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ORIGINAL PAPER

Combination of simvastatin administration and EPC transplantation enhances angiogenesis and protects against apoptosis for hindlimb ischemia

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Abstract The aim of this present study is to investigate the impacts of combinatorial simvastatin administration and endothelial progenitor cell (EPC) transplantation on therapeutic angiogenesis in an athymic nude mouse model of hind limb ischemia. Athymic nude mice were divided into four groups ($n = 10$/group): vehicle administration plus PBS injection (control), simvastatin administration plus PBS injection (simvastatin), vehicle administration plus EPC transplantation (EPC), and simvastatin administration plus EPC transplantation (combination). The combination therapy had the greatest laser Doppler blood perfusion imager (LDPI) index and capillary density among the four groups. Importantly, this combination therapy significantly reduced apoptosis of ischemic skeletal muscle cells in part through downregulation of Bax and upregulation of Bcl-2 compared with the other groups. Moreover, the combination therapy exhibited the highest efficacy of increasing the ratio of phospho-Akt to Akt among the four groups. Taken together, the simvastatin and EPC combination therapy promotes powerful angiogenesis in hindlimb ischemia. The combination therapy not only inhibites apoptosis of ischemic skeletal muscle cells partially via downregulation of Bax and upregulation of Bcl-2, but also activates Akt phosphorylation significantly. These efficacies may be mediated by the angiogenic potency of simvastatin, EPCs, and by the beneficial effects of simvastatin on transplanted EPCs as well.

Keywords Angiogenesis · Apoptosis · Endothelial progenitor cells · Peripheral arterial disease · Simvastatin

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Peripheral arterial disease (PAD) of the lower limbs is the third most important site of atherosclerotic disease alongside coronary heart disease and cerebrovascular disease. In recent years, PAD has received growing attention as an important cause of disability and of cardiovascular morbidity and mortality [1]. Pioneering studies indicated that infusion of in vitro expanded EPCs enhanced the neovascularization of ischemic hind limb [2–4]. On the basis of results in vitro and in vivo, a better therapeutic strategy is still needed to improve the angiogenic property of transplanted EPCs.

Recent experimental and clinical trials have demonstrated that statins exert vasculoprotective effects independent of cholesterol lowering. Statins were shown to not only promote proliferation, mobilization, adhesion, but also prevent senescence and apoptosis of EPCs [5–9]. These results suggest that statins may have beneficial effects on transplanted EPCs.

Accordingly, we investigated whether the combination therapy with simvastatin administration and EPC transplantation would augment functional neovascularization in an athymic nude mouse model of operatively induced unilateral hind limb ischemia.

Since apoptosis is relevant to both the cause and effect of hindlimb ischemia, characterizing its extent and regulation might provide a useful pathological measure of

disease progression. We compared the extent of apoptosis in the ischemic skeletal muscle cells in different groups. Since regulation of apoptosis is accomplished in part through the balance of proapoptotic to antiapoptotic Bcl-2 family [10], we also measured alterations in the levels of Bcl-2 and Bax via immunohistochemistry of these tissues [11].

Methods

Animal model of hindlimb ischemia

Female athymic nude mice (8 weeks old, weight 18–20 g; Shanghai Slac, China) were used in the present study. The right proximal femoral artery including the superficial and the deep branch as well as the distal saphenous artery were ligated with 6.0 silk suture under anesthesia with pentobarbital sodium (50 mg/kg, intraperitoneally) [2]. The left hindlimb was kept intact and used as the nonischemic limb. In this study, all mice received humane care in accordance with the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institute of Health (NIH publication 8523, revised 1996). The study protocol was approved by the Animal Care and Use Committee of Nanjing Medical University.

Cell cultures

Peripheral venous blood was harvested from healthy male human volunteers. The mononuclear cell fraction was isolated by Ficoll-Paque density gradient centrifugation as described previously [12]. Cells were plated on culture dishes coated with fibronectin (Roche, Germany) in M199 (JRH, USA) supplemented with 20% fetal bovine serum (Hyclone, Australia). After 4 days, nonadherent cells were removed by washing with PBS, adherent cells were cultured continually by the addition of fresh media for another 3 days.

Identification of EPCs

Direct fluorescent staining was used to detect dual binding of Fluorescein Ulex europaeus agglutinin I (UEA-I; Vector Laboratories, USA) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine -labeled acetylated low density lipoprotein (DiI-acLDL; Biomedical Technologies, USA.) on day 7. Cells were first incubated with DiI-acLDL (10 μg/ml) at 37°C for 4 h and then fixed with 3% paraformaldehyde for 10 min. After washed, the cells were reacted with UEA-1 (10 μg/ml) for 1 h. After the staining, samples were viewed with a laser scanning confocal microscope (LSM510, Zeiss, Germany). Cells displaying double positive fluorescence were identified as differentiating EPCs [3, 13].

