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Original article

Quercetin activates lysosomal function to ameliorate neuroinflammation and cognitive deficits in chronic cerebral hypoperfusion

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ABSTRACT

Chronic cerebral hypoperfusion (CCH) is a major contributor to vascular cognitive impairment (VCI), often resulting in progressive learning and memory deficits. Quercetin (Qu), a natural flavonoid with potent anti-inflammatory and antioxidant properties, demonstrates neuroprotective potential against ischemic injury, yet its mechanism of action remains incompletely understood. In this study, a rat model of CCH was established by bilateral common carotid artery occlusion, followed by Qu administration at doses of 20, 40, and 80 mg·kg^{-1}. In parallel, HT22 cells subjected to oxygen-glucose deprivation (OGD) were treated with Qu at 5, 10, or 20 μmol·L^{-1}. Behavioral tests, histological staining, ELISA, and Western blot were used to assess cognitive performance, neuronal injury, lysosomal activity, NF-κB pathway activation, and inflammatory cytokine levels. Hippocampal RNA sequencing was also performed. Our results demonstrate that Qu greatly improved cognitive performance and attenuated neuronal damage in both the cerebral cortex and hippocampus. Mechanistically, Qu improved lysosomal function and suppressed NF-κB-mediated neuroinflammatory response, leading to less production of pro-inflammatory cytokines. Notably, co-administration of a lysosomal inhibitor attenuated the neuroprotective effects of Qu, underscoring the functional importance of lysosomal activation. In conclusion, Qu alleviates CCH-induced cognitive decline and neurodegeneration by restoring lysosomal function and inhibiting NF-κB-driven neuroinflammation. These findings support the therapeutic potential of Qu for VCI and provide a rationale for its further development as a neuroprotective agent.

1. Introduction

Stroke is a leading cause of disability and death worldwide. It also contributes to long-term disability and vascular cognitive impairment (VCI) [1, 2]. People who have had a stroke are more likely to develop cognitive decline. As a result, VCI incidence increases [3]. This epidemiological shift imposes substantial burdens on healthcare systems and societal resources. It also makes patient management and social integration more difficult [2, 4, 5]. Current evidence indicates that systemic and neuro-inflammation resulting from chronic cerebral hypoperfusion (CCH) are widely recognized as major causes of ischemic brain damage [1, 3, 6].

Lysosomes are the main digestive compartments inside cells. They maintain homeostasis by breaking down damaged organelles and clearing abnormal protein aggregates [7, 8]. When lysosomes do not work properly, this dysfunction is seen in many neurodegenerative diseases and also contributes to lasting brain inflammation [9-11]. Impaired lysosomal function makes it harder to remove neurotoxic proteins, which worsens inflammatory conditions [12, 13]. This cascade speeds up neuronal injury and apoptosis. It creates a self-perpetuating loop that drives disease progression [14].

During CCH, lysosomal function declines [15]. This decline comes with impaired potassium channels and a drop intraluminal pH. These changes suppress or inactivate acidic hydrolase activity [16]. At the same time, oxidative stress and calcium overload damage the lysosomal membrane, increasing its permeability [17]. We therefore think that enhancing lysosomal function may help reduce CCH-induced damage.

Qu is a flavonoid that has drawn attention for its antioxidant and anti-inflammatory properties. It protects the heart and may have anticancer effects [18]. Recent studies show that Qu improves cognition and memory, particularly in individuals with Alzheimer's disease [19]. It also protects neurons in models of cerebral ischemia and traumatic brain injury [20]. These benefits come mainly from Qu's bioactive metabolites. These metabolites reduce oxidative stress, promote neuronal repair [21], and inhibit the

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NF-κB signaling [22].

We previously reported that Qu can enhance lysosomal function [23]. But it is unclear whether this mechanism is essential to cognitive recovery in VCI. To address this question, we designed the present study. We asked whether Qu activates lysosomes and reduces neuroinflammation in a validated VCI model. Our goal was to understand whether Qu could improve cognitive and memory functions and to support its future clinical application.

2. Materials and methods

2.1. Animals and cell culture

Male Sprague Dawley rats with a weight range of 230−250g were used for this experiment, with a sample size of n = 8 per group. Rats were acclimated to standard housing conditions for one week at a controlled temperature of 24 ± 2 °C, and they had free access to standard laboratory food and water. Also, HT22 mouse hippocampal neuronal cells were purchased from Wuhan Hycell Biotech and cultured in DMEM (Dulbecco's Modified Eagle Medium) obtained from Gibco, USA. The culture was supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, USA) and 1% streptomycin/penicillin (Beyotime), and the CO_2 concentration was maintained at 5%, with a constant temperature of 37 °C. All animal experimental procedures were approved by the Animal Care and Welfare Committee of Guangxi Medical University (Approval No. 201906013)

2.2. Antibodies and reagents

Qu (Q4951), Propidium iodide (P4170), Bafilomycin-A1 (Baf-A1) (19-148) and Chloroquine (CQ) (C6628) were all bought from Sigma-Aldrich (St. Louis, MO). LysoTracker red DND-99 (L7528), DQ red BSA (D12051) were purchased from Invitrogen. Dimethyl sulfoxide (DMSO) was obtained from Solarbio. NF-κB p65 (9936T), Cathepsin B (CTSB) (#31718), ATP6V1B2 (#14617) were obtained from Cell Signaling Technology, ATP6V1A (ab137574) was purchased from Abcam, Cathepsin D (CTSD) (SC-6486) was purchased from Santa Cruz, α-tubulin (T5168) was purchased from Sigma-Aldrich. HRP-conjugated secondary antibodies against rabbit (#7074), mouse (#7076), and goat (#91196) were purchased from Cell Signaling Technology.

2.3. Chronic cerebral hypoperfusion model establishment and treatment

Bilateral Common Carotid Arteries (BCCAO) method was used to create the CCH model. Sodium pentobarbital (50 mg·kg^{-1}) was administered *via* intraperitoneal injection for anesthesia. After making a midline incision on the neck, the BCCAO were separated from surrounding muscles and adjacent neural bundles, then both common carotid arteries were ligated with 3–0 silk sutures. Sham group had a cut in the front part but did not tie up the common carotid artery. Core body temperature was maintained at 37.5 °C using a heating pad during the entire operation and anesthesia recovery period to reduce mortality rate. Animals were randomly divided into six groups as follows: Sham group received saline without surgery; CCH group underwent BCCAO surgery; CCH + DMSO group underwent BCCAO followed by DMSO administration (DMSO concentration: 5% in saline, injection volume: 5 mL·kg^{-1}); and three CCH + Qu treatment groups underwent BCCAO and subsequently received Qu (dissolved in DMSO) via intraperitoneal injection at doses of 20, 40, and 80 mg·kg^{-1} for 2 weeks after surgery.

2.4. Oxygen and glucose deprivation

Glucose-free DMEM medium was used to simulate ischemic conditions in the cells, which had been pre-treated with varying concentrations of Qu (5, 10, and 20 μmol·L^{-1}) for 12 hours. Then the cell cultures were put into the condition of 95% N_2 and 5% CO_2 for 40 minutes.

2.5. Morris water maze (MWM)

Rats underwent five consecutive days of training with three trials per day. In each trial, they were placed at one of four starting points and allowed to swim for 120 seconds to locate a hidden platform. On day 6, a probe test was performed to assess spatial reference memory. The platform was removed, and rats swam freely for 120 seconds. Escape latency, platform crossings, and time in the target quadrant were recorded.

2.6. Elevated plus maze (EPM) test

On day 49 after BCCAO surgery, animals were subjected to the EPM test. The apparatus consisted of two open arms (50 cm × 10 cm) and two closed arms (50 cm × 10 cm × 40 cm) arranged in a cross shape, elevated 50 cm above the floor. Each rat was placed on the central platform (10 cm × 10 cm) facing an open arm and allowed to explore freely for 5 minutes under dim light.

2.7. Open-field test

On the same day as the EPM test, animals underwent the open-field test. Each rat was placed at the center of the arena and allowed to explore for 5 minutes. The arena was cleaned with 5% ethanol between trials. The following parameters were recorded: time spent in the central area, rearing frequency, and grid crossings.

