Analyzing the potential mechanism of Buyang Huanwu decoction for the treatment of salt-sensitive hypertension based on network pharmacology and in vivo experiments

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Abstract

Background: Buyang Huanwu decoction (BHD) is a traditional Chinese medicine herbal formula used for treating hypertension, particularly in the later stages of hypertension when it is associated with intracerebral hemorrhage. This study aims to investigate the treatment mechanism of BHD to provide a basis for its clinical application in hypertension treatment. Methods: Network pharmacology analysis and cell culture experiments were performed to explore the potential proteins and mechanisms of action of BHD against hypertension. Bioactive compounds related to BHD were screened, and relevant targets associated with hypertension and BHD were retrieved. Molecular docking technology was used to identify the effective signaling pathway based on the Kyoto Encyclopedia of Genes and Genomes and protein-protein interaction network cores. Lastly, the effects and mechanisms of BHD on salt-sensitive hypertensive endothelial cells were investigated.

Results: Ninety-three potential therapeutic targets for BHD and salt-sensitive hypertension were found to be closely associated with the PI3K/Akt/eNOS signaling pathway and oxidative stress. Cell experiments further indicated the pivotal role of endothelial cells in hypertension, and validation analysis showed that BHD significantly preserved cell morphology, suppressed oxidative stress reactions, activated the PI3K/Akt/eNOS signaling pathways, preserved normal endothelial cell function, and reduced cell apoptosis.

Conclusion: BHD effectively activates the PI3K/Akt/VEGF signaling pathway, attenuates oxidative stress-induced injury in endothelial cells exposed to high salt levels, and mitigates apoptosis, supporting the use of traditional Chinese medicine BHD in the treatment of salt-sensitive hypertension.

Keywords: BHD; salt-sensitive hypertension; network pharmacology; oxidative stress.
Hypertension is associated with several diseases, including stroke, coronary heart disease, heart failure, and kidney failure. In modern society, it is a prevalent condition that significantly impacts overall health and well-being [1]. Notably, reducing salt intake has been demonstrated to lower blood pressure in individuals with hypertension and pre-hypertension [2]. Several mechanisms have been proposed to elucidate the development of salt-dependent hypertension. These mechanisms encompass volume expansion, alterations in renal function, disruptions in sodium balance, impaired responses of the renin-angiotensin-aldosterone system and its associated receptors, central stimulation of sympathetic nervous system activity, and potential involvement of inflammatory processes [3]. According to estimates, over half of hypertensive patients display salt sensitivity. Factors contributing to salt sensitivity include aging, Black race, the presence of metabolic syndrome, obesity, and genetic polymorphisms [4].

Salt intake can accelerate cardiovascular damage in hypertensive individuals, primarily affecting endothelial cells, which play a crucial role in maintaining normal vascular function as they form a single-cell layer directly in contact with blood vessels. Beyond facilitating the supply of oxygen and nutrients to tissues, endothelial cells also participate in various physiological processes such as vasoconstriction, regulation of anticoagulant and procoagulant functions, angiogenesis, and modulation of vascular inflammatory responses. Hypertension can disrupt the function and structural integrity of vascular endothelium, leading to arterial damage, which may manifest as an intimal thickening, characterized by the accumulation of vascular smooth muscle cells and extracellular matrix deposition, accompanied by excessive production of reactive oxygen species (ROS) in the blood vessels [5].