Simvastatin administration and EPC transplantation

Simvastatin administration and EPC transplantation were performed immediately after hindlimb ischemia was created. Simvastatin (20 mg/kg per day) or vehicle (saline 0.9% IP) was administrationed every day by gavage for 21 days. After 7 days of culture, EPCs were detached by using 0.25% trypsin, harvested by centrifugation and resuspension in PBS. EPCs (5×10^6 cells per mouse) or PBS was injected into the ischemic thigh muscle with a 26-gauge needle at five different points. This protocol resulted in the creation of four groups ($n = 10$/group): (1) vehicle administration plus PBS injection (control group), (2) simvastatin administration plus PBS injection (simvastatin group), (3) vehicle administration plus EPC transplantation (EPC group), (4) simvastatin administration plus EPC transplantation(combination group). Simvastatin was kindly donated by Merck & Co., Inc., USA.

Laser Doppler blood perfusion analysis

Blood flow of the thigh was measured by using a laser Doppler blood perfusion imager (LDPI, PIM, Swenden) immediately after surgery, then at day 10, and at day 21 thereafter. After blood flow was scanned twice, the average flow values of the ischemic and nonischemic limbs were calculated by computer-assisted quantification. The LDPI index was determined as the ratio of ischemic to nonischemic hindlimb blood perfusion [4, 14].

Detection of capillary density

The effect on neovascularization was assessed under light microscopy (×200) by measurement of the number of endothelial cell capillaries in sections taken from the ischemic muscles. Four pieces of tissue specimens from each mouse were obtained from the adductor and semimembranous skeletal muscles after mice were sacrificed with an overdose of pentobarbital at day 21. Sections were prepared so that the muscle fibers were oriented transversely. Frozen tissue sections (5 μm) were stained for alkaline phosphatase by an indoxyltetrazolium method, and then counterstained with 0.5% eosin. Five fields from each sample were randomly selected for the counts by three independent blinded investigators. To ensure that the capillary density was not overestimated as a consequence of myocyte atrophy or underestimated because of interstitial edema, the capillary number adjusted per muscle fiber was used to compare the differences in capillary density among the four groups [15, 16].

Track of transplanted EPCs in ischemic hindlimb muscle

To assess whether transplanted male human EPCs survived and participated in the formation of capillary structure in the ischemic tissues of female mice, we used dual labeling with a synthetic probe specific for detection of the human Y chromosome *Sry* (sex-determining region of Y-chromosome) gene and immunostaining with anti-von Willebrand factor (vWF). Briefly, ischemic hindlimbs were obtained at day 21, embedded in optimal cutting temperature (OCT), snap-frozen in liquid nitrogen, and stored at −70°C. Pieces (5 μm) of the OCT blocks were collected on slides and fixed with 4% paraformaldehyde at 4°C for 5 min. Fluorescence in situ hybridization (FISH) was performed using a human *SRY* DNA FISH kit (Haoyang Biological Manufacture, China) according to the manufacturer's instructions. The probe sequence was 5′-CGCTT CACTC TATCC TGGAC GTTGC CTTTA-CTG-3′. Tissue sections were then stained with anti-vWF (Dako, USA). Samples were viewed with a laser scanning confocal microscope (LSM510, Zeiss, Germany). Cells manifesting both Sry positive and vWF positive were considered transplanted EPCs. Dual positive EPCs were counted in five randomly chosen high-power fields (HPFs) (×400) in each section and averaged by three independent blinded investigators. Four tissue sections of each mouse were evaluated. The result was expressed as EPCs per high power field [17, 18].

TUNEL assay

At day 21, tissue specimens from the ischemic adductor and semimembranous muscles were fixed in 4% paraformaldehyde and embedded in Paraffin. Paraffin sections were cut into 4 μm for the TUNEL assay using a in situ Cell Death Detection kit (Roche, Germany) according to the manufacturer's instructions. Cells in which the nucleus was stained brown were defined as TUNEL-positive. 1,000 cells were counted at ×400 magnification by three independent blinded investigators, and the percentage of apoptotic cells per total number of cells was determined [11, 19].

Immunohistochemical staining of Bax and Bcl-2 protein

Immunohistochemical staining of Bax and Bcl-2 protein was performed on 4 μm sections from adductor and semimembranous muscles at day 21 followed by streptavidin-peroxidase method according to the manufacturer's instructions (Zymed, USA). Cells stained brown were detected as positive cells. Thousand cells were counted at ×400 magnification by three independent blinded investigators, and the percentage of apoptotic cells per total number of cells was determined [11, 19].

