Original Paper

Mixed Aqueous Extract of *Salvia Miltiorrhiza* Reduces Blood Pressure through Inhibition of Vascular Remodelling and Oxidative Stress in Spontaneously Hypertensive Rats

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Key Words

Salvia miltiorrhiza • Hypertension • Vascular remodeling • Oxidative stress • Transforming growth factor β 1 • Collagen type I

Abstract

Background/Aims: *Salvia miltiorrhiza* (SM) contains four major aqueous active ingredients, which have been isolated, purified and identified as danshensu (DSS), salvianolic acid A (Sal-A), salvianolic acid B (Sal-B) and protocatechuic aldehyde (PAL), totally abbreviated as SABP. Although SM is often used to treat various cardiovascular diseases in traditional Chinese medicine, the efficacy and function of optimal compatibility ratio of SM's active ingredients (SABP) in the prevention and treatment of cardiovascular diseases remain uncertain. This study investigated antihypertensive effect and underlying mechanisms of SABP vs. SM lyophilized powder (SMLP) in spontaneously hypertensive rats (SHR) and to establish the ratio of the optimal compatibility of DSS, Sal-A, Sal-B and PAL in improving cardiovascular functions. **Methods:** The SHRs were treated with either SABP or SMLP and their systolic blood pressures (SBP) were monitored. The isolated thoracic aorta of SHRs was segregated for immunohistochemistry, Hematoxylin-Eosin stain and mRNA and protein expression of NOX4, TGF- β 1, Col-I, ET-1, α -SMA and Smad7. Moreover, the adventitial fibroblasts (AFs) were isolated and cultured from SD rats' aorta and the reactive oxygen species (ROS) production was determined after SABP or SMLP treatment. **Results:** SABP, but not SMLP, significantly reduced SBP, which were accompanied by the inhibited morphological changes in the thoracic aorta and the reduced mRNA and protein expression of NOX4, TGF- β 1, Col-I, ET-1 and α -SMA, but the increased Smad 7 expression in SHRs. Moreover, SABP also resulted in a decreased ROS production in AFs of SD rats. **Conclusions:** These results indicate that SABP, but not SMLP, treatment potentially inhibits hypertension through improvements of vascular remodeling and oxidative stress. The present study provides new evidence that the efficacy and function from

optimal compatibility ratio of SM active ingredients is much better than its lyophilized powder, which represents a strategy to develop SM's new beneficial effect in improving cardiovascular functions.

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Introduction

Hypertension is manifested by not only an increased arterial pressure, but also a complex multifactorial process involving vascular remodeling (VR) and functional alterations. This remodeling process encompasses extracellular matrix (ECM) deposition and medial layer thickening because of hypertrophy/hyperplasia and the migration of vascular smooth muscle cells [1]. Extensive data indicate a role for reactive oxygen species (ROS), oxidative stress (OS), and redox signaling in vascular damage and pathogenesis of hypertension [2]. ROS are generated by major enzyme-nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) in a regulated manner at low concentrations [1]. In stressed or pathological conditions, increased activity and/or expression of NOX, and/or decreased activation of antioxidant systems lead to oxidative stress [2]. ROS regulate many cellular processes in the vasculature in maintaining vascular tone and integrity (e.g., cell growth, contraction/dilation, migration, differentiation, and cytoskeletal organization) [3, 4].

Adventitial fibroblasts (AFs) differentiate into myofibroblasts (MFs) and this has long been thought to be a key step in vascular remodeling [5]. Phenotypic differentiation of AFs into MFs is characterized by the expression of α -smooth muscle actin (α -SMA) and the production of ECM proteins [6, 7]. Collagen type I (Col-I), the major component of ECM, and other ECM proteins can be deposited excessively in hypertension. Among various factors that induce Col-I expression, transforming growth factor-beta (TGF- β) is one of the most extensively studied [8, 9]. The synthesis and secretion of different Col-I polypeptides is controlled by two separate pathways: the TGF- β -activation protein pathway and its downstream Smad-signaling pathway [10]. In the Smad-dependent pathway, Smad7 is the important negative regulator to inactivate Smad protein transcriptional cofactors to the targeted DNA, resulting in decreased transcription of ECM genes such as Col-I [11, 12]. Recent research has reported that nebivolol (a β 1-adrenergic receptor blocker) attenuated vascular remodeling and collagen deposition associated with hypertension mechanism involving TGF- β , which promotes vascular remodeling in hypertension [13, 14].

