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*Original Article*

# Rescue of hypertension-related impairment of angiogenesis by therapeutic ultrasound

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**Abstract:** We examined the hypothesis that therapeutic ultrasound (TUS) treatment would rescue the hypertension-related inhibition of ischemia-induced angiogenesis. TUS protects against endothelial dysfunction, but it is little known that the effect of TUS treatment on angiogenesis inhibited by hypertension. 20-week-old male spontaneously hypertensive rats (SHRs) and Wistar-Kyoto rats (WKYs) were randomly allocated to 4 groups: SHR; TUS treated SHR (SHR-TUS); WKY and TUS treated WKY (WKY-TUS). After undergoing excision of the left femoral artery, the ischemic skeletal muscles were treated with extracorporeal TUS for 9 minutes of daily exposure (frequency of 1 MHz, intensity of 0.3 W/cm$^2$) for 14 consecutive days. We found that TUS normalized the blood perfusion in SHR-TUS accompanied by elevated capillary density. Similar results were found in the protein expression of angiogenic factors. TUS treatment also enhanced peripheral capillary density in WKY rats and restored the capillary rarefaction in hypertension by elevating the protein levels of endothelial nitric oxide synthase (eNOS), hypoxic inducible factor-1α (HIF-1α), vascular endothelial growth factor (VEGF) and phosphorylated Akt (p-Akt) *in vivo*. Our data demonstrated that TUS treatment ameliorated hypertension-related inhibition of ischemia-induced angiogenesis, at least in part, via an NO-dependent manner.

**Keywords:** Therapeutic ultrasound, angiogenesis, spontaneously hypertensive rat, hindlimb ischemia

## Introduction

Peripheral vascular occlusive disease (PVOD), one of the major health concerns throughout the world, was affecting a growing number of patients. Sufficient collateral vessel formation would re-establish blood perfusion and rescue ischemic tissue. Recent studies have demonstrated that PVOD accompanied with certain cardiovascular risk factors, hampered collateral development in animals with critical limb ischemia [1-3]. Hypertension, a leading hazard factor of atherosclerosis, dramatically impedes endothelial functions [4, 5]. Data from either patients or animals assay demostrated that PVOD accompanied with hypertension exhibited impaired angiogenic capacity in response to ischemic attack [6, 7].

Therapeutic ultrasound (TUS) is a physical wave form with a frequency of 1-10 MHz [8]. Low-intensity continuous ultrasound, a newly-invented TUS, has exhibited therapeutic potentials in current studies, including tumor ablation and bone regeneration [9]. Until recently, pro-angiogenic effects of TUS have been illustrated in endothelial cells [10], a mouse model of hindlimb ischemia [11], and a porcine model of chronic ischemic heart disease (IHD) [12]. However, little evidence indicates whether TUS have favorable impacts on tissue revascularization in hypertensive individuals.

In the present study, we introduced TUS to a spontaneously hypertensive rat (SHR) model of hindlimb ischemia, and investigated whether

**Table 1.** Effect of low-intensity continuous TUS on physiological parameters in WKY rats and SHRs

| Parameters | WKY | WKY-TUS | SHR | SHR-TUS |
|---|---|---|---|---|
| BW (g) | 293.7 ± 2.9 | 295.3 ± 2.8 | 296.5 ± 2.0 | 295.7 ± 2.5 |
| HR (beats/min) | 362.7 ± 4.3 | 357.8 ± 3.9 | 366.7 ± 2.8 | 365.8 ± 5.0 |
| GLU (mmol/L) | 6.4 ± 0.2 | 6.5 ± 0.1 | 6.4 ± 0.3 | 6.3 ± 0.3 |
| SBP (mmHg) | 132.1 ± 1.2 | 132.0 ± 1.0 | 202.5 ± 2.4** | 202.2 ± 3.0 |
| DBP (mmHg) | 98.0 ± 2.0 | 104.1 ± 2.8 | 153.4 ± 3.8** | 151.3 ± 5.7 |
| PP (mmHg) | 34.1 ± 1.8 | 27.9 ± 2.5 | 49.2 ± 4.6* | 50.9 ± 4.8 |

WKY: Wistar-Kyoto rat; WKY-TUS: Therapeutic ultrasound treated WKY; SHR: Spontaneously hypertensive rat; SHR-TUS: Therapeutic ultrasound treated SHR; BW: Body weight; HR: Heart rate; GLU: Glucose; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; PP: Pulse pressure; Values represent mean ± SEM; n = 6. $^{*}P < 0.05$ vs. WKY, $^{**}P < 0.01$ vs. WKY.

ischemia-induced angiogenesis could be ameliorated by TUS.

