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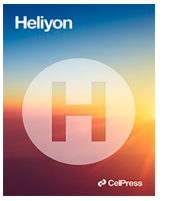

Research article

Zhijing powder manages blood pressure by regulating PI3K/AKT signal pathway in hypertensive rats

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ABSTRACT

Background: Zhijing Powder (ZJP) is a traditional Chinese medicine containing two kinds of Chinese medicine. Those studies analyze the molecular mechanism of ZJP in treating hypertension through network pharmacology, combined with animal experiments.
Methods: First, the effective ingredients and potential targets of the drug were obtained through drug databases, while the targets of disease obtained through disease target databases. The potential targets, cellular bioanalysis and signaling pathways were found in some platforms by analyzing collected targets. Further experiments were conducted to verify the effect and mechanism of drugs on cold and high salt in an induced-hypertension rat model.
Results: There are 17 effective components of centipedes and 10 of scorpions, with 464 drug targets obtained after screening. A total of 1263 hypertension targets were obtained after screening and integration, resulting in a protein-protein interaction network (PPI) with 145 points and 1310 edges. Gene ontology (GO) analysis shows that blood circulation regulation and activation of G protein-coupled receptors are mainly biological processes. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis shows that neuroactive ligand-receptor interaction, calcium signaling pathways, PI3K-AKT signaling pathways are the most abundant gene-enriched pathway. Animal experiments indicated that ZJP can reduce blood pressure (BP), affect expression of the PI3K-AKT signaling pathway, and improve oxidative stress in the body.
Conclusion: ZJP ameliorates oxidative stress and reduces BP in hypertensive rats caused by cold stimuli and high salt, revealing its effect on the expression of the PI3K/AKT signaling pathway in the rat aorta.

1. Introduction

Hypertension is a cardiovascular syndrome characterized by elevated pressure of circulating arteries as its main clinical manifestation, which is the leading risk factor of cardiovascular disease. According to statistics, about 1.38 billion adults suffered from high blood pressure (BP) in 2010 globally, and prevalence rate is still rising [1]. The etiology is complex, so it is likely that hypertension is caused by the interaction of genes, lifestyle, and environment [2–4]. Studies show that cold stimulation and high salt intake can activate the sympathetic and renin angiotensin-aldosterone system (RASS) [5]. Moreover, cold stimulation and a high salt diet allow

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the body to generate active oxygen, reducing bioavailability of nitric oxide, leading to vascular endothelial dysfunction and exacerbating hypertension [6,7]. The mechanism of hypertension is a challenging process associated with many factors. Although there has been some progress in the study of hypertension, the exact mechanism of treatment is still unclear. Therefore, a novel method must explore the optimal treatment mechanism.

Traditional Chinese medicine (TCM) has characteristics of multi-compound, multi-target, and multi-channel aspects [8]. Some Chinese medicine has significant anti-inflammatory, antioxidant, and other effects [9–11]. In addition, a unique feature of TCM is that it has unexpected therapeutic effects on complex diseases: there exists an early understanding of hypertension [12,13]. According to clinical manifestations of hypertension, it belongs to the category of "headache" or "dizziness" in Chinese medicine. TCM finds that pathogenic factors of hypertension mainly include 5 elements, e.g., wind, phlegm, stasis, fire, and deficiency, and a key to the onset of hypertension is disorder of yin and yang, gas and blood loss, and sputum's mutual resistance. Chinese medicine is a new way to study the treatment of hypertension.

Zhijing Powder (ZJP), a classic Chinese medicine, is composed of two animal medicine, and often used to regulate body functions such as alleviating blood coagulation, activating meridians, and relieving stasis [14–17]. Scorpion (*Buthus martensii Karsch Arthropoda; Arachricla; Scorpionida*; Chinese pinyin: Quan Xie), have a long history of medicinal use towards relieving wind and suppressing spasm, described in "Chinese Pharmacopoeia." Modern pharmacological studies show that the active ingredients in scorpions activate the PI3K/AKT signaling pathway [18], promoting the release of nitric oxide (NO) by endothelial cells [19]. NO is an important endothelial factor produced by nitric oxide enzyme (eNOS) [20], which promotes vascular diastolic changes, regulates vascular tension, permeability, and so on [21]. Centipedes (*Scolopendra subspinipes mutilans L. Koch; phylum arthropods; Chilopoda; scolopendridae*; Chinese pinyin: Wu Gong) function to dispel wind, relieve spasm, promote blood circulation and remove blood stasis, thus detoxifying and decreasing stagnation, recorded in Shen Nong Ben Cao Jing (Shennong's Herbal Classic) [22]. Furthermore, it was demonstrated that the medical composition of centipedes can reduce malondialdehyde (MDA) levels and increase superoxide dismutase (SOD) levels to achieve anti-inflammatory and antioxidant stress effects [23]. Although some knowledge has been gained on the medicinal components of centipedes and scorpions, the core mechanism of ZJP in treating hypertension is not clear.

Network pharmacology is a novel medicine design including system biology, network analysis, connectivity, redundancy, along with multi-effects [24]; it provides a basis for the study of molecular mechanisms for drug treatment of diseases by extracting target combinations, medicine, or compound combinations that identify key targets, bioactive compounds, and metabolic pathways [25]. It also provides a potential perspective to assess drug discovery, improvement in clinical outcomes, and comprehension of side effects and toxicity [26]. This is in line with a holistic view of Chinese medicine theory. Therefore, the purpose of this study is to use network pharmacology to identify bioactive ingredients and targets of ZJP. We conducted animal experiments to analyze its potential mechanisms, which will facilitate an in-depth understanding of the treatment for hypertension.

2. Materials and methods

2.1. Identification of active compounds of ZJP

The active compounds of ZJP are from the batman-TCM (http://bionet.ncpsb.org.cn/batman-tcm/) [27] database by searching for "WUGONG" and "QUANXIE" as keywords, the medical name by Pinyin, and combining relevant literature on "centipedes" and "scorpions" to supplement a grasp of its chemical composition. Organizing and analyzing the collected chemical components, using Swiss TargetPrediction (http://www.swisstargetprediction.ch/) [28] to draw a composition diagram, and save it in MDL SDfile format. The collected chemicals were entered into the SwissADME [29] for analysis, with GI absorption selected as high, and at least two chemicals with "YES" results in drug-likeness were used as active chemical components of ZJP.

