Research Square

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Isoquercitrin Ameliorates Post-Cardiac Arrest Brain Injury by Activating the ERK/NRF2 Pathway to Suppress Neuronal Apoptosis

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Abstract

Background

Isoquercitrin (ISO), a flavonoid glycoside derived from plants, has potent antioxidant, anti-inflammatory, and antiviral properties. In this study, we aimed to evaluate whether ISO suppresses neural apoptosis via the ERK/NRF2 Pathway in post-cardiac Arrest Brain Injury (PCABI).

Materials and Methods

Sprague-Dawley rats were used to develop a cardiac arrest /cardiopulmonary resuscitation (CA/CPR) model that mimics PCABI. Neurological deficit scoring (NDS) was performed to assess neurological function. Brain edema, infarct volume, and histological staining were performed to analyze cerebral injury. The Open Field Test (OFT), Y-Maze, and Elevated Plus Maze (EPM) were used as behavioral tests. The oxygen-glucose deprivation/reoxygenation (OGD/R) model was used in primary cortical neurons *in vitro*. Cell viability, gene expression, and protein levels were evaluated using CCK-8 assays, flow cytometry, qPCR, Western blotting, and ELISA.

Results

The ISO treatment significantly enhanced NDS scores, survival rates, and neurological function, with smaller brain edema and infarct volumes in PCABI rats, and preserved intact neuronal architecture. Additionally, ISO minimized Caspase-3 and Bax expression and maximized Bcl-2 expression, thereby restoring the pro- and anti-apoptotic protein balance and inhibiting apoptosis *in vivo* and *in vitro*. Mechanistically, ISO activated ERK/NRF2 signaling by enhancing ERK phosphorylation and nuclear NRF2 and heme oxygenase-1, whereas decreasing reactive oxygen species (ROS) and the levels of IL-1β, IL-6, and TNF-α, thereby reducing oxidative stress.

Conclusion

ISO acts as a neuroprotective agent against PCABI in the brain by activating the ERK/NRF2 pathway and preventing neuronal apoptosis. Therefore, it could be a potential therapeutic agent for PCABI.

Introduction

Post-cardiac arrest brain injury (PCABI) remains a long-term disability and a significant cause of mortality due to cardiac arrest (CA) [1]. In the United States, hospital CA occurs in over 290,000 adults annually, with a mean age of 66 years; 58% are men [2]. In recent years, 10–20% of CA patients recovered with good neurological function, and over 50% developed long-term disabilities [3]. Additionally, pro-inflammatory cytokines, extensive neural apoptosis, and oxidative stress from excessive

reactive oxygen species (ROS), glutamate excitotoxicity, mitochondrial dysfunction, and neuroinflammation contribute to PCABI [4].

PCABI mechanisms are characterized by primary injury (ischemic) resulting in interruption of both cardiac output and oxygen delivery to all vital organs [5]. Myocardial infarction, stroke, and peripheral vascular disease are involved in ischemic disorders resulting in disabilities and death. Therefore, during prolonged ischemia, anaerobic metabolism and lactate accumulation induce cellular ATP levels and intracellular pH decrease [6]. Secondary injury, known as reperfusion, followed by ongoing hypoxia, edema, and impaired cerebral autoregulation [1]. Though reperfusion is needed to restore oxygen essential for ischemic tissues, it can produce ROS against injury, leading to inflammation and cell death triggered by mitochondrial pathways [7]. Management guidelines for PCABI rely on targeted temperature management (TTM), which recommends selecting and maintaining a constant target temperature between 32 and 36°C for patients receiving TTM [8]. While TTM improves survival in out-of-hospital CA, the TTM2 trial (after out-of-hospital CA) showed that therapeutic hypothermia at 33°C did not reduce mortality or poor functional outcomes in unconscious patients with out-of-hospital CA (OHCA), compared with early fever treatment [9].

