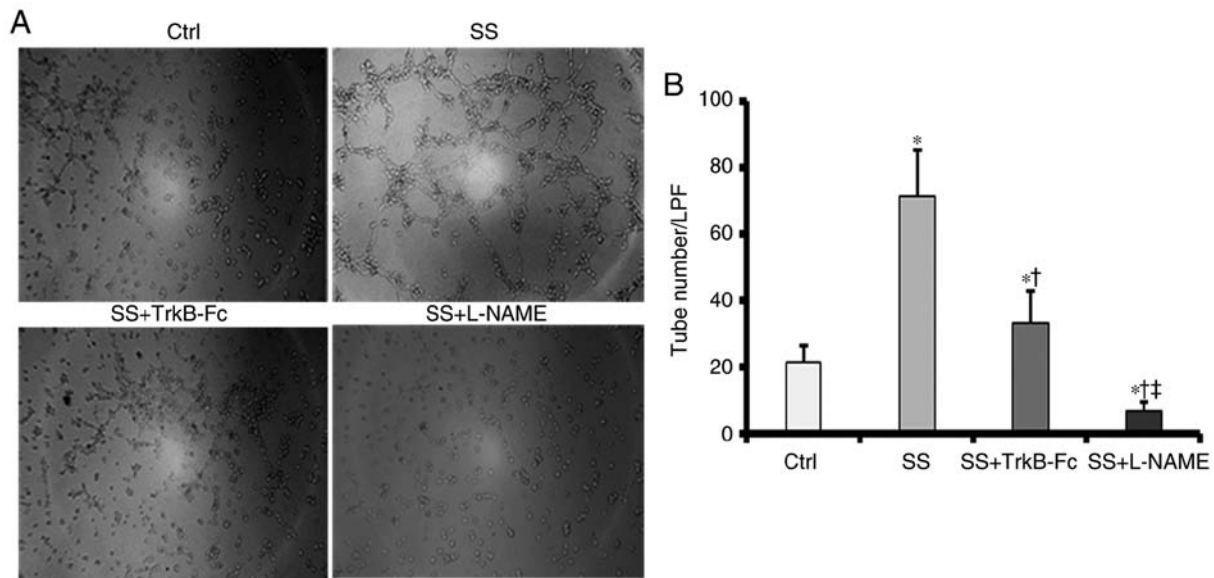


Figure 8. Effect of BDNF/TrkB activation on tube-forming capacity *in vitro*. Cultured HUVECs were incubated with or without L-NAME and TrkB-Fc and exposed to SS at 12 dyn/cm<sup>2</sup> for 6 h. Then, HUVECs were cultured in Matrigel and evaluated for *in vitro* angiogenesis with a tube formation assay. (A) Representative photomicrographs of HUVECs after exposure to SS with or without TrkB-Fc and L-NAME. Scale bar=50 μm. (B) Quantification of the number of tubes per LPF. \*P<0.05 vs. Ctrl, †P<0.01 vs. SS, ‡P<0.01 vs. SS+ TrkB-Fc. The values are presented as the mean ± standard error of the mean (n=6). Ctrl, control; SS, shear stress; TrkB, brain-derived neurotrophic factor/NT-3 growth factors receptor; FL, full-length; L-NAME, NG-nitro-L-arginine methyl ester; LPF, low-power field.

currently unknown. A previous study identified that increases in Ca<sup>++</sup> levels induced by neuronal stimulation caused transient TrkB activation that progressed to sustained activation (36). Substantial previous evidence also indicated that vascular endothelial cells increased their intracellular Ca<sup>++</sup> levels in accordance with the flow rate (38,39). Based on the present results and those of previous studies, we hypothesized that sustained TrkB activation resulted from SS-induced increases in Ca<sup>++</sup> levels. The data from the present study suggested that exercise-induced endogenous BDNF possessed greater bioactivity than exogenous BDNF, and confirmed the amplified effect of BDNF within endothelial cells exposed to increased shear force, indicating that endogenous BDNF expression depended on endothelial function *in vivo*; studies from Jin *et al* (40) and Prigent-Tessier *et al* (22) validated these conclusions. Based on these data and previous studies, we hypothesized that exogenous BDNF administration combined with exercise training may assist in alleviating circulatory disturbances in diabetic or hypertensive patients with poor vascular function. Therefore, the present study demonstrated the potential of utilizing BDNF to improve vascular function in clinical practice. However, additional animal and clinical experiments are warranted.