2.2. Chemical component target collection

The collected chemical components of the MDL SDfile file are entered in Swiss TargetPrediction for target prediction. Select "Homo sapiens", download all genetic target information, filter the downloaded gene information, and select the target with a probability greater than 0 as the genetic basis of its chemical component.

2.3. Target screening for hypertension

Disease targets are obtained from the Drugbank database (https://go.drugbank.com/) [30], the TTD database (http://db.idrblab.net/ttd/) [31], the DisGeNET database (https://www.disgenet.org/home/) [32], the GeneCard database (https://www.genecards.org/) [33], and the PharmGKB database (https://www.pharmgkb.org/) [34] by searching for "hypertension" as the key word. The above-mentioned databases collect the target, filter the information, and delete duplicate ideas.

2.4. Acquisition of drug-disease intersection targets

Using the intrinsic aggregate operation in Venn Graph (http://www.bioinformatics.com.cn/static/others/jvenn/example.html) in Microsystems, genetic target information of ZJP and hypertension were analyzed with common aspects of diseases and drugs: the Venn Graph was mapped.

2.5. Protein-protein interaction (PPI) network construction

The drug and disease common targets are stored in the STRING database [35], multiple proteins are selected, with the species limited to 'homo sapiens,' the unrelated free proteins in network are removed, and remaining parameters are constructed per default settings to build the PPI network map. To further screen core proteins. The TSV file obtained from the STRING database was processed by Cytoscape3.72 software [36], and core targets were then screened according to the medium degree value.

2.6. GO biological function and KEGG pathway analysis

The Metascape database (http://metascape.org) [37] was used for the Gene ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of drug-disease intersection targets.

2.7. Potential targets – pathway networks

The common targets of diseases and drugs, with predicted pathways are imported in Cytoscape 3.72 software to create a "Potential Targets - Pathways" network diagram.

2.8. Animal experiments

2.8.1. Experiment design

A total of 36 adult male Sprague-Dawley rats (age, 6 weeks; weight, 160–200 g) were obtained from the Laboratory Animal Center of Hebei Medical University. Rats were raised in plastic cages (n = 5/cage or n = 4/cage) and were given normal diets for an acclimation period of a week. All animals were approved by The Ethics Committee for Animal Experiments of Hebei University of Chinese Medicine (DWLL2020084). Animals were randomly divided into four groups (n = 8/group): the control group was fed tap water and ordinary food; the model group was fed tap water and an enriched salt diet (8%); the ZJP-alone group was fed tap water and ordinary food, while the ZJP treatment group was fed tap water and an enrichment salt diet (8%). In addition, the model and ZJP groups were regularly placed in a freezer at −10 °C ± 2 °C for 2 h (from 9:00am - 11:00am). After 6 weeks, the ZJP and ZJP-alone group were treated by gavage for 2 weeks, while the model and control groups had saline by gavage.

2.8.2. Oral gavage preparation

ZJP was composed of scorpions (10 g), and centipedes (10 g), and purchased from the National Medical Hall of Hebei University of Chinese Medicine (Shijiazhuang, China). The dosage of ZJP was based on the clinical efficacy dosage of Shimao Li, a master of TCM. Normal saline was purchased in Shijiazhuang NO.4 Pharmaceutical.

2.8.3. Measurement of blood pressure

Rats' BPs were measured by the rodent BP analysis system (Visitech Systems, lnc., Apex, NC, USA) in the afternoon (13:30–19:00) once a week. All rats were placed in a test room with a temperature of 25 °C for 30 min, put on the platform for monitoring and setting parameters—which was predicted five times, measured 10 times, with the data on BP taken from ten average levels.

2.8.4. Preparation of tissue and plasma

After the last administration, all rats fasted for 24 h: they were anaesthetized with sodium barbiturate (2%) in the abdominal cavity with blood through the femoral artery on both sides. The blood sample was centrifuged at 3000 rpm for 10 min after keeping it at room temperature for 2 h, extracted as the supernate, and poured into a 200 μL EP tube stored in a refrigerator at −80 °C. The thoracic aorta was dissected after opening the chest, with excess connective tissue in fixation fluid.

2.8.5. Histopathological analysis

The removed chest aorta was fixed, the conventional paraffin encapsulated, sliced with xylene dewax, ethanol wash, HE and Masson staining, routine dehydration, neutral resin sealing, using an optical microscope observation and photography. The National Institutes of Health ImageJ program was used for quantification. The arterial lumen and total vessel area were measured by the ImageJ program. The vessel area was calculated by subtracting the area of the arterial lumen and the total vessel area. To quantify the area of vascular fibrosis, Masson-stained images were converted to a gray scale in the ImageJ program, the stained area segmented with thresholding. The measured threshold area served as the fibrotic area.

2.8.6. Serological analysis

The activity of creatine kinase (CK, Catalog: A032-1-1), nitric oxide (NO, Catalog: A012-1-2), superoxide dismutase (SOD, Catalog: A001-3-1), hydrogen peroxide enzyme (MDA, Catalog: A003-1-1), glutathione peroxidase (GSH-PX, Catalog: A006-1-1), catalase (CAT, Catalog: A007-1-1) in the serum were detected by colorimetric assay with commercial kits, purchased from Nanjing Jiancheng Bioengineering Institute, China. The specific operation was in strict accordance with instructions.

2.8.7. Western-blot analysis

Take each rat's thoracic aortic tissue, PBS, and efficient liquid ice cracking protein sparing modified fast (PSMF) (including 1%),