2.8. Tissue preparation

Rats were deeply anesthetized, then brains were taken out by perfusing them through the heart with 4% formaldehyde solution at pH 7.4 in phosphate buffer. After fixation in 4% paraformaldehyde for 48 hours, the samples were dehydrated through graded alcohols, and embedded in paraffin. Hippocampus was cut into 4μm coronal sections for H&E staining and Nissl staining using a 0.1% cresyl violet solution.

2.9. Lineage tracing and 3D imaging

Transgenic R26-DreER; Nestin-CreER; R26-GFP rats underwent BCCAO on day 0 to induce cerebral hypoperfusion. Immediately after surgery, animals received daily intraperitoneal injections of Qu (80 mg·kg^{-1}) or vehicle (DMSO-saline) throughout the study; sham-operated controls received saline alone. On day 28 after BCCAO, tamoxifen (TAM) was administered to trigger Dre-rox recombination and activate GFP labeling in neural stem cells and their progeny. Tissues were collected at specified endpoints for confocal microscopic examination. The 3D drift AO scanning process was similar to that previously described [24].

2.10. Measurement of cell viability and cytotoxicity

Cell viability and cytotoxicity were evaluated using CCK-8 and LDH release assays. For the CCK-8 assay, cells were seeded into 96-well plates at 3 × 10^3 cells per well. After treatments, CCK-8 reagent was added to the culture medium, and the plates were incubated at 37 °C for 1 hour. Then the absorbance was measured at 450 nm by microplate reader. The LDH reaction was performed according to the manufacturer's instructions, and absorbance was measured at 490 nm.

2.11. Propidium iodide (PI) staining

HT22 cells were cultured in 24-well plates and incubated overnight under a 5% CO_2 atmosphere at 37 °C. After collecting the cells, they were washed twice with ice-cold PBS and then incubated in the dark with PI solution (5 mmol·L^{-1}). The fluorescence intensities of 10 000 cells per sample were measured by flow cytometry (Beckman Coulter, South Carolina).

2.12. Enzyme-linked immunosorbent assay (ELISA)

TNF-α, IL-1β, IL-6 and IL-10 levels were measured in hippocampal tissue homogenates and cell culture supernatants using ELISA kits (Elabscience) following the manufacturer's instructions. Equal amounts of supernatant were added to each well of a 96-well plate, then optical density was read at 450 nm with a microplate reader. Protein concentrations were calculated according to the standard curve.

2.13. Western blotting

Total protein was extracted from HT22 cells or rat brain tissue using cell lysis buffer (Beyotime) and quantified with BCA kit (Beyotime). Normalized protein samples were subjected to SDS-PAGE gels, then transferred onto PVDF membranes (Bio-Rad). Membranes were blocked with 5% nonfat milk in Tris-buffered saline at room temperature for one hour, then incubated overnight at 4 °C with specific primary antibodies. After washing, membranes were incubated with secondary antibodies for two hours. Immunoreactive bands were visualized using an ECL detection kit.

2.14. qRT-PCR analysis

Total RNA was extracted from HT22 cells by TRIzol reagent according to the manufacturer's instructions. Then, the RNA was reverse transcribed with a reverse transcription system in a 20 μL reaction mixture. Quantitative RT-PCR (qRT-PCR) was carried out using gene-specific primers (Table 1) to determine the expression levels of IL-6, IL-1β, TNF-α and IL-10, which were normalized to GAPDH levels.

Table 1 Gene-specific primer sequences used for qRT-PCR.

Gene	Primers
TNF-α	Forward-5′GCGACGTGGAACTGGCAGAAG, Reverse-5′GAATGAGAAGAGGCTGAGACATAGGC
IL-6	Forward-5′AGACTTCCATCCAGTTGCCTTCTTG, Reverse-5′CATGTGTAATTAAGCCTCCGACTTGTG

2.15. RNA sequencing analysis

Total RNA was extracted from hippocampal tissues of CCH model rats and Qu-treated groups with TRIzol reagent. RNA quality was evaluated by NanoDrop spectrophotometer and agarose gel electrophoresis. Sequencing libraries were prepared using the NEBNext Ultra RNA Library Prep Kit. mRNA was enriched by oligo beads, fragmented, and reverse-transcribed into cDNA. Libraries were sequenced on an Illumina NovaSeq 6000 platform. Raw data were processed using FastQC for quality control. Clean reads were aligned to the rat reference genome using HISAT2. Gene expression levels were quantified as FPKM, and differential expression analysis was carried out with DESeq2. Functional enrichment analysis was done through Gene Ontology terms.

2.16. Dual-luciferase reporter assay

HT22 cells were co-transfected with Luc-NF-κB and Renilla for 48 hours. Luciferase activity was measured by the dual-luciferase reporter assay system (Promega), according to the manufacturer's instructions, and firefly luciferase activity was normalized to Renilla luciferase activity.

2.17. LysoTracker Red staining

Lysosomal acidity was assessed through LysoTracker red and examined by flow cytometry. HT22 cells were incubated with 50 nmol·mL^{-1} LysoTracker red for 30 minutes, then washed and resuspended in PBS. The fluorescence intensities were measured at an excitation wavelength of 577 nm.

2.18. DQ-red BSA staining and confocal microscope

DQ-red BSA was used to evaluate the intra-lysosomal proteolysis, and cells were incubated for 1 hour at 37 °C with DQ-red BSA. Nuclei were stained by DAPI (Solarbio). Fluorescence intensities were taken and examined through confocal microscopy.

2.19. Statistical analysis

Statistical analysis was carried out by SPSS 26.0 software, and the results were shown as mean ± SD. Experimental group comparisons were made using Kruskal-Wallis tests, one-way ANOVAs, or Mann-Whitney tests where applicable, with a significance level of $P < 0.05$ determined through GraphPad Prism 8 software.

3. Results

3.1. Qu rescues spatial learning and memory deficits in CCH rats

To evaluate the possible neuroprotective effects of Qu on ischemic injury, we used the BCCAO model to induce CCH and examined spatial learning, memory, and behavioral conditions after Qu treatment (Fig. 1A). In the Morris water maze (MWM) test, both CCH and CCH + DMSO groups had longer escape latencies and path lengths after 5 days of training, which suggests serious spatial learning impairments (Fig. 1B). On the contrary, Qu treatment shortened escape latencies on day 6 (Fig. 1D), reduced the time to first platform crossing, and increased platform crossings, when compared to vehicle-treated CCH rats (Figs. 1C, 1E), indicating better retention of spatial memory after Qu treatment.

The open field test showed that CCH rats had motor impairment and anxiety-like behavior, such as less central area occupancy and exploration. Qu treatment restored central zone exploration and locomotor activity in a dose-dependent manner (Figs. 1F, 1H). Moreover, the rearing frequency was elevated in Qu-treated rats relative to CCH and vehicle controls (Fig. 1G), implying heightened exploratory motivation. The anxiolytic effect of Qu was further confirmed through EPM test. CCH rats spent less time in open arms, which is consistent with anxiety-like phenotypes (Fig. 1I). Qu reversed these behaviors (Figs. 1J–1K). These results show that Qu can reduce CCH-caused cognitive and anxiety-like deficits.