To gain insight into the mechanisms by which exercise activated the BDNF/TrkB axis, the effects of NO, an important inducer of exercise-associated angiogenesis in ischaemic myocardial tissue, were investigated (41). Several lines of evidence suggest that NO modulates BDNF expression and relaxation *in vivo* and *in vitro* (42,43). However, these studies were limited to nerve tissues. In the present study, it was demonstrated that exogenous L-NAME blocked exercise-induced BDNF/TrkB axis activation in heart tissue. To the best of our knowledge, the present study is the first to demonstrate that eNOS/NO participates in BDNF/TrkB axis

activation in organs other than the brain. A previous study has confirmed that high SS-induced increases in eNOS expression may be the basis of SS-mediated angiogenesis (44). The *in vitro* data from the present study indicated that an NO donor upregulated BDNF expression without TrkB-FL phosphorylation in HUVECs under static conditions, which may contribute to weak and short TrkB-FL activation in the absence of SS. Simultaneously, the eNOS inhibitor L-NAME blocked SS-induced BDNF/TrkB axis activation in HUVECs. These data, at least partially, explained the inhibitory effects of L-NAME observed in the animal studies.

In summary, the data from the present study indicate that exercise training activated the BDNF/TrkB (TrkB-FL and TrkB-T) axis in post-MI rat hearts and that only BDNF/TrkB-FL pathway activation is involved in exercise-induced angiogenesis. The laminar SS response to exercise may contribute to BDNF/TrkB-FL axis activation in an NO-dependent manner. Increased circulating BDNF concentrations may be important for activating TrkB-FL and were associated with improved cardiac function and angiogenesis in the ischaemic myocardium in post-MI rats subjected to exercise. These results provide novel insights not only into monitoring of cardiac function but also into the development of effective cardiac rehabilitation programmes for patients following MI events.

#### Acknowledgements

The authors would like to thank Dr Yu-Yu Yao (Department and Institute of Cardiology, Zhongda Hospital, Medical School of Southeast University) and Dr Yi-Ping Li (Department of Pathophysiology, Medical School of Southeast University) for their technical advice and support.



## Funding

The present study was supported by the Science and Technology Support, Social Development Research Fund, a division of Health Bureau, Jiangsu Province Government (grant no. BE2011793) awarded to Dr Nai-Feng Liu, and the Postgraduate Research Innovation Plan of Jiangsu Province (grant no. KYCX17\_0174) awarded to Dr Xi-Qiong Han.

## Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

## Authors' contributions

The study was conceived and designed by NFL, HJ and BLW. BLW conducted the experimental protocols with assistance from XQH and YX. The paper was written by BLW, with contributions from NFL and HJ.

## Ethics approval and consent to participate

The present study was approved by the Animal Care and Research Committee of Southeast University, and all experiments were conducted in accordance with the European Convention for the Protection of Vertebrates Used for Experiments and Other Scientific Purposes (European Union Directive 86/609EEC, 1986).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