Buyang Huanwu decoction (BHD) has a long history of use in preventing and treating cardiovascular and cerebrovascular diseases, particularly in the treatment of stroke, where its therapeutic effects are well-established. BHD exhibits beneficial effects in safeguarding vascular endothelium, combating atherosclerosis, regulating angiogenesis, controlling lipid metabolism, and providing neuroprotective benefits. In regards to vascular repair, BHD can improve oxidative stress-induced damage and promote the repair of endothelial cells [6]. Thus, it is of paramount importance to investigate the key molecular mechanisms underlying BHD’s protective effects on endothelial cells. Although the World Health Organization recommends a daily salt intake not exceeding 5 grams, most European countries surpass this recommended level [7]. Furthermore, elevated salt intake is associated with a higher incidence of target organ damage in hypertensive patients, particularly those with salt-sensitive hypertension (SSH), such as renal end-organ damage [8]. A significant increase in serum sodium ion concentration (2–4 mmol/L) from excessive salt consumption is a pivotal triggering factor for the rapid development of salt-induced hypertension [9]. There is also evidence suggesting that aberrations in sodium excretion within the kidneys of hypertensive patients can increase blood sodium levels [10]. Beyond the kidneys and central nervous system, the vascular endothelial system and the direct receptors for blood sodium concentration also play roles in blood pressure regulation [11]. Therefore, for the large population struggling to control their salt intake, it is important to explore and develop Chinese medicine as an alternative to antihypertensive medications to expand the spectrum of SSH prevention and treatment. BHD is frequently utilized for managing stroke sequelae and promoting cerebrovascular recovery post-stroke [12-14]. Therefore, investigations into the protective effects of BHD on vascular endothelial cells have been conducted [15, 16]. In clinical practice, BHD is also used for the treatment of certain patients with hypertension characterized by qi deficiency and blood stasis (a traditional Chinese medicine syndrome type, the main manifestations are fatigue and weakness, lack of breath and lazy speech, headache, palpitations, insomnia, chest and rib pain, and so on) [17]. BHD is a classical prescription in traditional Chinese medicine (TCM). Given that the therapeutic efficacy of BHD may result from the synergistic actions of multiple components rather than the independent effect of a single constituent, we aimed to identify the intervention target of BHD on salt-sensitive hypertensive endothelial cells through network pharmacology.

Network pharmacology is an emerging field focusing on utilizing multilayer networks to examine gene-drug interactions and disease phenotypes. It emphasizes the analysis of molecular correlation patterns between drugs and treatment targets from a systems-level and biological network perspective, making it especially relevant in various medical disciplines, including TCM [18]. In addition, network pharmacology provides valuable insights into the complex system of TCM and aids in the development of new drugs through clinically informed drug utilization, offering essential scientific and technological support. TCM’s effectiveness in treating complex disorders is attributed to its intricate chemical composition and molecular mechanisms, which target a wide range of cellular processes [19]. In this study, we investigate the protective mechanism of BHD against high salt-induced endothelial cell injury, screen potential molecular targets for hypertension treatment and provide insights into BHD’s component development and future applications.

Methods

Network pharmacology analysis
The Traditional Chinese Medicine System Pharmacology Database and Analysis Platform (TCMSP) database (http://tcmspw.com/tcmsp.php) was accessed to retrieve information about the chemical components and their associated targets for the seven TCM types that make up BHD [20]. Specific criteria were applied, requiring oral bioavailability to be equal to or greater than 30% and drug-likeness to be equal to or greater than 0.18 [21]. As the TCMSP database did not contain data for Phoretima aspergillum (E. Perrier), data were gathered on the effective components of Phoretima aspergillum (E. Perrier) through literature retrieval for subsequent data analysis [22, 23]. To identify the gene names of the target proteins, we utilized the UniProt database (https://www.uniprot.org/) and conducted an extensive search for potential disease target genes related to “salt-sensitive hypertension” across various databases, including GeneCards, OMIM, TTD, PharmGkb and DrugBank [24, 25]. Duplicate targets were removed from the search results, and target screening was based on median values. The component targets of BHD were then compared with the targets associated with SSH, and the results were visualized using a Venn diagram created with the R programming language. The shared components represented potential targets for treating SSH with BHD.

The Cytoscape v3.8.0 software was used to construct and analyze
the active component target network of BHD, where the nodes represent the active components of BHD and their intersection with SSH targets, while edges represent the connections between these components and targets. Key components for treating SSH with BHD were selected based on their correlation with the active components. Then, we uploaded the intersection targets to the STRING v11.0 database, specifying “*Homo sapiens*” as the species limitation, and set the interaction score to medium confidence (0.400) with default settings for other parameters. Free nodes were eliminated to establish the protein-protein interaction (PPI) network. Using the “network analyzer” tool in Cytoscape v3.8.0, we evaluated the PPI network’s topology and identified the core target responsible for BHD’s mechanism of action in SSH, characterized by values exceeding the median for degree, betweenness centrality, and closeness centrality [26].

In addition, the Metascape database was used to conduct gene biological processes of Gene Ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) signal pathway enrichment analyses of BHD anti hypertensive targets. Furthermore, a bioinformatics platform was used to visualize the analysis of biological functions and pathways. A significance level of \( P < 0.01 \) was utilized to screen the biological function and signaling pathways of anti hypertensive targets in BHD.