3.2. Qu alleviates neuropathological injury in the hippocampus of CCH rats

To further confirm the neuroprotective effect of Qu, we examined neuronal damage in the hippocampal CA1 area and cortex under ischemic injury conditions with H&E and Nissl staining. As shown in Fig. 2A, the CCH group had obvious neuronal degeneration such as pyknotic nuclei and shrinkage of cytoplasm compared to the normal morphology of the Sham group. Qu treatment dose-dependently alleviated these pathological changes. The Nissl staining confirmed these findings, showing a large decrease in apoptotic cells and an increase in Nissl bodies after Qu

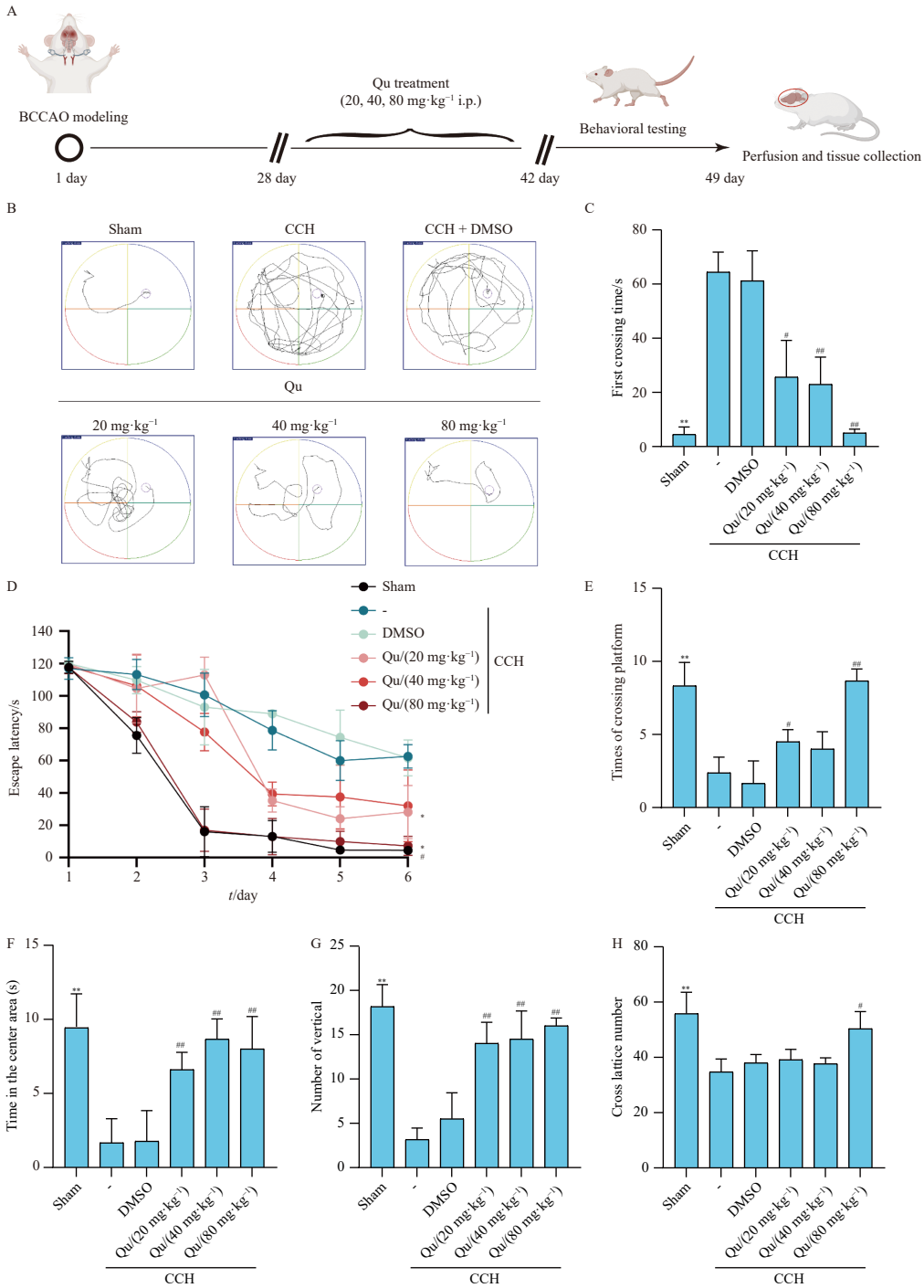
administration (Fig. 2B). We next explored Qu's neuroprotective mechanisms through an *in vitro* OGD model in HT22 cells. OGD greatly decreased cell viability; this decline was reversed by Qu at different concentrations (Fig. 2C). Consistently, Qu also reduced OGD-induced LDH release (Fig. 2D) and decreased cell death (Fig. 2E). All these results show that Qu provides strong protection for neurons from OGD injury.

3.3. Qu ameliorates CCH-induced deficits in dendritic development of adult-born hippocampal neurons

To assess whether Qu can affect hippocampal neurogenesis under CCH, we used the R26-DreER; Nestin-CreER; R26-GFP system with TAM induction to achieve permanent GFP labeling of Nestin + neural stem cells and their progeny (Figs. 3A–3B). Systematic morphological analysis showed that CCH greatly hindered the maturation of dendrites in the dentate gyrus, resulting in shorter total dendritic lengths and less complex branching structures. Qu treatment restored these structural parameters (Figs. 3C–3E), suggesting a strong protective effect of Qu on the development and integration of newborn neurons under chronic ischemic conditions.

3.4. Qu alleviates hippocampal neuroinflammation in CCH

In CCH, inflammation is well known to be an important mediator of tissue damage and neurodegeneration. In order to better understand the anti-inflammatory mechanisms behind Qu's neuroprotection, we looked into how it affected inflammatory cytokine expression in hippocampal neurons. Compared with the Sham group, CCH rats had higher levels of pro-inflammatory cytokines (IL-1β, TNF-α, and IL-6) and a significant decrease in the



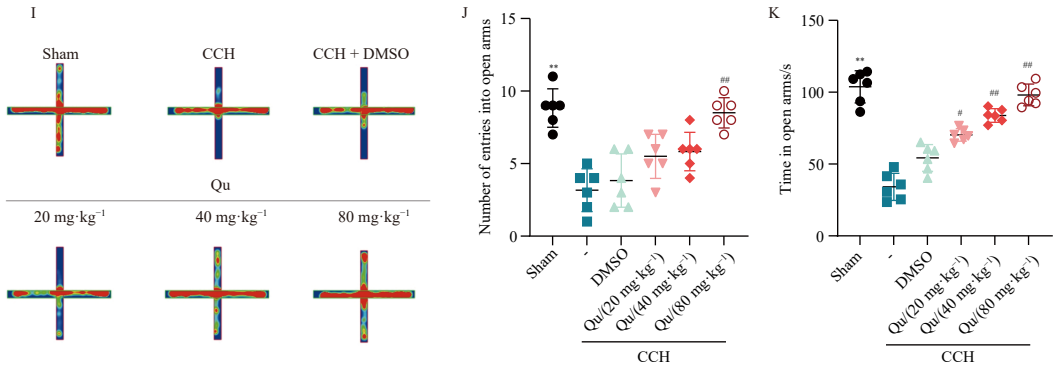


Fig. 1 Quercetin rescued spatial learning, memory, and behavioral deficits in CCH rats. (A) Sprague-Dawley rats underwent bilateral common carotid artery occlusion (BCCAO) surgery. After a 28-day model induction period, rats were treated with indicated doses of Quercetin (Qu) for 2 weeks. Cognitive function in the chronic cerebral hypoperfusion (CCH) animal model were assessed across Sham, CCH, CCH + DMSO, and CCH + Qu (20, 40, and 80 mg·kg^{-1}) groups by the following indicators: (B) Typical movement tracks, (C) Time to first platform crossing (seconds), (D) Escape latency (seconds), (E) The number of platform crossings, (F–H) Open field test: (F) Time spent in the central area, (G) Number of vertical movements, (H) Number of grid crossings. (I–K) Elevated plus-maze test: (I) Representative heatmaps, (J) Number of entries into open arms, (K) Time spent in open arms (seconds). Data are shown as mean ± SD; n = 8 per group. $^{**}P < 0.01$ *vs* CCH group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* CCH + DMSO group.

