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AKT1 and ferroptosis involved in the anti-cerebral infarction effect of Ginkgolide Injection

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Abstract

Introduction and Objective. Ferroptosis is essential for the occurrence and development of myocardial infarction. The aim of the study is to elucidate the molecular mechanism of Ginkgolide Injection against ischemic cerebral infarction (ICI), focusing on its multi-target regulation of ferroptosis via the AKT1 and NFE2L2.

Materials and Method. Human primary neurons and astrocytes were subjected to oxygen-glucose deprivation/reoxygenation (OGD/R) to mimic cerebral ischemia. Cells were treated with Ginkgolide Injection or transfected with AKT1-targeting siRNA. Targets of Ginkgolide Injection components against ICI-associated genes were retrieved from TCMSP, CTD, HERB, OMIM, and DisGeNET databases. A compound-target-disease network was constructed using Cytoscape, followed by PPI, GO, and KEGG analyses. Cell viability (CCK-8), lipid peroxidation assessment (C11-BODIPY581/591 staining), redox markers (GSH, ROS, MDA), and AKT1/Nrf2 signaling (RT-qPCR, Western blot) were analyzed.

Results. Ginkgolide Injection restored viability in OGD/R-injured neurons and astrocytes. Bilobalide in Ginkgolide Injection targeted 22 genes; Ginkgolide A targeted 32 genes; Ginkgolide B targeted 48 genes; Ginkgolide C targeted 30 genes; Ginkgolide J targeted 7 genes. A total of 55 targets for Ginkgolide Injection were common with ICI-associated genes. Network pharmacology identified AKT1 and NFE2L2 as core targets of Ginkgolide Injection against ICI. Ginkgolide Injection suppressed ferroptosis (reduced PTGS2 mRNA and lipid peroxidation) and activated AKT1 signalling in OGD/R-injured neurons and astrocytes. AKT1 knockdown abolished Ginkgolide's anti-ferroptotic effects, confirming AKT1's regulatory role.

Conclusions. Ginkgolide Injection mitigates cerebral infarction by co-activating AKT1 and NFE2L2 to suppress ferroptosis, highlighting its potential as a multi-target therapy for ischemic ICI.

Key words

cerebral infarction, Ginkgolide Injection, neurons, astrocytes, oxygen-glucose deprivation

INTRODUCTION

Ischemic cerebral infarction (ICI) triggers diverse neuropathological sequelae, ranging from focal tissue lesions and structural disruption to widespread neuronal death and functional impairment. These injuries manifest clinically as Alzheimer's disease-like neurodegeneration, motor dysfunction, cognitive decline, and intellectual deficits, among other neurological disorders [1]. Extensive mechanistic studies have identified three principal pathways driving ICI-induced neuronal injury. Direct neuron and astrocyte loss, a central contributor to post-infarct damage, has spurred research into neuroprotective strategies and regenerative therapies [2, 3]. Moreover, excessive reactive oxygen species (ROS) arise from ischemia-induced vascular occlusion, and the resulting oxidative stress intensifies neuronal injury and functional decline. [4]. Oxidative stress-response pathways, including ferroptosis, are actively explored as therapeutic targets to mitigate neuronal injury [4]. Investigations into ferroptosis and other oxidative stress-response pathways aim to mitigate these effects.

Ginkgolide Injection belongs to Chinese medicinal injection. It is a standard preparation composed of ginkgo diterpene lactone extract (Bilobalide, Ginkgolide A, Ginkgolide B, Ginkgolide C, and Ginkgolide J). Ginkgolide Injection received a Grade A recommendation for cerebral infarction treatment following rigorous clinical trials confirming its safety and therapeutic benefits [5]. It is recommended for use as a basic ICI clinical agent. Ginkgolide Injection as it has good efficacy in the treatment of cerebral infarction during the recovery period [5, 6]. In addition, Ginkgolide Injections can significantly enhance the efficacy of edaravone in acute ischemic stroke [7]. Ginkgolide Injection has been shown to restore ischemia/reperfusion-disrupted cerebral metabolism through multi-pathway regulation and confer

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neuroprotection, as revealed by metabolomic analysis [8]. However, the specific mechanism of Ginkgolide Injection in the treatment of cerebral infarction still needs to be validated.

The study explores the possible targets of Ginkgolide Injection against cerebral infarction using network pharmacology. Subsequently, oxygen-glucose deprivation and reoxygenation (OGD/R) models based on two types of cell lines, including neurons and astrocytes, were constructed. The expression and effects of AKT1 and NFE2L2 in OGD/R and Ginkgolide Injection treatment were observed.

