

Research Article

Hydroxysafflor Yellow A Alleviates Ischemic Stroke Through Modulation of GPX4-Associated Ferroptosis: Computational Simulation and Animal Validation

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Abstract

Background: Ferroptosis has been recognized as an important pathological mechanism underlying ischemic stroke (IS). However, the precise mechanisms underlying the neuroprotective effects of Hydroxysafflor yellow A (HSYA) in IS remain unclear. **Objective:** Therefore, this study aimed to investigate the protective effects of HSYA against IS and explore the underlying ferroptosis-related mechanisms. **Methods:** An integrated strategy combining network pharmacology, molecular docking, molecular dynamics simulations, and a middle cerebral artery occlusion (MCAO) mouse model was employed to elucidate the neuroprotective mechanisms of HSYA. Neurological deficit scores, infarct volume, histopathology, oxidative stress, and ferroptosis-related molecules were evaluated. For *in vivo* validation, mice were randomly assigned to the sham, MCAO, Ras-selective lethal (RSL), HSYA, and RSL + HSYA groups (n = 12 per group). Ras-selective lethal 3 (RSL3) was used as a glutathione peroxidase 4 (GPX4) inhibitor in the RSL and RSL + HSYA groups. **Results:** Network pharmacology identified GPX4 and Ferritin Heavy Chain 1 (FTH1) as potential ferroptosis-related targets of HSYA in IS. Docking analysis showed favorable binding affinities of HSYA toward GPX4 (−9.3 kcal/mol) and FTH1 (−9.1 kcal/mol), and molecular dynamics simulations further supported the stability of the HSYA–GPX4 and HSYA–FTH1 complexes. *In vivo* experiments showed that HSYA significantly improved neurological deficit score, reduced infarct volume, and alleviated histopathological damage in MCAO mice ($p < 0.01$ or $p < 0.05$). Moreover, HSYA significantly decreased malondialdehyde (MDA) and reactive oxygen species (ROS) levels while increasing superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities ($p < 0.01$ or $p < 0.05$). HSYA significantly upregulated the mRNA and protein levels of GPX4, FTH1, and mitochondrial ferritin (FTMT) ($p < 0.01$ or $p < 0.05$). Notably, HSYA significantly increased the relative protein expression of GPX4 compared with the MCAO group ($p < 0.01$). Furthermore, RSL3 partially attenuated the protective effects of HSYA on cerebral injury, oxidative stress, and the expression of GPX4, FTH1, and FTMT ($p < 0.01$ or $p < 0.05$). Collectively, the present study demonstrates that HSYA confers neuroprotection against IS, at least in part, through regulation of GPX4-associated ferroptosis pathways. **Conclusions:** HSYA provides significant neuroprotection against IS. These effects may be mediated, at least in part, through GPX4-associated ferroptosis pathways and the maintenance of iron homeostasis, thereby advancing understanding of its antioxidant mechanisms and supporting its therapeutic potential for IS.

Keywords: ischemic stroke; ferroptosis; network pharmacology; molecular dynamics simulation; hydroxysafflor yellow A; neuroprotective agents; phospholipid hydroperoxide glutathione peroxidase

1. Introduction

Stroke contributes markedly to worldwide disability and mortality, with its incidence rising particularly in low- and middle-income nations [1]. Although a great deal of time has been spent on researching neuroprotective drugs, only a few of them have proven to be truly effective. This indicates that it is necessary to further study the pathological mechanism of stroke and search for new therapeutic targets.

Iron-dependent ferroptosis is a relatively new type of controlled cellular death that is increasingly recognized as a key process in acute brain injury, including ischemic and hemorrhagic strokes [2,3]. It is distinguished by lipid peroxidation within cell membranes and is closely linked

to the depletion of glutathione peroxidase 4 (GPX4), a selenocysteine-containing enzyme essential for neutralizing phospholipid hydroperoxides [4]. Reduced GPX4 expression has been observed in acute ischemic stroke (IS) [5], indicating that dysfunction of the GPX4 antioxidant defense system may contribute to ischemic brain injury. Recent studies have identified GPX4, PRDX1, and PRDX6 as potential ferroptosis-related biomarkers for IS [6]. In addition, clinical studies have shown that ACSL4 and ferritin are associated with stroke severity and functional outcomes [7]. These findings further support the clinical relevance of ferroptosis and highlight ferroptosis-targeted intervention as a promising therapeutic strategy.



Besides, iron overload is also recognized as a major factor promoting ferroptosis. Previous studies have shown that iron overload exacerbates cerebral ischemia/reperfusion injury following transient middle cerebral artery occlusion (tMCAO) [8]. In contrast, iron chelation or depletion provides neuroprotection, reducing related brain damage [9]. It has been suggested that mitigating iron accumulation and strengthening the ferroptosis defense system may be promising approaches for stroke therapy [10]. For instance, in stroke patients, iron chelators such as deferoxamine mesylate (DFO) lower serum peroxide levels and inhibit the production of free radicals [11]. Likewise, selenium supplementation can upregulate GPX4 expression and alleviate post-stroke ferroptosis [12]. All in all, these findings point to ferroptosis inhibition as a key treatment option for IS.

Carthamus tinctorius L. has long been used in traditional Chinese medicine for the treatment. Classical medical texts, including the Compendium of Materia Medica and the Essential Prescriptions from the Golden Cabinet, document its clinical application in disorders associated with cardiovascular and cerebrovascular disorders [13]. Beyond its medicinal applications, safflower also possesses nutritional and industrial value. The corolla of safflower can be used to extract natural pigments for food coloring, and its seed oil, rich in linoleic acid, is widely applied in the field of health food. Modern nutritional research shows that safflower extract, as a functional food ingredient, can protect the heart by controlling inflammatory reactions and oxidative stress [14]. Hydroxysafflor yellow A (HSYA), the major bioactive compound isolated from safflower (chemical structure shown in Fig. 1), has been shown to suppress the generation of reactive oxygen species (ROS), a key driver of ferroptosis, and exhibits potent antioxidant activity. Furthermore, HSYA may influence ferroptosis by regulating ferritin and iron transporter proteins [13,15]. Although HSYA is a hydrophilic compound with limited permeability across the intact blood-brain barrier (BBB), pharmacokinetic studies have demonstrated that, under conditions of BBB disruption, HSYA can penetrate the BBB and accumulate in ischemic brain tissue, supporting its therapeutic potential in IS [16,17]. It remains unclear whether HSYA regulates GPX4-related antioxidant defense and thereby attenuates lipid peroxidation during cerebral ischemia–reperfusion injury. Addressing this question may elucidate how HSYA mitigates cerebral ischemia/reperfusion injury (CI/RI).

In recent years, advances in network pharmacology, molecular docking, and molecular dynamics (MD) simulations have provided powerful tools for identifying potential therapeutic targets, predicting compound–target interactions, and elucidating the molecular mechanisms of candidate drugs [18,19,20,21]. In this study, network pharmacology was used to identify the potential therapeutic targets and signaling pathways of HSYA in cerebral ischemia–

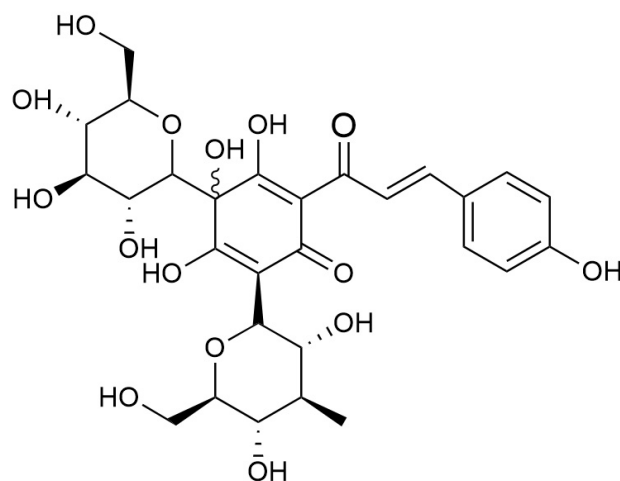


Fig. 1. The chemical structure of HSYA, with the molecular formula $C_{27}H_{32}O_{16}$ and a molecular weight of 612.5 g/mol. HSYA, hydroxysafflor yellow A.

reperfusion injury. Molecular docking and MD simulations were subsequently performed to evaluate the interactions between HSYA and key target proteins. Finally, these computational predictions were evaluated in an MCAO mouse model to investigate whether GPX4-associated ferroptosis-related mechanisms may be involved in the protective effects of HSYA against cerebral ischemia–reperfusion injury.

2. Materials and Methods

2.1 Materials

HSYA (purity $\geq 98\%$, CAS No. 78281-02-4, lot Nos. DST220921-017) was purchased from Chengdu Lemetian Medical Technology Co., Ltd. (Chengdu, China). Hematoxylin and eosin (H&E) staining solutions (H3136 and E4009; lot Nos. 20231125 and 20231117, respectively) and 2,3,5-triphenyltetrazolium chloride solution (TTC solution, 2%; G3005, lot No. 240004004) were from Solarbio (Beijing, China). A bicinchoninic acid (BCA) kit (P0012; lot No. 062122220811) was from Shanghai Biyun-tian Biotechnology Co., Ltd. (Shanghai, China). Primary antibodies against GPX4 (DF6701; lot No. 78p5366) and FTH1 (DF6278; lot No. 35y4418) were sourced from Affinity Biosciences Pty Ltd. (OH, USA), and anti-FTMT antibody (bs-20086R; lot No. BJ12283518) from Bioss Inc. (Beijing, China). HRP-linked anti-rabbit IgG (7074; lot No. 30) was obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Malondialdehyde (MDA, MB-5892A, lot No. 202503), reduced glutathione (GSH-PX, MB-5870A, lot No. 202503), ROS (MB-6180A, lot No. 202503), and superoxide dismutase (SOD, MB-6199A, lot No. 202503) assay kits were purchased from Jiangsu Enzyme Label Biotechnology Co., Ltd. (Jiangsu, China). Primers for mitochondrial ferritin (FTMT), GPX4, and ferritin heavy chain 1 (FTH1) were synthesized by San-

gon Biotech Biological Engineering Co., Ltd. (Shanghai, China).

2.2 Network Pharmacology

PharmMapper, SwissTargetPrediction, and SEA were used to estimate potential HSYA targets. Genes associated with ferroptosis and IS were gathered from the Fer- rDb and DisGeNET databases, respectively. These three gene sets were intersected using Venny 2.1 (<https://bioinfogp.cnb.csic.es/tools/venny/index.html>) to identify candidate targets. protein–protein interaction (PPI) networks were constructed with STRING (organism: *Homo sapiens*; confidence score ≥ 0.400) and visualized in Cytoscape 3.9.0 (<https://cytoscape.org/>) to screen core targets. The Cyto- Hubba plugin in Cytoscape was used to identify and rank hub genes based on the maximal clique centrality (MCC) algorithm, and the topological parameters of the hub genes were further analyzed. DAVID was employed to conduct Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment and Gene Ontology (GO) enrichment analysis (species: *Homo sapiens*) and visualized with R ggplot2.

2.3 Molecular Docking

HSYA structure in Structure Data File (SDF) format was optimized with Chem3D and subsequently translated to Protein Data Bank (PDB) format using OpenBabel (<https://openbabel.org/>). PyMol was applied for removing ligands, metal ions, and molecules of water from the protein structure before preparing it for docking. Molecular docking was performed using AutoDock Vina 1.2.7 (<https://vina.scripps.edu/>) with an exhaustiveness value of 16. Each docking calculation was independently repeated three times to ensure reproducibility, and the number of generated binding modes (num_modes) was set to the default value of 9. Incorporating hydrogen atoms, charge distribution, atomic type specification, and pdbqt file production are all part of this preparatory procedure. HSYA was used as the ligand and protein as the receptor in the docking process, and the best binding shape was predicted using a genetic algorithm. The final results were visualized using PyMol. The grid center coordinates were set as (25.706, –16.508, –17.168) with a grid box size of $21 \times 21 \times 21$ Å for GPX4, and (40.23, –4.969, 16.409) with a grid box size of $26.76 \times 26.76 \times 26.76$ Å for FTH1.

2.4 Molecular Dynamics Simulation

We evaluated the stability of the HSYA-protein complex using a multifaceted methodology. POCASA 1.1 was first used to forecast potential binding locations. To investigate different binding conformations, docking was done using DOCK 6.12 (<https://dock.compbio.ucsf.edu/>), Swiss-Dock (<https://www.swissdock.ch/>), and AutoDock Vina. The resultant poses were optimized and prioritized based on binding free energy utilizing RosettaVS. A multidimensional scoring function, incorporating van der Waals forces,

electrostatics, and solvation effects, was used to select the thermodynamically most favorable complexes. To further assess the stability of these ideal conformations, full-atom MD simulations were performed. Protein topology files were generated with the CHARMM36 force field, and ligand parameters were derived using the CGenFF 2.0.0 (<https://cgenff.com/>) server. In GROMACS 2022.4, the leading conformations were subjected to 100-nanosecond MD simulations. Root mean square deviation (RMSD), root mean square fluctuation (RMSF), and hydrogen bond interactions were analyzed to assess the complex's stability. Hydrogen bond occupancy and interaction duration between HSYA and target residues were also quantified. RMSD of Ca atoms was tracked during the simulation for trajectory analysis in order to guarantee system equilibration.