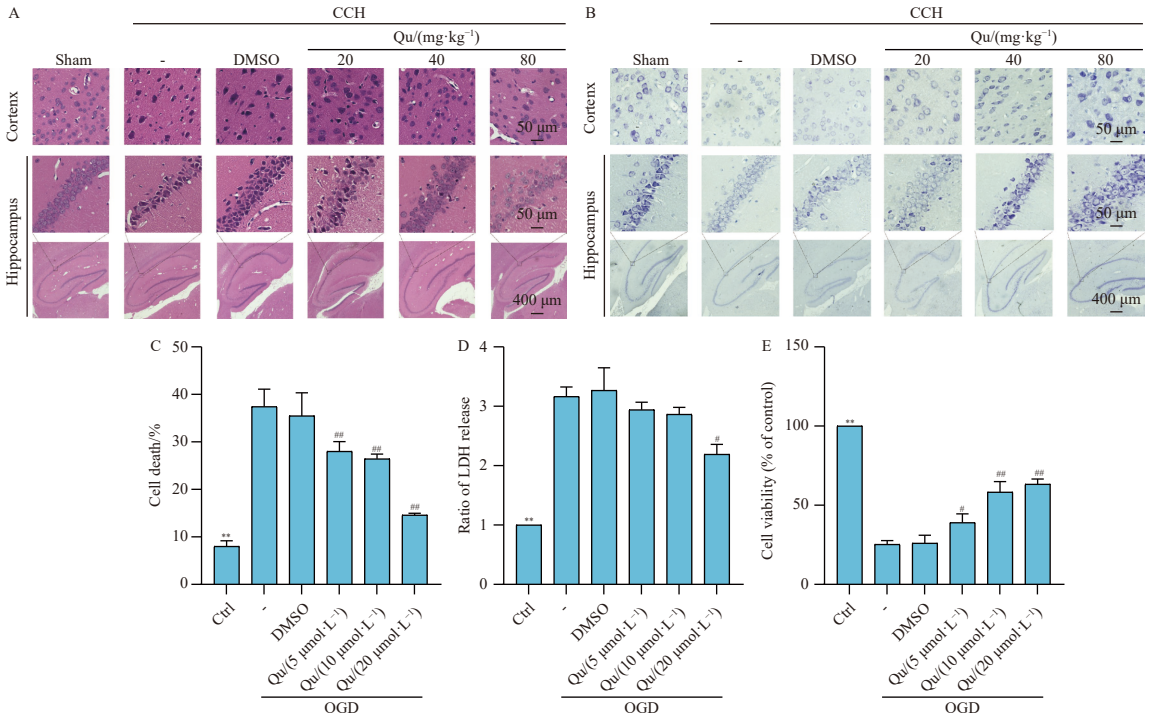


Fig. 2 Quercetin alleviates neuropathological injury in the hippocampus of CCH rats. (A–B) Neuronal damage in the hippocampal CA1 area and frontal cortex was assessed using H&E staining (A) and Nissl staining (B), with a scale bar of 50 μm (the middle and upper panel) and 400 μm (lower panel), respectively. HT22 cells were pretreated with Quercetin (Qu) for 24h, then subjected to oxygen-glucose deprivation (OGD), cell death was evaluated by CCK8 assay (C) and LDH release assay (D), and flow cytometry quantification of PI-positive cells (E). Results are shown as mean ± SD; n = 3. $^{**}P < 0.01$ *vs* OGD group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* OGD + DMSO group.

anti-inflammatory cytokine IL-10. Qu treatment greatly reversed these changes, inhibiting pro-inflammatory mediators and promoting anti-inflammatory cytokine production (Figs. 4A–4D). These findings suggest that Qu provides neuroprotection, at least in part by modulating the inflammatory response in CCH-induced neuronal injury.

Consistent with the *in vivo* findings, OGD-stimulated HT22 cells also showed increased secretion of IL-1β, TNF-α, and IL-6, and reduced IL-10 levels. Qu pretreatment dose-dependently normalized these cytokine levels (Figs. 4E–4H) and downregulated OGD-induced TNF-α and IL-6 mRNA expression level (Figs. 4I–4J). All these together show that Qu reduces neuroinflammation both *in vivo* and *in vitro*.

3.5. Qu inhibits neuroinflammation via the IκB/NF-κB pathway

To understand how Qu alleviates neuroinflammation, we looked at the NF-κB signaling pathway. Both CCH and CCH + DMSO groups showed noticeable rise in phosphorylated IκBα (p-IκBα) and p65 (p-p65) compared to Sham control. Qu treatment dose-dependently reduced these phosphorylation events (Figs. 5A–5B), indicating pathway inhibition.

Consistent with *in vivo* findings, OGD-stimulated HT22 cells also showed increased p-IκBα and p-p65 levels, which were concentration-dependently decreased by Qu treatment (Figs. 5C–5D). Dual-luciferase reporter assays further confirmed that Qu suppressed OGD-induced NF-κB transcriptional activation (Figs. 5E–5F). These results consistently suggest that Qu reduces neuroinflammation by inhibiting the NF-κB pathway.

3.6. Qu restores lysosomal gene expression in CCH

To explore how Qu alleviates CCH-induced neuroinflammation, we performed RNA sequencing and Transcriptome-wide analysis of hippocampal tissues from CCH rats (Fig. 6A). Qu treatment induced distinct molecular changes in gene expression

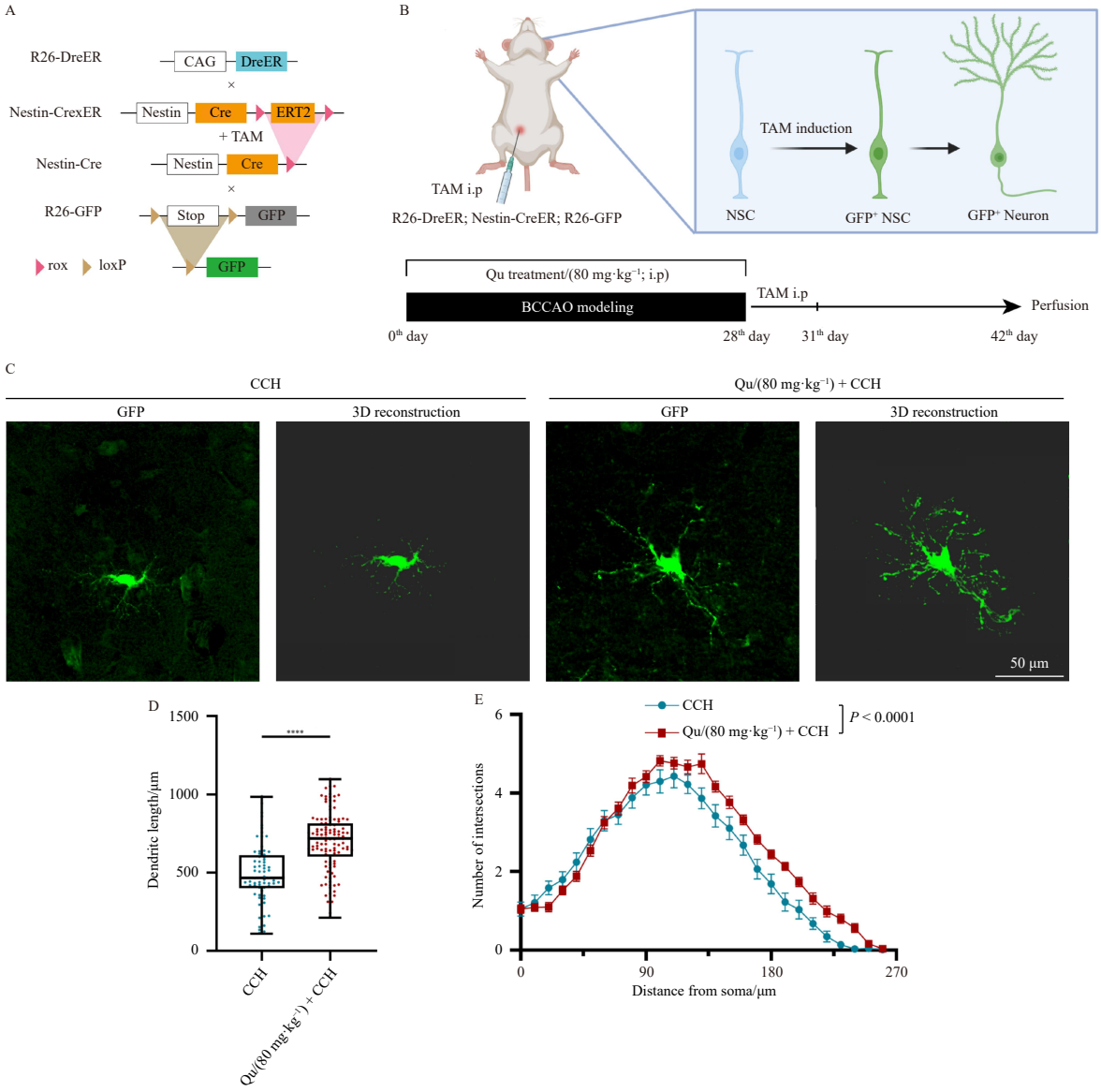


Fig. 3 Quercetin rescues CCH-induced deficits in adult-born hippocampal neurons. (A) Schematic showing the strategy for tracing cell cycles using dual recombinases. (B) Schematic showing the experimental design. (C) Representative images of GFP$^+$ newborn neurons in the adult DG of rats and their three-dimensional reconstruction images in the CCH group and quercetin (Qu) + CCH group. All scale bars are 50 μm. Quantification of the dendritic length (D) and Quantification of dendritic complexity (E) of GFP$^+$ newborn neurons in the adult DG of rats. Data are shown as mean ± SD. For CCH group, n = 67 neurons from 4 rats, for Qu + CCH group, n = 114 neurons from 4 rats. $^{****}P < 0.0001$ CCH *vs* Qu + CCH group.