MATERIALS AND METHOD

Chemicals and reagents. Primary human neurons, human astrocytes, Neuronal growth supplement, penicillin/streptomycin solution, Astrocyte growth supplements, Neuronal Medium, and Astrocyte Medium-Glucose Free were purchased from ScienCell Research Laboratories (USA), PBS (phosphate buffer saline), MEM-high glucose, DMEM-free of glucose, Cell Counting Kit-8 and Reduced Glutathione (GSH) Content Assay Kit were purchased from Solarbio (China). Lipid Peroxidation (MDA) Assay Kit (Sigma-Aldrich, USA), Reactive Oxygen Species Assay Kit, and Oxidized were purchased Small interfering RNA (siRNA) for AKT1 (siAKT1) and its negative control (siNC) were ordered from Abbexa (UK). Dharmafect siRNA transfection reagent was obtained from Thermo Scientific (UK).

Cell culture. Neuronal growth supplements and penicillin/streptomycin solution were added to the neuronal culture medium for culturing primary neurons. Astrocyte growth supplements and penicillin/streptomycin solution were added to the astrocyte culture medium for culturing astrocytes. Human microglia cells were cultured in DMEM-high glucose supplemented with penicillin/streptomycin solution. All cells were cultured at 37 °C in a humidified environment containing 5% CO_2.

Cell viability detection. For identification of proper experiment concentration, cell viability of human primary neurons and astrocytes was determined by the CCK-8 method under different concentrations of Ginkgolide Injection (0, 2.5, 5, 10, 20, and 30 μg/mL). Human primary neurons and astrocytes at a density of 4×10^3 cells/well were incubated with different concentrations of Ginkgolide Injection in 96-well plates for 24 hours, followed by the addition of 20 uL of the CCK-8 reagent. After another 4 hour-incubation, the absorbance at 450 nm was measured.

Oxygen-glucose deprivation and reoxygenation (OGD/R) treatment. Human primary neurons and astrocytes were plated at a density of (3×10^4) cells per ml in a 96-well plate. After discarding the original culture media, cells were washed twice with PBS and maintained in a glucose-free medium. The cells were divided into three groups: control, OGD/R group, and OGD/R +Injection, while the Ginkgolide Injection was dissolved into 1% dimethyl sulfoxide (DMSO), and added to the culture medium at a final concentration of 2 μg/mL. The cells were placed in a hypoxic incubator with a mixed gas of 94% N_2, 5% CO_2, and 1% O_2, and incubated at 37 °C for three hours for OGD damage. The culture medium was replaced with fresh normal culture medium and incubation carried out in a normoxic incubator at 37 for 24 hours for a reperfusion period. Control cells were cultured in a normoxic incubator with a medium containing glucose.

Network pharmacology approach. 1) Search for ingredient target – the targets for the active ingredients in Ginkgolide Injection [9] were collected from CTD (https://ctdbase.org/), HERB (http://herb.ac.cn/), ETCM 2.0 (http://www.tcmip.cn/ETCM2/front/#/), HIT (http://www.badd-cao.net:2345/search), TCMSP (https://www.tcmsp-e.com/#/home).
2) Search for disease genes – the ICI-associated genes were retrieved from OMIM (https://omim.org/), Genecards (https://www.genecards.org/), DISGENET (https://www.disgenet.com/) databases using the term 'ischemic cerebral infarction'. The genes from DISGENET were filtered using relevance scores greater than the average of 7.918.
3) Network building – a Ginkgolide Injection-chemical-target network was drawn by Cytoscape software.
4) The protein-protein interactions (PPI) were analyzed using the STRING database and displayed by the Cytoscape software.
5) GO and KEGG enrichment analyses were performed using Omicshare online tools.

Cell transfection. Cells were transfected with AKT1-targeting siRNA (siAKT1) or a non-targetting control siRNA (using DharmaFECT™ transfection reagent). Following a 48-hour incubation period, cells were harvested for quantification, and subsequent functional assays were performed.

RT-qPCR assay. Total RNA extracted from cells using PureLink RNA mini kit (Ambion, USA) was quantified by NanoDrop. A reverse transcription using Superscript II reverse transcriptase (Invitrogen, USA) was employed for cDNA production. qPCR was performed in triplicates using a diluted cDNA and PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, USA) on an Applied Biosystems 7500 Fast instrument. Three copies of each sample were repeated.

Western blot assay. Total protein was extracted from neurons and astrocytes using RIPA lysis buffer (HYC00825, HYCEZMBIO) supplemented with PMSF (HYP112) and protease inhibitor cocktail (HYP111–5). After centrifugation, protein concentrations were measured via BCA assay (P0009, Beyotime). Protein samples (30 μg) were separated on a 20% MOPS gel (M00930, Genscript) and transferred to PVDF membranes (IPVH00010, Millipore). Membranes were blocked (5 min) with Rapid Blocking Buffer (HYC00811), incubated overnight (4 °C) with primary antibody, washed (TBST, G0004, Servicebio), and probed with secondary antibody (1 h). Protein bands were detected using West ECL solution (HYC0316) and visualized on an Odyssey XF imager (LI-COR). Quantification was performed using ImageJ.

