exploring the ferroptosis mechanism of zhilong huoxue tongyu capsule for the treatment of intracerebral hemorrhage based on network pharmacology and in vivo validation

1. introduction

spontaneous intracerebral hemorrhage (ICH) refers to the hemorrhage within the brain parenchyma [1]. It is characterized by high mortality and disability rate with a globally rising prevalence [2]. Recently, the prevalence of ICH in young is increasing, and hypertension is the main cause of ICH in populations of Asia young adults [3], which has imparted a heavy social and economic burden. Although there is a great deal of attention focused on the development of effective treatment for ICH, there is still a lack of available class I level A treatment modalities for ICH patients [4].

Currently, the available treatment options for ICH employ supportive care management and/or surgical removal of hematoma [5], both of which are not ideal for significantly ameliorating ICH. Secondary brain injury (SBI) occurs due to neurotoxicity induced by deleterious byproducts of red blood cell lysis, especially free iron, which leads to inflammation, cytotoxicity, and oxidative damage [6, 7], has been critical effectors of neurological deficits after ICH [8]. Therefore, it is meaningful to figure out the mechanisms of SBI to develop an effective target-based treatment for ICH.

Traditional Chinese medicine is a characteristic module of medicine in China with a history of thousands of years. A
large number of Chinese herbal medicines have shown neuroprotective effects after ICH. ZL capsule is a patent traditional Chinese medicine consisting of five traditional Chinese medicines: *Huang qi*, *Di long*, *Da xue teng*, *Gui zhi*, and *Shui zhi*, the detailed information was shown in Table 1. Our previous results suggested that the ZL capsule could reduce the volume of intracranial hematoma in patients \((n=30)\) and improves stroke-related scale scores obviously [9]. Additionally, the ZL capsule was able to alleviate ICH injury outcomes such as inflammation, cerebral edema, neuronal apoptosis, and behavioral cognition in animal studies [10–12]. However, the specific mechanism of the ZL capsule for the treatment of ICH remains unknown.

Ferroptosis is an iron-dependent regulated form of cell death caused by the accumulation of lipid-based reactive oxygen species (ROS) [13]. It has been proven that ferroptosis could coexist with other types of programmed cell death (PCD), such as apoptosis and pyroptosis after ICH [14–16]. The inhibition of ferroptosis shows a therapeutic efficacy against ICH [17, 18]. And glutathione peroxidase 4 (GPX4) is a chief regulator to reducing lipid peroxidation in a glutathione (GSH) level-dependent manner and inhibits the subsequent ferroptosis [19]. Many studies have illustrated that the knock-down or inhibition of GPX4 contributes to lipid peroxidation and subsequent ferroptosis [19, 20]. To find whether the ZL capsule could alleviate ICH via ferroptosis, network pharmacology was performed. Network pharmacology has received great attention as an emerging research technology. The holistic concept, syndrome differentiation, and treatment of traditional Chinese medicine conform to the overall systematic characteristics of network pharmacology [21]. Network pharmacology could provide new insight and approaches for the treatment of disease and drug application.

In this study, we tried to find out the potential targets and mechanisms of the ZL capsule for the treatment of ICH via ferroptosis. First, we got the active ingredients of the ZL capsule, then screened the key targets to obtain GO and KEGG pathway enrichment analysis results. Finally, we screened the target to validate the results of network pharmacology. The flowchart of the experimental procedures of this study was shown in Figure 1.

2. Materials and Methods

2.1. Predicting Active Ingredients and Targets of ZL Capsule. To obtain the active ingredients and targets of the ZL capsule, the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP, https://tcmspw.com/tcmsp.php) [22] and the Uniport Protein Database (https://www.uniprot.org/) [23] were used. TCMSP is a database with the composition of herbal entries, which could help reveal the mechanisms of action of traditional Chinese medicine (TCM) and uncover the nature of TCM theory [22]. According to the characteristics of pharmacokinetics ADMET (absorption, distribution, metabolism, and excretion), the results retrieved from TCMSP were screened by two indexes: oral bioavailability (OB) ≥30% and drug-likeliness (DL) ≥0.18 to screen the effective active ingredients of ZL capsule [24, 25]. The active ingredients of *Shuizhi* and *Dilong* were screened from TCMID (https://47.100.169.139/tcmid/search/), and then screened in SwissADME to filter ingredients with low gastrointestinal (GI) absorption and SwissTargetPrediction [26] to predict the most probable protein targets [27]. In addition, known targets of compounds whose activity was not predicted were added from published articles. After screening, the information on protein targets was standardized in the Uniport Protein Database. Finally, human target proteins of the ZL capsule were obtained after integration.

