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The potential benefits of quercetin on brain ischemic injury in rats

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ABSTRACT

Cerebral ischemia/reperfusion (I/R) injury is a leading cause of neuronal damage, contributing to neurodegenerative diseases. This study explored the neuroprotective effects of quercetin (QCT), a natural flavonoid, in a rat I/R model. *In Silico* analysis was conducted using online databases to identify potential QCT targets related to brain ischemia. Forty male albino rats (220–250 g) were distributed into four groups as follows: the first group (Gp1): Sham group, Gp2: I/R untreated group, Gp3 and Gp4: I/R groups pretreated with QCT at 25 and 50 mg/kg b.wt, respectively for seven days before middle cerebral artery occlusion, followed by reperfusion for 24 hours. Brain injury and protection were evaluated *via* biochemical, histopathological, and ultrastructural assessments. Intersection analysis revealed 46 shared targets between QCT and cerebral ischemia, with epidermal growth factor receptor (EGFR), protein kinase B (AKT), proto-oncogene tyrosine-protein kinase Src (c-Src), glycogen synthase kinase 3 beta (GSK3β), and matrix metalloproteinase-9 (MMP9). Docking studies showed strong QCT binding to these proteins. I/R elevated Src and MMP9 levels, while reducing phosphorylated EGFR, AKT, and GSK3β levels, contributing to neuronal damage, mitochondrial swelling, endoplasmic reticulum (ER) stress, synaptic loss, and blood-brain barrier (BBB) disruption. QCT pretreatment, particularly at 50 mg/kg, prevents these effects by reducing Src and MMP9 levels, restoring EGFR/AKT/GSK3β phosphorylation, and preserving mitochondrial, ER, neuronal, and BBB integrity. QCT provides dose-dependent neuroprotection by modulating key proteins and preserving cortical neuron and BBB structure after I/R injury.

Keywords: Epidermal growth factor receptor; Blood brain barrier; EGFR/Akt/GSK3 β; Ischemia/reperfusion; Molecular docking; Quercetin; Src/MMP9; Ultrastructure

1. Introduction

Stroke is a devastating cerebrovascular disease, ranking as the second main cause of death and a major contributor to disability worldwide (Chen et al., 2024; Peng et al., 2024). Smoking, high blood pressure, high body mass index, and diabetes are factors that contribute to the occurrence and complications of ischemic stroke (Zhang et al., 2022b). Ischemic stroke, which accounts for approximately 80% of all stroke cases, is frequently characterized by fainting, unconsciousness, speech, and cognitive impairments, and unilateral paralysis (Chen et al., 2024). It occurs when blood supply to the brain is disrupted, leading to oxygen and nutrient deprivation, oxidative stress, inflammation, mitochondrial dysfunction, apoptosis, and increased blood-brain barrier (BBB) permeability that exacerbate cerebral damage (Xu et al., 2023; Chen et al., 2024). While the damage to brain tissue in the primary ischemic core is often irreversible, the surrounding penumbra, where nerve cell injury progresses due to oxygen deficiency and energy failure, offers a critical window for treatment strategy (Loginova et al., 2022; Peng et al., 2024). Reperfusion therapy, such as

thrombolysis and intravascular and endovascular thrombectomy, restores blood flow and prevents neuronal death, but is limited by narrow time windows and strict eligibility (Rehani and Ammanuel, 2020; Zhang et al., 2022b). However, despite its benefits, reperfusion itself can paradoxically aggravate brain injury by inducing cerebral ischemia/reperfusion (I/R) injury, which involves oxidative stress, mitochondrial dysfunction, inflammation, BBB disturbance, excitotoxicity, and neuronal destruction (Guo et al., 2021; Al-Obaidi et al., 2022). Furthermore, antiplatelet and anticoagulant treatments alone are often insufficient to prevent thrombosis, highlighting the importance of vascular protection strategies that preserve endothelial function and vascular integrity (Liu et al., 2021a).

Quercetin (QCT) is a flavonoid compound broadly identified in apples, berries, broccoli, grapes, brassica vegetables, onions, tea, and various seeds and leaves (Ardianto et al., 2023; Mahyar et al., 2025). Its favorable effects are principally related to its phenolic hydroxyl groups, providing antioxidant, anti-inflammatory, antibacterial, and antidiabetic properties (Hamza et al., 2016; Mahyar et al., 2025). Clinically, QCT has shown therapeutic potential in neurological disorders, metabolic syndrome, cardiovascular diseases, and COVID-19 (Zhang et al., 2022b). It promotes neuronal cell proliferation, hinders oxidative enzymes, and modulates antioxidant and anti-inflammatory pathways, thereby countering oxidative stress from neural damage (Mehany et al., 2022; Li et al., 2023). QCT can prevent inflammatory cytokine secretion, decreasing platelet aggregation and limiting inflammatory thrombosis (Zhang et al., 2022b; Li et al., 2023). QCT demonstrates neuroprotection in cerebral ischemia through antioxidant, iron-chelating, and antiferroptotic mechanisms that support BBB stability, vascular repair, and neuronal survival (Peng et al., 2024). *In silico* studies including network pharmacology and molecular docking demonstrate that QCT has a strong binding affinity for multiple proteins implicated in brain ischemic injury (Li et al., 2024c). Such approaches offer valuable insights into QCT's multi-target mechanisms, justifying their use prior to or in parallel with experimental validation (Li et al., 2023; Li et al., 2024c) . Thus, the current study investigated QCT ability to mitigate ischemic brain injury by modulating cerebral ischemia-related pathways.

Materials and methods

In Silico identification and molecular docking of quercetin targets associated with brain ischemia

Brain ischemia-related genes were obtained from DisGeNET (https://disgenet.com/) and GeneCards (https://www.genecards.org/) using "ischemia brain". QCT targets were predicted *via* Swiss Target Prediction (http://www.swisstargetprediction.ch/) from its SMILES structure retraces from PubChem (CID: 5280343). Common targets between QCT and ischemia were identified using Venny 2.1 and analyzed for protein-protein interactions in STRING (https://string-db.org/). Cytoscape v3.10.3 was used to calculate network metrics (degree, closeness, MNC), and the top five hub proteins were selected for validation. Gene ontology (GO) terms and KEGG pathway enrichment studies were achieved with ShinyGO v0.77 to identify main pathways (http://bioinformatics.sdstate.edu/go77/). The proteins EGFR (PDB ID: 1M17), GSK3β (3SAY), AKT1 (4EJN), Src (4F5B), and MMP9 (4H82) 3D structure were obtained from the Protein Data Bank in PDB format. Each protein structure was opened in Discovery Studio Visualizer 2021, where potential binding sites were predicted using the "Define and Edit Binding Site" protocol. The site with the largest volume and most well-defined cavity was selected, and the corresponding active site coordinates were recorded. Protein preparations was done using UCSF Chimera X 1.9, involving eliminating water molecules and co-crystallized ligands, adding hydrogen atoms, and assigning charges. The final structures were saved in PDB format for docking (Shapovalov and Dunbrack, 2011). The QCT SMILES code generated the 3D ligand structure on the SwissDock web server (https://www.swissdock.ch/) (Eberhardt et al.,

2021; Bugnon et al., 2024). The AutoDock Vina engine achieved docking, and the docking was restricted to the predefined active site predicted by Discovery Studio by setting the grid box coordinates. The docking results were analyzed based on binding free energy and clustered poses. The most favorable binding conformation was visualized in Discovery Studio Visualizer 2021 to assess interactions and hydrogen bonding.