Molecular docking technology was used to investigate the mechanism of action of active components within BHD and their respective targets in the treatment of hypertension. In combination with KEGG analysis, the core gene VEGF/akt1/NOS3 was identified as a potential candidate. The 2D structures of quercetin and kaempferol, compounds influencing this gene, were retrieved from PubChem. Subsequently, we performed 3D optimization of these structures using Chem3D software. To obtain the VEGF/akt1/NOS3 protein, UniProt was used, and pre-treatment steps, including dehydration and hydrogenation, were conducted using the pmv1.5.6 software. Then, the selected compounds were docked with the target protein through the utilization of autodocktools-1.5.6 software to explore their interactions and potential mechanisms in the context of hypertension treatment.

### Main reagents and chemicals

PCR primers were obtained from Beijing Bomad Biotechnology Co., Ltd. (Beijing, China), while the reverse transcription and amplification kits were acquired from Beijing Kangwei Century Biotechnology Co., Ltd. (Beijing, China). Western blotting, BCA protein concentration assay kit and ultrasensitive ECL chemiluminescence kit were purchased from the Biyuantian Institute of Biotechnology (Shanghai, China), TBS from Solarbio Co., Ltd. (Beijing, China), the PAGE Gel Rapid Preparation kit from Shanghai Ya Enzyme Biotechnology Co., Ltd. (Shanghai, China), an apoptosis kit (KGA107) from Jiangsu KGI Biotechnology Co., Ltd. (Nanjing, China), the human peroxynitrite anion (ONO0-) kit from Andi Huatai Biotechnology Co., Ltd. (Beijing, China), endothelial nitric oxide synthase (eNOS) and phospho-endothelial nitric oxide synthase (p-eNOS) antibodies from Jiangsu ProTech Biological Research Center Co., Ltd. (Nanjing, China), and GP81, F38, P-P38, HSP27, P-HSP27, Akt and vascular endothelial growth factor (VEGF) antibodies were purchased from Hu&an Biotechnology Co., Ltd. (Hanzhou, China).

BHD consists of the following components: 30 g of Astragalus Radix, 4.5 g of Paoniae Radix Rubra, 3 g of Chuanxiong Rhizoma, 6 g of Angelicae Sinensis Radix, 3 g of Phellertina, 3 g of Persicae Semen and 3 g of Carthami Flos, and were obtained in granule form from the Affiliated Hospital of Liaoning University of Traditional Chinese Medicine. They were thoroughly mixed and melted completely, and after a 10 min wait, the ECM solution was rotated for evaporation and concentration at 200 rpm at a temperature of 50 °C. The concentrated drug was then freeze-dried in a vacuum condensation dryer for 40 h, followed by grinding into a powder and subsequent freeze-drying. The resulting lyophilized powder was dissolved in ultrapure water to prepare the liquid, which was subsequently filtered, sterilized using a 0.22 Syringe-driven filter, and stored in a refrigerator at −20 °C for future use.

### Chemical composition of BHD

Ultra-performance liquid chromatography-electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-ESI-Q-TOF-MS) was conducted by combining Waters UPLC HCLASS with Waters Xevo XS Q-TOF-MS, performed on an ACQUITY UPLC BEH C18 separation column (100 mm × 2.1 mm, 1.7 µm) at a column temperature of 40 °C, with an injection volume of 5 µL. The mobile phase consisted of both (A) 0.1% formic acid aqueous solution and (B) acetonitrile. The separation of BHD components was achieved through gradient elution at a flow rate of 0.4 mL/min as follows: 0 to 15 min, transitioning from 5% to 50% B; 15 to 20 min, shifting from 50% to 80% B; and 20 to 25 min, maintaining 80% B. MS conditions encompassed positive and negative ion modes (sensitivity mode) with the following settings: spray probe voltage at 3,000 V, sample conical hole voltage at 40 V, solvent gas temperature set to 500 °C, solvent gas flow rate at 1,000 L/h, source temperature at 150 °C, collision energy ranging from 25 to 40 eV, scanning frequency at 0.2 s, and scanning range from m/z 100 to 1,200.

### Cell culture

Human umbilical vein endothelial cells (HUVEC) (Guangzhou Chiniot Biotechnology Co., Ltd., Guangzhou, China). Delivery date: 08 April, 2016) were used. The cells were cultured in DMEM containing 10% fetal bovine serum and 0.45 mmol/L aldobetone for three days, then stimulated with different concentrations of NaCl (135, 145, 155 mmol/L) to determine the most appropriate concentration for modeling. Following pre-treatment with varying concentrations of BHD for 24 h, NaCl at a concentration of 150 mmol/L was introduced to stimulate the endothelial cells for 3 h, during which changes in various indices were observed.