2.2. Screening of Targets for ICH. To obtain the ICH-related genes comprehensively, we searched target genes from the Human Gene Database (GeneCards database, https://www.genecards.org/) with the keywords "Intracerebral Hemorrhage" and "Cerebral Hemorrhage," and set the species to "Homo sapiens."

2.3. Screening of Targets for Ferroptosis. The related targets of ferroptosis were searched from GeneCards and the Online Mendelian Inheritance in Man (OMIM) database (https://www.omim.org), with the keyword "Ferroptosis."

2.4. Protein-Protein Interaction Network Construction. Protein-Protein Interaction (PPI) plays a key role in the pathogenesis of disease, so the modulation of protein-protein complexes has relevant clinical significance [28]. In this part, the target genes of the ZL capsule for the treatment of ICH were imported into the STRING database (https://string-db.org/) [29] to get the PPI network mapping in the analysis mode of "multiple protein" with the species limited to "Homo sapiens" mode. After mapping, the "tsv" format file was imported into Cytoscape to build diagrams for data visualization and integration [30]. The network analyzer plug-in in Cytoscape was used for calculating and ranking the topological parameters to screen the target genes.

2.5. GO and KEGG Pathway Enrichment Analysis. To elucidate the pathway and mechanism of the ZL capsule for the treatment of ICH, we collected intersectant targets for GO enrichment and KEGG enrichment analysis in Metascape (https://metascape.org/gp/index.html) for further analysis [31]. Metascape combines gene annotation, interactome analysis, functional enrichment, and membership search to leverage over 40 independent knowledgebases within one integrated portal. GO enrichment is a system used to describe the function of gene in different aspects, such as molecular function (MF), cell composition (CC), and biological process (BP). KEGG enrichment could analyze the pathway involved in the genes. The difference was considered to be statistically significant at \(P < 0.01\).
2.6.1. Animals and Grouping.

2.6.2. ICH Model.

2.6.3. Behavioral Studies.

2.6.4. Drug and Dosing.

2.6.5. Quantitative Real Time PCR.

2.6.6. Western Blot Analysis.
Table 2: Zea-longa score.

<table>
<thead>
<tr>
<th>Zea-longa score</th>
<th>Behavioural performance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>The rats’ behavior without any symptoms of neurological deficit (normal).</td>
</tr>
<tr>
<td>1</td>
<td>The rats have dysfunction in stretching the left forelimb (mild neurological deficit).</td>
</tr>
<tr>
<td>2</td>
<td>The rats walk in circles and cannot go straight (moderate neurological deficit).</td>
</tr>
<tr>
<td>3</td>
<td>The rats lean to the opposite side when standing or crawling (severe neurological deficit).</td>
</tr>
<tr>
<td>4</td>
<td>The rats lost conscious and unable to walk.</td>
</tr>
</tbody>
</table>

Figure 1: Flowchart of the experimental procedures of network pharmacology for the treatment of ZL capsule in ICH via ferroptosis.
Beyotime, Shanghai, China) overnight at 4°C. The membranes were then washed with TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (dilution, 1: 5000; goat anti-rabbit; Beyotime, Shanghai, China) for 2 h at room temperature. The protein bands were visualized via enhanced chemiluminescence (ECL) kit (Beyotime, Shanghai, China), and the relative protein quantity was determined using ImageJ software (National Institutes of Health, USA).

2.7. Statistical Analysis. All data were presented as mean± standard deviation (SD) and analyzed with SPSS 24.0 software. One-way ANOVA was used to analyze data obtained from multiple groups. P value < 0.05 was considered statistically significant.

