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Systems pharmacology and experimental evaluation to investigate the therapeutic targets of Chinese medicine QiShenYiQi in the treatment of ischemic stroke

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Author contributions

Wang XM, and Ding Y conceived and designed the study and supported the funding. Bai M and Cui N collected and analyzed data, and drafted the manuscript. Meng Q, Wang YW, and Wen AD provided guidance and revised the manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare no conflicts of interest.

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Peer review information

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Abbreviations

QSYQ, QiShenYiQi; IS, ischemic stroke; TCMSP, Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform; NCBI, National Center for Biotechnology Information; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MCAO, middle cerebral artery occlusion; PFA, paraformaldehyde; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IL-1β, interleukin-1 beta; PPI, protein-protein interaction; TTC, 2,3,5-triphenyl-tetrazolium chloride; H&E, hematoxylin and eosin; TUNEL, deoxynucleotidyl transferase dUTP nick end labeling; ELISA, enzyme-linked immunosorbent assay; TCM, traditional Chinese medicine; SD, standard deviation; STAT3, signal transducer and activator of transcription 3; JUN, Jun proto-oncogene; CXCL8, C-X-C motif chemokine 8; MMP9, matrix metalloproteinase 9; TP53, tumor protein P53; CASP3, caspase 3; MAPK1, mitogen-activated protein kinase 1; VEGFA, vascular endothelial growth factor A.

Citation

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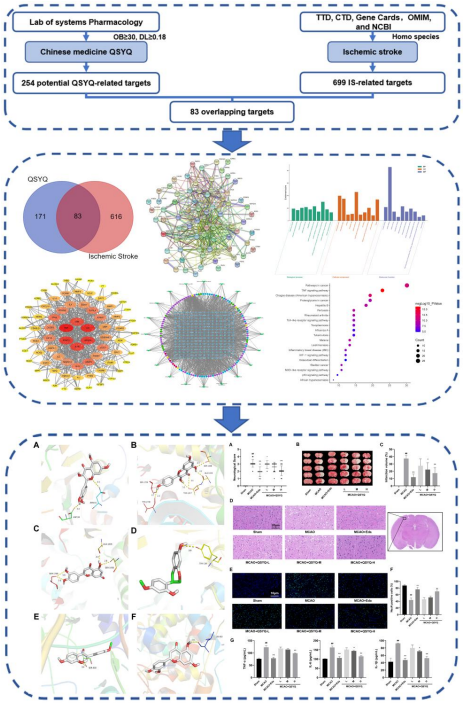
Abstract

Background: QiShenYiQi (QSYQ) is commonly accepted to treat ischemic stroke (IS) in clinical settings, yet the underlying mechanism of action of QSYQ is largely unknown. **Methods:** By combining systems pharmacology with experimental assessment, we examined the key targets, bioactive components, and mechanisms of QSYQ against IS. **Results:** Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform predicted a total number of 254 targets that were potentially related to QSYQ, whereas 699 targets associated with IS were gathered from Therapeutic Target Database, Comparative Toxicogenomics Database, Gene Cards, Online Mendelian Inheritance in Man, and National Center for Biotechnology Information databases, and 83 of these targets overlap with QSYQ-related targets. Importantly, through the analysis of Gene Ontology functional annotation, Kyoto Encyclopedia of Genes and Genomes pathway enrichment, and protein-protein interaction network, we identified 20 related signaling pathways along with 4 hub genes. Subsequently, our molecular docking results revealed that QSYQ might interact with PTGS2, PTGS1, SCN5A, and HSP90AB1. We observed dose-dependent beneficial effects of QSYQ in significantly improving neurological function and alleviating histopathological damage in middle cerebral artery occlusion model, while decreasing infarct volume. Notablely, QSYQ markedly downregulates tumor necrosis factor-α, interleukin-6, and interleukin-1 beta. Overall, this study demonstrates the synergetic effects of QSYQ on regulating multi-targets in IS through inhibiting inflammatory processes and neuronal apoptosis, these findings may expand the understanding of QSYQ and provide guidance for its clinical application in treating IS. **Conclusion:** Current study reveals the protective roles of QSYQ against IS through modulating PTGS2/PTGS1/SCN5A/HSP90AB1 and TNF signaling pathways.

Keywords: QiShenYiQi; ischemic stroke; systems pharmacology; experimental assessment



Highlights

Neuroprotection: QiShenYiQi (QSYQ) improves neurological function and reduces brain damage in a stroke model.
Anti-inflammatory effects: QSYQ downregulates tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β), indicating anti-inflammatory activity.
Multi-target regulation: QSYQ's synergistic effects on multiple targets in ischemic stroke (IS) are highlighted.
Clinical application: Study provides insights for QSYQ's clinical use in treating IS.

Medical history of objective

The QSYQ decoction is derived from "*Zheng Zhi Zhun Xian: Lei Fang*", Volume One, a work penned by the Ming Dynasty medical scholar Wang Ken Tang, completed in the year 1602. This decoction is well-known for its capacity to enhance energy levels, improve digestion, maintain hydration, and balance bodily functions to reduce excessive perspiration. It is primarily indicated for the treatment of Qi deficiency and Yang collapse, as well as for conditions characterized by a faint pulse and cold extremities (Qi deficiency refers to a state where the body's vital energy or "Qi" is insufficient, leading to various symptoms such as fatigue, spontaneous perspiration, shortness of breath, and a weak pulse. It is a common concept in traditional Chinese medicine (TCM) and is often associated with a lack of energy to perform daily activities. "Yang collapse" in TCM refers to a critical condition where the body's Yang energy is severely depleted or collapsing. This state indicates an extreme deficiency of Yang, which is associated with warmth, activity, and the body's vital functions).

Background

Cerebral stroke, commonly known as a cerebrovascular accident, typically represents a sudden event of either ischemic or hemorrhagic brain damage due to a disruption in the blood supply to the brain. Of these, IS accounts is considered to be the most prevalent type, accounting for roughly 80% of all clinical cerebral stroke cases [1]. IS has become a serious threat to human well-being as it poses a significant risk of developing disability, illnesses, and fatalities [2]. However, its clinical application of intravenous thrombolysis for acute IS is limited due to its limited therapeutic window, which is less than 4.5 h, along with the possibility of other hemorrhagic issues [3, 4]. Therefore, it is urgently needed to develop novel therapies to treat IS with improved safety and efficacy.

TCM has a long-standing reputation for its significant therapeutic benefits in treating IS, owing to its extensive clinical use throughout history [5–7]. As a modern Chinese herbal medicine, QSYQ dropping pill has been granted authorization by China's State Food and Drug Administration. It is widely prescribed to patients with cerebrovascular diseases, which are identified as "deficiency of Qi" and "blood stasis" (blood stasis is a pathological condition in TCM characterized by the disruption of blood circulation, leading to the formation of stagnant blood in the body. This can manifest as fixed pain, purplish discoloration of the skin, and the presence of masses. Blood Stasis can be caused by various factors including Qi deficiency, emotional stress, cold, or trauma, and it is considered a significant factor in the development of many diseases within TCM theory) in Chinese medicine [8, 9]. The main ingredients of QSYQ include: Huangqi (HQ, *Stragali Radix*), Danshen (DS, *Salviae Miltiorrhizae Radix et Rhizoma*), Sanqi (SQ, *Notoginseng Radix et Rhizoma*), and Jiangxiang (JX, *Dalbergiae Odoriferae Lignum*) [10, 11]. The possible healing effects of QSYQ pills in the treatment of IS and diabetic nephropathy have been reported recently [8]. Nonetheless, the underlying mechanisms of QSYQ remains elusive.