## Materials and methods

### *Animals*

20-week-old male SHRs and WKY rats were housed in an environmentally controlled breeding room, and allowed to have *ad libitum* access to food and water. All operative procedures were approved by the Shanghai Jiao Tong University Animal Care and Use Committee.

### *Induction of ischemic hindlimb*

The hindlimb ischemic rat model was induced as previously described [13-15]. Briefly, 20-week-old male SHRs and WKY rats were anesthetized (150 mg/kg chloral hydrate, i.p.), and the left femoral artery between the inguinal ligament proximally and the popliteal fossa distally was surgically excised. Then, the animals were randomly classified to 4 groups: SHR (n = 6); TUS treated SHR (SHR-TUS, n = 6); WKY (n = 6); and TUS treated WKY (WKY-TUS, n = 6).

### *Cardiovascular parameters*

With the aid of a computerized tail-cuff system (MPA-2000, Alcott Biotech, Shanghai, China), resting heart rate (HR) and blood pressure (BP) of rats were obtained in conscious condition. BP, HR and body weight (BW) were measured before and after TUS treatment.

### *Glucose assay*

Non-fasting blood glucose values of all rats were obtained in a tail-vein blood glucose measurement manner via an automated One Touch Ultra Glucometer (Johnson & Johnson, New Jersey, USA) before and after TUS treatment.

### *Hindlimb ischemia assay*

Hindlimb ischemic score was evaluated as previously described [16, 17]. Briefly, 14 days after hindlimb ischemic surgery, rats were assessed and scored as followed: 0, there was no obvious necrosis or defect; 1, necrosis was observed; 2, there existed amputation under ankle; 3, visible amputation presented above ankle.

### *TUS treatment*

TUS was generated by an ultrasonic generator, which was provided by Institute of Acoustics of Tongji University. TUS was transmitted by a cylindrical transducer with 2-cm diameter, with a frequency of 1.0 MHz and a density of 0.3 W/cm$^2$. The ischemic area of the rats among TUS groups were exposed to active TUS for 9 minutes per day, while the non-TUS groups were treated with inactive TUS for a consecutive 14 days after surgery.

### *Thermal infrared imaging (TIRI) analysis*

To evaluate the blood perfusion, the TIRI analyzer (Prism-DS 50137, FLIR Systems) was employed to measure the skin temperature of both hindlimbs [18, 19]. The little blood perfusion will give rise to a low skin temperature near the blood vessel, and vice versa. The TIRI measurements were performed pre-operation, immediately after surgery (day 0) and day 14 after operation. Different color pixels represented the diverse temperature values, dark-to-purple Interval denoted Low temperature, whereas red-to-white Interval expressed high temperature. To lessen the effects of ambient temperature, the ischemic/nonischemic limb temperature ratios were adopted to express blood perfusion.

### *Muscle histology and immunofluorescence analysis*

Fourteen days after operation, all rats were sacrificed and skeletal muscles were fixed in 4% paraformaldehyde. Five-mm thick histological sections were cut before hematoxylin and eosin (H&E) staining was carried out to evaluate myocyte morphology. Immunofluorescence staining for capillary density was per-

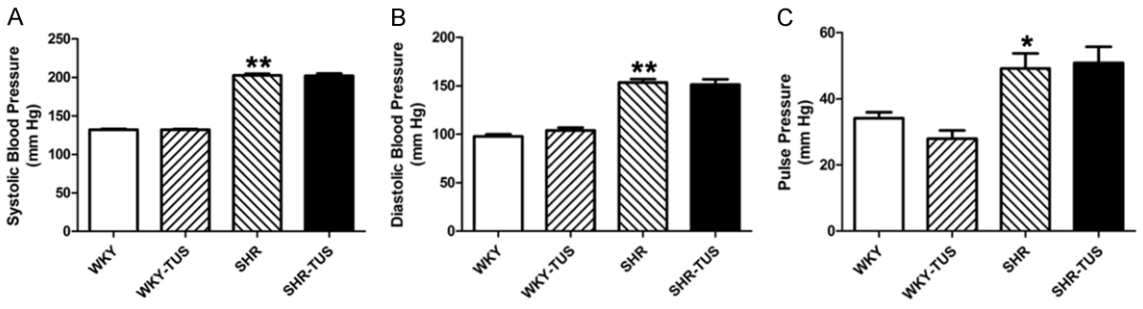


**Figure 1.** Blood pressure after TUS therapy. The data of (A), SBP, (B), DBP and (C), PP were collected on day 14. Values represent mean ± SEM; n = 6, $^{*}P < 0.05$ vs. WKY, $^{**}P < 0.01$ vs. WKY.

