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Original Article

Therapeutic ultrasound plus pulsed electromagnetic field improves recovery from peripheral arterial disease in hypertension

Zhao-Yang Lu[1*], Hong-Sheng Zhou[2*], Zhi-Xiao Su[1,3], Jia Qi[1,4], Jian Zhang[4], Guan-Hua Xue[5], Yue Li[6], Chang-Ning Hao[5], Yi-Qin Shi[7], Jun-Li Duan[1]

Departments of [1]Gerontology, [4]Pharmacy, Xinhua Hospital, School of Medicine, Shanghai Jiaotong University, Kongjiang Road 1665, Shanghai 200092, China; [2]Shanghai Acoustics Laboratory, Chinese Academy of Science, Xiaomuqiao Road 456, Shanghai 200032, China; [3]Shanghai Key Laboratory of New Drug Design, School of Pharmacy, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China; [5]Department of Vascular Surgery, Renji Hospital, School of Medicine, Shanghai Jiaotong University, Dongfang Road 1630, Shanghai 200127, China; [6]Division of Pulmonary and Critical Care Medicine, Department of Medicine, School of Medicine, University of Maryland, 20 Penn Street, HSF-2, Room #S112, Baltimore 21201, MD, USA; [7]Department of Nephrology, Zhongshan Hospital, Fudan University, Fenglin Road 180, Shanghai 200032, China. [*]Equal contributors.

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Abstract: The objective of this investigation was to evaluate the therapy effect of combined therapeutic ultrasound (TUS) treatment and pulsed electromagnetic field (PEMF) therapy on angiogenesis in hypertension-related hindlimb ischemia. After subjecting excision of the left femoral artery, spontaneously hypertensive rats (SHRs) were randomly distributed to one of four groups: SHR; TUS treated SHR (SHR-TUS); PEMF treated SHR (PEMF-TUS); and TUS plus PEMF treated SHR (SHR-TUS-PEMF). Wistar-Kyoto rats (WKYs) with femoral artery excision were regarded as a control group. At day 14 after surgery, the TUS plus PEMF united administration had the greatest blood perfusion accompanied by elevated capillary density and the lowest TUNEL index. Interestingly, the united administration up-regulated the angiogenic factors expression of phosphorylated Akt (p-Akt), phosphorylated endothelial nitric oxide synthase (p-eNOS), vascular endothelial growth factor (VEGF), anti-apoptotic protein of Bcl-2 and down-regulated pro-apoptotic protein levels of Bax and Cleaved caspase-3 *in vivo*. Our results demonstrated that the united administration could significantly rescue hypertension-related inhibition of ischemia-induced neovascularization partly by promoting angiogenesis and inhibiting apoptosis.

Keywords: Therapeutic ultrasound, pulsed electromagnetic field, peripheral arterial disease, spontaneously hypertensive rat

Introduction

Peripheral arterial obstructive disease (PAOD) is caused by atherosclerotic of peripheral arteries, and the lower extremity is the predilection site. Hypertension, a major hazard element for atherosclerosis, can accelerate the progression of cardiovascular diseases (CVDs) [1, 2]. The clinical prognosis for patients with this form of artery disease is poor. In spite of surgical treatment or endovascular intervention has gained tremendous improvement in restoring blood flow, clinical symptoms may persistent or recur. It seems new therapeutic schedules for PAOD represent a continuing requirement.

Therapeutic ultrasound (TUS) is a form of physical wave, usually at a frequency of 1-10 MHz. Previous investigations have demonstrated TUS can improve endothelial cells function [3], promote angiogenesis in a rat model of intracerebral hemorrhage and a hindlimb ischemic mouse model [4, 5]. Pulsed electromagnetic field (PEMF), a form of electromagnetic stimulation, has been reported can improve endothelial function [6, 7], accelerate fracture healing

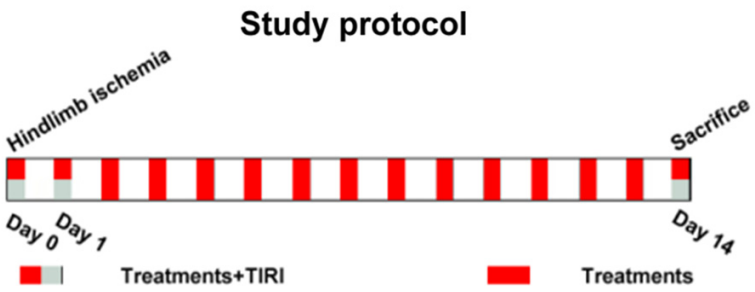

Figure 1. Experimental protocol and sampling time points. TIRI indicates thermal infrared imaging.

[8], as well as stimulate angiogenesis in an acute hindlimb ischemic rat model [9].

