

# Quercetin Protects Against Global Cerebral ischemia–reperfusion Injury by Inhibiting Microglial Activation and Polarization

Naigeng Wang<sup>1</sup>, Fei Li<sup>2</sup>, Jing Du<sup>3</sup>, Jianhong Hao<sup>1</sup>, Xin Wang<sup>2</sup>, Yueru Hou<sup>3</sup>, Zhenguo Luo<sup>1</sup> 

<sup>1</sup>Department of Anesthesiology, Honghui Hospital, Xi'an Jiaotong University, Xi'an, Shaanxi, People's Republic of China; <sup>2</sup>Department of Neurosurgery, Tangdu Hospital, The Fourth Military Medical University, Xi'an, Shaanxi, People's Republic of China; <sup>3</sup>Second Clinical Medical College, Shaanxi University of Chinese Medicine, Xianyang, Shaanxi, People's Republic of China

Correspondence: Zhenguo Luo, Email [luozhenguo@stu.xjtu.edu.cn](mailto:luozhenguo@stu.xjtu.edu.cn)

**Background:** This study aims to investigate the protective effect of quercetin against global cerebral ischemia–reperfusion (GCI/R) injury in rats and elucidate the underlying mechanism.

**Methods:** A GCI/R injury rat model was established using a four-vessel occlusion (4-VO) method. An oxygen–glucose deprivation/reoxygenation (OGD/R) injury model was induced in BV2 cells. The extent of injury was assessed by evaluating neurological deficit scores (NDS) and brain water content and conducting behavioral tests. Pathomorphological changes in the prefrontal cortex were examined. Additionally, the study measured the levels of inflammatory cytokines, the degree of microglial activation and polarization, and the protein expression of Toll-like receptor 4 (TLR4) and TIR-domain-containing adaptor inducing interferon- $\beta$  (TRIF).

**Results:** Quercetin pretreatment significantly ameliorated neurological impairment, improved learning and memory abilities, and reduced anxiety in rats subjected to GCI/R injury. Furthermore, quercetin administration effectively mitigated neuronal injury and brain edema. Notably, it suppressed microglial activation and hindered polarization toward the M1 phenotype. Simultaneously, quercetin downregulated the expression of TLR4 and TRIF proteins and attenuated the release of IL-1 $\beta$  and TNF- $\alpha$ .

**Conclusion:** This study highlights the novel therapeutic potential of quercetin in alleviating GCI/R injury. Quercetin demonstrates its neuroprotective effects by inhibiting neuroinflammation and microglial activation while impeding their transformation into the M1 phenotype through modulation of the TLR4/TRIF pathway.

**Keywords:** quercetin, cerebral ischemia, neuroprotection, neuroinflammation

## Introduction

Global cerebral ischemia is a prevalent cause of death and disability, occurring in various clinical situations, such as cardiac arrest.<sup>1</sup> Restoring cerebral blood perfusion as soon as possible is an urgent matter for cerebral ischemia patients. However, the secondary injury caused by restoring perfusion, that is, global cerebral ischemia–reperfusion (GCI/R) injury, poses a significant challenge for patients.<sup>2,3</sup> Cerebral ischemia–reperfusion injury involves various pathological processes, including calcium overload, neuronal apoptosis, oxidative stress, and inflammation. In particular, the inflammatory response has been recognized as a major contributor to neuronal injury.<sup>2,4</sup> Therefore, mitigating inflammation is essential for alleviating GCI/R injury.

Microglia are immune cells that reside in the central nervous system (CNS) and play a crucial role in CNS development and maintaining a balanced microenvironment.<sup>5</sup> After cerebral ischemia, microglia undergo rapid activation and polarization.<sup>6,7</sup> The M1 phenotype of microglia secretes various proinflammatory cytokines, including interleukin 1 $\beta$  (IL-1 $\beta$ ), IL-6, and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), which can contribute to neuronal damage.<sup>8,9</sup> On the other hand, the M2 phenotype of microglia secretes a range of anti-inflammatory cytokines, such as IL-4 and IL-10, promoting endocytosis and ultimately alleviating neuronal damage.<sup>6,10</sup> Toll-like receptor-4 (TLR4), as a transmembrane protein, is a key molecule in the microglia-mediated inflammatory response.<sup>11</sup> In vitro experiments have confirmed that inhibition of the TLR4 signaling pathway can increase the

level of M2 markers, decrease the level of M1 markers, and alleviate the lipopolysaccharide-induced inflammatory response.<sup>12</sup> Therefore, investigating the regulation of microglial phenotypes after GCI/R injury could offer a viable strategy for preventing GCI/R injury.

Quercetin is a naturally occurring flavonoid present in various plants, particularly in fruits and vegetables.<sup>13</sup> Studies have demonstrated that quercetin possesses several biological properties, including antioxidant and anti-inflammatory properties.<sup>14,15</sup> As a safe and reliable dietary supplement, quercetin has been shown to reduce neuroinflammation and prevent various neurological diseases.<sup>16</sup> In addition, quercetin has been found to improve the microglia-mediated inflammatory response through the induction of endogenous antioxidant enzymes, thereby preventing obesity-induced neuroinflammation.<sup>17</sup> In adult mice subjected to chronic stress, oral administration of quercetin at a dose of 30 mg/kg/day was found to reduce neuroinflammation in the cortex and hippocampus.<sup>18</sup> However, the effects and mechanisms of quercetin on neuroinflammation after GCI/R injury have not been previously reported.

In this study, we hypothesized that quercetin may play a neuroprotective role by modulating microglial polarization to reduce neuroinflammation. With this objective, we utilized a four-vessel occlusion (4-VO) animal model and an oxygen-glucose deprivation/reoxygenation (OGD/R) model. The objective was to verify the protective effect of quercetin on GCI/R injury and explore the potential underlying mechanism.

## Methods and Materials

### Animals and Experimental Groups

Adult male Sprague–Dawley (SD) rats weighing 250–300 g were procured from the Laboratory Animal Center of the Fourth Military Medical University in China. These rats were housed in a controlled environment with a natural 12-hour light-dark cycle, a temperature range of 20–25°C, and humidity maintained at 50–65%. They had unrestricted access to food and water. All experimental protocols strictly followed the guidelines for the Care and Use of Laboratory Animals from the National Institutes of Health and received ethical approval from the Animal Ethics Committee of Xi'an Jiaotong University (No.202003053).