Among therapeutic agents, N-acetylcysteine and NMDA antagonists have shown potential as adjunctive treatments for a wide range of medical conditions, particularly chronic diseases. Still, toxicity and lack of target engagement have impeded clinical translation [10, 11]. NAC shows potential as an adjunctive treatment for a wide range of medical conditions, particularly chronic diseases. It may be beneficial for polycystic ovary syndrome, endometriosis, male infertility, cataracts, glaucoma, dry eye syndrome, Parkinsonism, multiple sclerosis, Alzheimer’s disease, stroke outcomes, non-acetaminophen-induced acute liver failure, Crohn’s disease, ulcerative colitis, schizophrenia, bipolar disorder, and obsessive-compulsive disorder [11]. Other approaches, such as targeted overexpression of nuclear factor erythroid 2-related factor 2 (NRF2), are considered promising therapeutic strategies for the treatment of neurodegenerative disorders [12]. Additionally, the KEAP1-NRF2 pathway is the principal protective response to oxidative and electrophilic stresses. Under basal conditions, KEAP1 targets NRF2 for ubiquitination, thereby inducing stress that allows NRF2 to accumulate in the cell, translocate to the nucleus, and promote antioxidant transcription [13]. Electrophiles and oxidants modify cysteine thiols on Keap1 and Nrf2, thereby inhibiting Nrf2 ubiquitination and promoting Nrf2 activation and induction, thereby increasing stress resistance for cell survival, because Nrf2 knockout increases susceptibility to toxicity and disease processes in mice [14].

ISO exhibits higher pharmacological activity than rutin due to its specific antioxidant properties, which play essential roles in anti-allergic and anti-aging effects and help preserve damaged cells by eliminating free radicals [15]. In focal cerebral ischemia models, such as middle cerebral artery occlusion (MCAO), ISO reduced infarct volume by 40%, improved neurological scores, and attenuated oxidative stress markers, effects attributed to its modulation of signaling pathways like PI3K/Akt and MAPK [16]. MCAO and PCABI share the same biological mechanism, ischemia-reperfusion injury, that causes injury. ISO

exerts neuroprotective effects on PCABI; however, no research has yet tested the efficacy of ISO on PCABI in the context of ERK/NRF2 pathway interactions.

In this study, we examined the protective effects of ISO against PCABI in a rat model, both *in vivo* and *in vitro*. CA/CPR was employed on rats to induce PCABI. Neurological and behavioral assessments, histological analyses, and molecular assays were used to evaluate whether ISO suppresses neuronal apoptosis and oxidative stress in PCABI. Additionally, *in vitro* OGD/R models were used to assess the cellular mechanisms underlying ERK/NRF2 signaling in primary cortical neurons. Furthermore, CCK-8 assays, flow cytometry, qPCR, Western blotting, and ELISA were used to evaluate cellular viability and the mechanisms underlying neural activity.

Materials and Methods

Reagents and Antibodies

Various reagents were included, such as RPMI-1640 medium (Gibco™, Thermo Fisher Scientific, Cat# 11875-093), fetal bovine serum (Sigma-Aldrich, Cat# F6178), 0.25% trypsin-EDTA(Gibco™, Thermo Fisher Scientific, Cat# 25200-056), Neurobasal-A medium(Gibco™, Thermo Fisher Scientific, Cat# 10888-022), B-27 supplement (Gibco™, Thermo Fisher Scientific, Cat# 17504-044), GlutaMAX (Gibco™, Thermo Fisher Scientific, Cat# 35050061), CCK-8 kit (Beyotime, Cat# C0038), Annexin V-FITC/PI apoptosis kit (Yeasen, Cat# 40302ES), TUNEL kit (Beyotime, Cat# C1088), hematoxylin-eosin (Beyotime, Cat# C0105S) and Nissl staining kits (Molecular Probes™, Cat# N21480), 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich, Cat# T8877) and ISO (Thermo Scientific Chemicals, Cat# 471801000). Primary antibodies targeted p-ERK (Cell Signaling Technology, cat#4695), ERK (Proteintech, cat#66192-1-Ig), NRF2 (Cell Signaling Technology, cat#20733), HO-1 (Proteintech, cat#10701-1-AP), Bax (Proteintech, cat#60267-1-Ig), Bcl-2 (Proteintech, cat#26593-1-AP), and Caspase-3 (Proteintech, cat#82202-1-RR). HRP-conjugated goat anti-rabbit and anti-mouse IgG were used as secondary antibodies (Cell Signaling Technology, Cat# 7074). All reagents were stored at the recommended temperatures (− 20°C for ISO, 4°C for antibodies) and prepared fresh according to the manufacturers' protocols. Antibody specificity was confirmed using positive and negative controls.