Salvia miltiorrhiza (SM) is a highly valued traditional Chinese medicine (TCM) and widely applied in clinics with a remarkable effect on cardiovascular and cerebrovascular diseases [15-19]. As an antioxidant, its effects of "promoting blood circulation and removing blood stasis" which function as "Decoction of Four Drugs" were documented in the ancient books of TCM. The traditional application mode of the SM is decoction. So the active ingredient should be based in aqueous substances. But it's containing hundreds of chemical substances and lack of accurately mechanism of action. The component ratio of SM is relatively fixed. Recent decades, amounts of researches about aqueous extract of SM have shown lots of new findings. For example, both salvianolic acid A (Sal-A) and danshensu (DSS) have the effect of preventing cardiac remodeling in SHR [20-22]. DSS and salvianolic acid B (Sal-B) could reduce OS in primary rats with paracetamol-induced toxicity [23]. A mixed aqueous extract of *Salvia miltiorrhiza* and *Panax notoginseng* showed antihypertensive effects on SHR [24]. Sal-A and Sal-B also may lower fasting blood glucose, reduce obesity and obesity-related metabolic disorders by suppressing adipogenesis [25, 26]. Chinese herbal medicine compatibility is the predominant form for clinical medication. The TCM effective component combination is the new model of Chinese herbal medicine compatibility. The effective component combination have many merits including exact ingredients, clear principles, better targeting, stable quality, high security, and enhance therapeutic effect [27, 28]. SM has four major aqueous extract components: DSS, Sal-A, Sal-B, and protocatechuic aldehyde (PAL), collectively abbreviated SABP [29]. This is a new compatibility of SM effective monomer. The monomer component of TCM is a current research focus [30-32] and could

be a potential alternative medicine of SM.

Therefore, the current study was undertaken to compare the effects between SABP and *salvia miltiorrhiza* lyophilized powder (SMLP) on hypertension and vascular remodeling through measurement of histological changes and changes in protein and mRNA levels of NOX4, TGF- β 1, Col-I, endothelin 1(ET-1), α -SMA, and Smad 7 in the thoracic aorta of SHRs following SABP or SMLP administration. We also detected the level of ROS in AFs derived from thoracic aorta of SD rat.

Materials and Methods

Chemicals and reagents

Injection of DSS, Sal-A, Sal-B, and PAL was purchased respectively from Shanghai Fu Life Industry Co. Ltd., China. Aqueous extract of *Salvia miltiorrhiza* lyophilized powder (SMLP) for injection was purchased from the Harbin Pharmaceutical Group Traditional Chinese Medicine Plant. Perindopril were purchased from Servier (Tianjin) Pharmaceutical Co. Ltd., China.

Experimental animals and treatment

All animal protocols were approved and conducted according to the recommendations from the Research Subcommittee of the Hebei Medical University on Animal Care and Use and the Chinese Council on Animal Care. Thirty two male SHRs (16weeks of age) weighing 250-300 g were used in the current experiment. Eight male Wistar Kyoto (WKY) rats (16 weeks of age) weighing 250-300 g were used as age-matched controls. All rats were purchased from Vital River Laboratory Animal Technology Co. Ltd, China. SHRs and Wistar rats were housed in a climate-controlled environment (12 h light–dark cycles at 22°C) with access to rodent chow and water ad libitum.

Thirty two SHRs were divided into four groups: the hypertension model (SHR) group; the SMLP group; the perindopril treatment (PD) group; the optimal compatibility and dose rate of SM aqueous extract (SABP) group. There was an additional normal control (WKY) group. The SHR and WKY group received a daily intraperitoneal (i.p.) injection of an equal volume of saline. The SMLP group received daily i.p. injection of SMLP (1 g/kg/d). The PD group received daily i.p. injection of PD (0.4 mg/kg/d). The SABP group received daily i.p. injection of SABP (DSS: 5 mg/kg/d, Sal-A: 0.233 mg/kg/d, Sal-B: 10 mg/kg/d, PAL: 17 mg/kg/day) in which uniform and orthogonal design formulas were applied to divide into groups and composition, screening the optimal compatibility proportion. Drugs were dissolved in normal sodium. After a 1-week adaptation, the animals were administered i.p. injection for 8 weeks.

At 25 weeks of age, all rats were deprived of food for 10 h and euthanized. Blood samples for the determination were taken from venae cava inferior. A portion of the thoracic aorta collected from each rat was excised and frozen immediately in liquid nitrogen to prepare it for polymerase chain reaction (PCR) and Western blotting assays. The remaining portion of thoracic aorta from each rat was fixed for hematoxylin and eosin (HE) staining, Immunohistochemistry (IHC) staining.