2.5 Animal Experiment

2.5.1 Animals

Specific pathogen-free male C57BL/6 mice (6–8 weeks old, 21–23 g) were obtained from the Animal Experiment Center of Zhejiang Chinese Medical University (Production License No. SCXK [Shanghai] 2022-0004). The Animal Ethical and Welfare Committee of Zhejiang Chinese Medical University gave its approval to the procedure (Approval No. IACUC-20220516-15) and supervised throughout by the same committee. All animal experiments were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and are reported in compliance with the ARRIVE guidelines. Before experimentation, mice were acclimatized for seven days at a temperature of 20–25 °C, with a 12-hour light/dark cycle, 40–45% humidity, and unlimited access to food and drink.

2.5.2 MCAO Modeling and Drug Treatment

Mice were allocated to five groups using a random-number table ($n = 12$): Sham, MCAO, HSYA, RSL, and RSL+HSYA. tMCAO was induced to produce focal cerebral ischemia. Experimental evaluations were performed after 1 h of occlusion and 24 h of reperfusion. Mice were pretreated with atropine (0.04 mg/kg, subcutaneously) and subsequently anesthetized with Zoletil 50 (40 mg/kg, intraperitoneally). The MCAO model was established according to previously published protocols [22]. During MCAO surgery, body temperature was maintained at 37 ± 0.5 °C using a thermostatically controlled heating pad. Anesthetic depth was assessed by monitoring the respiratory rate and the absence of the pedal withdrawal reflex. Mice were continuously observed throughout the procedure to ensure stable respiration and physiological condition.

The experimental design is detailed in Fig. 2. Based on prior studies and preliminary experiments [23], the HSYA group received tail vein injections of HSYA (20 mg/kg) once daily for three consecutive days after surgery. Mice in the RSL group received intraperitoneal injections

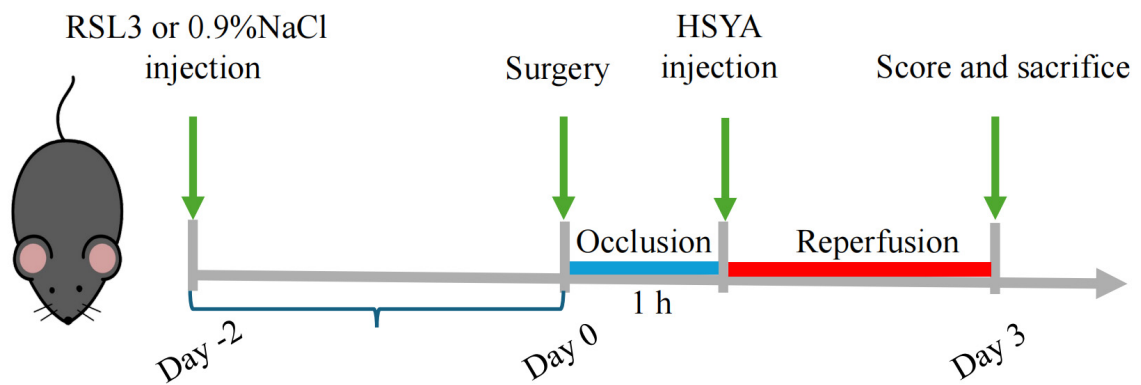


Fig. 2. Flowchart of the experimental procedure. HSYA, Hydroxysafflor yellow A; RSL3, RAS-selective lethal 3.

of Ras-selective lethal 3 (RSL3) (5 mg/kg) once daily for two consecutive days before surgery [24]. The RSL + HSYA group received both treatments in the same regimen. RSL3 was administered before MCAO surgery to ensure sufficient inhibition of GPX4 activity prior to ischemic injury, thereby facilitating evaluation of the contribution of GPX4-associated ferroptosis to the neuroprotective effects of HSYA. Sham and MCAO groups were given equal volumes of sterile saline. Mice were housed under controlled conditions with ad libitum access to chow and water. Blood collection was performed under anesthesia. Following deep anesthesia, mice were euthanized by exsanguination. Death was confirmed by the absence of spontaneous respiration and heartbeat. Animals that died during surgery or failed MCAO modeling were excluded from subsequent analyses. The $n = 12$ per group refers to the final number of animals included after exclusions. Initially, 12, 18, 17, 17, and 18 mice were assigned to the Sham, MCAO, HSYA, RSL, and RSL + HSYA groups, respectively. No animals were excluded from the Sham group, whereas 6 mice in the MCAO group, 5 in the HSYA group, 5 in the RSL group, and 6 in the RSL + HSYA group were excluded because of perioperative death or unsuccessful MCAO modeling. Thus, 12 mice per group were included in the final analyses. Of these 12 mice, 6 were used for neurological deficit scoring; among them, 3 were subsequently used for TTC staining and the other 3 for H&E staining. The remaining 6 mice were used for ELISA and RT-qPCR analyses, and 3 of these 6 mice were also used for Western blotting.

2.5.3 Neurological Deficit Score

Neurological deficits were evaluated three days after ischemia using the Longa scoring scale: 0, no neurological deficit; 1, lack of full extension of the left forepaw; 2, consistent left-turning behavior; 3, severe circling to the left; 4, inability to walk or loss of consciousness [25]. Higher scores indicate more severe behavioral impairments. Neurological deficit scoring was performed under blinded conditions.

2.5.4 TTC Staining and Infarct Volume Analysis

Brains were sectioned into six 2-mm-thick coronal slices and immersed in 2% TTC solution (37 °C, 30 min). Infarct areas were quantified using ImageJ software v1.8.0.112 (<https://imagej.net/>). Infarct volume (%) = [(volume of the contralateral hemisphere – volume of the non-infarcted ipsilateral hemisphere) / volume of the contralateral hemisphere] $\times$ 100%. TTC image analysis and infarct volume quantification were performed under blinded conditions.

2.5.5 Histological Staining

Paraffin-embedded sections (4 μ m) underwent dewaxing, rehydration, and staining using hematoxylin and eosin (H&E), followed by mounting and evaluation under light-based microscopy. Histopathological damage was evaluated using a semiquantitative scoring system (Supplementary Table 1). Histopathological evaluation and scoring were performed under blinded conditions.

2.5.6 Enzyme-Linked Immunosorbent Assays (ELISAs)

Serum concentrations of SOD, MDA, GSH-Px, and ROS were quantified using commercial ELISA kits. Blood samples from the animals were allowed to stand for 1 h before centrifugation for 15 minutes at room temperature at 4500 rpm. The resulting supernatant was used for biochemical analysis.

2.5.7 WB (Western Blotting)

GPX4, FTH1, and FTMT were detected by WB. A BCA test was used to measure the total protein concentration, followed by SDS-PAGE and transfer to PVDF membranes. Primary antibodies against GPX4, FTH1, FTMT, and β -actin (diluted 1:1000) were used to probe the blots overnight at 4 °C. HRP-conjugated secondary antibodies (1:5000) were then used for two hours at 25 °C. Blots were blocked (5% non-fat milk in Tris-buffered saline with Tween 20 (TBST), 1 hour, room temperature). After measuring band intensities, the ratio of the target band's gray

Table 1. Primer sequence.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
<i>GPX4</i>	AAGTCCTAGGAAACGCCCG	CATCGCGGGATGCACACAAG
<i>FTMT</i>	GCCAGATCAACCTGGAGTTGT	CAGTCATCACGGTCTGGTTTCTTTA
<i>FTH1</i>	CAAGCACATCAGCTCTGCAC	TCCAGGTTGATTTGGCGGTT
<i>GAPDH</i>	ACCCTTAAGAGGGATGCTGC	CCAATACGGCCAAATCCGTT

value to that of β -actin was used to determine the relative amounts of protein expression.

2.5.8 Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

RNA concentrations were measured using spectrophotometry after total RNA was extracted using TRIzol as instructed. After reverse transcription (15 min, 42 °C), enzymes were inactivated (5 min, 85 °C). PCR amplification was carried out in 96-well plates in the following manner: 40 cycles of 95 °C for 15 s and 60 °C for 60 s, followed by initial denaturation at 95 °C for 10 min. Primer sequences are listed in Table 1.

2.6 Statistical Analysis

Statistical analyses were performed using SPSS 24.0 (<https://www.ibm.com/spss>). Data are presented as mean $\pm$ standard deviation (SD). Normality was assessed using the Shapiro–Wilk test. Homogeneity of variance was evaluated using Levene’s test. For normally distributed data with homogeneous variances, differences among groups were analyzed using one-way analysis of variance followed by the Student-Newman-Keuls q test for multiple comparisons. For data that did not satisfy the assumptions of normality or homogeneity of variance, the Kruskal–Wallis test was used. $p < 0.05$ was considered statistically significant.

3. Results

3.1 Network Pharmacology

3.1.1 Candidate HSYA Targets for Treating IS

A total of 314 HSYA-related gene targets, 62 ferroptosis-associated genes, and 1159 IS-related genes were collected from the corresponding databases. Venn analysis was performed using Venny 2.1, which identified 10 overlapping targets among HSYA, ferroptosis, and IS (Fig. 3).

3.1.2 PPI Network

With a minimal interaction score of 0.4, the ten overlapping targets were utilized in STRING to build the PPI network, which was then visualized using Cytoscape (Fig. 4). Hub genes were ranked using the MCC algorithm in the CytoHubba plugin, with GPX4 and FTH1 ranking first and second, respectively (**Supplementary Table 2**). These findings identified GPX4 and FTH1 as potential hub proteins mediating the neuroprotective effects of HSYA through ferroptosis-related signaling pathways in IS.

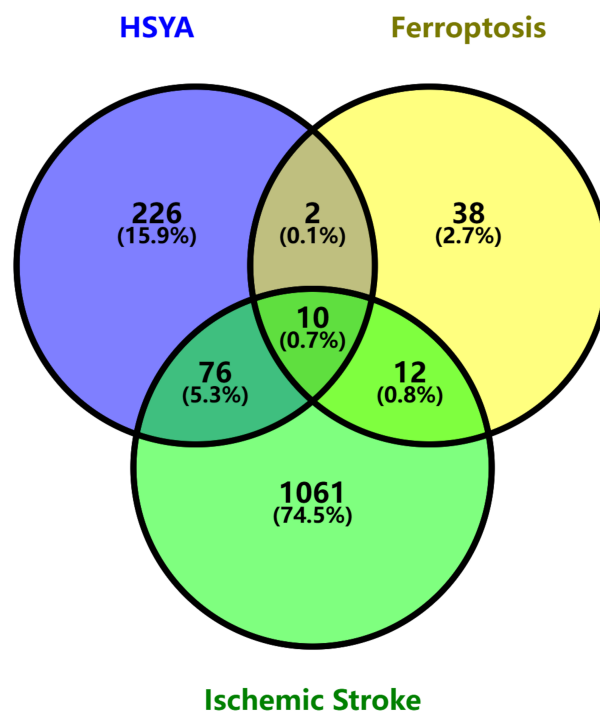


Fig. 3. Venn diagram showing the overlapping targets among HSYA, ferroptosis, and ischemic stroke. HSYA, hydroxysafflor yellow A; IS, ischemic stroke.

3.1.3 GO Enrichment Analysis

GO analysis of 10 main HSYA targets produced enrichment in 29 terms, including 5 cellular components (CCs), 5 molecular functions (MFs), and 19 biological processes (BPs). These results suggest that the potential effects of HSYA may be associated with biological processes relevant to ferroptosis, oxidative stress responses, and iron handling. Visualization with the ggplot2 package showed that the 10 shared targets were particularly enriched in BP terms (negative regulation of ferroptosis, oxidative stress response), CC terms (cytoplasm, intracellular lumen), and MF terms (iron ion binding, protein binding) (Fig. 5).

3.1.4 KEGG Enrichment Analysis

Using DAVID, the KEGG pathway enrichment of the 10 key targets mentioned above was examined. Seven pathways were significantly enriched ($p < 0.05$) (Fig. 6), further suggesting the potential involvement of ferroptosis-related signaling in the neuroprotective effects of HSYA.

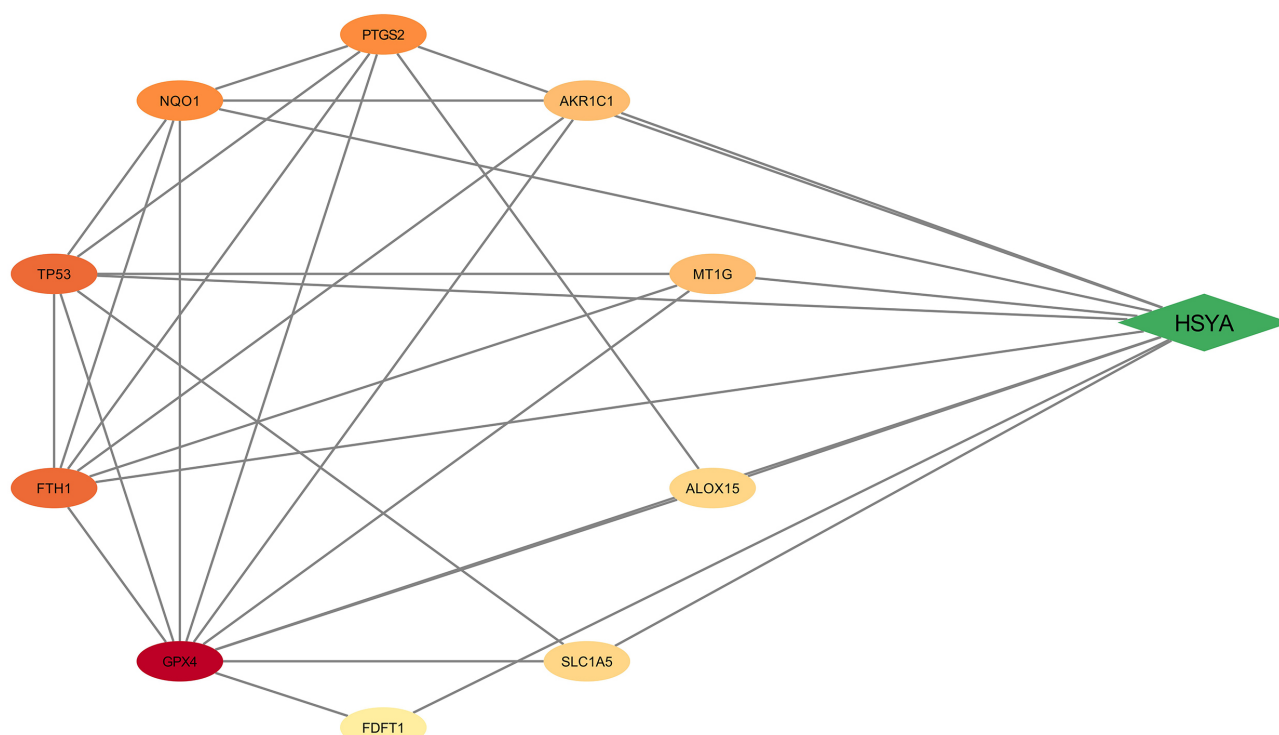


Fig. 4. Integrated network of HSYA and the protein–protein interactions among the overlapping targets associated with ferroptosis and ischemic stroke. HSYA, hydroxysafflor yellow A.