2.2. Animals and PCABI Model

In all experiments, 8-10-week-old male Sprague-Dawley rats (250–280 g) were purchased from Zhejiang Vital River Laboratory Animal Technology Co., Ltd. (Certificate number: SCXK 2019-0001). The rats were maintained at 21 ± 2°C and 55 ± 5% humidity under a 12-hour light/dark cycle. Food and water were freely available to all rats except during periods of food or water deprivation. This experiment was conducted at Hainan Medical University and was approved by the Ethics Committee of Hainan Medical University, in accordance with the guidelines for the Care and Use of Laboratory Animals. Animals were divided into groups (n = 6/group), and 2–3% isoflurane (RWD Life Science, Cat# R510-22) was used to anesthetize rats for surgical procedures with a precision vaporizer. The toe pinch reflex was used to

monitor the depth of anesthesia. Animals were then euthanasia by carbon dioxide (CO_2) inhalation at a flow rate of 20–30% chamber volume per minute, followed by cervical dislocation to confirm death.

PCABI was induced using the CA/CPR model. Rats were anesthetized with 5% isoflurane, intubated with a 14-G catheter, and mechanically ventilated. Monitored parameters included rectal temperature, heart rate, and MAP, using a multi-channel system. 2 mg/kg intravenous Vecuronium bromide was used to induce muscle relaxation, and 4 min of asphyxia-induced CA was performed, confirmed by MAP ≤ 20 mmHg. CPR was initiated by manual chest compressions, epinephrine, and a ventilator until restoration of spontaneous circulation (ROSC), which was MAP 60 mmHg or more over 5 min (**Table S1**). Wounds were sutured, and piperacillin-tazobactam was administered to rats daily for 3 days after ROSC to prevent infection. Anesthesia, intubation, and femoral catheterization were performed on sham rats without asphyxia or CPR **(Table S2)**.

Rats were divided into five groups: Sham, PCABI, PCABI + 25 μg/ml ISO, PCABI + 50 μg/ml ISO, and PCABI + 100 μg/ml ISO (n = 6/group). Control groups, such as Sham and PCABI, received 1 ml/d of PBS. In contrast, the remaining groups were administered 10 mg/kg/d ISO in PBS immediately after ROSC and daily for 7 days, as specified in the protocols. Exclusion criteria included < 5% of animals because of unsuccessful ROSC within 5 min or surgical complications.

Neurological Deficit Score (NDS)

The NDS was assessed every 24 hours after ROSC to evaluate neurological function. Two blinded investigators (Cohen's $\kappa > 0.85$) confirmed the assessments. Scores ranged from 0 to 80, with 0 indicating brain death and 80 normal functions, based on evaluations of consciousness, cranial nerve reflexes, motor function, sensory function, and behavior.

Behavioral Assessments

Y-maze

A Y-maze apparatus with three equal Plexiglass arms (40 x 10 x 20 cm) at 120-degree angles, with a central triangular platform, was used to test spatial working memory and exploratory behavior. After 60 minutes of acclimatization in the testing room to reduce stress associated with transportation, each subject was placed at the far end of an initial arm, turned toward the center, and then left to move freely on all three arms for 8 minutes. To remove any odor cues, the maze was thoroughly washed with 70% ethanol and dried between trials. An overhead camera recorded behavioral performance, which was analyzed with EthoVision XT or ANY-maze tracking software. At the end of the analysis, the sequence and total arm entries were recorded; an entry was defined as all four paws in the arm. The primary endpoint, the percentage of spontaneous alternation, was divided into the percentage of actual alternations (sequential entries into three arms) divided by the possible alternations (total arm entries

and two). Total arm entries were also recorded as an index of general locomotor activity to ensure that motor deficits did not bias the memory results. A one-way ANOVA was conducted to test differences among the groups.