## References

1. Barbacid M: Neurotrophic factors and their receptors. *Curr Opin Cell Biol* 7: 148-155, 1995.
2. Chao MV: Neurotrophins and their receptors: A convergence point for many signalling pathways. *Nat Rev Neurosci* 4: 299-309, 2003.
3. Monteggia LM, Barrot M, Powell CM, Berton O, Galanis V, Gemelli T, Meuth S, Nagy A, Greene RW and Nestler EJ: Essential role of brain-derived neurotrophic factor in adult hippocampal function. *Proc Natl Acad Sci USA* 101: 10827-10832, 2004.
4. Okada S, Yokoyama M, Toko H, Tateno K, Moriya J, Shimizu I, Nojima A, Ito T, Yoshida Y, Kobayashi Y, *et al*: Brain-derived neurotrophic factor protects against cardiac dysfunction after myocardial infarction via a central nervous system-mediated pathway. *Arterioscler Thromb Vasc Biol* 32: 1902-1909, 2012.
5. Kermani P, Rafii D, Jin DK, Whitlock P, Schaffer W, Chiang A, Vincent L, Friedrich M, Shido K, Hackett NR, *et al*: Neurotrophins promote revascularization by local recruitment of TrkB<sup>+</sup> endothelial cells and systemic mobilization of hematopoietic progenitors. *J Clin Invest* 115: 653-663, 2005.
6. Donovan MJ, Lin MI, Wiegand P, Ringstedt T, Kraemer R, Hahn R, Wang S, Ibañez CF, Rafii S and Hempstead BL: Brain derived neurotrophic factor is an endothelial cell survival factor required for intramyocardial vessel stabilization. *Development* 127: 4531-4540, 2000.
7. Cao L, Zhang L, Chen S, Yuan Z, Liu S, Shen X, Zheng X, Qi X, Lee KK, Chan JY and Cai D: BDNF-mediated migration of cardiac microvascular endothelial cells is impaired during ageing. *J Cell Mol Med* 16: 3105-3115, 2012.
8. Liu Y, Sun L, Huan Y, Zhao H and Deng J: Application of bFGF and BDNF to improve angiogenesis and cardiac function. *J Surg Res* 136: 85-91, 2006.
9. Feng N, Huke S, Zhu G, Tocchetti CG, Shi S, Aiba T, Kaludercic N, Hoover DB, Beck SE, Mankowski JL, *et al*: Constitutive BDNF/TrkB signaling is required for normal cardiac contraction and relaxation. *Proc Natl Acad Sci USA* 112: 1880-1885, 2015.
10. Fulgenzi G, Tomassoni-Ardori F, Babini L, Becker J, Barrick C, Puvarel S and Tessarollo L: BDNF modulates heart contraction force and long-term homeostasis through truncated TrkB.T1 receptor activation. *J Cell Biol* 210: 1003-1012, 2015.
11. Fukushima A, Kinugawa S, Homma T, Masaki Y, Furihata T, Yokota T, Matsushima S, Abe T, Suga T, Takada S, *et al*: Decreased serum brain-derived neurotrophic factor levels are correlated with exercise intolerance in patients with heart failure. *Int J Cardiol* 168: e142-e144, 2013.
12. Swardfager W, Herrmann N, Marzolini S, Saleem M, Shammi P, Oh PI, Albert PR, Daigle M, Kiss A and Lancôt KL: Brain derived neurotrophic factor, cardiopulmonary fitness and cognition in patients with coronary artery disease. *Brain Behav Immun* 25: 1264-1271, 2011.
13. Circulating brain-derived neurotrophic factor concentrations and the risk of cardiovascular disease in the community. *J Am Heart Assoc* 5: e002098, 2016.
14. Möbius-Winkler S, Uhlemann M, Adams V, Sandri M, Erbs S, Lenk K, Mangner N, Mueller U, Adam J, Grunze M, *et al*: Coronary collateral growth induced by physical exercise: Results of the impact of intensive exercise training on coronary collateral circulation in patients with stable coronary artery disease (EXCITE) trial. *Circulation* 133: 1438-1448, 2016.
15. Leosco D, Rengo G, Iaccarino G, Golino L, Marchese M, Fortunato F, Zincarelli C, Sanzari E, Ciccarelli M, Galasso G, *et al*: Exercise promotes angiogenesis and improves beta-adrenergic receptor signalling in the post-ischaemic failing rat heart. *Cardiovasc Res* 78: 385-394, 2008.
16. Tang XY, Hong HS, Chen LL, Lin XH, Lin JH and Lin Z: Effects of exercise of different intensities on the angiogenesis, infarct healing, and function of the left ventricle in postmyocardial infarction rats. *Coron Artery Dis* 22: 497-506, 2011.
17. Tinken TM, Thijssen DH, Hopkins N, Dawson EA, Cable NT and Green DJ: Shear stress mediates endothelial adaptations to exercise training in humans. *Hypertension* 55: 312-318, 2010.
18. Duncker DJ and Bache RJ: Regulation of coronary blood flow during exercise. *Physiol Rev* 88: 1009-1086, 2008.
19. Marsh SA and Coombes JS: Exercise and the endothelial cell. *Int J Cardiol* 99: 165-169, 2005.
20. Zoladz JA and Pilc A: The effect of physical activity on the brain derived neurotrophic factor: From animal to human studies. *J Physiol Pharmacol* 61: 533-541, 2010.
21. Lee HW, Ahmad M, Wang HW and Leenen FH: Effects of exercise training on brain-derived neurotrophic factor in skeletal muscle and heart of rats post myocardial infarction. *Exp Physiol* 102: 314-328, 2017.
22. Prigent-Tessier A, Quirié A, Maguin-Gaté K, Szostak J, Mossiat C, Nappey M, Devaux S, Marie C and Demougeot C: Physical training and hypertension have opposite effects on endothelial brain-derived neurotrophic factor expression. *Cardiovasc Res* 100: 374-382, 2013.
23. Nakahashi T, Fujimura H, Altar CA, Li J, Kambayashi J, Tandon NN and Sun B: Vascular endothelial cells synthesize and secrete brain-derived neurotrophic factor. *FEBS Lett* 470: 113-117, 2000.
24. Hiraoka Y, Kishimoto C, Takada H, Nakamura M, Kurokawa M, Ochiai H and Shiraki K: Nitric oxide and murine coxsackievirus B3 myocarditis: Aggravation of myocarditis by inhibition of nitric oxide synthase. *J Am Coll Cardiol* 28: 1610-1615, 1996.
25. Jiménez-Maldonado A, de Álvarez-Buylla ER, Montero S, Melnikov V, Castro-Rodríguez E, Gamboa-Domínguez A, Rodríguez-Hernández A, Lemus M and Murguía JM: Chronic exercise increases plasma brain-derived neurotrophic factor levels, pancreatic islet size, and insulin tolerance in a TrkB-dependent manner. *PLoS One* 9: e115177, 2014.
26. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>-ΔΔC<sub>T</sub></sup> method. *Method* 25: 402-408, 2001.