In vivo study

Experimental design

In The study protocol was permitted under ethical application number DMU-SCI-CSRE-24-7-06, following regulations from institutional animal care guidelines, Damanhur University, Faculty of Science. Forty adult male albino rats (220–250 g) were used and housed under standard environments, randomly assigned to four equal groups. Group 1 (Gp1): sham-operated, Gp2: I/R untreated, Gp3: pretreated with QCT (25 mg/kg) followed by I/R induction, and Gp4: pretreated with QCT (50 mg/kg) followed by I/R induction. QCT (Sigma-Aldrich Company, St. Louis, MO, USA) was dissolved in saline and administered orally for 7 days before I/R induction (Yang et al., 2022). The last QCT dose was given 1.0 hour before ischemia induction.

Induction of ischemia/reperfusion

Middle cerebral artery obstruction technique was utilized to promote I/R using a modified Longa et al. (1989) method under ketamine-xylazine anesthesia at 80 mg/kg and 10 mg/kg, respectively. The internal carotid artery was exposed by making a midline neck incision, and a filament was inserted to block the middle cerebral artery. Blunt-tipped scissors and hemostats were used to assist vessel occlusion and control bleeding during the procedure. After 1.0 hour of occlusion, the filament was carefully withdrawn to permit reperfusion. Sham animals underwent the same surgery without occlusion or filament insertion.

Sacrifice and sample collection

Twenty-four hours post-reperfusion, animals were euthanized according to ethical guidelines by an overdose of sodium pentobarbital. The brain specimens were rapidly excised, and the cerebral cortex was carefully dissected. Cortex samples were immediately fixed in 10% formalin for histological study or stored at –20°C for protein analysis. For ultrastructural examinations, rats underwent perfusion fixation with $_4F_1G$ fixative (4% formaldehyde and 1% glutaraldehyde in phosphate buffer, pH 7.4), after which the cortex tissues were dissected and processed for transmission electron microscopy.

Quantification of target proteins by ELISA

Cortex tissues were cleaned in cold 1X phosphate-buffered saline (0.01 M, pH 7.4) to eliminate blood, minced, and homogenized on ice in PBS with protease inhibitors at a 1:9 ratio. Homogenates were spun at 5,000 ×*g* at 4°C for 5–10 minutes, and supernatants were gathered for analysis. Levels of epidermal growth factor receptor (EGFR), Src proto-oncogene, non-receptor tyrosine kinase (Src), protein kinase B (AKT1), glycogen synthase kinase-3 beta (GSK3β), and matrix metalloproteinase-9 (MMP-9), were quantified by commercial ELISA kits according to the manufacturers' procedures: rat EGFR ELISA Kit (ELK Biotechnology, Denver, CO, USA; Cat: ELK2225), rat Src ELISA Kit (Abbexa LTD, Cambridge, UK; Catalogue number: abx547297), rat PKB ELISA Kit (Assay Genie, Dublin, Ireland; Catalogue number: RTFI01064), rat GSK3β ELISA Kit (FineTest®, Wuhan, Hubei, China; Catalogue number: ER0060), and rat MMP-9 ELISA Kit (Elabscience®, Houston, TX, USA; Catalogue number: E-EL-R3021). Phospho-EGFR, phospho-GSK3β, and phospho-Akt levels were measured using MyBioSource rat ELISA kits (San Diego, CA, USA; Catalogue numbers: MBS1600643, MBS730623, and MBS775153, respectively).

Light microscopy sample preparation

Cortex tissues fixed in 10% formalin for 24–48 hours at room temperature were then dehydrated through graded alcohols (50, 70, and 100%), cleared in xylene, and embedded in paraffin wax. A microtome cut 4–5 µm sections mounted on glass slides, deparaffinized, rehydrated, stained with hematoxylin, and counter-stained with eosin

(Bancroft and Layton, 2019). Microscopic images were captured using an Olympus CX41 microscope (Tokyo, Japan) connected to a Canon EOS 1200D digital camera.

Transmission electron microscopy sample preparation

Cortex tissues were perfusion-fixed with 4F1G fixative (4% formaldehyde, 1% glutaraldehyde in phosphate buffer, pH 7.4) and then immersed in fresh fixative at 4°C. Cortical tissues were post-fixed in 1% osmium tetroxide, dehydrated in ethanol, cleared in propylene oxide, infiltrated, and embedded in epoxy resin (Reynolds, 1963). Ultrathin sections (70–90 nm) were cut and mounted on copper grids. Then, lead citrate and uranyl acetate were used to stain the grids, and grids were examined using a JEOL JSM1400-PLUS TEM (Faculty of Science, Alexandria University, Egypt).

Statistical methods

Data were expressed as mean ± SD and evaluated using GraphPad Prism 8. One-way ANOVA followed by Tukey's post hoc test was applied.

3. Results

Identification of hub genes and functional pathways in I/R injury and QCT treatment

Several databases were searched for molecular targets linked to ischemia injury and QCT. A total of 1542 disease-related targets and 100 QCT-related targets were retrieved. Intersection analysis revealed 46 common targets, indicating potential therapeutic targets of QCT in I/R injury (Fig. 1). Common targets were analyzed using STRING, yielding a PPI network with 46 nodes and 227 edges ($p < 1e-16$) (Fig. 2A). Topological analysis *via* Cytoscape and CytoHubba identified EGFR, Src, AKT1, GSK3β, and MMP9 as the top five hub genes, highlighting their key roles in ischemia injury and QCT's protective effects (Fig. 2B).

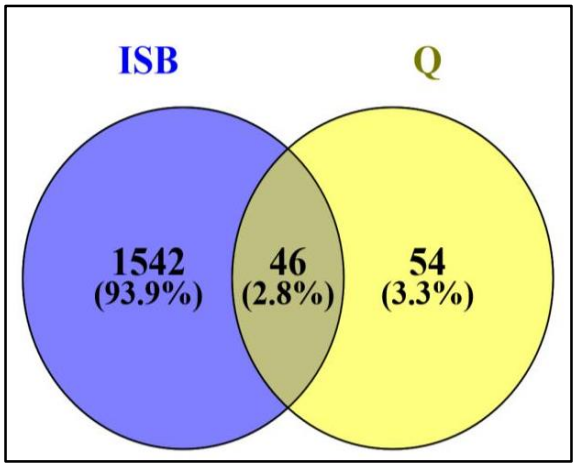

Fig. 1. Intersection of quercetin and ischemia-related targets. Venn diagram of ischemia-associated and quercetin-related targets showing 1588 ischemia-associated targets and 100 quercetin-related targets with 46 overlapping common targets identified.

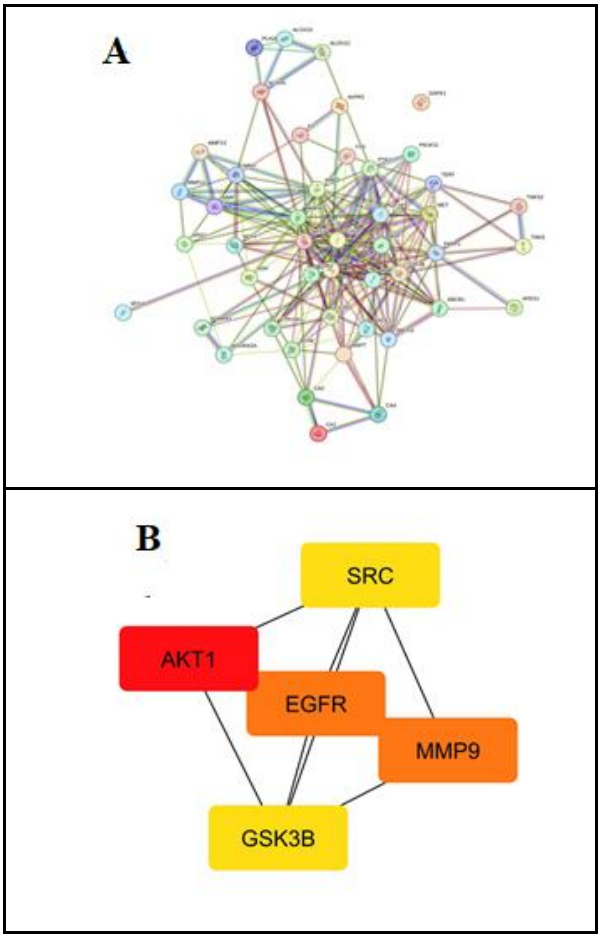

Fig. 2. Network analysis of quercetin and ischemia-related targets. (A) Protein-protein interaction (PPI) network of common target proteins constructed using the STRING database. The network contains 46 nodes representing proteins and 227 edges indicating their interactions. (B) The top five hub genes were identified by degree of centrality. EGFR, SRC, AKT1, GSK3β, and MMP9 showed the highest connectivity, indicating key roles in the studied network.