Elevated Plus Maze (EPM)

The anxiety-like behavior was assessed using the EPM, which consisted of two closed arms (35 x 5 cm) and two open arms (35 x 5 cm) extending from a central 5 x 5 cm platform. Fifteen cm-high walls surrounded the closed arms, whereas there were no walls around the open arms; the entire setup was suspended 50 cm above the floor. Before the test, animals were acclimatized to the soundproof testing chamber for 1 h. Subsequently, each animal was placed on a central platform facing a closed arm and allowed to move freely in the maze for 5–10 minutes. Between tests, the apparatus was washed entirely with 70% ethanol to remove olfactory cues. An overhead camera was used to capture behavioral metrics, and the data were analyzed using ANY-maze or EthoVision XT video-tracking software. Anxiety-like performance was assessed using metrics such as the percentage of time in open arms and the number of entries into open arms. In addition, the number of entries in closed arms was used to evaluate the overall locomotor activity. Vertical exploration was also assessed by quantifying rearing rate and head dipping at the edges of the open arms.

Open Field Test (OFT)

An OFT was also performed to assess locomotor and exploratory behavior in a 100 × 100 × 40 cm arena made of high-density, non-porous PVC, and the floor was divided into a peripheral region and a central area. Rats were brought to the room 1h before the test. Before each 5-minute trial, a single rat was put in the center of the arena and allowed to move freely for 5 minutes under 300 lux lighting. Total locomotor and anxiety-like activities within the central zone (25% of the total area) were recorded using an overhead camera and quantified using EthoVision XT or ANY-maze tracking software. All rats were tested once, and the arena was dried using 70 percent ethanol and then dried between trials to remove olfactory stimuli. The primary readouts were time spent in the center zone, total distance traveled, and rearing frequency.

2.4. Brain Edema and Infarct Volume

Brain water content was measured 24 hours after ROSC using the wet-dry method to assess edema. Male Sprague-Dawley rats (250–280 g) were anesthetized with 5% isoflurane for 2 minutes. Brains were rapidly removed, excluding the olfactory bulbs, cerebellum, and brainstem. Each hemisphere was separated, weighed wet, then dried at 110°C for 24 hours. After drying, the hemispheres were weighed again. The percentage of brain water content (BWC) was determined by using the following formula: BWC (%) = (wet weight - dry weight)/wet weight × 100. All experiments were conducted in triplicate to ensure accuracy.

Infarct volume was measured using TTC staining. Brains were cut into 2-mm sections with a brain matrix. Slices were incubated in 2% TTC (Sigma, Invitrogen, New York, USA) in PBS at 37°C for 15–20 minutes in the dark. After fixation in 10% neutral-buffered formalin for 6 hours, infarct areas were analyzed with ImageJ Software. Edema correction was applied using the formula: corrected infarct volume = measured infarct volume × (contralateral hemisphere volume / ipsilateral hemisphere volume).

2.5. Histological staining

The brain tissue was fixed in 4% paraformaldehyde, dried in 70% ethanol, embedded in paraffin, and sectioned at 5 μm. In the case of hematoxylin and eosin (H&E) staining, sections were incubated in hematoxylin for 5 minutes, rinsed, and then incubated in eosin for 2 minutes. The slides were then dried, placed, and viewed under a Leica DM2000 microscope (Leica Microsystems, Wetzlar, Germany).

For Nissl staining, sections were incubated in 1% cresyl violet at 37°C for 10 minutes, then dehydrated to enable quantification of Nissl bodies.

Apoptosis was detected by TUNEL staining using a TUNEL kit. Permeabilization of the samples was performed with 0.1% Triton X-100, and the samples were blocked with 5% BSA at room temperature. Cells were incubated with the TUNEL reaction mixture at 37°C for 1 hour in the dark. Following DAPI counterstaining (Abcam, ab285390), coverslips were mounted on glass slides. Fluorescence microscopy (Zeiss Axio Observer) was employed for visualization and image acquisition.