Lipid peroxidation assessment via C11-BODIPY581/591 staining. Lipid peroxidation in cells was monitored using the C11-BODIPY581/591 fluorescent probe (Thermo Fisher Scientific, USA). For fluorescence microscopy, cells were incubated with 2 μM C11-BODIPY581/591 in culture medium for 30 min at 37 °C. Fluorescence signals were captured using a confocal microscope (excitation/emission: 581 nm/591 nm for intact probe; 488 nm/510 nm for oxidized probe). For flow cytometric quantification, 5×10^5 cells per well were seeded in

6-well plates and pretreated with dimethyl sulfoxide (DMSO, vehicle control) or SAS (2.5 mM) for 12 hours. Cells were harvested by trypsinization, washed twice with ice-cold PBS, and stained with 2 μM C11-BODIPY581/591 in Hanks' balanced salt solution (HBSS) at 37 °C for 15 min. After centrifugation (300 ×g, 5 min), cells were resuspended in HBSS and analyzed immediately on a flow cytometer (BD FACSCanto II). Lipid peroxidation was quantified as the ratio of green fluorescence (oxidized probe, 510 ± 20 nm) to red fluorescence (non-oxidized probe, 610 ± 20 nm), reflecting oxidative degradation of polyunsaturated fatty acids.

Glutathione (GSH) quantification. Intracellular glutathione levels were measured using the QuantiChrom™ GSH Assay Kit (BioAssay Systems, DIGT-250, USA). In brief, neurons and astrocytes were lysed, and supernatants were incubated with 5,5 -dithiobis(2-nitrobenzoic acid) (DTNB), which reacts with free thiol groups in GSH to generate a yellow-coloured 2-nitro-5-thiobenzoic acid (TNB) derivative. Absorbance was measured spectrophotometrically at 412 nm using a standard curve generated with known concentrations of GSH (0–50 μM). GSH levels were normalized to total cellular protein content.

ROS generation assay. Reactive Oxygen Species Assay Kit (DHE) (C1300–2, Applygen, China) was employed for ROS generation assay. After removing the culture medium, cells were washed twice with serum-free culture medium, collected via centrifugation, and resuspended in PBS. The cell suspension was adjusted to a density of 1×10^6 cells/mL and incubated with 10 μM dihydroethidium (DHE) probe at 37 for 30 min in the dark. The results of fluorescence detection (excitation 500 nm/ emission 600 nm) were represented by fold changes versus fluorescence values of the control group.

Malondialdehyde (MDA) quantification. Intracellular MDA levels were quantified using the Lipid Peroxidation (MDA) Assay Kit (Sigma-Aldrich, USA). Briefly, cellular lysates were incubated with thiobarbituric acid (TBA) under acidic conditions (95 °C, 60 min) to form the MDA-TBA adduct. Following centrifugation to remove precipitated proteins, the pink-coloured adduct was measured spectrophotometrically at 532 nm using a microplate reader (BioTek Synergy H1, USA). MDA concentrations were interpolated from a standard curve (0–20 μM) and normalized to the values in the control group.

Evaluation of iron level. Cultured cells under different experimental conditions were washed and scraped in lysis buffer. Cell pellets were obtained by centrifugation at 300 × g for 5 min at 4 , followed by lysis in RIPA buffer (with protease inhibitors) by sonication on ice. The lysates were clarified by centrifugation and the supernatant were collected for protein concentration determination using the BCA assay. For total iron measurement, 50 μL of supernatant was mixed with 200 μL of Iron Reducer Buffer, provided in the Iron Assay Kit (MAK025; Sigma-Aldrich, USA), and incubated at room temperature for 30 min, protected from light. The absorbance was measured at 593 nm. The iron concentration in each sample was calculated based on the standard curve.

Statistical analysis. Data analysis was performed using the SPSS 12.0 and GraphPad Prism 10.1 software. One-way or two-way ANOVAs followed by *post-hoc* tests were used. Significance was set at $P<0.05$.

RESULTS

Ginkgolide injection rescued OGD/R-induced viability loss in primary neural cells. CCK-8 results showed that the viability of human primary neurons and astrocytes was inhibited by Ginkgolide Injection in a dose-dependent manner (Fig. 1A and 1B). Human primary neurons and astrocytes subjected to OGD/R exhibited significantly reduced cell viability compared to normoxic controls (Figure 1C-D). Ginkgolide Injection treatment effectively rescued these OGD/R-induced deficits, restoring viability to near-baseline levels in both cell types and normalizing cell type-specific survival disparities (Fig. 1C-D).