F-actin was visualized through immunofluorescence staining

HUVEC were cultured in laser confocal dishes until they reached approximately 30% confluence. Subsequently, the samples were washed three times with PBS at 37 °C. The cells were then fixed with 4% paraformaldehyde for 30 min, followed by another three washes with PBS at 37 °C. Next, HUVEC were permeabilized using 0.05% Triton X-100 for one hour and washed again three times with PBS at 37 °C. Subsequently, a 10% BSA solution was applied for one hour for blocking, followed by three additional washes with PBS at 37 °C. To visualize F-actin distribution, staining was conducted using 100 µg/L Rhodamin-Phalloidin, which was incubated overnight at 4 °C. Afterward, the samples were repeatedly washed with PBS, and DAPI staining was performed. The distribution of F-actin was visualized using a laser confocal microscope.

### Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

HUVEC were lysed using TRIzol, total mRNA was extracted using the extraction method, and the mRNA was reverse transcribed into cDNA, followed by amplification using qPCR. The amplification protocol involved the following steps: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. Table 1 provides a list of the primers used for the amplification process. The fusion reaction was performed according to the specified conditions of the ABI 7500 instrument, and the qPCR results were analyzed utilizing the 2^(-△△Ct) method.

### Western blot analysis

HUVEC were lysed in 1 mL of RIPA lysate, followed by centrifugation at 12,000 rpm at 4 °C for 20 min, and the supernatant was collected. Protein concentration was determined using the BCA method. Subsequently, the separated and concentrated proteins were transferred onto a PVDF membrane, which was blocked with PBS containing 5% skimmed milk powder for 1 h at room temperature (RT) and then incubated overnight with primary rabbit antibodies against the following proteins: gg91 (HUAIO, Lot:H00221, 1:1,000), P38 (HUAIO, Lot:H661867008, 1:1,000), P38 (HUABIO, Lot:20231129002)

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Determining nitric oxide (NO), ONOO
Following the treatment of cells with high salt and BHD, cell supernatants were collected, and the contents of NO and ONOO were determined using an ELISA kit.

Apoptosis detection
HUVEC were seeded in 6-well plates at a volume of 1 mL per well. Once the cells had adhered to the well, various concentrations of BHD were added and incubated for 24 h. Then, the cells were exposed to 150 mmol/L high salt for 3 h. Afterward, the cells were detached, collected using trypsin without EDTA and centrifuged at 1,000 revolutions for 5 min. The cells were washed twice with PBS to remove any nonspecific fluorescence interference. Experimental groups, including a negative group and a single positive control group, were established. In each group, 500 μL of binding buffer was added to suspend the cells uniformly using a pipette. Then, 5 μL of Annexin V-FITC and 5 μL of propidium iodide were added, and the cells were incubated for 5 min at room temperature in the dark. Flow cytometry was employed for detection.

Statistical analysis
The data are presented as mean ± standard deviation (μ ± s). One-way analysis of variance (ANOVA) was conducted using SPSS 21.0. If the assumption of homogeneity of variance was met, the LSD test was employed; otherwise, Tamhane’s T2 test was utilized. Statistical significance level was set at P < 0.05.

Results
The chemical composition of BHD
The total ion current diagram of BHD was constructed using UHPLC-ESI-Q-TOF-MS. In the positive ion mode, a total of 103 compounds were detected, while in the negative ion mode, 124 compounds were identified (Figure 1).

Analysis of network pharmacology of BHD against SHH
Active components and targets screening in BHD. A search in the TCMSP database to identify all seven active ingredients of BHD and their associated genes based on oral bioavailability and drug-likeness parameters yielded the following results: 20 active components from Astragalus Radix, two active components from Paeoniae Radix Rubra, 29 active components from Chuanxiong Rhizoma, seven active components from Angelicae Sinensis Radix, 23 active components from Pheretima, 22 active components from Carthami Flos, and 20 active components from Persicae Semen related to their respective targets. An analysis of UniProt identified a total of 242 target genes associated with BHD.