Primary Cortical Neuron Culture and OGD/R Model

The primary cortical neurons were obtained from Sprague-Dawley rat embryos. Embryos were decontaminated with 75% ethanol, decapitated, and brains removed in ice-cold PBS with 2% penicillin-streptomycin. Cells were resuspended in Neurobasal-A medium containing 1% penicillin-streptomycin, 2% B27, and 0.5 mM L-glutamine and plated on poly-L-lysine-coated plates. Cultures were maintained at 37°C in 5 per cent CO2. The culture medium was replaced every 3 days. The phase-contrast microscopy was used to monitor neural morphology.

Oxygen-glucose deprivation (OGD) analysis was used to simulate PCABI. Neuronal cultures were maintained in glucose-free DMEM and then transferred to a hypoxic chamber flushed with 95% nitrogen and 5% carbon dioxide at 37°C for 3 h. Experimental groups were made (control (OGD/reoxygenation), OGD/reoxygenation with 25 μg/ml ISO, OGD/reoxygenation with 50 μg/ml ISO, and OGD/reoxygenation with 100 μg/ml ISO). ISO was administered during the reoxygenation phase, and the oxygen level was maintained at 1%.

Cell Counting Kit-8 (CCK-8) assay

The Cell Counting Kit-8 (CCK-8) assay was used to determine cell viability. In 100 µL of culture medium, cells were seeded in 96-well plates between 5,000–10,000 cells per well and allowed to incubate for 24 hours at 37°C in 5% CO2. After treatments, 100 µL of CCK-8 solution was added to each well, and the plates were incubated for 4 h at 37°C. A microplate reader was used to measure formazan production at 450 nm (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cell viability was calculated relative to untreated controls by subtracting the background absorbance from the absorbance of wells containing medium and CCK-8 reagent only. Each experiment was done thrice.

Flow Cytometry

The levels of cellular apoptosis were measured using the Annexin V-FITC/Propidium Iodide (PI) Apoptosis Detection Kit (BD Biosciences, USA) according to the manufacturer's instructions. Cells were gathered, rinsed with cold phosphate-buffered saline (PBS) twice and resuspended in 1X Binding Buffer containing approximately 1 x 10^6 cells/mL. One hundred µL of the suspension was transferred to a sterile flow tube and incubated with 5 µL of Annexin V-FITC and 5 µL of PI at room temperature in the dark. After incubation, 400 µL of 1X Binding Buffer was added to each tube, and samples were analyzed at 1 hour via BD FACSVerse™ flow cytometer. Data analysis used FlowJo software and gating to identify viable (Annexin V -/PI -), early apoptotic (Annexin V -/PI -), late apoptotic (Annexin V +/PI +), and necrotic (Annexin V -/PI +) cells. The apoptosis percentage was the combined early- and late-apoptotic percentages.

ROS Assay

The reactive oxygen species detection kit (DCFDA; Sigma-Aldrich, Cat. #287810) was used to measure ROS levels in cells. Cells cultured in DMEM were washed twice with a buffer prepared by diluting the given 10x buffer, and 5 mM 2', 7'-dichlorodihydrofluorescein diacetate was added to each well and incubated at 37°C in the dark. Cells were washed. Live-cell microscopy was performed using a fluorescein (FITC) filter set (EVOS FL, Thermo Fisher Scientific), and signals in digital cell images were analyzed and compared using ImageJ.

ELISA

The cell supernatants were centrifuged at 1,000 rpm for 5 minutes. Specific kits were used to analyze IL-1β, IL-6, and TNF-α (Nanjing Jiancheng). To be accurate, measurements were done three times at 450 nm.

qPCR

The TRIzol Reagent (Thermo Fisher Scientific, USA) was used to extract total RNA, and 10 g of total RNA was reverse-transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Germany) according to the manufacturer's instructions. qPCR was measured using SYBR Green (Bio-Rad) on a 7500 Real-Time PCR System (Thermo Fisher Scientific, USA). qPCR conditions were: 95°C 3 minutes, 40 cycles of 95°C 1 minute. Specificity was ascertained through the melting curve. Primers are listed in

Table S3. The samples were analyzed three times, and relative expression was determined using the $2^{-\Delta\Delta Ct}$ method.

