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Salvia miltiorrhiza augments endothelial cell function for ischemic hindlimb recovery

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Abstract: *Salvia miltiorrhiza* (*Salvia miltiorrhiza*) root, as a traditional herb, is widely applied to pharmacotherapy for vascular system disease. In this study, we elucidate the therapy mechanism of *Salvia miltiorrhiza* by using a model of hindlimb ischemia. Blood perfusion measurement showed that intravenous administration of the Water Extract of *Salvia miltiorrhiza* (WES) could facilitate damaged hindlimb blood flow recovery and blood vessel regeneration. *In vitro* mRNA screen assay in cultured human umbilical vein endothelial cells (HUVECs) show that WES induced increased *NOS3*, *VEGFA*, and *PLAU* mRNA levels. Endothelial NOS (eNOS) promoter reporter analysis revealed that WES and the major ingredients danshensu (DSS) could enhance eNOS promoter activity. Additionally, we found that WES and its ingredients, including DSS, protocatechuic aldehyde (PAI), and salvianolic acid A (SaA), promoted HUVECs growth by the endothelial cell viability assays. A mechanistic approach confirmed that WES augments HUVECs proliferation through the activation of extracellular signal-regulated kinase (ERK) signal pathway. This study reveals that WES promotes ischemic remodeling and angiogenesis through its multiple principal ingredients, which target and regulate multiple sites of the network of the blood vessel endothelial cell regenerating process.

Keywords: angiogenesis; endothelial cell; endothelial nitric oxide synthase; hindlimb ischemia; *Salvia miltiorrhiza* (Danshen).

1 Introduction

Peripheral arterial disease (PAD) is caused by hyperlipidemia, hypertension, diabetes mellitus, and chronic kidney disease. It is characterized by occlusion of peripheral blood vessels and interruption of circulatory flow. Developing novel treatment strategies is required due to the lack of effective therapies for improving the perfusion of the limbs in patients with PAD after expecting immediate application of vascular surgery. Currently, people focus on seeking therapeutic agents to alleviate occasion and promote angiogenesis for PAD treatment (Annex 2013; Cooke and Losordo 2015; Eelen et al. 2020).

Vascular endothelial growth factor (VEGF)-mediated angiogenesis, as a therapeutic target, is crucial for restoring blood flow in ischemic tissue. Endothelial cells (ECs) are the most important cells that participate in multiple processes, including the perception of hypoxia or nutrition-deprived conditions, proliferation, and migration, which are necessary for formation of new blood vessels during development and in adult tissue injury or damaged conditions (Simons et al. 2016). Endothelial NOS (eNOS) in the vascular endothelium is main source to generate NO that critical for regulate blood flow and maintenance of optimal vascular function (Tejero et al. 2019).

S. miltiorrhiza is widely used for several cardiovascular disease therapy, including stroke, myocardial ischemia, and atherosclerosis in China. Oral administration of *S. miltiorrhiza* bunge and radix *puerariae* protect rats from streptozotocin-induced diabetic vascular injury by inhibiting increased expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), NOX2, and NOX4 in the aorta (Zhao et al. 2019). Ingredients from *S. miltiorrhiza*, including caffeic acid (CA), dihydroxyphenyl lactic acid (DLA), magnesium lithospermate B (MLB), salvianolic acid A (SaA), salvianolic acid B (SaB), and tanshinone IIA (Tan-IIA), exert anti-oxidant function and alleviate oxidative damage effect during tissue ischemia/reperfusion by regulating endogenous radical scavengers (Han et al. 2017).

Recent reports have indicated that the traditional Chinese formula with *S. miltiorrhiza* can be used to treat PAD (Koon et al. 2021; Yan et al. 2021). *S. miltiorrhiza* is composed of several active ingredients. Sodium tanshinone

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IIA sulfonate from *S. miltiorrhiza* improves blood perfusion recovery via decreased reactive oxygen species (ROS) level and microRNA-133a (miR-133a) expression in the ischemic hindlimb in diabetic mice (Chen et al. 2019). However, sodium tanshinone IIA sulfonate is a water-soluble derivative of tanshinone IIA, which takes up a small fraction of the total weight of the compounds detected and identified in the water extract of *S. miltiorrhiza* (Liu et al. 2013). Hence, further mechanistic research is required before widespread applications of *S. miltiorrhiza* to clinical PAD therapeutics.

In this study, we demonstrate that administering mice with a water extract of the traditional herb *S. miltiorrhiza* promotes functional recovery from hindlimb ischemia. Furthermore, we describe the different chemical ingredients from *S. miltiorrhiza* that could boost ECs' function by provoking ERK signaling-mediate proliferation and enhancing eNOS expression.

2 Results

2.1 *S. miltiorrhiza* treatment improving recovery in a hindlimb ischemia model

To test whether *S. miltiorrhiza* promotes angiogenic activity, we used the commercial Water Extraction of *S. miltiorrhiza* (WES) in this study. Six major ingredients were identified from WES (Table 1) in previous research, including danshensu (DSS), protocatechuic aldehyde (PAI), lithospermic acid (LA), rosmarinic acid (RA), salvianolic acid A (SaA), and salvianolic acid B (SaB) (Liu et al. 2013). The concentration of individual ingredients from WES was listed in Table 1, within the range of 1.19–12 µg/mL.