3. Results

3.1. Active Ingredients of ZL Capsule. The total ingredients of the ZL capsule were searched through TCMSP and TCMID databases. The detailed information is outlined in Table 3. Based on integrated results of the two databases, Da xue teng included 4 active ingredients, 17 in Huangqi, 6 in Guizhi, 1 in Shuizhi and 4 in Dilong. It was found that Sargentodoxa cuneate and Cinnamomum cassia Presl contain two similar ingredients (A&B in Table 3). After removing the duplicates, a total of 30 non-repeating ingredients were retrieved.

3.2. Target Retrieval and Analysis. Based on identified active ingredients, we further searched the related potential targets. A total of 1351 targets were retrieved, and all the targets’ names were standardized in the Uniport Protein Database [20]. After eliminating the duplicate targets, 539 targets remained (Figure 2(c)). Next, we searched ICH-related targets in Genecards database using the keywords, “Intracerebral Hemorrhage” and “Cerebral Hemorrhage,” and we obtained 5379 targets for ICH. After eliminating the duplicate targets, 4338 targets remained. Moreover, we searched the targets of ferroptosis in the Genecards and OMIM database, which displayed 408 ferroptosis-related non-redundant targets.

In order to screen out the target genes of the ZL capsule for ICH, we intersected the targets of the ZL capsule with that of ICH, and a total of 300 overlapping target genes were searched. The related target genes between ICH and ferroptosis were intersected, and 249 target genes were selected. The related target genes between ferroptosis and ZL capsule were intersected, and 40 target genes were selected. To further explore the mechanism of the ZL capsule through ferroptosis in the treatment of ICH, we intersected the intersectant target again and got 33 key target genes. The intersectant targets can be seen in Figure 2(a) and supplementary materials.

3.3. PPI Network for the Targets of ZL Capsule. In order to analyze the function between known protein and predicted protein, we conducted a PPI network analysis in the STRING database. More than 33 key genes were imported into STRING, with specie selection as “Homo sapiens.” According to the results of STRING, 33 nodes, 180 edges, and 10.9 average node degrees were identified. The results were then imported into Cytoscape in the form of “tsv” to construct the figure of the PPI network. The analysis for the rank of the genes according to the value of degree was performed via network Analyzer tool in Cytoscape. As shown in Figure 2(b), the size of each circular node represented the importance of each gene. The bigger size the of the node, the higher the corresponding degree value. According to the degree value, top 10 genes that were found to be influential targets in the treatment of ZL capsule via ferroptosis are as follows: TP53 [25], TNF [23], EGFR [22], PTEN [22], MYC [21], JUN [21], PTGS2 [18], RELA [16], HMOX1 [15] and GSK3B [15].

3.4. GO Enrichment and KEGG Analysis. To investigate the molecular mechanism of the ZL capsule through ferroptosis, GO enrichment and KEGG pathway analysis were carried out on the 33 key genes in the Metascape database. The GO analysis described the function of key targets from three aspects: biological process (BP), cellular component (CC), and molecular function (MF). The detailed results of the GO enrichment analysis were outlined in Figure 3(a). The results indicated that the biological processes of the target ZL capsule for the treatment of ICH via ferroptosis mainly included the response to an inorganic substance (GO: 0010035), response to xenobiotic stimulus (GO: 0009410), regulation of apoptotic signaling pathway (GO: 2001233), negative regulation of phosphate metabolic process (GO: 0045936), gland development (GO: 0048732) and cellular response to organonitrogen compound (GO: 0071417). The enriched molecular functional ontologies mainly included ubiquitin protein ligase binding (GO: 0031625), DNA-binding transcription factor binding (GO: 0140297), kinase binding (GO: 0019900), oxidoreductase activity (GO: 0016491), cadherin binding (GO: 0045296), and phosphoprotein binding (GO: 0051219). The cellular component analysis showed that cell body (GO: 0044297), membrane raft (GO: 0045121), focal adhesion (GO: 0005925), transcription regulator complex (GO: 0005667), vesicle lumen (GO: 0031983), and PML body (GO: 0016605).

Moreover, the KEGG pathway was analyzed for the 33 key genes, as displayed in Figure 3(c) and Table 4. The identified influential pathways include pathways in cancer (hsa05200), hepatitis B (hsa05161), human cytomegalovirus infection (hsa05163), IL-17 signaling pathway (hsa04657), fluid shear stress and atherosclerosis (hsa05418), chemical carcinogenesis—reactive oxygen species (hsa05208), and ferroptosis (hsa04216).