Western Blot

Total protein was extracted using RIPA lysis buffer (Thermo Fisher Scientific, USA) supplemented with protease and phosphatase inhibitors (Roche, Germany). Proteins (2040 µg) were separated by 12% SDS-PAGE and transferred to PVDF membranes (Millipore, USA). The 5% non-fat milk in TBST was used to block membranes for 1 hour at room temperature. Overnight incubation at 4°C was performed with primary antibodies (1:1000). Membranes were washed and incubated with goat anti-rabbit IgG conjugated with HRP (Cell Signaling Technology, Cat#7074, 1:1000) after washing, and incubated at room temperature (1 hour). Protein bands were visualized using the ECL system (Thermo Fisher Scientific, USA), quantified with ImageJ, and 2-actin was used as a loading control.

Statistical Analysis

Statistical analyses were performed using SPSS version 25.0. Normality was assessed with the Shapiro-Wilk test, and homogeneity of variance with Levene's test. Results are reported as mean ± standard deviation. One-way ANOVA with Tukey's post-hoc test was used for comparisons among three or more groups, and unpaired t-tests for two-group comparisons. Non-parametric data were analyzed with the Kruskal-Wallis test followed by Dunn's test. Pearson's or Spearman's correlation coefficients were calculated to assess relationships between variables. Experimental data were also analyzed using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

Results

ISO Enhances Neurological Recovery and Survival in PCABI Rats

Kaplan-Meier survival analysis showed significant differences among experimental groups (Fig. 1A). The survival rate of the PCABI group was 20 percent at 72 hours, and this indicates the intensity of the injury caused by CA/CPR. ISO treatment improved survival to 37% in the PCABI + 25 µg/ml ISO group, 50% in the PCABI + 50 µg/ml ISO group, and 72% in the PCABI + 100 µg/ml ISO group at 72 hours ($P < 0.01$ vs. PCABI). These results show that administration of ISO improves survival in PCABI rats and provides strong protective effects.

PCABI rats showed behavioral dysfunction in multiple tests (Fig. 1B–D). Y-maze analysis showed a marked decrease in novel arm exploration compared with Sham ($P < 0.0001$; Fig. 1B), indicating impaired spatial memory. Treatment with 50 µg/ml ISO improved exploration to 85% of Sham and a significant increase over the PCABI group ($P < 0.001$, Fig. 1B). ISO also improved exploratory behavior, as shown by the time ratio in PCABI, Sham, and PCABI + 50 µg/ml ISO ($P < 0.001$). Elevated plus maze analysis

showed a reduction in open arm time in PCABI rats compared with Sham ($P < 0.0001$, Fig. 1C). ISO at 50 μg/ml increased open arm time ($P < 0.01$, Fig. 1C), consistent with anxiolytic effects. In the open field test, PCABI rats showed decreased central zone activity compared with Sham ($P < 0.0001$; Fig. 1D), whereas ISO at 50 μg/ml increased central zone activity ($P < 0.001$; Fig. 1D), without affecting total locomotor distance ($P > 0.05$). Laser Doppler blood flow imaging was performed to evaluate cerebral perfusion. The cerebral blood flow and perfusion volume were significantly lower in PCABI rats (Fig. S1, S2), with markedly impaired NDS compared to Sham ($P < 0.0001$; Fig. 1E), which progressed over a 72-hour time course, as shown in Fig. S3. Deficits were observed in consciousness, motor function, and behavior. ISO at 25 μg/ml increased NDS in a dose-dependent manner compared with PCABI ($P < 0.0001$), with 50 μg/ml providing optimal recovery of consciousness ($P < 0.01$). Brain edema increased water content in PCABI rats compared with Sham ($P < 0.001$; Fig. 1F), whereas 50 μg/ml ISO reduced water content ($P < 0.01$). TTC staining showed 29.7 ± 3.4% of hemisphere volume was infarcted in PCABI brains versus 0% in Sham ($P < 0.0001$, Fig. 1G). Infarct volume decreased at 50 μg/ml of ISO ($P < 0.001$), partially at 25 μg/ml and 100 μg/ml. ISO treatment reduces PCABI-related dysfunction, edema, and infarction dose-dependently.