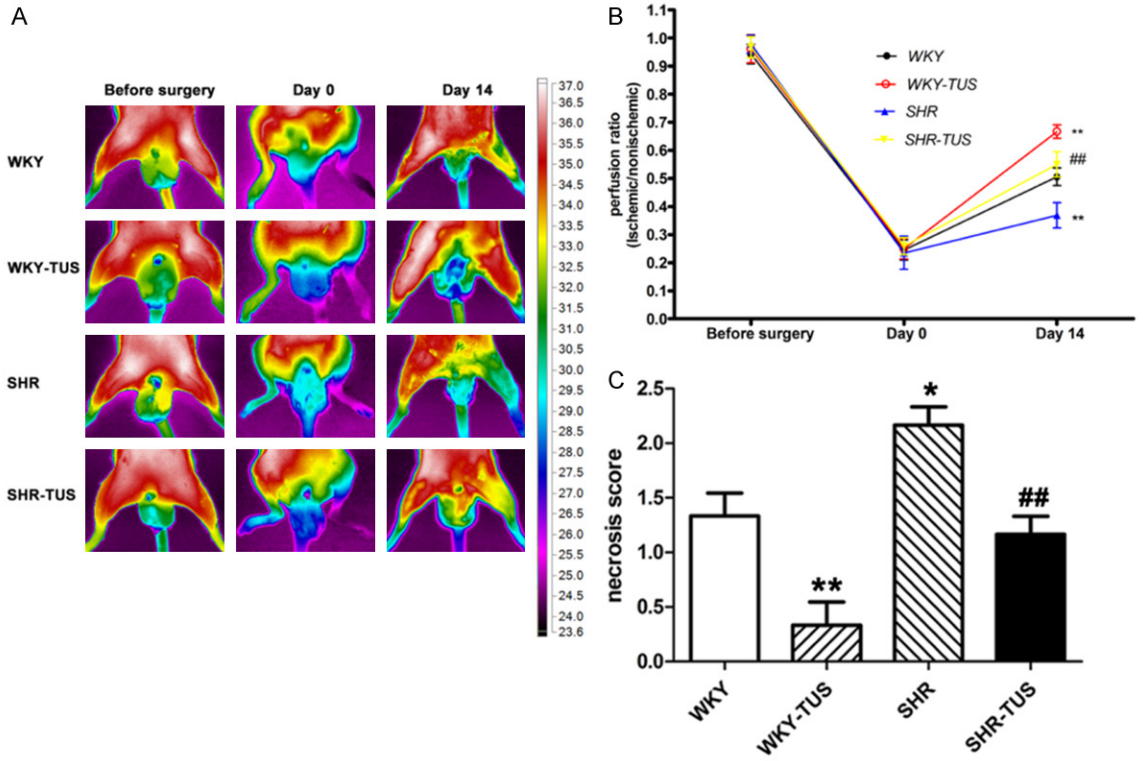


**Figure 2.** TUS elevated the blood flow restoration of the ischemic hindlimb in hypertension. A. Representative TIRI images of hindlimb blood flow in four groups. B. Reperfusion values were represented as changes in ischemic/non-ischemic limb perfusion before surgery, day 0 and day 14 after the hindlimb ischemia operation. C. Necrosis scores were evaluated in terms of a recognized evaluation standard on day 14. Values represent mean ± SEM; n = 6, $^{*}P < 0.05$ vs. WKY. $^{**}P < 0.01$ vs. WKY. $^{\#\#}P < 0.01$ vs. SHR.

formed as described previously [20]. Briefly, heat-mediated antigen retrieval procedure and blocking step were performed. The sections were labeled with goat anti-CD31 antibody (BD Biosciences, Franklin Lakes, NJ) at 4°C overnight followed with an anti-goat antibody (Invitrogen, Carlsbad, CA, USA) incubated at room temperature for 45 minutes. Ten randomly selected fields from each section were imaged, and the vessel density was reported as numbers of capillary per field (320 × magnification) [21, 22].