Although the isolated effect of TUS or PEMF has been described, little evidence exists concerning the profits of the use of TUS and PEMF in combination in hypertensive individuals. Based on such, the objective of this investigation was to estimate the effects of TUS and PEMF, employed individually and in coordination, on reperfusion in a SHR model with PAOD.

Materials and methods

Animal care

Twenty-week-old, male WKY rats and SHRs were used for experiments in this investigation. All animals were housed at a controlled breeding room (temperature at 22°C and an automatic 12-h light-dark cycle), and allowed to get common rat chow and water freely. All animal studies and operative procedures were approved by the Shanghai Jiao Tong University Animal Care and Use Committee.

Rat hindlimb ischemia model

Briefly, all WKY rats and SHRs were anesthetized using ketamine (50 mg/kg, intra-peritoneal administration) and diazepam (5 mg/kg, intra-peritoneal administration). After skin incision, the entire left femoral artery and vein (between the inguinal ligament proximally and the popliteal fossa distally) were isolated and excised.

Experimental procedures

All WKY rats and SHRs were randomly distributed into 5 experimental groups: A) WKY (n = 6); B) SHR (n = 6); C) TUS treated SHR (SHR-TUS, n = 6); D) PEMF treated SHR (SHR-TUS, n = 6); and E) TUS plus PEMF treated SHR (SHR-TUS-PEMF, n = 6). All rats were underwent unilateral hindlimb ischemia as illustrated above. Rats started to receive treatment on the day of operation, and the administration lasts to the end of the experiment. Before ischemic operation and after surgery at day 14, all study rats were weighed, resting heart rate (HR) and systemic blood pressure (BP) were captured in the conscious condition by a computerized tail-cuff method (MPA-2000, Alcott Biotech, Shanghai, China), and blood glucose (GLU) levels were obtained via tail-vein blood with One Touch Ultra Glucometer machine (Johnson & Johnson, New Jersey, USA).

Acoustic devices

TUS was emitted by a specific ultrasonic generator (kindly designed and made by Institute of Acoustics of Tongji University). TUS (at a frequency of 1.0 MHz and a density of 0.3 W/cm^2) was transported through a 2-cm-diameter cylindrical transducer.

Electromagnetic devices

PEMF was transmitted by a commercial healing device-get from Biomobie (Shanghai, China) Regenerative Medicine Technology. Electromagnetic fields parameters were summarized as follows: asymmetric, with 4.5 ms pulses at 30±3 Hz, and magnetic flux density increasing from 0 to 5 mT in 400 μs.

Treatments

All WKY rats and SHRs were housed in specially designed cages, the Rats in TUS group were exposed to active TUS for 9 minutes/day, the rats in PEMF group were exposed to active PEMF for 32 minutes/day (4 cycles, 8 minutes/cycle), and TUS plus PEMF group rats were treated with active TUS (9 minutes/day) plus PEMF (32 minutes/day). All the treatments continued until 14 days after surgery (**Figure 1**). The administration time and density was chosen based on our previous experiments dis-

Table 1. Effect of TUS plus PEMF on physiological parameters in normotensive and hypertensive rats

	WKY	SHR	SHR-TUS	SHR-PEMF	SHR-TUS-PEMF
SBP (mmHg)					
Before surgery	130.74±2.89	182.12±8.38^{++}	181.16±3.79	182.34±3.78	180.56±3.75
14 day	128.88±3.04	184.75±3.33^{++}	181.73±3.99	182.40±3.87	183.33±3.75
DBP (mmHg)					
Before surgery	101.36±4.04	135.08±5.28^{++}	134.54±3.75	137.05±5.89	134.33±2.63
14 day	100.76±3.84	134.42±5.90^{++}	136.03±5.43	137.31±4.96	137.66±3.83
PP (mmHg)					
Before surgery	29.38±1.35	47.04±4.12^{+}	46.62±4.17	45.29±4.30	46.23±4.07
14 day	28.12±1.44	50.33±3.20^{+}	45.70±3.96	45.09±3.96	45.67±5.47
Weight (g)					
Before surgery	322.00±6.68	329.67±7.17	325.83±6.11	317.17±8.01	318.00±5.89
14 day	324.17±6.88	334.67±8.27	331.67±7.57	321.67±8.33	321.67±6.54
HR (beats/min)					
Before surgery	359.33±8.59	350.00±9.67	355.67±9.28	349.50±8.31	359.50±7.75
14 day	351.50±7.64	356.00±7.49	362.17±8.72	357.5±8.80	352.50±9.33
GLU (mmol/L)					
Before surgery	5.78±0.23	5.63±0.31	5.58±0.18	5.53±0.22	5.53±0.16
14 day	5.65±0.21	5.53±0.38	5.63±0.19	5.48±0.27	5.45±0.18

Values are represented as mean ± SEM. $^{+}P < 0.05$ vs. WKY. $^{++}P < 0.01$ vs. WKY.

playing efficient treatment and minimal side-effect [4, 7, 10, 11].