Gene ontology and enrichment analyses

GO showed that common targets and hub genes were involved in oxidative stress response, cell death regulation, and stress responses (Fig. 3A). Cellular components involved axon, neuron projection, and perinuclear region of cytoplasm (Fig. 3B). Molecular functions included protein tyrosine kinase activity, metallopeptidase activity, and ATP binding (Fig. 3C). KEGG analysis revealed enrichment in PI3K-AKT pathways, key to survival, inflammation, and tissue repair in ischemia-reperfusion injury (Fig. 3D).

Docking results

QCT exhibited notable binding affinities with all target proteins. The strongest binding was observed with MMP9 (–10.011 kcal/mol), followed by AKT1 (–9.547 kcal/mol), EGFR (–8.494 kcal/mol), and GSK3β (–8.047 kcal/mol). The Src has the lowest binding affinity (–6.120 kcal/mol). These results indicated that QCT may act as a multi-target modulator with high binding potential, especially toward MMP9 and AKT1. Key interactions included: hydrogen bonds with GLN227, LEU187, LEU188, ALA189, TYR245 (MMP9); SER205, ASP292 (AKT1); MET749, MET769 (EGFR); VAL135, PRO136, GLU137 (GSK3β); and ARG158, ARG178, THY182 (Src), and hydrophobic interactions with VAL223, HIS226, MET247, TYR248 (MMP-9); TRP80, VAL270, LEU264, LYS268 (AKT1); LEU694, VAL702, ALA719, LEU820 (EGFR); ILE62, VAL70, ALA83, LEU188 (GSK3β); and ALA188, LEU206 (Src) (Fig. 4).

Quercetin modulates EGFR/AKT/GSK3β signaling in I/R injury

The I/R untreated group exhibited a significant elevation in total EGFR and GSK3β levels and decreased AKT expression (p<0.001) if compared to the sham group (Fig. 5). Pretreatment with 25 mg/kg of QCT (QCT25) significantly reduced EGFR and GSK3β levels and elevated AKT levels compared to the I/R untreated group (p< 0.01); however, these levels did not fully normalize to sham levels (p< 0.01). 50 mg/kg of QCT (QCT50) pretreatment produced a more pronounced effect, significantly restoring AKT, while reducing EGFR and GSK3β levels closer to sham values (p< 0.05). Furthermore, phosphorylated EGFR (p-EGFR), AKT (p-AKT), and GSK3β (p-GSK3β) levels were significantly reduced in the I/R untreated group compared to sham group (p<0.001). QCT25 pretreatment partially restored their phosphorylation levels (p<0.01) when compared to the I/R untreated group, while QCT50 led to a greater restoration in comparison to the I/R untreated group (p<0.001), approaching sham group values (p<0.05).

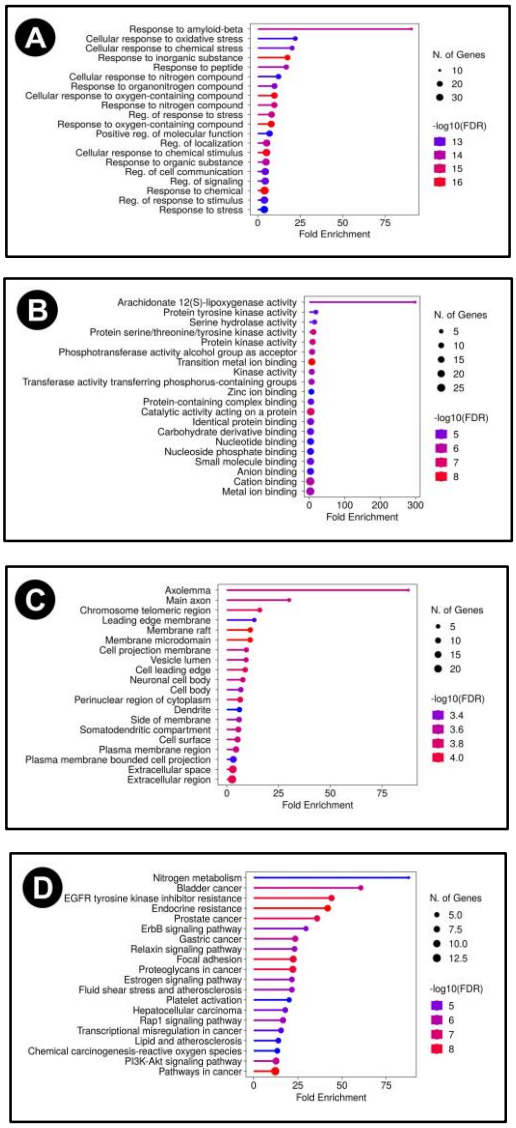

Fig. 3. Enrichment analysis of common targets and hub genes**.** Biological processes (A), Molecular functions (B), Cellular components (C), and KEGG pathways (D).

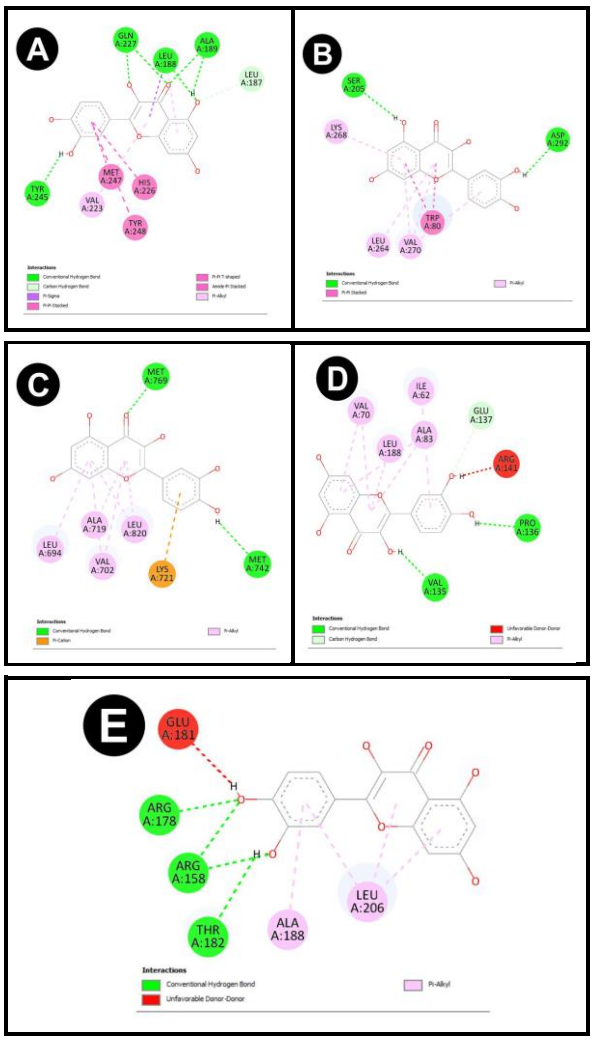

Fig. 4. Docking poses of quercetin with (A) MMP9, (B) AKT1, (C) EGFR, (D) GSK3β, and (E) Src showing key interactions.