(Fig. 6B). GO enrichment analysis identified upregulation of lysosome-associated genes as one of the most enriched terms (Fig. 6C). Therefore, we chose to focus on the lysosomal pathway. Heatmap visualization of the top 15 differentially expressed genes (DEGs) within this pathway showed consistent upregulation in the Qu-treated group (Fig. 6D).

Given the close functional connection between the lysosome and autophagy, we next examined whether this lysosomal activation was part of a broader autophagic response. Comparative analysis of lysosomal-related genes (36 genes) and autophagy-related genes (24 genes) showed only 6 overlapping genes (Fig. 6E). As shown in the heatmap of Fig. 6F, Qu treatment elevated lysosomal genes expression but failed to produce a upregulation of autophagy-specific genes or the 6 shared genes. This finding suggests that the transcriptional effect of Qu under CCH is specific to the lysosomal pathway.

3.7. Qu enhances lysosomal function in neuronal cells

To determine whether Qu enhances lysosomal function, we assessed lysosomal activity both *in vitro* and *in vivo*. In OGD-stimulated HT22 cells, LysoTracker Red and DQ-BSA staining showed a decrease in lysosomal activity (Figs. 7A–7C), which was also accompanied by the reduction of some important lysosomal markers such as ATP6V1A, ATP6V1B2, CTSB and CTSD. Qu administration could reverse the down-regulation of these lysosomal proteins caused by OGD in hippocampal tissue (Fig. 7D–7E). We further confirmed our findings using the CCH rat model. Western blot analysis of hippocampal tissues from CCH rats confirmed that Qu reversed the CCH-induced decrease in lysosomal markers (Figs. 7F–7G). These results show that Qu enhances lysosomal function under chronic hypoperfusion.

3.8. Lysosomal inhibition abolishes Qu-mediated neuroprotection

To determine whether lysosomal function is necessary for the neuroprotective effect of Qu, we used Baf-A1 and CQ to pharmacologically block lysosomal activity. LysoTracker Red and DQ-BSA staining were utilized to confirm the successful lysosomal inhibition (Figs. 8A–8C). Lysosomal inhibition greatly reduced anti-

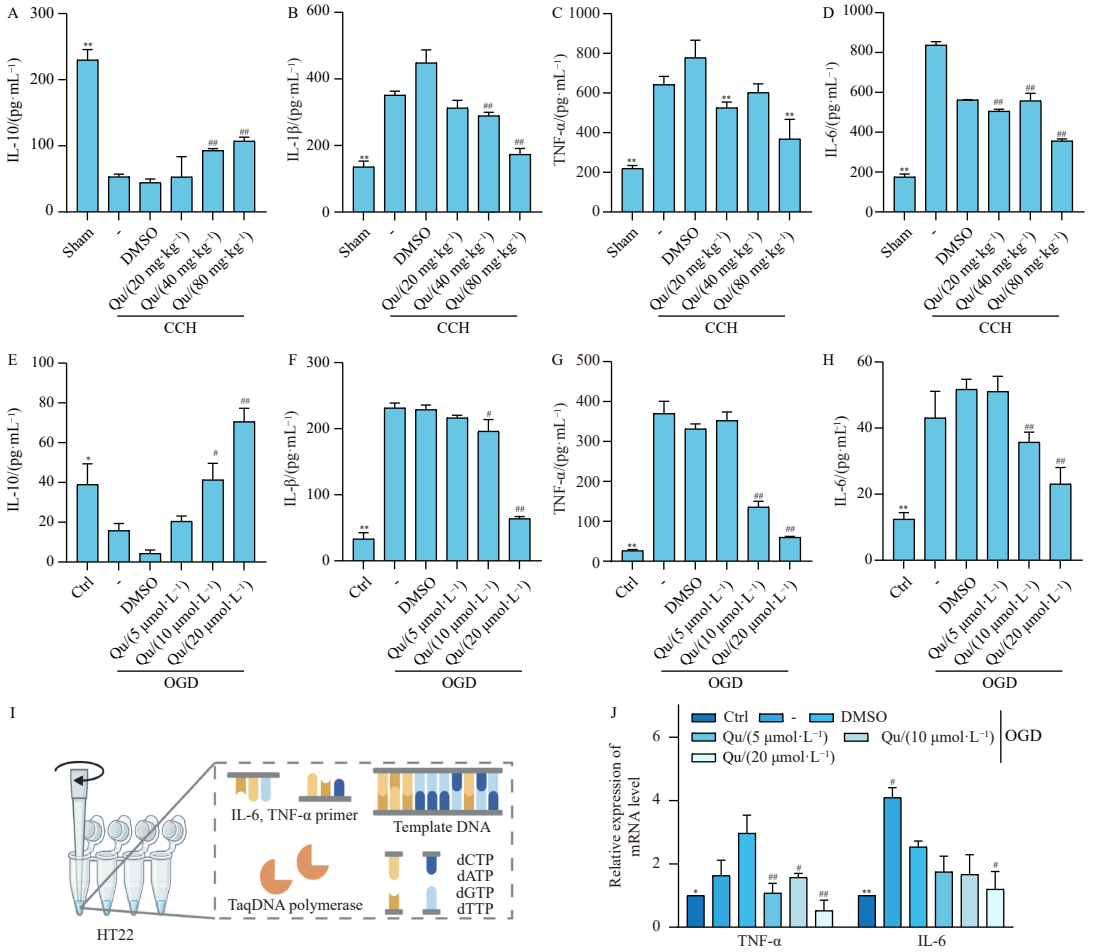
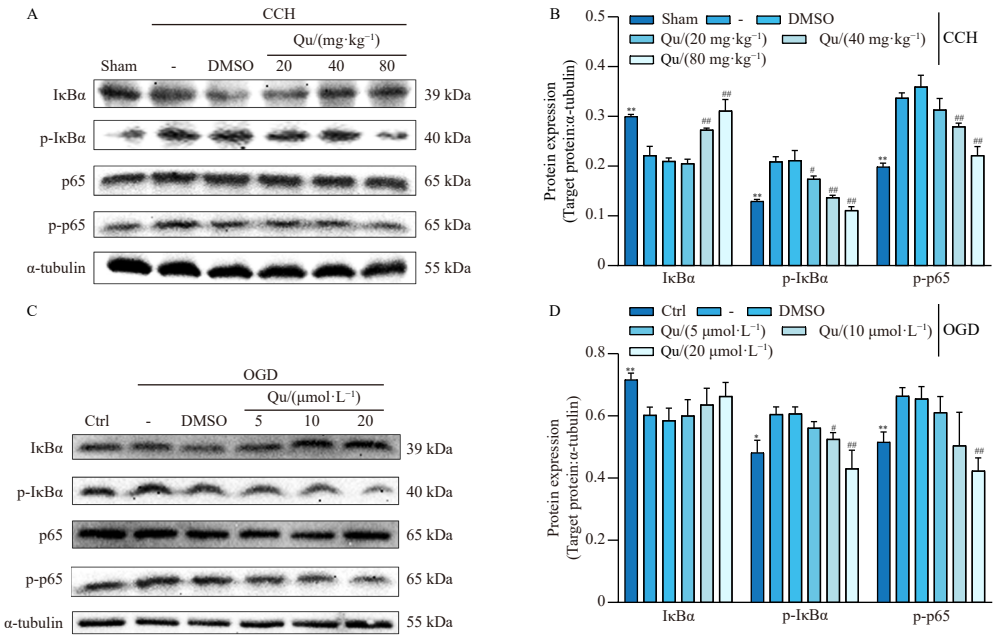