Thermal infrared imaging (TIRI) analysis

TIRI was performed, as we previously illustrated [10, 12, 13]. Blood perfusion Data was presented as ischemic/non-ischemic limb ratio.

Necrosis assay

Gross hindlimb ischemic score was determined as previously described [14], and the criteria as follows: 1, there existed no necrosis; 2, there were minor necrosis on the nail bed; 3, all digits could capture necrosis; 4, at least one digit was lost; and 5, more than two digits was lost or severe foot amputation.

Histological and immunofluorescence analysis

At postoperative day 14, gastrocnemius muscles from bilateral were collected and weighed, fixed in 4% paraformaldehyde. Muscle sections (Five-μm thick) were dyed with hematoxylin and eosin (H&E) to estimate myocyte morphology. For immunofluorescence assay, tissue sections were performed using anti-CD31 antibody (BD Biosciences, Franklin Lakes, NJ). Ten randomly selected fields in 4 independent sections (× 400 magnification) were counted, and the vessel density was represented as number of capillaries/field [11, 15, 16].

TUNEL assay

To examine the skeletal muscles apoptosis, the TUNEL technique was performed by a commercially available TUNEL kit (Promega, Madison, Wisconsin, USA). The number of total nuclei and the TUNEL^{+} nuclei (× 400 magnification) were reckoned by three independent investigators. TUNEL positive cell density was represented as TUNEL-positive nuclei per 10^3 nuclei.

Western blot analysis

Protein extracts from mid-anterior gastrocnemius were subjected to SDS-PAGE, electrotransfer, and then blotted with anti-eNOS, anti-p-eNOS (Ser1177), anti-Akt, anti-p-Akt (Ser473), anti-bcl-2, anti-bax, anti-caspase3, anti-cleaved caspase3 (Cell Signaling Technology, Danvers, MA, USA), anti-vascular endothelial growth factor (VEGF) and anti-GAPDH (Beyotime, Haimen, China). Then, the membranes were incubated with IRDye800CW conjugated secondary antibody. Targeted genes were detected by Odyssey imaging system (LICOR).

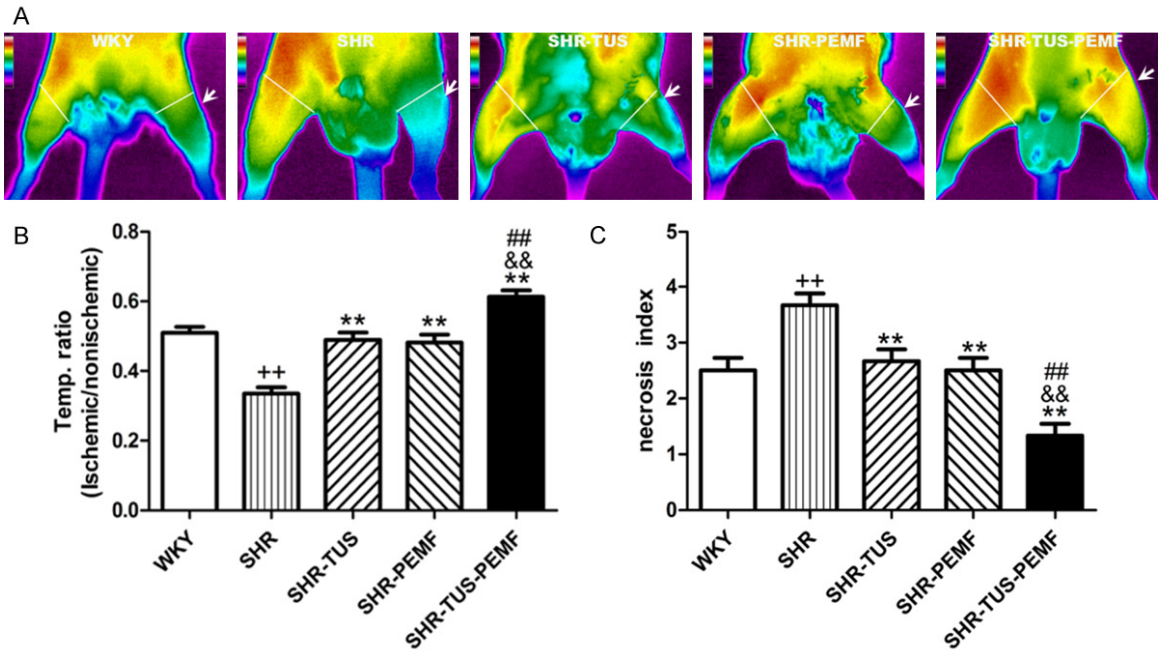

Figure 2. The united administration increased ischemic hindlimb blood flow and prevented ischemic tissue necrosis in hypertension. At day 14 after ischemia surgery, TIRI assay (A and B) and necrosis severity index (C) were performed to determine angiogenic actions. Values are represented as mean ± SEM. $^{++}P < 0.01$ vs. WKY. $^{**}P < 0.01$ vs. SHR. $^{\&\&}P < 0.01$ vs. SHR-TUS. $^{\#\#}P < 0.01$ vs. SHR-PEMF.