3.5. Pathway Network of ZL Capsule Active Ingredients for ICH Treatment Targets via Ferroptosis. In order to figure out the key targets and compounds, Cytoscape was performed to build the ZL capsule active ingredients- ICH key targets-top 11 Pathway network diagram, and the analysis results were arranged, as shown in Figure 3(b), Tables 5 and 6. The degree value of quercetin was found to be 18, Betweenness Centrality
was 0.23778696, and Closeness Centrality was 0.51239669. It was indicated that quercetin was the main active ingredient in the ZL capsule for the treatment of ICH. Further, the degree value of crocetin was found to be 6, Betweenness Centrality was 0.08568377, and Closeness Centrality was 0.41059603. For kaempferol, the degree value was 6, Betweenness Centrality was 0.01941172, and Closeness Centrality was 0.41059603. In addition to gene targets, the degree value of PTGS2 was 24, Betweenness Centrality was 0.44995211, and Closeness Centrality was 0.55357143. It was suggested that PTGS2 serve as the major target in the ZL capsule for ICH. For RELA, the degree value was 10, Betweenness Centrality was 0.04458046, and Closeness Centrality was 0.41891892. Similarly, the degree value of JUN was 9, Betweenness Centrality was 0.03443671, and Closeness Centrality was 0.41333333. The degree value of TP53 was 8, Betweenness Centrality was 0.0687080, and Closeness Centrality was 0.43055556. Taking together the above three parameters, PTGS2 and TP53 were identified to be the major targets.

Based on our results and previous research studies regarding the mechanism of ferroptosis, we selected TP53 as the final target of validation. As the core target in ferroptosis, we further proved that GPX4 participates in the treatment of ZL capsules for ICH via ferroptosis.

### 3.6. Expression Levels of Key Targets in the Treatment of ZL Capsule for ICH in Rats

#### 3.6.1. The Results of Behavioral Tests after ICH in Rats

ICH-induced neurological deficits in rats were assessed by Zea-Longa score and NSS. The Zea-Longa score of the ICH model was significantly increased as compared to the Sham group ($P < 0.05$, Figure 4(b)), but ZL capsule-treated rats had shown decreased score ($P < 0.05$, Figure 4(b)), indicating the ameliorating effect of ZL capsule on neurological damage after ICH. Moreover, the NSS in ICH group was dramatically increased as compared to the Sham group ($P < 0.05$, Figure 4(a)), and the treatment with ZL capsules in ICH rats decreased the NSS level ($P < 0.05$, Figure 4(a)).

#### 3.6.2. Real-Time Quantitative PCR of TP53

The results of mRNA expression levels of TP53 were displayed in Figure 4. Compared with the Sham group, the mRNA expression level
of TP53 in the ICH group was significantly increased ($P < 0.05$, Figure 4(c)), whereas, the ZL capsule group showed a dramatically decreased expression level of TP53 ($P < 0.05$, Figure 4(c)).

3.6.3. Western Blot of TP53 and GPX4. For the multifaceted verification, the western blot was performed to investigate the protein expression of TP53 and GPX4, and the results are shown in Figure 4(d). In the ICH group, the TP53 protein expression was upregulated as compared to the Sham group, however, the ZL capsule treatment group demonstrated a significantly decreased protein expression level of TP53 ($P < 0.05$, Figure 4(f)). Moreover, the expression level of GPX4 was significantly decreased in the ICH group as compared to the Sham group, whereas, upregulated in the ZL capsule treatment group ($P < 0.05$, Figure 4(e)).
Figure 3: (a) The BP, CC and MF enrichment score in GO enrichment analysis of ZL capsule. (b) The ZL capsule active ingredients- ICH key targets- Top 11 pathway network diagram. Different shapes represent different nodes. The circle is the active ingredients, the inverted triangle is the top 11 pathway, and the diamond is the gene target. The node size represents its degree value. The larger the size, the more important the node is. (c) The bubble diagram of top 11 pathway in KEGG enrichment of ZL capsule. The size and color of circulars represent the importance of related pathway. The larger the circular size and the redder the color, the more important the pathway is.
4. Discussion