Isolation and Culture of SD Rat Thoracic Aorta Adventitial Fibroblast

Male SD rats, 6-8 weeks of age, were obtained from the Experimental Animal Center of Hebei Medical University. The method of culture of thoracic aorta AFs was as described previously in our laboratory [33].

Cell proliferation assay

When the cells had grown to confluence, they were transferred serially into 96-well plates ($4-5 \times 10^4$ cells/well). The proliferation of AFs growth was determined using an MTT assay according to the previously described method [34]. Cell proliferation was expressed as the percentage of the absorbance of treated cells relative to the control cells. Administration of the concentration of SABP was as follow: DSS: 1.5×10^{-4} mol/L, Sal-A: 7×10^{-6} mol/L, Sal-B: 3×10^{-4} mol/L, PAL: 5×10^{-4} mol/L.

ROS measurement

In the cardiovascular system, the major ROS are superoxide hydrogenperoxide (H₂O₂) [1]. Superoxide levels were determined by dichlorodihydrofluorescein diacetate (DCFH, Cayman Chemical Company, USA.)

on the thoracic aorta Afs. This study was divided into four groups: control; SABP (concentration same as above), Ang II (1×10^{-6} mol/L); Ang II plus SABP.

Cells were incubated with DCFH-DA (1×10^{-5} mol/L) and drugs in 6-well culture dishes ($2-3 \times 10^4$ cells/well) at 37°C for 20 min. After staining, cells were detected with a fluorescence microscope (Olympus, Tokyo, Japan) with a 10× objective and transferred to a 96-well white-walled plate to detect the fluorescent signal (502/30 nm) with a fluorescence microplate reader.

Blood Pressure Measurement

Systolic blood pressure (SBP) was determined in conscious rats using an indirect tail-cuff plethysmography (BP-100A, Chengdu Taimeng Software Co. Ltd., Chengdu, China). Briefly, SBP was measured every 2 weeks during the experiment for 9 weeks. Rats were preheated at 40°C for 10 min, and five stable consecutive measurements of blood pressure were averaged.

Measurement of Ang II, ET-1, MDA, SOD and TGF- β 1 levels in plasma

Total Superoxide Dismutase (SOD) was measured by the xanthine oxidase method, and malondialdehyde (MDA) was measured by the 2-thiobarbituric acid (TBA) method [35] (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Angiotensin II (Ang II), TGF- β 1, and ET-1 concentrations in plasma were measured by double-antibody sandwich enzyme-linked immunosorbent assay (ELISA KIT, Shanghai Elisa Biotech Co. Ltd., China).

Measurement of Col-I, ET-1, NOX4 and TGF- β 1 in Thoracic aortas by Immunohistochemistry (IHC) and Hematoxylin-Eosin (HE) stain

Thoracic aortic segments separated for Immunohistochemistry processing were fixed immediately after excision in buffered paraformaldehyde solution (4%) for 1 day. Five-micrometer thick sections were cut. Immunohistochemistry staining was performed for NOX4, TGF- β 1, ET-1, and Col-I. The primary antibody used for the stainings was a monoclonal antibody. NOX4 (1:100), TGF- β 1 (1:100), and Col-I (1:100) were purchased from Proteintech Group, Inc., China. ET-1 (1:100) was purchased from Bioworld Technology, Inc., China. Binding was visualized with biotinylated secondary antibody followed by the avidin-biotin-peroxidase detection system (Universal Vectastain ABC Elite Kit, Beijing Zhong Shan-Golden Bridge Biological Technology CO., LTD) using DAB as a chromogen. Progress of the immunoreaction was monitored under a light microscope and the reaction was stopped by the removal of DAB. Samples were analyzed with an electron microscope (Olympus, Tokyo, Japan) with 20× objective.

Thoracic aortas were cut to 5- μ m thick sections, and routinely processed for HE staining. The pathological examinations were performed with a microscope (Olympus, Tokyo, Japan) with a 40× objective.

RNA Preparation and Quantitative Real-time PCR

Total RNA was extracted from intact aorta by TRIzol (Invitrogen) and 1 μ g of RNA was subjected to reverse transcription using a first-strand cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. Real-time PCR analysis was done with the ABI 7500 FAST system, using the Platinum SYBR Green qPCR SuperMix UDG Kit (Invitrogen), according to the manufacturer's instructions. Total RNA isolation, reverse transcription of the RNA, and all PCR experiments were performed as described [36]. As an internal control, GAPDH primers were used for RNA template normalization. All PCRs were performed in triplicate. The primers were purchased from Shanghai GENEray Biotechnology Co., Ltd, Shanghai, China. The primer sequences were as described in Table 1.