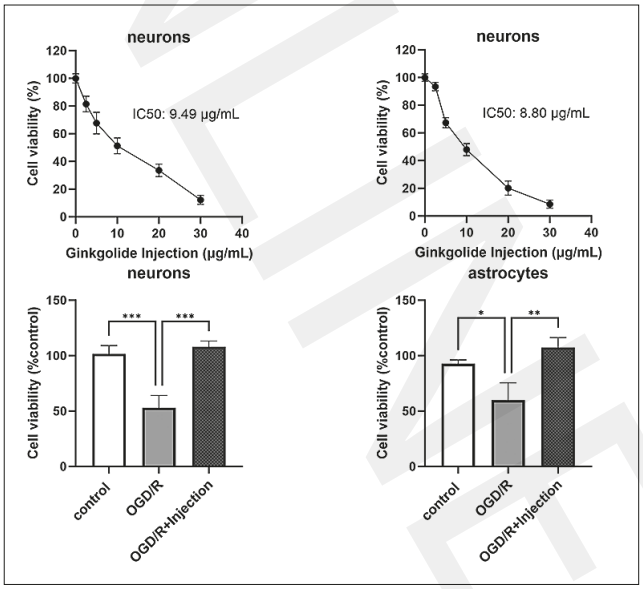

Figure 1. Cell viability detection. (A-B) Cells were treated with increasing concentrations of Ginkgolide Injection for 24 hours, and the cell viability rates were measured by CCK-8 assay. (C-D) Cell viability was measured under 2 μg/mL Ginkgolide Injection or not using CCK-8 assay in human primary neurons and astrocytes.
n = 3; *$p<0.05$; **$p<0.01$; ***$p<0.001$

Exploration of targets of ginkgolide injection. Key pharmacological and molecular properties data of the active ingredients in Ginkgolide Injection are listed in Table 1. After database retrieval, Bilobalide was predicted to target 22 genes; Ginkgolide A was predicted to target 32 targets; Ginkgolide B was predicted to target 48 targets; Ginkgolide C and Ginkgolide J were predicted to target 30 and 7 targets, respectively (Fig. 2A). Among these targets, 55 common nodes were obtained for targets of Ginkgolide Injection against ICI-related genes (Fig. 2B). Then a Ginkgolide Injection-ingredient-target-ICI network was constructed, which contained 62 nodes and 144 edges (Fig. 2C).

Table 1. Key pharmacological and molecular properties data of the main chemicals in Ginkgolide Injection

Chemicals	Molecular weight	Oral bioavailability (%)	Drug-likeness
Bilobalide	326.33	84.41	0.35
Ginkgolide A	408.44	13.82	0.74
Ginkgolide B	424.44	44.38	0.73
Ginkgolide C	440.44	48.32	0.72
Ginkgolide J	424.44	44.83	0.74

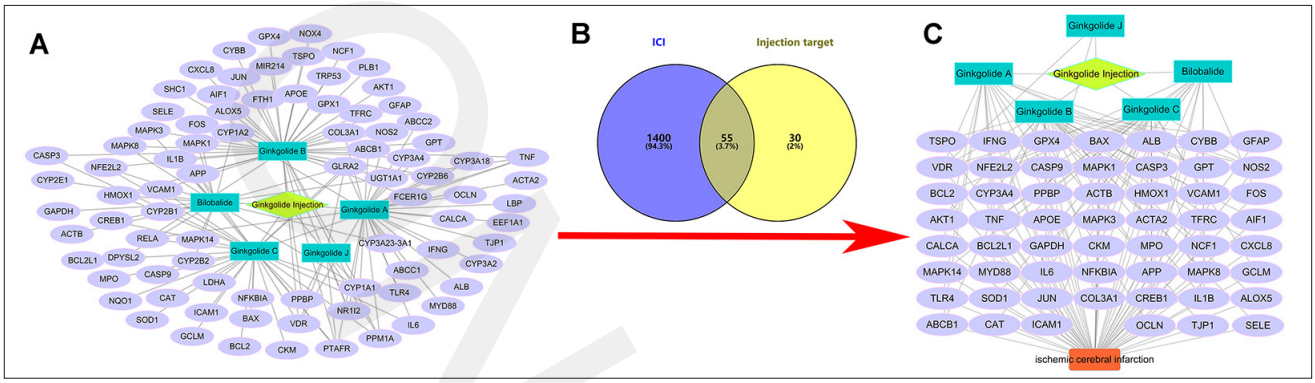

Figure 2. Construction of networks. (A) Ginkgolide Injection-chemical-target network. (B) VENN diagram of Ginkgolide Injection targets and ischemic cerebral infarction-associated genes. (C) Ginkgolide Injection-ingredient-target-ICI network