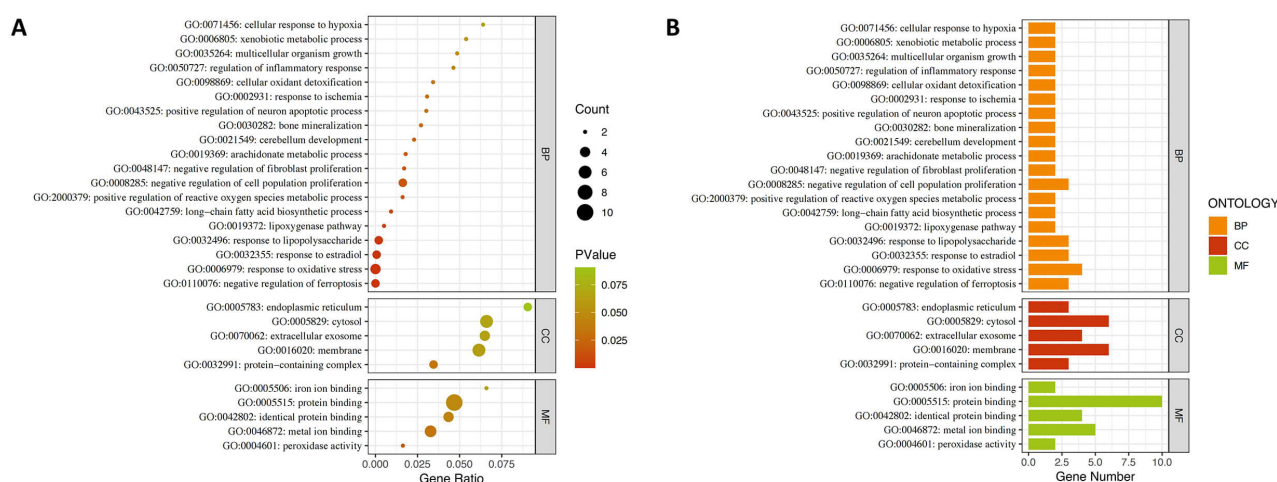


Fig. 5. GO enrichment analysis results of core targets. (A) Bubble plot of enriched GO terms. (B) Bar plot showing the gene number for each enriched GO term. BP, biological process; CC, cellular component; GO, Gene Ontology; MF, molecular function.

3.2 Molecular Docking

3.2.1 Docking of Key Protein Targets in the PPI Network

Potential binding between HSYA and the top-ranked proteins from the PPI network was assessed using molecular docking simulations. Binding energies for all ligand–target complexes were below $-5.0 \text{ kcal} \cdot \text{mol}^{-1}$ (Fig. 7, Table 2).

3.2.2 Molecular Docking Analysis of Ferroptosis-Related Proteins

Building on the network pharmacology findings and prior literature, GPX4 and FTH1 were hypothesized to be potential ferroptosis-related targets associated with the protective effects of HSYA in IS. Docking simulations were therefore performed with these proteins to evaluate the potential interactions of HSYA with ferroptosis-related targets. For GPX4 (PDB ID: 2OBI), the docking grid was

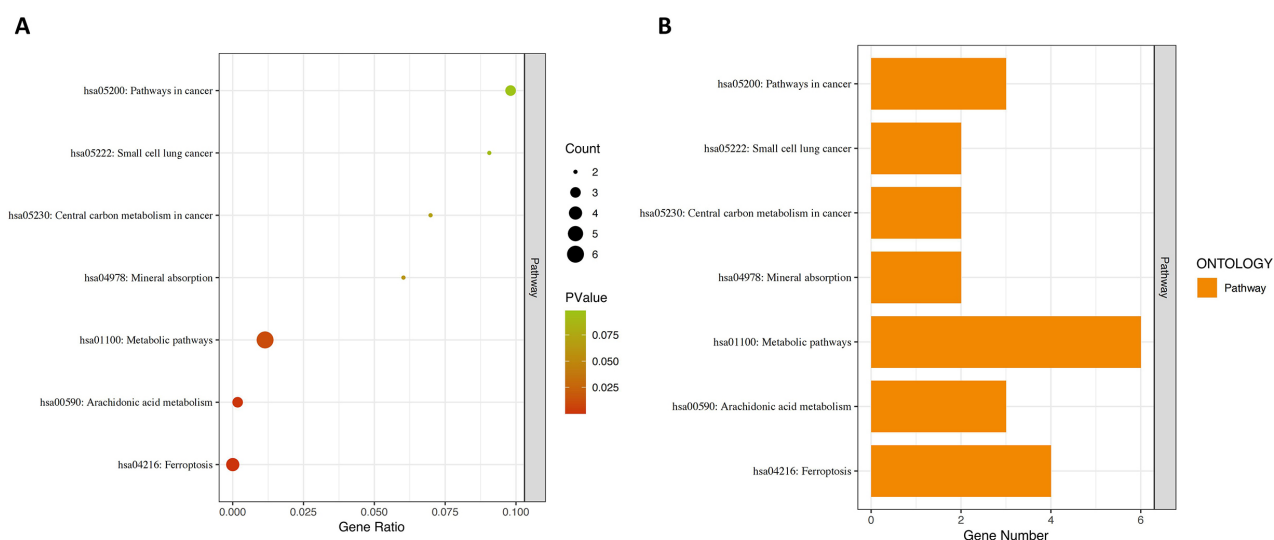


Fig. 6. The top 7 most enriched KEGG pathways. (A) Bubble plot of enriched KEGG pathways. (B) Bar plot showing the gene number for each enriched KEGG pathway. KEGG, Kyoto Encyclopedia of Genes and Genomes.

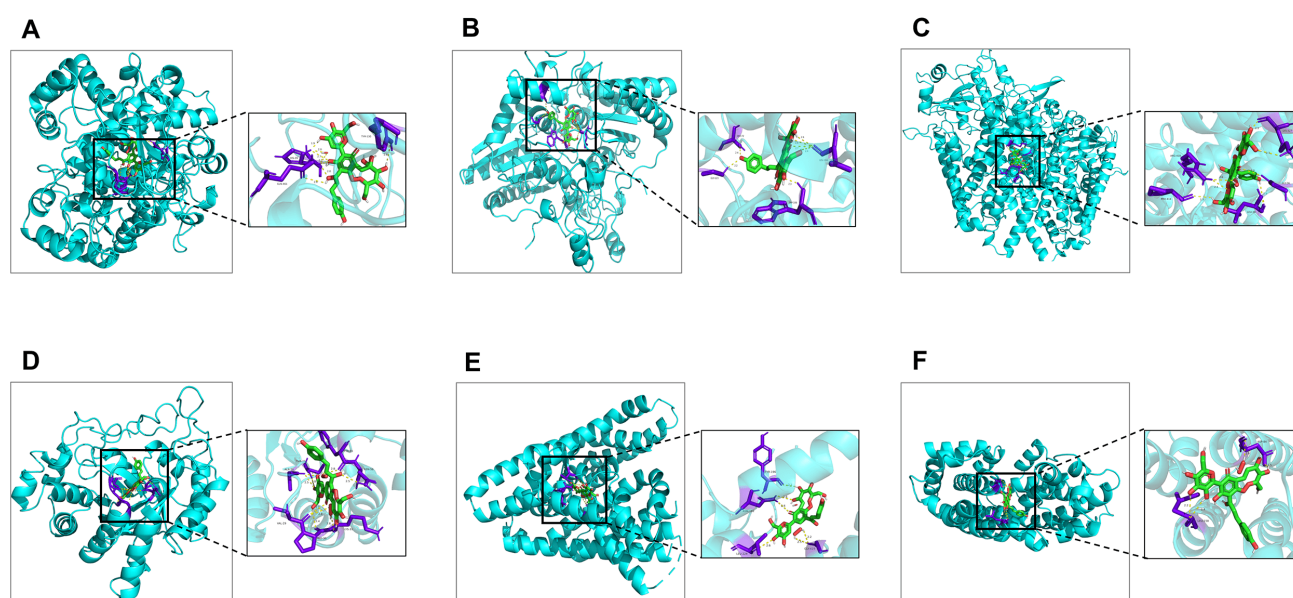


Fig. 7. Molecular docking conformations of HSYA with the top-ranked target proteins. (A) PTGS2 (PDB ID: 5F19). (B) NQO1 (PDB ID: 8RFN). (C) ALOX15 (PDB ID: 5ME0). (D) AKR1C1 (PDB ID: 3C3V). (E) SLC1A5 (PDB ID: 5LM4). (F) FDFT1 (PDB ID: 3V66).

centered on the Cys46 active-site residue. HSYA exhibited favorable binding affinities with both GPX4 and FTH1, with binding energies of -9.3 kcal/mol and -9.1 kcal/mol, respectively (Fig. 8, Table 2).

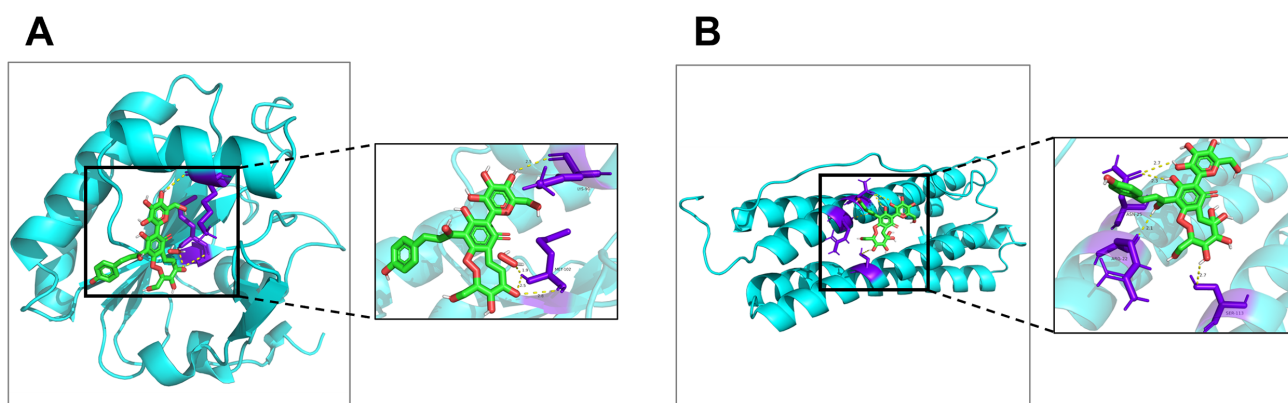
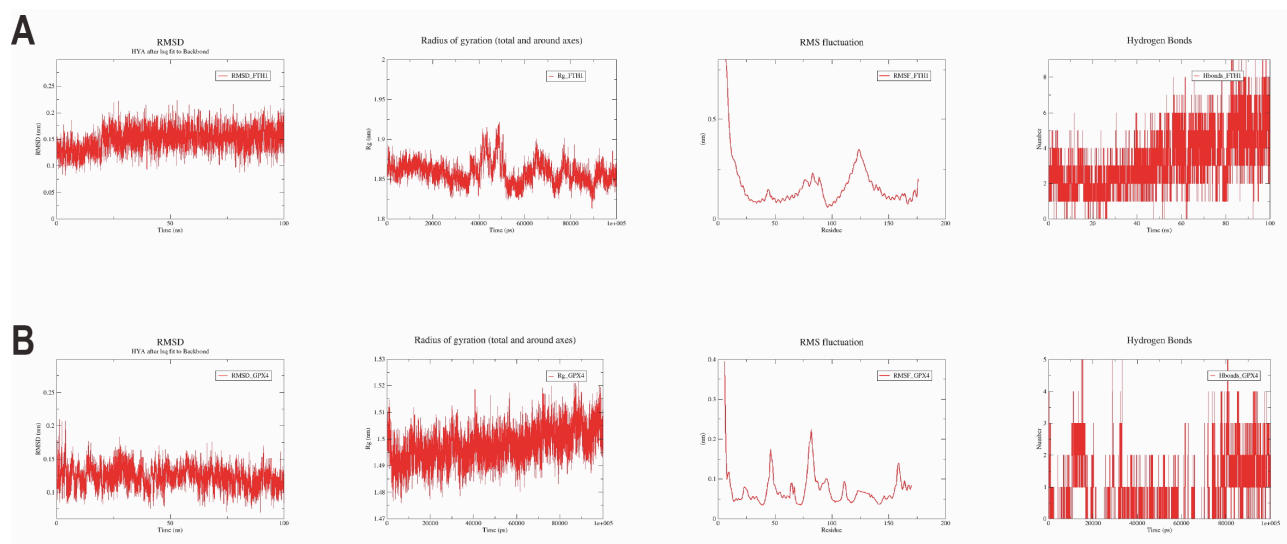
3.3 Molecular Dynamics

GROMACS was then used to perform 100 ns MD simulations for the HSYA–FTH1 and HSYA–GPX4 complexes to characterize their dynamic behavior and structural stability (Fig. 9). For the FTH1–ligand complex, the RMSD profile demonstrated only minor conformational re-

arrangements during the first 0–25 ns, after which the system reached a dynamic equilibrium. The mean RMSD stabilized at approximately 0.15 nm. The radius of gyration (Rg) values remained within 1.82–1.87 nm, with occasional fluctuations between 35 and 55 ns. Residue-level RMSF analysis revealed heterogeneous flexibility across the FTH1 sequence. The functional loop region's residues 100–150 showed the largest mobility (peaking at ~ 0.35 nm). Hydrogen bond analysis further showed that the number of hydrogen bonds remained stable, averaging three to six throughout the simulation.

