## Original Article Atorvastatin plus therapeutic ultrasound improve postnatal neovascularization in response to hindlimb ischemia via the PI3K-Akt pathway

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Received January 17, 2019; Accepted April 19, 2019; Epub May 15, 2019; Published May 30, 2019

Abstract: Statins and therapeutic ultrasound (TUS) have been shown to ameliorate angiogenesis on ischemic hindlimb animals and promote human umbilical vein endothelial cells (HUVECs) tube formation and proliferation. Here, we evaluate the therapeutic effect of TUS in combination with atorvastatin (Ator) therapy on angiogenesis in hindlimb ischemia and HUVECs. After subjecting excision of the left femoral artery, all mice were randomly distributed to one of four groups: Control; Ator treated mice (Ator); TUS treated mice (TUS); and Ator plus TUS treated mice (Ator+TUS). At day 14 post-surgery, the Ator plus TUS treatment cohort had the greatest blood perfusion, accompanied by elevated capillary density. In vitro, Ator plus TUS augmented tube formation, migration and proliferative capacities of HUVECs. Additionally, the united administration upregulated expression of angiogenic factors phosphorylated Akt (p-Akt), phosphorylated endothelial nitric oxide synthase (p-eNOS), as well as vascular endothelial growth factor (VEGF), both in vivo and in vitro. These benefits could be blocked by either phosphoinositide 3-kinase (PI3K) or eNOS inhibitor. Our data indicated that the united administration could significantly enhance ischemia-mediated angiogenesis and exert a protective effect against ischemic/hypoxia induced damage among HUVECs through up-regulating VEGF expression and activating the PI3K-Akt-eNOS pathway.

Keywords: Statin, therapeutic ultrasound, angiogenesis, eNOS, VEGF

#### Introduction

Peripheral arterial disease (PAD), caused by narrowing of the arteries in the limbs, is increasing in incidence and prevalence as our population is ageing and as diabetes is becoming more prevalent [1]. Treatments for PAD include medications that reduce the increased risk of cardiovascular events and help improve blood flow, as well as endovascular or surgical repair or the bypass of blocked arteries [2]. Atorvastatin (Ator), a lipid lowering agent, possesses various pleiotropic vasculoprotective effects [3]. Preclinical studies suggest that statins may promote angiogenesis in ischemic limbs through mechanisms that may be mediated by activation of Akt signaling and endotheliumderived nitric oxide (NO) production in normocholesterolemic animals [4, 5]. Treatment with a low dose of atorvastatin improves angiogenic responsiveness and was dependent on the activation of PI3K/Akt signaling pathway [6]. Numerous studies have documented that statins can promote endothelial cells proliferation, migration in vitro and in vivo [7-9].

Therapeutic ultrasound (TUS) is a form of physical wave, usually at a frequency of 1-10 MHz. TUS may be used for physiotherapy and can exert biological effects through either thermal or mechanical mechanisms. Previous investigations have demonstrated TUS can improve endothelial cell function and promote angiogenesis in a rat model of intracerebral hemorrhage and a hindlimb ischemic mouse model [10-12]. It has been demonstrated that the molecular mechanisms underlying the pro-angiogenic qualities of TUS involves the up-regulation of endothelial nitric oxide synthase (eNOS) and vascular endothelial growth factor (VEGF) [13, 14].

Although the isolated effect of atorvastatin or TUS has been described, little evidence exists concerning the benefits of combined administration of atorvastatin and TUS in hindlimb ischemia individuals. Based on such, the objective of this investigation was to explore the effects of TUS and atorvastatin, employed individually and in coordination, on reperfusion in a model with hindlimb ischemia.

## Materials and methods

## Animals

C57BL/6 mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China), housed in a temperature- and humiditycontrolled room with a 12:12 hour light: dark cycle, and provided with ad libitum access to water and food. All animal studies and operative procedures were approved by the Shanghai Jiao Tong University Animal Care and Use Committee. All surgeries were performed under anesthesia, and all efforts were made to minimize suffering.