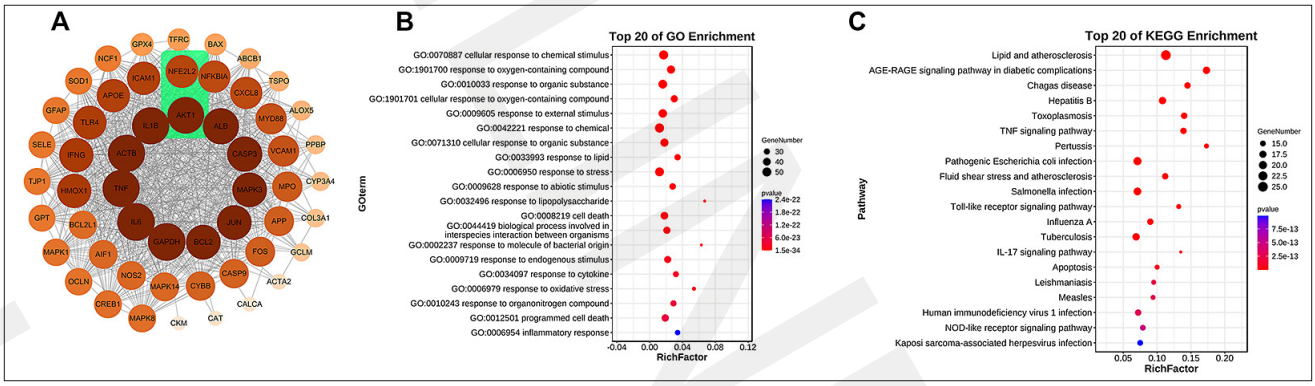

Figure 3. Gene annotation. (A) PPI network of Ginkgolide Injection targets against ischemic cerebral infarction. (B) Top 20 GO enriched terms. (C) Top 20 KEGG pathways