*Western blotting*

Western blotting was employed to analyze the levels of protein expression in the ischemic skeletal muscles as described previously [23-25]. Briefly, equal amounts of tissues were prepared, homogenized, electrotransfe-

rred, and then immunoblotted with anti-HIF-1α (Santa Cruz Biotechnology, California, USA), anti-eNOS, anti-Akt, anti-p-Akt (Ser473) (Cell Signaling Technology, Danvers, MA, USA), anti-VEGF (Beyotime, Haimen, China) and anti-GAPDH (Sigma, St. Louis, MO, USA). Targeted genes were detected with a FluorChem E data system (Cell Biosciences, Santa Clara, CA) and the densitometry by ImageJ software. Skeletal muscles GAPDH were performed as an internal control to standardize the results.

*Statistical analysis*

Data were reported as mean ± 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

*Physiological parameters*

The BW, HR, glucose, SBP, DBP, and PP values of the groups WKY, WKY-TUS, SHR and SHR-TUS are summarized in **Table 1**, After 2 weeks of TUS treatment, we didn't observe difference of basal HR, mean BW and random blood glucose among 4 individual groups.

Although, the SBP, DBP, PP of SHR and SHR-TUS were higher than their WKY counterparts, statistical differences were not observed in WKY-TUS and SHR-TUS groups compared with their untreated groups (**Figure 1**).

*Effect of TUS on hindlimb blood flow restoration in SHR*

To study whether TUS advanced tissue recovery, we assessed the necrosis scores of the ischemic limbs 14 days after surgery. We found that the ischemic scores of TUS-treated rats were significantly lower than that in non-treated ones either between WKY groups or SHR groups, indicating that TUS effectively enhanced skin wound healing and blood perfusion after ischemic episode (**Figure 2C**).

In the help of TIRI, blood perfusion among all groups were measured before surgery, immediately after surgery (day 0) as well as post-operative day 14. As expected, the induction of ischemia was followed by a dramatic decrease in hindlimb blood flow in both WKY and SHR. Compared with untreated ischemic WKY rats, the blood perfusion of untreated ischemic SHRs were significantly suppressed. Whereas, after 2-week-treatment of TUS, significant improvements of blood perfusion could be detected in WKY-TUS group and blood flow was normalized in SHR-TUS group relative to WKY group (**Figure 2A** and **2B**).

*TUS improved histologic recovery in SHR*

H&E sections indicated regular myocyte morphology, there were no obvious adipose cell or inflammatory cell infiltration, also muscle cells rounding, gathered nucleuses were not detected (**Figure 3**). To further prove the elevated blood perfusion was due to the newly-generated capillaries, the quantitative data of capillary density were determined. Anti-CD31 staining revealed that TUS augmented angiogenesis in TUS-treated rats. When compared with WKY rats, SHRs displayed capillary rarefaction. However, TUS exposure effectively elevated the capillary density in SHR-TUS group 14 days after operation (**Figure 4**).

*TUS increased angiogenic factors in SHR*

To deep elucidate the underlying molecular mechanism of TUS-induced angiogenesis in SHR, we subsequently measured the expressions of eNOS, VEGF, HIF-1α and p-Akt in skeletal muscles at day 14 after surgery. When compared with untreated WKY, TUS increased all these protein expressions in WKY-TUS (**Figure 5**). As expected, angiogenic factors were evidently retarded in SHR compared with WKY. Interestingly, this inhibition can be rectified by TUS in SHR-TUS (**Figure 5**), indicating that VEGF, eNOS, HIF-1α and p-Akt could act synergistically for appropriate vascular growth.

## Discussion

The major findings of the current study are (1) ischemia induced angiogenesis and regional blood perfusion were impaired in SHR, and the hypertension-related impairment of angiogenesis was associated with down-regulated pro-angiogenic factors in the ischemic tissues; (2) TUS, a noninvasive and non-pharmacological intervention, can improve angiogenic factors levels and blood perfusion in hypertensive rats.