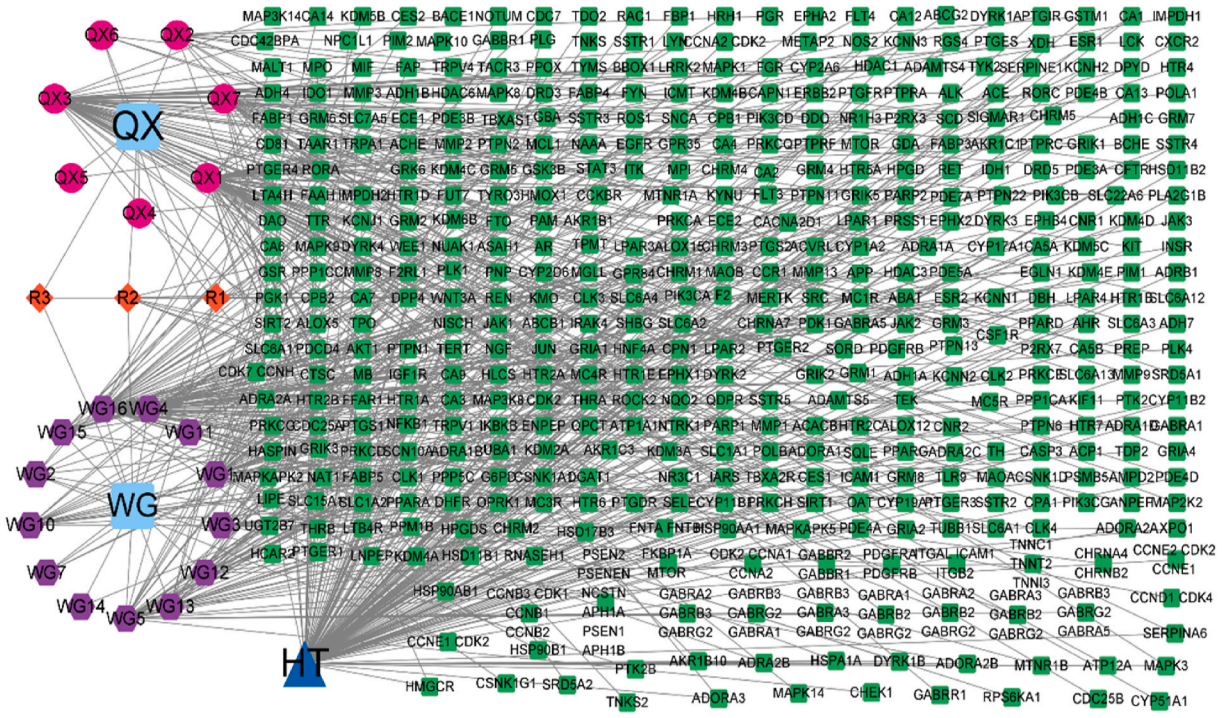

Fig. 1. Relationships among the active components and targets of centipedes and scorpions. Pink represents centipede active components, purple represents the scorpion active components, orange represents components shared by centipedes and scorpions, green represents targets of centipedes and scorpions. There are 17 active components of centipede and 10 active components of scorpion, and 464 drug targets. The code numbers of drugs are presented in Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4 °C after centrifuging to clear total protein in the BCA kit(Catalog: G2026, Servicebio technology Co., Ltd., Wuhan, China) to determine protein concentration, as per protein concentration calculation sample, with 95 °C water bath pot boil for 10 min-electrophoresis, transfer, skimmed milk powder for a closed parallel fight after the incubation, and the specific protein was anti-PI3K (Catalog: bs-2067R, Beijing Bioss Biotechnology Co., Ltd, Beijing, China, 1: 600 dilution), anti-AKT (Catalog: bs-0115R, Beijing Bioss Biotechnology Co., Ltd, Beijing, China, 1: 600 dilution), and anti-GAPDH (Catalog: bs-2188R, Bioss Biotechnology Co., Ltd, Beijing, China, 1: 2000 dilution). The polyvinylidene fluoride (PVDF) membrane was placed on the smooth surface of the glue-making glass backplane, and luminescence droplets were evenly added to the PVDF membrane with a 200 μL pipetting gun. Exposure was opened with ImageLab software, and target protein bands were detected. The gray value of each strip was measured with ImageJ software and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference. The ratio of PI3K/AKT protein to the gray value of the internal reference with GAPDH protein band was the relative expression level, compared to the normal control group for statistics.

2.8.8. Statistical analysis

IBM SPSS24.0 software (Chicago, IL, USA) was used for data analysis, and all measurement data were expressed as mean ± SD ($x \pm s$). One-way analysis of variance was used for comparison between multiple groups, while the LSD test was used for pairwise comparison of variance between groups, the Dunnett-t test was used for uneven variances, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Screening of active components and targets in ZJP

Through batman-TCM and the literature, 23 effective chemical components of centipedes and 16 effective components of scorpions were found. Targeted screening based on GI absorption and Drug-likeness, and non-targeted screening were conducted, with 17 effective components of centipedes and 10 effective components of scorpions. There were 514 targets for centipedes and 229 targets for scorpions, anticipated by Swiss TargetPrediction, and a total of 743 targets were obtained. After deleting repeated targets, a total of 464 drug targets were obtained. A network of component-targets was constructed by Cytoscape3.72 (Fig. 1) .

Table 1
List of active components.

Number	Active components
Centipedes	
WG1	(3S) – 1,2,3,4-tetrahydro-carbo-line-3-carboxylic acid
WG2	3,8-Dihydroxyquinoline (Jineol)
WG3	7,8-Dimethylpyrrolidine
WG4	8-Hydroxy-1h-2-benzopyran
WG5	N-Acetyl-2-phenylethylamine
WG6	Alanine
WG7	Hypoxanthine
WG8	Uracil
WG9	Proline
WG10	Glycerin1-monostearate
WG11	Centipede alkali b
WG12	Scolosprine A
WG13	Vanillic acid
WG14	Valine
WG15	Acetylcholine
WG16	Indole-3-acetamide
Scorpions	
QX1	(−)4-(2′-Iso-octanoicacid)-6-hydroxy-1-methyl cyclohexene
QX2	(−)22E,24-3-Cholestone-4,22(23)-diene-25-alcohol
QX3	2β-,22-Dihydroxy,3-acetoxyl,20-methoxy-cardenolidol
QX4	3β-Acetoxyl,2,14,22-trihydroxy,19-hydroxymethyl,9α,5β,14β-card20(22) enolide
QX5	Alanine
QX6	Glycerol
QX7	Trigonelline
QX8	Leucine
QX9	Uracil
QX10	Proline

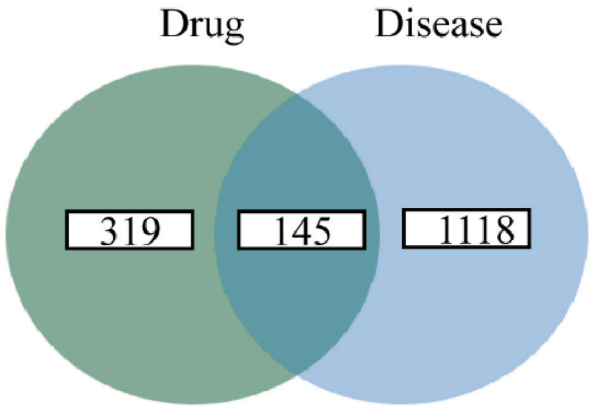

Fig. 2. Intersection targets of diseases and drugs; Green represents drug targets. Blue represents disease targets; There are 464 drug targets, 1263 disease targets and 145 intersection targets. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.2. Screening of hypertension targets

Through TTD, DrugBank, PharmGKB, DisGeNET, and GeneCard database input "Hypertension", a total of 1263 targets were retrieved, screened, and integrated.