Table 2. Molecular docking of HSYA and ischemic stroke-related targets.

Targets	PDB ID	Molecular binding energy (kcal·mol ⁻¹)
PTGS2	5F19	−12.8
NQO1	8RFN	−11.8
ALOX15	5ME0	−11.6
AKR1C1	3C3V	−11.2
SLC1A5	5LM4	−11.2
FDFT1	3V66	−10.8
GPX4	2OBI	−9.3
FTH1	2CIH	−9.1
TP53	2B1P	−8.6
MT1G	1JJD	−7.8

**Fig. 8. Molecular docking results of HSYA with GPX4 and FTH1.** (A) GPX4 (PDB ID: 2OBI). (B) FTH1 (PDB ID: 2CIH). FTH1, ferritin heavy chain 1; GPX4, glutathione peroxidase 4; HSYA, Hydroxysafflor yellow A.**Fig. 9. Molecular dynamics simulations of the HSYA–FTH1 and HSYA–GPX4 complexes over 100 ns.** From left to right: RMSD, radius of gyration, RMS fluctuation, and hydrogen bonds. (A) HSYA–FTH1 complex. (B) HSYA–GPX4 complex. RMSD, root mean square deviation; Rg, radius of gyration; RMSF, root mean square fluctuation; HSYA, hydroxysafflor yellow A; GPX4, glutathione peroxidase 4; FTH1, ferritin heavy chain 1.

The HSYA–GPX4 complex showed a relatively stable dynamic profile with an average RMSD of about 0.1 nm over the simulation. The Rg remained between 1.48

and 1.51 nm. RMSF analysis showed region-specific differences, especially inside the functional loops containing residues 35–55 and 75–100, which showed the maximum

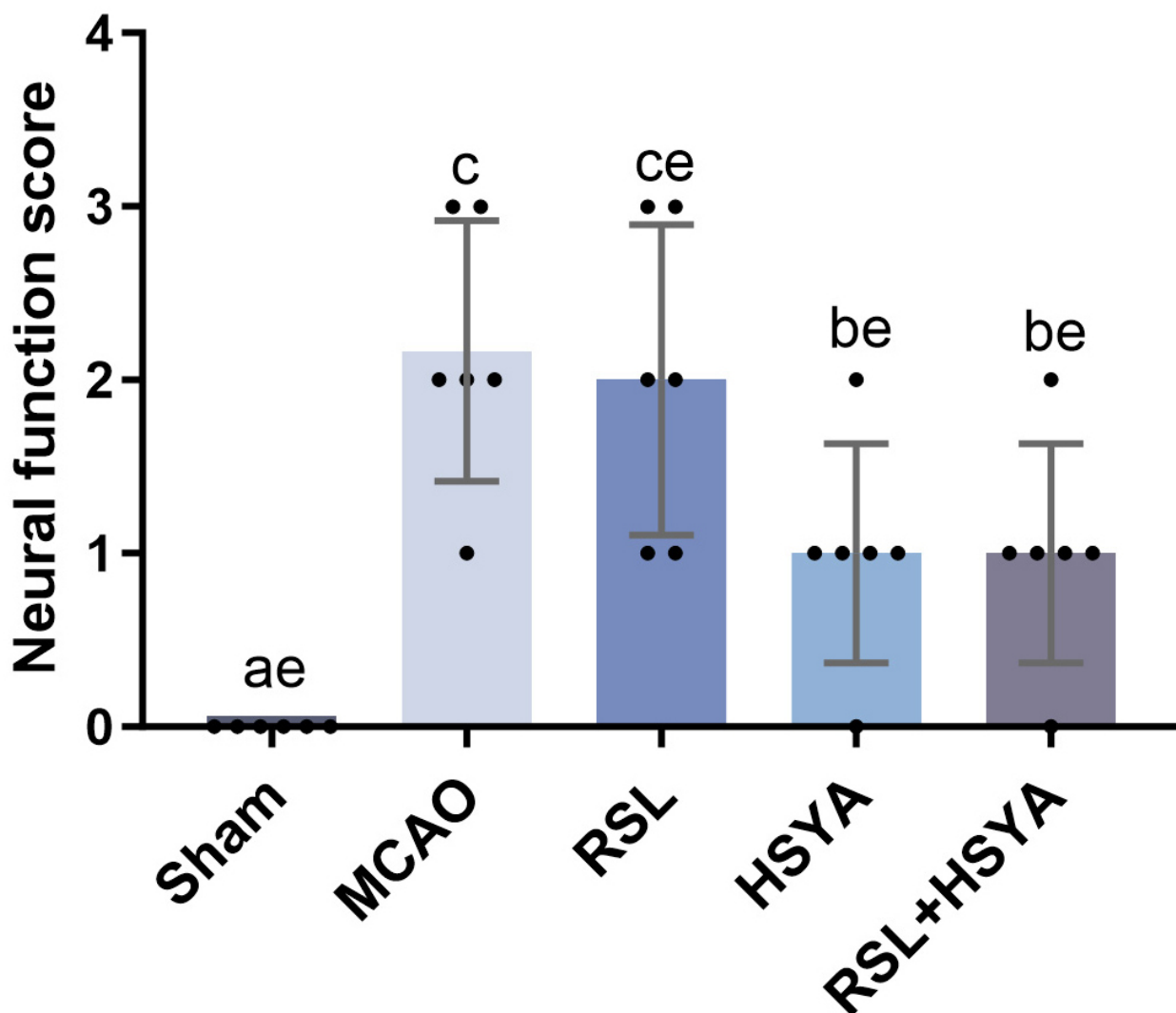


Fig. 10. Neurological deficit scores of mice (Longa scoring system) (n = 6). Note: Data are presented as mean ± SD. Group differences were analyzed using one-way ANOVA followed by the Student–Newman–Keuls test, or the Kruskal–Wallis test when appropriate. Adjacent letters indicate $p < 0.05$, non-adjacent letters indicate $p < 0.01$, and identical letters indicate no significant difference ($p > 0.05$). Higher scores indicate more severe neurological impairment. HSYA, hydroxysafflor yellow A; MCAO, middle cerebral artery occlusion; RSL, RSL3-treated group.

flexibility (up to ~0.2 nm). Hydrogen bond analysis showed that an average of two to three hydrogen bonds was maintained throughout the simulation.

3.4 Animal Model Verification

3.4.1 HSYA Improves Neurological Function

Compared with sham-operated mice, MCAO mice exhibited significantly higher neurological deficit scores ($p < 0.01$; Fig. 10). While there was no significant difference between the MCAO and RSL groups ($p > 0.05$), the administration of HSYA considerably decreased these scores as compared to the MCAO group ($p < 0.05$). No significant difference in neurological deficit scores was observed between the RSL group and the HSYA + RSL group ($p > 0.05$).

3.4.2 HSYA Reduces the Infarcted Cerebral Area

The MCAO group showed a significant increase in infarct volume compared to the sham group, as shown by TTC staining ($p < 0.01$; Fig. 11). While HSYA significantly reduced the infarct area in comparison to the MCAO group ($p < 0.01$). Combined administration of HSYA and RSL3 resulted in a further decrease in infarct size compared with RSL group ($p < 0.01$).

3.4.3 HSYA Improves Serum Biochemical Parameters in MCAO Mice

Serum ROS and MDA concentrations were significantly higher in the MCAO group, whereas SOD and GSH-Px levels were significantly lower than those in the sham group ($p < 0.01$), as determined by ELISA (Fig. 12). com-

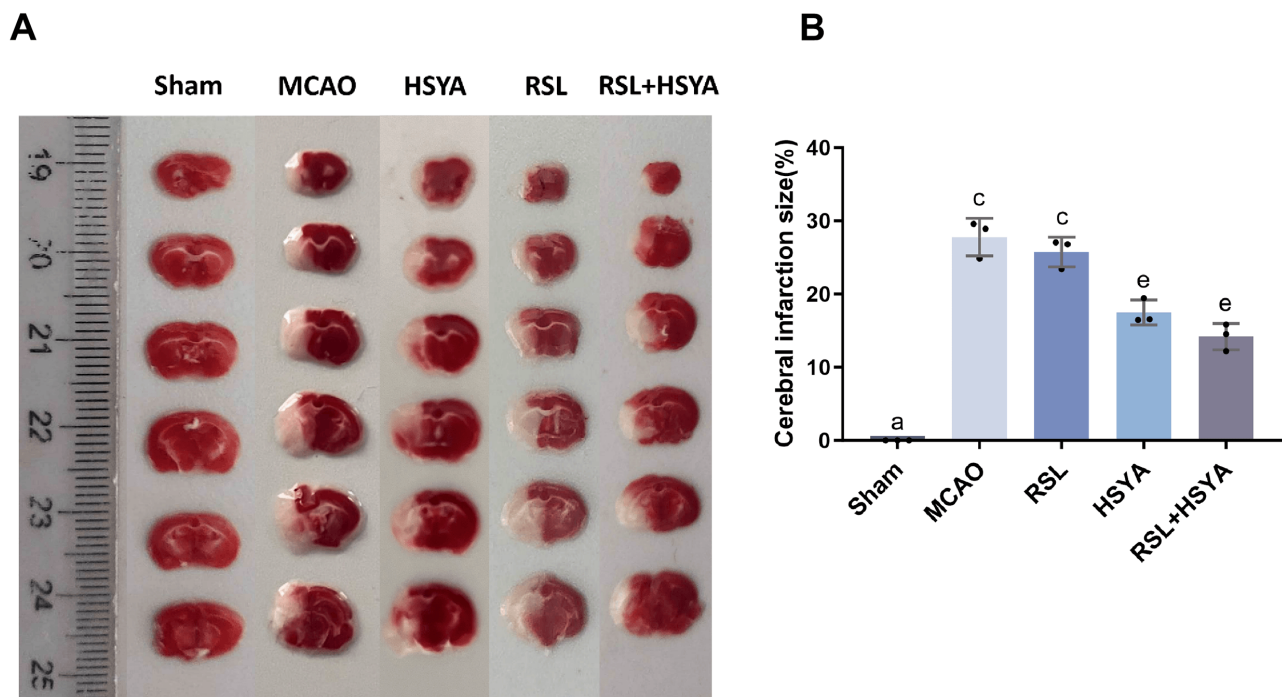


Fig. 11. TTC staining results (n = 3). (A) representative TTC staining images. (B) Quantification of cerebral infarct volume. Note: Data are presented as mean $\pm$ SD. Group differences were analyzed using one-way ANOVA followed by the Student–Newman–Keuls test, or the Kruskal–Wallis test when appropriate. Adjacent letters indicate $p < 0.05$, non-adjacent letters indicate $p < 0.01$, and identical letters indicate no significant difference ($p > 0.05$). HSYA, hydroxysafflor yellow A; MCAO, middle cerebral artery occlusion; RSL, RSL3-treated group; TTC, 2,3,5-triphenyltetrazolium chloride.

pared with the MCAO group, the RSL group exhibited significantly lower MDA levels and significantly higher GSH-Px levels ($p < 0.01$ or $p < 0.05$). HSYA treatment significantly reduced MDA and ROS levels while increasing SOD and GSH-Px levels in MCAO mice ($p < 0.01$ or $p < 0.05$). Notably, compared with the RSL group, the RSL+HSYA group showed a further reduction in MDA levels to values comparable to those of the sham group, accompanied by a significant increase in SOD levels ($p < 0.01$ or $p < 0.05$), whereas no further significant changes were observed in ROS or GSH-Px levels.

3.4.4 HSYA Improves Pathological Brain Damage in MCAO Mice

When comparing the cerebral parenchyma of MCAO and RSL mice to that of sham animals, histological investigation using H&E staining (Fig. 13) showed significant vascular congestion, bleeding, and perivascular edema ($p < 0.01$). HSYA therapy significantly decreased the histopathological scores of the cerebral cortex and hippocampus ($p < 0.01$), improved tissue morphology and alleviated neuronal structural damage. Marked differences in histopathological scores were seen between the HSYA and MCAO groups, as well as between the HSYA+RSL and RSL groups ($p < 0.01$).

3.4.5 HSYA Modulates GPX4-Related Gene and Protein Expression

WB and RT-qPCR analyses (Figs. 14,15, **Supplementary Figs. 1–4**) demonstrated marked reductions in GPX4, FTH1, and FTMT expression at both the mRNA and protein levels in the MCAO group relative to the sham group ($p < 0.01$ or $p < 0.05$). HSYA administration markedly restored the expression of these molecules ($p < 0.01$ or $p < 0.05$), whereas RSL group produced no detectable changes compared with the MCAO group ($p > 0.05$). In mice receiving combined HSYA and RSL3 treatment, protein levels of GPX4, FTH1, and FTMT were significantly lower than those in the HSYA group ($p < 0.01$ or $p < 0.05$), while the corresponding mRNA levels remained unchanged, suggesting that RSL3 partially attenuated the regulatory effects of HSYA on ferroptosis-related proteins.