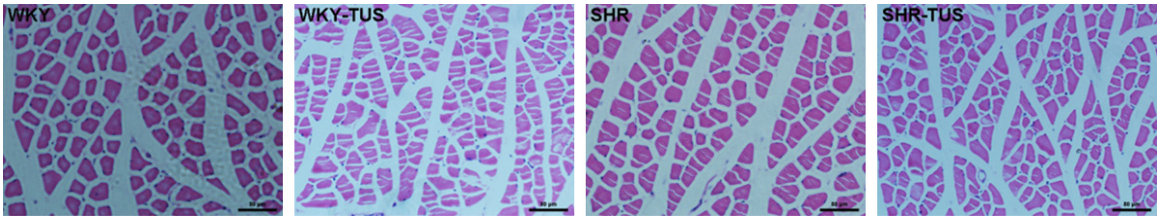


**Figure 3.** Representative H&E-staining images of ischemic hindlimb muscles of all groups. There was no proof of muscle cells rounding, gathered nucleuses, adipose cells or inflammatory cells infiltration indicating significant atrophy or necrosis (320 × magnification, Scale bar = 50 μm).

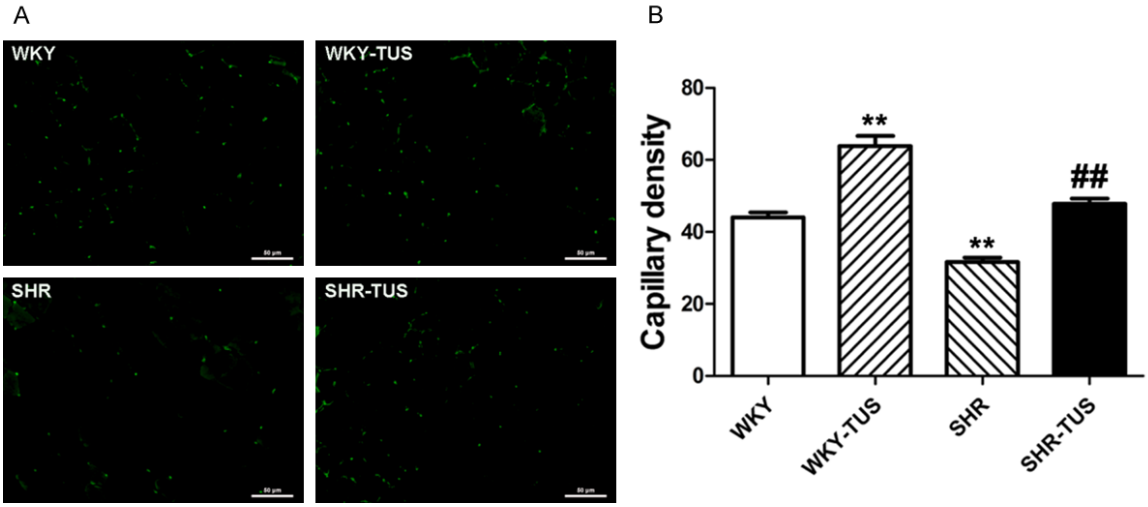


**Figure 4.** TUS augmented capillary growth of the ischemic hindlimb in hypertension. CD31-positive cells were stained in ischemic skeletal muscles at postsurgical day 14. A. Representative photographs of immunofluorescence staining with an anti-CD31 of 4 groups (320 × magnification, Scale bar = 50 μm). B. Quantitative analysis. Values represent mean ± SEM of capillary density (capillaries/field) in 4 individual groups of rats, n = 6, $^{**}P < 0.01$ vs. WKY. $^{\#\#}P < 0.01$ vs. SHR.

*Hypertension impaired ischemia-induced angiogenesis in vivo*

In the current investigation, angiogenesis in response to ischemia was significantly retarded in SHR. Several possible mechanisms should account for the impaired angiogenesis in the hypertension context. First, hypertension-related endothelial dysfunction and decreased eNOS expression may account for the impaired angiogenesis, for the proliferation and migration of ECs are essential and initial processes for angiogenesis. Moreover, studies have revealed that compensatory angiogenic response after ischemic attack was severely attenuated in eNOS-knockout animals [26-28]. Therefore, reduced synthesis of NO in the hypertensive state may partially explicate the inhibited angiogenesis. Consistent with this hypothesis, the expression of eNOS protein were all dramatically reduced in the hypertension rats compared with their WKY counterparts in our current investigations. Second, hypertension-induced oxidative stress accelerated cellular toxicity, exacerbated endothelial dysfunction and apoptosis associated with rarefaction of microvessels [7, 29-31]. Moreover, hypertension itself may directly restrain EC activity. Taken together, EC dysfunction and decreased eNOS production in hypertension seem to be responsible for the impaired angiogenesis in the Hypertension background.