Table 1: Content of nine major ingredients in danhong.

No.	Ingredient	Molecular formula	Molecular weight	Content in danhong solution (µg/mL)
1	Danshensu	DSS C ₉ H ₁₀ O ₅	198.17	12
2	Protocatechuic aldehyde	PAI C ₇ H ₆ O ₃	138.12	1.53
3	Lithospermic acid	LA C ₂₇ H ₂₂ O ₁₂	538.46	1.19
4	Rosmarinic acid	RA C ₁₈ H ₁₆ O ₈	360.31	1.76
5	Salvianolic acid A	SaA C ₂₆ H ₂₂ O ₁₀	494.452	2.81
6	Salvianolic acid B	SaB C ₃₆ H ₃₀ O ₁₆	718.62	7.7
	Total			26.99

First, we determined the effects of WES on the blood flow in the ischemic hindlimb after femoral artery ligation using laser speckle contrast imaging. Complete excision of the artery was ligated and dissected in rats to make the hindlimb ischemia model. Figure 1 shows a similar decline in the distal hindlimb blood flow in both WES-treated and the control saline-treated groups after ligation. In the WES-treated group, the rats were repetitively administrated with WES that showed faster recovery of hindlimb perfusion after femoral artery ligation compared to the control saline-treated animals (Figure 1A). Accordingly, quantitative analysis (Figure 1B) demonstrated that the impaired ischemic reserve capacity significantly differed between the two groups at 7, 14, 21, and 28 days after the operation. Overall, the studies revealed that repetitive intravenous administration of water extract of *S. miltiorrhiza* improves ischemic hindlimb recovery and blood flow perfusion *in vivo*.

2.2 mRNA expression profile of related gene in WES-treated endothelial cells

Restore blood flow from acute ischemic limb rely on the vessel and capillary angiogenesis. To better understand the mechanism of WES to improve post-ischemic hindlimb blood flow, we conducted a transcriptional profile of functional gene-related angiogenesis in cultured ECs (Figure 2). HUVECs were treated with WES or the same volume of saline, then stimulated with phorbol 12-myristate 13-acetate (PMA) or IL-1β protein, respectively. PMA is an endogenous activator of protein kinase C (PKC) and has been used as a model agent to provoke diverse responses such as cellular growth and differentiation (Parekh et al. 2000). The results show that mRNA expression patterns of cell adhesion molecules *ICAM-1* and *VCAM-1* were increased after PMA or IL-1β stimulation, and WES slightly blocks PMA or IL-1β-induced *VCAM1* and *ICAM1* levels. Meanwhile, WES suppressed the PMA and IL-1β-induced *TNF-α* mRNA level enhancement. *TNF-α* as a pro-inflammatory cytokine indicated the inflammatory status of cells. These results indicate that WES alleviated ischemia-induced tissue inflammation.

On the other hand, the relative mRNA expression of *VEGFA* and *PLAU* was prompted by either WES or PMA/IL-1β treatment, and the highest expression appeared in WES plus PMA/IL-1β treatment group (Figure 2A and B). mRNA expression of *NOS3* was explicitly elevated in WES-treated groups. *VEGFA* signal triggers downstream enhancement of *NOS3* and *PLAU* expression, which plays a vital role in angiogenesis. *ENOS(NOS3)* generated nitric oxide (NO) is required for proper endothelial cell migration, proliferation,