4. Discussion

IS remains a major global health challenge because of its high mortality and long-term disability despite advances in current therapeutic strategies. The progression of ischemic brain injury is orchestrated by multiple interconnected pathological processes, including oxidative stress, inflammation, apoptosis, and ferroptosis [26]. Ferroptosis is characterized by iron-dependent lipid peroxidation and has emerged as an important pathological mechanism un-

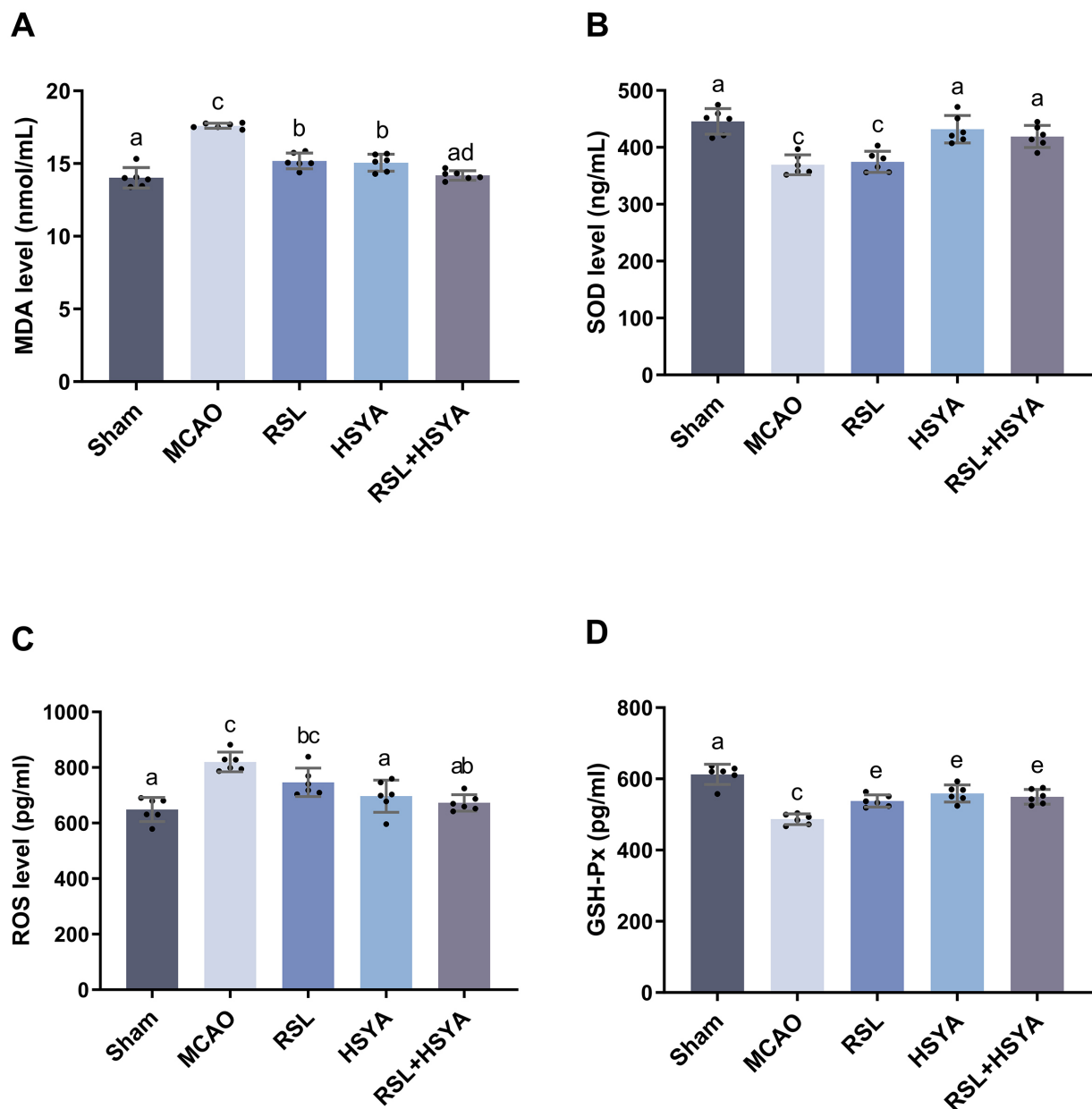


Fig. 12. Serum levels of oxidative stress-related indicators in mice from different groups ($n = 6$). (A) MDA. (B) SOD. (C) ROS. (D) GSH-Px. Note: Data are presented as mean $\pm$ SD. Group differences were analyzed using one-way ANOVA followed by the Student–Newman–Keuls test, or the Kruskal–Wallis test when appropriate. Adjacent letters indicate $p < 0.05$, non-adjacent letters indicate $p < 0.01$, and identical letters indicate no significant difference ($p > 0.05$). HSYA, hydroxysafflor yellow A; MCAO, middle cerebral artery occlusion; RSL, RSL3-treated group; ROS, reactive oxygen species; MDA, malondialdehyde; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase.

derlying cerebral ischemia–reperfusion injury [27,28,29]. Therefore, identifying novel neuroprotective agents capable of modulating ferroptosis-related pathways may provide promising therapeutic strategies for IS. To explore the mechanisms underlying the neuroprotective effects of HSYA against IS, network pharmacology, molecular docking, MD simulations, and an MCAO mouse model were integrated in the present study (Fig. 16).

In the present study, network pharmacology was first employed to investigate the potential mechanisms underlying the therapeutic effects of HSYA against IS. By integrating HSYA-, ferroptosis-, and IS-related targets, ten common targets were identified. Subsequent PPI network analysis revealed that GPX4 and FTH1 occupied central positions within the network, suggesting that they may serve as key targets through which HSYA regulates ferroptosis-related processes. Molecular docking analysis

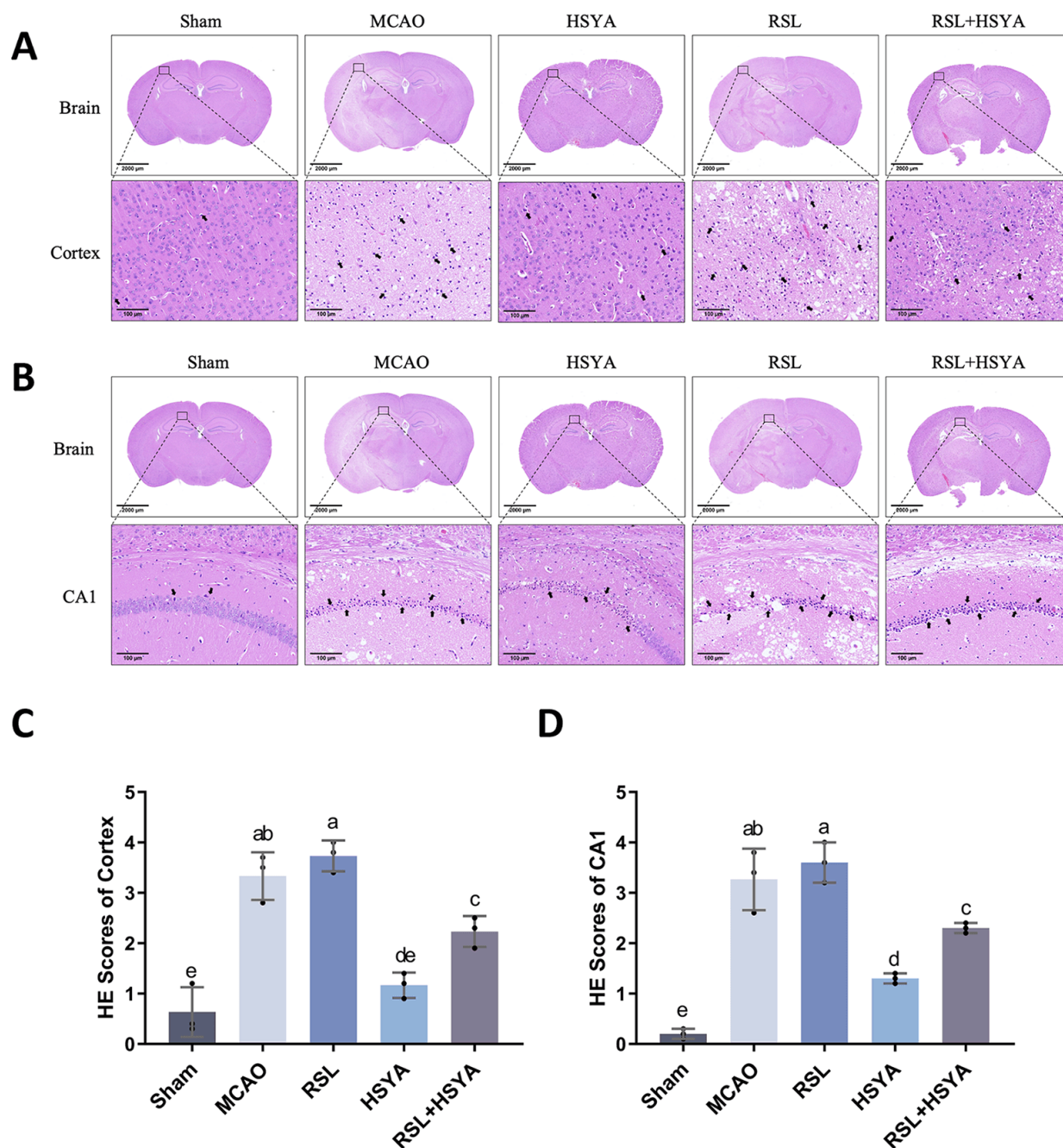


Fig. 13. Histopathological changes and injury scores in brain tissues of mice from different groups. (A) Representative H&E images of the cerebral cortex (10 $\times$, 2000 μ m; 200 $\times$: 100 μ m). (B) Representative H&E images of the hippocampus (10 $\times$, 2000 μ m; 200 $\times$: 100 μ m). (C) Semi-quantitative H&E cortical staining score (n = 3). (D) Semi-quantitative H&E hippocampus staining score (n = 3). Note: Data are presented as mean $\pm$ SD. Group differences were analyzed using one-way ANOVA followed by the Student–Newman–Keuls test, or the Kruskal–Wallis test when appropriate. Adjacent letters indicate $p < 0.05$, non-adjacent letters indicate $p < 0.01$, and identical letters indicate no significant difference ($p > 0.05$). CA1, cornu ammonis 1; HE, hematoxylin and eosin; HSYA, hydroxysafflor yellow A; MCAO, middle cerebral artery occlusion; RSL, RSL3-treated group.

further demonstrated favorable binding affinities of HSYA toward both GPX4 and FTH1. Both values were lower than $-5.0 \text{ kcal} \cdot \text{mol}^{-1}$, indicating that HSYA may form stable interactions with these proteins and providing a structural basis for its potential biological activities.

MD simulations were performed for the HSYA–GPX4 and HSYA–FTH1 complexes to assess the stability and dynamic behavior of the predicted protein–ligand interactions. The simulations showed durable hydrogen-bond interactions under equilibrium conditions and globally stable