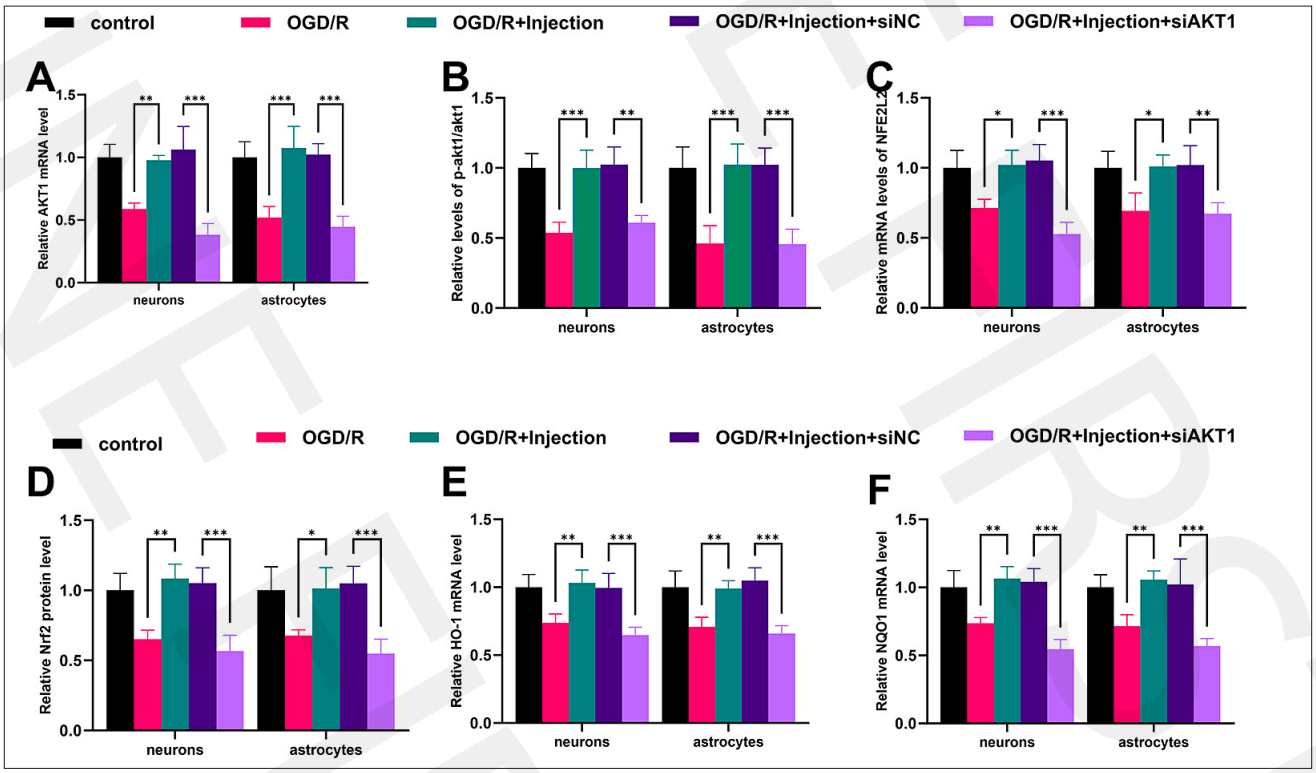

Figure 4. AKT1 and NFE2L2 were target genes of Ginkgolide Injection in OGD/R-injured neural cells. (A) RT-qPCR assay was performed to analyze AKT1 expression in human primary neurons and astrocytes transfected with siRNA and treated with Ginkgolide Injection. (B) Western blot analysis of p-Akt1/Akt1 ratio in human primary neurons and astrocytes. (C) RT-qPCR assay was performed to analyze NFE2L2 expression in human primary neurons and astrocytes transfected with siRNA. (D) Western blot analysis of Nrf2 in human primary neurons and astrocytes. (E) mRNA levels of HO-1 were measured by RT-qPCR. (F) mRNA levels of NQO1 were measured by RT-qPCR. *p<0.05, **p<0.01, ***p<0.001

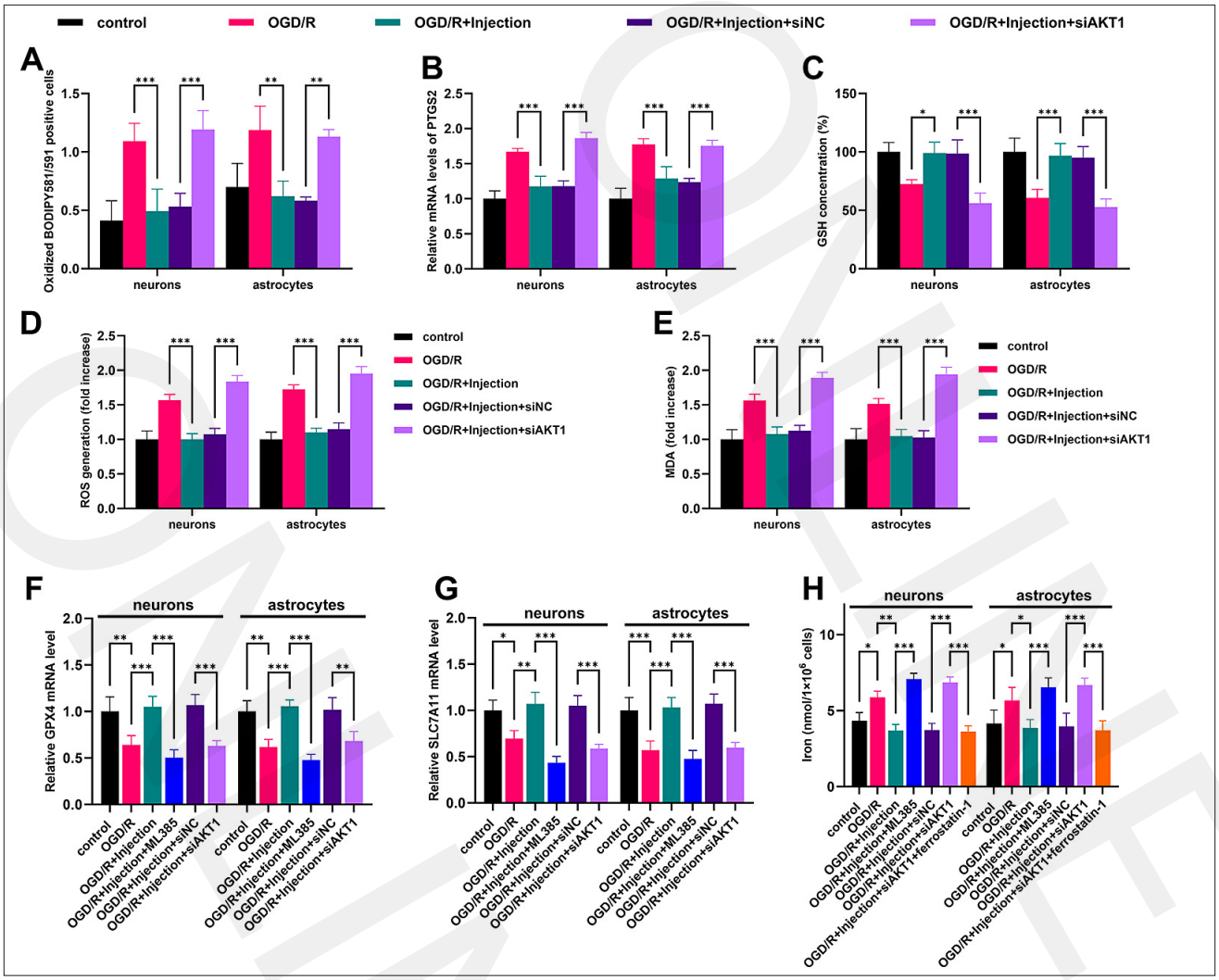

Figure 5. AKT1 mediates anti-ferroptotic effects of Ginkgolide Injection in OGD/R-injured neural cells. (A) Lipid peroxide production was assessed by flow cytometry using C11-BODIPY581/591. (B) mRNA levels of PTGS2 were measured by RT-qPCR. (C) Total intracellular GSH levels were measured by QuantiChrom GSH assay. (D) ROS were measured by commercial kit. (E) Malondialdehyde (MDA) formation was detected colourimetrically using the thiobarbituric acid reactive substance (TBARS) assay. (F) mRNA levels of GPX4 were measured by RT-qPCR. (G) mRNA levels of SLC7A11 were measured by RT-qPCR. (H) Levels of total iron were detected using a commercialized reagent kit.
*p<0.05; **p<0.01; ***p<0.001