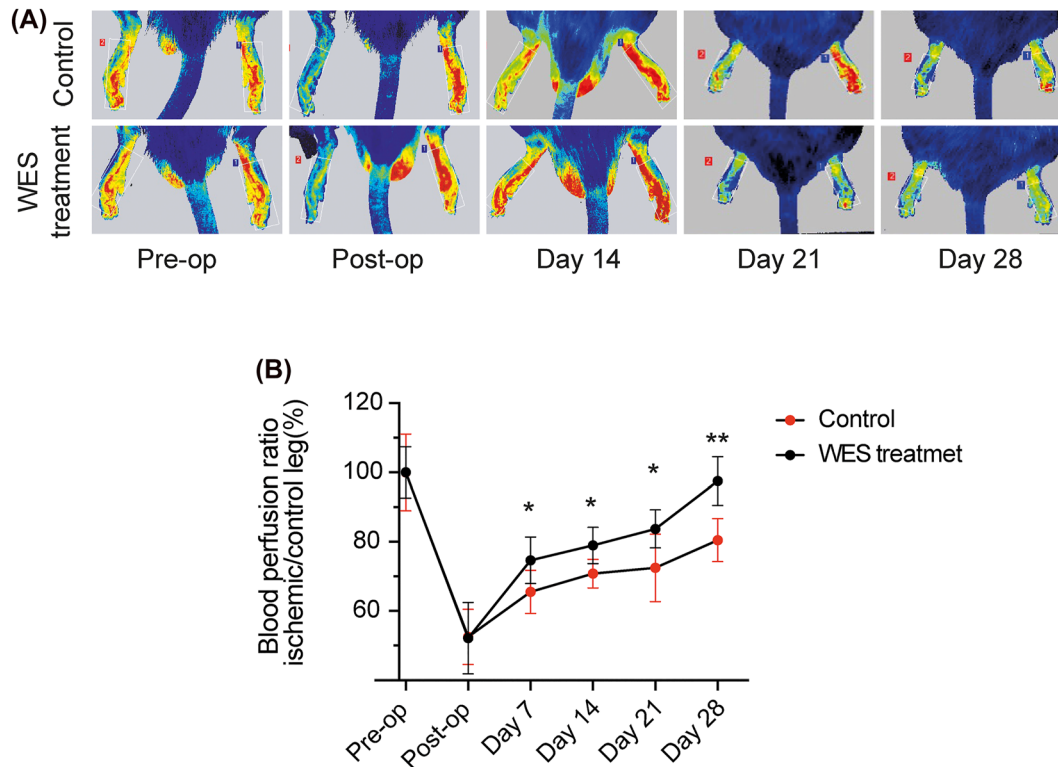


Figure 1: WES promotes recovery from ischemia in an ischemic hindlimb model. (A) Representative perfusion images with the region of interest (ROI) before and at different time points after femoral artery ligation in WES-treated and control rats. The blue-red color indicates the intensity of blood flow. Red is the strongest. Pre-operation (Pre-op); Post-operation (Post-op). (B) Quantification of perfusion data. Changes in perfusion are shown as a ratio of the right (ischemic) to left (control) hindlimb perfusion ($n = 6$). Values are mean \pm SD. P values were calculated with Student's unpaired t -test. * $p < 0.05$; ** $p < 0.01$.

and differentiation in wound repair and angiogenesis. A previous study reports that the eNOS knockout mice significantly delayed wound closure and reduced endothelial cell sprouting (Lee et al. 1999).

The urokinase-type plasminogen activator system, including urokinase-type plasminogen activator (uPA), its receptor uPAR, and its inhibitor plasminogen activator inhibitor-1 (PAI-1), participates in VEGF-induced angiogenesis processes through modulation of activating cellular proteolysis, increasing permeability, and endothelial cell proliferation and migration (Breuss and Uhrin 2012). The immunoblotting result showed that WES could enhance uPA (PLAU) protein expression (Figure 2C), consistent with increased mRNA levels (Figure 2A and B). We further investigated the role of each ingredient of WES on NO release. As shown in Figure S1, WES or DSS, PAI, LA, RA, SaA, and SaB treated cells all resulted in various induction of cell NO release, to a degree similar to that eNOS enhance result (Figure 3). WES and DSS appeared to affect the NO induction. Based on these studies, we confirmed the WES could provoke eNOS protein level and induce NO release with the ingredient of DSS.

Taken together, our results suggested that the effect of WES helping ischemic limb recovery was involved in expanding specific gene expression to improve wound repair conditions and enhance endothelial lumen regeneration.

2.3 WES promotes eNOS expression

Given that WES is capable of enhancing the transcriptional level of eNOS (NOS3) (Figure 2A and B), we assessed the protein expression following WES pretreated HUVECs and non-pretreated cells (Figure 3A). The immunoblotting result showed that WES could enhance eNOS protein expression. WES-mediated increase of eNOS levels is in a time-dependent manner. Thus, WES appeared to trigger the VEGF-initiated expression of eNOS.

eNOS is a pivotal protein to generate a NO to regulate vascular tone and maintain endothelial integrity, expressed in the vascular endothelium. We observed the protein and mRNA levels enhanced by WES. Next, we use a reporter plasmid containing the eNOS promoter region and LUC gene to depict whether WES regulated NOS3 transcription in ECs.