The SBI after ICH is complicated and multifactorial [8], the key factors that accelerate SBI included hemin, hemoglobin, iron and thrombin, which lead to the occurrence of neuroinflammation, oxidative stress and neuronal death after ICH [38]. And iron deposition could contribute to oxidative stress and lipid peroxidation via Fenton reaction and subsequent ferroptosis [39]. It has been proven that iron overload and accumulation of lipid peroxides and ROS are the basic biochemical characteristics of ferroptosis [40], which has been confirmed to be involved in the onset and development of ICH. Therefore, the occurrence of ferroptosis is closely related to the SBI after ICH. In this study, we attempt to identify the mechanism of the ZL capsule against ICH via ferroptosis with the combination of a network pharmacology approach and experimental validation. And there are 30 active ingredients in the ZL capsule and 539 related gene targets. After topology analysis, quercetin, crocetin, kaempferol, beta-sitosterol, formononetin and isorhamnetin were the important active ingredients found in the ZL capsule for the treatment of ICH. The corresponding gene targets identified include TP53, PTGS2, RELA, TNF, etc., which were found to be involved in BP, MF, CC and pathway enrichment. The main pathways included ferroptosis, IL-17 signaling pathway and VEGF signaling pathway. Further experimental verification suggested that the ZL capsule engages ferroptosis inhibition via regulating TP53 for the treatment of ICH.

ZL capsule is a patent traditional Chinese medicine that is composed under the medication rule of Xuan Fu theory and Feng medicine, and the clinical application of the ZL capsule has been widely accepted. A systematic review and meta-analysis of the ZL capsule in the treatment of acute cerebral infarction (ACI) suggested that the ZL capsule could ameliorate short-term outcomes of ischemic stroke [41]. Moreover, studies have shown that for treating cerebrovascular diseases, the multi-components of the ZL capsule exert their action via multi-targets of anti-inflammatory, anti-apoptosis, and pro-angiogenesis pathways [9]. Previous studies have reported the coexistence of ferroptosis,

Table 4: Top 11 related pathways in metascape for the ICH treatment of ZL capsule via ferroptosis.

<table>
<thead>
<tr>
<th>GO</th>
<th>Description</th>
<th>Count</th>
<th>%</th>
<th>Log10(P)</th>
<th>Log10(q)</th>
<th>Hits</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa05200</td>
<td>Pathways in cancer</td>
<td>17</td>
<td>51.52</td>
<td>−20.99</td>
<td>−18.57</td>
<td>CASP8, NQO1, EGFR, GSK3B, GSTP1, HMOX1, JUN, MYC, NFE2L2, MAPK1, PTEN, PTGS2, RB1, RELA, TGFBI, TGFBI2, TP53</td>
</tr>
<tr>
<td>hsa05161</td>
<td>Hepatitis B</td>
<td>10</td>
<td>30.3</td>
<td>−14.91</td>
<td>−12.85</td>
<td>CASP8, JUN, MYC, MAPK1, RB1, RELA, TGFBI1, TGFBI2, TNF, TP53</td>
</tr>
<tr>
<td>hsa05163</td>
<td>Human cytomegalovirus infection</td>
<td>10</td>
<td>30.3</td>
<td>−13.47</td>
<td>−11.63</td>
<td>CASP8, EGFR, GSK3B, MYC, MAPK1, PTGS2, RB1, RELA, TNF, TP53</td>
</tr>
<tr>
<td>hsa04657</td>
<td>IL-17 signaling pathway</td>
<td>8</td>
<td>24.24</td>
<td>−13.07</td>
<td>−11.44</td>
<td>CASP8, GSK3B, JUN, MAPK1, PTGS2, RELA, TNF, TFNAIP3</td>
</tr>
<tr>
<td>hsa05418</td>
<td>Fluid shear stress and atherosclerosis</td>
<td>8</td>
<td>24.24</td>
<td>−11.69</td>
<td>−10.39</td>
<td>NQO1, GSTP1, HMOX1, JUN, NFE2L2, RELA, TNF, TP53</td>
</tr>
<tr>
<td>hsa05208</td>
<td>Chemical carcinogenesis - reactive oxygen species</td>
<td>8</td>
<td>24.24</td>
<td>−10.04</td>
<td>−8.9</td>
<td>NQO1, EGFR, HMOX1, JUN, NFE2L2, MAPK1, PTEN, RELA</td>
</tr>
<tr>
<td>hsa04216</td>
<td>Ferroptosis</td>
<td>5</td>
<td>15.15</td>
<td>−9.08</td>
<td>−7.99</td>
<td>ALOX15, ACSL4, HMOX1, SLC3A2, TP53</td>
</tr>
<tr>
<td>hsa01524</td>
<td>Platinum drug resistance</td>
<td>4</td>
<td>12.12</td>
<td>−5.91</td>
<td>−5.22</td>
<td>CASP8, GSTP1, MAPK1, TP53</td>
</tr>
<tr>
<td>hsa05012</td>
<td>Parkinson disease</td>
<td>5</td>
<td>15.15</td>
<td>−5</td>
<td>−4.39</td>
<td>HSPA5, NFE2L2, SNCA, TP53, UCHL1</td>
</tr>
<tr>
<td>hsa04217</td>
<td>Necroptosis</td>
<td>4</td>
<td>12.12</td>
<td>−4.57</td>
<td>−3.98</td>
<td>ALOX15, CASP8, TNF, TFNAIP3</td>
</tr>
<tr>
<td>hsa04370</td>
<td>VEGF signaling pathway</td>
<td>3</td>
<td>9.09</td>
<td>−4.43</td>
<td>−3.86</td>
<td>HSPB1, MAPK1, PTGS2</td>
</tr>
</tbody>
</table>