Statistical analysis

Data were presented 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

Table 1 summarizes systolic blood pressures (SBP), diastolic blood pressures (DBP), pulse pressures (PP), body weight (BW), heart rate (HR) and blood glucose (GLU) of all the groups before operation and at postoperative day 14. There existed no significant differences of basal HR, mean BW and random GLU among 5 individual groups at both of the determination points ($P > 0.05$). Although, all of the three blood pressure parameters in SHR group and SHR treatment groups were higher than that in WKY group ($P < 0.05$ versus WKY group), there were no statistical differences among SHR group and SHR treatment groups at any of the time points ($P > 0.05$).

Hindlimb perfusion

Firstly, we examined whether hypertension affects angiogenic responses as an answer to hypoxic injury, all of the rats were subjected to skeletal ischemia, and the ratio of ischemic/nonischemic temperature were estimated. At postoperative day 14, SHR group showed a lower temperature ratio compared to WKY group (SHR 0.34±0.02 versue WKY 0.51±0.02, $P < 0.01$), indicating that hypertension mitigated angiogenesis as an answer to hypoxia. However, the inhibiting effect of hypertension could be strongly reversed by the unilateral treatment or the united administration, as the temperature ratio was higher in the TUS treatment group (0.49±0.02, $P < 0.01$ versus SHR), the PEMF group (0.48±0.03, $P < 0.01$ versus SHR) and the united administration group (0.61±0.03, $P < 0.01$ versus SHR) than in SHR group. And the ratio of the united administration group reached the highest among the 5 groups (**Figure 2A** and **2B**).

Meanwhile, the pedal necrosis index was employed as described previously to assess the tissue necrosis of the 5 groups. We found that the necrosis index of SHR group ($P < 0.01$ versus WKY) exhibited a significant increase rela-

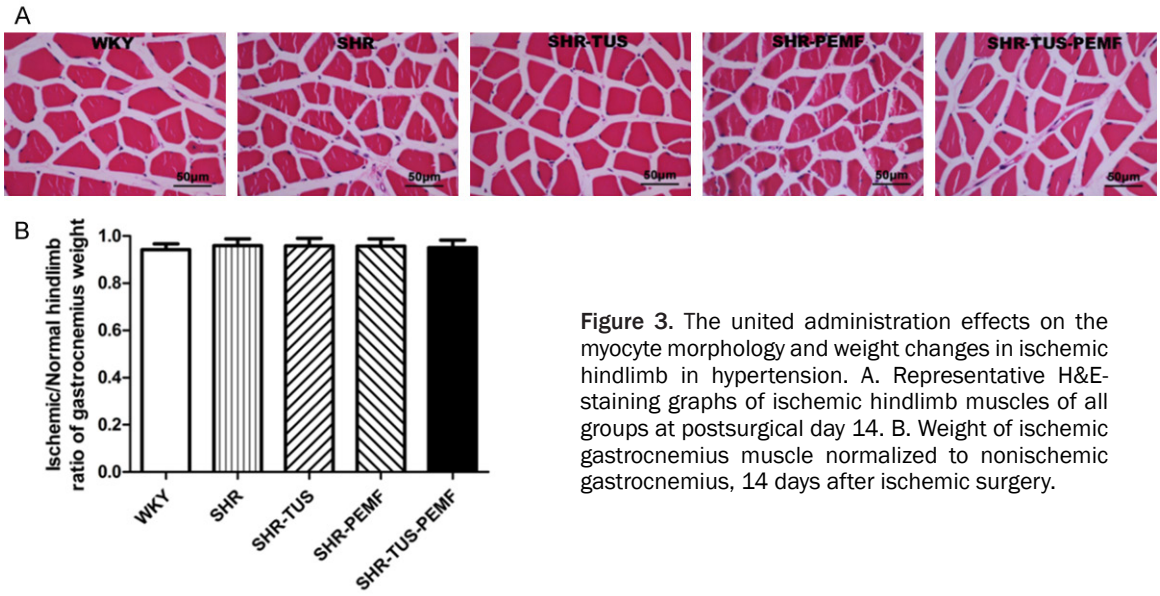

Figure 3. The united administration effects on the myocyte morphology and weight changes in ischemic hindlimb in hypertension. A. Representative H&E-staining graphs of ischemic hindlimb muscles of all groups at postsurgical day 14. B. Weight of ischemic gastrocnemius muscle normalized to nonischemic gastrocnemius, 14 days after ischemic surgery.

tive to WKY group, and the treatments can effectively restore this trend ($P < 0.01$ versus SHR), the necrosis index was the lowest in the united administration group among the 5 groups (**Figure 2C**).