Quercetin modulates Src/MMP9 signaling in I/R injury

Src and MMP9 protein levels were significantly elevated in the I/R untreated group ($p < 0.001$) when compared to the sham group levels, indicating activation of matrix-degrading pathways (Fig. 6). Pretreatment with QCT at both 25 mg/kg (QCT25) and 50 mg/kg (QCT50) significantly reduced Src compared to I/R untreated group ($p<0.001$), although levels persisted elevated compared to the sham group ($p<0.05$). Similarly, QCT25 pretreatment significantly lowered MMP9 levels compared to the I/R untreated group ($p< 0.001$), but levels remained elevated relative to sham ($p< 0.01$). QCT50 pretreatment normalized the MMP9 level, showing no significant difference from the sham group ($p> 0.05$).

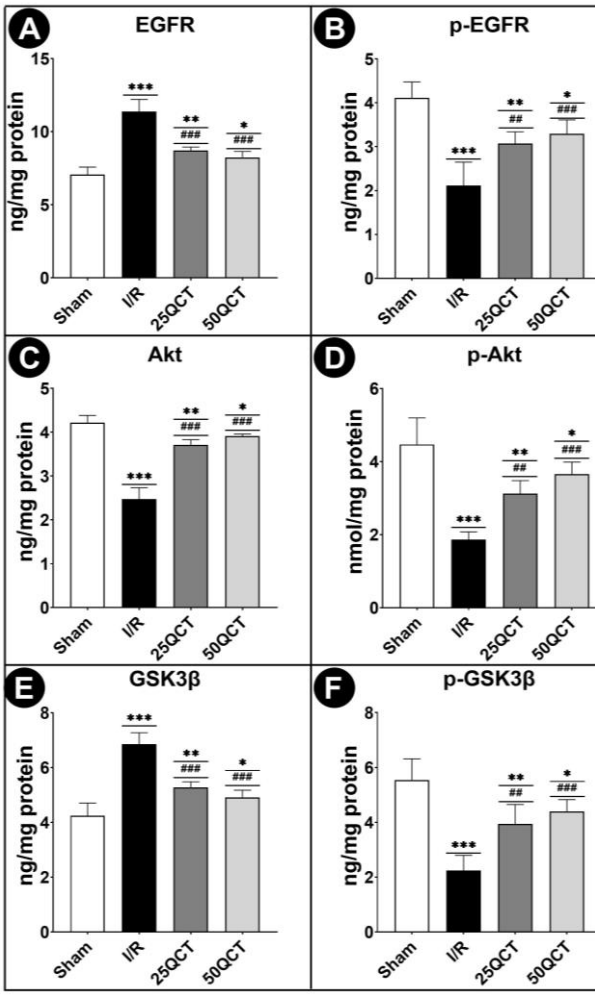

Fig. 5. Effect of I/R and QCT pretreatment on EGFR/Akt/GSK3β signaling. Data are presented as mean ± SD. Significance vs. Sham: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). Significance vs. I/R: $p < 0.01$ ($^{\#\#}$), $p < 0.001$ ($^{\#\#\#}$). I/R: Ischemia/Reperfusion; QCT25: Quercetin 25 mg/kg; QCT50: Quercetin 50 mg/kg.

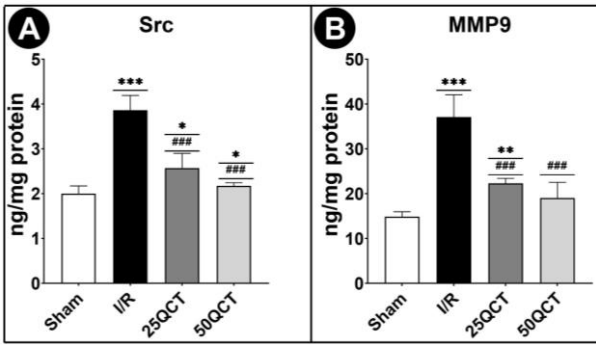

Fig. 6. Effect of I/R and QCT pretreatment on Src/MMP9 signal. Data are presented as mean ± SD. Significance vs. Sham: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). Significance vs. I/R: $p < 0.001$ ($^{\#\#\#}$). I/R: Ischemia/Reperfusion; QCT25: Quercetin 25 mg/kg; QCT50: Quercetin 50 mg/kg.

Histological investigations

Examination of the histological section of the cerebral cortex tissue in the sham group revealed typical cortical architecture with well-organized neuronal layers, intact pyramidal neurons, and clear nuclei with prominent nucleoli with normal neuropil background (Fig. 7A). In contrast, the I/R untreated group exhibited marked histopathological alterations, including neuronal degeneration with intensely eosinophilic cytoplasm, pyknotic nuclei, vacuolization, pericellular holes and perivascular edema, and disruption of cortical layering, along with prominent neuropil vacuolization (Fig. 7B). Pretreatment with QCT25 showed moderate neuroprotective effects, as evidenced by reduced neuronal damage, few pyknotic cells, and partial preservation of cortical structure. However, some areas still exhibited mild vacuolation and disorganization (Fig. 7C). Notably, pretreatment with QCT50 resulted in substantial histological improvement, preservation of normal cortical architecture, reduced neuronal degeneration, and minimal signs of edema or vacuolation (Fig.7D). Most neurons appeared morphologically intact, suggesting that QCT effectively mitigates I/R-induced histological damage dose-dependently.

Ultrastructure investigations of neurons, neutrophil, and capillaries

The sham group's TEM micrographs showed neurons with intact nuclear envelopes, prominent nucleoli, well-developed Golgi apparatus, and abundant Nissl bodies (rough endoplasmic reticulum "ER" clusters), indicating active protein synthesis. The mitochondria exhibited normal size and dense cristae (Fig. 8A and B). The neuropil contained regularly arranged myelinated axons, compact myelin sheaths, and healthy unmyelinated axons. In the I/R untreated group, neurons appeared shrunken with electron-dense nuclei with chromatin condensation and dilated irregularly shaped nuclear envelopes showing frequent invaginations (Fig. 8C and D). The rough endoplasmic reticulum was markedly dilated, reflecting cellular stress and impaired protein synthesis.

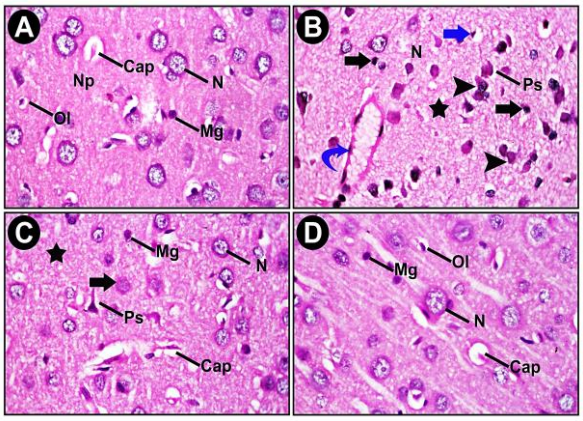

Fig. 7. Histological evaluation of cerebral cortex. (A) Sham group: normal neurons with clear nuclei (N), intact neuropil (Np), evenly distributed microglia (Mg), oligodendrocytes (Ol), and capillaries (Cap). (B) I/R group: shrunken pyknotic neurons (head arrow), wide pericellular space (Ps), neuropil vacuolization (star), hypertrophic microglia (black arrow), degenerated oligodendrocytes (blue arrow), and congested capillaries (blue curved arrow). (C) QCT25 pretreatment group: few damaged neurons (black arrow), mild dilated pericellular space (Ps), mild neuropil vacuolization (star), microglial cell (Mg), and moderately preserved capillaries (Cap). (D) QCT50 pretreatment group: nearly normal cortex neurons (N), minimal neuropil vacuolization (Np), quiescent microglia (Mg), preserved oligodendrocytes (Ol), and intact capillaries (Cap). (H&E staining , X 1000)