The rats were categorized randomly into five groups: the sham group, the GCI/R group, the G-L group (GCI/R group pretreated with 10 mg/kg quercetin), the G-M group (GCI/R group pretreated with 30 mg/kg quercetin), and the G-H group (GCI/R group pretreated with 50 mg/kg quercetin).

### Quercetin Pretreatment

Quercetin (98.02% purity) was purchased from MedChemExpress (HY-18085, Monmouth Junction, USA). Quercetin was dissolved in 10% dimethylsulfoxide (DMSO) and further diluted with saline. The pretreatment groups of rats were intragastrically administered quercetin at doses of 10, 30, or 50 mg/kg once daily for 5 consecutive days before the surgical procedure. Similarly, the rats in the sham and model groups were intragastrically administered an equal volume of saline once daily for 5 consecutive days before the surgical procedure.

### GCI/R Rat Model

The GCI/R injury model was established using the 4-VO method, as previously described.<sup>19</sup> Prior to surgery, SD rats underwent a 12-hour fasting period and were then anesthetized with 10% chloral hydrate (3 mL/kg). A heating pad was used to maintain a constant body temperature of 37.0±0.5°C. A midline incision was made at the first cervical vertebra to expose the transverse wing and reveal the bilateral alar foramina. The vertebral arteries were permanently occluded by inserting a heated electric iron tip directly into the bilateral alar foramina. After 24 hours, the rats were anesthetized with sevoflurane, and arterial clamps were used to isolate and clamp the bilateral common carotid arteries. Ten minutes later, the clamps were removed to initiate reperfusion. In the sham group, the rats underwent isolation of the bilateral common carotid arteries without coagulation of the vertebral arteries.

## Neurological Deficit Score

Following 48 hours of reperfusion, each group of rats (n=7) was assessed for neurological deficits using the neurological deficit score (NDS) developed by Geocadin.<sup>20</sup> The NDS had a total score of 80 points, where lower scores indicated more severe neurological impairment, while higher scores indicated milder neurological impairment.

## Brain Water Content

The brain water content was evaluated using the dry–wet weight method. Rats (n=6) were anesthetized with isoflurane and then decapitated to quickly obtain brain tissue. The samples were immediately weighed to determine the wet weight. Following this, the samples were dried in a 100°C oven for 48 hours and then reweighed to ascertain the dry weight. The brain water content was calculated using the following formula: brain water content = [(wet weight - dry weight)/wet weight] × 100%.

## Morris Water Maze Test

The Morris water maze test was conducted 48 hours following reperfusion. The experimental procedures comprised two parts: the place navigation test and the probe trial test. The entire experiment lasted six days, with the initial five days devoted to the place navigation test and the final day allocated for the probe trial test. Before commencing the experiment on the sixth day, the platform situated in the target quadrant was removed. Subsequently, rats were placed in the water from the quadrant opposite the original platform's location. The measurements recorded included the escape latency (EL), the number of crossings in the target quadrant, and the residence time of the rats in the target quadrant within one minute.

## Open Field Test

Forty-eight hours after reperfusion, the open field test was performed to examine the impact of quercetin on anxiety in rats postinjury. The background of the test was partitioned into 16 equally sized small squares using tracer system software. The squares located near the box edges were designated as the surrounding area, while the remaining squares were labeled as the central area. Each rat was placed at the center of the box, and its movement trajectory and time spent in the central zone were recorded for a duration of 10 minutes.

## Tissue Processing and Slice Preparation

Rats were anesthetized with pentobarbital sodium, followed by transcardial perfusion using 150–200 mL of 0.1 M phosphate-buffered saline (PBS) at pH 7.2–7.4. Subsequently, perfusion with 200 mL of 4% paraformaldehyde (PFA) was performed immediately after the liver turned white. The brains were postfixed overnight in 4% PFA and then transferred to a sucrose solution for gradual dehydration. Using OCT as an embedding medium, the brains were coronally sectioned at a thickness of 20 µm using a Leica microtome. These sections were preserved in a cryoprotectant solution (60% glycerol) and stored at –20°C for further processing.

## Hematoxylin and Eosin (HE) Staining

Following 48 hours of reperfusion, the prefrontal cortex was subjected to HE staining to visualize pathological alterations in a sample size of 6 rats. To ensure consistency, brain sections were washed three times with 0.1 M PBS at pH 7.2–7.4 and stained using an HE staining kit (#G1120, Solarbio, China). The quantification of HE-positive cells in the prefrontal cortex was performed blindly using light microscopy.

## Nissl Staining

Following 48 hours of reperfusion, the prefrontal cortex region was examined to assess neuronal morphological changes. The sections underwent the same washing procedure detailed for HE staining. The experiments followed the manufacturer's instructions for the Nissl staining kit (#G1432, Solarbio, China). Light microscopy was used to analyze five distinct fields of view for quantification of all Nissl-positive neurons.

## Immunofluorescence Staining

Rat brains were sectioned, and the resulting slices were washed three times in 0.1 M PBS for subsequent experiments. The slices were then incubated with 0.3% Triton X-100 for 20 minutes, followed by another three washes with 0.1 M PBS. After a 1-hour incubation in 2% donkey serum albumin, the slices were subjected to overnight incubation at 4°C with the respective primary antibodies: Guinea pig anti-Iba-1 (1:500; Synaptic Systems, Germany), mouse anti-CD86 (1:100; Santa Cruz, USA), and rabbit anti-CD206 (1:500; Cell Signaling Technology, USA). Nuclei were labeled using 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). To visualize the target proteins, the sections were incubated with Alexa-488 (green) or Alexa-594 (red) conjugated donkey anti-rabbit or donkey anti-mouse secondary antibodies for 2 hours. Changes in microglial morphology and the numbers of Iba-1, CD86, and CD206 positive cells were tracked using confocal laser scanning microscopy.

## Western Blotting (WB) Analysis

The cerebral cortex tissue was dissolved in RIPA buffer (Beyotime, Nantong, China), which contained an inhibitor. The tissues were ground, sonicated, and then centrifuged to obtain supernatants. Total protein expression was quantified using the BCA kit (Beyotime, Nantong, China). Subsequently, protein samples were electrophoresed and transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking with 5% bovine serum albumin for 1.5 hours, the membranes were incubated overnight with primary antibodies. The primary antibodies included rabbit anti-TLR4 (1:1000; Abcam, USA), rabbit anti-TIR-domain-containing adaptor inducing interferon- $\beta$  (TRIF) (1:1000; Proteintech, China), mouse anti-CD86 (1:500; Santa Cruz, Europe), rabbit anti-CD206 (1:1000; Cell Signaling Technology, USA), mouse anti-Iba-1 (1:1000; Synaptic Systems, Germany), and mouse anti- $\beta$ -actin (1:2000; Proteintech, China). Following primary antibody incubation, the membranes were incubated for 2 hours with the appropriate horseradish peroxidase-conjugated secondary antibody. The protein bands were quantified using ImageJ 1.8.0 in the presence of chemiluminescence.