PPI, GO, and KEGG pathway enrichments for common genes. PPI analysis was continued on the 54 targets for Ginkgolide Injection against ICI (Fig. 3A). GO and KEGG enrichment analysis were then performed on these targets. The GO enrichment analysis showed that these 54 target genes were related to ischemic cerebral infarction-related biological processes, such as response to oxidative stress and inflammatory response (Fig. 3B). The KEGG pathway showed that these 54 target genes were enriched in multiple pathways, including lipid and atherosclerosis, and fluid shear stress and atherosclerosis (Fig. 3C).

AKT1 and NFE2L2 were target genes of Ginkgolide Injection in OGD/R-injured neural cells. To study the potential role of AKT1 in the mechanism of Ginkgolide Injection against OGD/R, the expression of AKT1 in human primary neurons and astrocytes was down-regulated, using siRNA targeting AKT1. Ginkgolide Injection increased the AKT1 levels and the ratio of p-Akt1/Akt1 in neurons and astrocytes, whereas the siRNA targeting AKT1 reduced the AKT1 levels and ratio of p-Akt1/Akt1 (Fig. 4A-B). Ginkgolide Injection can increase the NFE2L2 mRNA levels in neurons and astrocytes, while the siRNA targeting AKT1 significantly inhibited the NFE2L2 mRNA levels in both cell lines (Fig. 4C). The Nrf2 protein levels (Fig. 4D) and Nrf2-dependent gene expression (HO-1 and NQO1) (Fig. 4E-F) significantly increased by Ginkgolide Injection treatment, but reduced by AKT1 siRNA.

AKT1 mediates anti-ferroptotic effects of Ginkgolide Injection in OGD/R-injured neural cells. Given the critical role of AKT1 and NFE2L2 in ferroptosis [10, 11], the anti-ferroptotic effects of Ginkgolide Injection in OGD/R-injured neural cells were then explored. Compared to the OGD/R group, in the human primary neurons and astrocytes treated with Ginkgolide Injection, a notable decrease in the number of ferroptotic cell deaths was observed. Next, more ferroptotic cell death in AKT1-knockdown neurons and astrocytes were observed, compared to the negative control (Fig. 5A). Further confirmation of the effects of Ginkgolide Injection and AKT1 on ferroptosis was assessed via higher mRNA levels of prostaglandin endoperoxide synthase 2 (PTGS2),

a key ferroptosis marker (Fig. 5B). In addition, treatment with Ginkgolide Injection significantly elevated total GSH levels while reducing ROS accumulation and increasing MDA formation in both neurons and astrocytes; AKT1-knockdown reversed the differences in these measurements (Fig. 5C-E). In addition, the expressions of GPX4 and SLC7A11 were significant. Under Ginkgolide Injection, the down-regulated GPX4 and SLC7A11 were increased; whereas the NRF2 inhibitor ML385 or AKT1 siRNA reduced these increases (Fig. 5F-G). In the OGD/R model group, the iron content increased, while Ginkgolide Injection treatment decreased the iron content (Fig. 5H). However, the NRF2 inhibitor ML385 or AKT1 siRNA offset this reduction, while ferrostatin-1 can suppress the increase caused by AKT1 siRNA (Fig. 5H).

DISCUSSION

The mechanisms of cerebral infarction-induced neurologic injury are complex, implicating various biomarkers and molecular pathways [1]. Current disease definitions are based on phenotype rather than disease mechanism. Therefore, in the case of complex diseases, the current 'one disease-one-target-one drug' dogma is hardly able to produce comprehensive therapeutic results. In fact, the etiology of ICI is a network of many signalling factors [12]. A network pharmacology approach allows the use of two or more drugs to act on mechanisms of the same causal signalling disease module, thereby targeting key network proteins in a synergistic manner [13].

Chinese medicine compound preparations are often complex combinations of ingredients, and network pharmacology approaches are useful for such complex drug combinations. For example, network pharmacology analysis indicates that bioactive compounds in Chuanxiong Tongluo Capsules potentially modulate cerebral infarction recovery through multi-target regulation of calcium, cAMP, cGMP-PKG, and TNF signalling pathways. Key molecular targets include INS, ALB, IL-6, VEGFA, TNF, and TP53, suggesting a systems-level pharmacological mechanism [14]. Ginkgolide Injection has been reported to inhibit the activation of REK1/2 signalling pathway and play a synergistic role with aspirin in cerebral ischemic stroke [15]. In the current study, it was found that Ginkgolide Injection can target AKT1 and NFE2L2, which are associated with ICI.