Fig. 4 Quercetin mitigates hippocampal neuroinflammation in Chronic cerebral hypoperfusion and oxygen-glucose deprivation conditions. (A–D) Effects of quercetin (Qu) on inflammation-related cytokines in the hippocampal area under chronic cerebral hypoperfusion (CCH) were determined by Western blot and ELISA: IL-10 (A), IL-1β (B), TNF-α (C), and IL-6 (D). ELISA data are shown as mean ± SD; $n = 3$. $^{**}P < 0.01$ *vs* CCH group; $^{\#\#}P < 0.01$ *vs* CCH + DMSO group. (E–H) HT22 cells were subjected to oxygen-glucose deprivation (OGD) and then treated with Qu. Levels of IL-10 (E), IL-1β (F), TNF-α (G), and IL-6 (H) were measured by ELISA. (I–J) mRNA levels of TNF-α and IL-6 were analyzed by qRT-PCR. Data are shown as mean ± SD; $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* the OGD group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* the OGD + DMSO group.

inflammatory effects of Qu. Specifically, lysosomal inhibition eliminated the increase in IL-10 and the decrease in TNF-α, IL-6, and IL-1β caused by Qu treatment (Figs. 8D–8G). At the same time, lysosomal inhibition also reversed Qu-mediated suppression of NF-κB (Figs. 8H–8I). Moreover, lysosomal inhibition blocked the protective effect of Qu on cell survival (Figs. 8J–8I). All these results show that improving lysosomal function is one key mechanism by which Qu alleviates neuroinflammation and



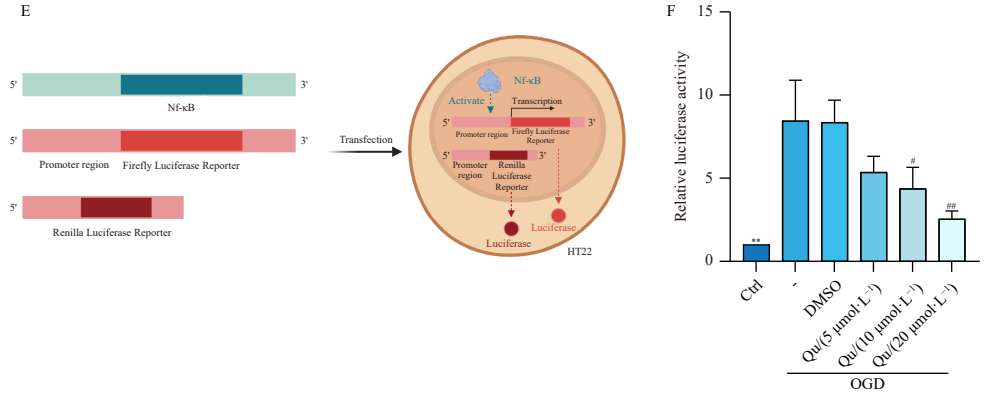


Fig. 5 Quercetin inhibits neuroinflammation by targeting the NF-κB signaling pathway. (A–B) Rats with chronic cerebral hypoperfusion (CCH) were treated with quercetin (Qu) at indicated doses (20, 40, 80 mg·kg^{-1}). Protein expression levels of NF-κB pathway-related proteins in hippocampal tissue were analyzed by Western blot (A), with quantitative analysis shown in (B). Data are presented as mean ± SD; n = 8, $^{*}P < 0.05$ $^{**}P < 0.01$ *vs* CCH group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* CCH + DMSO group. (C–D) HT22 cells were subjected to oxygen-glucose deprivation (OGD) and then treated with Qu at indicated concentrations. Protein expression levels of the same NF-κB pathway components were analyzed by Western blot (C), with quantitative analysis shown in (D). (E–F) After indicated treatments, relative NF-κB luciferase activity was measured by a dual-luciferase reporter assay. Data are shown as mean ± SD; n = 3, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* OGD group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* OGD + DMSO group.

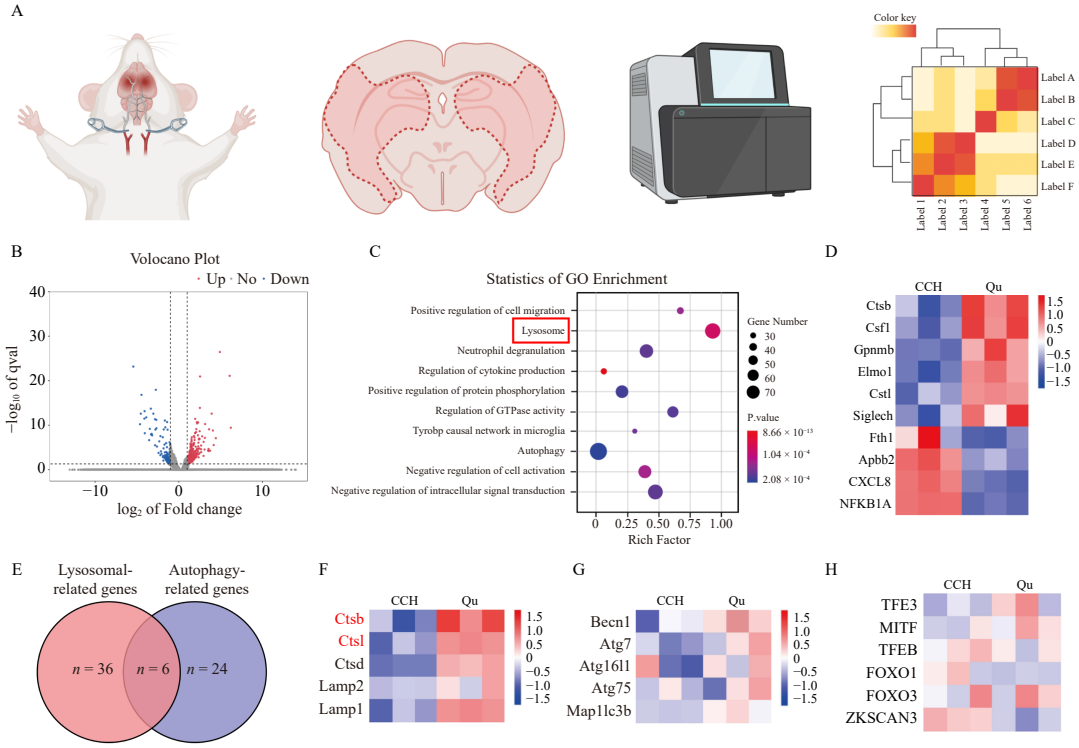


Fig. 6 Transcriptomic profiling reveals Quercetin enhances lysosomal gene expression in Chronic cerebral hypoperfusion model rats. (A) Experimental workflow of hippocampal tissue collection from chronic cerebral hypoperfusion (CCH), and Quercetin (Qu)-treated (80 mg·kg^{-1}) rats (n = 8/group), followed by RNA sequencing and bioinformatics analysis. (B) Volcano plot displays differentially expressed genes (DEGs) between CCH and Qu-treated group. (C) GO enrichment analysis of the top 10 biological processes. (D) Expression heatmap of the top 15 DEGs. (E) Venn diagram showing the intersection between lysosomal-related (36 genes) and autophagy-related (24 genes) genes, revealing 6 overlapping genes. (F–H) Heatmap shows expression patterns of the top 5 DEGs from the lysosomal pathway (F), the top 5 from the autophagy pathway (G), and the 6 overlapping genes (H).

promotes neuronal survival after ischemic injury.