Table 5: Top 6 active ingredients of ZL capsule in above related 11 pathways.

<table>
<thead>
<tr>
<th>ID</th>
<th>MolID</th>
<th>Compound</th>
<th>Degree</th>
<th>BetweennessCentrality</th>
<th>ClosenessCentrality</th>
</tr>
</thead>
<tbody>
<tr>
<td>HQ7</td>
<td>MOL000098</td>
<td>Quercetin</td>
<td>18</td>
<td>0.23778696</td>
<td>0.51239669</td>
</tr>
<tr>
<td>SZ</td>
<td></td>
<td>Crocetin</td>
<td>6</td>
<td>0.08568377</td>
<td>0.41059603</td>
</tr>
<tr>
<td>HQ6</td>
<td>MOL000422</td>
<td>Kaempferol</td>
<td>6</td>
<td>0.01941172</td>
<td>0.41059603</td>
</tr>
<tr>
<td>A</td>
<td>MOL000358</td>
<td>Beta-sitosterol</td>
<td>4</td>
<td>0.01428604</td>
<td>0.40522876</td>
</tr>
<tr>
<td>HQ16</td>
<td>MOL000392</td>
<td>Formononetin</td>
<td>3</td>
<td>0.00604758</td>
<td>0.38509317</td>
</tr>
<tr>
<td>HQ10</td>
<td>MOL000354</td>
<td>Isorhamnetin</td>
<td>3</td>
<td>0.00570997</td>
<td>0.38509317</td>
</tr>
</tbody>
</table>

Table 6: Top 7 targets of ZL capsule in above 11 pathways.

<table>
<thead>
<tr>
<th>Targets</th>
<th>Degree</th>
<th>BetweennessCentrality</th>
<th>ClosenessCentrality</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTGS2</td>
<td>24</td>
<td>0.44995211</td>
<td>0.55357143</td>
</tr>
<tr>
<td>RELA</td>
<td>10</td>
<td>0.04458046</td>
<td>0.41891982</td>
</tr>
<tr>
<td>JUN</td>
<td>9</td>
<td>0.03443671</td>
<td>0.41333333</td>
</tr>
<tr>
<td>TP53</td>
<td>8</td>
<td>0.068708</td>
<td>0.43055556</td>
</tr>
<tr>
<td>TNF</td>
<td>8</td>
<td>0.04903199</td>
<td>0.42465753</td>
</tr>
<tr>
<td>CASP8</td>
<td>8</td>
<td>0.03937065</td>
<td>0.43333333</td>
</tr>
<tr>
<td>GSK3B</td>
<td>8</td>
<td>0.05341178</td>
<td>0.38271605</td>
</tr>
</tbody>
</table>
autophagy and necrosis in mouse brain tissues after ICH [16], and the inhibition of ferroptosis contribute significantly to the alleviation of neurological outcomes post-ICH [42]. So, it is meaningful to figure out the role of the ZL capsule in the treatment of ICH.