## Rat hindlimb ischemia model and treatments

Briefly, all mice were exposed to 2% isoflurane (Forane, Abbott, Queensborough, UK) through a vaporizer. Unilateral hindlimb ischemia was surgically induced as previously described [13]. Then, all mice were divided into 4 groups: (1) left femoral artery resection without any treatment (control group, n = 6), (2) left femoral artery resection treated with TUS (TUS group, n = 6), (3) left femoral artery resection treated with atorvastatin (Ator group, n = 6), (4) left femoral artery resection treated with TUS and atorvastatin (TUS+Ator group, n = 6). The mice in TUS group were exposed to active TUS for 9 minutes/day with an energy flux density of 0.3 W/cm<sup>2</sup> at a frequency of 1.0 MHz, the mice in Ator group received intragastric feeding with atorvastatin (10 mg/kg/day, Sigma), and TUS+ Ator group mice were treated with active TUS plus atorvastatin (10 mg/kg/day). TUS were produced by a device with applicator designed and made by Institute of Acoustics, Chinese

Academy of Science (Shanghai, China). All the treatments started on the day of operation, and the administration lasts to the end of the experiment.

## Thermal infrared imaging (TIRI) analysis

The blood perfusion was detected indirectly by the temperature of the limbs [13]. At five predetermined time points (before and immediately after surgery and at postoperative days 3, 7 and 14) we performed two consecutive thermal infrared scans over the same region of interest. After two scans, the average temperature of the ischemic and nonischemic (normal) hindlimbs were computed from histograms of the colored pixels.

## Immunohistochemistry analysis

Animals were euthanized after finishing infrared spectrum imaging on postoperative day 14. The thigh adductor muscle was immersed in 4% formaldehyde and embedded in paraffin for immunohistochemistry analysis. Capillary density in each cross-section (5 µm) was labeled by immunofluorescence staining as previously reported [15]. Briefly, after procedures of antigen retrieval and blocking, incubation with goat anti-CD31 antibody (BD, Franklin Lakes, NJ) and secondary antibody (Invitrogen, Carlsbad, CA, USA) were successively performed. 10 random microscopic fields (× 320 magnification) in each mouse were counted, and the density of capillary was determined as the number of capillaries/field.

## Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from American Type Culture Collection (Manassas, VA, USA) and were cultivated in DMEM medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin. The cells were maintained in a humidified atmosphere incubator (HF 212UV; Health Force Development Ltd, UK) with 5%  $CO_2$  at 37°C, passaged regularly. Each condition was assessed in triplicate.

#### Ischemia/hypoxia (I/H) model and treatments

According to previous study, HUVECs were cultured under the ischemic-hypoxia (I/H) conditions for 48 hr [14, 16]. Then the cells were divided into 6 groups: without any treatment (I/H group), the cells treated with TUS (9 min/ day for 3 consecutive days, 1 MHz, 0.3 W/cm<sup>2</sup>, TUS group), the cells treated with atorvastatin (10 nmol/L for 3 consecutive days, Ator group), the cells treated with TUS and atorvastatin with/without LY294002 or L-NAME for consecutive 3 days (Ator+TUS group, Ator+TUS+ LY294002 group, Ator+TUS+L-NAME group).

## Cell viability assay

Cell viability was assessed by CCK-8 assay. Briefly, after HUVECs were received appropriate treatments and seeded in 96-well plates, 10 µl CCK-8 (Dojindo Lab, Tokyo, Japan) solution was added to each well at a 1/10 dilution, followed by a further 2 h incubation. Absorbance was measured at 450 nm with a microplate reader (ELX 800; Bio-Tek, Winooski, VT, USA). The mean optical density of four wells in different groups was used to calculate the percentage of cell viability.

## Matrigel tubule formation assay

HUVECs were detached by trypsinization and, after neutralization of trypsin, were counted and resuspended. HUVECs were seeded at  $2 \times 10^4$  cells per well in Matrigel (BD Biosciences) pre-coated 96-well plates (Ibidi). Tube formation was quantified 6-8 h later. Digital images of endothelial tubes were obtained using a phase-contrast microscope (Leica). Tube formations were measured blind by an independent observer, giving total tube length per image.

## Transwell migration assay

24-well Boyden Transwell chambers (Corning, Cambridge, MA) were employed to determine cells migration. In brief, original incubation mediators (600  $\mu$ L) were added to lower chambers, corresponding pretreated HUVECs (1 × 10<sup>4</sup>/well/100  $\mu$ L) were reseeded in upper chambers in serum-free Medium. 24 hours later, migrated HUVECs were counted with light microscopy (× 100 magnification).