*TUS restored the hypertension-mediated harm of angiogenesis*

In the present study, TUS rescued the impaired angiogenesis in spontaneous hypertensive rats. The enhanced angiogenesis by TUS was documented by the decreased limb necrosis

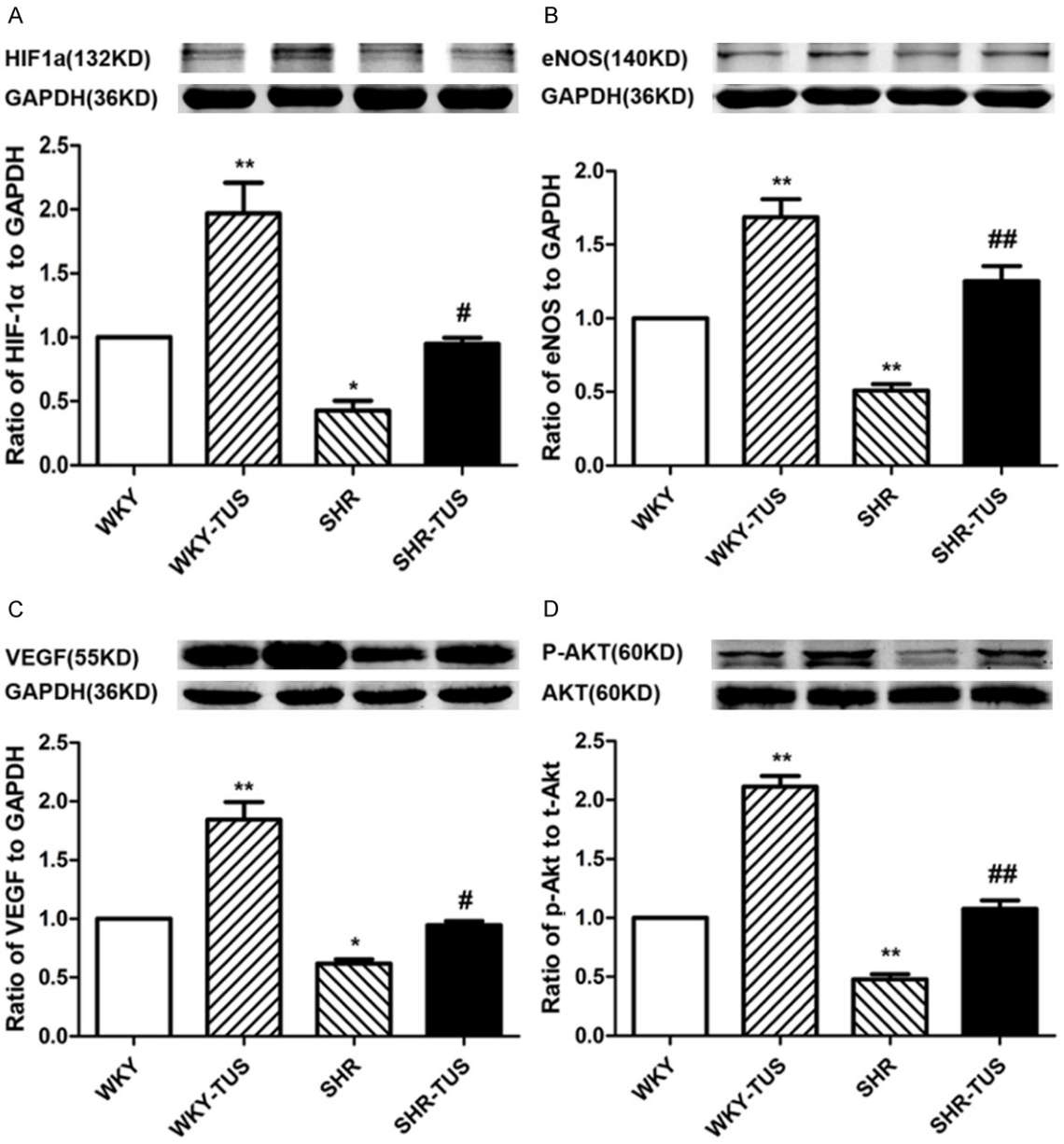


**Figure 5.** TUS effects on angiogenic factors expression in ischemic skeletal muscles in hypertension. Fourteen days after surgery, protein content of (A) HIF-1α, (B) eNOS, (C) VEGF and (D) phosphorylated Akt (p-Akt), total Akt (Akt) were analyzed by western blot, genes were standardized to ischemic internal GAPDH and represented as fold of WKY. Values represent mean ± SEM; n = 6, $^{*}P < 0.05$ vs. WKY. $^{**}P < 0.01$ vs. WKY. $^{\#}P < 0.05$ vs. SHR. $^{\#\#}P < 0.01$ vs. SHR.

scores, increased ischemic/nonischemic blood perfusion ratio by the TIRI analysis, increased capillary density compared with the untreated SHR group. Moreover, TUS significantly elevated the angiogenic factors of VEGF, eNOS and HIF-1α via activating PI3K-Akt signal pathway in the ischemic tissues.

Previous investigations have demonstrated that postnatal angiogenic response is retarded in animals and human beings of hypertension [6, 7], and such impairment can be partially reversed by Lowering blood pressure [32]. However, the pro-angiogenic effect of TUS was not due to changes in blood pressure, since significant statistical differences were not obtained in blood pressure between SHR group and TUS treated SHR group, while blood pressure values were remarkably higher in SHR individuals than in WKY rats.

Ultrasound has been extensively adopted in medical field, both as a diagnostic appliance, as well as a therapeutic tool. TUS can generate many kinds of biological effects *in vitro* and *in vivo* [33], including facilitating angiogenic cells proliferation and migration capacities [34, 35], increasing revascularization of rats suffered from skeletal muscle ischemia [8], and improving capillary density of porcine subjected to chronic ischemic heart disease [12]. Apart from proangiogenic potentials, relevant researches have demonstrated that the low energy ultrasound wave irradiation performs a variety of effects, including anti-inflammatory effects, antioxidant responses and antiapoptotic effects [36-39].