Systems pharmacology acts as a powerful tool for analyzing drug-target interactions and has been broadly utilized to explore the intricate pharmacological mechanisms underlying TCM. Here, for the first time, we investigated the regulation pathways of QSYQ for IS by systematic pharmacology, molecular docking, and experimental assessment methods. The study's methodology was encapsulated in a summary (as depicted in Figure 1). We initially employed a systematic pharmacological strategy to identify the principal bioactive constituents and core targets of QSYQ. Concurrently, we carried out an analysis of the protein-protein interaction (PPI) network and subsequently executed enrichment analyses. We then constructed a network that integrated the components, targets, and pathways to elucidate the complex interactions within QSYQ. To further our understanding, we developed a comprehensive network model that deciphered the multifaceted interactions involving multiple compounds, targets, and pathways inherent to QSYQ.

Materials and methods

Collection of the pharmacokinetic information

To assess pharmacological and molecular properties, we used keywords "Huangqi, Danshen, Sanqi, and Jiangxiang" on TCM Systems Pharmacology Database and Analysis Platform (TCMSP, http://tcmspw.com/tcmsp.php).

Prediction of IS-related targets

Online Mendelian Inheritance in Man (http://www.omim.org/), Therapeutic Target Database (http://db.idrblab.net/ttd/), Gene Cards (http://www.genecards.org/), Comparative Toxicogenomics Database (http://ctdbase.org/), and National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) were all selected to obtain IS targets. "IS" was the keyword used in all databases, and candidate targets were selected if they were commonly associated with IS.

Construction of PPI network

We analyzed all QSYQ-related and IS-related targets, then extracted the overlapping genes, that is, co-expression targets, by Draw Veen Diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/), then entered them into STRING 11.0 (https://string-db.org/), and "*Homo sapiens*" was chosen among species for its medium confidence (0.7), indicated by the lowest interaction score. We obtained the drug-disease target PPI network. We next performed a visual analysis through Cytoscape 3.8.0 (National Institute of General Medical Sciences, Bethesda, MD, USA). Finally, the values of nodes were determined, and the degree value higher than mean value (12.64) was used as selection criteria for critical targets.

Gene function and pathway enrichment analysis

We utilized DAVID Bioinformatics Resources (https://david.ncifcrf.gov/) for conducting a Gene Ontology (GO) analysis on all targets that were co-expressed, setting a rigorous significance threshold at $P < 0.05$. Additionally, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, also adhering to the same stringent *P*-value threshold of less than 0.05.

Network construction

Having gathered all the necessary data from the aforementioned procedures, we identified the principal bioactive components in QSYQ, their associated co-expressed genes, and the foremost 20 pathways identified in the KEGG enrichment analysis, with a significance level set at $P < 0.05$. These elements were then compiled into a QSYQ-target-pathway network, which was depicted using the Cytoscape 3.8.0 software.

Molecular docking

We obtained the crystallographic strKEGuctures of the four most central genes (receptors) from the RCSB Protein Data Bank (http://www.rcsb.org/). Additionally, we sourced the 3D structures of

QSYQ (ligands) from TCMSP. We then fed the information into PyMOL 2.4 (University of California, San Francisco, CA, USA) (https://pymol.org/) and AutoDock 1.5.6 (http://autodock.scripps.edu/) to remove water molecules and heteroatoms, while adding charges and hydrogen atoms. Next, we used AutoDockTools binding energy to predict ligand-receptor binding conformations, and we used one of the molecular docking results to assess the ligand-receptor binding energy potential (affinity ≤ -5 kcal/mol). Lastly, final conformation was selected according to the optimal binding energy, and visualized using PyMOL 2.4.

Design of in vivo experiment

Specific pathogen free grade male Sprague-Dawley rats, with the license number SYXK (Shaanxi) 2022-001, weighting between 230 to 280 g and at an age of 8 weeks, were procured from the Experimental Animal Center of the Fourth Military Medical University (Xi'an, China). All experimental operations involving rats were consistent with the standard guidelines [12]. These rats were maintained in a regulated environment with a temperature maintained at 25 ± 2 °C, a relative humidity between 45–75%, and under a 12-h alternating light-dark cycle. They had free access to a standard chow diet and water ad libitum. The welfare of the animals and experimental protocols were in compliance with and sanctioned by the Animal Ethics Committee at the Fourth Military Medical University (No: XJ20210205011). After 10 days of acclimatization to growth, rats were established as middle cerebral artery occlusion (MCAO) model and randomly divided into six groups, namely (1) Sham group (n = 12), (2) MCAO group (n = 12), (3) MCAO + low-dose QSYQ group (n