The main mechanisms of ferroptosis include lipid peroxidation, the antioxidant system and iron metabolism after ICH [14]. In our studies, ZL capsules have shown a neuroprotective effect after ICH in rats. It is significant to determine whether ZL capsules could alleviate ICH by modulating the mechanism of ferroptosis. The occurrence and onset of ferroptosis are closely related to oxidative stress [43], therefore the ability of antioxidant is important for the inhibition of ferroptosis. Regarding the effective ingredients of the ZL capsule, quercetin is a naturally occurring flavonoid and popularly known as a nutritional anti-oxidant. It has been verified that quercetin is involved in the protection against ICH through suppressing oxidant activity, inflammatory response, and apoptosis [44, 45]. Crocetin is derived from Crocus sativus stigmas and has shown antioxidant and neuronal protection in many studies [46]. Trans-crocin 4 (TC4), which quickly hydrolyzes to crocetin after oral administration, can penetrate the BBB without hydrolysis, further mechanism needs to be explored [47]. Kaempferol is a natural flavonoid in plants, with anti-oxidant, anticancer, and anti-inflammatory properties in treating cardiovascular diseases, neurodegenerative diseases, and cerebral ischemic reperfusion injury [48, 49]. Importantly, it has been confirmed that kaempferol ameliorated ferroptosis in ODG/R via Nrf2/SLC7A11/GPX4 axis [50]. From our findings, we can conclude that the active ingredients of the ZL capsule could inhibit ferroptosis after ICH via attenuating oxidative stress.

TP53 is a tumor suppressor protein and is involved in the cellular response to stresses, including hypoxia, DNA damage, and oncogene activation [51]. Previous results suggest that TP53 could regulate ferroptosis in a bidirectional manner. On the one hand, TP53 contributes to ferroptosis via inhibiting SLC7A11 expression or promoting SAT1 and GLS2 expression. On the other hand, TP53 could also suppress ferroptosis through the inhibition of DPP4 activity [51]. TP53 is a tumor suppressor protein and is involved in controlling cell survival and division under various pressures [54], such as the...
metabolism of lipids [55], polyamines [56], iron, and ROS production [57]. Interestingly, many studies have found that TP53 can influence the redox state, thereby modifying the metabolic processes of cells [58] and promoting ferroptosis in oxidative stress conditions [59]. In view of these studies, TP53 is an important regulator for ferroptosis after ICH. Our results have shown that the ZL capsule could down-regulate the expression of TP53 and inhibit subsequent ferroptosis after ICH. In order to explain the role of TP53 between ferroptosis and oxidative stress after ICH, further studies are needed.

To sum up, network pharmacology was used in this study, and further validation experiments to explore the potential mechanism of the ZL capsule for the treatment of ICH via ferroptosis were performed. Finally, network pharmacology analyzed that the ZL capsule could alleviate ICH via a multi-target and multi-pathway manner, the results of validation experiments have shown that the ZL capsule could ameliorate ICH via inhibiting ferroptosis through regulating TP53, but more efforts are needed to figure out further mechanism about TP53 and ferroptosis after ICH.

5. Conclusion

In this study, we have proven the protective effect of ZL capsules after ICH in rats with the application of network pharmacology and validation experiments. And the active ingredients of the ZL capsule may attenuate oxidative stress after ICH, and further inhibit ferroptosis by modulating the TP53 signaling pathway. These results could support the clinic application of ZL capsules and provide a new sight for the research of ZL capsules and other herbal medicines.

Data Availability

The data that support the findings of this study are available from the first author upon request.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

Authors’ Contributions

This work is finished under the guidance of Prof. Sijin Yang and Houping Xu. Lixia Wang and Li Wang proposed the idea of this article. Lixia Wang conducted the experiments and wrote the original draft. Wei Ren and Linshen Mao edited the manuscript. Chen Zhou investigated the literature. Maryam Mazhar critically edited and revised the manuscript.

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Supplementary Materials

The supplemental file of network pharmacology-related data in the article. (Supplementary Materials)

References

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