Western blot analysis

Two grams of the sample was obtained from both ischemic adductor and semimembranous muscles of each mouse at day 21 and collected together to each group. Whole-cell lysates were isolated with a RIPA buffer and centrifuged at 12,000 rpm for 10 min at 4°C to separate soluble from insoluble fractions. Proteins (30 μg per lane) were denatured, loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel and electroblotted onto a nitrocellulose membrane. Each membrane was incubated with primary antibody against phospho-Akt (1:1,000, Santa Cruz, USA) or Akt (1:1,000, Santa Cruz, USA) at 4°C overnight. The membrane was then incubated with peroxidase labeled with secondary antibody (Santa Cruz, USA) at a dilution of 1:1,000 at 37°C for 1 h. Positive protein bands were visualized with an ECL kit (Amersham, USA) and measured by densitometry.

Statistics

All values were expressed as mean ± SEM. Student's unpaired t test was used to compare differences between every two groups. Comparisons of parameters among three or four groups were made by 1-way ANOVA, followed by Scheffé multiple comparison test. Comparisons of the time course of the LDPI index were made by 2-way ANOVA for repeated measures, followed by Scheffé multiple comparison tests. A probability value <0.05 was considered statistically significant.

Results

Identification of EPCs

EPCs were characterized as adherent cells, positive for both DiI-acLDL and UEA-1 under a laser scanning confocal microscope. The double positive cells were recognized as differentiating EPCs (Fig. 1).

Combination therapy increases blood perfusion

The limb perfusion was severely reduced after surgery in all four groups. Over the subsequent 21 days, blood perfusion of the ischemic hindlimb notably improved in the treatment groups (Fig. 2a). The laser Doppler perfusion index was significantly higher in the simvastatin group, the EPC group and the combination group than in the control

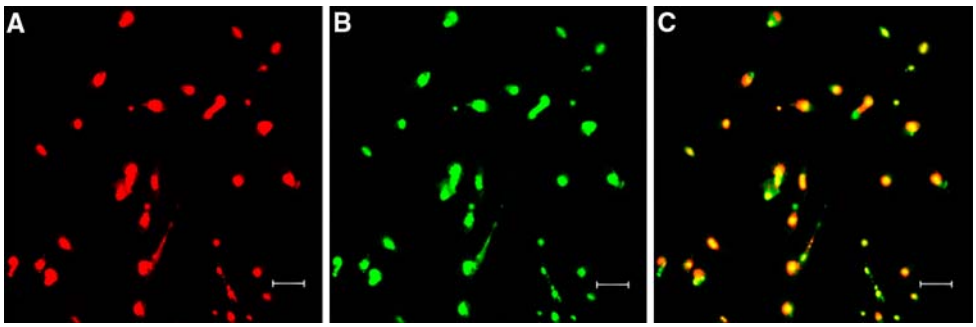

Fig. 1 Identification of EPCs. The representative micrograph of DiI-acLDL positive cells (**a**, red), UEA-1 positive cells (**b**, green) and the double positive cells for DiI-acLDL and UEA-1 (**c**, yellow) (×200). Bars: 50 μm