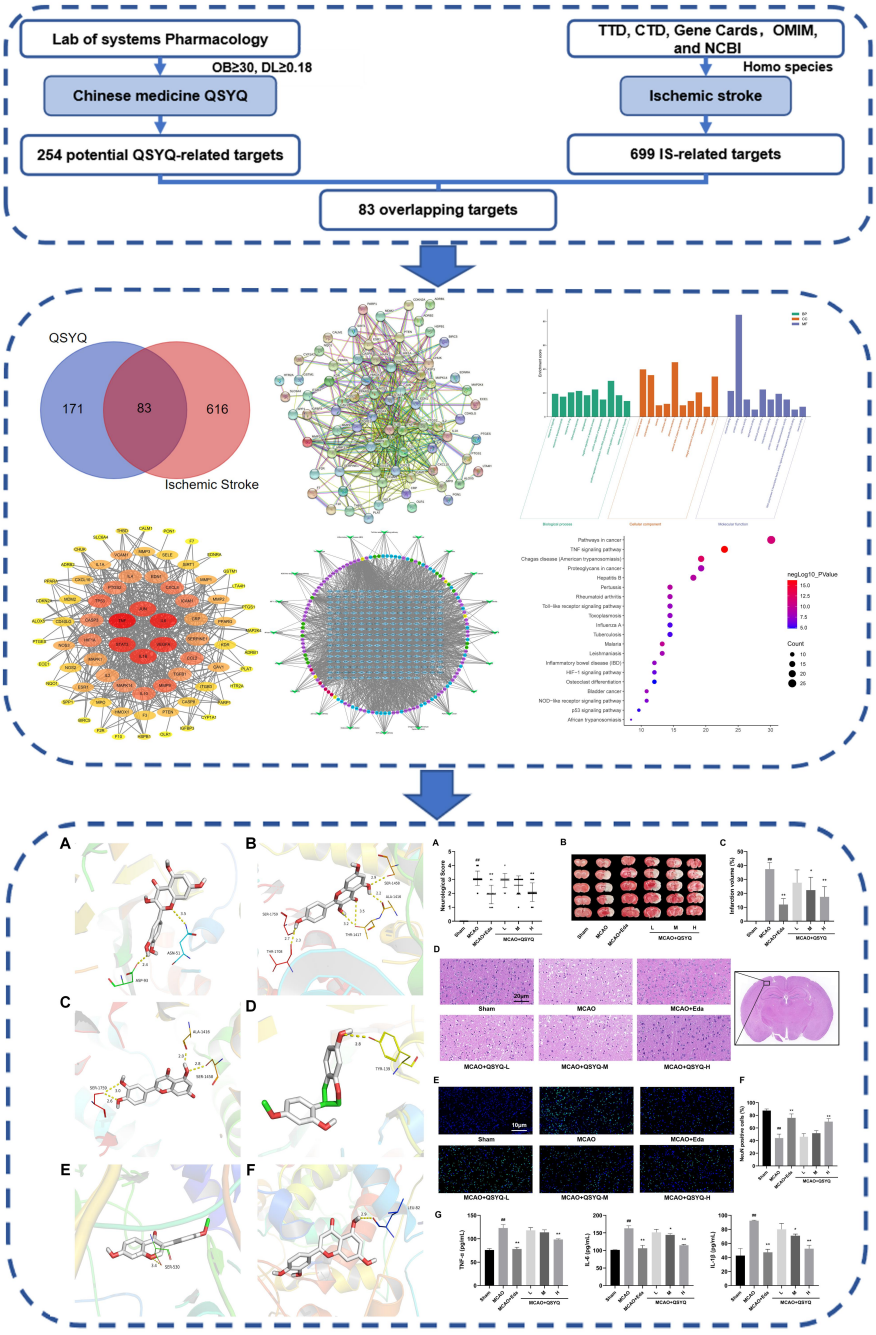


Figure 1 The analytical process of systems pharmacology. TTD, Therapeutic Target Database; CTD, Comparative Toxicogenomics Database; OMIM, Online Mendelian Inheritance in Man; QSYQ, QiShenYiQi; MCAO, middle cerebral artery occlusion; NCBI, National Center for Biotechnology Information; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IL-1β, interleukin-1 beta.

= 12, 50 mg/kg body weight/d), (4) MCAO + medium-dose QSYQ group (n = 12, 100 mg/kg body weight/d), (5) MCAO + high-dose QSYQ group (n = 12, 200 mg/kg body weight/d) and (6) Positive Control group (edaravone, n = 12, 3 mg/kg body weight/d). In the QSYQ group (the standard ratio of HQ:DS:SQ:JX was 148.01:70.35:70.35:11.97) treatment group, the medication was mixed with saline to create a solution with a density of 50 mg per milliliter for oral administration via gavage. These clinically relevant dosing choices were adopted from human dosages used in clinic [13, 14]. Administer the drug immediately after suture plug was pulled out. The positive control group was administered intravenously. Rats in the Sham and MCAO groups were given 0.9% saline at a dosage of 10 mL/kg via oral gavage daily for three consecutive days.

Surgery of MCAO model

The rats were anesthetized by administering a 1% sodium pentobarbital solution intraperitoneally at a dosage of 40 mg/kg and then secured to the surgical platform. A 2-cm incision was made on the right side of the neck to expose the common, external, and internal carotid arteries, which were then carefully dissected. Subsequently, a 4-0 nylon suture (diameter ranging from 0.22 to 0.24 mm) was introduced into the common carotid artery at the bifurcation site. To induce MCAO, the filament was advanced through the internal carotid artery to the circle of Willis, and the thread was tied off. After a 2-h period, reperfusion was initiated. Laser Doppler flowmetry (Moor Instruments, specifically in Devon, United Kingdom) was utilized to measure regional cerebral blood flow in the IS model. Animals that did not achieve an 80% reduction in blood flow or those that succumbed after ischemia were not included in the study [15, 16].

Neurological deficit score

Evaluation of neurological deficit was based on the five-point evaluation system [17, 18]. It is usually evaluated after 72 h of reperfusion. The scoring system for neurological deficits was as follows: 0 points indicated no visual impairment; 1 point signified a mild focal neurological impairment, characterized by an inability to fully extend the left forepaw; 2 points represented a moderate focal neurological impairment, evident when the rat's head tilted to the left upon tail elevation; 3 points indicated a severe focal neurological impairment, with the rat falling to the left; and 4 points meant the rat was unable to walk without assistance and exhibited a reduced level of consciousness.

2,3,5-triphenyl-tetrazolium chloride (TTC) staining

72 h after surgery, rats were sacrificed under anesthesia and injected with 1% sodium pentobarbital through the intraperitoneal injection. The harvested brains were chilled at −20 ˚C for 20 min prior to being sectioned into 2-mm thick slabs. Subsequently, the sections were stained with TTC (obtained from Sigma-Aldrich, St. Louis, MO, USA) for 30 min at a temperature of 37 ˚C, followed by an overnight incubation in 4% paraformaldehyde (PFA, Jiangsu Huda Chemical Technology Co., Ltd., Changzhou, China). Healthy brain tissue showed rosy appearance, while infarct volume appeared to be white. Finally, we used image pro plus 6.0 system (Media Cybernetics Image Technology, Rockville, MD, USA) to take and analyze images for infarct volume measurement. The subsequent Equation (1) was utilized to account for edema when calculating the infarct volume:

$$\text{Infarct Volume Ratio} = (\text{Left Side Normal Tissue} - \text{Right Side Normal Tissue})/\text{Normal Tissue on the Left Side} \times 100\% \quad (1)$$

Hematoxylin and eosin (H&E) staining

To estimate histological damage, rat brains were harvested 72 h after surgery. Then, we placed the brain in 4% PFA and fixed it for 24 h. Subsequently, the brain tissue was dehydrated and embedded in paraffin for sectioning into 5 μm coronal slices. The sections were then stained with H&E (sourced from Servicebio, Wuhan, China), and examined for histopathological alterations under an optical microscope (manufactured by Nikon, Tokyo, Japan). The neural cell viability was assessed through H&E staining, which highlighted the nuclei. Neurons affected by ischemia exhibited characteristics such as nuclear pyknosis and somatic contraction.

Deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

We performed TUNEL staining to measure neuronal apoptosis. 72 h after reperfusion, the brain tissue samples were obtained and fixed in 4% PFA, paraffin-embedded, sectioned and stained using a TUNEL kit (Servicebio, Wuhan, China). TUNEL-positive cell counts were conducted in a blinded fashion on 5 μm coronal sections from the ischemic penumbra, employing a fluorescence microscope (Nikon, Tokyo, Japan) for the analysis.

Enzyme-linked immunosorbent assay (ELISA) assay

We collected blood samples through the abdominal aorta 72 h after surgery, and processed the blood sample by centrifugation to collect serum. We then performed ELISA to determine the concentration of inflammatory cytokines (TNF-α, IL-6, and IL-1β, Xinbosheng Biotechnology Co., Ltd., Shenzhen, China).