## Western blot analysis

NuPAGE 1 × LDS Sample Buffer (Invitrogen) was used to extract the total proteins of cells. Proteins from muscle tissues were extracted in RIPA buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA and 10 mM Tris-HCl, pH 7.0) containing phosphatase and protease inhibitor cocktail (Thermo Scientific). The protein extracts were subjected to centrifugation at 12000 g for 15 min and loaded onto sodium dodecyl sulfate-polyacrylamide gels and then transferred to nitrocellulose membranes (PALL). After blocking with 5% nonfat milk in TBS (Amresco. Solon, OH, USA) containing 0.1% Tween-20, the membranes were incubated with anti-eNOS. anti-p-eNOS (Ser1177), anti-Akt, anti-p-Akt (Ser473), anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA), and anti-vascular endothelial growth factor (VEGF) (Bevotime, Haimen, China) overnight at 4°C, followed by soaking in HRP-conjugated secondary antibodies and detected by Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA). Signals were quantified by densitometry using a Bio-Rad Image Laboratory system.

## Statistical analysis

Data were presented as mean  $\pm$  SEM. Two-way ANOVA was performed to evaluate the strain and condition factors. Tukey's post-hoc test was employed for multiple comparisons when a statistical significance was obtained with ANOVA. *P* values < 0.05 were considered significant.

## Results

Ator plus Tus increase hindlimb blood perfusion and vascular density in the hindlimb ischemia mouse model

A low dose of atorvastatin can significantly increase regional blood flow in hindlimb ischemic rats [6]. In our previous study, we found that TUS also augmented the blood perfusion and increased capillary density [13]. To investigate the effect of atorvastatin plus TUS in combination in hindlimb ischemia individuals, we recorded the representative photographs of infrared spectrum (before and immediately after surgery and at postoperative days 3, 7 and 14) (**Figure 1**).

Before surgery, the difference of left/right hindlimb temperature was  $\approx$  0 in all groups. Immediately after operative induction of the left hindlimb ischemia, temperature of the ischemic limb markedly decreased, ranging from 1.8 to 2.3°C. This decrease immediately after



**Figure 1.** A. Representative TIRI analyses of hindlimb temperature in each group. B. Progressive recoveries of temperature within 14 days after induction of limb ischemia were observed in 4 groups. C. Anti-CD31 immunohistochemical staining in an ischemic hindlimb muscle at day 14 after induction of ischemia. D. CD31 positive blood vessels were counted in ten microscopic fields from each section. Date are the mean  $\pm$  SEM (n = 6) (\**P* < 0.01) vs. control (\**P* < 0.05) vs. Ator. Scale bar indicated 100 µm.



Figure 2. Effect of atorvastatin with/without TUS on the expression of p-eNOS, eNOS, p-Akt, Akt and VEGF in an ischemic hindlimb. At day 14 after ischemia surgery, pro-angiogenic actions indicated by the expression of p-eNOS (B), p-Akt (C) and VEGF (D) was measured Western-blotting (A). Values are represented as mean  $\pm$  SEM, \*SEM, \*P < 0.001.

induction of unilateral limb ischemia did not differ among the 4 groups, indicating that the severity of the ischemia created was comparable among the 4 groups. Representative images of the hindlimb temperature before and immediately after surgery and at postoperative days 3, 7, and 14 are shown in **Figure 1A**. TIRI analyses disclosed progressive recovery of the temperature within 14 days after surgery (**Fi**- gure 1B). The temperature in Ator group decreased significantly at postoperative days 3 and 7 which did not differ from controls. However, minus was significantly higher in Ator+TUS group than controls and Ator in day 7 and 14. The hindlimb temperature improved significantly in the Ator+TUS united group compared with the control group at postoperative days 7 and 14 (Figure 1A, 1B).



**Figure 3.** Ischemic-hypoxia hampered the angiogenic capacity and reduced angiogenic factor expression in HU-VECs. A. The tube formation of HUVECs. Ischemic-hypoxia reduced tube-like length of HUVECs. B. Cell migration for unstimulated HUVECs versus after exposure to ischemic-hypoxia for 48 hr. Ischemic-hypoxia reduced the migration of HUVECs. C. The protein levels of p-Akt, p-eNOS, and VEGF were determined by Western blot analysis. D. Cell proliferation for unstimulated HUVECs versus after exposure to ischemic-hypoxia for 48 hr. Ischemic-hypoxia hampered the proliferation of HUVECs. Scale bars, 500  $\mu$ m. \**P* < 0.05.

Quantitative analyses counting the CD31-positive vessel under light microscopy (Olympus, Tokyo, Japan) revealed that the capillary densities were significantly higher (P < 0.01) in ischemic hindlimbs with 10 mg/kg/day atorvastatin for 2 weeks in comparison to the controls (**Figure 1C, 1D**). As expected, the highest density exhibited in the united administration group (P < 0.01 versus Ator). The results suggest that Ator induced neovascularization, and combined with TUS represented an effective adjunct intervention of therapeutic neovascularization in ischemic hindlimbs.