## Quantitative Polymerase Chain Reaction (qPCR) Analysis

The mRNA expression levels of inflammatory cytokines in the rat prefrontal cortex (n=6) were determined using qPCR. First, mRNA was extracted from the tissue using TRIzol reagent (Cat No. 15596026, Thermo Fisher Scientific, Waltham, USA). Subsequently, the mRNA was reverse transcribed into complementary DNA (cDNA) using the Evo M-MLV RT Mix Kit (Cat No. AG11728, Accurate Biology, China). qPCR was performed on the Bio-Rad CFX Real-Time PCR System according to the RT-qPCR kit protocols (Cat No. DY20301, Deeyee, China), utilizing a two-step method. The  $2^{-\Delta\Delta CT}$  method was employed to calculate quantitative mRNA levels. Primer sequences are listed in Table 1.

## BV2 Microglial Cell Culture and Treatments

BV2 cells (Corning Inc., Corning, USA) were cultured in DMEM high-glucose medium supplemented with 10% fetal bovine serum (Corning) at 37°C in a 5% CO<sub>2</sub> incubator under sterile conditions. The effect of quercetin and TRIF inhibitors on BV2 cell

**Table 1** Primer Sequences of Inflammatory Cytokines

Primer Name	Primer Sequences (5'–3')
GAPDH Forward Primer	ACAGCAACAGGGTGGTGGAC
GAPDH Reverse Primer	TTTGAGGGTGCAGCGAACTT
IL-1 $\beta$ Forward Primer	CAGCTATGGCAACTGTCCCT
IL-1 $\beta$ Reverse Primer	AACAGGTCATTCTCCTCACTGT
TNF- $\alpha$ Forward Primer	GTCCCAACAAGGAGGAGAAGTT
TNF- $\alpha$ Reverse Primer	CTCCGCTTGGTGGTTTGCTA
IL-4 Forward Primer	CTTACGGCAACAAGGAACACC
IL-4 Reverse Primer	AGACCGCTGACACCTCTACA
IL-10 Forward Primer	GGGAGAGAAGCTGAAGACCC
IL-10 Reverse Primer	ACACCTTTGTCTTGGAGCTTATTA

survival was evaluated by the CCK-8 assay. BV2 cells were seeded at  $1 \times 10^5$  cells per well in 96-well plates. After 24 hours, the cells were treated with different concentrations of quercetin (0  $\mu\text{M}$ , 10  $\mu\text{M}$ , 20  $\mu\text{M}$ , 30  $\mu\text{M}$ , 40  $\mu\text{M}$ ) and TRIF inhibitor (0  $\mu\text{M}$ , 10  $\mu\text{M}$ , 30  $\mu\text{M}$ , 50  $\mu\text{M}$ ) for 12 hours. Then, 10  $\mu\text{L}$  CCK-8 reagent was added to each well, and the cells were incubated for 1 h at  $37^\circ\text{C}$ . Cell survival was calculated by measuring the absorbance of each well at 450 nm on a microplate reader.

The cells were categorized into four groups: control group, OGD/R group, quercetin treatment group, and quercetin plus TRIF inhibitor group. The OGD/R-induced BV2 cell model was established following a previously described method.<sup>21</sup> The original culture medium was discarded, and the cells were washed once with PBS. Subsequently, glucose-free DMEM was added, and the cells were incubated at  $37^\circ\text{C}$  with 2%  $\text{O}_2$  and 5%  $\text{CO}_2$  for 4 hours to simulate oxygen-glucose deprivation. After the deprivation period, the medium was promptly replaced with DMEM high-glucose culture medium and maintained under normal conditions for 12 hours before subsequent experiments. In the quercetin-pretreated group, cells were initially exposed to quercetin for 6 hours prior to OGD/R modeling. For the quercetin plus TRIF inhibitor group, cells were first pretreated with quercetin for 6 hours, followed by the addition of TRIF inhibitor along with oxygen-glucose deprivation. Immediately afterward, DMEM high-glucose culture medium was added, and the cells were maintained under normal conditions for 12 hours.

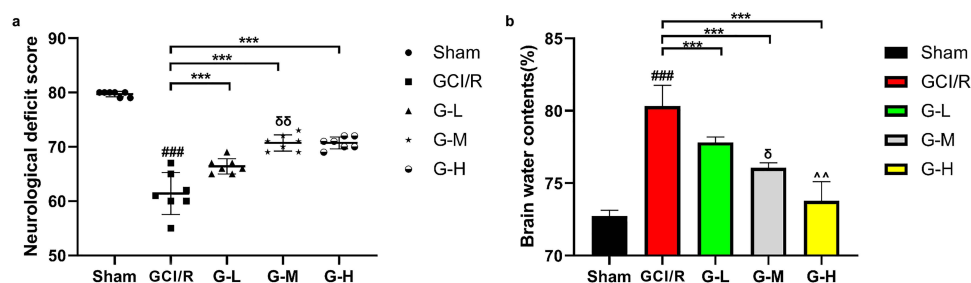
## Statistical Analysis

The statistical analysis was performed using SPSS v26.0 (SPSS Inc., Chicago, USA). All data, except for the NDS, are presented as the mean  $\pm$  standard deviation. Significant differences between the experimental groups were assessed using one-way analysis of variance (ANOVA) with Tukey's post hoc test. The NDS values are presented as interquartile ranges and medians, and the nonparametric Mann–Whitney *U*-test was employed to compare the differences between groups. A significance level of  $P < 0.05$  was considered statistically significant.

## Results

### Quercetin Effectively Improved Neurological Impairment and Reduced Brain Edema After GCI/R

We used the NDS and brain water content to investigate the neuroprotective effect of quercetin in GCI/R injury. Figure 1a demonstrates that the NDS of rats in the GCI/R group was significantly increased compared to that of rats in the sham group. However, pretreatment with quercetin resulted in a significant decrease in the NDS. The neurological scores of the rats in the G-M group were higher than those in the G-L group. However, there was no significant difference in neurological function scores between G-M and G-H rats. Furthermore, brain water content was measured to evaluate the extent of brain edema following GCI/R. According to Figure 1b, the brain water content was  $72.7\% \pm 0.5\%$  in the sham group and  $80.3\% \pm 0.2\%$  in the GCI/R group, with a statistically significant difference between the two groups. Additionally, the brain water content was  $77.8\% \pm 0.4\%$  in the G-L group,  $76.1\% \pm 0.3\%$  in the G-M group, and  $73.8\% \pm 0.3\%$  in the G-H group ( $p < 0.01$  vs. GCI/R group).