Cerebral infarction can affect various types of cells in the brain, such as neurons and astrocytes [16]. Astrocytes can be activated into reactive astrocytes, which protect the nervous system through a series of processes and improve the survival rate of neurons in the nervous system [17]. In a novel *in vitro* rat cortical capillary endothelial cell-astrocyte-neuron network model, Ginkgo biloba extract and Ginkgolide B are found to increase the neuron cell viability, ameliorate cell injury, and inhibit the cell apoptotic rate after OGD/R injury [18]. In the current study, it was found that the cell viability of neurons and astrocytes was reduced in OGD/R models of neurons and astrocytes. In line with that result, OGD/R can induce marked structural and molecular remodelling across all major neural cell populations, including neurons (decreased), astrocytes (decreased), microglia, and oligodendrocytes, within organotypic spinal cord slice cultures [19]. In these OGD/R models, it was found that Ginkgolide Injection can reduce the increased cell viability of astrocytes and neurons.

The network pharmacology analysis in this study implies that Ginkgolide Injection may treat ICI in a multi-target and multi-pathway manner. Specifically, it was found that Ginkgolide Injection can increase the levels of AKT1 and NFE2L2 in OGD/R cell models. Emerging evidence implicates ferroptosis, a form of iron-dependent cell death driven by lipid peroxidation, in the pathophysiology of cerebral infarction. Neuronal ferroptosis is exacerbated during ischemia-reperfusion injury due to glutathione depletion, ROS accumulation, and impaired antioxidant defences [19, 20]. Ginkgolide B has been reported to bind to GPX4 and activate the GPX4-GSH pathway, thus alleviating the MACO/R-induced brain injury [21]. The current study revealed that Ginkgolide Injection can interact with GPX4 and NFE2L2, thus reducing ferroptosis and OGD/R-induced neuronal cell death. Notably, AKT1 and NFE2L2 (encoding the transcription factor Nrf2) are pivotal regulators of ferroptosis resistance [22]. AKT1 activation phosphorylates and stabilizes Nrf2, enabling its nuclear translocation to upregulate antioxidant genes (e.g., GPX4, SLC7A11) that neutralize lipid peroxides and suppress ferroptotic cascades [23, 24]. Data from the current study demonstrates that Ginkgolide Injection treatment enhances both phosphorylated AKT1 (p-AKT1/AKT1 ratio) and Nrf2 protein levels in OGD/R-injured cells, thereby restoring redox homeostasis (increased GSH, reduced ROS/MDA) and attenuating ferroptosis (Fig. 5). These findings align with prior studies showing that Nrf2 activators mitigate ischemic brain damage by inhibiting ferroptosis [25–27]. By co-activating AKT1 and Nrf2 signalling, ginkgolide orchestrates a dual defence against OGD/R-induced ferroptosis, potentiating antioxidant capacity while suppressing lipid peroxidation. This mechanistic synergy likely underpins its neuroprotective efficacy in cerebral infarction, as further validated by *in vivo* functional recovery experiments.

Limitations of the study. This study has several limitations. First, the mechanistic evidence linking AKT1 to Nrf2 regulation remains incomplete; while AKT1 knockdown reduced NFE2L2 mRNA and Nrf2 protein levels, key functional evidence, such as direct measurement of Nrf2 nuclear translocation or rescue experiments, is lacking, which prevented a definitive causal conclusion. Second, the network pharmacology analysis is primarily descriptive and hypothesis-generating; the criteria for target selection and confidence scoring are insufficiently detailed, and predicted targets beyond AKT1 were not experimentally validated. Finally, the absence of *in vivo* validation limits the physiological relevance of the findings, as all conclusions are derived from *in vitro* models that cannot replicate systemic complexity.

CONCLUSIONS

The study integrated network pharmacology and experimental validation to decode the anti-ischemic mechanism of Ginkgolide Injection. AKT1 and NFE2L2 (Nrf2) were identified as pivotal targets through which Ginkgolide exerted neuroprotection. In OGD/R-injured neural cells, Ginkgolide upregulated AKT1 phosphorylation and Nrf2 expression, thereby enhancing antioxidant defences and

inhibiting ferroptosis. These findings bridge the gap between Ginkgolide's multi-target pharmacology and its therapeutic efficacy, offering mechanistic insights into its clinical benefits for cerebral infarction. However, limitations include the reliance on *in vitro* models; future studies should validate these pathways *in vivo* and explore synergistic interactions with other neuroprotective pathways.

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