Western blot analysis

Intact thoracic aortas were homogenized in ice-cold RIPA lysis buffer (1 μ g/mL leupeptin, 5 μ g/mL aprotinin, 100 μ g/mL PMSF, 1 mmol/L sodium orthovanadate, 1 mmol/L EGTA, 1 mmol/L EDTA, 1 mmol/L NaF, and 2 mg/mL β -glycerolphosphate). Aorta homogenates were incubated on ice for 20 minutes then centrifuged at 20,000g for 20 minutes in 4°C. The supernatant was collected and the protein concentration was determined using the bicinchoninic acid (BCA) method (GENEray Biotechnology, Shanghai, China). Equal amounts of protein samples were electrophoresed through a 7.5% SDS-polyacrylamide gel and then transferred onto immobilon-P polyvinylidenedifluoride (PVDF) membrane (Millipore) using wet transfer at 100 V for 90 minutes at 4°C. Non-specific binding sites were blocked by 5% non-fat milk or 1% BSA in 0.05%

Table 1. Primer sequences and product lengths of NOX4, TGF- β 1, α -SMA, Smad7, Col-I, ET-1

Primer	Sequence	Length (bp)
NOX4	Forward: 5'-ATACCAGAAATGAGGATCCCAGAAAG-3'	676
	Reverse: 5'-GTGGACACCAAATGTTGCTTTGGTTTCA-3'	
TGF- β 1	Forward: 5'-TGCGCTGCAGAGATTCAAG-3'	82
	Reverse: 5'-AGGTAACGCCAGGAATTGTTGCTA-3'	
α -SMA	Forward: 5'-ACGGCATCATCACCAACTGG-3'	316
	Reverse: 5'-AGACGCATGATGGCATGAGG-3'	
Smad7	Forward: 5'-GTGTTGCTGTGAATCTTACGGG-3'	227
	Reverse: 5'-GAAGAAGTTGGGAATCTGAAAGC-3'	
Col-I	Forward: 5'-TGCCGTGACCTCAAGATGTG -3 '	462
	Reverse: 5'-CACAAAGCGTGCTGTAGGTGA-3'	
ET-1	Forward: 5'-GCTCCTCCTTGATGGACAA-3 '	182
	Reverse: 5'-TTTGGTGAGCAGACTGGC-3'	
GAPDH	Forward: 5'-ATGGTCTACATGTTCCAGTA-3'	590
	Reverse: 5'-TCAGATCCACAACGGATACA-3'	

Tween-20 tris-buffered saline (TBST) and then incubated overnight at 4°C with primary antibody anti-NOX4 (1:500), anti-TGF- β 1 (1:500), anti- α -SMA (1:1000), anti-Col-I (1:1000), anti-ET-1 (1:1000), and anti-Smad7 (1:500). The blots were incubated with appropriate secondary antibodies with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Proteintech, Chicago, IL, USA) or HRP-conjugated rabbit anti-goat antibody (Proteintech, Chicago, IL, USA) at a 1:3000 dilution for 1 hour at room temperature. All blot washes were performed in TBST. Blots were developed with an enhanced chemiluminescence detection system (Sagecreation, Beijing, China). Densitometry was performed using a lane-1 system (Sagecreation, Beijing, China).

Statistical Analysis

Results were expressed as means $\pm$ SEM. Each observation was reproduced from at least six different animals. Statistical comparisons were made using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests (GraphPad Software, San Diego, CA, USA). P values < 0.05 indicate a statistically significant difference.

Result

Inhibitory Effect of SABP on Proliferation in SD Rat Thoracic Aorta Adventitial Fibroblasts

Traditional Chinese medicine SM contains four major aqueous active ingredients, they have been shown to be DSS, Sal-A, Sal-B and PAL. This study was to explore the effect of the compatibility proportion rule and the ratio of the optimal compatibility of DSS, Sal-A, Sal-B and PAL on the prevention and treatment of cardiovascular diseases. Uniform and orthogonal design formulas were applied to divide into groups and composition, screening the optimal compatibility proportion. Taking SD rat thoracic aorta adventitial fibroblast as a screening model, and the effect of the combination of active components on the cell proliferation was tested. Results were not shown. The optimal compatibility and dose rate of DSS, Sal-A, Sal-B and PAL were found to be named SABP. The optimal compatibility and dose rate of SABP are 1.5×10^{-4} : 7×10^{-6} : 3×10^{-4} : 5×10^{-4} mol/L. The results revealed that SABP had the strongest inhibitory effect on SD rat thoracic aorta adventitial fibroblast than the other groups (Fig. 1).