3.3. Drug-disease intersection genes

Based on the results of 2.1 and 2.2, 145 common target information for diseases and drugs was obtained by the aggregate operation Venn Graph in Microsystems (http://www.bioinformatics.com.cn/static/others/jvenn/example.html) (Fig. 2).

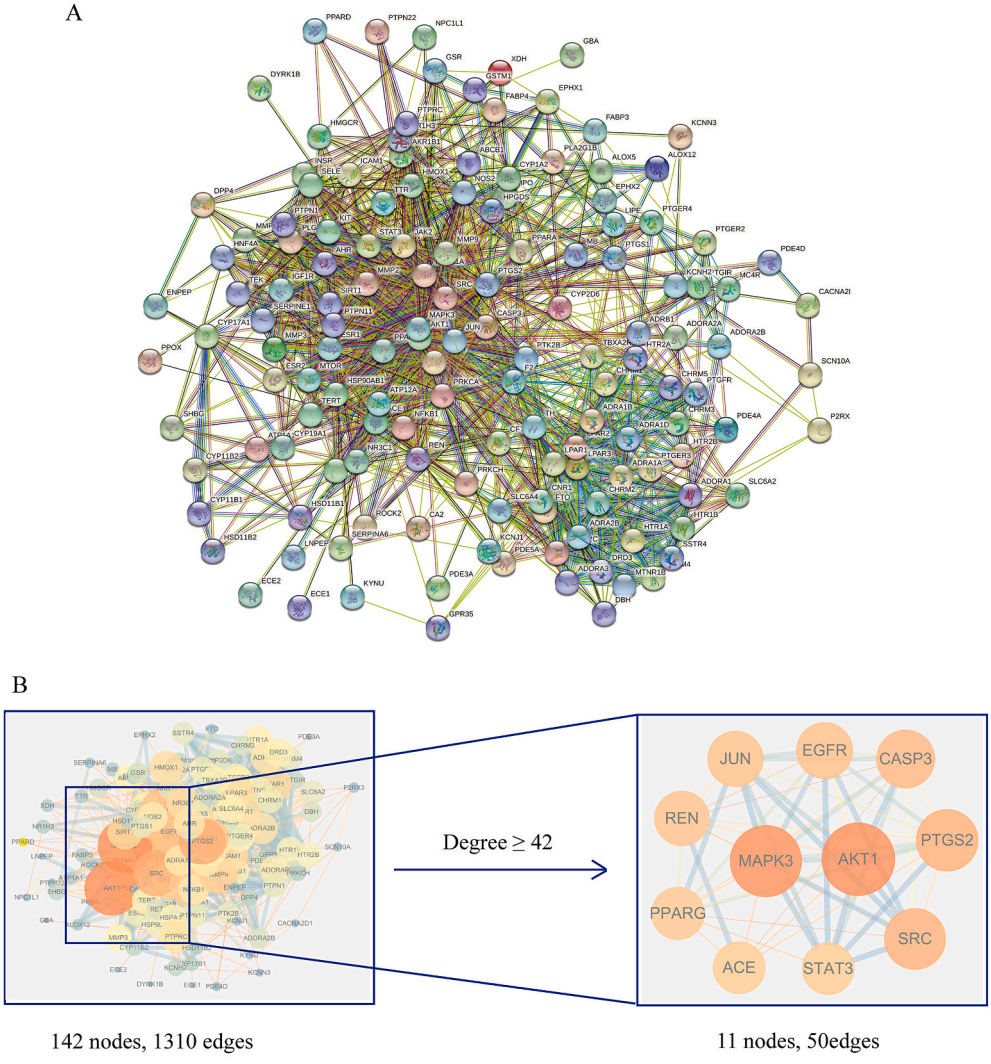

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Fig. 3. (A) PPI network of ZJP in treating hypertension. 142 targets in this network showed the protein interaction, and 1310 edges indicated the interaction between proteins. (B) Screening and visualization of core targets. A total of 11 core targets were screened, which were AKT1, MAPK3, PTGS2, SRC, CASP3, EGFR, JUN, REN, PPARG, STAT3, ACE. (C) The ranking of the degree values of these targets.

3.4. Construction and analysis of protein-protein interaction (PPI) networks

The String platform was used to preliminarily construct the protein interaction network for common targets of diseases and drugs, shown in Fig. 3A. The network diagram consists of 145 nodes and 1310 edges. Nodes represent proteins and edges represent the interaction between proteins. The higher the network connection, the closer the relationship between the proteins. Moreover, 11 core targets were screened out when the medium degree value was set to greater than 42, via Cytoscape3.72 software, (Fig. 3B and C).

3.5. GO biological function and KEGG pathway analysis

The drug-disease intersection genes were imported to Metascape for GO biological function and KEGG pathway analysis. GO analysis shows that rich intersection genes are mainly involved in regulation of blood circulation, postsynaptic response, activation of G protein-coupled receptors, and other biological processes. KEGG pathway analysis shows that the neuroactive ligand-receptor interaction, calcium signaling pathway, Phospholipase D signaling pathway, and PI3K-AKT signaling pathway are those with the most enriched genes. Combining KEGG analysis with results of 3.3, a potential target-pathway network was constructed through Cytoscape (Fig. 4).

3.6. Analysis of animal experimental results

3.6.1. Effects of ZJP on blood pressure

Before treatment, there was no difference in BP among all groups ($P > 0.05$). After a high-salt diet and cold stimulation, BP of the model group and ZJP group was significantly increased in the second week ($P < 0.05$) and peaked in the fifth week ($P < 0.05$). After treatment, the BP of the model group was significantly increased, compared to the control group and ZJP-alone group ($P < 0.05$); assessing the model group, BP of the ZJP group was significantly decreased 2 weeks after administration ($P < 0.05$), (Fig. 5).