By employing the keyword “salt-sensitive hypertension”, we conducted searches in the GeneCards, OMIM, TTD, PharmGkb, and DrugBank databases, resulting in the retrieval of 591, 56, 219, 10, and

Table 1 The primer sequences used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences</th>
<th>Length</th>
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<tr>
<td>VEGF</td>
<td>Forward: 5'-GATTCTGGGCTCCTCCTT-3'</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-TGGCTTGCTGCTTACCT-3'</td>
<td>144</td>
</tr>
<tr>
<td>Akt</td>
<td>Forward: 5'-CGACACGGGAAAGTTA-3'</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CTCCACGGTAGCACTGA-3'</td>
<td>41</td>
</tr>
<tr>
<td>eNOS</td>
<td>Forward: 5'-CAGACACGGACATCCTCC-3'</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GCACCATGACACATT-3'</td>
<td>66</td>
</tr>
<tr>
<td>Gp91</td>
<td>Forward: 5'-CTCTATTAAGGGTGTG-3'</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-AGACTTGTATGAGGC-3'</td>
<td>70</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: 5'-TGCTCTCCAGATACCAA-3'</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GCACCACCAACTGTAG-3'</td>
<td>66</td>
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Figure 1 The total ion chromatogram of BHD through the UHPLC-ESI-Q-TOF-MS

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74 targets, respectively. After removing duplicate targets and combining the data, a total of 779 disease-related targets were obtained. Subsequently, 93 common targets were identified as potential targets for BHD in the context of SSH (Figure 2a, 2b).

**Constructing the active ingredient target network.** The network diagram illustrates the intersection between TCM components and disease targets. BHD is distinguished by its multitude of components and targets in the treatment of hypertension. Additionally, the active components can exert their effects on multiple targets, and the same target can correspond to multiple active components (Figure 3).

**Constructing the PPI network.** The results of the constructed PPI network analysis revealed 14 core targets of BHD, namely JUN, PTGS2, AKT1, TP53, CAT, VEGF, HIF1A, CXCL8, ICAM1, CASP3, IL1B, MMP9, CCL2 and NOS3, with the potential for treating SSH (Figure 4).

**GO and KEGG enrichment analyses.** GO and KEGG enrichment analyses were conducted to examine the functions of the 93 proteins within the compound-component-target network. A significance threshold of $P < 0.01$ was applied to identify biologically relevant processes and metabolic pathways. The R4.1.2 software was used for a more detailed analysis of the specific functions of the 14 core targets in conjunction with the “clusterprofiler”, “org.hs.eg.db”, “enrichplot”,

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**Figure 2** Venn diagram of drug-disease common targets. (a) The search for targets of SSH. (b) The target of BHD and SSH. BHD and disease shown by green and pink circles.

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**Figure 3** Active ingredient-target network. In the figure, purple squares represent *Persicae Semen*, red squares denote *Paeoniae Radix Rubra*, blue squares signify *Chuanxiong Rhizoma*, pink squares represent *Pheretima*, yellow squares indicate *Astragali Radix*, cyan squares denote *Carthami Flos*, green squares depict *Angelicae Sinensis Radix*, and the wathet square designates the targets associated with BHD and SSH.

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and “ggplot2” packages to generate scatter plots for GO enrichment analysis. The analysis revealed that the biological processes influenced by these genes include responses to nutrient levels, oxidative stress, xenobiotic stimuli, cellular responses to chemical stress, lipopolysaccharides, oxygen levels, molecules of bacterial origin, wound healing, hypoxia, and decreased oxygen levels. In terms of cellular components, the genes were found to be associated with membrane rafts, membrane microdomains, focal adhesions, cell-substrate junctions, endocytic vesicles, apical parts of cells, caveolae, plasma membrane rafts, vesicle lumens, and sarcolemma. Moreover, the molecular functions of these genes encompassed DNA-binding transcription factor binding, heme binding, tetrapyrrole binding, RNA polymerase II-specific DNA-binding transcription factor binding, serine-type endopeptidase activity, serine-type peptidase activity, serine hydrolase activity, nuclear receptor activity, ligand-activated transcription factor activity, and steroid hormone receptor activity (Figure 5a).

The KEGG analysis yielded a total of 156 gene pathways related to the targets of BHD, with the first 30 pathways listed in Figure 5b. Among them, 106 gene pathways were found to be statistically significant ($P < 0.01$). The KEGG results highlighted the involvement of BHD in pathways primarily related to vascular processes, including lipid and atherosclerosis, fluid shear stress and atherosclerosis, AGE-RAGE signaling pathway in diabetic complications, chemical carcinogenesis, ROS, MAPK signaling pathway, human cytomegalovirus infection, HIF-1 signaling pathway, proteoglycans in cancer, IL-17 signaling pathway, and TNF signaling pathway. Thirty of these pathways are of particular significance (Figure 5b).