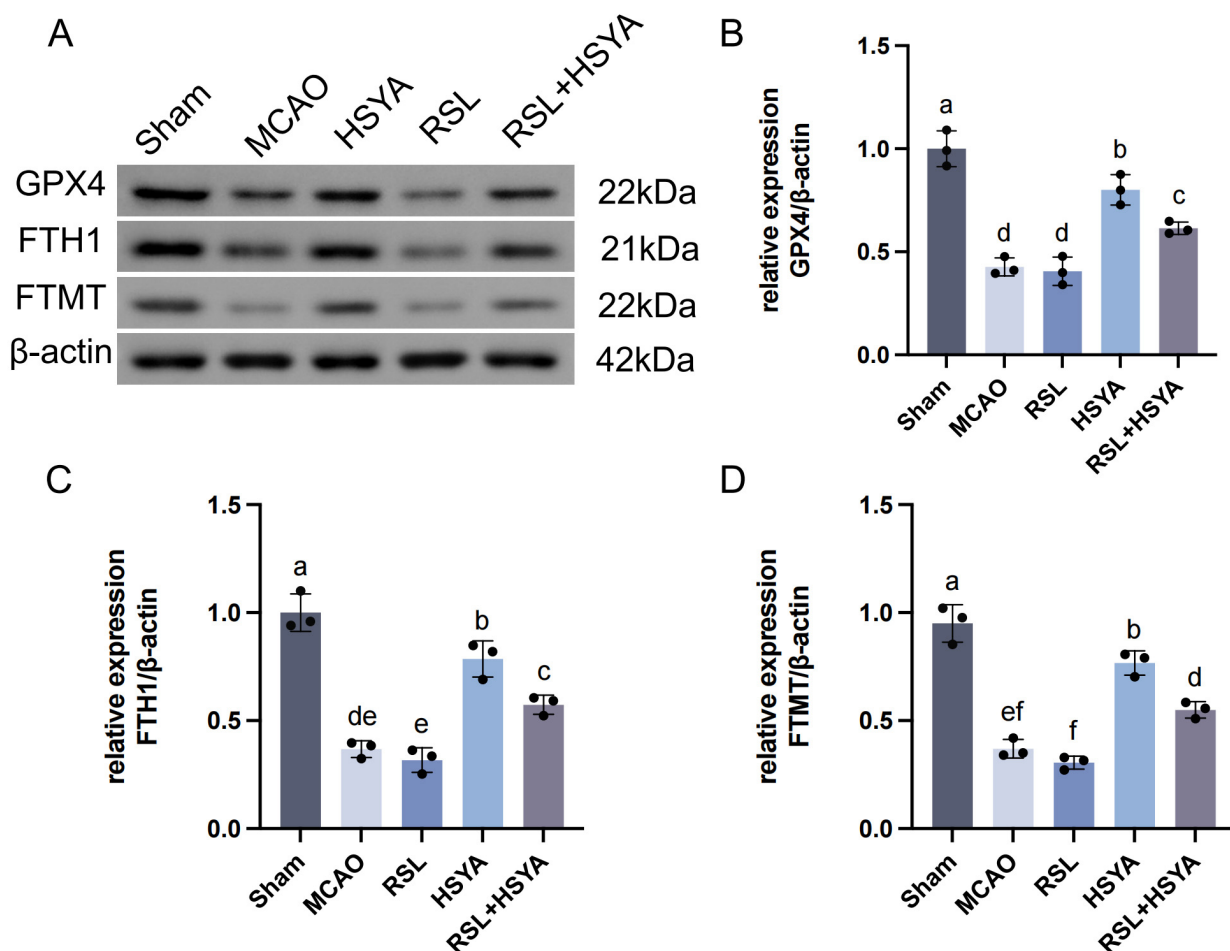


Fig. 14. Protein expression levels of GPX4, FTH1, and FTMT in brain tissues of mice determined by Western blotting (n = 3). (A) Representative images of GPX4, FTH1 and FTMT. (B–D) Quantitative analysis of relative GPX4, FTH1, and FTMT protein expression normalized to β-actin. Note: Data are presented as mean ± SD. Group differences were analyzed using one-way ANOVA followed by the Student–Newman–Keuls test, or the Kruskal–Wallis test when appropriate. Adjacent letters indicate $p < 0.05$, non-adjacent letters indicate $p < 0.01$, and identical letters indicate no significant difference ($p > 0.05$). FTH1, ferritin heavy chain 1; FTMT, mitochondrial ferritin; GPX4, glutathione peroxidase 4; HSYA, hydroxysafflor yellow A; MCAO, middle cerebral artery occlusion; RSL, RSL3-treated group.

protein-ligand complexes with limited flexibility in specific functional areas. Together with the molecular docking results, these findings support the reliability of the predicted interactions between HSYA and its ferroptosis-related protein targets.

More precisely, both complexes attained stable conformations over time, according to a 100-ns MD simulation of the HSYA-FTH1 and HSYA-GPX4 systems. RMSD analysis showed that FTH1 underwent modest conformational adjustments during the early phase (0–25 ns) before stabilizing, whereas GPX4 maintained consistently low fluctuations, indicating a relatively rigid structure. Rg analysis showed that GPX4 exhibited a slight, continuous expansion, suggesting subtle ligand-induced conformational changes, whereas FTH1 showed brief mid-simulation fluctuations, possibly reflecting local conformational rear-

rangements. RMSF analysis identified pronounced flexibility within functionally important loop regions of both proteins. Specifically, residues 100–150 of FTH1 and residues 35–55 and 75–100 of GPX4 exhibited marked fluctuations, consistent with conformational adaptability during ligand interaction. Hydrogen-bond analysis corroborated these findings: the HSYA–FTH1 complex maintained a relatively large number of hydrogen bonds, which may contribute to the stability of the complex, while GPX4 relied on fewer but strategically positioned hydrogen bonds to sustain its active conformation. Collectively, the molecular docking and MD simulation results provide computational support for GPX4 and FTH1 as plausible ferroptosis-related targets of HSYA. However, these computational findings do not establish direct physical binding between HSYA and GPX4.

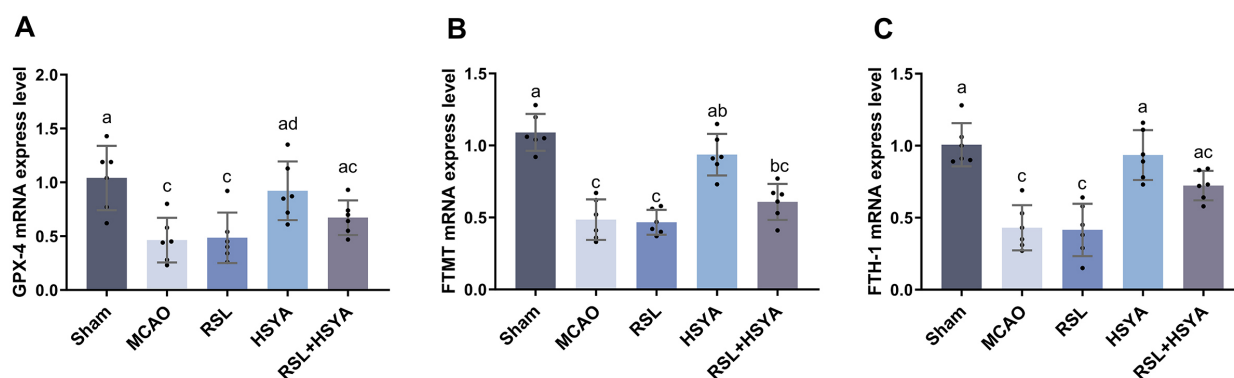


Fig. 15. Relative mRNA expression levels of GPX4, FTMT, and FTH1 in the brain tissues of mice from different groups determined by RT-qPCR (n = 6). (A) GPX4. (B) FTMT. (C) FTH1. Note: Data are presented as mean $\pm$ SD. Group differences were analyzed using one-way ANOVA followed by the Student–Newman–Keuls test, or the Kruskal–Wallis test when appropriate. Adjacent letters indicate $p < 0.05$, non-adjacent letters indicate $p < 0.01$, and identical letters indicate no significant difference ($p > 0.05$). FTH1, ferritin heavy chain 1; FTMT, mitochondrial ferritin; GPX4, glutathione peroxidase 4; HSYA, hydroxysafflor yellow A; MCAO, middle cerebral artery occlusion; RSL, RSL3-treated group; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

To further validate the computational findings, *in vivo* experiments were conducted to evaluate the neuroprotective effects of HSYA. The results demonstrated that HSYA significantly improved neurological deficits, reduced cerebral infarct volume, and attenuated histopathological damage, indicating a significant neuroprotective effect against IS, which is consistent with previous studies [30,31]. Given that oxidative stress is a fundamental pathological process underlying both cerebral ischemia-reperfusion injury and ferroptosis, we further investigated the effects of HSYA on oxidative stress-related parameters.

Oxidative stress is recognized as a major driving force of ferroptosis. During cerebral ischemia-reperfusion, excessive generation of ROS triggers lipid peroxidation, thereby exacerbating neuronal injury [30,32]. MDA is a major product of lipid peroxidation, whereas SOD and GSH-Px are key endogenous antioxidant enzymes that maintain cellular redox homeostasis [33,34,35]. In the present study, MCAO mice exhibited significantly increased serum levels of MDA and ROS, accompanied by markedly decreased SOD and GSH-Px levels, indicating that cerebral ischemia-reperfusion induced oxidative stress. HSYA treatment significantly reduced MDA and ROS levels while restoring SOD and GSH-Px activities, suggesting that HSYA alleviates lipid peroxidation and enhances endogenous antioxidant defenses. These findings are consistent with previous studies and further support the antioxidant and neuroprotective properties of HSYA [30].

GPX4 is an important GSH-dependent antioxidant enzyme and a member of the 170 amino acid GPX enzyme family that includes selenium. GPX4 has a remarkable ability to neutralize lipid peroxides and preserve cellular redox equilibrium because of its unique amino acid composition and spatial structure. GPX4 lowers membrane damage by reducing the accumulation of lipid peroxidation prod-

ucts, inhibiting ferroptosis, and preventing cell death by catalyzing the reduction of phospholipid hydroperoxides to their corresponding hydroxylated derivatives. This reduction process is entirely dependent on an adequate supply of GSH, which in turn requires continuous replenishment of intracellular cysteine. When GSH synthesis or recycling is impaired, GPX4 activity is diminished, lipid peroxides accumulate unchecked, and cells undergo ferroptotic death [36]. In the present study, MCAO markedly reduced GPX4 expression at both the mRNA and protein levels, whereas HSYA treatment significantly restored GPX4 expression, suggesting that HSYA may enhance the resistance of brain tissue to oxidative stress and lipid peroxidation by restoring GPX4 expression. Furthermore, under RSL3 treatment, HSYA still increased GPX4 protein expression, although no significant change was observed in the corresponding mRNA level. This observation suggests that the regulation of GPX4 by HSYA may not rely solely on transcriptional regulation but may also involve post-transcriptional mechanisms. However, the precise molecular mechanisms underlying these effects remain to be elucidated.

Taken together with the findings from network pharmacology, molecular docking, and MD simulations, our results further suggest that GPX4 may represent a potential molecular target underlying the neuroprotective effects of HSYA. GPX4 is a key regulator of ferroptosis that prevents lipid peroxidation and maintains cellular redox homeostasis [36,37,38]. Decreased GPX4 expression or activity promotes the accumulation of lipid peroxides and consequently triggers ferroptosis, whereas restoration of GPX4 function enhances cellular antioxidant capacity and alleviates neuronal injury [39,40]. Previous studies have shown that kaempferol attenuates OGD/R-induced neuronal injury by activating GPX4-related signaling pathways [40], whereas selenium suppresses ferroptosis by promoting GPX4 ex-

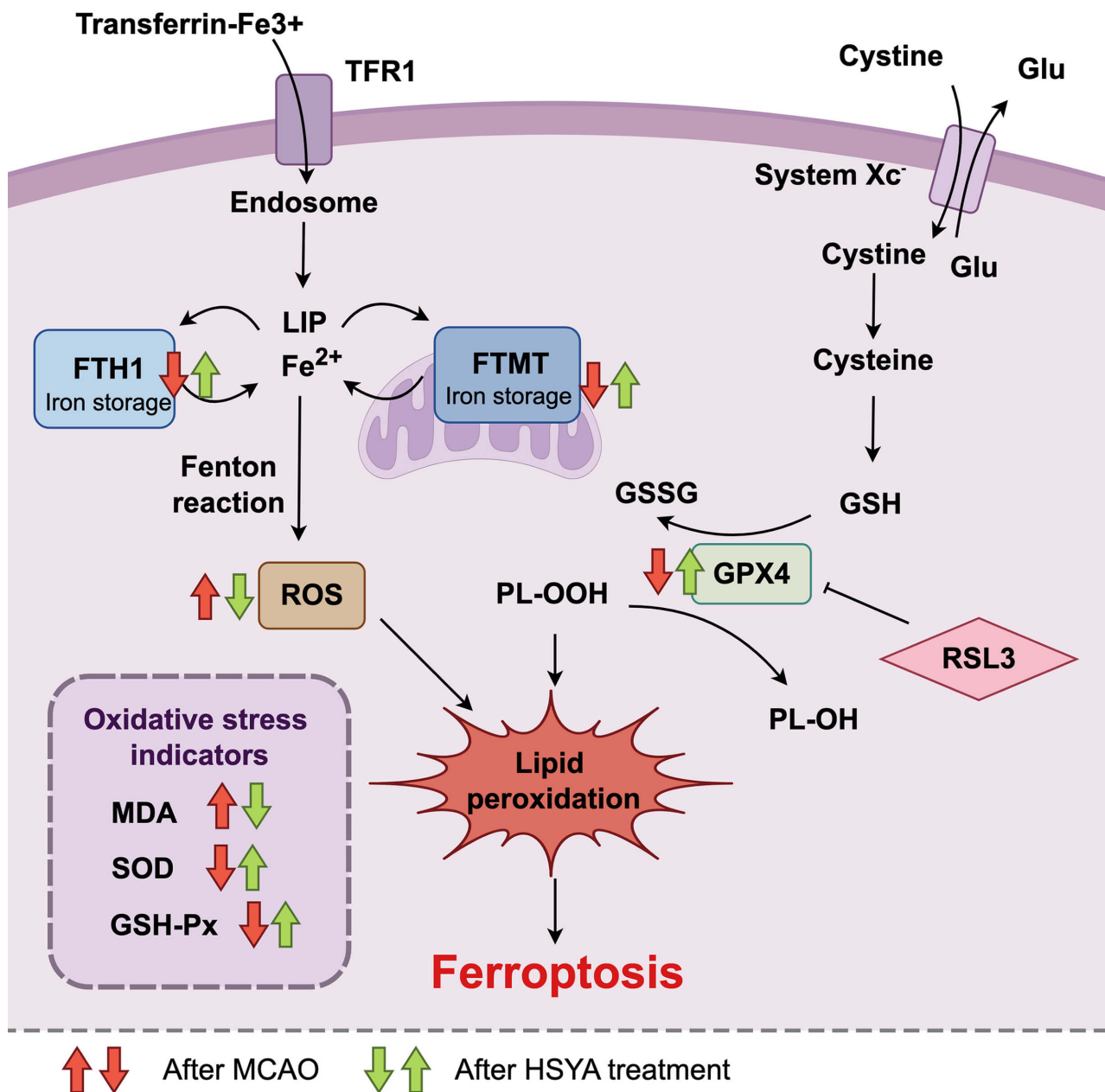


Fig. 16. Proposed mechanism underlying the neuroprotective effects of HSYA against ischemic stroke. HSYA may attenuate ferroptosis-related changes and oxidative stress following cerebral ischemia/reperfusion injury. Under ischemic conditions, impaired iron homeostasis increases the LIP, promotes ROS generation through the Fenton reaction, and triggers lipid peroxidation. HSYA upregulates GPX4, FTH1, and FTMT, thereby enhancing antioxidant defense, maintaining iron homeostasis, reducing lipid peroxidation, and ultimately suppressing ferroptosis. Red arrows indicate changes after MCAO, whereas green arrows indicate changes after HSYA treatment. RSL3 inhibits GPX4 activity. FTH1, ferritin heavy chain 1; FTMT, mitochondrial ferritin; GPX4, glutathione peroxidase 4; GSH, glutathione; GSH-Px, glutathione peroxidase; GSSG, oxidized glutathione; LIP, labile iron pool; MCAO, middle cerebral artery occlusion; MDA, malondialdehyde; PL-OH, phospholipid alcohol; PL-OOH, phospholipid hydroperoxide; ROS, reactive oxygen species; RSL3, Ras-selective lethal small molecule 3; SOD, superoxide dismutase; System Xc⁻, cystine/glutamate antiporter; TFR1, transferrin receptor 1. Figure created with Figdraw (www.figdraw.com; ID: OWIYRd46f2).

pression [12]. These findings further support the critical role of GPX4 in ferroptosis regulation. In the present study, HSYA restored GPX4 expression, attenuated oxida-

tive stress, and alleviated ischemic brain injury, whereas co-treatment with the GPX4 inhibitor RSL3 partially reversed these protective effects. Therefore, our findings sug-

gest that GPX4-associated ferroptosis is at least partially involved in the neuroprotective effects of HSYA.