3.6.2. Effects of ZJP on chest aorta histopathology

Compared to normal aortic structure of the control and the ZJP-alone group, the aortic intima structure of the model group was disorganized, the endothelium was incomplete, with protrusions and defects, while the middle membrane was thickened, the boundary between the outer membrane and middle membrane was poor, and the increased lumen area was accompanied by disorganized, exacerbated fibrosis. Compared to the model group, the intima structure of the aorta in the ZJP group was improved to various degrees, the intima was smooth, the endothelium was basically intact, and the boundary between the outer and middle membranes was clear, (Fig. 6).

3.6.3. Effects of ZJP on oxidative stress and serum CK levels

Compared to the control and ZJP groups, the activities of SOD, CAT, and GSH in the model group were significantly decreased, while content of MDA and CK was significantly increased ($P < 0.05$). In terms of the model group, SOD, CAT and GSH activities in the ZJP group were increased, while MDA content was decreased ($P < 0.05$), (Fig. 7).

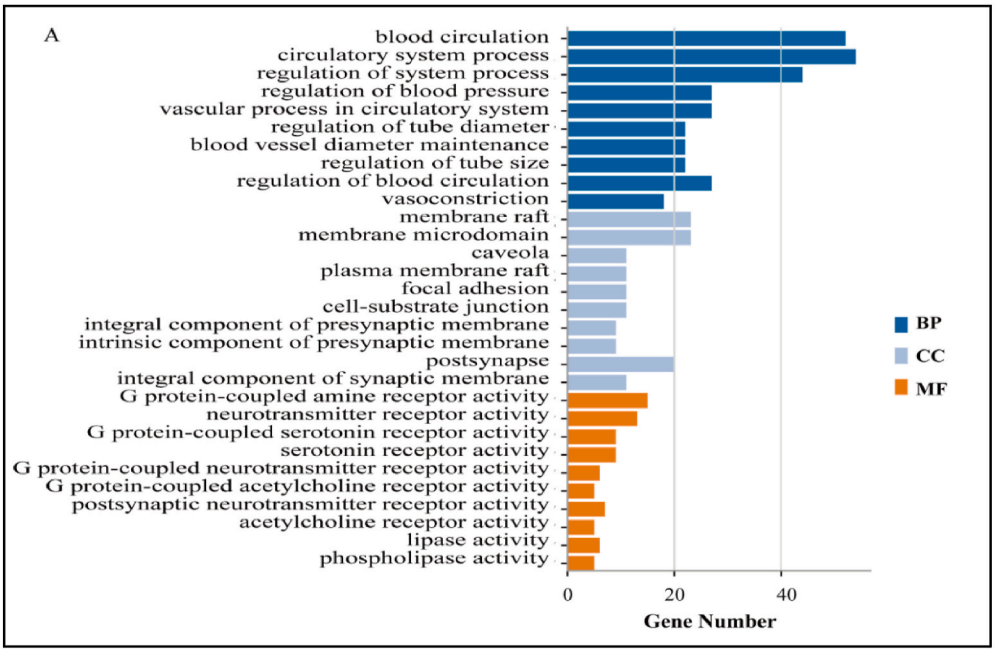
3.6.4. Effects of ZJP on PI3K and AKT expressions levels

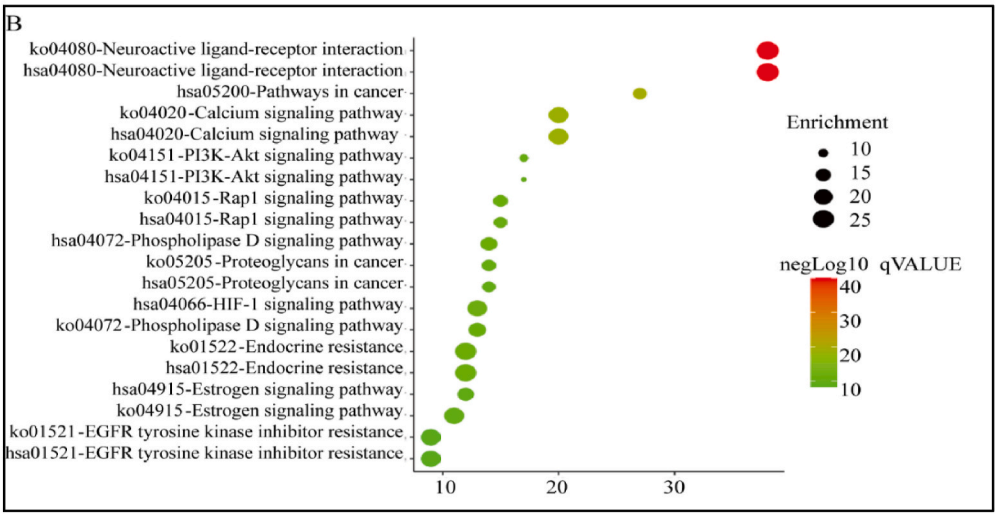
In regard to the control and ZJP groups, protein expression of PI3K and AKT in the model group were downregulated ($P < 0.05$); compared to the model group, protein expression levels of PI3K and AKT in ZJP group were upregulated ($P < 0.05$), (Fig. 8).

4. Discussion

ZJP is a traditional Chinese medicine (TCM) prescription, used to relieve spasm by collaterals, with studies showing that centipedes and scorpions in their composition improve vascular permeability and regulate vascular tone [12,38,39]. Yet, there is no relevant research to discuss the molecular mechanism of treating hypertension with ZJP. In this study, potential targets, pathways, and biological functions of ZJP in treating hypertension were analyzed through network pharmacology (Fig. 4). In addition, this research group found that ZJP can improve oxidative stress and reduce BP in animal experiments, affecting expression of PI3K/AKT in the aorta of rats, with hypertension induced by cold and high salt.

The complex ingredients, targets and multi proteins of TCMs will create more dialogue between proteins, increasing the difficulty of studying the mechanism of action of TCMs [40]. Network pharmacology analysis indicates that different proteins or genes can regulate the same disease, and some proteins can regulate multiple diseases, consistent with the characteristics of TCM treating diseases [24,41]. This research explored the molecular mechanism of ZJP to treat hypertension by network pharmacology and screened potential core gene to treat hypertension, including AKT1, MAPK3, PTGS2, SRC, CASP3, EGFR, JUN, REN, PPARG, STAT3, ACE, and AKT1 is the protein with the strongest role. AKT, whose encoded protein is a serine/threonine kinase in the protein kinase B (PKB) family, was a target of platelet-derived growth factor and activated phosphatidylinositol 3 kinase (PI3K) [42,43].