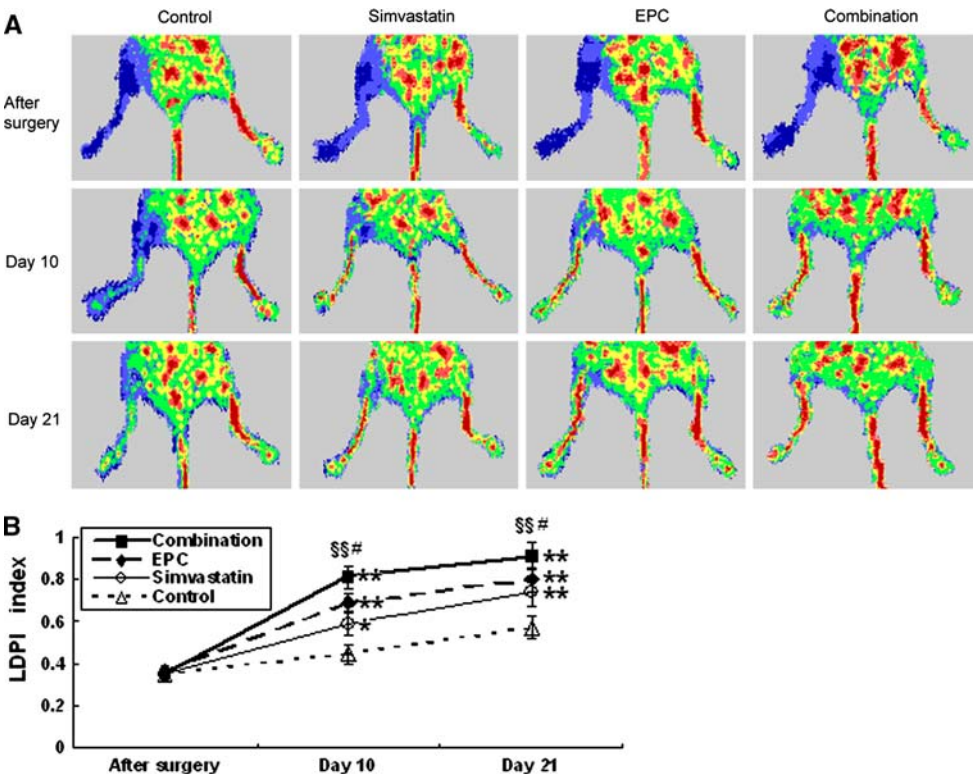

Fig. 2 The combination therapy increased blood perfusion. (**a**) Representative examples of serial laser Doppler perfusion images. Hues indicating regions from lower to higher perfusion are as follows: dark blue, blue, green, yellow, red, dark red. (**b**) Quantitative analysis with LDPI index showed the significantly greatest improvement in the combination group among the four groups. Data are mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control; $^{\S\S}P < 0.01$ versus simvastatin; $^{\#}P < 0.05$ versus EPC

group (0.59 ± 0.06, 0.68 ± 0.07 and 0.81 ± 0.07 versus 0.46 ± 0.05, respectively, $P < 0.05$ or $P < 0.01$) on day 10 after treatment and showed further improvement afterwards (0.74 ± 0.07, 0.80 ± 0.07 and 0.91 ± 0.06 versus 0.57 ± 0.06, respectively, $P < 0.05$ or $P < 0.01$) on day 21. The LDPI index was the highest in the combination group among the four groups (Fig. 2b). The normal value of LDPI index was 1.00 ± 0.03 in this study.

Combination therapy enhances capillary density

Representative photomicrographs of histological sections stained with alkaline phosphatase in the ischemic tissues are shown in Fig. 3a. Quantitative analyses showed that the capillary/muscle fiber ratio, an index of neovascularization, was the highest in the combination group, followed by the EPC group, the simvastatin group and the control group (0.69 ± 0.07, 0.49 ± 0.05 and 0.45 ± 0.04 versus 0.26 ± 0.03, respectively, $P < 0.01$) (Fig. 3b). The normal ratio of capillary/muscle fiber was 0.81 ± 0.07.

Differentiation of EPCs after transplantation

Twenty-one days after transplantation, double-positive (yellow) for *Sry* (green) and vWF (red) cells were shown in the EPC and the combination group, but absent in the control group and the simvastatin group. Some transplanted EPCs formed vascular structures (Fig. 4a). The number of *Sry*/vWF double-positive cells was significantly higher in the combination group than in the EPC group (8.3 ± 1.5/HPF versus 5.1 ± 0.99/HPF, $P < 0.01$) (Fig. 4b).

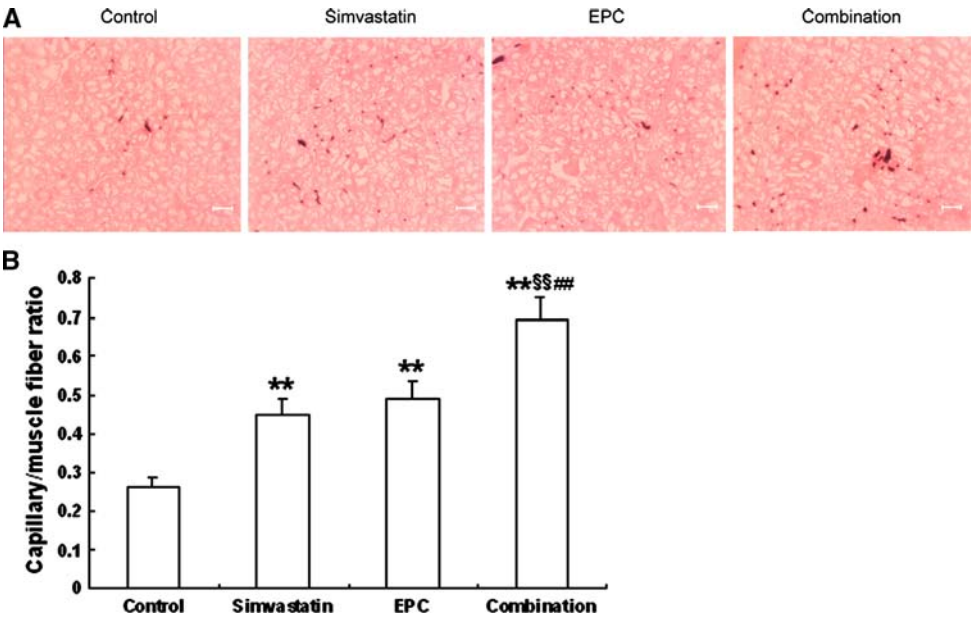

Fig. 3 The combination therapy enhanced the capillary density. (**a**) Representative photomicrographs of histological sections in ischemic skeletal muscles (×200). (**b**) Quantitative analysis revealed a notably increased capillary/muscle fiber ratio in the combination group among the four groups. Data are mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control; $^{\S\S}P < 0.01$ versus simvastatin; $^{\#\#}P < 0.01$ versus EPC. Bars: 50 μm