Histologic and angiogenic responses

To evaluate the changes of myocyte morphology in respond to hypoxic injury, H&E sections were performed. The results showed approximately normal myocyte morphology, with significant muscle cells rounding, obvious centralized nuclei, pronounced inflammatory cell or adipose cell were not captured in all 5 groups (**Figure 3A**). Meanwhile, there was no notable difference in the weight ratio of the ischemic/nonischemic gastrocnemius muscle at the end of the investigation in all 5 groups (**Figure 3B**).

Anti-CD31 immunofluorescence staining of endothelial cells (**Figure 4A** and **4B**) revealed capillary rarefaction in SHR group ($P < 0.01$ versus WKY) relative to WKY group. As expected, the capillary density was significantly restored in 3 treatment groups ($P < 0.01$ versus SHR), and the highest density exhibited in the united administration group.

To further illustrate the potential mechanism underlying the retardment of ischemia-induced angiogenic response in SHR, we determined the contents of p-Akt, Akt, p-eNOS, eNOS, and VEGF protein expressions in ischemic skeletal muscles among the 5 groups at day 14 after femoral artery ligation. Protein expressions of p-Akt, p-eNOS, and VEGF were obviously lower in SHR group ($P < 0.01$ versus WKY) than that in WKY group. The unilateral treatment or the united administration significantly restored all the angiogenic factors ($P < 0.05$ versus SHR), and the protein levels were the highest in the united therapy group among the 5 groups (**Figure 4C-E**).

Apoptosis analyses

To examine whether the elevated perfusion rate in the treatment groups was related to the decrease in cellular apoptosis in respond to hypoxic episode in vivo, TUNEL technique was employed to detect the apoptotic cells. The apoptotic ratio, represented by TUNEL positive nucleus per 10^3 total nuclei, was remarkably elevated in the SHR group ($P < 0.01$ versus WKY) when compared to WKY group. The treatments significantly reduced the apoptotic ratio ($P < 0.01$ versus SHR), and the ratio of apoptosis in the united treatment group was the lowest among the 5 groups (**Figure 5A** and **5B**).

The Bcl-2 family proteins, Bax and Bcl-2, are strong regulators of apoptosis. Expression changes of these proteins demonstrate the damage of the survival programs. To identify the protein expression changes in the ischemic skeletal muscles in SHR, and the repair capacity of treatments in response to hypoxia, west-