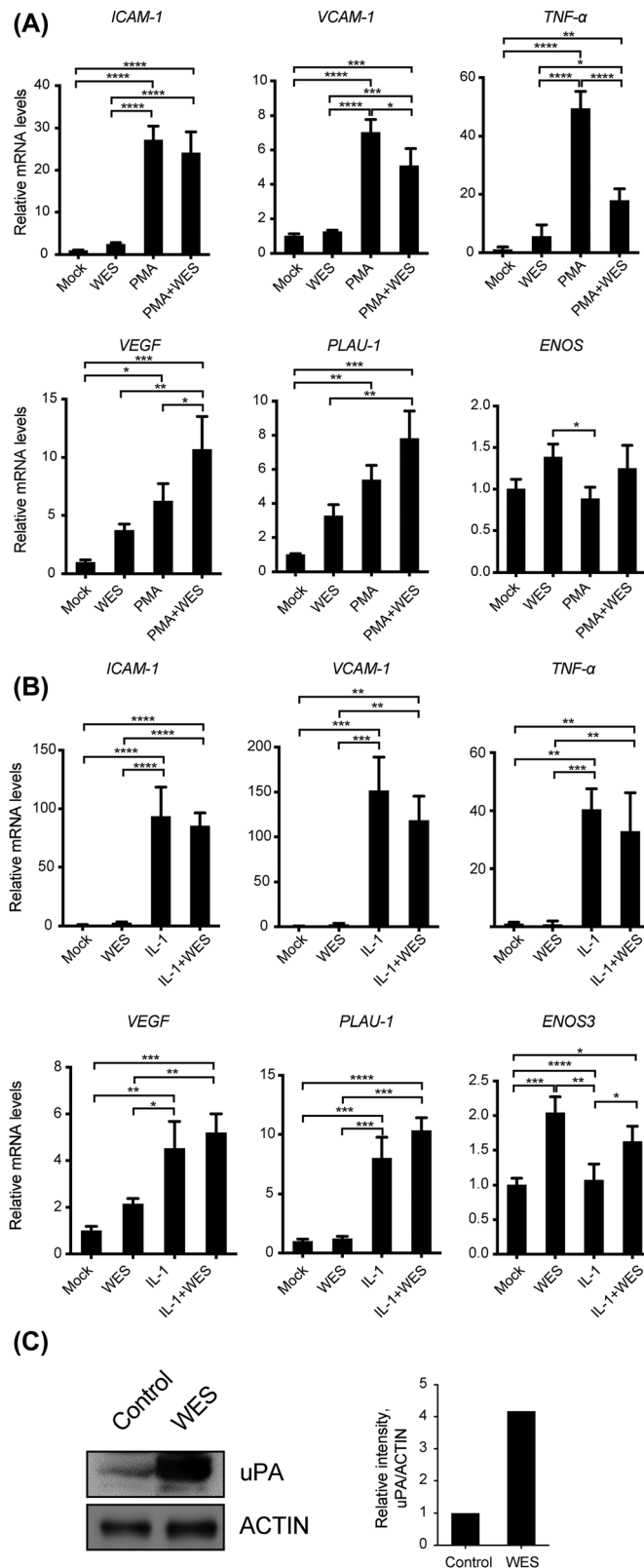


Figure 2: WES regulated the transcriptional profile of angiogenesis-related genes in HUVECs. Cells were pretreated with 1% WEH in a culture medium (24 h) and then stimulated by (A) PMA (50 ng/mL) or (B) IL-1 β recombinant protein (20 ng/mL) for 4 h. cells were harvested for *VCAM-1*, *ICAM-1*, *TNF- α* , *VEGF*, *PLAU*, and *NOS3* mRNA quantification by real-time PCR. (C) WES stimulates uPA expression. HUVECs were pretreated with WES (final concentration 1% V/V in cell culture media) for 12 h. The graphs represent data from one of three independent experiments, and each was done in triplicate \pm SD. *P* values were calculated using ordinary one-way ANOVA with Tukey's multiple comparisons test. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

As displayed in Figure 3B, the activity of the eNOS promoter was enhanced by WES treatment in a dose-dependent manner. The six major ingredients (DSS, PAI, LA, RA, SaA, and SaB) account for most of the total weight of the

compounds detected and identified in WES (Liu et al. 2013). To further demonstrate the role of each essential ingredient of WES, we treated cells individually to determine their function by promoter activity analysis. We use the

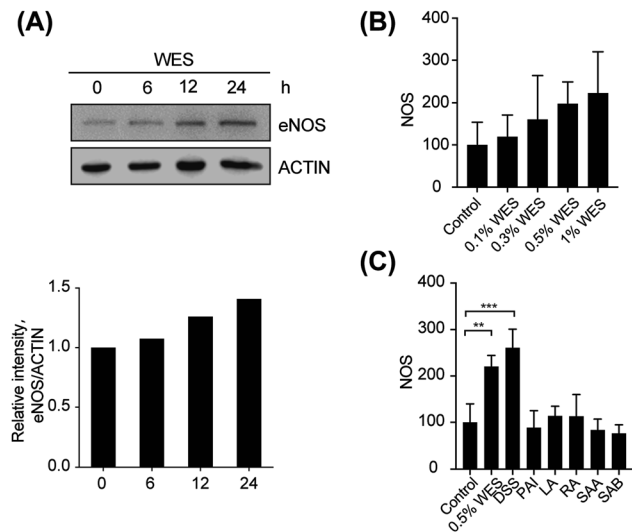


Figure 3: WES modulates eNOS expression. (A) WES stimulates eNOS expression. HUVECs were pretreated with WES (final concentration 1% V/V in cell culture media) for the indicated time. (B and C) after co-transfection of HUVECs with both the pGL2 enhancer-F1 and the Renilla luciferase plasmid, HUVECs were treated with WES or indicated concentration of WES or DSS, PAI, LA, RA, SaA, and SaB for 24 h. Activity is expressed as firefly luciferase activity/Renilla luciferase activity. (B) The eNOS promoter activity of HUVECs treated with WES significantly increased compared with the control cells in a dose-dependent manner. (C) The eNOS promoter activity of HUVECs treated with DSS, PAI, LA, RA, SaA, and SaB. Results are averaged from two independent experiments, each done in triplicate \pm SD. $**p < 0.01$; $***p < 0.001$; for comparison control versus other treatments, ordinary one-way ANOVA with Tukey's multiple comparisons test was used to calculate p values.

individual chemical of these six most abundant ingredients based on the concentrations (Table 1) detected in WES. As shown in Figure 3C, the pure chemical DSS got a similar result to WES, effectively stimulating *NOS3* promoter activation. In contrast, RA, PAI, LA, SaA, and SaB failed to activate the *NOS3* promoter. Our results indicate that WES is capable of increasing eNOS protein expression via the DSS from WES to enhance *NOS3* promoter activity. Interestingly, the efficiency of WES lower than single chemical DSS at the same concentration of WES in activation of *NOS3* promoter. We hypothesized that it is possible to contain unknown antagonists that lead to inhibition of the function of DSS since WES is a mixer of many ingredients.