Statistical analysis

Data analysis was conducted using GraphPad Prism version 8.3.0 (GraphPad Software Inc, San Diego, CA, USA). The outcomes are presented as the mean ± standard deviation (SD) derived from a minimum of three independent experiments. To assess differences between two groups, an unpaired two-tailed Student's t-test was utilized, while one-way ANOVA was utilized for comparing multiple groups. To further investigate significant differences among groups, a Bonferroni correction was applied for post hoc analysis, with statistical significance defined as $P < 0.05$.

Results

Pharmacokinetic information of QSYQ

The pharmacological and molecular characteristics of QSYQ were sourced from the TCMSP. In line with the TCMSP database's parameter details and filtering criteria, the oral bioavailability and drug-likeness for potentially effective drugs were identified to be at least 30 and 0.18, respectively. Approximately 130 principal active components were identified.

Candidate targets

TCMSP predicted a total number of 254 targets that were potentially related to QSYQ, whereas 699 targets associated with IS were gathered from Therapeutic Target Database, Comparative Toxicogenomics Database, Gene Cards, Online Mendelian Inheritance in Man, and National Center for Biotechnology Information databases. Among these targets, we identified 83 shared targets that were co-expressed (Figure 2A).

PPI network analysis

We performed PPI network analysis using STRING 11.0, and the results were displayed in Figure 2B. Circular nodes represented proteins in PPI network, and the lines between nodes represented protein interactions. More lines between proteins indicated stronger interaction. Among the 83 genes analyzed, 75 of them showed protein interaction, while the other 8 (*ADRA2A, ADRA2B, ADRA2C, PPARD, NR3C2, DIO1, ABCG2*, and *MAP2*) were not associated with other nodes. The targets with a degree above their mean value are in Table 1. As shown in Figure 2C, darker and redder nodes indicated greater degree.

GO and KEGG pathway enrichment analysis

We employed DAVID Bioinformatics Resources to ascertain functional and pathway enrichment data for the genes illustrated in Figure 3A. With the significance level established at $P < 0.05$, we gathered a total of 557 GO annotations: 443 were related to biological processes, 41 to cellular components, and 73 to molecular functions. They primarily focused on responses to hypoxia, extracellular space,

enzyme binding, etc. As summarized in Table 2, Figure 3B, KEGG pathway analysis revealed top ten relevant signaling pathways (Q value < 0.05), which heavily concentrated in TNF signaling, Chagas disease (American trypanosomiasis), cancer, Malaria, Pertussis, etc.

QSYQ-target-pathway network

There were about 397 nodes in QSYQ-target-pathway network (Figure 3C). The color and shape of the nodes denote various bioactive substances, targets, and pathways, including four types of compounds (Green ellipse: *Astragalus*; Purple ellipse: *Salvia miltiorrhiza*; Red ellipse: *Panax notoginseng*; Blue ellipse: *Dalbergiae Odoriferae Lignum*; Yellow ellipse: repeating elements;) and 252 targets (Blue diamond) and twenty KEGG pathways (Green inverted triangle). Furthermore, the significance of nodes within the network was denoted by their degree of connectivity with other nodes.

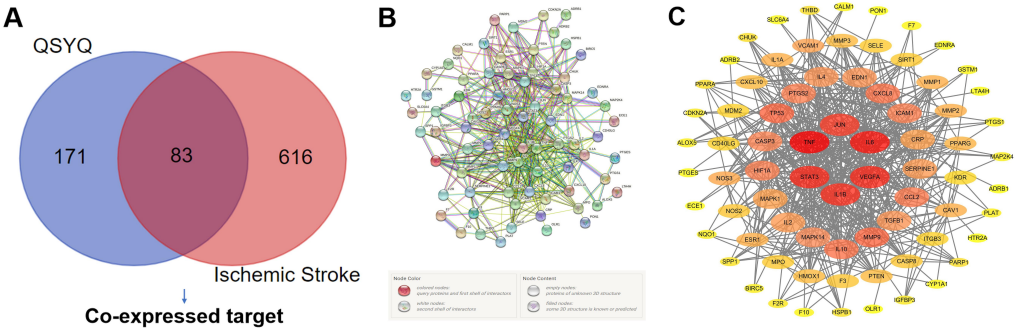


Figure 2 The intersection of QSYQ and IS and the PPI Network. (A) Common targets between QSYQ and IS, 83 targets that are common to Chinese Medicine QSYQ and IS. (B) In the PPI network, nodes signify proteins, while edges denote interactions between them. (C) Network visualization of key targets, where proteins with more connections are depicted in darker shades. QSYQ, QiShenYiQi.