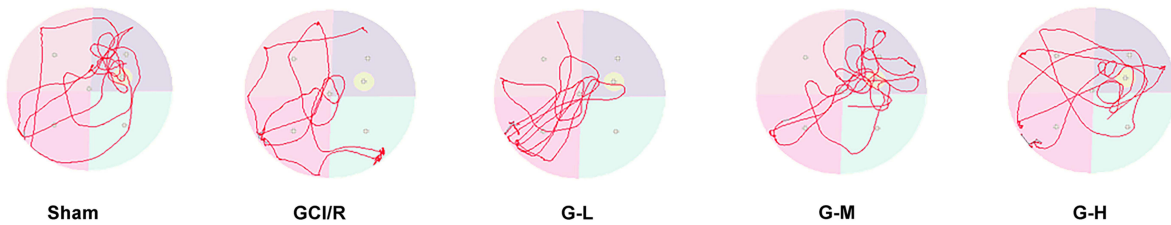
**Figure 1** Quercetin pretreatment ameliorated GCI/R injury. Neurological deficit scores were assessed 48 hours after reperfusion (a). Statistical analysis of brain water content in rats was conducted (b). Compared with the sham group, ### $P < 0.001$ . Compared with the GCI/R group, \*\*\* $P < 0.001$ . Compared with the G-L group,  $\delta P < 0.05$ ,  $\delta\delta P < 0.01$ . Compared with the G-M group, ^^ $P < 0.01$ .



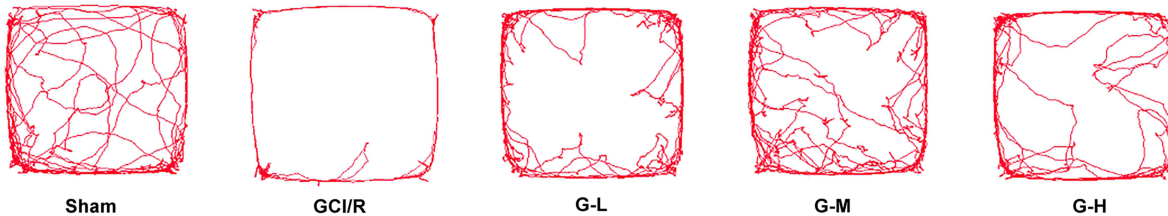
## Quercetin Effectively Improved Memory Impairment and Anxiety After GCI/R

The effect of quercetin on learning and memory in rats following GCI/R was investigated using a water maze. The swimming trajectories of rats in each group during the Morris water maze test are depicted in Figure 2a. Additionally, we employed the open field test to assess anxiety levels in all rat groups. The motor trajectories of rats in each group during the open field test are shown in Figure 2b. All five groups of rats reached the platform within 60 seconds without guidance, EL gradually decreased with the increase of observation days; rats pretreated with quercetin exhibited an active search for the platform upon entering the water, characterized by a reduction in EL (Figure 2c). Compared to rats

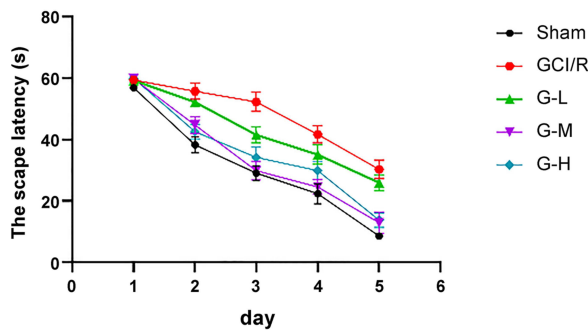
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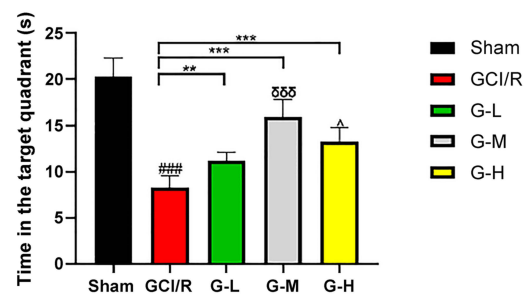
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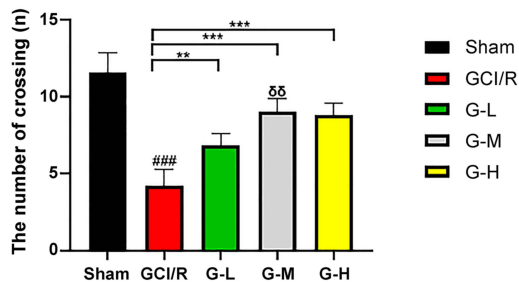
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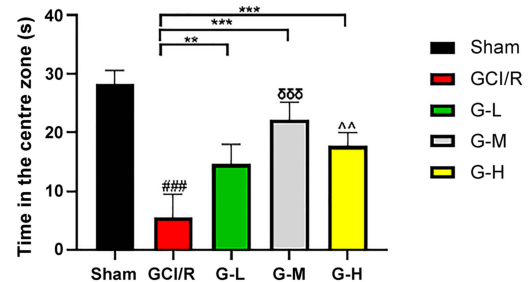
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**Figure 2** Quercetin enhanced the behavior of rats after 48 hours of reperfusion. Trajectories of rats in the Morris water maze test (a). Trajectories of rats in the open field test (b). The escape latency (c). Time spent in the target quadrant (d). The number of target quadrant crossings (e). Time in the central zone (f). Compared with the sham group, #### $P < 0.001$ . Compared with the GCI/R group, \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Compared with the G-L group,  $\delta\delta P < 0.01$ ,  $\delta\delta\delta P < 0.001$ . Compared with the G-M group,  $\Delta P < 0.05$ ,  $\Delta\Delta P < 0.01$ .