Effects of SABP on Blood Pressure in SHRs

The systolic blood pressure (SBP) level of SHRs significantly increased ($P < 0.01$) in comparison with that of WKY. The SBP level decreased gradually with SABP treatment from week 17 to 25 of age in comparison with that of SHRs ($P < 0.01$), and reached to the lowest level in 25 weeks. Compared with PD treatment group, SABP treatment had similar anti-hypertensive effect ($P > 0.05$). However, SABP treatment did not reduce the SBP level to the range in WKY (Fig. 2). In contrast, the SMLP treatment did not significantly reduce the SBP level that was similar to that of SHRs ($P > 0.05$) (Fig. 2).

Effects of SABP on ROS levels in SD Rat Thoracic Aorta Adventitial Fibroblasts

Ang II significantly increased ROS generation in aorta adventitial fibroblasts in comparison with controls as seen in Fig. 3A ($P < 0.05$). Treatment with SABP completely abolished ROS generation induced by Ang II, and was similar to control. The ROS level as shown by fluorescence microscope in aorta adventitial fibroblasts demonstrated the same effect as that shown above (Fig. 3B), suggesting the role of SABP in Ang II-induced responses.

Effects of SABP on Vascular Remodeling in Thoracic Aorta

The pathological damage in thoracic aorta of SHRs was revealed through histomorphometry. It was observed the treatment of SABP can significantly improve and

Fig. 1. The inhibition rate of the four major aqueous active ingredients of SM on SD Rat thoracic aorta adventitial fibroblast cells by permutations and combinations. Data are expressed as the mean $\pm$ SEM, $n = 8$, $\Delta\Delta P < 0.01$ vs. each group.

Fig. 2. Development of SBP in the five experimental groups during an 8-week period. Five time points of SBP were measured using tail-cuff apparatus measurement in each group. Data are expressed as the mean $\pm$ SEM, $n = 8$. ** $P < 0.01$ vs. SHR group; ## $P < 0.01$ vs. WKY group; # $P < 0.05$ vs. WKY group.

Fig. 3. Effects of SABP on ROS levels in SD rat thoracic aorta AFs. (A) The fluorescence intensity of ROS level by fluorescence microplate reader in SD rat thoracic aorta AFs. (B) Representative figure of ROS levels in SD rat thoracic aorta AFs (DCFH staining $\times 100$). Data are expressed as the mean $\pm$ SEM, $n = 8$, ** $P < 0.01$ vs. Ang II group.

Fig. 4. Effects of SABP on vascular remodeling in SHR thoracic aorta. (A) The tunica media thickness of the thoracic aorta. (B) The adventitia thickness of the thoracic aorta. (C) The vessel wall thickness/ radius (VT/VA) of the thoracic aorta in all groups. Data are expressed as the mean ± SEM, n = 8. ** P < 0.01 vs. SHR group; ## P < 0.01 vs. WKY group.

Fig. 5. Effects of SABP on Ang II, ET-1, MDA, SOD and TGF-β1 Levels in the SHR Plasma. (A) Plasma levels of Ang II. (B) Plasma levels of ET-1. (C) Plasma levels of MDA. (D) Plasma levels of SOD. (E) Plasma levels of TGF-β1. Data are expressed as the mean ± SEM, n = 8. ** P < 0.01 vs. SHR group; * P < 0.05 vs. SHR group; ΔΔP < 0.01 vs. each group.

repair the pathological damage to the arterial wall of SHRs and SABP has a protective effect on the pathological damage of the arterial wall of SHRs. Thoracic aorta sections were processed for HE staining and the results were showed in Fig. 8. After SABP treatment, tunica media thickness (MT) ($P < 0.05$) (Fig. 4A), adventitia thickness of the thoracic aorta (AT) ($P < 0.05$) (Fig. 4B), and vessel wall thickness/ radius (VT/VA) ($P < 0.05$) (Fig. 4C) were reduced significantly compared with the SHRs. SABP treatment, but not SMLP treatment, had similar effect with PD treatment ($P > 0.05$).

Fig. 6. Effects of SABP on the mRNA expression levels of α -SMA, Col-I and ET-1 in thoracic aorta of the five experimental groups. (A) The ET-1 mRNA level on the thoracic aorta. (B) The Col-I mRNA level on the thoracic aorta. (C) The α -SMA mRNA level on the thoracic aorta. Data are expressed as the mean $\pm$ SEM, n = 8. ** P < 0.01 vs. SHR group.