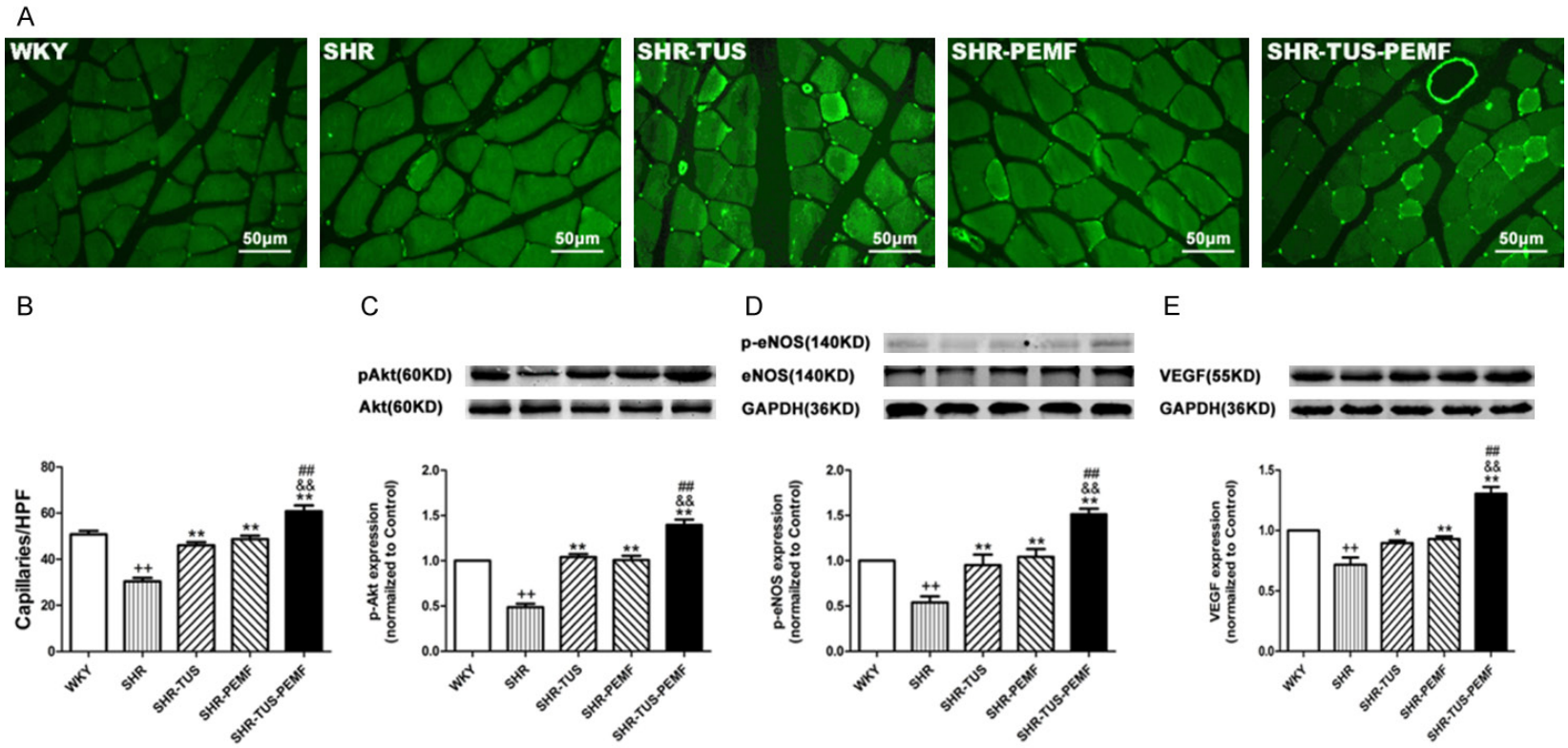

Figure 4. The united administration improved capillary growth and angiogenic factors expression in ischemic hindlimb skeletal in hypertension. At day 14 after ischemia surgery, anti-CD31 immunofluorescence staining (A and B), and Western blot assay to contents of p-Akt (C), p-eNOS (D), and VEGF (E) in the ischemic muscles were examined to estimate pro-angiogenic actions. Values are represented as mean ± SEM. $^{++}P < 0.01$ vs. WKY. $^{*}P < 0.05$ vs. SHR. $^{**}P < 0.01$ vs. SHR. $^{\&\&}P < 0.01$ vs. SHR-TUS. $^{\#\#}P < 0.01$ vs. SHR-PEMF.

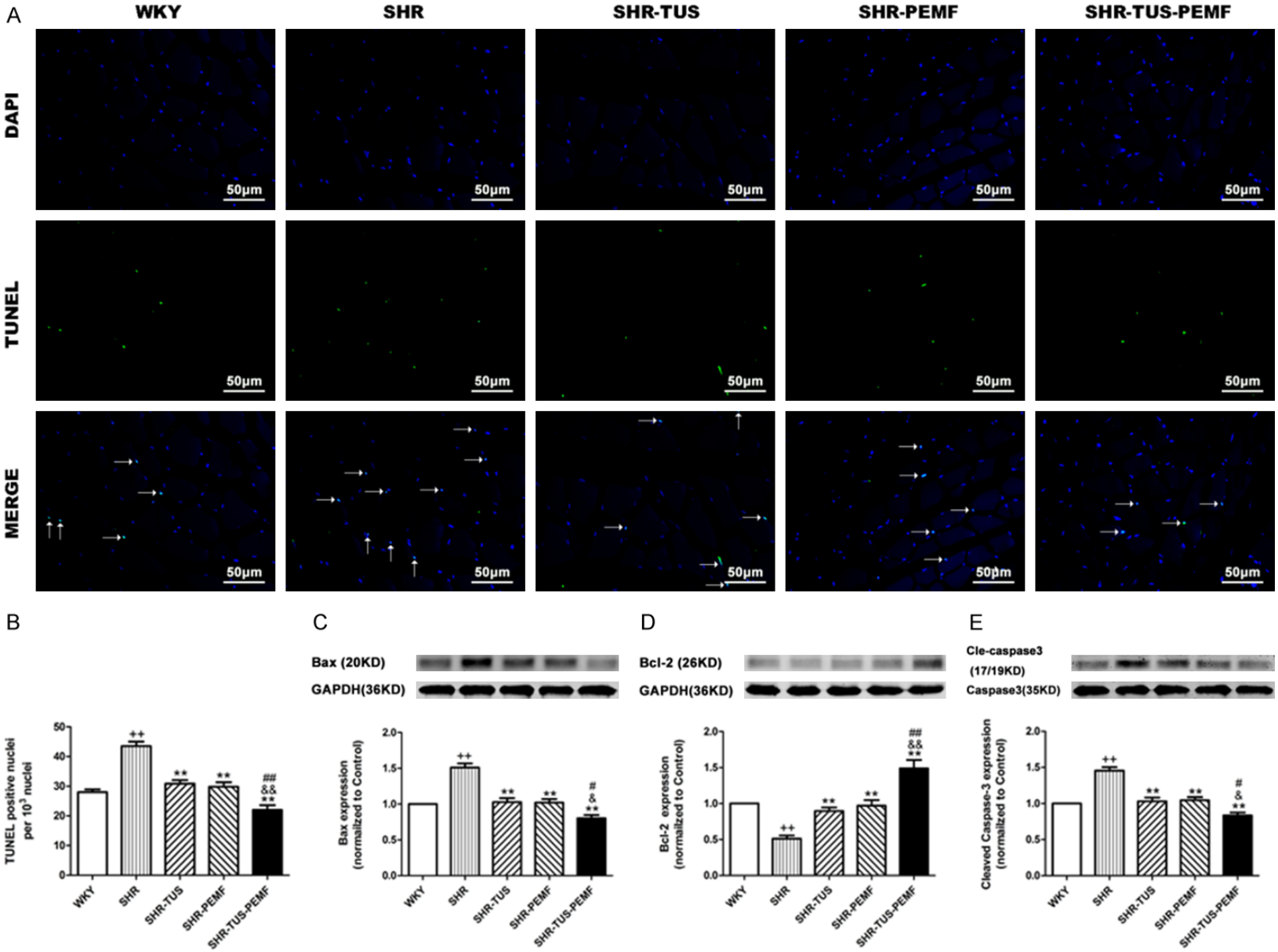
A
WKY
SHR
SHR-TUS
SHR-PEMF
SHR-TUS-PEMF
DAPI
TUNEL
MERGE
50μm
B
TUNEL positive nuclei per 10³ nuclei
C
Bax (20KD)
GAPDH(36KD)
Bax expression (normalized to Control)
D
Bcl-2 (26KD)
GAPDH(36KD)
Bcl-2 expression (normalized to Control)
E
Cle-caspase3 (17/19KD)
Caspase3(35KD)
Cleaved Caspase-3 expression (normalized to Control)