The cytoplasm was also electron-dense, accompanied by swollen mitochondria with disrupted cristae, fragmented and swollen Golgi apparatus, and a marked reduction and dispersal of Nissl bodies. Cytoplasmic vacuolization was evident, indicating severe neuronal injury. Neuropil showed vacuolization and disrupted synaptic profiles, reflecting widespread damage to neuronal processes and glial elements, degenerated myelinated axons, and damaged unmyelinated axons. In the QCT25 pretreatment group, neurons showed partial ultrastructural improvement. The cytoplasm remained electron-dense but less intense than the I/R untreated group neuron cytoplasm (Fig. 8 E and F). The rough endoplasmic reticulum was mildly dilated, and the Golgi apparatus appeared less fragmented, though still swollen. Nissl bodies were more clustered than the I/R untreated group, and mitochondrial swelling was reduced, indicating moderate recovery of cellular function. Neuropil vacuolization was reduced, synaptic structures appeared more preserved than in the I/R untreated group, and

myelinated and unmyelinated axons were moderately vacuolated and disorganized. In the QCT50 pretreatment group, neurons exhibited near-normal ultrastructure. The cytoplasm appeared less electron-dense, indicating reduced cellular stress (Fig. 8G and H). The rough endoplasmic reticulum, Nissl bodies, and Golgi apparatus were well preserved with normal morphology. The mitochondria appeared healthy, with intact cristae and regular size. Neuropil was mostly intact, with preserved synaptic contacts and myelinated and unmyelinated axons, indicating substantial neuroprotective effects.

Capillary ultrastructure in the sham group revealed intact endothelial cells with thin cytoplasm and continuous basement membranes. The endothelial nuclei were regular, and the capillary lumens were clear and patent. The perivascular space was minimal or absent, indicating no edema (Fig. 9A). Examination of capillaries in the I/R untreated group exhibited endothelial cell swelling with electron-dense cytoplasm and irregular, disrupted endothelial lining (Fig. 9B). The endothelial nuclei exhibited disintegration and fragmentation, indicating severe cellular injury. Basement membranes appeared thickened and irregular. Capillary lumens were narrowed or partially collapsed, sometimes occluded by swollen endothelial cells. Pericyte processes were disrupted, and tight junctions were compromised, reflecting microvascular damage and increased permeability. Surrounding the capillaries was a prominent perivascular space, appearing as a translucent clear area around the capillary, suggesting edema or fluid accumulation. Capillaries showed moderate preservation in the QCT25 pretreatment group compared to the I/R untreated group (Fig. 9C). Endothelial swelling was reduced, and the cytoplasm remained electron dense. Basement membrane thickening was present but less pronounced. The perivascular space was reduced when compared to the I/R untreated group, but it is still mildly evident. In the QCT50 pretreatment group, capillaries exhibited near-normal ultrastructure with intact endothelial cells and nuclei, electron-dense cytoplasm, tight junction, thin, continuous basement membranes, and wide lumens (Fig. 9D). The perivascular space was minimal or absent, reflecting the resolution of edema.

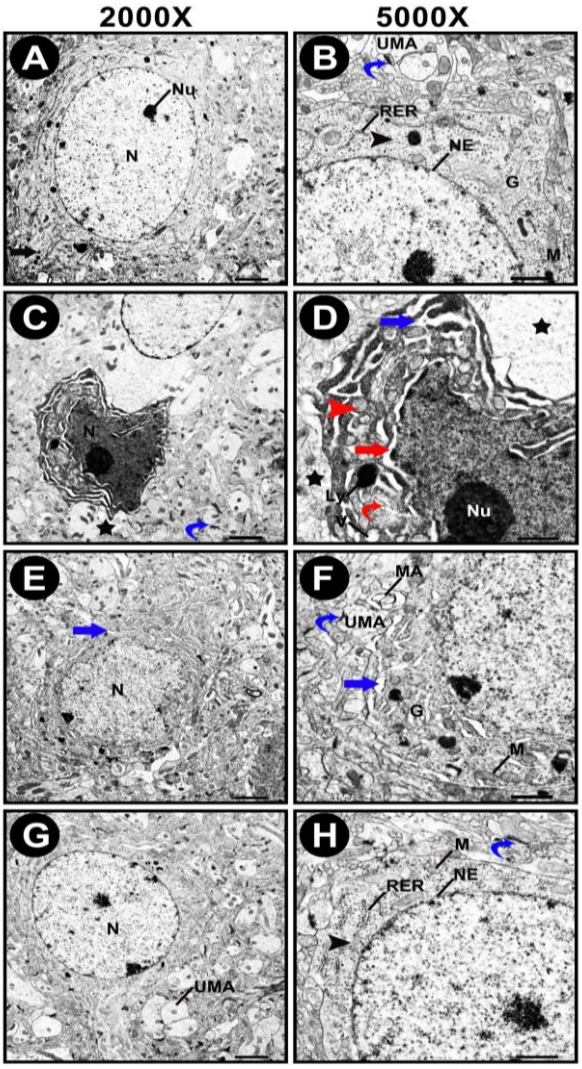

Fig. 8. Ultrastructural alterations of cerebral cortical neurons in experimental groups. Sham group (A-B): Neurons have nuclei (N) with intact envelopes (Ne), prominent nucleoli (N), abundant Nissl bodies (head arrow), rough endoplasmic reticulum (RER), Golgi apparatus (G), and mitochondria (M). Neuropil has organized unmyelinated axons (UMA) and preserved synapses (blue curved arrow). I/R group (C-D): Shrunken neurons with electron-dense nuclei, irregular nuclear envelopes (red arrow), dilated rough endoplasmic reticulum (blue arrow), fragmented Golgi (curved red arrow), swollen mitochondria (red head arrow), lysosomes (Ly), and cytoplasmic vacuolization (V). Vacuolated neuropil, degenerated unmyelinated axons (star) and reduced synapsis (blue curved arrow). QCT25 (E-F): neurons with regular nuclei (N), mildly dilated rough endoplasmic reticulum (blue arrow) , Golgi apparatus (G), clustered Nissl bodies (black head arrow), mild mitochondrial swelling (M). Regular myelinated axon (MA), unmyelinated axons (UMA), and synaptic cleft (blue curved arrow). QCT50(G-H): neurons exhibit near-normal nuclei (N) with intact nuclear envelopes (NE), regular rough endoplasmic reticulum (RER) and Golgi apparatus (G), abundant Nissl bodies (black head arrow), and mitochondria (M). Regular synapses (blue curved arrow), myelinated axons (MA) with compact sheaths and intact unmyelinated axons (UMA).

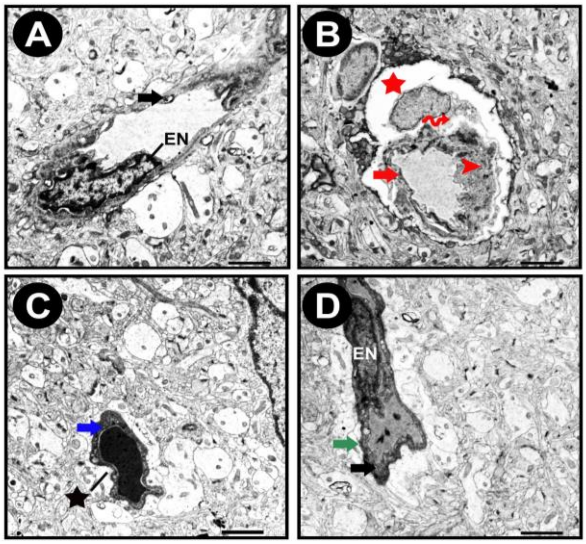

Fig. 9. Ultrastructural Alterations of Cortical Capillaries in Experimental Groups (X 2500). (A) Sham group: capillaries with intact endothelial lining (EN), and thin basement membrane (black arrow). (B) I/R group: swollen endothelial cells with disintegrated nuclei (red head arrow), thickened basement membrane (red arrow), damaged pericyte (red zigzag arrow), and prominent perivascular space (red star). (C) QCT25 pretreatment group: mildly thickened basement membrane (blue arrow) and mildly perivascular space (black star). (D) QCT50 pretreatment group: near-normal capillary structure with intact endothelium (EN), continuous basement membrane (black arrow), tight junction (green arrow).