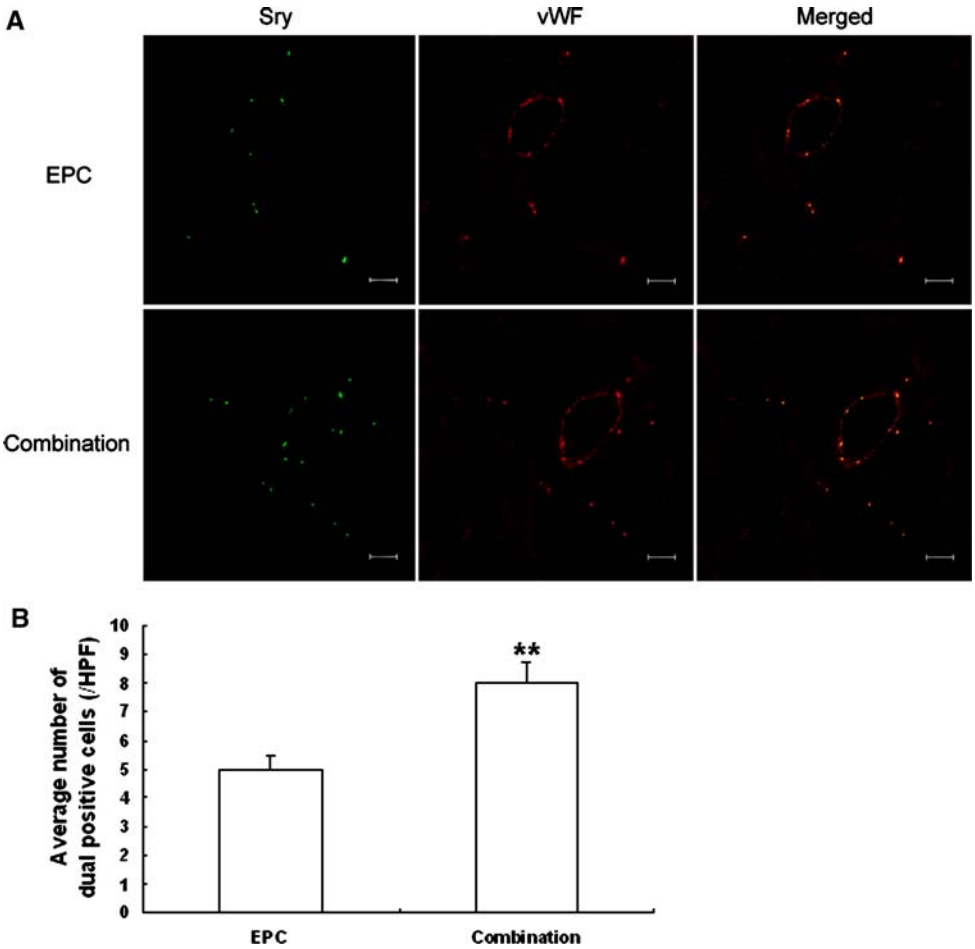

Fig. 4 Differentiation of transplanted EPCs. (**a**) The representative micrograph of FISH analysis of the *Sry* positive cells (green), vWF positive cells (red) and double positive cells for Sry and vWF (yellow) on hind limb sections of female athymic nude mice in the EPC and the combination group (×400). (**b**) Average number of Sry/vWF double-positive cells was significantly higher in the combination group than in the EPC group. Data are mean ± SEM. $^{**}P < 0.01$ versus EPC. Bars: 25 μm. HPF indicates high power field

Combination therapy protects ischemic skeletal muscle cells against apoptosis

TUNEL-positive nuclei staining was detectable in skeletal muscle cells in both the control and the treated groups (Fig. 5a). The percentage of TUNEL-positive cells markedly decreased in the simvastatin group (25.62 ± 2.62%), the EPC group (23.43 ± 2.55%) and the combination group (14.43 ± 1.42%) compared with the control group (37.24 ± 3.83%, $P < 0.01$). The percentage of apoptotic