Figure 5. The united administration effects on cellular apoptosis and apoptosis-related factors in ischemic hindlimb skeletal in hypertension. 14 days after ischemia surgery, TUNEL assay (A and B), and Western blot assay to levels of Bax (C), Bcl-2 (D), and Cleaved caspase-3 (E) in the ischemic tissues were determined to evaluated apoptotic actions. Values are represented as mean ± SEM. $^{++}P < 0.01$ vs. WKY. $^{**}P < 0.01$ vs. SHR. $^{\&}P < 0.05$ vs. SHR-TUS. $^{\&\&}P < 0.01$ vs. SHR-TUS. $^{\#}P < 0.05$ vs. SHR-PEMF. $^{\#\#}P < 0.01$ vs. SHR-PEMF.

ern blotting was performed. The results showed an obvious increase in the protein levels of pro-apoptotic (Bax) in SHR group ($P < 0.01$ versus WKY) relative to WKY group, treatments could remarkably reverse this situation ($P < 0.01$ versus SHR) and the united administration showed the best-the protein expression reached the lowest among all the groups. An opposite tendency was detected in protein expression of anti-apoptotic (Bcl-2), and the united administration induced the highest Bcl-2 ($P < 0.01$ versus SHR) expression in all groups (**Figure 5C** and **5D**).

To further examine the downstream signaling cascades involving apoptosis, contents of cleaved caspase-3 and total caspase-3 were detected. Cleaved caspase-3 was significantly elevated in SHR group ($P < 0.01$ versus WKY) relative to WKY group. Treatments led to reduced levels of cleaved caspase-3 relative to the SHR group ($P < 0.01$ versus SHR), and the united administration group showed the lowest expression in all groups (**Figure 5E**).

Discussion

In this investigation, we demonstrated that the united administration of TUS plus PEMF led to a more notable improvement in response to hindlimb ischemia than TUS treatment or PEMF treatment in SHR. Furthermore, we testified that the united administration rescued ischemic muscle cells from apoptosis in SHR partly by downregulating Bax expression, reducing caspase-3 activity and upregulating Bcl-2 level more efficiently than TUS or PEMF alone.

Hypertension is a main dangerous element for CVDs [1], and a number of possible mechanisms could be responsible for retarded angiogenesis in hypertension circumstance. First, angiogenic potential is damaged in SHR relative to WKY [17]. The rarefaction of arterioles and capillaries has been documented in the microcirculation of hypertension individuals and animal models of hypertension [18, 19]. And literatures reported rarefaction emerged in the early stage of hypertension with a genetic susceptible [20]. These results indicate that rarefaction as well as the decreased angiogenic response after hypoxic issue is more likely to the result of innately reduced angiogenic potential than an adaptive result to the changed mechanical stresses in hypertension. Second, endothelial function is damaged in SHR compared with WKY. The activation, migration and proliferation of ECs are initial processes of angiogenesis [21]. Previous observations documented that decreased eNOS bioavailability in response to hypertension in both humans and animals led to endothelial cell (EC) dysfunction, and overexpression of eNOS could be profitable for ischemic muscle reperfusion during hypertension [22], implying that endothelial dysfunction may contribute to retarded hypertension-related angiogenesis. Third, certain angiogenic protein expressions are downregulated in SHR versus WKY. Romero-Vásquez et al. reported that the level of hepatocyte growth factor (HGF) was lower in SHR than that in WKY [23]. Possibly, decreased angiogenic factor levels may have contributed to inhibited angiogenesis in hypertension episode.