Discussion

Ischemic stroke is the highly dominant cerebrovascular disease, causing localized brain tissue necrosis due to acute ischemia and hypoxia, posing a major global threat to health and quality of life across all ages (Duan et al., 2024). A common consequence of ischemic stroke is I/R injury, which causes additional damage upon restoration of blood flow due to oxidative stress, inflammation, and apoptosis (Guo et al., 2021). Current therapies for ischemic stroke are limited, as neuronal death in the infarct core leads to irreversible functional loss, driving increased research into the pathways of neuron death and the neuroprotective agents identification (Peng et al., 2024). Ischemic brain injury triggered by hypoxia hypoglycemia leads to inflammation, calcium overload, and oxidative damage, ultimately culminating in neuronal apoptosis (Chen et al., 2024). The BBB disturbance during ischemic stroke results in neurological dysfunction and hemorrhage, mainly due to the damage of tight junction proteins and adhesion molecules (Liu et al., 2021a). Multiple intracellular signaling pathways are implicated in brain I/R injury. Key pathways include EGFR/PI3K/Akt (Yu et al., 2016; Zhou et al., 2015), Akt/GSK3β (Duan et al., 2025), and Src/MMP9 (Li et al., 2024c; Sun et al., 2021), all of which represent potential therapeutic targets for attenuating I/R-induced neuronal damage. QCT is a flavonoid widely distributed in foods like green tea, onion, pepper, and apples, and it is known for its antioxidant, anti-inflammatory, antiapoptotic, and neuroprotective activities (Li et al., 2025b; Yang et al., 2025). QCT reduces cerebral infarct volume, decreases brain edema, protects BBB integrity, inhibits neuronal apoptosis, and improves neurological function in animal models of I/R by decreasing oxidative stress and inflammation (Li et al., 2023; Yang et al., 2022). Based on the multifactorial nature of I/R injury and the multi-targeted therapeutic potential of QCT, this study aimed to investigate the mechanisms underlying QCT-mediated neuroprotection in a cerebral I/R model.

Network pharmacology integrates data, metadata target prediction analysis, and simulations to map drug-target-pathway relationships and reveal drug-target interactions. At the same time, molecular docking, a key tool in drug discovery, simulates drug-receptor binding to aid in novel drug design (Lv et al., 2022). Using this approach, in the present study, QCT exerts its protective effect on ischemic brain injury through targets such as EGFR, Src, AKT1, GSK3β, and MMP9. Similarly, Li et al. (2023) reported identification of 28 common targets, including AKT1, EGFR, Src, and MMP9, through which QCT may modulate microglia/macrophage polarization, neuroinflammation, apoptosis, and oxidative stress after cerebral I/R injury Docking scores and binding energies indicate binding strength, with scores ≤ −5 suggesting potential activity; lower binding energy reflects stronger binding affinity (Liu et al., 2024b). In this study, molecular docking established that QCT's strong binding affinity (−10.01 to −6.120 kcal/mol) to MMP9, AKT1, GSK3β, EGFR, and Src supported its role as a multi-target agent. Similarly, Li et al. (2025b) reported that QCT can alleviate neurodegenerative disorders

through multiple therapeutic pathways. Neurogenesis is regulated by multiple factors, with epidermal growth factor (EGF) signaling being crucial for neural stem cell proliferation and dedifferentiation (Chen et al., 2019). The transmembrane EGFR is a tyrosine kinase receptor that plays a key role in ischemic injury by activating downstream pathways like PI3K/AKT upon EGF binding (Zhou et al., 2015; Li et al., 2025b). Gou et al. (2020) reported that the PI3K/AKT signaling pathway influences neurogenesis by regulating transcription factor activation and gene expression, with AKT as a PI3K effector, playing a pivotal role in apoptotic hippocampus neuronal death following cerebral ischemia. The serine/threonine kinase AKT stimulates survival and inhibits cell death (Duan et al., 2025). Among its three isoforms (AKT1, AKT2, and AKT3), AKT1 is particularly prevalent in the brain (Gao et al., 2022). AKT phosphorylation is crucial for activating the AKT signaling pathway (Liu et al., 2024a). The inhibition of AKT activity is linked to neuronal death after cerebral I/R, whereas enhanced AKT activity promotes antiapoptotic and neuroprotective effects (Yu et al., 2016).

In this study, I/R injury led to elevated EGFR expression but reduced total AKT and phosphorylated forms (p-EGFR, p-AKT), consistent with prior findings showing decreased p-AKT in hypoxic-ischemic conditions (Liu et al., 2021b; Liu et al., 2024a). Moreover, high serum AKT levels in the brain I/R group were reported by Fang et al. (2024). However, transient EGFR activation can be neuroprotective, sustained activation due to severe injury can lead to neurotoxic effects (Nakano et al., 2023). Conversely, QCT pretreatment (dose-dependent) decreased EGFR expression, while increasing p-EGFR, AKT, and p-AKT levels in a dose-dependent effect, indicating enhanced EGFR/AKT pathway activation in the I/R group. Activating EGFR can reduce cerebral I/R injury, suggesting EGFR's signaling protective role (Tang et al., 2018). EGFR stimulates cell proliferation, improves neural precursor cell proliferation in vivo, and reduces brain damage in models of ischemic stroke (Chen et al., 2019). An increase in tyrosine 845 phosphorylation of EGFR and EGFR protein expression was noticed in the hippocampus 24 hours after I/R in rats treated with delta opioids (Chen et al., 2021). QCT dose-dependently promotes phosphorylation of AKT and PI3K, contributing to protection against neurological outcomes following ischemic stroke (Li et al., 2023). AKT1 activation can reduce I/R injury in the kidneys, liver, and heart (Gao et al., 2022). Ruan et al. (2021) reported that metformin triggers the PI3K/AKT signaling pathway, inhibiting inflammation, oxidative stress, and apoptosis while enhancing PI3K phosphorylation to reduce neurological deficits caused by I/R damage. EGFR /PI3K/AKT activation stimulates angiogenesis, cell survival, proliferation, differentiation, and migration, reducing apoptosis and necrosis and offering neuroprotection after ischemic injury (Tang et al., 2018; Chen et al., 2020; Xu et al., 2023).

Molecular docking is a vital computational tool in drug design that predicts ligand binding modes, screens potential drug candidates, and estimates binding affinities with target proteins(Guedes et al., 2014). In the present study, the high binding activity of QCT with EGFR and AKT is consistent with previous studies (Zu et al., 2021; Li et al., 2025b). QCT may exert neuroprotective effects by modulating EGFR-mediated MAPK signaling, which regulates angiogenesis, neuronal survival, and repair, highlighting its potential to promote recovery and cognitive improvement (Zu et al., 2021). Therefore, QCT's ability to activate EGFR/AKT suggests a mechanism by which it could counteract the negative effects of I/R injury on these pathways.