**Verification of core components in BHD through molecular docking.** To further validate the reliability of the key target-active ingredient network, molecular docking was conducted with three target proteins, namely VEGF, AKT1 and NOS3, and seven TCM ingredients. The molecular docking results demonstrated that the active ingredients (baicalin, beta-carotene, elagic acid, luteolin, and quercetin) in Huangqi, Chishao, and Honghua exhibited strong binding affinity with the core target VEGF. Similarly, the active ingredients in Huangqi, Honghua, and Chishao (baicalin, beta-carotene, kaempferol, luteolin, and quercetin) displayed strong binding affinity with the core target AKT1. Moreover, the active ingredients (nicotinic acid and quercetin) in Huangqi, Honghua, and Dihong exhibited strong binding affinity with the core target NOS3. In all cases, the binding energies were less than $-7$ kcal/mol, with an energy difference of less than 5. Taken together, these results indicate stable docking binding and robust activity for these 12 pairs of molecules (Figure 6).

**BHD inhibits oxidative stress of endothelial cells induced by salt stress**

When compared to the control group, the model group demonstrated a significant decrease in NO concentration in the endothelial cell supernatant, accompanied by a significant increase in ONOO$^-$ concentration ($P < 0.05$). However, when compared to the high-salt group, the medium-dose BHD group (20 mg/mL for 24 h) exhibited a significant increase in NO concentration and a decrease in ONOO$^-$ concentration ($P < 0.05$). These findings suggest that BHD can mitigate the oxidative stress response induced by salt stress in endothelial cells (Figure 7).
Figure 5 Functional analyses of BHD against hypertension. (a) GO analysis of key targets. The circle color represents the Q value, and the circle size indicates the count. (b) KEGG analysis of core targets. The circle color represents the Q value, and the circle size indicates the count.
BHD inhibits salt-induced endothelial cell injury
In normal cells, F-actin is mainly distributed around the cell periphery. However, compared to the normal group, a high salt concentration can lead to a significant increase in F-actin in endothelial cells, shortening, thickening and retraction to the center. BHD can restore the normal morphology of endothelial cells. High salt exposure leads to increased GP91 mRNA and protein expression, along with the activation of phosphorylation in P38 and HSP27 proteins (P < 0.05). In contrast, the medium-dose BHD group (20 mg/mL for 24 h) inhibited GP91 mRNA expression and protein levels, as well as reduced phosphorylation of P38 and HSP27 (P < 0.05) (Figure 8).

Effect of BHD pre-treatment on PI3K/Akt/eNOS pathway in endothelial cells
Compared to the normal group, the model group exhibited a significant reduction in VEGF and eNOS mRNA expression, VEGF protein levels, and the phosphorylation of p-Akt and p-eNOS proteins (P < 0.05). However, when compared to the model group, the medium-dose BHD group demonstrated a significant increase in the expression of VEGF and eNOS mRNA, elevated VEGF protein expression, and enhanced phosphorylation of p-Akt and p-eNOS proteins (P < 0.05). These results suggest that BHD enhances the endothelial protective effect of the PI3K/Akt/eNOS signaling pathway by activating VEGF in endothelial cells (Figure 9).

Effect of BHD pre-treatment on endothelial cell apoptosis
Compared to the normal group, the model group exhibited a significant increase in the proportion of early and late apoptosis (P < 0.05). However, in comparison to the model group, BHD demonstrated the ability to inhibit endothelial cell apoptosis and provide cytoprotective effects (P < 0.05) (Figure 10).
Discussion

Herein, we employed a network pharmacological approach to investigate the potential mechanism of BHD in treating SSH and to elucidate its protective effects on endothelial cells under high-salt conditions. Our findings provide valuable insights into potential interventions for the prevention and treatment of SSH. Our GO and KEGG analyses reveal the therapeutic mechanism of BHD, emphasizing its role in ameliorating oxidative stress induced by salt overload and highlighting the significance of the VEGF/PI3K/Akt pathway in this mechanism.