27. Siemerink MJ, Klaassen I, Vogels IM, Griffioen AW, Van Noorden CJ and Schlingemann RO: CD34 marks angiogenic tip cells in human vascular endothelial cell cultures. *Angiogenesis* 15: 151-163, 2012.
28. Frisén J, Verge VM, Fried K, Risling M, Persson H, Trotter J, Hökfelt T and Lindholm D: Characterization of glial trkB receptors: Differential response to injury in the central and peripheral nervous systems. *Proc Natl Acad Sci USA* 90: 4971-4975, 1993.
29. Suh GY, Les AS, Tenforde AS, Shadden SC, Spilker RL, Yeung JJ, Cheng CP, Herfkens RJ, Dalman RL and Taylor CA: Hemodynamic changes quantified in abdominal aortic aneurysms with increasing exercise intensity using mr exercise imaging and image-based computational fluid dynamics. *Ann Biomed Eng* 39: 2186-2202, 2011.
30. Rodriguez I and Gonzalez M: Physiological mechanisms of vascular response induced by shear stress and effect of exercise in systemic and placental circulation. *Front Pharmacol* 5: 209, 2014.
31. Wragg JW, Durant S, McGettrick HM, Sample KM, Egginton S and Bicknell R: Shear stress regulated gene expression and angiogenesis in vascular endothelium. *Microcirculation* 21: 290-300, 2014.
32. Papaioannou TG and Stefanadis C: Vascular wall shear stress: Basic principles and methods. *Hellenic J Cardiol* 46: 9-15, 2005.
33. Barnes JM, Nauseef JT and Henry MD: Resistance to fluid shear stress is a conserved biophysical property of malignant cells. *PLoS One* 7: e50973, 2012.
34. Galie PA, Nguyen DH, Choi CK, Cohen DM, Janmey PA and Chen CS: Fluid shear stress threshold regulates angiogenic sprouting. *Proc Natl Acad Sci USA* 111: 7968-7973, 2014.
35. Ueda A, Koga M, Ikeda M, Kudo S and Tanishita K: Effect of shear stress on microvessel network formation of endothelial cells with in vitro three-dimensional model. *Am J Physiol Heart Circ Physiol* 287: H994-H1002, 2004.
36. Guo W, Ji Y, Wang S, Sun Y and Lu B: Neuronal activity alters BDNF-TrkB signaling kinetics and downstream functions. *J Cell Sci* 127: 2249-2260, 2014.
37. Ji Y, Lu Y, Yang F, Shen W, Tang TT, Feng L, Duan S and Lu B: Acute and gradual increases in BDNF concentration elicit distinct signaling and functions in neurons. *Nat Neurosci* 13: 302-309, 2010.
38. Ando J, Ohtsuka A, Korenaga R and Kamiya A: Effect of extracellular ATP level on flow-induced  $Ca^{++}$  response in cultured vascular endothelial cells. *Biochem Biophys Res Commun* 179: 1192-1199, 1991.
39. Yamamoto K, Korenaga R, Kamiya A and Ando J: Fluid shear stress activates  $Ca(2+)$  influx into human endothelial cells via P2X4 purinoceptors. *Circ Res* 87: 385-391, 2000.
40. Jin H, Chen Y, Wang B, Zhu Y, Chen L, Han X, Ma G and Liu N: Association between brain-derived neurotrophic factor and von Willebrand factor levels in patients with stable coronary artery disease. *BMC Cardiovasc Disord* 18: 23, 2018.
41. Calvert JW: Cardioprotective effects of nitrite during exercise. *Cardiovasc Res* 89: 499-506, 2011.
42. Cui X, Chopp M, Zacharek A, Ning R, Ding X, Roberts C and Chen J: Endothelial nitric oxide synthase regulates white matter changes via the BDNF/TrkB pathway after stroke in mice. *PLoS One* 8: e80358, 2013.
43. Li ST, Pan J, Hua XM, Liu H, Shen S, Liu JF, Li B, Tao BB, Ge XL, Wang XH, *et al*: Endothelial nitric oxide synthase protects neurons against ischemic injury through regulation of brain-derived neurotrophic factor expression. *CNS Neurosci Ther* 20: 154-164, 2014.
44. Kolluru GK, Sinha S, Majumder S, Muley A, Siamwala JH, Gupta R and Chatterjee S: Shear stress promotes nitric oxide production in endothelial cells by sub-cellular delocalization of eNOS: A basis for shear stress mediated angiogenesis. *Nitric Oxide* 22: 304-315, 2010.