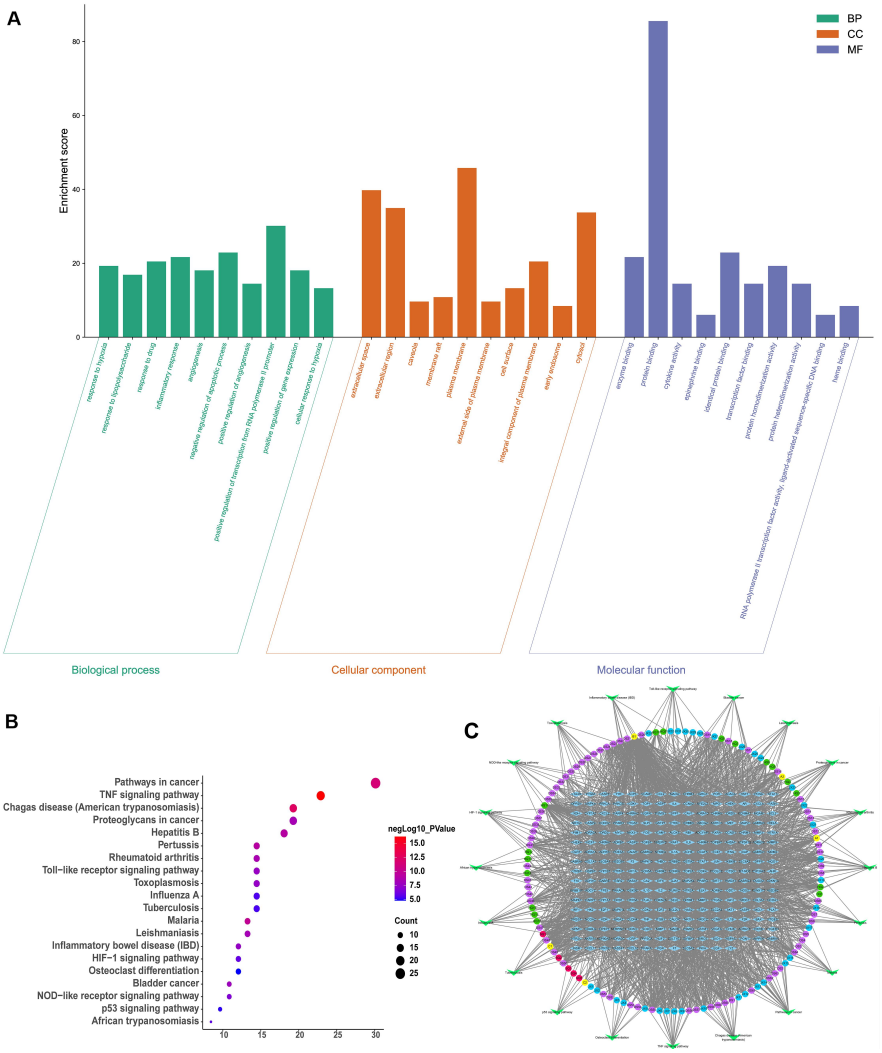


Figure 3 Enrichment analysis and network diagram. (A) GO enrichment analyses. GO term analysis: biological process (BP), cellular component (CC), and molecular function (MF), respectively. (B) KEGG pathway enrichment analyses. (C) Visualized network diagram of component-target-pathway. Green ellipse: *Astragalus*; Purple ellipse: *Salvia miltiorrhiza*; Red ellipse: *Panax notoginseng*; Blue ellipse: *Dalbergiae Odoriferae Lignum*; Yellow ellipse: repeating elements; Blue diamond: common targets; Green inverted triangle: signal pathways.

Table 1 Top 10 targets with degree higher than the average value in the PPI network

No.	Name of target	Degree
1	TNF	40
2	IL6	37
3	STAT3	34
4	VEGFA	34
5	IL1B	33
6	JUN	31
7	TP53	26
8	MMP9	26
9	IL10	24
10	CXCL8	24

Table 2 The enriched KEGG pathway and the related genes

Pathway	Genes	Fold enrichment	*P* value
TNF signaling pathway	*MAP2K4, JUN, EDN1, VCAM1, CHUK, MMP3, PTGS2, MAPK14, SELE, TNF, MMP9, ICAM1, CXCL10, IL6, CASP8, IL1B, CASP3, CCL2, MAPK1*	22.89	6.18E−17
Chagas disease (American trypanosomiasis)	*IL10, MAP2K4, JUN, TGFB1, CXCL8, NOS2, CHUK, SERPINE1, MAPK14, TNF, IL2, IL6, CASP8, IL1B, CCL2, MAPK1*	19.28	3.50E−13
Pathways in cancer	*CXCL8, PTEN, PTGS2, HIF1A, EDNRA, CASP8, CASP3, MAPK1, JUN, TGFB1, NOS2, CHUK, CDKN2A, MMP1, MMP2, F2R, STAT3, MMP9, VEGFA, IL6, MDM2, BIRC5, PPARG, TP53, PPARD*	30.12	3.34E−12
Malaria	*IL10, IL6, TGFB1, VCAM1, CXCL8, CD40LG, IL1B, CCL2, SELE, TNF, ICAM1*	13.25	1.12E−10
Pertussis	*IL10, IL1A, IL6, JUN, CXCL8, NOS2, IL1B, CASP3, MAPK1, CALM1, MAPK14, TNF*	14.46	5.28E−10
Hepatitis B	*MAP2K4, JUN, TGFB1, CXCL8, CHUK, STAT3, PTEN, TNF, MMP9, IL6, CASP8, CASP3, BIRC5, MAPK1, TP53*	18.07	5.86E−10
Rheumatoid arthritis	*IL1A, IL6, JUN, TGFB1, CXCL8, MMP1, IL1B, MMP3, CCL2, TNF, ICAM1, VEGFA*	14.46	3.07E−09
Proteoglycans in cancer	*TGFB1, ITGB3, MMP2, CAV1, STAT3, MAPK14, HIF1A, ESR1, TNF, MMP9, VEGFA, CASP3, MDM2, KDR, MAPK1, TP53*	19.28	4.62E−09
Leishmaniasis	*IL10, IL4, IL1A, JUN, TGFB1, NOS2, IL1B, MAPK1, MAPK14, PTGS2, TNF*	13.25	5.15E−09
Bladder cancer	*CXCL8, CDKN2A, MMP1, MMP2, MDM2, MAPK1, TP53, MMP9, VEGFA*	10.84	1.34E−08

Molecular docking results

QSYQ and the top 4 hub genes selected were analyzed for molecular docking using PyMOL 2.4 and AutoDock 1.5.6 (Figure 4). Binding energies between QSYQ and target proteins vary from −9.1 to −4.5 kcal/mol. Under the filtering criteria for docking outcomes, a mere four target proteins exhibited binding energies above −5 kcal/mol in interaction with QSYQ, as listed in Table 3. Lastly, when comparing with targets selected by the KEGG enrichment, 4 targets (5F1A, 6Y3C, 6LQA, and 3NMQ) were identified. Several hydrogen bonds, as indicated by the yellow dotted lines, were observed among QSYQ and core targets. In addition, QSYQ has been shown to bind to ASP-93 and ASN-51 binding sites of HSP90AB1, THR-1708, THR-1417, SER-1759, SER-1458, and ALA-1416 binding sites of SCN5A, SER-1759, SER-1458, and ALA-1416 binding sites of SCN5A, TYR-139 binding sites of PTGS2, SER-530 binding sites of PTGS1, LEU-82 binding sites of HSP90AB1.

Amelioration by QSYQ of brain injury induced by MCAO rats

Before the rats were sacrificed, we did not observe any neurological deficits in Sham group, but severe neurological deficits were recorded in MCAO group ($P < 0.01$). Important, edaravone and QSYQ-treated (200 mg/kg) group showed significant decrease in neurological deficit scores when comparing to MCAO group ($P < 0.01$, Figure 5A), and QSYQ high-dose group showed better improvement than the low-dose QSYQ group and medium-dose QSYQ group. TTC staining showed more extensive lesions on ipsilateral cerebral hemispheres in MCAO animals (Figure 5B). As revealed by infarct volumes, we detected a larger infarct volume in MCAO group in comparison to Sham group ($P < 0.01$), suggesting successful MCAO model was generated. QSYQ (200 mg/kg) treatments substantially reduced the cerebral infarct volume by more than 50% (from 37.42% to 17.43%, $P < 0.01$, Figure 5C). In addition, the neuroprotective effect of QSYQ high-dose group was close to positive drug edaravone. These data revealed that the protection of QSYQ against cerebral ischemia. Next, as shown by H&E staining, cells did not show noticeable morphological change in Sham group, while apparent changes were observed after ischemia, including disorganized neuron arrangement, pyknotic nucleus, and neuronal loss. Nonetheless, such detrimental effects were mitigated by QSYQ (200 mg/kg) treatment (Figure 5D). TUNEL staining revealed a high number of apoptotic cells within the cerebral infarction area of MCAO rats. Notably, the extent of apoptosis in this region was considerably lessened in rats that received QSYQ (200 mg/kg) treatment (Figure 6A, 6B). In conclusion, QSYQ can reduce brain damage after cerebral ischemia, enhance the neuroprotective effect, and contribute to the recovery of brain function. Therefore, QSYQ has an excellent protective effect against stroke.