Previous studies have demonstrated that HSYA exerts neuroprotective effects through multiple signaling pathways, including Nrf2, sirtuin 1 (SIRT1), nuclear factor kappa B (NF- κ B), which are critically involved in the regulation of oxidative stress, inflammation, and ferroptosis [16,30]. In particular, activation of the Nrf2 signaling pathway not only contributes to the maintenance of intracellular GSH homeostasis but also regulates GPX4 expression, thereby inhibiting lipid peroxidation and attenuating ferroptosis [41,42,43,44]. In addition, the SIRT1 and PI3K signaling pathways can improve mitochondrial function, enhance cellular antioxidant capacity, and may promote GPX4 expression by regulating Nrf2 or other downstream molecules [45,46,47]; In contrast, excessive activation of the NF- κ B signaling pathways exacerbates neuroinflammation and oxidative stress [48], thereby promoting lipid peroxidation and ferroptosis, which may be associated with impaired GPX4-mediated antioxidant defense [49,50,51]. Therefore, the restoration of GPX4 expression observed in this study may not represent an isolated event, but rather a downstream consequence of the coordinated regulation of multiple upstream signaling pathways by HSYA. Although our findings suggest that the regulation of ferroptosis pathway through GPX4 is an important mechanism underlying the neuroprotective effects of HSYA, whether HSYA indirectly regulates GPX4 expression and activity through classical signaling pathways such as Nrf2, SIRT1, and NF- κ B remains to be further investigated.

In addition to lipid peroxidation, disruption of iron homeostasis is another critical mechanism driving ferroptosis. FTH1 and FTMT, two important members of the ferritin family, are responsible for iron storage in the cytoplasm and mitochondria, respectively [52]. By limiting the accumulation of free Fe²⁺ and suppressing the Fenton reaction, they restrict lipid peroxidation and contribute to the regulation of ferroptosis [52,53]. It has been reported that reduced expression of FTH1 and FTMT impairs cellular iron-buffering capacity, resulting in iron overload, enhanced lipid peroxidation, and increased ferroptosis, whereas their upregulation helps maintain iron homeostasis and alleviates ferroptosis-related injury [54]. Therefore, we further evaluated the effects of HSYA on the expression of FTH1 and FTMT to investigate its potential role in regulating iron homeostasis-related molecules. In the present study, MCAO markedly reduced both the mRNA and protein expression levels of FTH1 and FTMT, suggesting that cerebral ischemia-reperfusion injury may be accompanied by impaired iron-storage capacity and disruption of iron homeostasis. HSYA treatment significantly restored the expression of FTH1 and FTMT at both the mRNA and protein levels, indicating that HSYA may contribute to the maintenance of cellular iron homeostasis, which is consistent with its protective effects against ox-

idative stress and cerebral injury. Unlike the classical iron chelator deferoxamine [15], which inhibits ferroptosis primarily by chelating intracellular labile iron and reducing cellular iron burden [55,56], HSYA may limit free iron accumulation by restoring FTH1 and FTMT expression and enhancing iron-storage capacity in the cytoplasm and mitochondria. Notably, RSL3 alone had no significant change on FTH1 or FTMT expression, whereas co-treatment with RSL3 significantly reduced their protein expression levels compared with the HSYA group, without affecting the corresponding mRNA levels. These findings suggest that the regulatory effects of HSYA on FTH1 and FTMT may not rely solely on transcriptional regulation but may also involve post-transcriptional regulatory mechanisms. However, the precise molecular mechanisms underlying these effects remain to be elucidated.

In summary, by integrating network pharmacology, molecular docking, MD simulations, and *in vivo* experiments, the present study elucidated the neuroprotective effects of HSYA against IS and explored its underlying mechanisms. HSYA improved neurological function, alleviated cerebral injury and oxidative stress, and regulated the expression of ferroptosis-related molecules, including GPX4, FTH1, and FTMT. Combined with the computational analyses and experimental validation, our findings suggest that the neuroprotective effects of HSYA may be partially attributed to the modulation of GPX4-associated ferroptosis-related pathways and the maintenance of iron homeostasis. These findings deepen our understanding of the mechanisms underlying the neuroprotective effects of HSYA and support ferroptosis as a promising therapeutic target for IS.

Despite these encouraging findings, several limitations of the present study should be acknowledged. First, additional ferroptosis-specific markers, such as lipid ROS, ACSL4, SLC7A11, 4-HNE, and iron accumulation, were not evaluated. Second, although molecular docking and MD simulations suggested potential interactions between HSYA and the predicted target proteins, direct binding validations (e.g., surface plasmon resonance, Microscale thermophoresis and Isothermal titration calorimetry) were not performed. In addition, the molecular docking analysis lacked RMSD calculation and validation using known ligands, while the MD simulations did not include MM/PBSA or MM/GBSA binding free-energy calculations, solvent-accessible surface area, or principal component analysis. Therefore, the thermodynamic properties and detailed interaction mechanisms of the predicted protein–ligand complexes require further investigation. Third, neurological outcomes were evaluated primarily using the Longa neurological deficit score, whereas additional behavioral assessments and long-term functional recovery were not investigated. Fourth, pharmacokinetic evaluation, dose–response analysis, and assessment of the blood–brain barrier penetration of HSYA were not performed, limiting the comprehensive characterization of its pharmacological proper-

ties. Finally, the spatial localization of GPX4 and FTH1 in ischemic brain tissue was not evaluated by immunohistochemistry, and their region- and cell-specific expression patterns require further investigation.

5. Conclusions

Taken together, our findings suggest that HSYA effectively attenuates cerebral ischemia-reperfusion injury, as reflected by improved neurological function, reduced cerebral infarct volume, alleviated oxidative stress, and mitigated histopathological damage. By integrating network pharmacology, molecular docking, MD simulations, and *in vivo* experimental validation, our findings suggest that GPX4 and FTH1 may represent potential ferroptosis-related targets of HSYA. Furthermore, HSYA regulated the expression of GPX4, FTH1, and FTMT, suggesting that its neuroprotective effects may be partially associated with the modulation of GPX4-associated ferroptosis-related pathways and the maintenance of iron homeostasis. These findings provide new experimental evidence for elucidating the therapeutic mechanisms of HSYA in IS and support its further investigation as a potential therapeutic candidate for IS. However, the precise molecular mechanisms by which HSYA regulates GPX4-associated signaling pathways and iron homeostasis remain to be further elucidated.

Abbreviations

HSYA, Hydroxysafflor yellow A; CA1, cornu ammonis 1; CI/RI, cerebral ischemia/reperfusion injury; FTH1, ferritin heavy chain 1; FTMT, mitochondrial ferritin; GPX4, glutathione peroxidase 4; GSH, glutathione; GSH-Px, glutathione peroxidase; H&E, hematoxylin and eosin; HSYA, hydroxysafflor yellow A; IS, ischemic stroke; LIP, labile iron pool; MCAO, middle cerebral artery occlusion; MDA, malondialdehyde; PL-OH, phospholipid alcohol; PL-OOH, phospholipid hydroperoxide; ROS, reactive oxygen species; RSL, Ras-selective lethal small molecule; SOD, superoxide dismutase; System Xc⁻, cystine/glutamate antiporter; TFR1, transferrin receptor 1.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

YC: Data curation, Methodology, Visualization, Writing—original draft. TC: Data curation, Investigation, Writing—original draft, Validation. LY: Conceptualization, Funding acquisition, Investigation, Writing—review & editing. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All animal experiments were approved by the Animal Ethical and Welfare Committee of Zhejiang Chinese Medical University (Approval No. IACUC-20220516-15). All animal experiments were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were reported in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

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Not applicable.

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/Pharmazie55314>.

References

- [1] Owolabi MO, Thrift AG, Mahal A, Ishida M, Martins S, Johnson WD, et al. Primary stroke prevention worldwide: translating evidence into action. *The Lancet. Public Health*. 2022; 7: e74–e85. [https://doi.org/10.1016/S2468-2667\(21\)00230-9](https://doi.org/10.1016/S2468-2667(21)00230-9)
- [2] Chen T, Shi R, Suo Q, Wu S, Liu C, Huang S, et al. Progranulin released from microglial lysosomes reduces neuronal ferroptosis after cerebral ischemia in mice. *Journal of Cerebral Blood Flow and Metabolism : Official Journal of the International Society of Cerebral Blood Flow and Metabolism*. 2023; 43: 505–517. <https://doi.org/10.1177/0271678X221145090>
- [3] Friedmann Angeli JP, Krysko DV, Conrad M. Ferroptosis at the crossroads of cancer-acquired drug resistance and immune evasion. *Nature Reviews. Cancer*. 2019; 19: 405–414. <https://doi.org/10.1038/s41568-019-0149-1>
- [4] Yang WS, SriRamaratnam R, Welsch ME, Shimada K, Skouta R, Viswanathan VS, et al. Regulation of ferroptotic cancer cell death by GPX4. *Cell*. 2014; 156: 317–331. <https://doi.org/10.1016/j.cell.2013.12.010>
- [5] Zhu L, Feng Z, Zhang J, Du L, Meng A. MicroRNA-27a Regulates Ferroptosis Through SLC7A11 to Aggravate Cerebral ischemia-reperfusion Injury. *Neurochemical Research*. 2023; 48: 1370–1381. <https://doi.org/10.1007/s11064-022-03826-3>
- [6] Zhang LM, Liang XL, Xiong GF, Xing XL, Zhang QJ, Zhang BR, et al. Analysis and identification of oxidative stress-ferroptosis related biomarkers in ischemic stroke. *Scientific Reports*. 2024; 14: 3803. <https://doi.org/10.1038/s41598-024-54555-2>
- [7] Yeh SJ, Chen CH, Lin YH, Tsai LK, Lee CW, Sun Y, et al. Ferroptosis biomarkers predict the outcomes of patients with acute ischemic stroke undergoing endovascular thrombectomy. *Scientific Reports*. 2025; 15: 39510. <https://doi.org/10.1038/s41598-025-23289-0>
- [8] García-Yébenes I, Sobrado M, Moraga A, Zarruk JG, Romera VG, Pradillo JM, et al. Iron overload, measured as serum ferritin,