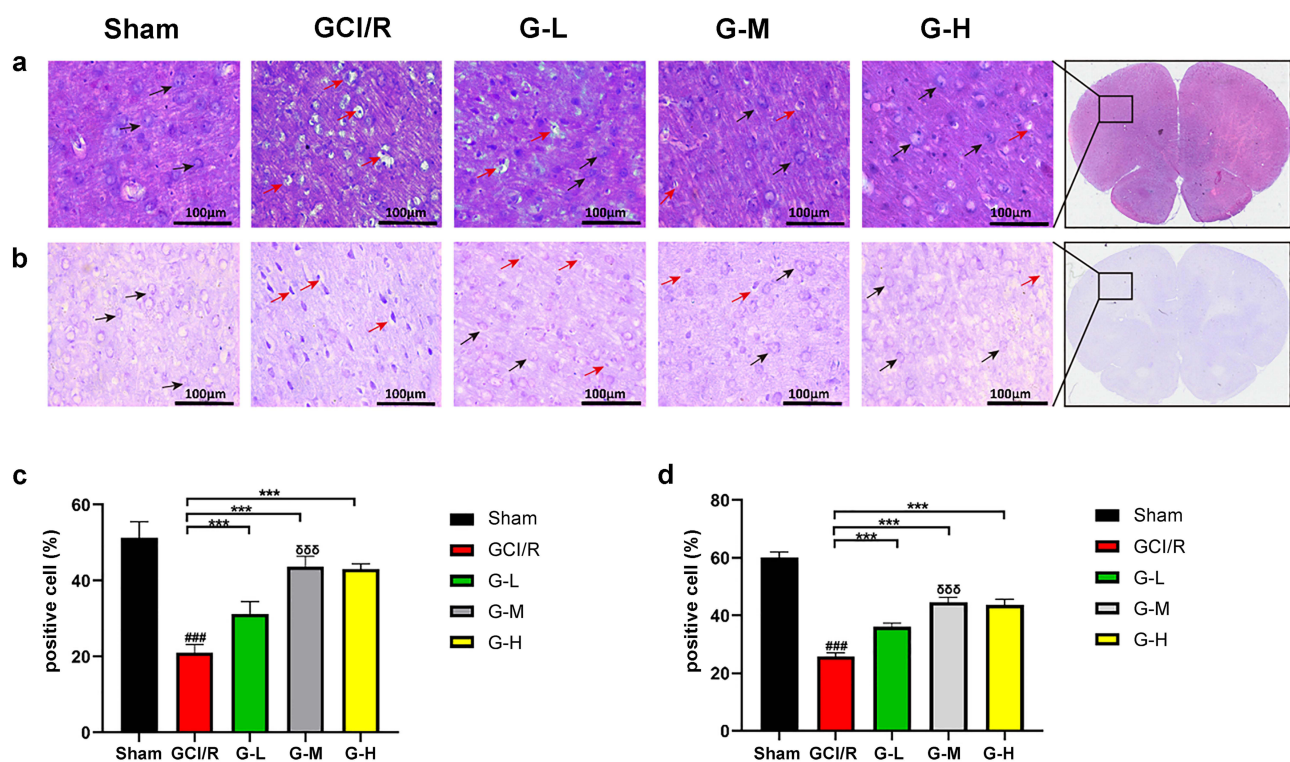
undergoing sham surgery, those in the GCI/R group exhibited impaired spatial learning and memory, however, the quercetin pretreated rats showed significant improvements in spatial learning and memory, this improvement was characterized by a significant increase in the target quadrant residence time (Figure 2d) and the number of crossings (Figure 2e). The experimental findings also revealed a significant decrease in central zone residence time for rats in the GCI/R group compared to the sham group. Conversely, rats in the quercetin pretreatment intervention groups showed a gradual extension of central zone residence time compared to the GCI/R group (Figure 2f).

## Quercetin Effectively Reduced Neuronal Injury in the Prefrontal Cortex

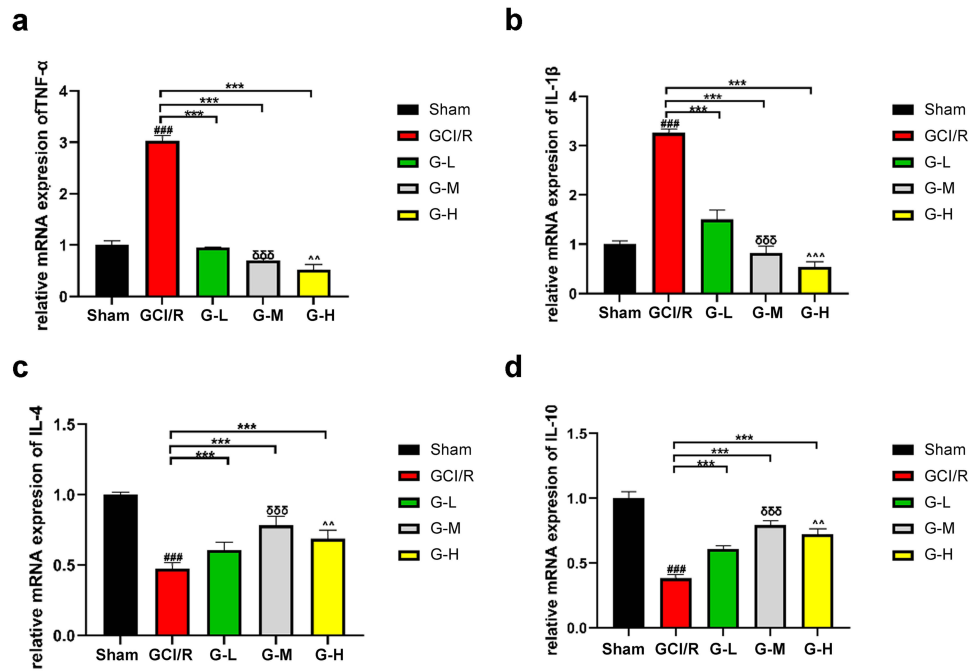
Neuronal injury in the prefrontal cortex following GCI/R injury was evaluated using HE and Nissl staining techniques. The prefrontal cortex of the model group showed significant neuronal cell atrophy, blurred contours, and an abnormally disordered texture. Additionally, there were many cavities visible, which indicated that the region had experienced severe edema and liquefaction necrosis (Figure 3a and b). In the prefrontal cortex of sham rats, neurons showed normal morphology, clear contour, uniform cytoplasmic staining, and dense structure. Importantly, clear neuronal contours were observed in the rat prefrontal cortex after quercetin therapy (Figure 3a and b). Moreover, quercetin pretreatment resulted in a significantly higher number of positive neurons compared to the GCI/R group (Figure 3c and d). These results suggested that quercetin significantly improves neuronal damage after GCI/R.