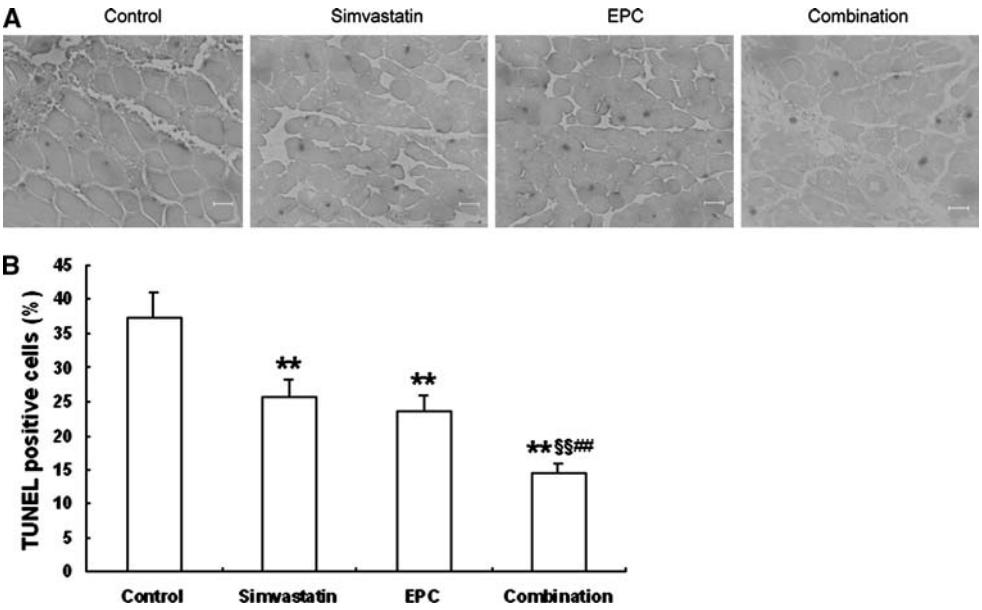

Fig. 5 The combination therapy reduced apoptosis. (**a**) Representative photomicrographs of TUNEL-positive nuclei staining (brown) in ischemic skeletal muscle cells (×400). (**b**) The percentage of apoptotic cells was the lowest in combination group among the four groups. Data are mean ± SEM. $^{**}P < 0.01$ versus control; $^{\S\S}P < 0.01$ versus simvastatin; $^{\#\#}P < 0.01$ versus EPC. Bars: 25 μm

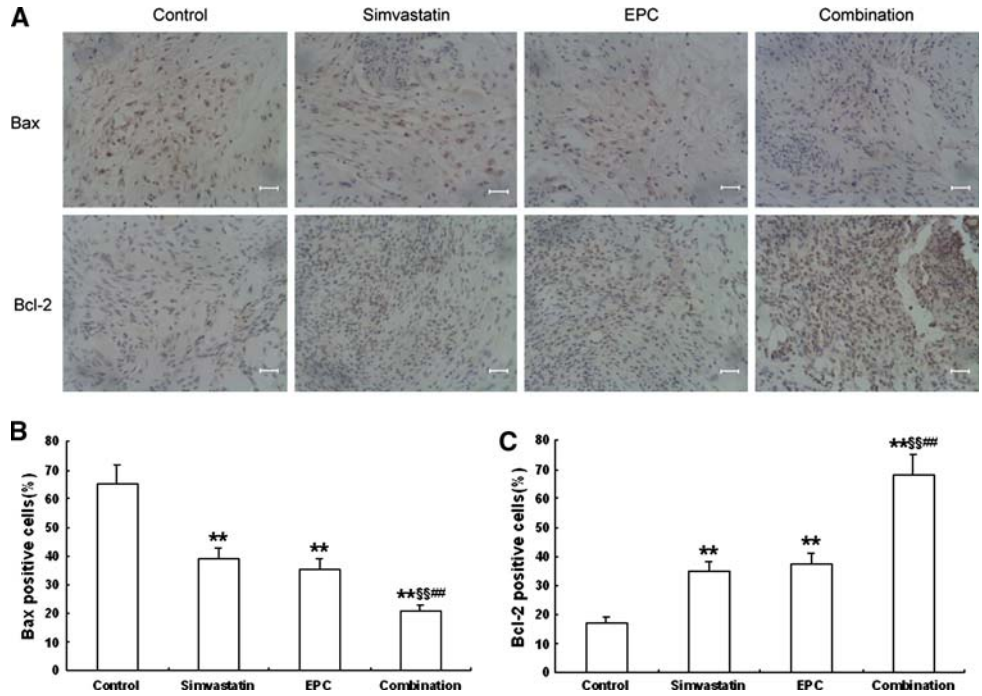

Fig. 6 The combination therapy promoted Bcl-2 and inhibited Bax expression. (**a**) Representative photomicrographs of Bax-positive staining (brown) and Bcl-2-positive staining (brown) in ischemic skeletal muscles cells (×400). (**b**) The percentage of Bax-positive cells was the lowest in combination group among the four groups. C, The percentage of Bcl-2-positive cells was the highest in combination group among the four groups. Data are mean ± SEM. $^{**}P < 0.01$ versus control; $^{\S\S}P < 0.01$ versus simvastatin; $^{\#\#}P < 0.01$ versus EPC. Bars: 25 μm

cells was the lowest in the combination group among the four groups (Fig. 5b).