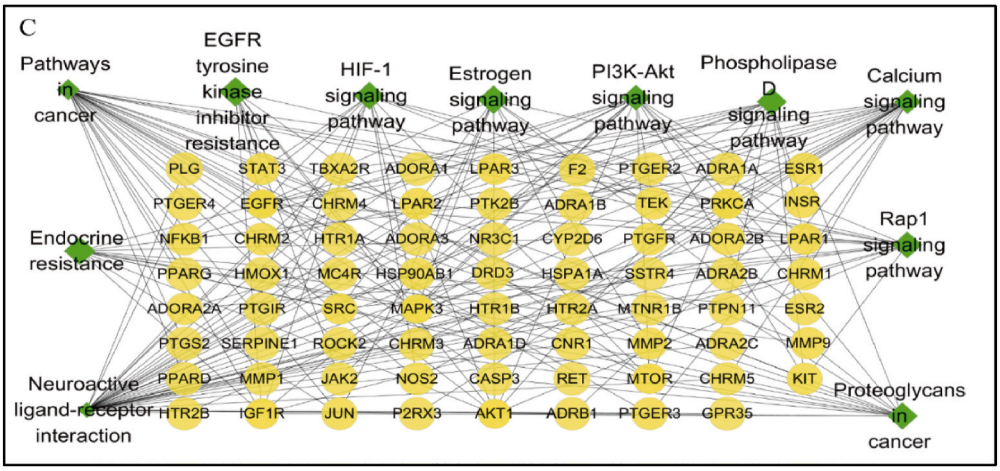


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Fig. 4. (A) Gene ontology (GO) enrichment analysis. (B) KEGG pathway enrichment analysis. (C) Network relationship map of pathways and targets.

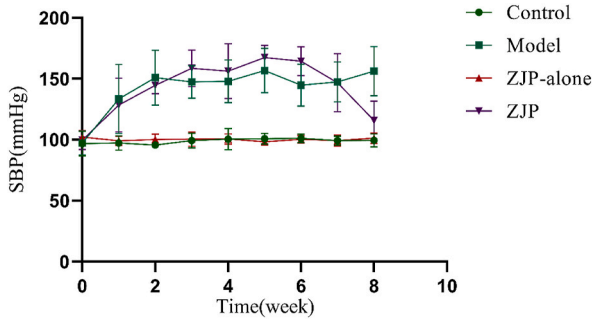

Fig. 5. ZJP treatment of hypertensive rats induced by combined high salt and cold stimuli. Differences of SBP among groups were not statistically significant during week of adaptive feeding ($P > 0.05$). SBP of Model and ZJP group was significantly higher in the second week ($P < 0.05$) and peaked at the fifth week after modeling ($P < 0.05$). Two weeks after treatments, SBP of ZJP group had decreased significantly versus Model group, #, $P < 0.05$, n = 8.

AKT comprises three closely related, highly conserved cellular homologs termed AKT1, AKT2, and AKT3 [44]. Besides, AKT1 is the predominant isoform expressed in endothelial cells [45]. Through GO analysis, this research group found that the treatment of hypertension by ZJP mainly involved blood circulation, process of the circadian system, membrane microdomain, G protein coupled amine receptor activity, and other biological processes. KEGG analysis predicted HIF-1 signaling pathway, neuroactive ligand-receptor interaction, calcium signaling pathway, PI3K/AKT signaling pathway, and estrogen signaling pathway as potential pathways for treating hypertension with ZJP, among which the most enriched pathways included neuroactive ligand-receptor interaction, PI3K/AKT signaling pathways, and calcium signaling pathways. It is worth noting that the activity of eNOS can be regulated by PI3K/AKT signaling to influence NO production and endothelial dysfunction from NO reduction; this is an important feature of hypertension [46]. The disadvantage of network pharmacology is that it is limited by available experimental and database data, which cannot be ignored. Taking advantage of existing data, mining ingredients of drugs, predicting targets of action, and experimental verification of drug action by the corresponding model (animal or cell), using them in combination, the two can dissect the mechanism of TCM herbal network pharmacology.

The network pharmacology analysis of the present study indicated that the mechanism of ZJP in treating hypertension was closely related to the PI3K/AKT signaling pathway. Extensive literature points to the involvement of the PI3K/AKT signaling pathway in the molecular mechanism of cold induced hypertension [47,48]. Cold stimulation is the only naturally induced form of experimental hypertension [49]. Sun also found that BP elevation during cold exposure is sodium dependent and that majority features of cold-induced hypertension are similar to salt-induced hypertension [49]. Olli et al. [50] found that the increase in diastolic BP was significantly higher in high salt diets than normal diets during acute whole-body cold exposure. This experiment induced a hypertensive rat model by cold, compounded with high salt. Salt sensitivity is related to the enhancement of sympathetic nervous system activity. Cold stimulation causes sympathetic nervous excitement through the central nervous system, increases peripheral adrenaline, and increases heart rate and blood pressure. Renal hemodynamics, renal tubular sodium and water treatment will also be affected [51, 52]. Our data corroborates that cold combined with high salt stimulation could increase the BP of rats, and the present research group found that their BP was significantly higher in the second week ($P < 0.05$), leveling off after BP peaked in the fifth week ($P < 0.05$) (Fig. 5), coinciding with previous experiments.

Studies have shown that hypertension induced by cold and high salt stimulation can result in impaired vascular endothelial function in rats [53–55]. The manifestation of hypertension is often accompanied by vascular endothelial dysfunction and inadequate repair ability [56]. In this experiment, HE and Masson staining show that the structure of aortic intima in the model group was disordered by fibrosis, the endothelium was incomplete, the arterial lumen became larger, and the vascular wall was thickened (Fig. 6). Despite enlargement of arterial lumen, the blood pressure remained elevated, which is associated with increased fibrosis, leading to reduced arterial elasticity. Studies have illustrated that vascular fibrosis affects the elasticity of blood vessels [57], as the deposition of collagen fibers is involved in vascular remodeling [58,59]. The literature suggests that hypertension-related endothelial dysfunction is closely associated with reduced bioavailability of NO, which can mediate guanosine cyclophosphate to promote vasodilation, regulate vascular tone, and vascular remodeling and angiogenesis [21,60]. There is evidence that NO is involved in the pathological mechanism of cold-induced hypertension [61]. Our experimental results corroborated that the plasma level of NO in the model group was significantly lower than that in the control group ($P < 0.05$) (Fig. 7B). Evidence shows that the reduction of NO production and bioavailability is related to the balance between the superoxide anion (O^{2-}), lipid free radicals, and SOD and other endogenous antioxidant defense mechanisms, which determines oxidative stress [62]. Studies show that oxidative stress can accelerate the degradation of NO and lead to decline of NO bioavailability [56]. This was demonstrated by experimental results compared to the control and ZJP group, the activities of SOD, GSH, and CAT in the rat serum of the model group decreased and the MDA level increased