VEGF plays an important role in enhancing endothelial cell survival and promoting angiogenesis by facilitating Akt-dependent eNOS phosphorylation and the subsequent production of NO [27, 28]. NO is essential for maintaining vascular tone, as it inhibits leukocyte adhesion to the endothelium and platelet aggregation, thereby preserving the integrity of the vascular endothelial wall and overall vascular health. It exerts multiple positive effects, such as reducing vascular NO bioavailability and impairing vasodilation capacity, contributing to the pathophysiology of hypertension. NOS catalyzes the formation of NO from O2 and arginine, with eNOS being the predominant isof orm within the vascular wall. Activation of eNOS agonists through receptors rapidly activates the enzyme, often in response to shear stress, while its activity is regulated by important allosteric modulators. Beyond its vasodilatory and anti-proliferative properties, NO also plays a vital role in counte racting the effects of molecules such as angiotensin II, endothelin, and ROS. NO diffuses in gaseous form to the underlying smooth muscle, where it interacts with various molecular receptors, including soluble guanylate cyclase. A reduction in NO levels can potentially lead to an increase in ROS levels [29]. ROS, in turn, plays a significant role in mediating inflammation in the body. Excessive ROS can attack and disrupt the antioxidant enzyme system, exacerbating the inflammatory response [30, 31]. Thus, maintaining a delicate balance between NO and angiotensin II in the vasomotor center is crucial for regulating vasosympathetic tension. This study used network pharmacological experiments to screen for the protective effects of BHD on endothelial cell function in SSH and validated the relevance of the VEGF/PI3K/Akt signaling pathway. Overall, these results indicate that the therapeutic and protective mechanisms of BHD are closely associated with the activation of this signaling pathway, the promotion of NO production, and the inhibition of oxidative stress.

Numerous studies have indicated that excessive salt intake can lead to endothelial cell damage and increase the risk of cardiovascular and cerebrovascular disorders, including hypertension [32–35]. Elevated salt concentrations can stimulate changes in the polysaccharide-protein complex, known as the effective salt barrier, present on the endothelial cell membrane, causing endothelial cells to

![Figure 8 BHD inhibits salt-induced endothelial cell injury. F-actin is marked with Rhodamin-Phalloidin in red. Nucleus is marked with DAPI in blue. 600× magnification. *P < 0.05 and **P < 0.005 vs. normal or NaCl group, n = 3.](https://www.tmrjournals.com/tmr)
Figure 9 Effect of BHD pre-treatment on the PI3K/Akt/eNOS pathway in endothelial cells. *P < 0.05 and †P < 0.005 vs. normal or NaCl group, n = 3.

Figure 10 BHD inhibits endothelial cell apoptosis. *P < 0.05 and ‡P < 0.005 vs. normal or NaCl group, n = 3.

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transition from a state of sodium release to one of sodium absorption. Research has shown that even a modest increase in extracellular sodium concentration, ranging from 5 to 15 mmol/L, can induce alterations in endothelial cell morphology and function, leading to reduced nitrite production in these cells [36, 37]. Specifically, when the extracellular sodium concentration reaches 142 mmol/L, eNOS activity decreases by approximately 25% compared to a concentration of 137 mmol/L, consistent with the results obtained in our recent study [38]. When the extracellular sodium ion concentration ranged from 145 to 155 mmol/L, it resulted in reduced eNOS expression in endothelial cells and decreased nitric oxide content in the cell supernatant, indicating the occurrence of endothelial cell injury.

Oxidative stress induced by excessive salt intake is the primary molecular mechanism underlying vascular endothelial cell injury in conditions of high salt consumption. This oxidative stress triggers a reorganization of cytoskeletal proteins in aortic endothelial cells by modulating the p38-HSP27 signaling pathway, resulting in a decrease in the expression of p-eNOS and subsequent inhibition of NO release. When the extracellular sodium ion concentration in endothelial cells increases, it leads to structural changes and thickening of the endothelial cytoskeleton, which is accompanied by an increase in ONOO− levels in the cell supernatant, a decrease in NO levels, and an upregulation in the expression of the NADPH oxidase subunit gp91 protein. These changes collectively indicate oxidative stress in endothelial cells following exposure to high salt levels. The regulatory components, p-p38/p38 and p-HSP27/HSP27, are also significantly activated, resulting in the recombination of F-actin. To confirm that BHD can enhance NO levels via the VEGF/Pi3K/Akt pathway and achieve antioxidant stress, we pre-administered BHD in the extracellular fluid and observed that BHD could fully mitigate alterations in the endothelial cytoskeleton, as well as the expression of p-eNOS, gp91, p-p38/p38 and p-HSP27/HSP27 induced by high salt stimulation. Furthermore, BHD could restore the levels of NO and ONOO− in the cell supernatant. Collectively, these findings suggest that BHD exerts antioxidant stress and inhibits F-actin recombination through the p38 pathway, resulting in increased eNOS expression and enhanced NO release in vascular endothelial cells.