2.4 WES promotes endothelial cells proliferation by activating ERK signal pathway

Evaluation of ECs proliferation rates has been used to identify, validate, and develop many angiogenesis regulators

(Nowak-Sliwinska et al. 2018). Thus, the effect of WES on the proliferation of ECs was evaluated in HUVECs. Based on the cell viability assay, we observed that WES effectively induced HUVECs growth in a dose-dependent manner (Figure 4A). It is known that VEGF-mediated downstream ERK signal pathways regulate endothelial cell proliferation (Uchiba et al. 2004). For further test underlying mechanism, protein and phosphorylation of ERK1/2 levels were tested at 0, 0.5, 1, and 2 h after WES treated HUVECs. As shown in Figure 4B, Immunoblotting exhibited that WES could stimulate phosphorylation of ERK1/2 signaling at 0.5 h. This result indicates that WES applied outside the cell could target the ERK1/2 signal pathway to facilitate ECs proliferation.

The six major ingredients (DSS, PAI, LA, RA, SaA, and SaB) from WES were also tested. As shown in Figure 4C, when HUVECs were incubated with the LA, RA, and SaB separately, the cells maintained a low proliferation rate over a wide concentration range of 0.01–4 μ M. In contrast, incubation of HUVECs with PAI and SaA led to the promotion of cell growth in a dose-dependent manner, similar to WES's pattern. In addition, DSS could enhance cell proliferation at the concentration of 4 μ M. Cross-check with the previous data (Table 1), 1% WES compose 0.6 μ M DSS, 0.11 μ M PAI, 0.02 μ M LA, 0.05 μ M RA, 0.06 μ M SaA, 0.01 μ M SaB. Since the concentrations of DSS, PAI, and SaA from cell culture medium containing WES (1% in culture medium) were much lower than those concentration thresholds to promote HUVECs proliferation (see Figure 4C), we assume that it is a combination efficacy by multiple active ingredients combined. These results suggest that WES facilitated ECs growth via active ERK1/2 signaling and relies on multiple active ingredients, including DSS, PAI, and SaA.

2.5 Multi-ingredients of WES target multiple signal sites

With the evidence provided above, we can conclude that the WES treatment augments ischemic perfusion recovery, which depends on the signaling activation of classical VEGFA-mediated eNOS, uPA, and ERK1/2 pathways, as well as their downstream ECs proliferation and function promotion. Many therapeutic agents alleviated ischemia pathologies by boosting VEGFA synthesis (Aghazadeh et al. 2022). VEGF-A signal stimulates VEGF receptor-1/2-mediated eNOS and iNOS expression, further inducing angiogenesis and endothelial function via NO production (Kroll and Waltenberger 1998). Given that WES can boost eNOS levels in HUVECs, to assess the mechanism, we analyzed eNOS promoters' functional activation after WES treatment (Zhang et al. 1995) (Figure 5). For individual ingredients