Fig. 7. Effects of SABP on the protein expression levels of ET-1, Col-I, and α -SMA in thoracic aorta of the five experimental groups. (A) Representative figure of the protein expression levels of ET-1, Col-I, and α -SMA in the thoracic aorta by Western blot. (B) The ET-1 protein expression level on the thoracic aorta. (C) The Col-I protein expression level on the thoracic aorta. (D) The α -SMA protein expression level on the thoracic aorta. Data are expressed as the mean $\pm$ SEM, n = 8. ** P < 0.01, * P < 0.05 vs. SHR group.

Effects of SABP on Ang II, ET-1, MDA, SOD and TGF- β 1 Levels in the Plasma

The levels of Ang II and ET-1 in plasma of SABP treated were significantly lower than that of SHRs ($P < 0.05$), and there were no differences in the levels of Ang II and ET-1 between the SABP and WKY groups (Fig. 5A, B). MDA and SOD levels in plasma indirectly reflect the level of oxidative stress, the level of SOD ($P < 0.05$) in plasma of the SABP treatment was significantly increased whereas the level of MDA ($P < 0.05$) was significantly reduced when compared with those in the SHRs. There was no difference in the levels of MDA and SOD between the SABP and WKY (Fig. 5C, D). TGF- β plays a key role in driving the differentiation of fibroblasts to MFs, which helps promote cell proliferation and ECM deposition. The plasma level of TGF- β 1 in the SABP treatment group was significantly decreased compared with the SHR group ($P < 0.05$), but there was no difference compared with the WKY (Fig. 5E). SABP treatment, but not SMLP treatment, had similar effect with PD treatment ($P > 0.05$).

Effects of SABP on mRNA and protein expression of ET-1, Col-I and α -SMA

The mRNA expression of ET-1, Col-I and α -SMA ($P < 0.05$) were significantly up-regulated in SHR compared with WKY. SABP treatment significantly reduced the increased mRNA expression of ET-1, Col-I and α -SMA ($P < 0.05$) (Fig. 6). Western blot analysis also revealed that SHRs exhibited higher protein expression of ET-1, Col-I, and α -SMA ($P < 0.05$) than the WKY. Treatment with SABP in SHRs significantly inhibited the protein expression of ET-1, Col-I, and α -SMA ($P < 0.05$) (Fig. 7). IHC staining analysis was also used to examine the

Fig. 8. Representative figure of aortic cross-section stained with HE ($\times 400$) and IHC ($\times 200$) in the five experimental groups. The immunohistochemistry were performed with anti-Col-I, anti-ET-1, anti-NOX4 and anti-TGF- $\beta 1$ antibody. Bars = 50 μ m.

Table 2. The average optical density value of NOX4, TGF- $\beta 1$, ET-1, and Col-I by IHC Staining on SHR vascular wall in the five experimental groups. (Mean $\pm$ SEM, n = 8). ** $P < 0.01$ vs. SHR group; ## $P < 0.01$ vs. WKY group; $^{\Delta} P > 0.05$ vs. SHR group

Groups	NOX4	TGF- $\beta 1$	ET-1	Collage- I
WKY	74.88 $\pm$ 3.198	43.03 $\pm$ 2.398	70.4 $\pm$ 4.18	113.1 $\pm$ 6.721
SHR	118.0 $\pm$ 3.775	77.01 $\pm$ 3.053	114.2 $\pm$ 5.97	150.8 $\pm$ 6.129
SMLP	97.88 $\pm$ 3.93 ^{##}	63.38 $\pm$ 3.77 ^{##}	93.67 $\pm$ 8.57 ^{##} $^{\Delta}$	138.8 $\pm$ 6.84 ^{##} $^{\Delta}$
PD	68.25 $\pm$ 8.154 ^{**}	42.38 $\pm$ 3.139 ^{**}	73.2 $\pm$ 3.992 ^{**}	112.5 $\pm$ 5.074 ^{**}
SABP	74.5 $\pm$ 3.645 ^{**}	45.147 $\pm$ 4.362 ^{**}	72.4 $\pm$ 3.059 ^{**}	114.3 $\pm$ 7.142 ^{**}

expression of Col-I and ET-1. Analyzed by the Image-Pro Plus software, the SHRs displayed higher levels of Col-I ($P < 0.05$) and ET-1 ($P < 0.05$) when compared to the WKY group (Fig. 8). Treatment with SABP also significantly inhibited the expression of Col-I ($P < 0.05$) and ET-1 ($P < 0.05$) (Fig. 8 and Table 2), and PD treatment had similar effects ($P > 0.05$). In contrast, SMLP treatment did not show significant inhibitory effects on the expression of ET-1, Col-I and α -SMA in the SHRs ($P > 0.05$).