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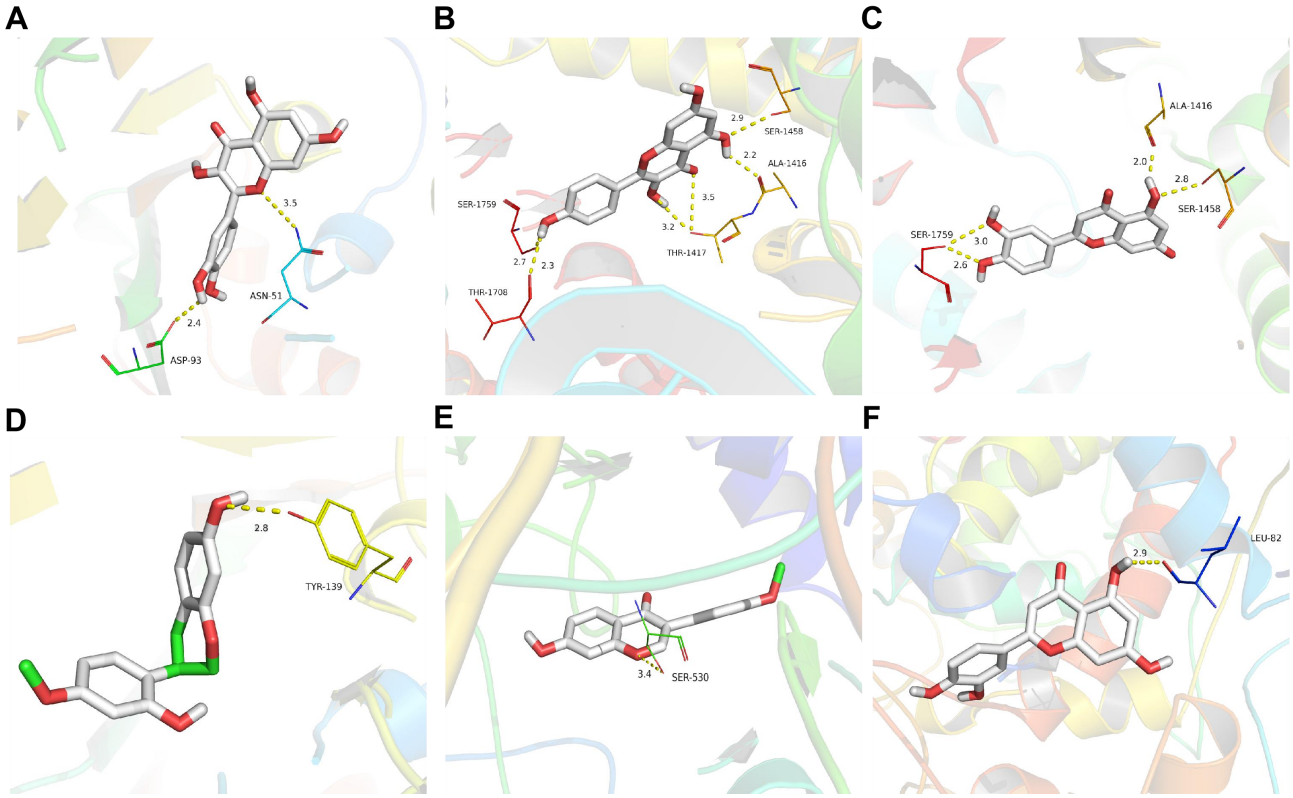

Figure 4 3D docking pose of QSYQ with (A) HSP90AB1, (B) SCN5A, (C) SCN5A, (D) PTGS2, (E) PTGS1, (F) HSP90AB1, respectively. The white sticks represent quercetin, kaempferol, luteolin, formononetin, quercetin, and (-) -Vestitol.

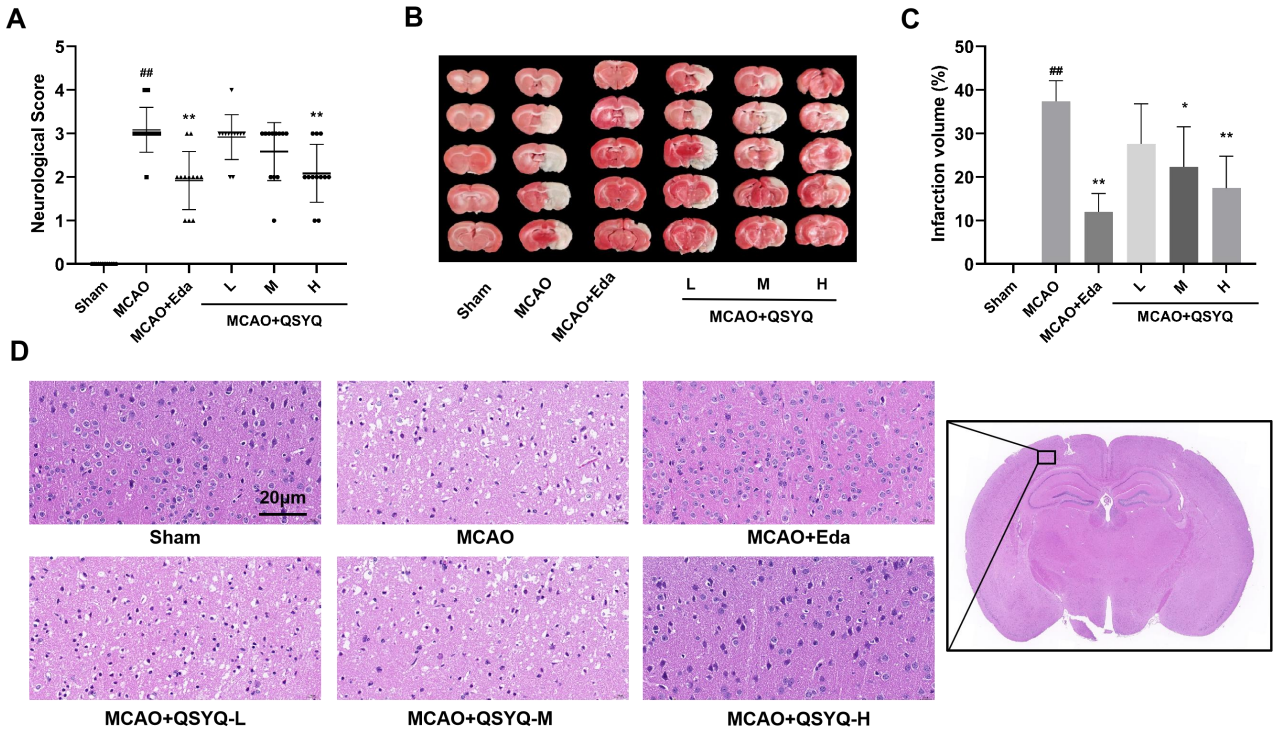

Figure 5 QSYQ mitigates brain damage caused by MCAO. (A) Scatter plot depicting neurological deficits in the Sham, MCAO, edaravone, and QSYQ (50, 100, 200 mg/kg) treatment groups (median value shown, n = 12). (B) Cerebral infarct volumes among rats in each group. (C) Statistical evaluation of infarct volume ratios across groups. (D) H&E staining of coronal sections from the ischemic cerebral cortex. Scale bar = 20 μm. Data, excluding the neurologic score, were presented as mean ± SD. $^{\#\#}P < 0.01$, versus Sham group; $^{*}P < 0.05$, $^{**}P < 0.01$, versus MCAO group. MCAO, middle cerebral artery occlusion; QSYQ, QiShenYiQi.