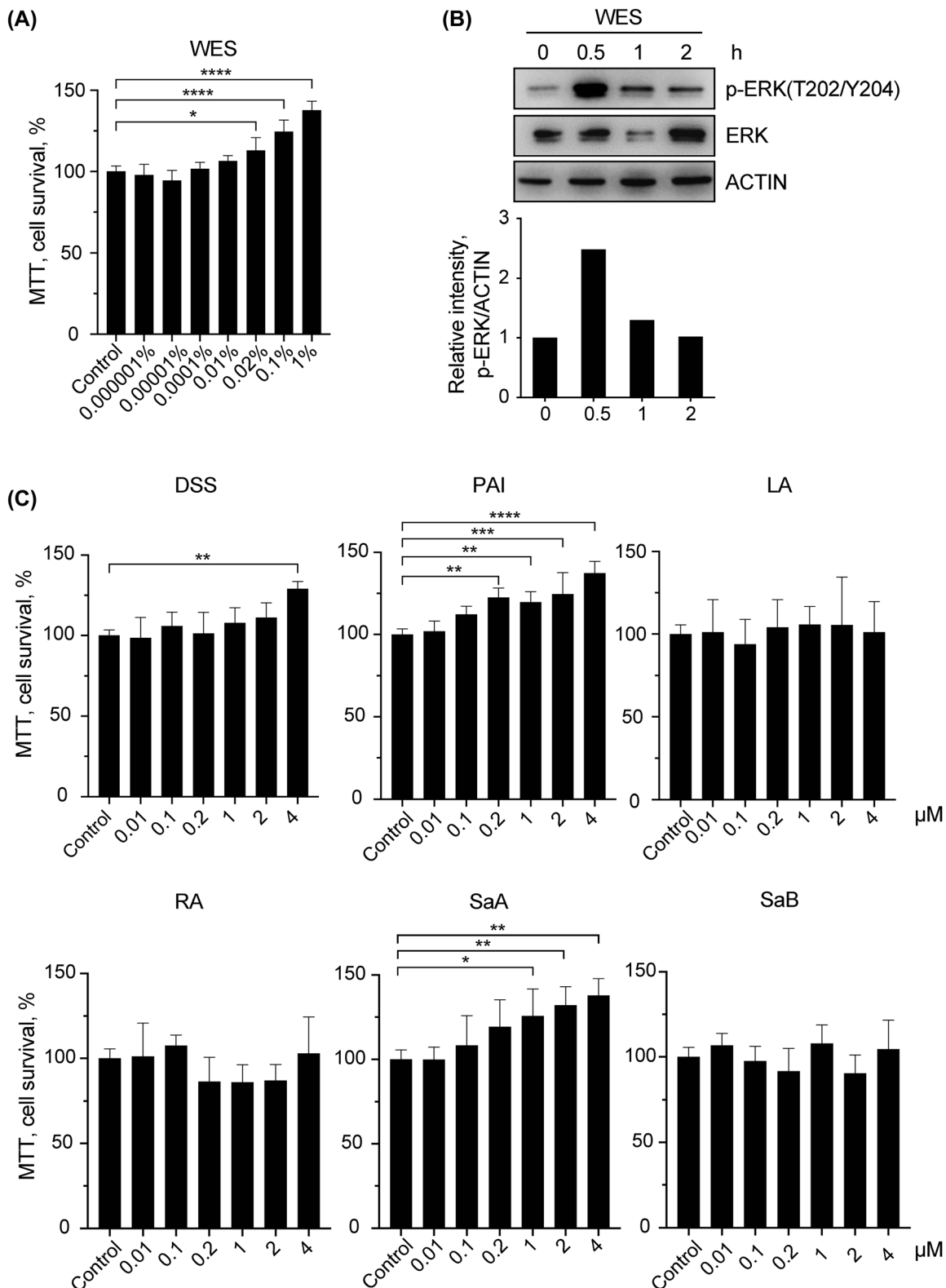


Figure 4: WES promotes HUVEC proliferation through activation of the ERK1/2 signal pathway. WES stimulates HUVECs proliferation. (A) The cell viability of HUVECs treated with WES significantly increased compared with the control cells in a dose-dependent manner. (B) WES regulates cellular ERK signal pathways to stimulate cell proliferation. HUVECs were pretreated with WES (final concentration 1% V/V in cell culture media) for the indicated time. Immunoblot showing phosphorylation of ERK1/2 protein. MTT assay for WES, DSS, PAI, LA, RA, SaA, and SaB. HUVECs were treated with WES (final concentration 1% V/V in cell culture medium) for the indicated time. (C) The cell viability of HUVECs treated with DSS, PAI, LA, RA, SaA, and SaB. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; for comparison control versus other treatments, ordinary one-way ANOVA with Tukey's multiple comparisons test was used to calculate p values.

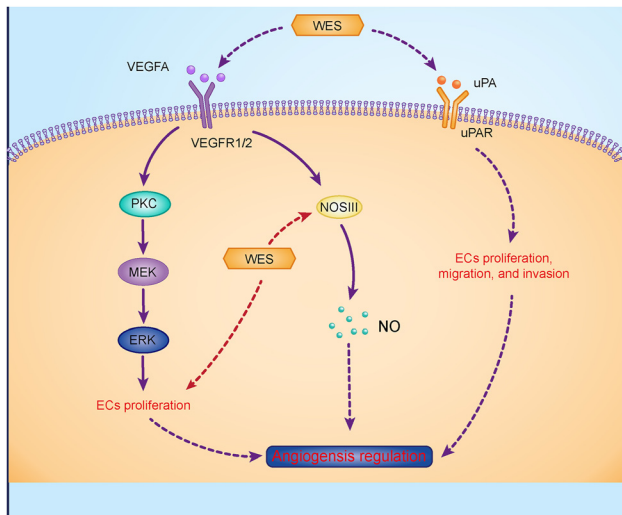


Figure 5: WES promotes multiple sites in angiogenesis. WES exerts its angiogenesis effect at VEGFA/eNOS/ERK/uPA sites. In VEGFA-independent angiogenesis, WES active VEGF signaling, triggers downstream VEGF-mediated uPA and eNOS response, activation of intracellular ERK1/2 kinases pathways, leading to ECs proliferation, vascular regeneration, and alleviated limb ischemia.

analysis, our data revealed that DSS is in charge of active eNOS transcriptional function, and DSS, SaA, and PAI confer WES promote cell proliferation function. Together, our data demonstrated that WES markedly enhances the VEGFA-mediated provoke ECs regeneration function in angiogenesis by multiple active ingredients.

3 Discussion

Traditional Chinese Medicine (TCM) contains crude forms of various natural products from plants, animals, and minerals composed of numerous chemical compounds. Most TCM is a complex drug containing multiple active ingredients. Indeed, the practical chemical components of a TCM prescription are often not thoroughly investigated and identified. One tremendous advantage of TCM is that its effectiveness and safety have been proven in clinical practice. Hence, it has attracted increasing attention to clarify the effective components of a TCM drug and to understand its underlying mechanisms, such as to figure out the multi-targets of the drug and how it affects disease-relevant signal networks. It would not only promote generating new “pure” chemical drugs that would have discarded non-inactive parts of herbs from a TCM prescription but also rend us a deeper understanding of the signal network behind a disease.

On the other hand, TCM is composed of plenty of useless ingredients, increasing the likelihood of undesired side effects, such as hepatotoxicity and nephrotoxicity. Although TCM has a successful history as an effective medical intervention in China, the theory behind those natural product combinations is difficult to accept by modern medicine, as it is more likely to be metaphysics rather than pharmacology. The most famous active entity is artemisinin, a signature ingredient derived from *Artemisia annua* L., which saved hundreds of millions of people’s life from severe malaria (Neill 2011).