GSK3β, another serine/threonine kinase greatly expressed in the CNS, is vital in regulating gene expression and cell division and differentiation (Kisoh et al., 2017; Duan et al., 2025). GSK3β is regulated primarily through inactivation *via* phosphorylation at specific residues (Song et al., 2015). The AKT pathway, acting upstream of GSK3β (Chen et al., 2018), controls its signaling activity by inhibiting GSK3β through phosphorylation

(Kisoh et al., 2017). In the current study, the I/R untreated group exhibited increased GSK3β levels, but decreased levels of its phosphorylated form (p-GSK3β), consistent with prior reports of increased GSK3β during cerebral ischemia (Zhang et al., 2022c) and reduced p-GSK3β/GSK3β ratios in I/R model (Liu et al., 2021b). The AKT inactivation and GSK3β activation are linked to neuronal apoptosis (Ambacher et al., 2012).

QCT alleviates Parkinson's disease pathology by triggering the PI3K/AKT/GSK3β activity, suppressing neuroinflammation, and preventing apoptosis, making it a promising multi-target therapeutic agent (Li et al., 2025b). In the present study, pretreatment with different doses of QCT led to a dose-dependent reduction in GSK3β levels and increased p-GSK3β expression. Molecular docking further revealed strong binding affinities between QCT and AKT and GSK3β, consistent with the findings of Li et al. (2025b). The QCT activates PI3K/Akt/GSK-3β pathway and inhibits GSK-3β activity, thereby reducing oxidative stress and suppressing neuronal apoptosis (Li et al., 2025b). QCT pretreatment downregulated the expression of GSK3β compared to the I/R group (Jin et al., 2019). AKT phosphorylation enhances glucose metabolism and energy production post-ischemia, while activation of the downstream AKT/GSK3β pathway attenuates oxidative stress, neuron apoptosis, and BBB damage, collectively exerting neuroprotection effect (Ajzashokouhi et al., 2023; Duan et al., 2025; Liu et al., 2024a). EGFR phosphorylates triggers AKT and GSK3β activity and terminates in apoptosis during oxidative stress and axonal injury (Mansour et al., 2022). GSK3β inhibition significantly alleviates post-stroke damage by reducing neurological deficits, promoting neuronal survival, and decreasing infarct volume (Zhang et al., 2022c). Similarly, tanshinone I exerts neuroprotective activity by activating the AKT/GSK3β pathway (Liu et al., 2021b), and the Ac2-26 peptide reduces neuronal loss *via* the AKT1/GSK3β pathway (Ju et al., 2024). GSK-3β inhibition reduces BBB permeability and apoptosis in I/R model (Liu et al., 2020). These results suggest that QCT's overall effect on GSK3β in I/R injury is likely mediated through its upstream activation of AKT, leading to GSK3β inactivation and subsequent neuroprotection.

P-glycoprotein (P-gp), a key BBB transporter on endothelial cells; in cerebral ischemia, Src signaling reduces P-gp function and promotes MMP-9 expression, while Src inhibition restores MMP9 regular expression, increases tight junction proteins, and improves BBB integrity (Li et al., 2024c). The Src family of non-receptor tyrosine kinases, broadly distributed in the brain, have an important role in gene transcription, adhesion, cell division, and angiogenesis (Yan et al., 2022; Zan et al., 2014). Src is activated by bioactive molecules released during hypoxia, promoting apoptosis (Loginova et al., 2022; Zhang et al., 2024b). In the current study, an increased level of Src in the I/R untreated group was recorded, aligning with reports of excessive Src activation in neurons after ischemic insults (Yan et al., 2022; Li et al., 2024a; Tang et al., 2024). Src activation promotes tyrosine phosphorylation of VE-cadherin, weakening cell junctions and increasing vascular permeability (Tang et al., 2023), and it is activated under ischemic conditions, promoting inflammation and BBB disruption (Liu et al., 2021a). Src overexpression worsened brain damage and cognitive impairment caused by the I/R model by triggering the MAPK signaling pathway and increasing its related proteins' phosphorylation (Li et al., 2022). In the present study, pretreatment with QCT reduced Src levels in a dose-dependent way.

The molecular docking result showed strong binding between QCT and Src binding sites. QCT can protect against cerebral ischemic injury by modulating BBB integrity and the Src/P-gp/MMP9 signaling pathway (Li et al., 2024c). Molecular docking and dynamics simulations showed stable, tight binding of QCT to Src (Li et al., 2024c). The inhibition of Src may offer a promising future therapy for ischemic brain injury (Loginova et al., 2022). Notably, the Y845 site on EGFR is a major phosphorylation site for Src, indicating a direct molecular link between these two signaling proteins (Zhou et al., 2015). EGFR

phosphorylation, phosphorylating Src, and inhibiting apoptosis suggest that EGFR activation is crucial in preventing neuronal apoptosis after ischemic injury (Ishizawar and Parsons, 2004; Li et al., 2024a). Inhibition of Src kinase can reduce hypoxia-induced apoptosis and brain damage and enhance survival and neurological outcomes in hypoxic models and I/R (Loginova et al., 2022; Yan et al., 2022). Using Src inhibitors has significantly reduced Src expression, cerebral edema, infarct size, and neurological dysfunction in vascular cognitive models (Gao et al., 2024). MMP-9 is a gelatinase B expressed by neurons, glia, and immune cells (Lakhan et al., 2013; Jin et al., 2019). A key protease induces extracellular matrix degradation and BBB integrity loss (Wu et al., 2022). MMP9 upregulation after brain injury increases BBB permeability, neuronal necrosis, apoptosis, and neurological dysfunction (Wu et al., 2020). In the current study, the I/R untreated group exhibited increased MMP9 levels and damaged capillary endothelium and tight junction. Similarly, several studies reported that inhibition of the AKT/GSK3β signaling triggers MMP9 upregulation and increased BBB permeability by damaging extracellular matrix components and tight junction-related proteins, thus worsening brain I/R injury (Lakhan et al., 2013; Jin et al., 2019; Jiao-Yan et al., 2021).

In the current study, QCT pretreatment markedly decreased MMP9 expression in comparison to the I/R untreated group while preserving the tight junction's capillaries, thus reducing I/R injury. Molecular docking revealed the highest QCT–MMP9 affinity, suggesting that MMP9 is a strong molecular target for QCT. These results suggest that QCT's neuroprotection against BBB dysfunction involves inhibiting MMP-9 and restoring tight junction proteins (Jin et al., 2019). QCT protects against BBB disruption during focal ischemia by reducing brain edema, inhibiting MMP9 activity, and lowering oxidative stress, thereby preserving extracellular matrix integrity and improving functional outcomes; these findings suggest its potential as a neuroprotective agent in focal ischemic stroke (Lee et al., 2011). Similarly, preventive salvianolic acid A treatment attenuates BBB injury and tight junction damage caused by ischemic stroke by reducing Src and MMP9 expression (Liu et al., 2021a). These results suggest that QCT's effect on the brain barrier in I/R injury is likely attributed to the inactivation of Src and MMP9 and subsequent neuroprotection. BBB disruption, commonly associated with stroke, contributes to the progression of cerebral ischemic injury, as it functions as a key component of the multicellular neurovascular unit involved in the pathophysiology of I/R damage (Jiao-Yan et al., 2021). The integrity of the BBB is important in protecting neurons from harmful substances (Jin et al., 2019). In ischemic stroke, reduced blood flow leads to oxygen and nutrient deprivation, causing brain cell damage, BBB disruption, inflammation, oxidative stress, and activation of enzymes like MMP9 that degrade tight junctions and worsen brain injury (Jin et al., 2019; Liu et al., 2021a).