Mechanism of atorvastatin plus TUS in the hindlimb ischemia mouse model

To further examine the underlying mechanisms of atorvastatin plus TUS-induced angiogenesis, we evaluated the expression of Akt, eNOS and



Figure 4. CCK8 assay of HUVECs in response to ATOR and/or TUS. Values are mean  $\pm$  SEM, \**P* < 0.05.

VEGF protein expression in ischemic muscles among 4 groups at day 14 after femoral artery ligation (**Figure 2**). Protein expressions of p-Akt, p-eNOS, and VEGF were noticeably higher in unilateral or united group when compared to control (P < 0.001), and protein levels were highest in the united group among the 4 cohorts (**Figure 2B-D**). The united administration allowed better angiogenic factors expression than atorvastatin separately.

Ischemic-hypoxia hampered the angiogenic capacity and reduced angiogenic factors expression in HUVECs

Previous studies have shown that compared with normoxic HUVECs, hypoxic HUVECs showed enhanced migration and angiogenesis in vitro, but prolonged hypoxia reduced both the endothelial cell tube-like length and the angiogenic capacity of HUVECs when tested by Matrigel assay [16, 17]. We first evaluated the effect of ischemic-hypoxia on HUVECs and observed that exposure of HUVECs to ischemichypoxia for 48 hr caused an obvious alteration in in vitro HUVECs viability, migration, and tube formation (**Figure 3A**, **3B**, **3D**). Moreover, chemic-hypoxia reduced p-Akt, p-eNOS, and VEGF levels in HUVECs (**Figure 3C**).

#### Ator and TUS facilitated the HUVECs proliferation

The effects of atorvastatin plus TUS on HUVECs proliferation were assayed by CCK-8 assay (**Figure 4**). Atorvastatin or TUS was able to enhance the proliferation of HUVECs as have been previously described [7, 14]. In present

study, we found that the united administration facilitated the HUVECs proliferation better than atorvastatin or TUS separately.

## Ator and TUS augmented tube formation

In tube formation assay, the HUVECs-formed micro-tubes were lengthened in response to ATOR and/or TUS exposure. Atorvastatin plus TUS increased tubule formation induced by I/H in HUVECs. Moreover, this benefit could be markedly attenuated using eNOS inhibitor L-NAME or PI3K inhibitor LY-294002 (Figure 5).

## Ator and TUS enhance migration in vitro

The transwell migration assay was performed to evaluate HUVECs migration ability in response to atorvastatin and TUS. Both atorvastatin- and TUS-treated group showed more migrated HUVECs than control group, and the united administration allowed better HUVECs migration than atorvastatin or TUS separately (**Figure 6**).

# Ator and TUS enhanced angiogenic factors in vitro

To clarify the proangiogenic effects of atorvastatin on eNOS, Akt and VEGF activities in ischemic/hypoxia-treated HUVECs, we assessed the expression of eNOS, Akt and VEGF in treated HUVECs. As illustrated in **Figure 7B-D**, administration of atorvastatin upregulated phosphorylation of eNOS at Ser1177, phosphorylation of Akt at Ser437 and VEGF in ischemic/hypoxia-treated HUVECs. When combined with TUS, HUVECs experienced greater angiogenic factor expression than atorvastatin separately. The united benefits can be neutralized by administration of L-NAME and LY-294002 (**Figure 7**).

## Discussion

In this paper, we studied the effect of Ator plus TUS both in vivo and in vitro. The present study confirmed the presence of neovascularization in the ischemic hindlimbs of rats treated with Ator or TUS. Interestingly, the united administration of Ator plus TUS lead to a more notable improvement in response to hindlimb ischemia than Ator or TUS separately. Furthermore, we testified that the united administration rescued ischemic muscle cells through enhancing endo-



**Figure 5.** Tube formation ability was evaluated by Matrigel assay. A. Representative images of tube formation in HUVECs by stimulated atorvastatin or TUS, and united groups treated with LY294002 or L-NAME. B. Quantitative analysis of tube length. Values are mean  $\pm$  SEM, \*P < 0.001. Scale bar indicated 500 µm.