ISO Attenuates Neuronal Apoptosis in PCABI Rats

Histological analysis of PCABI brains showed severe neuronal damage **(**Fig. 2A**)**. Hematoxylin and eosin (H&E) staining revealed disorganized cortical and hippocampal neurons, with pyknotic nuclei and cytoplasmic swelling. This resulted in a 30% reduction in cell number relative to shams, which showed normal, tightly packed neurons with large nuclei ($P < 0.001$). Treatment of 50 μg/ml ISO maintained 70 percent of normal neuronal morphology, with more distinct nuclei and less swelling than PCABI ($P < 0.01$). The Nissl staining revealed that the density of Nissl bodies was lower in PCABI than in the sham ($P < 0.0001$). An ISO concentration of 50 μg/ml increased Nissl body density ($P < 0.01$). TUNEL staining indicated a significant increase in apoptotic cells, with TUNEL-positive neurons in PCABI versus sham controls ($P < 0.0001$; Fig. 2B, C). The dose-dependent effect of ISO at 50 μg/ml and 100 μg/ml significantly inhibited apoptosis ($P < 0.001$). Quantitative qPCR showed increased Caspase-3 and Bax mRNA levels and decreased PCABI compared with sham. On the other hand, ISO 50 μg/ml increased Caspase-3 and Bax and decreased Bcl-2 ($P < 0.01$; Fig. 2D). Similar results were observed in Western blot analysis, with increased Caspase-3 and Bax protein in PCABI, whereas Bcl-2 was reduced. ISO treatment restored Caspase-3, Bax, and Bcl-2 protein levels to sham levels ($P < 0.001$; Fig. 2E). The most important apoptosis marker, the Bax/Bcl-2 ratio, was higher in PCABI than in sham, with a lower ratio at 50 μg/ml ISO. These findings suggest that ISO inhibits apoptosis, maintains neuronal architecture, and balances pro- and anti-apoptotic proteins in response to PCABI-induced neural loss.

ISO Activates ERK/NRF2 Signaling in PCABI Rats

qPCR showed significant downregulation of ERK, NRF2, and HO-1 mRNA in PCABI compared to sham ($P < 0.001$, Fig. 3A). In contrast, 50 μg/ml ISO upregulated ERK, NRF2, and HO-1 ($P < 0.01$, Fig. 3A), with more minor effects at 25 μg/ml ISO and 100 μg/ml ISO. Western blot showed a reduction in p-ERK/ERK

ratio, nuclear NRF2 and HO-1, while cytosolic NRF2 was increased ($P < 0.001$, Fig. 3B), indicating impaired signaling and reduced nuclear translocation in PCABI. Treatment with 50 μg/ml ISO enhanced p-ERK, nuclear NRF2, and HO-1, while cytosolic NRF2 was decreased ($P < 0.001$, Fig. 3B). Nuclear NRF2 levels were significantly associated with HO-1 expression ($P < 0.001$), and inversely with TUNEL positive cells ($P < 0.01$), consistent with the cytoprotective role of NRF2. These results suggest that ISO promotes nuclear accumulation of NRF2 and restores ERK phosphorylation.

ISO Protects Primary Cortical Neurons from OGD/R Injury

CCK-8 assays showed decreased neuronal viability in OGD/R compared with control ($P < 0.0001$; Fig. 4A). ISO treatment increased viability at 50 μg/ml ($P < 0.01$), with moderate effects at 25 μg/ml and 100 μg/ml. ELISA showed that OGD/R significantly increased IL-1β, IL-6, and TNF-α compared to control ($P < 0.001$), while ISO (50 μg/ml) reduced IL-1β, IL-6, and TNF-α ($P < 0.01$, Fig. 4B). DCFDA showed increased ROS levels and ROS MFI in OGD/R compared to control ($P < 0.0001$, Fig. 4C, D). ISO at 50 μg/ml decreased ROS levels and ROS MFI ($P < 0.001$), with similar trends at 25 μg/ml and 100 μg/ml. Fluorescence intensity and quantitative analysis of ROS MFI levels across different groups are presented in Fig. S4. Flow cytometry showed that apoptosis was increased in OGD/R neurons compared to controls ($P < 0.0001$; Fig. 4E, F), with early and late apoptosis. ISO reduced total apoptosis ($P < 0.001$) by primarily decreasing early apoptosis. qPCR showed an increase in Caspase-3 3 and Bax in OGD/R, while Bcl-2 was decreased ($P < 0.001$, Fig. 4G). In contrast to ISO at 50 μg/ml, which decreased Caspase-3 and Bax with an increase of Bcl-2 ($P < 0.01$). Western blot confirmed an increase of Caspase-3 and Bax protein with a decrease of Bcl-2 in OGD/R. ISO increased Caspase-3, Bax, and Bcl-2 to control levels ($P < 0.001$, Fig. 4H). These findings show that ISO acts as an anti-apoptotic, antioxidant, and anti-inflammatory agent in PCABI.