In the present study, blood capillaries of the I/R untreated group exhibited congestion, perivascular edema, along with endothelium and junction damage. Similarly, Zhang et al. (2022a) reported blood vessels congestion, and perivascular edema marking BBB disturbance. The endothelium is the key component of the BBB; its deterioration marks ischemic cerebrovascular disease and worsens tissue injury in acute ischemic stroke (Liu et al., 2021a). The capillary endothelial cells showed swelling and irregular thickening (Jin et al., 2019). Endothelial cells' tight junctions are important in controlling BBB permeability, and I/R damage degrades tight junction proteins and junction adhesion molecules, leading to disruption of the BBB and causing hemorrhage (Jin et al., 2019; Li et al., 2024c). In the current study, QCT pretreatment preserved blood capillary integrity after I/R injury, as evidenced by reduced perivascular space, less endothelial thickening, and maintained tight junctions, with more pronounced effects observed at the 50 mg/kg dose. In the QCT-treated group, endothelial cells exhibited a more flattened morphology with decreased vesicle and vacuole formation, along with diminished cellular swelling; additionally, QCT suppressed the widening of

the perivascular space, disruption of tight junctions, and capillary endothelial ultrastructural damage after cerebral I/R injury (Jin et al., 2019).

QCT preserves BBB following cerebral ischemia by reducing brain edema, preserving extracellular matrix integrity through MMP9 inhibition, mitigating redox imbalance, and reducing bad neurological outcomes (Lee et al., 2011). QCT can elevate the levels of tight junction proteins in the BBB, suggesting its role in maintaining BBB integrity (Li et al., 2021). These results suggest that QCT preserves BBB integrity after ischemia by reducing endothelial damage, preventing MMP9 increase, mitigating oxidative stress, and maintaining tight junctions.

In the present study, I/R injury resulted neuron appeared shrunken with intensely stained cytoplasm and nuclei similar to Weng et al. (2023) observation. Similar "dark stained neurons" are seen in cortex of I/R models, reflecting acute neuronal necrosis (Li et al., 2025a). I/R injury resulted in cellular shrinkage, mitochondrial swelling, ER dilation, and nuclear chromatin condensation. Cortical neuronal mitochondria under I/R conditions exhibit swelling and increased electron density, followed by matrix disintegration (Solenski et al., 2002). Hypoxic-ischemic brain damage is associated with mitochondrial alterations, including reduced size, loss or absence of cristae, and rupture of the outer mitochondrial membrane (Lin et al., 2022). By 24–48 h reperfusion, hippocampal neurons had swollen, dark mitochondria with no identifiable cristae and dilated rough ER cisternae forming vacuoles (Pagnussat et al., 2007). Ischemic neurons display ER stress (dilated cisternae, detached ribosomes) and mitochondrial vacuolization (Yang et al., 2025). Inhibition of the PI3K/AKT pathway induces mitochondrial transformation from elongated to rounded shapes, contributing to oxidative stress-induced mitochondrial damage (Li et al., 2024b). Nonphosphorylated GSK-3β protects mitochondria during I/R, but ER stress reduce GSK-3β activity leading to increased mitochondria membrane permeability trigger cytochrome-c release and cell death (Zhang et al., 2024a). Mitochondrial oxidative stress leads to mitochondrial fission dysfunction and neuronal apoptosis (Meng et al., 2025). Persistent disruption in protein folding within the endoplasmic reticulum due to pathological factors leads to the buildup of misfolded proteins, ER stress, triggering degradation pathways, and if unresolved, results in cell death (González et al., 2022). I/R-induced oxidative stress can trigger ER stress, contributing to endothelial cell injury by disrupting protein folding, activating the unfolded protein response, and impairing antioxidant defenses, ultimately leading to apoptosis (Li et al., 2021). In the I/R group, hippocampal neurons showed contracted, triangular, or highly irregular cell bodies with dark-stained cytoplasm, pyknotic nuclei, chromatin clumping, damaged nuclear membranes and organelles, and vacuole formation (Guo et al., 2021). Ischemic brain neurons showed prominent apoptotic characteristics and hyperpigmentation (Mahyar et al., 2025). Prolonged ER stress, triggered by I/R injury, activates the unfolded protein response and pro-apoptotic pathways, eventually leading to neuron death (Yang et al., 2025).

In cerebral ischemic injury, QCT reduces brain edema, protects the BBB, inhibits neuronal apoptosis, and shifts microglia/macrophages from pro-inflammatory to anti-inflammatory activities (Li et al., 2023). A previous study highlight QCT's potential as a neuroprotective agent for mitigating early brain damage and improving cognitive outcomes following ischemic stroke (Ardianto et al., 2023). QCT pretreatment in I/R injury preserved mitochondrial and ER structures and maintained ribosome density on the ER membrane (Yang et al., 2025). QCT supports neuronal health and function by maintaining mitochondrial integrity, which is essential for energy production and cell survival(Mahyar et al., 2025). TEM on cortical penumbra neurons in hyperglycemic I/R rats found that QCT restored ER and mitochondrial structure (Yang et al., 2025). QCT pretreatment improves the preservation of neuronal structures, particularly in vulnerable regions like hippocampal neurons, which are highly

susceptible to ischemia (Mahyar et al., 2025). Akt translocates to mitochondria where it inhibits apoptosis by phosphorylating proapoptotic proteins, preventing mitochondrial outer membrane permeabilization, and preserving mitochondrial integrity (Yu and Miyamoto, 2021). QCT protects neurons from I/R injury by preserving mitochondrial and ER structure, reducing apoptosis, maintaining BBB integrity, and supporting energy production to enhance neuronal survival.

Conclusion

In cerebral ischemia, reduced EGFR activation impairs AKT phosphorylation, leading to persistent GSK-3β activity, which triggers pro-apoptotic signaling, chromatin condensation, and neuronal death. Concurrently, ER stress diminishes GSK-3β's protective mitochondrial role, increasing mitochondrial membrane permeability, cytochrome-c release, and apoptosis. This mitochondrial damage is further intensified by prolonged ER stress, which activates the unfolded protein response and exacerbating neuronal loss. In parallel, I/R activate Src kinase, upregulating MMP9 expression, which degrades extracellular matrix and tight junction proteins, resulting in BBB disruption, perivascular edema, and vascular leakage. These interconnected disruptions in EGFR/AKT/GSK3β and Src/MMP-9 signaling pathways collectively contribute to neuronal apoptosis and cerebrovascular pathology (Fig. 10). Pretreatment with QCT reverses these effects by restoring EGFR/AKT signaling, inhibiting GSK-3β overactivation, and suppressing Src/MMP-9 expression. Additionally, phosphorylated AKT translocates to mitochondria, where it blocks apoptosis by inactivating pro-apoptotic proteins and maintaining mitochondrial membrane integrity. Through these combined actions, QCT preserves neuronal function, maintains BBB integrity, and improves neurological outcomes following ischemia/reperfusion injury (Fig. 10). These effects are dose-dependent, with greater protection at 50 mg/kg. Further studies are required to define the optimal dose and fully clarify the underlying mechanisms. Thus, QCT improves neurological outcomes and holds promise as a therapeutic agent for ischemic stroke.

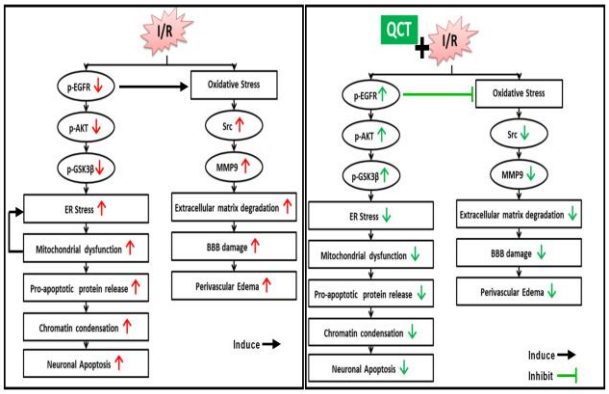

Fig. 10. Proposed molecular mechanism of ischemia-induced neuronal and vascular injury and the ameliorative effect QCT.

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