4. Discussion

Our research provided evidence that Qu protects against CCH-induced cognitive impairment by enhancing lysosomal function and suppressing NF-κB-driven neuroinflammation (Fig. 9). These results place lysosomal activation as a potential upstream strategy to reduce neuroinflammation in VCI. Lysosomal dysfunction is increasingly recognized in neurodegenerative and cerebrovascular diseases [8, 11, 25]. Consistent with our previous work, we show that Qu improves the expression of lysosomal-associated proteins and enhances acidification [23, 26], indicating that restoration of lysosomal function is a primary mechanism of Qu-mediated neuroprotection.

A key question is whether Qu can target the brain despite its low bioavailability. Recent evidence suggests that Qu and its active metabolites reach brain tissue at sufficient concentrations [27]. Our most novel finding is that lysosomal potentiation is a necessary upstream event for suppressing NF-κB-driven neuroinflammation. This cause-effect relationship is supported by experiments showing that pharmacological inhibition of lysosomes (with bafilomycin A1 or chloroquine) abolishes the anti-inflammatory and neuroprotective effects of Qu. Beyond its anti-inflammatory effects, Qu may protect neurons through other mechanisms. For example, Qu activates the Wnt/β-catenin pathway, re-

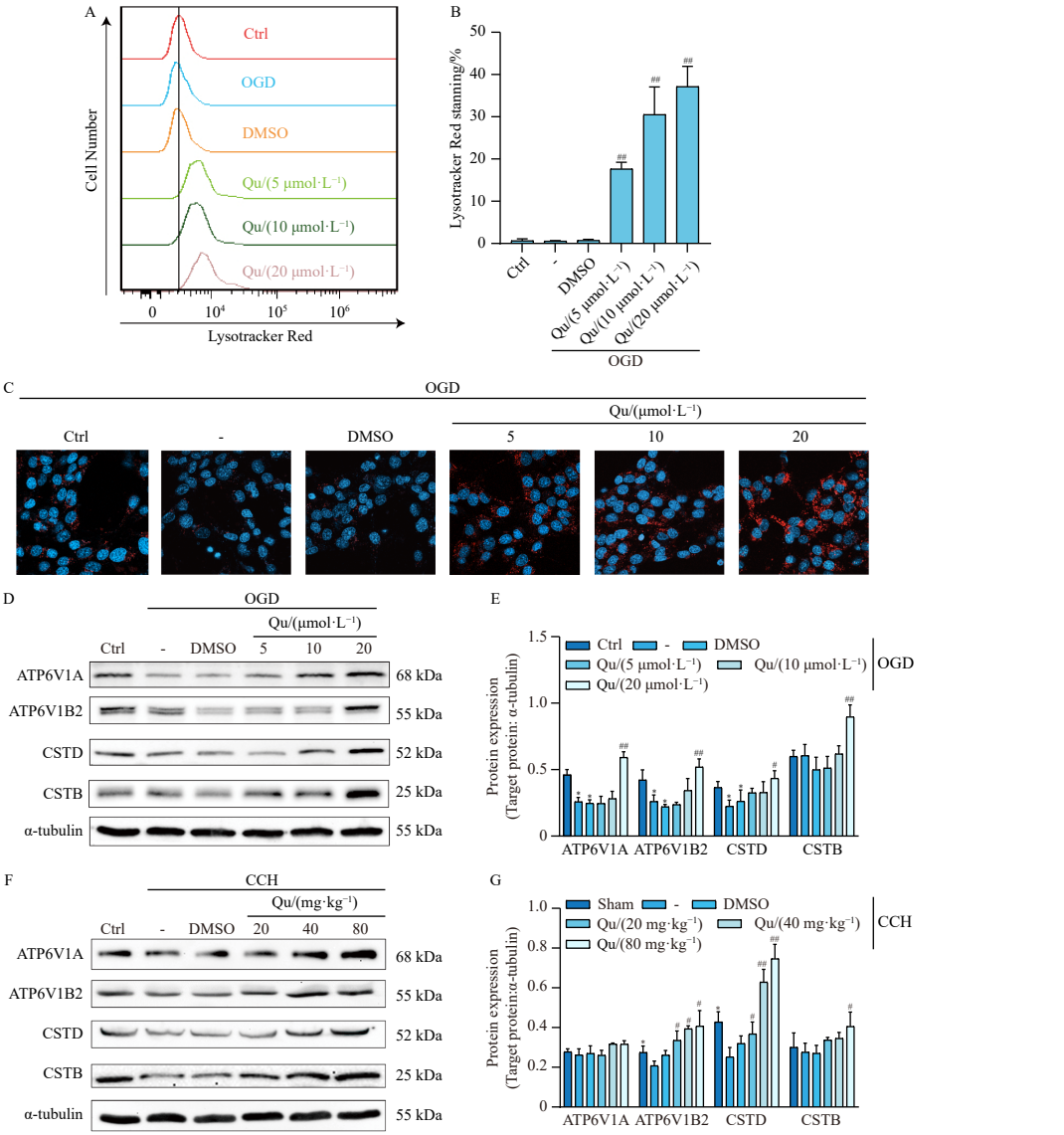
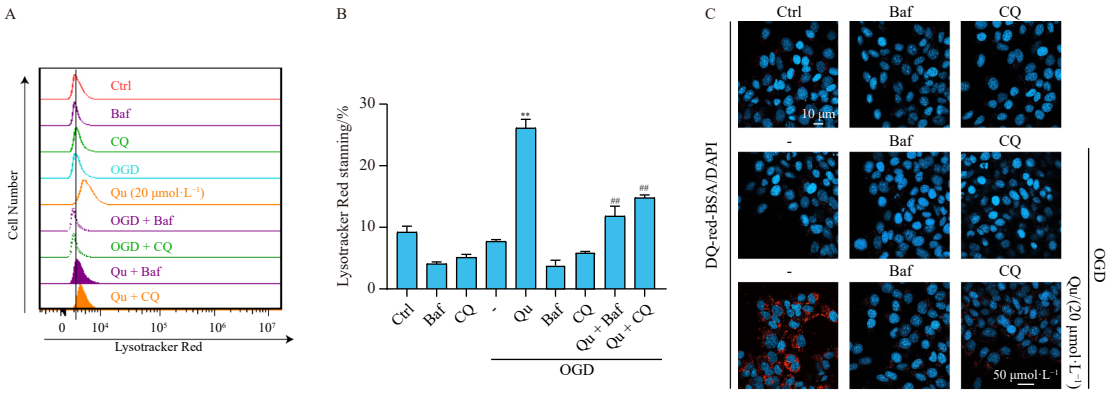


Fig. 7 Quercetin enhances lysosomal function in nerve Cells. (A–B) Lysosomal acidification in HT22 cells after OGD and quercetin (Qu) treatment (with or without chloroquine (CQ) or bafilomycin A1 (Baf)) was assessed using LysoTracker red staining and observed through flow cytometry. (C) Lysosomal activity in HT22 cells was measured by DQ-BSA red staining. All scale bars are 10 μm. (D–E) Lysosome-related protein expression in HT22 cells was evaluated by Western Blot. Data are shown as mean ± SD; n = 3, $^{*}P < 0.05$ *vs* OGD group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* OGD + DMSO group. (F–G) Lysosomal protein expression in the hippocampus of CCH rats was detected by Western Blot. Data are shown as mean ± SD; $^{*}P < 0.05$ *vs* CCH group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* CCH + DMSO group.

ducing blood-brain barrier damage [28], and inhibits voltage-gated sodium channels, lowering excitotoxicity after ischemic injury [29].