Effects of SABP on NOX4

NOX4 was originally identified as an NADPH oxidase homolog expressed in the vascular wall [37]. The level of NOX4 mRNA ($P < 0.05$) was significantly higher in the SHR than that of WKY. SABP treatment significantly reduced the mRNA expression levels of NOX4 ($P < 0.05$) (Fig. 9A). The Western blot revealed that the protein expression of NOX4 ($P < 0.05$) was upregulated in SHRs. SABP treatment attenuated this increased expression of NOX4 ($P < 0.05$) (Fig. 9B, C). NOX4 protein ($P < 0.05$) was also reduced after SABP treatment as shown by IHC staining (Fig. 8) and (Table 2). SABP treatment had similar effect with PD treatment ($P > 0.05$), but no significant difference was observed in SMLP treated SHRs ($P > 0.05$).

Effects of SABP on TGF- $\beta 1$ and Smad7

The Smad pathway is the primary signaling pathway downstream of TGF- β , and Smad7 is the critical inhibitory element of this pathway. Quantitative Real-time PCR and Western blotting revealed the levels of TGF- β mRNA and protein ($P < 0.05$) were significantly higher in the SHR than that in the WKY, SABP treatment significantly reduced the levels of TGF- β mRNA (Fig. 10A) and protein (Fig. 10C) $P < 0.05$). IHC staining showed e similar results. In contrast, the level of the Smad7 mRNA and protein ($P < 0.01$) in the thoracic aorta were

Fig. 9. Effects of SABP on NOX4 in thoracic aorta of the five experimental groups. (A) The NOX4 mRNA level on the thoracic aorta. (B) Representative figure of the protein expression levels of NOX4, TGF- β 1, and Smad7 in the thoracic aorta by Western blot. (C) The protein level of NOX4 on the thoracic aorta. Data are expressed as the mean $\pm$ SEM, $n = 8$. ** $P < 0.01$ vs. SHR group; * $P < 0.05$ vs. SHR group.

Fig. 10. mRNA and protein level of TGF- β 1 and Smad7 in thoracic aorta of the five experimental groups. (A) The TGF- β 1 mRNA level on the thoracic aorta in each group. (B) The Smad7 mRNA level on the thoracic aorta. (C) The protein level of TGF- β 1 on the thoracic aorta. (D) The protein level of Smad7 on the thoracic aorta. Data are expressed as the mean $\pm$ SEM, $n = 8$. ** $P < 0.01$ vs. SHR group; * $P < 0.05$ vs. SHR group.

significantly down-regulated in SHR compared with WKY. SABP treatment significantly increased the mRNA (Fig. 10B) and protein (Fig. 10D) expression of Smad7 ($P < 0.05$), which was similar to PD treatment ($P > 0.05$). However, SMLP had no significant effect on the SHRs ($P > 0.05$).

Discussion

In this study, we demonstrated that compared with SMLP, SABP, a mixture of aqueous extract of SM, has the most efficient anti-hypertensive effect in all dose ratios of four aqueous components by uniform and orthogonal design formulas. We further revealed that SABP effectively alleviated hypertension, vascular remodeling, and oxidative stress in SHRs. This anti-hypertension effect was mediated, at least in part, by inhibition of the oxidative stress level and TGF- β signal transduction pathway.

Salvia miltiorrhiza is a topical Chinese herbal medicine (CHM) for improving the cardiovascular function. Research indicates that the active ingredients of SM are hydrophilic

phenolic acids. SABP are the effective aqueous extract components of SM and it is the new model of Chinese herbal medicine compatibility. This mixture of the four monomers might contribute toward novel drug of anti-hypertensive effects from the medicinal herbs.

Recently, emerging evidence shows that the vascular adventitia is the “first responder” and that adventitial remodeling is the initiator of VR in a variety of cardiovascular diseases [5, 38]. In our experiment, we first observed the effect of SABP on AFs *in vitro*. As expected, SABP, the new effective component compatibility of SM had the strongest inhibitory effects on AFs proliferation as compared to each element applied alone (Fig. 1).

In the present study, we also demonstrated that SABP attenuated SBP significantly on SHRs, which is consistent with previously report [24]. Ang II and ET-1 are two of the strongest substantial agents to induce vascular contraction. Furthermore, we found that Ang II and ET-1 levels also were reduced in plasma by administration of SABP. Ang II is important effector of the renin-angiotensin-aldosterone system (RAAS). ET-1 is released from the endothelium. In a future study, we will further investigate the accommodation role of SABP in RAAS disturbance and endothelium derangement in SHR arteries.