Apoptosis is a significant indicator of high-salt-induced damage to endothelial cells. Typically, alterations in oxidative stress can trigger an increase in mitochondrial membrane permeability, facilitating the release of cytochrome C from the mitochondrial matrix into the cytoplasm. This released cytochrome C, in conjunction with Apaf1 and caspase-9, forms apoptotic bodies that subsequently activate caspase-3, the principal executor of apoptosis, ultimately leading to programmed cell death [39]. Our experiments included apoptosis-related assessments through flow cytometry, and the results demonstrated that BHD could inhibit cell apoptosis, showcasing its efficacy in protecting endothelial cells (Figure 10). BHD is a well-established formula for the treatment of post-stroke sequelae. In light of the prominent role of TCM in combating oxidative stress, inflammation and apoptosis, it is worth exploring whether BHD can permeate the blood-brain barrier and exert anti-inflammatory effects within the brain [40, 41]. Furthermore, the results of molecular docking (Figure 6) indicate that the active components of TCM, such as Huangqi, Chishao, Honghua, and Dilong, can bind to VEGF, AKT, and NO3, suggesting that these proteins may serve as potential targets for BHD treatment. In summary, these findings highlight the ability of BHD to suppress endothelial cell apoptosis, mitigate endothelial damage, and protect vascular endothelial cells in high-salt environments by modulating the PI3K/Akt/eNOS signaling pathway. The PI3K/Akt/eNOS signaling pathway is a crucial pathway in vascular endothelial cells that regulates vascular relaxation and contraction and plays a pivotal role in various cellular processes, including cell growth, survival, differentiation, glucose transport and metabolism. Dysfunction of vascular endothelial cells is closely associated with the onset, progression and treatment of hypertension.

Vascular endothelial cell damage is not only a significant pathological consequence of hypertension but also a contributing factor to its development. In endothelial cells, the PI3K/Akt/eNOS signaling pathway controls endothelial function, reduces vascular tension, and influences vascular remodeling. PI3K, an intracellular phosphatidylinositol kinase, and Akt, the principal downstream molecule in the PI3K signaling pathway, play critical roles in this pathway. Phosphorylated Akt promotes the phosphorylation of eNOS, which catalyzes the production of NO from L-arginine within endothelial cells, a process vital for vascular relaxation, inhibition of smooth muscle cell proliferation, and prevention of platelet adhesion and aggregation. Given the relatively short half-life of NO (approximately 6–30 s), the production of NO in the endothelium predominantly relies on the activity of the key enzyme eNOS. The PI3K/Akt signaling pathway also plays a role in the central regulation of hypertension induced by high salt intake. Under pathological conditions induced by high salt consumption, a decrease in nitric oxide synthesis, impaired release or increased inactivation can result in endothelial dysfunction, compromised endothelium-dependent vasodilation and elevated blood pressure.

Our present study reports BHD’s significant protective effect on endothelial cells. Employing a network pharmacology approach, we identified common targets between BHD’s active components and SSHT. Through molecular docking, our research focused on the PI3K/Akt/eNOS signaling pathway activated by VEGF, and experimental validation confirmed BHD’s therapeutic efficacy. In summary, our findings confirm BHD’s role in safeguarding vascular endothelial cells in SSHT by increasing NO levels through the activation of the PI3K/Akt/VEGF signaling pathway, thus exerting antioxidative stress effects. These suggest that addressing both cellular oxidative stress responses and high-salt-induced endothelial injury could be pivotal in the prevention and treatment of hypertension.

**Conclusion**

A high-salt diet exacerbates the onset and progression of hypertension and triggers endothelial damage, primarily through the induction of oxidative stress. Vascular endothelial dysfunction plays a pivotal role in hypertension development, making it a significant factor. BHD can activate the PI3K/Akt/VEGF signaling pathway, thereby inhibiting oxidative stress-induced injury in endothelial cells caused by high salt and ultimately reducing apoptosis. Taken together, these findings establish an experimental foundation for the traditional Chinese medicine approach to SSHT.

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