PAD is a chronic vascular disease characterized by obstructions in circulation to the lower extremities. It is known as critical limb ischemia (CLI) and is the most severe stage of PAD (Duff et al. 2019). PAD, especially CLI, with increased risk of cardiovascular events and amputation in patients, also causes pain, cramping walking, and brings health care burden due to high readmissions rates (Agarwal et al. 2017).

TCMs are composed of multiple therapeutic compounds targeting convergent and divergent signal pathways. In this approach, we used a water extract of *S.miltiorrhiza* (WES), a Chinese herb that is widely used for relieving damage of ischemia/reperfusion injury and restoring blood flow to ischemic tissue. In this study, we demonstrated that WES alleviated responses to acute ischemia by restoring impaired blood flow. The results revealed that WES regulated vascularization and angiogenesis processes, including cell differentiation, invasion, and proliferation-related signal pathway-ERK1/2 signaling. WES blocked PMA/IL-1 β -stimulated enhancement of *ICAM-1*, *VCAM-1*, and *TNF- α* mRNA. The result is consistent with previous data that Danshen aqueous extract could inhibit serum TNF- α secretion (Liang et al. 2013).

Furthermore, WES promotes VEGF-initiated enhancement of eNOS and uPA. eNOS is a vasoprotective enzyme that converts arginine to nitric oxide (NO) and is primarily expressed in vascular endothelial cells, further managing blood vessel tone. eNOS knockout mice showed impaired VEGF and ischemia-initiated blood flow recovery resulting in critical limb ischemia. eNOS-derived NO is vital in arteriogenesis, angiogenesis, and mural cell recruitment to immature angiogenic sprouts (Yu et al. 2005). Our data shows that DSS promotes eNOS levels by activating the eNOS promotor.

Recent research shows that NOS3 directly interacts with MAP kinases (Solone et al. 2022). Enhancement of ERK activity in mice or *in vitro* cultured endothelial cells could raise the expression of arterial marker genes and promotes arterial branching (Deng et al. 2013). Previous research revealed that DSS could stimulate myocardial angiogenesis,

increase VEGF, VEGFR-2, and MMP-9, promote endothelial progenitor cell proliferation, and protect ischemic myocardium after coronary obstruction (Li et al. 2014). Very likely, the DSS from WES facilitated the proliferation and function of ECs through active eNOS and eNOS-mediated ERK1/2 signaling.

Another WES administration mechanism that promotes blood flow recovery is related to uPA signaling. VEGF-initiated angiogenesis required integrin redistribution in endothelial cells. It was modulated by the urokinase plasminogen activator/urokinase receptor (uPA/uPAR) system, a key VEGF step that controls endothelial cell migration (Alexander et al. 2012). Upon exposure of HUVECs to VEGF, uPAR recruited the low-density lipoprotein receptor-related protein 1 (LRP-1) to VEGFR2, further induced VEGFR2 internalization, transduce VEGF signal to cellular multiple signal pathways, and initial angiogenesis-related process involved cell proliferation, migration, and invasion (Herkenne et al. 2015).

Previous studies show that these bioactive hydrophilic constituents (DSS, SaA and B, PAI, etc.) from *Salvia miltiorrhiza* contribute to cardiovascular protective actions (Li et al. 2018). In this study, we prove that six principal ingredients of WES synergistically or independently exert function in the angiogenesis process. In future studies, it will be essential to determine the mechanisms that underlie the functional *S. miltiorrhiza* and its active ingredients in regulating the ischemic blood flow recovery associated with ECs growth and function.

Many insufficient ingredients exhibit therapeutic value when they combine with other compounds; hence, building mathematical models to qualitatively and quantitatively simulate the additive or synergistic effects of those agents is an urgent work in the future study, which will shed light on the details of the mechanisms of WES on ischemic hindlimb recovery.

4 Materials and methods

4.1 Reagents

Water Extract of *S. miltiorrhiza* (WES) was obtained from Buchang Pharmacy and the same batches (Lots) of the product were used in this study (Liu et al. 2013). PMA (phorbol 12-myristate 13-acetate) was purchased from Cayman Chemical (Cat# 10008014-1; Ann Arbor, MI, USA). Recombinant Interleukin-1 β protein (IL-1 β protein) was purchased from R&D Systems (Minneapolis, MN, USA). Danshensu, protocatechuic aldehyde, lithospermic acid, rosmarinic acid, salvianolic acid A, and salvianolic acid B were purchased from Beijing Sciherb (Beijing, China, purity $\geq 98\%$).

The following antibodies were used for immunoblots: monoclonal β -actin (Cat# A5441) was purchased from Sigma Chemical Co. (St. Louis, MO, USA); p44/42 MAPK (ERK1/2, Cat# 4695), and phospho-p44/42 MAPK (ERK1/2, Thr202/Tyr204, Cat# 4370) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-eNOS/NOS Type III (Cat# 610296) antibody was purchased from BD Transduction Laboratories™ (San Diego, CA, USA). Anti-uPA antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

4.2 Animal hindlimb ischemia model

Handling of animals was performed by following protocols approved by the China Academy of Chinese Medical Sciences (No. of approval: 2020B045). Male Sprague Dawley (SD) rats (8–10 weeks, weighing 200–220 g) were purchased from the Vital River Laboratories (Beijing, China). Rats were used to develop the hindlimb ischemia model followed and adjusted from previous reports (Niiyama et al. 2009). Briefly, after anesthetization (sodium pentobarbital 150 mg/kg) of the rats, hindlimb ischemia was made by left femoral artery ligation. The acquisition of perfusion images of a limb using a pericam perfusion speckle imager (PeriCam PSI System, Perimed, Sweden). The data were analyzed as the ratio of values in the right/left (R/L) hindlimb.