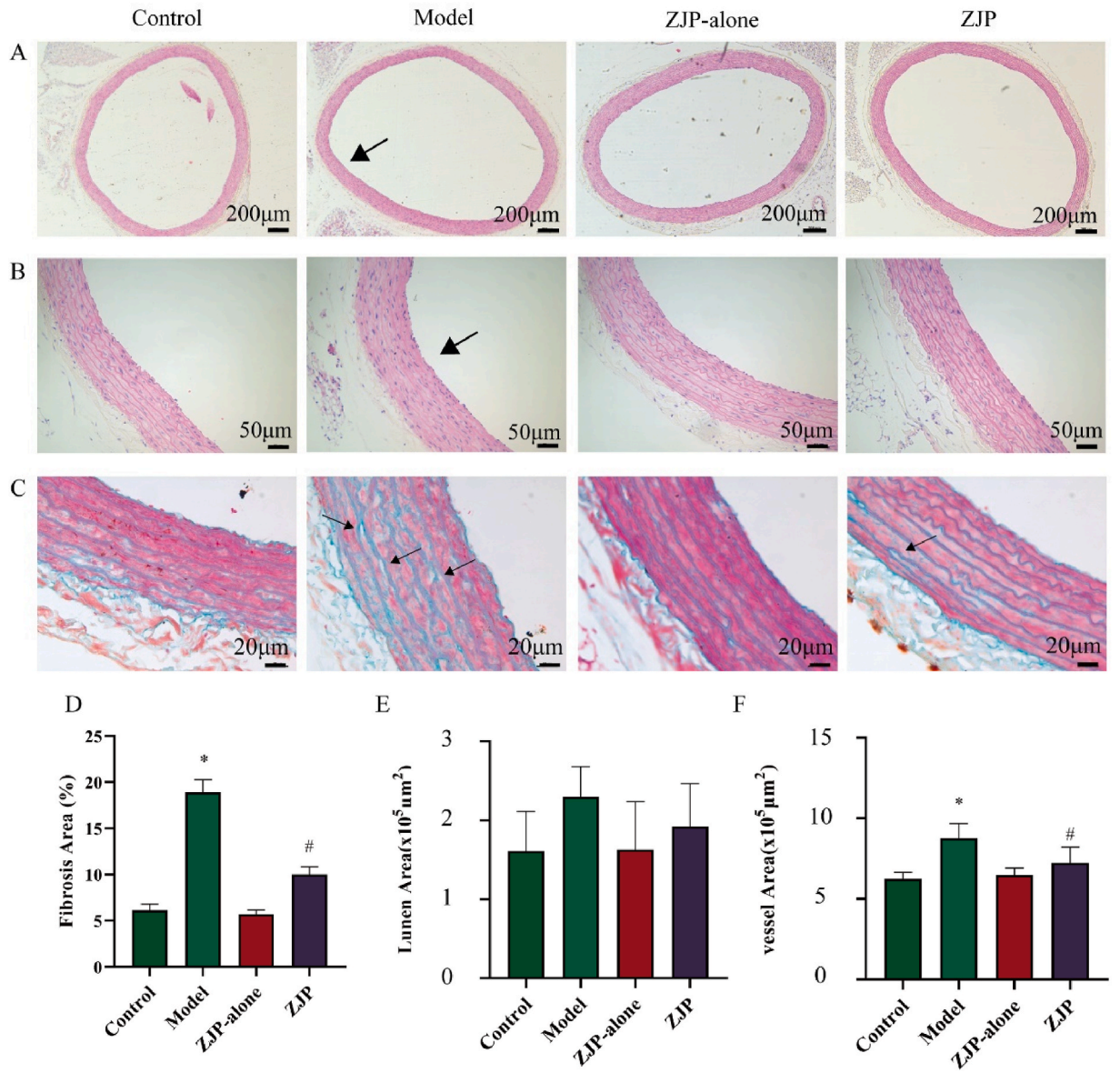

Fig. 6. Effect of ZJP improved vascular injury in hypertensive rats. (A) Histopathology with hematoxylin and eosin staining (50×) of the aorta. (B) Histopathology with hematoxylin and eosin staining (200×) of the aorta. (C) Masson staining of aorta (400×). (D) Quantification of vascular fibrosis by ImageJ software. (E) and (F) Lumen area of arteries and area of vessel wall measured by ImageJ software. *, $P < 0.05$ vs. Control group. #, $P < 0.05$ vs Model group, n = 5.

($P < 0.05$), with no significant difference between the ZJP and control group, or the ZJP-alone group ($P > 0.05$) (Fig. 7). Thus, ZJP has no adverse reaction after treatment from the comparison of ZJP-alone group and other groups: these results indicate that ZJP can alleviate oxidative stress caused by cold and high salt stimulation and improve vascular endothelial dysfunction from reduced NO bioavailability.

NO is an important endodermal factor produced by eNOS [20]. Reduced eNOS activation can reduce NO production and decrease bioavailability [6,63]. Protein kinase AKT, a key mediator of PI3K downstream signal transduction pathway, can directly activate eNOS through phosphorylation of serine and promote NO production [6,64]. The expression of PI3K/AKT protein in rat aorta was detected by Western blot for the effect of ZJP on PI3K/AKT signaling pathway in the rat aorta. Experimental results show that the expression of PI3K/AKT protein in the model group was significantly lower than that of the control or ZJP group ($P < 0.05$), as there was a difference between the ZJP and control group, and the ZJP-alone group ($P > 0.05$). The results indicate a tendency for increased PI3K/AKT activity by ZJP, and it was pointed out in the literature that the reduction of NO production by downregulation of the PI3K/AKT/eNOS signaling pathway is an important mechanism in the decline of endogenous vascular repair ability in hypertension [46]. In contrast, the increased production of NO illustrates the upregulation of the PI3K/AKT signaling pathway. Results suggest that