VR is a major pathological change associated with hypertension and is viewed as an accurate predictor of worsening vascular function [1]. Sal-A attenuates vascular remodeling in a pulmonary arterial hypertension rat model [39]. Importantly, our results showed that SABP reversed VR in SHRs. AFs differentiation to MFs is the critical physiopathologic feature of VR and expressed tunica media thickening and α -SMA increase. This effect is stronger in SHRs [2]. We revealed that the VR, MT, and AT of the thoracic aorta were significantly reduced by treatment with SABP (Fig. 4). The SABP have the effect of reducing the α -SMA expression and inhibiting AFs proliferation. Large amounts of ECM proteins are produced from AFs, including collagen. But this effect of MFs is stronger than AFs [40]. Previous studies in our laboratory have confirmed that AFs can produce ET-1 [33]. We measured the Col-I and ET-1 expression at both the transcriptional and translational levels and found that they were markedly down-regulated in SABP. Thus, administration of SABP reversed the AFs differentiation to MFs, and then reduced them to secrete Col-I and ET-1, thereby reducing the vessel-wall thickness and adventitial ECM deposition. These effects are agreed with that previously reported [41].

In hypertension, perturbations in ROS signaling are associated with endothelial dysfunction, impaired vascular tone, and arterial remodeling [42]. Studies have shown that SM hydrophilic extracts effectively ameliorate mitochondrial oxidative stress in human microvascular endothelial cells [43]. Sal-B has been shown to be the primary mediator of cellular functions, especially through its antioxidative and free radical scavenging effects [41]. The MDA content reflects the levels of free radicals and oxidative stress while the level of SOD reflects the ability of scavenging free radicals [44]. We found that SABP significantly decreased the MDA level and increased the SOD level in plasma (Fig. 5). The result was demonstrated that SABP has the antioxidative effect. A large body of evidence supports that AFs is a major and first site of vascular ROS production in vascular damage [45]. SABP could significantly attenuated ROS content in AFs (Fig. 3). NOX4 is an important NADPH oxidase generating ROS which is expressed on the vascular wall [37]. SABP inhibited both NOX4 mRNA and protein in the thoracic aorta (Fig. 9). NOX1, NOX2, and NOX5, the other subtypes of NOX, are distributed in different vessels. Their effects in hypertension need to be researched further and more deeply.

TGF- β 1 is a key mediator of the process of activating AFs differentiation to MFs, and is a powerful initiating factor to stimulate the synthesis and deposition of collagen in MFs [46]. SABP substantially reduced the TGF- β 1 level in plasma (Fig. 5) and the expression in the thoracic aorta (Fig. 10). The TGF- β signaling pathway, which can be Smad-dependent, has been considered the most important pathway involved in the activation of fibroblasts [47]. The Smad family has three types: inhibitory Smad (I-Smad), receptor-activated Smad (R-Smad), and common Smad (Co-Smad). Smad7, the I-Smad has a fundamental role in negative direction accommodation. Our data showed that SABP could upregulate the expression of Smad7 in mRNA and protein levels (Fig. 10). Recently, research has reported

that Sal-B has strongly inhibited Smad3 phosphorylation, which is R-Smad [30]. Our results demonstrate the same effect on the TGF- β 1/Smad pathway.

The TGF- β signaling pathways also have a Smad-independent pathway (e.g., the MAPK-P38, Ras, and Raf pathways) [31]. Recent reports indicate that TGF- β can activate NOX in all type of vascular. Reversely, NOX4 can regulate the expression of TGF- β [48]. Thus, they influence each other, and the accurate mechanism should be researched more deeply.

Collectively, the main findings of the present study reveal that SABP plays an anti-hypertension and ameliorative VR role via regulation of the oxidative stress and the TGF- β /Smad-signaling pathway. However, the exact extent by which each pathway is downregulated by SABP and contributes to its effects is difficult to estimate because there is complicated cross-talk between the two pathways. Hence, more in-depth studies of their roles and the Smad-independent pathway are still needed.

Conclusion

In conclusion, our study is the first to demonstrate that treatment with SABP, but not SMLP, remarkably alleviated SBP and vascular remodeling by suppressing oxidative stress and the TGF- β /Smad pathway on SHRs. These results indicate that the efficacy and function from optimal compatibility ratio of SM active ingredients (SABP) is much better than its lyophilized powder (SMLP), which represents a strategy to develop SM's antihypertensive effect.

Ethics approval and consent to participate

All animal protocols were approved and conducted according to the recommendations from the Research Sub-Committee of Hebei Medical University on Animal Care and Use and the Chinese Council on Animal Care.

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Disclosures Statement

No conflicts of interest, financial or otherwise, are declared by the author(s).

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