## Quercetin Effectively Inhibited Inflammatory Responses in the Prefrontal Cortex

The expression of inflammatory cytokines in the prefrontal cortex was detected using qPCR analysis 48 hours after reperfusion. As shown in (Figure 4) compared to the sham group, the expression of proinflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ ) was significantly increased (Figure 4a and b), and the expression of anti-inflammatory cytokines (IL-4, IL-10) was significantly decreased (Figure 4c and d). After quercetin pretreatment, there was a notable reduction in the expression of TNF- $\alpha$  and IL-1 $\beta$



**Figure 3** Quercetin pretreatment attenuated neuronal damage in the prefrontal cortex after 48 hours of reperfusion. HE staining showing morphological changes in neurons (a). Black arrows show cells with normal and well-defined contours. Red arrows indicate blurred cell contours and enlarged spaces with surrounding tissue. Nissl staining showing morphological changes in neurons (b). Black arrows indicate intact neuronal morphology. Red arrows indicate damaged neurons. Percentage of HE-positive cells (c). Percentage of Nissl-positive cells (d). Scale bar = 100  $\mu$ m. Compared with the sham group,  $###P < 0.001$ . Compared with the GCI/R group,  $***P < 0.001$ . Compared with the G-L group,  $\delta\delta\delta P < 0.001$ .



**Figure 4** Quercetin effectively inhibited inflammatory responses in the prefrontal cortex after 48 hours of reperfusion. qPCR analysis of TNF- $\alpha$  (a), IL-1 $\beta$  (b), IL-4 (c) and IL-10 (d) protein expression. Compared with the sham group, ####P < 0.001. Compared with the GCI/R group, \*\*\*P < 0.001. Compared with the G-L group, δδδP < 0.001. Compared with the G-M group, ^^P < 0.01, ^^P < 0.001.

(Figure 4a and b), accompanied by a substantial increase in the expression of IL-4 and IL-10 (Figure 4c and d). Based on the above changes in inflammatory cytokines, quercetin effectively ameliorated the inflammatory response after GCI/R.

## Quercetin Effectively Inhibited the Activation and Polarization of Microglia in the Prefrontal Cortex

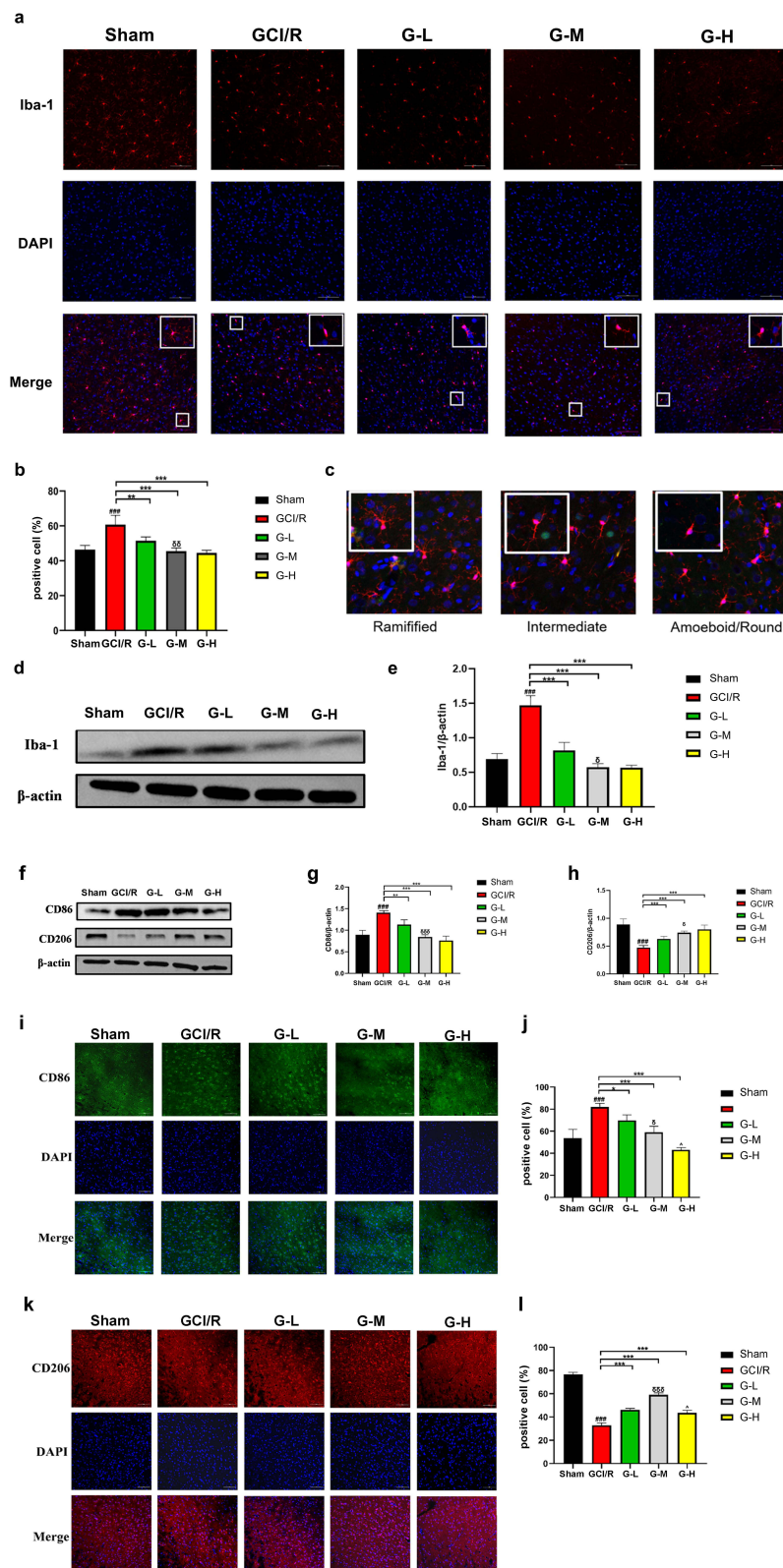
Distinct morphologies of microglia are thought to correspond to different functional states. The resting state is characterized by branched microglia, while the active state is characterized by amoeba/circular microglia. As shown in the figures, the microglia in the prefrontal cortex of the sham operation rats mainly showed branching morphology (Figure 5a–c). Forty-eight hours after ischemia–reperfusion injury, the microglia were dominated by amoeba/circular microglia (Figure 5a–c). Quercetin pretreatment resulted in a decrease in amoeboid microglia and an increase in branched microglia (Figure 5a–c). In addition, the expression of the microglial activation marker Iba-1 was also changed. Figure 5d and e show that the expression level of Iba-1 protein in the GCI/R group was significantly increased, while that in the quercetin group was significantly decreased.