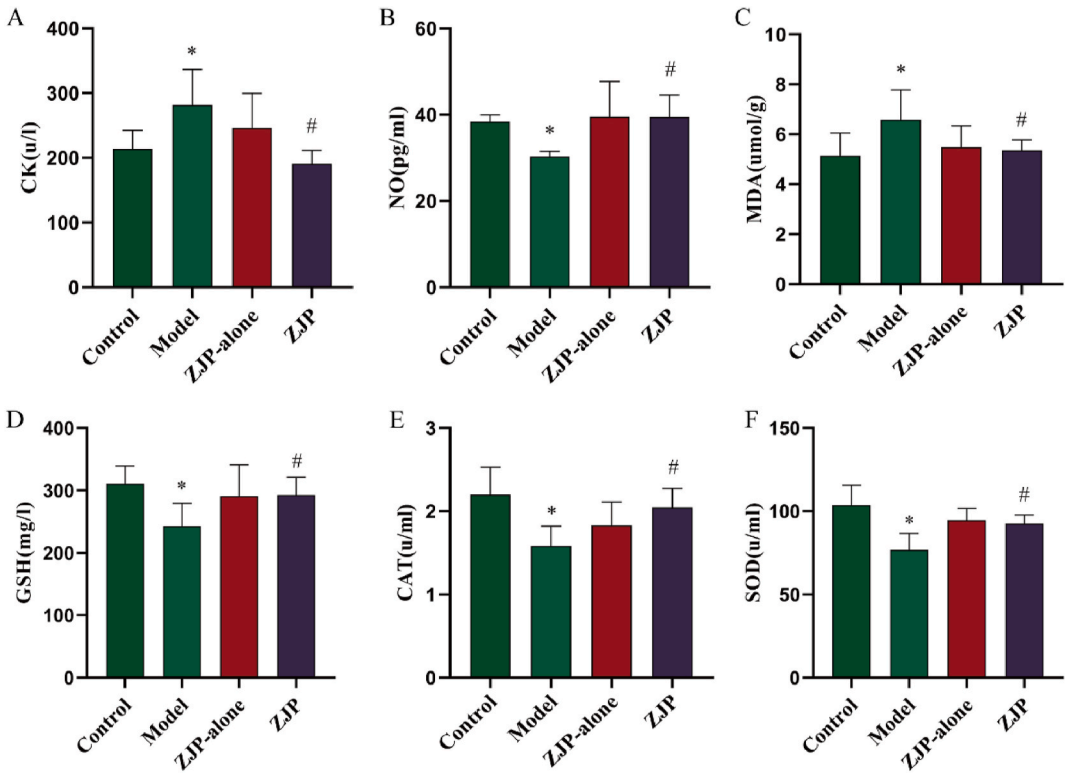

Fig. 7. ZJP ameliorated vascular oxidation stress of hypertension rats. Activity of (A) CK, (B) NO, (D) GSH, (E) CAT, and (F) SOD, and the content of (C) MDA, were detected by ELISA. *, $P < 0.05$ vs. Control group. #, $P < 0.05$ vs Model group, n = 7.

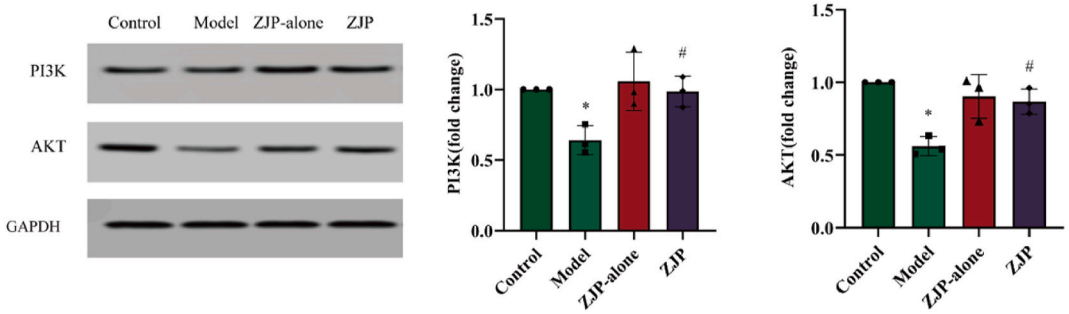

Fig. 8. Effects of ZJP on PI3K, AKT Expressions Levels Assessment of aortic expression of PI3K and AKT in rats by Western blot. *, $P < 0.05$ vs. Control group. #, $P < 0.05$ vs Model group, n = 3.

ZJP can regulate NO production by affecting PI3K/AKT protein expression in the aorta of hypertensive rats, induced by cold and high salt stimulation.

Both the development and treatment of hypertension are complex processes involving multifactorial and multiple signaling pathways. The autonomic nervous system and endocrine mechanisms such as RASS and vasopressin all have regulatory effects on blood pressure. One of these directions was selected for this study as a breakthrough due to the complexity of hypertension. We will investigate more pathways and mechanisms related to hypertension, not only PI3K/AKT signaling pathway and oxidative stress, and test more diversely experimental indexes, e.g., isolated vascular ring tension detection. ZJP is a clinically effective traditional Chinese medicine prescription with few side effects, which will be followed by clinical trials when its future research becomes more intensive.

Our study suggests that cold and high salt stimulation can cause vascular damage and oxidative stress in rats, while inducing hypertension. ZJP can reduce hypertension induced by cold and high salt stimulation and improve the morphology and function of the hypertensive aorta, which may be related to ZJP with expression of the PI3K/AKT signaling pathway, while improving oxidative stress.

Authors' contributions

YW and MqZ also HL participated in the design of the experiments. YW and BW also XZ performed the experiments and collection of data. YW and PfZ also PpC carried out the collation and analysis of the data. YW and MqZ made a final confirmation of all data. All authors have read the article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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List of abbreviations

ZJP	Zhijing Powder
TCM	Traditional Chinese Medicine
NO	Nitric oxide
eNOS	Nitric oxide enzyme
MDA	Malondialdehyde
SOD	Superoxide dismutase
CAT	Catalase
GSH	Glutathione
PPI	Protein-protein Interaction
GO	Gene ontology
RASS	Renin-Angiotensin-Aldosterone-System
KEGG	Kyoto Encyclopedia of Genes and Genomes
CK	Creatine Kinase
PBS	Phosphate buffered saline
WG	Centipede (Chinese pinyin: Wu Gong)
QX	Scorpion (Chinese pinyin: Quan Xie)
BP	Blood pressure
PVDF	Polyvinylidene fluoride
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

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