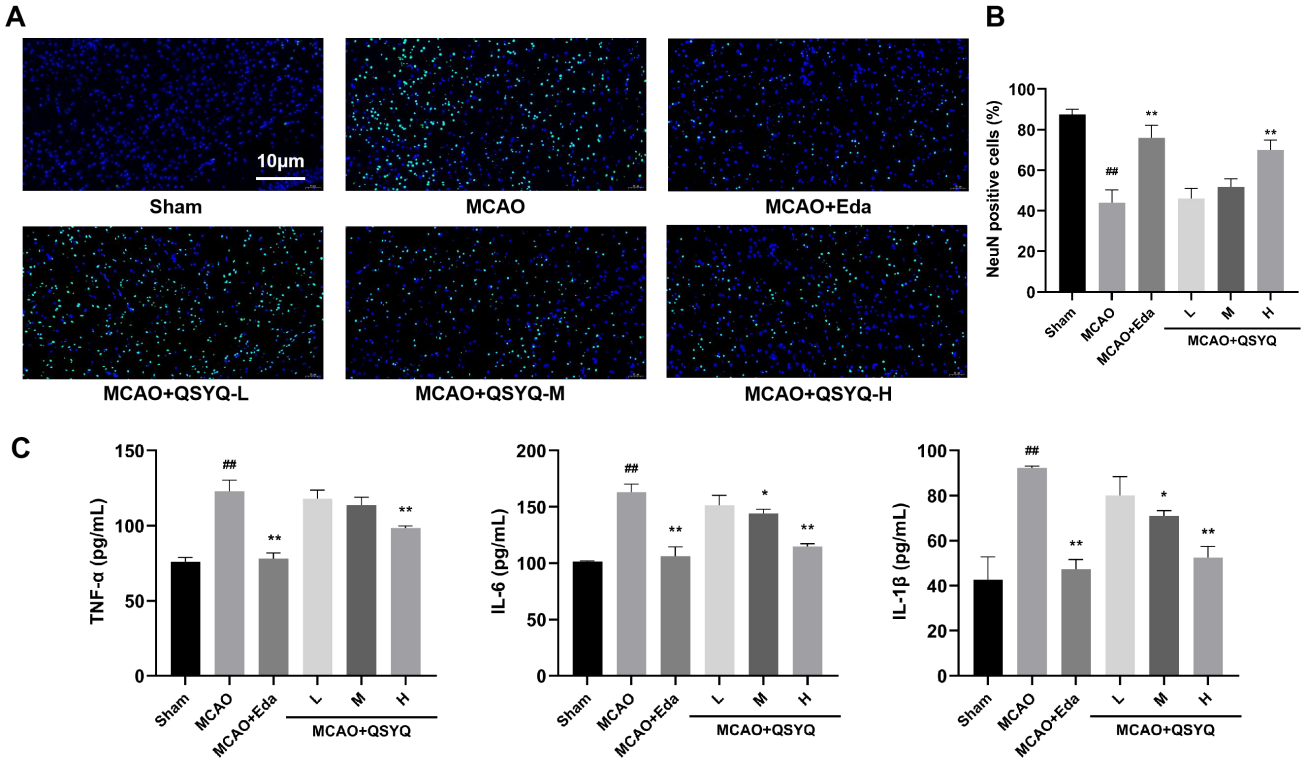

Figure 6 Effects of QSYQ on neuronal apoptosis, neuronal survival, and inflammatory cytokine levels in MCAO rats. (A) TUNEL assays were conducted on brain tissues from rats across all groups. Scale bar = 10 μm. (B) Statistical analysis of NeuN-positive cells. (C) The accumulation of TNF-α, IL-6, and IL-1β were decreased in the ischemic penumbra of rats in each group that received QSYQ treatment (n = 3). Data were presented as the mean ± SD. $^{\#\#}P < 0.01$, compared with the Sham group; $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the MCAO group. QSYQ, QiShenYiQi; MCAO, middle cerebral artery occlusion; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IL-1β, interleukin-1 beta.

Table 3 The docking information of QSYQ with the four targets

Gene	Binding energy (kcal/mol)				
	Quercetin	Kaempferol	Luteolin	(-)-Vestitol	Formononetin
PTGS2	−5.3	−5.5	−6.2	−4.5	−5.9
PTGS1	−4.9	−5.0	−5.4	−4.5	−4.9
SCN5A	−7.9	−8.0	−8.3	−7.2	−7.7
HSP90AB1	−9.1	−8.9	−9.1	−7.3	−9.1

Regulation the expression of inflammatory cytokines by QSYQ in MCAO rats

To assess the inflammatory reaction in the cerebral cortex of ischemic brains, an ELISA kit (Xin Bosheng Biotechnology Co., Ltd., Shenzhen, China) was employed for quantifying inflammatory marker levels. There was a significant increase in the levels of TNF-α, IL-6, and IL-1β within the infarcted brain region of MCAO rats compared to the control Sham group ($P < 0.01$). However, QSYQ (200 mg/kg) significantly inhibited the expression these cytokines ($P < 0.01$, vs. MCAO group, Figures 6C).

Discussion

Stroke continues to be among the top contributors to mortality and neurological impairment globally, even with extensive endeavors to manage risk factors and enhance emergency treatment for sufferers [19]. It's important to highlight that, as of now, no small molecule or biological therapy has received approval from the Food and Drug Administration to facilitate the recovery process post-stroke. The absence of approved treatments aimed at enhancing functional recovery following IS is partly due to the focus on inadequate single-agent approaches that target only one molecular pathway or a single therapeutic target [20]. The existing therapeutic approach to IS does not embrace a multi-target and multi-pathway strategy, which could be contributing to the less-than-ideal treatment outcomes observed in recent years [21]. Therefore, the development of multi-molecular and multi-target therapeutics is of great significance for the treatment of IS. QSYQ is widely used in clinical practice to treat cardiovascular diseases [22]. TCMs adhere to the concept of "syndrome differentiation and treatment". According to TCM theory, stroke is categorized under the pattern of "Qi deficiency and blood stasis", predominantly affecting the circulatory system. Therefore, the pathogenesis of this disease indicates that the treatment strategy of TCM should be closely linked, called "treating different diseases with the same" [23, 24]. QSYQ, a contemporary herbal remedy, is extensively utilized in China for treating individuals with conditions associated with Qi deficiency and blood stasis [8, 25]. Current research in animal models has confirmed that the components of QSYQ can increase survival, decrease the size of brain infarctions, and improve neuromotor functions in rats subjected to cerebral ischemia/reperfusion injury [25, 26]. Clinically, QSYQ combined with conventional western medicine therapy had a certain effect on chronic heart failure patients, and is extensively applied in managing conditions of the heart and blood vessels [27]. In our research, we employed network pharmacology, molecular docking, and experimental assessments to explore the molecular mechanisms of QSYQ in IS. We first acquired all pharmacological and molecular properties of QSYQ from TCMSP. 65 ingredients (such as isoflavanone,