We used immunofluorescence and WB analysis to investigate the impact of quercetin on microglial polarization following GCI/R injury. WB analysis revealed a significant increase in the protein levels of the M1 marker CD86, while the M2 marker CD206 exhibited a decrease in the GCI/R group compared to the sham group (Figure 5f–h). Compared to the GCI/R group, quercetin pretreatment suppressed the expression of the M1 marker CD86 and increased the levels of the M2 marker CD206 (Figure 5f–h). Likewise, the immunofluorescence results demonstrated a significant decrease in the number of CD86-positive cells and a substantial increase in the number of CD206-positive cells following quercetin pretreatment when compared to the GCI/R group (Figure 5i–l).

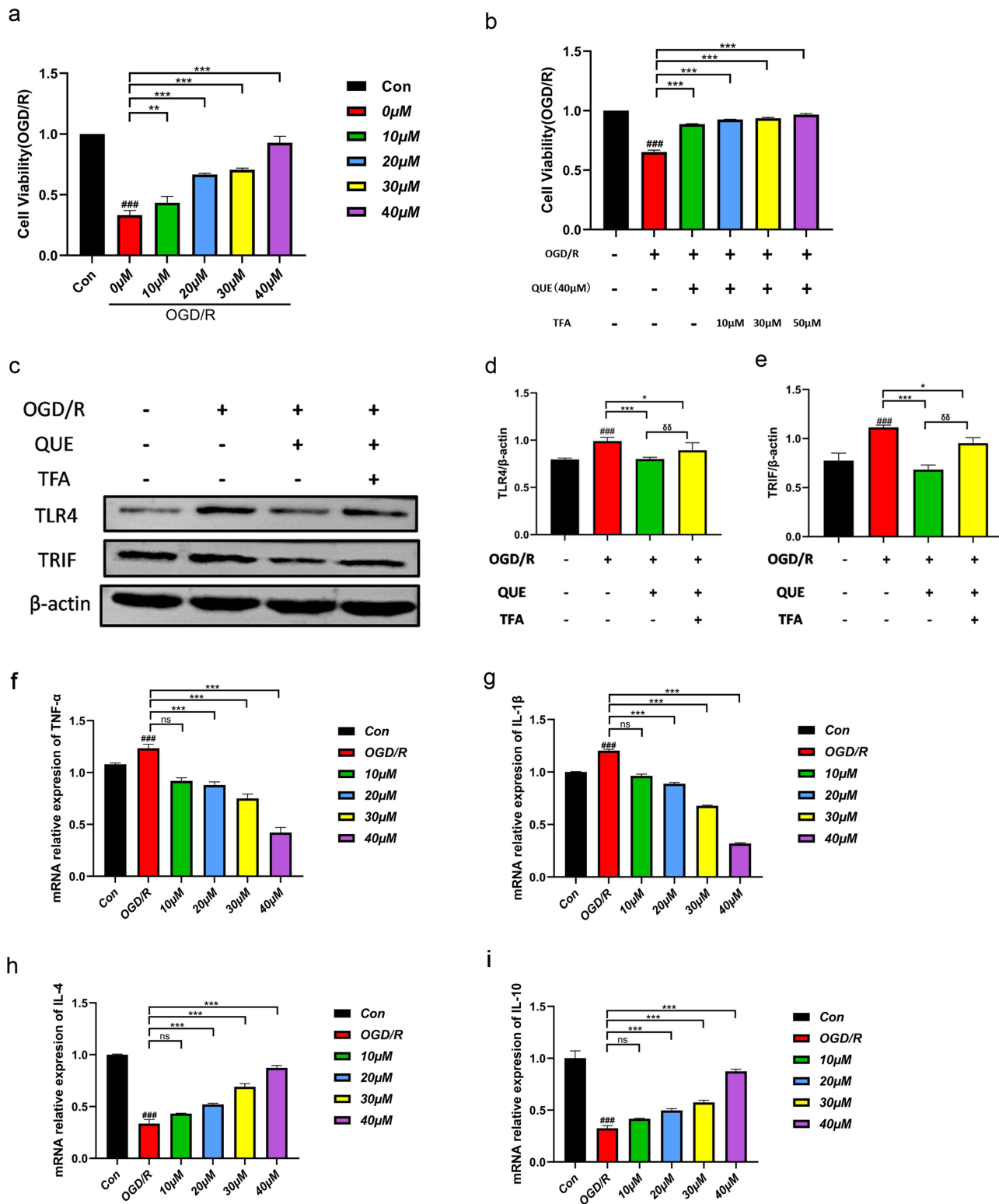
## Quercetin Regulated Microglial Polarization via the TLR4/TRIF Signaling Pathway

To further investigate whether microglial polarization is mediated by the TLR4/TRIF signaling pathway, we conducted WB to measure the protein expression of TLR4 and TRIF in BV-2 cells following a 12-hour exposure to OGD/R. Initially, we determined the optimal concentrations of quercetin and the TRIF inhibitor [pepinh-TRIF (TFA)] through CCK-8 experiments (Figures 6a and b). As shown in (Figures 6c–e) quercetin decreased the expression of TLR4 and





**Figure 5** Quercetin substantially inhibited microglial activation and polarization in the prefrontal cortex 48 hours after reperfusion. Immunofluorescence and quantitative analysis showed the expression of activated Iba1-labeled microglia (a and b). The different morphologies of microglia (c). WB and quantitative analysis of the expression level Iba-1 (d and e). WB and quantitative analysis of the expression levels of CD86 and CD206 (f-h). Immunofluorescence and quantitative analysis showed the expression of CD86 (i and j) and CD206 (k and l). Scale bar = 100 μm. Compared with the sham group, ####P < 0.001. Compared with the GCI/R group, \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001. Compared with the G-L group, <sup>δ</sup>P < 0.05, <sup>δδ</sup>P < 0.01, <sup>δδδ</sup>P < 0.001. Compared with the G-M group, <sup>Δ</sup>P < 0.05.