In current study, the united administration of TUS plus PEMF restored the retarded angiogenesis in SHR. The improved angiogenesis by united administration was documented by the reduced hindlimb necrosis index, increased ischemic/nonischemic skin temperature ratio, augmented capillary density, consistent with upregulated proangiogenic factors relative to the untreated SHR. Moreover, united administration significantly rescued cells from apoptosis, accompanied by elevated pro-apoptotic proteins and decreased anti-apoptotic factor in the ischemic tissues in treated SHR. This result remained significant that united administration did not provoke visible inflammation and notable atrophy in skeletal muscle with H&E staining and skeletal muscle weight. The fact that TUS plus PEMF did not affect the blood pressure indicates that the protective effect of united administration on angiogenesis in response to ischemic issue is independent to the alteration of blood pressure.

Protein kinase Akt, a downstream effector of PI3K, is a multifunctional regulator of cell survival, protein synthesis and angiogenesis [24], and the eNOS activity can be upregulated by the activated Akt. Activated PI3K-Akt-eNOS signaling pathway can robustly improve the survival of cells, and act a key role in angiogenesis [25]. In view of this, the contents of angiogenesis protein p-Akt, p-eNOS in ischemic skeletal muscles were examined. We demonstrated that both of the pro-angiogenic factors were significantly decreased in SHR group relative to WKY group. Both the TUS and the PEMF obviously restored the levels of protein expressions, and the united administration showed the best effect. VEGF is a main regulator of ECs survival and angiogenesis, and eNOS can stimulate the expression of VEGF [26]. In accordance with this finding, the result of the current investigation showed that ischemic muscle VEGF levels from SHRs were significantly lower than that in WKY rats, with capillary rarefaction. The treatments significantly elevated the level of VEGF, restored capillary density, and the united administration enhanced the greatest among the treated groups.

Previous study has demonstrated that hindlimb ischemia led to myonuclei apoptosis [27]. We determined the extent of myonuclei apoptosis in SHR and found a significant increase of apoptotic myonuclei relative to WKY. Two main pathways can trigger cell apoptosis: one is the death receptor pathway, involving Fas, tumor necrosis factor (TNF) receptors, and TNF-related ligand; another is the mitochondrial pathway, including Bax and Bcl-2 [28]. Currently, little evidence indicates the death receptor pathway involved the muscle cell apoptosis, investigations were mainly focused on the mitochondrial pathway. As a matter of fact, we captured a prominent increase in pro-apoptotic protein expression of Bax, and the effector molecule of cleaved caspase-3 in SHR ischemic skeletal muscles. It is the Bax and Bcl-2 interactions, determine the onset of apoptosis. As expected, a lower expression of antiapoptotic protein Bcl-2 in SHR ischemic muscles was detected. Both the TUS and the PEMF significantly rescued the myonuclei from apoptosis, and the effect of united administration is the best.

In summary, the present investigation illustrated the effect of the TUS and the PEMF united administration on hindlimb recovery via regulation of angiogenesis and apoptosis during hindlimb ischemia in SHR. The TUS and the PEMF united administration may represent an effective adjunct intervention of therapeutic neovascularization in hypertension individuals. Further optimization of the treatment parameters may be conducive to increase efficacy of therapeutic angiogenesis.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yi-Qin Shi, Department of Nephrology, Zhongshan Hospital, Fudan University, Fenglin Road 180, Shanghai 200032, China. Tel: +86-21-64041990; Fax: +86-21-6404-1990; E-mail: jennyshiyiqin@gmail.com; Dr. Jun-Li Duan, Department of Gerontology, Xinhua Hospital, School of Medicine, Shanghai Jiaotong University, Kongjiang Road 1665, Shanghai 200092, China. Tel: +86-21-25071115; Fax: +86-21-65795173; E-mail: duanjunlixh@163.com; Dr. Chang-Ning Hao, Department of Vascular Surgery, Renji Hospital, School of Medicine, Shanghai Jiaotong University, Dongfang Road 1630, Shanghai 200127, China. Tel: +86-21-2507-7715; Fax: +86-21-6549-3951; E-mail: gilbertha-ocn@gmail.com; Dr. Yue Li, Division of Pulmonary and Critical Care Medicine, Department of Medicine, School of Medicine, University of Maryland, 20 Penn Street, HSF-2, Room #S112, Baltimore 21201, MD, USA. Tel: +1-410-706-2572; E-mail: YueLi@som.umaryland.edu

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