For WES treatments, rats were injected with 2.6 mL/kg WES or an equivalent volume of saline 30 min before the operation. WES or control saline was administered by intravenous injection once a day for total 28 consecutive days to mice of the corresponding group. The recommended dosages of WES are 0.29 mL/kg for human intravenous administration.

4.3 Cell culture and stimulation

Human umbilical vein endothelial cells (HUVECs) were purchased from ScienCell (Catalog #8000, ScienCell Research Laboratories, Carlsbad, USA). Cells were cultured in 100-mm dishes coated with 50 μ g/mL fibronectin, (Catalog #8248, ScienCell Research Laboratories, Carlsbad, USA), supplemented with an endothelial cell culture pack from ScienCell (ScienCell Research Laboratories, Carlsbad, USA; Endothelial Cell Medium, ECM, Cat. #1001; Endothelial Cell Growth Supplement, ECGS, Cat #1052, 5% fetal bovine serum, 100 units/mL penicillin, and 100 μ g/mL streptomycin). The cells used for the experiments were between passages two and five.

Cells were plated in 6-well plates overnight, followed by pre-incubation with 1% WES (V/V), saline, or indicated chemicals in culture media for 24 h, then stimulated with PMA at 50 ng/mL or IL-1 β recombinant protein at 20 ng/mL in the indicated time. Cells were harvested for the following experiment.

WES solution endotoxin content was verified by Toxin Sensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, NJ, USA) according to the manufacturer's instructions to avoid artifacts caused by contamination. The endotoxin level from WES was less than five EU/mL.

4.4 Immunoblotting

HUVECs were grown in 100-mm fibronectin (ScienCell Research Laboratories, CA, USA), coated plates and collected in RIPA buffer (150 mM NaCl, 1% Triton-100, 0.1% SDS, 2 mM EDTA, 20 mM NaPO₄, 50 mM NaF, 1% deoxycholic acid), and protease. Protein concentration was determined using a BCA protein assay kit (Solarbio, Beijing, China). Equal amounts of total protein were loaded in each lane of a 10% SDS-PAGE gel and

subsequently transferred to polyvinylidene difluoride membranes. Immunoblot analyses were performed using the antibodies as indicated.

4.5 RNA extraction and quantitative RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription from 1 µg of RNA was performed using the ProtoScript II reverse transcriptase (New England Biolabs, Beverly, MA, USA) and poly (T) primer. Quantitative real-time PCR was performed in the AB 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the DNA binding dye SYBR green (Real-time PCR Master Mix, TIANJIN, Beijing, China). All primer sets were tested by the relative standard curve method (Applied Biosystems) with $R^2 > 0.990$, and an amplification difference of less than 10% between endogenous control and target genes was used for analysis. Cycling parameters were as follows: 50 °C for 2 min; 95 °C for 15 min; 40 cycles of 95 °C for 30 s and 60 °C for 1 min. Melting curve analysis was performed post-RT-PCR, and gene expression fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method. Triplicate samples were measured, and arithmetic means were calculated. Levels of target gene mRNAs were normalized to those of the housekeeping genes GAPDH.

HUVECs were harvested for *GAPDH*, *PLAU-1*, *ICAM-1*, *VCEM-1*, *NOS3*, *TNF-α*, and *VEGFA* mRNA level measurement by quantitative real-time PCR after indicated treatment (for primer details, see Supplementary Table S1).

4.6 Cell viability assay

Cell viability was determined by MTT assay. Cells were plated at 1000–1500 cells per well in a 96-well plate and then treated with WES or indicated amounts of chemicals for 48 h. Then, 20 µL (5 mg/mL in PBS) MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma Chemical Co., St. Louis, Mo.) was added to each well. After another 4 h, 150 µL DMSO was added to dissolve formazan crystals in each well, and the absorbance was recorded at 570 nm.

4.7 NO release measurement

NO release was determined by the quantitation of nitrite. HUVECs were plated at 1500 cells per well in a 96-well plate and then treated with indicated amounts of reagent for 24 h. Whole nitrite levels from the cell-cultured media were assessed by a Measure-iT™ High-Sensitivity Nitrite Assay Kit (Invitrogen). Then nitrite percentage was calculated by assuming 100% to be measured by the background level of NO released by the control cells in cell culture media.

4.8 Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). Data are presented as means ± SD. Statistical analysis was performed using a two-tailed Student's *t*-test, and a *p*-value < 0.05 was considered of significant difference. A one-way ANOVA (with Dunnett's post-test) or repeated-measures ANOVA with multiple testing corrections are used to compare several groups. The figure asterisk indicates the statistical significance (**p* < 0.05; ***p* < 0.01; ****p* < 0.001).

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