**Figure 6** Quercetin alleviated microglia-mediated neuroinflammation via TLR4/TRIF CCK-8 assay showing the effects of quercetin (a) and TRIF inhibitor (b) on microglial viability. Western blotting (c) and quantitative analysis of the protein expression of TLR4 (d) and TRIF (e). Meanwhile, the effect of quercetin on TNF-α (f), IL-1β (g), IL-4 (h) and IL-10 (i) mRNA expression was detected by qPCR. Compared with the sham group, ####P < 0.001. Compared with the OGD/R group, \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001. Compared with the quercetin treatment group, <sup>δδ</sup>P < 0.01.

TRIF proteins after OGD/R treatment. In addition, treatment with TRIF inhibitors (TFA) effectively counteracted the downregulation of TLR4 and TRIF expression induced by quercetin. Meanwhile, the effect of quercetin on the mRNA expression of TNF- $\alpha$ , IL-1 $\beta$ , IL-4 and IL-10 was detected by qPCR. Compared with the OGD/R group, quercetin pretreatment decreased the expression of M1 markers (TNF- $\alpha$  and IL-1 $\beta$ ) and improved the expression of M2 markers (IL-4 and IL-10) (Figure 6f–i). Based on these results, we can conclude that quercetin regulates microglial polarization through the TLR4/TRIF signaling pathway.

## Discussion

Quercetin, a natural polyphenol with various bioactivities,<sup>22</sup> has gained increasing attention in recent years due to its protective effect on I/R injury through various mechanisms.<sup>23</sup> A recent report showed that intraperitoneal injection of quercetin can notably improve neurological function deficits following MCAO in rats.<sup>24</sup> Another study reported that quercetin can alleviate ischemic hypoxic brain damage in newborn mice.<sup>25</sup> Despite these findings, the effect of quercetin on GCI/R injury remains insufficiently explored. In this study, we observed that quercetin alleviated injury, reduced brain edema, improved behaviors, and inhibited the neuroinflammatory response following GCI/R injury in rats. Additionally, quercetin demonstrated the ability to mitigate the neuroinflammatory response in the rat prefrontal cortex by efficiently inhibiting the activation and polarization of microglia. This specific mechanism is achieved through the inhibition of the TLR4/TRIF signaling pathway. Despite these findings, the impact of q

Neuroinflammation is the main factor in nerve injury in cerebral ischemia patients after restoring blood flow and greatly increases the difficulty of clinical treatment.<sup>4,26</sup> After blood flow is interrupted, the inflammatory cascade is activated, leading to neuronal apoptosis, increased blood–brain barrier permeability, brain edema, and further aggravating brain injury.<sup>27</sup> In a mouse model of Alzheimer's disease, rutin significantly reduced the expression levels of IL-1 $\beta$  and TNF- $\alpha$ , inhibited neuroinflammation, and significantly improved cognitive function in mice.<sup>28</sup> In this study, quercetin pretreatment significantly decreased TNF- $\alpha$  and IL-1 $\beta$  levels in rats after GCI/R, while increasing IL-4 and IL-10 levels. Furthermore, *in vitro* experiments using BV-2 cells to simulate OGD/R conditions confirmed that quercetin significantly inhibited the mRNA expression of TNF- $\alpha$  and IL-1 $\beta$ , and up-regulated mRNA expression of IL-4 and IL-10. Collectively, these findings from both *in vitro* and *in vivo* studies suggested that quercetin alleviated GCI/R injury by reducing the expression of proinflammatory cytokines and enhancing the expression of anti-inflammatory cytokines, aligning with the outcomes of previous research.<sup>24</sup>

Activation of microglia and production of proinflammatory cytokines are features of neuroinflammation.<sup>29</sup> Microglia are rapidly activated during ischemic stroke. In fact, activated microglia exhibit two distinct phenotypes with opposing functions: The M1 phenotype can generate proinflammatory cytokines and mediators, leading to cell apoptosis and secondary damage.<sup>30</sup> Conversely, activated M2 cells produce anti-inflammatory cytokine and neurotrophic factors and contribute to repairing damaged tissue.<sup>31,32</sup> Transforming microglia from the M1 phenotype to the M2 phenotype is considered a promising strategy for treating cerebral I/R injury.<sup>33</sup> In an *in vivo* experiment, it was found that icaritin alleviated cerebral I/R injury by inhibiting M1 polarization of microglia and promoting M2 polarization.<sup>4</sup> In our study, quercetin pretreatment inhibited microglial activation after GCI/R injury, inhibited the expression of CD86 and promoted the expression of CD206. Furthermore, we observed that quercetin significantly downregulated the mRNA expression levels of proinflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$  and upregulated the mRNA expression levels of M2 markers such as IL-4 and IL-10 following reperfusion injury. These findings suggest that quercetin may play an anti-neuroinflammatory role by regulating microglial polarization.

TLR4 is a widely expressed innate immune receptor in the central nervous system that can induce the production of various inflammatory factors after activation.<sup>34</sup> Thus, blocking the activation of TLR4 is an effective pathway for neuroprotection.<sup>35</sup> Recent evidence suggests that several anti-inflammatory treatments targeting TLR4 activation have demonstrated the ability to mitigate the damage caused by I/R injury.<sup>36–38</sup> TLR4 is the sole receptor that triggers both the myeloid differentiation factor 88 (MyD88) and TRIF pathways.<sup>39</sup> While previous the study has confirmed quercetin's role in regulating microglial polarization via the TLR4/myd88 pathway,<sup>25</sup> its impact on the TLR4/TRIF pathway remained unclear. In this study, we found that following OGD/R injury, quercetin downregulated the expression of TLR4 and TRIF proteins in BV2 cells. Notably, we observed that TFA effectively counteracted quercetin's inhibitory effects on TLR4 and TRIF proteins. Meanwhile, the mRNA expression levels of the M1 phenotype markers TNF- $\alpha$  and IL-1 $\beta$  were significantly downregulated after quercetin pretreatment, while the mRNA expression levels of the M2

phenotype markers IL-4 and IL-10 were upregulated. These findings suggest that quercetin may inhibit M1 microglial polarization and promote M2 microglial polarization through the TLR4/TRIF pathway, thereby inhibiting neuroinflammation, which is consistent with the reduction in neuroinflammation in traumatic brain injury rats by acupuncture regulating the microglial phenotype by inhibiting the TLR4/TRIF pathway.<sup>40</sup>

## Conclusion

In this study, we demonstrated the efficacy of quercetin in treating GCI/R injury and its possible mechanism. Specifically, our findings revealed that quercetin exhibited anti-neuroinflammatory effects by inhibiting M1 microglial polarization and promoting M2 microglial polarization via the TLR4/TRIF pathway.

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## Disclosure

The authors declare no conflicts of interest in this work.

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