7-O-methylisomucronulatol, formononetin, FA, and Mairin, etc.) belong to HQ, 20 ingredients (such as przewalskin b, formyltanshinone, miltionone II, epidanshenspiroketallactone, and prolithospermic acid, etc.) originate from DS, 8 ingredients (Stigmasterol, beta-sitosterol, ginsenoside rh2, Diop, and, quercetin, etc.) derive from SQ, and 37 ingredients (such as Sativanone, (3r)-5'-methoxyvestitol, violanone, Dalbergin, and isoduartin, etc.) stem from JX. Intriguingly, numerous studies have documented that these aforementioned bioactive compounds exert powerful effects against IS via multiple targets/pathways molecular mechanism, which further supported our findings [28–36]. Secondly, by searching through multiple drug/disease databases, we retrieved 83 overlapping targets to build PPI network, and 75 of nodes showed PPI. Further topological analysis of PPI network revealed 10 core targets with higher degree values, which were primarily associated with inflammatory responses (TNF, IL6, signal transducer and activator of transcription 3 (STAT3), IL1B, Jun proto-oncogene (JUN), IL10, C-X-C motif chemokine 8 (CXCL8), and matrix metalloproteinase 9 (MMP9)), immune response (IL6, STAT3, IL1B, IL10, CXCL8), apoptosis (JUN, STAT3, tumor protein P53 (TP53), caspase 3 (CASP3), B-cell lymphoma/leukemia-2, and mitogen-activated protein kinase 1 (MAPK1)), as well as vascular endothelial function (vascular endothelial growth factor A (VEGFA), nitric oxide synthase 3, and endothelin 1). Inflammation is a characteristic feature of stroke pathology and plays a role in worsening brain damage [37]. JUN (c-Jun), a component of the AP-1 transcription factor complex, is crucial in the processes of neuronal apoptosis and inflammation. Typically, c-Jun becomes highly activated following a cerebral ischemic event, with its expression markedly elevated in the cerebral cortex. An escalating body of research has identified that various natural compounds possess anti-inflammatory properties in the context of cerebral ischemia. These effects are attributed to their ability to modulate the underlying inflammatory mechanisms and the influence of inflammatory mediators in the pathogenesis of IS [38]. In addition, it has been revealed that the adaptive immune system is activated within 24 h after cerebral ischemia. This encompasses inflammatory effects mediated by T cells and B cells, which trigger the release of multiple interleukins and cytokines, thus regulating the inflammatory response [39]. Validated that the STAT3 protein in endothelial cells is a promising therapeutic target for preventing post-stroke endothelial dysfunction, and that blocking the STAT3 signaling pathway could mitigate brain damage caused by cerebral ischemia [40–42].

As revealed by KEGG analysis, QSYQ could protect against IS through modulating TNF signaling, Chagas disease, Pathways involved in cancer, malaria, pertussis, hepatitis b, rheumatoid arthritis, proteoglycans in cancer, leishmaniasis, and bladder cancer, etc. Collectively, the pathways identified here are highly related with cellular processes such as anti-oxidant, anti-apoptosis, anti-inflammatory responses [43, 44]. Several previous publications displayed that decreased TNF-α, IL-6, and IL-1β levels can protect against cerebral ischemic damage [45–47].

We next performed molecular docking analysis to explore the relationships among core components and their key targets. As revealed by our analysis, the majority of key component-targets had binding affinities below −5 kcal/mol, indicative of strong interactions, thereby reinforcing the credibility of our systems pharmacology predictive approach. Then, 4 hub genes were used for molecular docking, and revealed 16 potential targets for effective binding to QSYQ. These data indicated that QSYQ might effectively interact with PTGS2, PTGS1, SCN5A, and HSP90AB1.

The component-target-pathway network suggested that the cerebral protective (anti-IS) effects of QSYQ is a complex network of cellular processes, which requires modulation of various targets associated with multiple pathways. Through the topological analysis of network, five key components with higher degree values are identified: quercetin, kaempferol, luteolin, formononetin, and (-) -Vestitol. The previous study has shown that quercetin improved neuronal count in the hippocampal subfields by down-regulating iNOS and caspase-3 activity after Cerebral ischemia reperfusion [48–51]. Kaempferol exhibited neuroprotective effects against brain damage and neuroinflammation through suppressing NF-κB and STAT3 pathways [52–54]. Furthermore, the study indicated that luteolin has the potential to treat IS by inhibiting MMP9 and activating PI3K/Akt signaling cascade [55, 56]. To sum up, these results demonstrate therapeutic potential of QSYQ core components to treat IS.

Lastly, by using rat MCAO model with histological analyses, we verified the accuracy of our systems pharmacology approach. Interestingly, QSYQ dose-dependently improved neurological function, ameliorated pathological damage, lowered infarct volume and rate of impaired neurons of MCAO rats. The neuroprotective effect of QSYQ high-dose group was close to that of positive drug edaravone. Therefore, our experimental data. Meanwhile, QSYQ markedly reduced the levels of inflammatory factors within the infarcted tissue of MCAO rats. In line with previous reports, our findings revealed the protective effect of QSYQ on cerebral ischemia and suggested that these pathways are major contributors to the pathogenesis of ischemic brain damage [13, 57–59].

Limitation

Though preliminary findings highlight the promise of QSYQ in facilitating post-stroke rehabilitation, certain limitations persist. Firstly, the outcomes of network pharmacology could be influenced to some extent by the choice of various databases, timeliness of the collected components and targets. Secondly, because there are differences between the compounds of QSYQ retrieved in the database and the components entered into the blood, the correlation between the efficacy and mechanism of QSYQ needs to be further verified through experiments at various levels. To sum up, it would be worthwhile to assess the favorable impacts and underlying mechanisms of QSYQ on the rehabilitation of motor functions in a long-term post-stroke recovery animal model, as this aligns more closely with the clinical relevance for actual patient care.

Conclusion

Current study represented the first attempt to investigate the mechanism of QSYQ in treating IS by systematic and comprehensive analysis. The components, targets, and 20 important KEGG pathways of QSYQ were evaluated by network pharmacology. Importantly, our molecular docking analysis identified 16 potential targets that can effectively bind to QSYQ. Altogether, our results provided better understanding on the molecular mechanisms of QSYQ, which may guide future research and development of anti-IS drugs. Our experimental and network pharmacological analyses illustrated that the mechanisms of QSYQ in ischemic brain injury is mainly consist of two therapeutic modules: mitigating neuronal apoptosis and suppressing inflammatory processes. More importantly, TNF signaling pathway may be the most critical targets for QSYQ protection against ischemic brain damage.

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