Combination therapy inhibits Bax and promotes Bcl-2 expression

Immunohistochemical staining showed that Bax or Bcl-2 immunoreactivity was found in the control and the treated groups (Fig. 6a). The percentage of Bax positive cells was significantly reduced in the simvastatin group, the EPC group and the combination group compared with the control group (39.03 ± 4.27%, 35.51 ± 3.60% and 20.84 ± 2.05% versus 65.14 ± 6.47%, respectively, $P < 0.01$). The percentage of Bcl-2 positive cells was higher in the simvastatin group, the EPC group and the combination group than in the control group (34.70 ± 3.52%, 37.24 ± 3.74% and 68.16 ± 6.25% versus 17.29 ± 1.81%, respectively, $P < 0.01$). The percentage of Bax-positive cells was the lowest (Fig. 6b) and the percentage of Bcl-2-positive cells was the highest (Fig. 6c) in the combination group among the four groups.

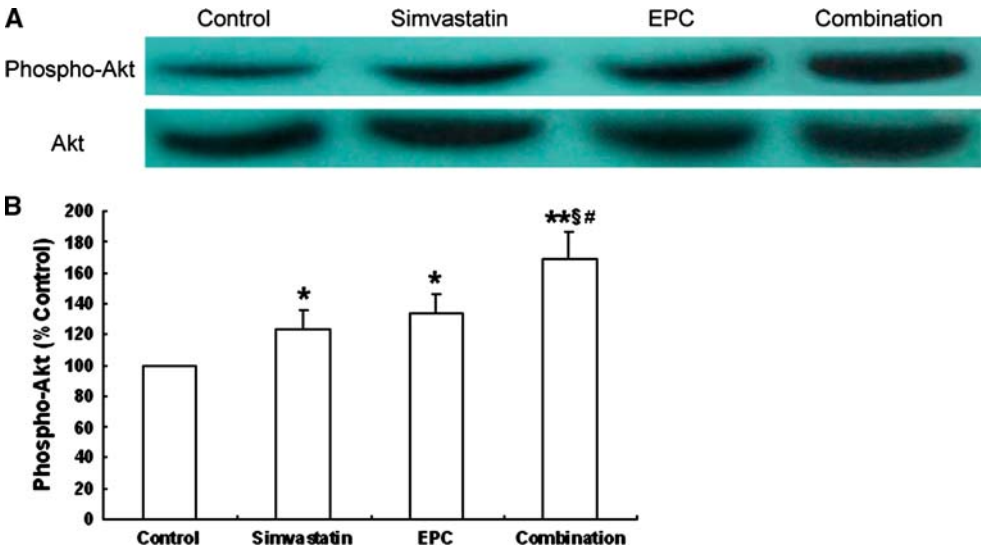

Fig. 7 The combination therapy activated Akt phosphorylation. (**a**) Representative western blots for expression of phospho-Akt, Akt. Total Akt served as a loading control. (**b**) The ratio of phospho-Akt to Akt was the highest in the combination group among the four groups. Each value was the mean ± SEM of triplicate determinations. Data are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control; $^{\S}P < 0.05$ versus simvastatin; $^{\#}P < 0.05$ versus EPC

Combination therapy activates Akt phosphorylation

As shown in Fig. 7a, significant increases in Akt phosphorylation and the ratio of phospho-Akt to Akt were observed in the treated groups compared with the control group. The phospho-Akt/Akt was the highest in the combination group among the four groups (Fig. 7b).

Discussion

In the present study, we demonstrated that the combinatorial simvastatin administration and EPC transplantation caused significantly greater improvement in hindlimb ischemia than simvastatin administration or EPC transplantation alone. The combination therapy stimulated transplanted EPCs survival notably compared with EPC transplantation. Furthermore, we demonstrated that the combination therapy protected ischemic skeletal muscle cells against apoptosis in part via downregulation of Bax and upregulation of Bcl-2 more effectively than simvastatin administration or EPC transplantation alone. We also proved that the combination therapy activated Akt phosphorylation markedly.