- increases brain damage induced by focal ischemia and early reperfusion. *Neurochemistry International*. 2012; 61: 1364–1369. <https://doi.org/10.1016/j.neuint.2012.09.014>
- [9] Chen J, Yang L, Geng L, He J, Chen L, Sun Q, et al. Inhibition of Acyl-CoA Synthetase Long-Chain Family Member 4 Facilitates Neurological Recovery After Stroke by Regulation Ferroptosis. *Frontiers in Cellular Neuroscience*. 2021; 15: 632354. <https://doi.org/10.3389/fncel.2021.632354>
- [10] Li XN, Shang NY, Kang YY, Sheng N, Lan JQ, Tang JS, et al. Caffeic acid alleviates cerebral ischemic injury in rats by resisting ferroptosis via Nrf2 signaling pathway. *Acta Pharmacologica Sinica*. 2024; 45: 248–267. <https://doi.org/10.1038/s41401-023-01177-5>
- [11] Selim M. Treatment with the iron chelator, deferoxamine mesylate, alters serum markers of oxidative stress in stroke patients. *Translational Stroke Research*. 2010; 1: 35–39. <https://doi.org/10.1007/s12975-009-0001-0>
- [12] Alim I, Caulfield JT, Chen Y, Swarup V, Geschwind DH, Ivanova E, et al. Selenium Drives a Transcriptional Adaptive Program to Block Ferroptosis and Treat Stroke. *Cell*. 2019; 177: 1262–1279.e25. <https://doi.org/10.1016/j.cell.2019.03.032>
- [13] Bai X, Wang WX, Fu RJ, Yue SJ, Gao H, Chen YY, et al. Therapeutic Potential of Hydroxysafflor Yellow A on Cardio-Cerebrovascular Diseases. *Frontiers in Pharmacology*. 2020; 11: 01265. <https://doi.org/10.3389/fphar.2020.01265>
- [14] Fan L, Zhao HY, Xu M, Zhou L, Guo H, Han J, et al. Qualitative evaluation and quantitative determination of 10 major active components in *Carthamus tinctorius* L. by high-performance liquid chromatography coupled with diode array detector. *Journal of Chromatography. a*. 2009; 1216: 2063–2070. <https://doi.org/10.1016/j.chroma.2008.03.046>
- [15] Wu Y, Yin L, Wang Z, Yuan S, Ma D, Wen C, et al. Hydroxysafflor yellow A inhibits neuronal ferroptosis and ferritinophagy in ischemic stroke. *Biochimica et Biophysica Acta. Molecular Basis of Disease*. 2025; 1871: 167867. <https://doi.org/10.1016/j.bbadis.2025.167867>
- [16] Zhao F, Wang P, Jiao Y, Zhang X, Chen D, Xu H. Hydroxysafflor Yellow A: A Systematical Review on Botanical Resources, Physicochemical Properties, Drug Delivery System, Pharmacokinetics, and Pharmacological Effects. *Frontiers in Pharmacology*. 2020; 11: 579332. <https://doi.org/10.3389/fphar.2020.579332>
- [17] Sheng C, Peng W, Xia Z, Wang Y. Plasma and cerebrospinal fluid pharmacokinetics of hydroxysafflor yellow A in patients with traumatic brain injury after intravenous administration of Xuebijing using LC-MS/MS method. *Xenobiotica; the Fate of Foreign Compounds in Biological Systems*. 2020; 50: 545–551. <https://doi.org/10.1080/00498254.2019.1668983>
- [18] Chauhan N, Jain S, Gupta P, Dwivedi J, Paliwal S, Sharma S, et al. Identification of a novel GSK-3 β inhibitor for Alzheimer's disease using In-Silico prediction and experiment cycling, validated in a streptozotocin-induced Alzheimer's disease mouse model. *Naunyn-Schmiedeberg's Archives of Pharmacology*. 2026; 399: 11557–11579. <https://doi.org/10.1007/s00210-026-05073-7>
- [19] Jain S, Bhole R, Labhade S. Unveiling the anti-Alzheimer potential of flavonoids: A comprehensive in-silico study. *Vietnam Journal of Chemistry*. 2026; 64: 299–312. <https://doi.org/10.1002/vjch.70051>
- [20] Zhang XD, Liu B, Zou YF, Hu WX, Lü YB, Li CY, et al. An efficient strategy for the rapid exploration of lipase inhibitors derived from the *Lindera* aggregate leaves by utilizing hybrid lipase-catalytic zeolitic imidazolate framework reactor combined with HPLC-Q-TOF-MS/MS and molecular docking techniques. *Food & Medicine Homology*. 2025. <https://doi.org/10.26599/FMH.2026.9420097>
- [21] Jin R, Zhao Z, Zhang Q, Sun YU, Wang W, Peng D, et al. An anti-inflammatory active ingredients in *Poriae cutis*: Screening based on network pharmacology. *Die Pharmazie*. 2024; 79: 72–81. <https://doi.org/10.1691/ph.2024.3647>
- [22] Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke*. 1989; 20: 84–91. <https://doi.org/10.1161/01.str.20.1.84>
- [23] Hou Y, Yan Z, Wan H, Yang J, Ding Z, He Y. A Combination of Astragaloside IV and Hydroxysafflor Yellow A Attenuates Cerebral Ischemia-Reperfusion Injury via NF- κ B/NLRP3/Caspase-1/GSDMD Pathway. *Brain Sciences*. 2024; 14: 781. <https://doi.org/10.3390/brainsci14080781>
- [24] Chen H, Qi Q, Wu N, Wang Y, Feng Q, Jin R, et al. Aspirin promotes RSL3-induced ferroptosis by suppressing mTOR/SREBP-1/SCD1-mediated lipogenesis in PIK3CA-mutant colorectal cancer. *Redox Biology*. 2022; 55: 102426. <https://doi.org/10.1016/j.redox.2022.102426>
- [25] Zhang J, Han M, Wang S, Wu R, Zhao Q, Chen M, et al. Study on the anti-mitochondrial apoptosis mechanism of *Erigeron breviscapus* injection based on UPLC-Q-TOF-MS metabolomics and molecular docking in rats with cerebral ischemia-reperfusion injury. *Journal of Ethnopharmacology*. 2024; 319: 117310. <https://doi.org/10.1016/j.jep.2023.117310>
- [26] Cheng Y, Song Y, Chen H, Li Q, Gao Y, Lu G, et al. Ferroptosis Mediated by Lipid Reactive Oxygen Species: A Possible Causal Link of Neuroinflammation to Neurological Disorders. *Oxidative Medicine and Cellular Longevity*. 2021; 2021: 5005136. <https://doi.org/10.1155/2021/5005136>
- [27] She X, Lan B, Tian H, Tang B. Cross Talk Between Ferroptosis and Cerebral Ischemia. *Frontiers in Neuroscience*. 2020; 14: 776. <https://doi.org/10.3389/fnins.2020.00776>
- [28] Zhang Y, Lu X, Tai B, Li W, Li T. Ferroptosis and Its Multifaceted Roles in Cerebral Stroke. *Frontiers in Cellular Neuroscience*. 2021; 15: 615372. <https://doi.org/10.3389/fncel.2021.615372>
- [29] Tuo QZ, Lei P. Ferroptosis in ischemic stroke: Animal models and mechanisms. *Zoological Research*. 2024; 45: 1235–1248. <https://doi.org/10.24272/j.issn.2095-8137.2024.239>
- [30] Yu L, Jin Z, Li M, Liu H, Tao J, Xu C, et al. Protective potential of hydroxysafflor yellow A in cerebral ischemia and reperfusion injury: An overview of evidence from experimental studies. *Frontiers in Pharmacology*. 2022; 13: 1063035. <https://doi.org/10.3389/fphar.2022.1063035>
- [31] Huang P, Wu SP, Wang N, Seto S, Chang D. Hydroxysafflor yellow A alleviates cerebral ischemia reperfusion injury by suppressing apoptosis via mitochondrial permeability transition pore. *Phytomedicine: International Journal of Phytotherapy and Phytopharmacology*. 2021; 85: 153532. <https://doi.org/10.1016/j.phymed.2021.153532>
- [32] Wang J, Lv C, Wei X, Li F. Molecular mechanisms and therapeutic strategies for ferroptosis and cuproptosis in ischemic stroke. *Brain, Behavior, & Immunity - Health*. 2024; 40: 100837. <https://doi.org/10.1016/j.bbih.2024.100837>
- [33] Ayala A, Muñoz MF, Argüelles S. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxidative Medicine and Cellular Longevity*. 2014; 2014: 360438. <https://doi.org/10.1155/2014/360438>
- [34] Cordiano R, Di Gioacchino M, Mangifesta R, Panzera C, Gangemi S, Minciullo PL. Malondialdehyde as a Potential Oxidative Stress Marker for Allergy-Oriented Diseases: An Update. *Molecules (Basel, Switzerland)*. 2023; 28: 5979. <https://doi.org/10.3390/molecules28165979>
- [35] Jomova K, Alomar SY, Alwasel SH, Nepovimova E, Kuca K, Valko M. Several lines of antioxidant defense against oxidative stress: antioxidant enzymes, nanomaterials with mul-

- multiple enzyme-mimicking activities, and low-molecular-weight antioxidants. *Archives of Toxicology*. 2024; 98: 1323–1367. <https://doi.org/10.1007/s00204-024-03696-4>
- [36] Xie Y, Kang R, Klionsky DJ, Tang D. GPX4 in cell death, autophagy, and disease. *Autophagy*. 2023; 19: 2621–2638. <https://doi.org/10.1080/15548627.2023.2218764>
- [37] Yang WS, Stockwell BR. Ferroptosis: Death by Lipid Peroxidation. *Trends in Cell Biology*. 2016; 26: 165–176. <https://doi.org/10.1016/j.tcb.2015.10.014>
- [38] Li J, Cao F, Yin HL, Huang ZJ, Lin ZT, Mao N, et al. Ferroptosis: past, present and future. *Cell Death & Disease*. 2020; 11: 88. <https://doi.org/10.1038/s41419-020-2298-2>
- [39] Kajarabille N, Latunde-Dada GO. Programmed Cell-Death by Ferroptosis: Antioxidants as Mitigators. *International Journal of Molecular Sciences*. 2019; 20: 4968. <https://doi.org/10.3390/ijms20194968>
- [40] Yuan Y, Zhai Y, Chen J, Xu X, Wang H. Kaempferol Ameliorates Oxygen-Glucose Deprivation/Reoxygenation-Induced Neuronal Ferroptosis by Activating Nrf2/SLC7A11/GPX4 Axis. *Biomolecules*. 2021; 11: 923. <https://doi.org/10.3390/biom11070923>
- [41] Abdalkader M, Lampinen R, Kanninen KM, Malm TM, Liddell JR. Targeting Nrf2 to Suppress Ferroptosis and Mitochondrial Dysfunction in Neurodegeneration. *Frontiers in Neuroscience*. 2018; 12: 466. <https://doi.org/10.3389/fnins.2018.00466>
- [42] Kerins MJ, Ooi A. The Roles of NRF2 in Modulating Cellular Iron Homeostasis. *Antioxidants & Redox Signaling*. 2018; 29: 1756–1773. <https://doi.org/10.1089/ars.2017.7176>
- [43] Jiang X, Yu M, Wang WK, Zhu LY, Wang X, Jin HC, et al. The regulation and function of Nrf2 signaling in ferroptosis-activated cancer therapy. *Acta Pharmacologica Sinica*. 2024; 45: 2229–2240. <https://doi.org/10.1038/s41401-024-01336-2>
- [44] Qu Z, Zeng J, Zeng L, Li X, Zhang F. Esculetin triggers ferroptosis via inhibition of the Nrf2-xCT/GPx4 axis in hepatocellular carcinoma. *Chinese Journal of Natural Medicines*. 2025; 23: 443–456. [https://doi.org/10.1016/S1875-5364\(25\)60853-3](https://doi.org/10.1016/S1875-5364(25)60853-3)
- [45] Liu X, Hou M, Lv X, Lan W, Li X, Han X, et al. Proanthocyanidins alleviate ovarian ischemia reperfusion injury by inhibiting ferroptosis through the SIRT1/Nrf2/GPX4 pathway. *European Journal of Pharmacology*. 2026; 1024: 178864. <https://doi.org/10.1016/j.ejphar.2026.178864>
- [46] Duan Y, Luo Y, Han X, Yu H, Liu H, Zhou Y, et al. SIRT1 deficiency promotes age-related heart failure through enhancing ferroptosis via GATA4-HADHA-GPX4 axis. *Cell Death & Disease*. 2026; 17: 343. <https://doi.org/10.1038/s41419-026-08634-z>
- [47] Gao N, Huang Z, Xie J, Gao S, Wang B, Feng H, et al. Cryptotanshinone alleviates cerebral ischemia reperfusion injury by regulating ferroptosis through the PI3K/AKT/Nrf2 and SLC7A11/GPX4 signaling pathway. *Journal of Ethnopharmacology*. 2025; 348: 119800. <https://doi.org/10.1016/j.jep.2025.119800>
- [48] Cheng B, Dong Y, Li X, Yu H, Feng Y, Wang Y, et al. Glycyrrhizic acid inhibited inflammatory response in LPS-stimulated microglial BV2 cells via MAPK, Akt and NF-κB signaling pathways. *Die Pharmazie*. 2025; 80: 55–59. <https://doi.org/10.1691/ph.2025.5532>
- [49] Mohan S, Alhazmi HA, Hassani R, Khuwaja G, Maheshkumar VP, Aldahish A, et al. Role of ferroptosis pathways in neuroinflammation and neurological disorders: From pathogenesis to treatment. *Heliyon*. 2024; 10: e24786. <https://doi.org/10.1016/j.heliyon.2024.e24786>
- [50] He Y, Wang J, Ying C, Xu KL, Luo J, Wang B, et al. The interplay between ferroptosis and inflammation: therapeutic implications for cerebral ischemia-reperfusion. *Frontiers in Immunology*. 2024; 15: 1482386. <https://doi.org/10.3389/fimmu.2024.1482386>
- [51] Chen B, Liu H, Xie Y, Yu J. TPX2 promotes ferroptosis in LPS-induced C28/I2 chondrocytes via NF-κB p65-mediated down-regulation of GPX4 and SLC7A11. *Molecular Biology Reports*. 2025; 53: 171. <https://doi.org/10.1007/s11033-025-11273-w>
- [52] Zhang X, Xiang Y, Wang Q, Bai X, Meng D, Wu J, et al. Regulation of iron metabolism in ferroptosis: From mechanism research to clinical translation. *Journal of Pharmaceutical Analysis*. 2025; 15: 101304. <https://doi.org/10.1016/j.jpha.2025.101304>
- [53] Chen X, Yu C, Kang R, Tang D. Iron Metabolism in Ferroptosis. *Frontiers in Cell and Developmental Biology*. 2020; 8: 590226. <https://doi.org/10.3389/fcell.2020.590226>
- [54] Wang P, Cui Y, Ren Q, Yan B, Zhao Y, Yu P, et al. Mitochondrial ferritin attenuates cerebral ischaemia/reperfusion injury by inhibiting ferroptosis. *Cell Death & Disease*. 2021; 12: 447. <https://doi.org/10.1038/s41419-021-03725-5>
- [55] Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE, et al. Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell*. 2012; 149: 1060–1072. <https://doi.org/10.1016/j.cell.2012.03.042>
- [56] Zhang L, Luo YL, Xiang Y, Bai XY, Qiang RR, Zhang X, et al. Ferroptosis inhibitors: past, present and future. *Frontiers in Pharmacology*. 2024; 15: 1407335. <https://doi.org/10.3389/fpha.2024.1407335>