Given the integral relationship between lysosomes and autophagy, considered how Qu affects autophagy in ischemic brain injury. Ischemic stress increases autophagosome formation, but lysosomal function declines, leading to impaired autophagosome-lysosome fusion and autophagosome accumulation, which ultimately causes neuronal death [30, 31]. Our RNA-seq data indicate that Qu does not broadly activate the autophagic response but instead promotes expression of lysosome-related proteins, increasing lysosomal capacity and restoring abnormal autophagy. We therefore propose that the neuroprotective effect of Qu depends



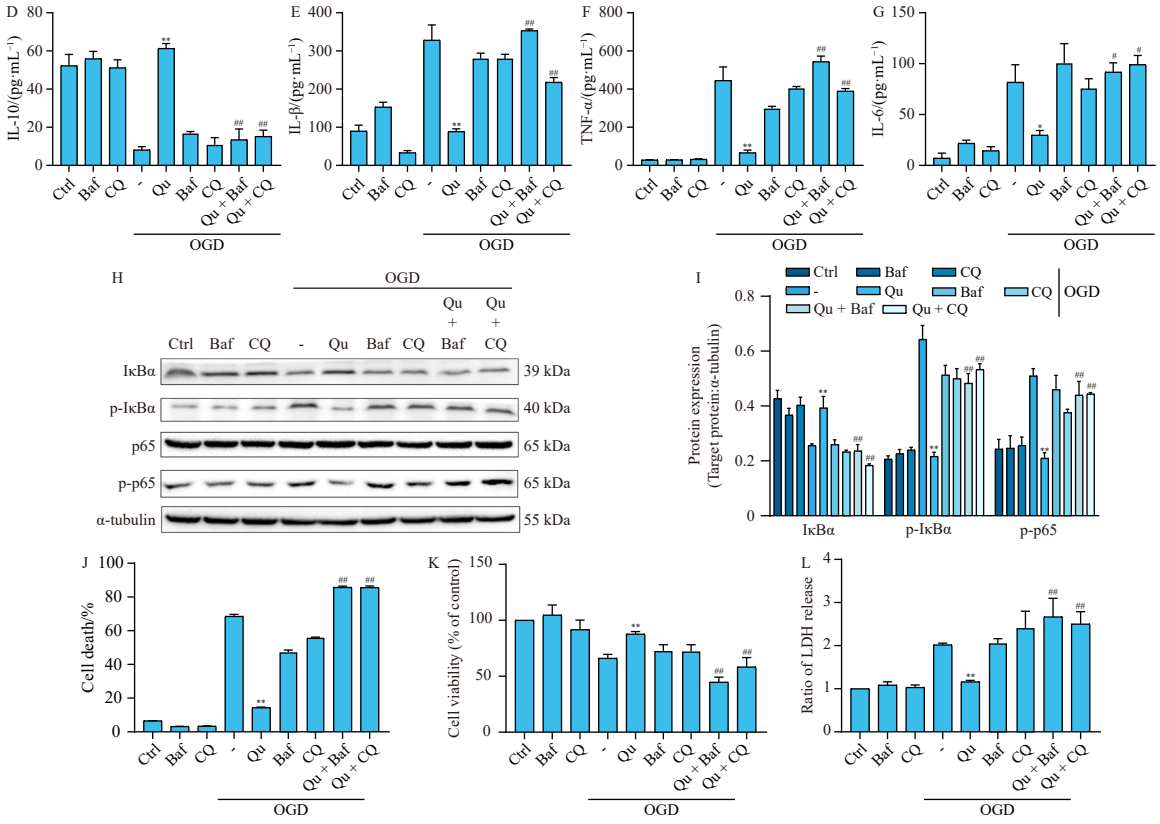


Fig. 8 Lysosomal inhibition abolishes Qu-induced neuroprotection. (A–B) HT22 cells were subjected to oxygen-glucose deprivation (OGD) and then treated with quercetin (Qu), with or without the lysosomal inhibitors chloroquine (CQ) or bafilomycin A1 (Baf). LysoTracker Red and fluorescence intensities was monitored by flow cytometry; (C) Lysosomal activity in HT22 cells was assessed using confocal microscopy after DQ-BSA red staining. All scale bars are 10 μm. (D–G) After indicated treatment, levels of IL-10, IL-1β, TNF-α, and IL-6 were measured using ELISA kits. (H–I) Expression levels of NF-κB signaling proteins were evaluated by Western Blot. (J–L) HT22 cell death was assessed by CCK-8 assay (J), PI staining assays (K), and LDH release assay (I). Data are presented as mean ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* OGD group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* Qu-treated group.

more on enhancing lysosomal function than on regulating autophagy initiation.

The precise mechanism by which Qu improves lysosomal function remains unclear. Based on our results and other studies, we propose several possibilities. As a reactive oxygen species scavenger, Qu may directly repair CCH-induced oxidative damage to the lysosomal membrane and help maintain acidic pH [32]. Post-transcriptional regulation could explain why Qu shows neuroprotective effects even when TFEB activation is modest. Qu might also stimulate lysosomes by modulating lysosome-related post-translational modifications, possibly acting as a molecular chaperone that boosts hydrolase activity or V-ATPase-mediated acidification [33]. Our data suggest that under CCH conditions, Qu acts mainly through TFEB-independent pathways, although a potential role for TFEB cannot be fully excluded and requires further investigation. Future studies should directly compare lysosomal membrane stability, internal pH, and hydrolase activity, and use proteomics to examine post-translational modifications.

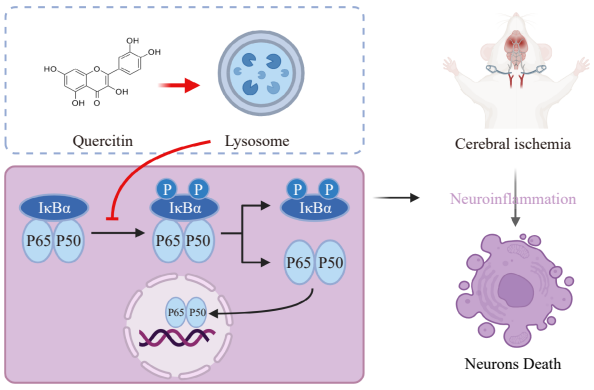


Fig. 9 Schematic diagram of the molecular mechanism by which Quercetin alleviates neuronal injury by enhancing lysosomal function and blocking the NF-κB signalling cascade. Quercetin (Qu) improves lysosomal function, which in turn inhibits the phosphorylation of IκBα by the IKK complex and its ubiquitin-dependent degradation. This prevents the nuclear translocation of p65/p50 and ultimately mitigating NF-κB dependent inflammatory responses caused by cerebral ischemia and exerting neuroprotective effects.

Traditionally, neuroinflammation has been attributed mainly to microglia and astrocytes [34]. But our results suggest a neuron-centric anti-inflammatory mechanism. CCH causes neuronal lysosomal dysfunction, triggering inflammatory signaling inside neurons that recruit and activate glial cells. Qu restores the neuronal clearance system and reduces pro-inflammatory signals. Qu may also affect other glial cell types, promoting microglial M2 polarization via the PI3K/Akt/NF-κB pathway [35] and exerting direct anti-inflammatory effects on astrocytes [36].

The mechanism by which Qu restores lysosomal function remains unclear. It may involve regulation of intra-lysosomal ion balance (H^+ and Ca^{2+}) [37,38] which could also reduce IKK/NF-κB activation by interfering with NLRP3 inflammasome formation, a known sensor of lysosomal stability [39]. Our research has shown that doses of 20–80 mg·kg^{-1} of Qu can produce therapeutic effects *in vivo*, and there is also direct evidence that Qu can cross the blood-brain barrier and reach brain tissue in its active form [40], and this dosage range achieves effective brain concentrations [41]. Qu also protects against neuronal toxicity *via* PI3K/Akt/GSK-3β pathway in Parkinson's disease models [42]. But the low water solubility and extensive metabolism limit its clinical application [43]. Future efforts should develop improved delivery methods, such as nanoparticles or lipid carriers.

Qu also showed beneficial effects on neuroinflammation and memory deficits in our CCH model, though its therapeutic effects

require testing under more complex conditions [44]. An important remaining question is whether Qu-induced lysosomal activation affects other disorders involving protein aggregation, ferroptosis, and mitochondrial dysfunction, such as Alzheimer's and Parkinson's diseases [45-47]. Addressing this question will help define both the mechanism of action and the therapeutic range of Qu.

In summary, our study shows that Qu can alleviate cognitive deficits after cerebral hypoperfusion by improving lysosomal function and inhibiting NF-κB-driven neuroinflammation, providing a rationale for developing lysosome-targeting therapies for VCI.

Declaration of competing interests

The authors declare that they have no competing interests.

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