EPCs contribute to reendothelialization and neovascularization after endothelium injury and tissue ischemia [20]. Meanwhile, simvastatin promotes angiogenesis in ischemic limbs [21]. Further studies have revealed that statins raise EPC numbers and improve function additionally including an increase in proliferative, promigratory, proadhesive, antisenescent and antiapoptotic capacity in vitro [5, 6, 9, 22, 23]. Consistent with these researches, our study showed that administration of simvastatin or local infusion of EPCs increased blood perfusion and capillary density in hindlimb ischemia. Furthermore, a combination of simvastatin and EPC significantly enhanced angiogenesis compared with simvastatin administration or EPCs transplantation alone. In addition, observations indicated that significantly more transplanted EPCs were found in the combination group than in the EPC group, which demonstrated that simvastatin increased transplanted EPCs survival in ischemic tissues in vivo. Therefor the angiogenic potency of the combination therapy may not only attribute to the influences of simvastatin and transplanted EPCs on the ischemic hindlimb respectively, but also to the beneficial effects of simvastatin on the proliferation, migration, adhesion, senescence and apoptosis of transplanted EPCs.

Previous studies have showed that the fundamental cellular process of apoptosis contributes to myocyte loss in many forms of ischemic injury [24–26]. Apoptosis is dramatically activated by ischemia in skeletal muscle cells. Therefore, modulating apoptosis in ischemic skeletal muscle may present a novel therapeutic target in peripheral arterial disease [27, 28].

Our observations manifested that simvastatin administration was capable of reducing apoptosis involving suppressing Bax expression, promoting Bcl-2 expression and thus increasing the ratio of bcl-2 to Bax. These benefits may be ascribed to simvastatin's effects on vasculature and inflammation [21, 29, 30]. EPC transplantation can protect skeletal muscle cells against apoptosis and cause regeneration [27]. In conformity with these findings, our data further documented that the antiapoptotic effect was regulated by inhibiting Bax, promoting Bcl-2 and raising the Bcl-2/Bax ratio in hindlimb ischemia. Recently, accumulating evidence revealed that transplanted bone marrow stem cells (BMSCs) provide protection by paracrine

mechanisms involving release of a wide array of cytokines such as vascular endothelial growth factor, basic fibroblast growth factor, insulin-like growth factor, etc. that exert their effects on surrounding cells [31–34]. It has been demonstrated that Bcl-2 and Bax can be regulated by these mediators [35–38]. Since BMSCs are multipotent and can differentiate into several distinct cell types, including EPCs, therefore the transplanted EPCs are inferred to mediate their protection by paracrine mechanism [39]. Strikingly, the combination therapy conspicuously enhanced antiapoptotic capacity than simvastatin administration or transplantation of EPCs alone. The combination therapy generated the most potent effects on down-regulation of Bax, up-regulation of Bcl-2, and thereby increasing the bcl-2/Bax ratio among the treated groups. These results identify novel molecular mechanisms underlying the protective effects of the combination therapy during ichema injury. Accordingly most ischemic skeletal muscle cells may survive and their impaired function can be improved.

The phosphatidylinositol 3-kinase-Akt signaling pathway is known to play an important role in angiogenesis [40–42]. Our research also confirmed that simvastatin administration induced phosphorylation of Akt in ischemic tissues of hind limbs. Interestingly, EPCs transplantation can also enhanced phosphorylation of Akt in ischemic limbs. This finding is in line with a recently study by Christian Kupatt et al. who has demonstrated EPC-mediated stimulation of the PI3-kinase/AKT pathway [39]. The mechanism is elucidated that EPCs not only promote neovascularization in the ischemic area, but in addition, EPCs, providing a rich source of secreted factors via paracrine mechanisms, also contribute at early time points to several protective processes by stimulating cell survival and controlling inflammation. Of note, the combinatorial simvastatin and EPC therapy significantly activated Akt phosphorylation than simvastatin administration or EPC transplantation alone. Thus the combination therapy enhances the greatest neovascularization via phosphorylation of Akt in the treated groups.

A concern for this study was that whether simvastatin administration might induce tumor formation. Though an increased risk of cancer was reported in some animal experiments during pronounced cholesterol lowering, which demonstrated that statins could augment the frequency of certain cancers in rodents, especially when used at high doses [43], experimental evidences also indicated that statins inhibited tumor growth [44–47]. Furtheremore, the majority of large trials with statins did not show significant differences in the incidence of cancer in comparison with placebo groups [48–51]. The usage of low-dose simvastatin might be less susceptible to causing cancer in this present research.

In summary, the simvastatin and EPC combination therapy promotes powerful angiogenesis in hindlimb ischemia. The combination therapy not only inhibites apoptosis of ischemic skeletal muscle cells partially via downregulation of Bax and upregulation of Bcl-2, but also activates Akt phosphorylation significantly. These efficacies may be mediated by the angiogenic potency of simvastatin, EPCs, and by the beneficial effects of simvastatin on transplanted EPCs as well. Therefore, the combination therapy has ascendency as a new strategy to attain a sufficient degree of therapeutic neovascularization.

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