The precise mechanisms of TUS enhanced tissue angiogenesis in the ischemic limb remains a brisk field of research. Previous investigation showed that TUS can augment calcium channels open in ECs membrane [40], leading to an abundance of $Ca^{2+}$ influx, which is important to enhance cell proliferation [41]. Elevated intracellular $Ca^{2+}$ can activate and upregulate the expression of eNOS, and therefore sufficient and sustained release of NO, which is a crucial mediator to enhance peripheral vessels perfusion. Furthermore, in accord with the previous study [11], we also revealed that the HIF-1α-Akt signal pathway was activated in hypertensive rats subjected to ischemia.

Recent investigations have revealed that endothelial mechanical stress can induce sustained eNOS synthesis, and insufficient perfusion and NO would result in peripheral microvessel rarefaction in hypertension [42, 43]. As a matter of fact, the suppressed NO activity inhibited angiogenesis in hypertension episode [44]. TUS can deliver mechanical energy to endothelial cells to regulate the activity of eNOS [45, 46], and augmented eNOS can upregulate VEGF expression [47], which is a major regulator of ECs survival and a robust agonist to sustain angiogenesis [48]. Additionally, HIF-1α could be activated by intracellular NO through the PI3K-Akt-eNOS signaling pathway.

Another possible pro-angiogenic mechanism of TUS is to improve endothelial function. Previous study [49] have revealed that regenerating ECs in ischemic tissues were generally dysfunctional, and our previous studies have indicated that TUS can promote ECs migration, proliferation and tube formation through PI3K-Akt-eNOS signal pathway [50]. Other acoustic-effects, including anti-inflammatory [36], antioxidant [51], antiapoptotic [52] and pro-satellite cell differentiation [53], may also conduce to the protective effects of ultrasound in the current investigation, and further study of the detailed mechanism of revascularization by TUS is extremely needed.

In conclusion, our present experiment uncovered that angiogenesis of the ischemic skeletal muscles were retarded in SHR compared with their WKY counterparts, as we expected, this suppression can be restored by TUS, partially, by an NO-dependent manner. The enhanced multiple angiogenic pathways and normalized blood perfusion in response to TUS for hypertensive peripheral arterial disease (PAD) may represent an attractive clinical approach to stimulate ischemic angiogenesis and restore lower limb perfusion.

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## Disclosure of conflict of interest

None.

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