**Figure 6.** Ator plus TUS promote the migration of HUVECs. HUVECs were plated in transwell and stimulated by Ator and/or TUS for 3 days, and the united group treated with L-NAME or LY294002. Migrated cells were stained (A) and quantitative analysis of migrated cells (B). Values are mean  $\pm$  SEM, \**P* < 0.001. Scale bar indicated 200 µm.

thelial proliferation, tube formation, migration and secretion via acting on the Akt-eNOS-VEGF pathway of endothelial cells.

We and others showed that endotheliumderived NO is a critical modulator for angiogenesis [18, 19]. Reduced oxygen levels (hypoxia) reduced endothelial NOS (eNOS) activity and eNOS phosphorylation at Ser<sup>1177</sup> [20], which then led to endothelial dysfunction. Further, studies have demonstrated that overexpression of eNOS could be beneficial for ischemic muscle reperfusion [4]. Previous studies have demonstrated that TUS also promote angiogenesis by stimulating Akt-eNOS-VEGF axis [13, 14]. Our previous studies have demonstrated that TUS treatment increased protein levels of eNOS and p-Akt, both in vivo and in vitro. TUS

therapy promotes postnatal neovascularization through multiple angiogenic pathways in mice model of ischemic hindlimb [21]. In view of this, the contents of angiogenesis proteins p-Akt and p-eNOS in ischemic skeletal muscles were examined. We demonstrated that both TUS and Ator clearly restored the levels of protein expression, and the united administration showed the greatest effect. VEGF is a main regulator of endothelial cells survival and angiogenesis, and eNOS can stimulate the expression of VEGF [22]. In accordance with this finding, the result of the current investigation showed that ischemic muscle VEGF levels from untreated group were significantly lower than that in TUS and/or Ator treated rats, with capillary rarefaction. The treatments significantly elevated the level of VEGF, restored capillary



Figure 7. Atorvastatin plus TUS resulted in upregulation of eNOS phosphorylation, Akt phosphorylation and VEGF in ischemic/hypoxia-treated HUVECs. Treatment with L-NAME or LY294002 decreased the eNOS, Akt and VEGF activation of untied group. Values are mean  $\pm$  SEM, \*P < 0.05.

density, and the united administration exhibited the greatest effect among the treatment groups. In conclusion, the united administration of TUS plus atorvastatin promoted the angiogenesis in ischemic limb. The improved angiogenesis by the untied administration was documented by increased ischemic skin temperature, augmented capillary density, consistent with upregulated proangiogenic factors relative to the untreated group. Moreover, Ator plus TUS significantly increased the expression of angiogenic cytokines, such as p-eNOS, p-Akt and VEGF in the ischemic hindlimb. The increases in multiple angiogenic cytokines indicated that atorvastatin plus TUS might protect ischemic hindlimb tissue, and these cytokines might be subsequently increased.

In order to further investigate the possible regulatory mechanism by which TUS plus Ator protects HUVECs from ischemic/hypoxia in vitro, the PI3K-Akt-eNOS pathway was examined. The eNOS activity can be upregulated by activated Akt, activated PI3K-Akt-eNOS signaling pathway can robustly improve the survival of cells, and act a key role in angiogenesis. Statins increase endothelial cells via the PI3-kinase/Akt (PI3K/Akt) pathway and phosphorvlated Akt at Ser473 and subsequently led to increased expression of eNOS [23, 24]. Previous studies also showed that TUS enhanced phosphorylation of the endogenous Akt substrate, eNOS, inhibited apoptosis and accelerated vascular structure formation in vitro in an Akt-dependent manner [14, 25]. In the current study, Ator or TUS significantly rescued cells from ischemic/hypoxia, accompanied by elevated protein expressions, such as p-eNOS, p-Akt and VEGF, and the united group allowed better angiogenic factors expression than atorvastatin or TUS separately. The united benefits can be neutralized by administration of L-NAME

and LY-294002. The results indicated that Ator plus TUS triggered angiogenesis via activation of Akt-eNOS-VEGF axis.

In summary, the present investigation illustrated the effect of Ator and TUS united administration on hindlimb recovery via regulation of angiogenesis during hindlimb ischemia. These findings highlighted the powerful therapeutic potential of atorvastatin plus TUS for a safe and simple healing response with better bold perfusion in patients with critical limb ischemia.

## Acknowledgements

This study was supported by the Chinese Natural Science Foundation grants (11574210, 11774232 and 81870476); Science Foundation of Shanghai Science and Technology Committee (18401900500); Xinhua Hospital Program (15LC18).

#### Disclosure of conflict of interest

None.

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