ISO Activates ERK/NRF2 Pathway in OGD/R Neurons

qPCR showed that mRNA expression levels of ERK, NRF2, and HO-1 were significantly downregulated compared to control ($P < 0.001$). 50 μg/ml of ISO upregulated ERK, NRF2, and HO-1 ($P < 0.01$, Fig. 5A). Western blot showed a decrease in p-ERK/ERK, nuclear NRF2, and HO-1 in OGD/R, along with increased cytosolic NRF2 ($P < 0.001$). Conversely, ISO increased p-ERK, nuclear NRF2, and HO-1, whereas cytosolic NRF2 was reduced ($P < 0.001$; Fig. 5B). The correlations of apoptosis ($P < 0.01$) with nuclear NRF2 and of ROS ($P < 0.001$) with cytosolic NRF2 confirmed the protective role of this pathway. These results suggest that ERK/NRF2 activation is a central mechanism in ISO neuroprotection.

Discussion

PCABI remains a leading cause of mortality and long-term disability caused by ischemia and reperfusion of the brain following resuscitation [5]. This study strongly indicates that ISO, a flavonoid, activates the ERK/NRF2 signaling pathway by suppressing neural apoptosis, reducing oxidative stress, and preventing inflammation in PCABI. Our results were based on both *in vivo* and *in vitro* studies using a rat model,

including CA/CPR and OGD/R experiments. 50 μg/ml of ISO in PCABI rats results in a marked reduction in spatial memory, as assessed by Y-Maze analysis; in anxiety regulation, as evaluated by the EPM; and in exploratory behavior, as assessed by the OFT. Dose-dependent of ISO reduced NDS, brain edema, and infarct size in PCABI, indicating an improvement of neurological function, especially with 50 μg/ml than 25 μg/ml and 100 μg/ml. We found that an ISO treatment of 50 μg/ml is the optimal dose, improving neurological activity and behavioral tests by exerting a neuroprotective effect against PCABI, consistent with a previous study on synaptic and cognitive dysfunction and behavioral impairments after global cerebral ischemia [17].

The PCABI brains were histologically disorganized, with respect to cortical and hippocampal neurons, pyknotic nuclei, cytoplasmic swelling, reduced Nissl bodies, and increased apoptosis. Conversely, ISO preserved normal morphology, with more distinct nuclei, less swelling, more Nissl bodies, and less apoptosis, confirming that ISO suppresses neuronal apoptosis in PCABI rats and thereby preserves neuronal morphology and function.

ISO-modulated apoptotic pathways decrease Caspase-3 and Bax and increase Bcl-2, thereby reducing the Bax/Bcl-2 ratio. Therefore, Caspase-3 mediates cell death, whereas Bax promotes mitochondrial permeabilization and Bcl-2 inhibits this pathway. Molecular analysis in the *in vitro* OGD/R rat model reveals downregulation of ERK, NRF2, and HO-1 mRNA expression in PCABI, whereas ISO increases their mRNA expression. Additionally, ISO enhanced ERK phosphorylation, nuclear NRF2, and HO-1, whereas PCABI reduced these markers. Therefore, ISO promotes the restoration of ERK phosphorylation and the nuclear accumulation of NRF2. However, HO-1 is a pleiotropic protein with a heme-degradation function that leads to the formation of antioxidant bile pigments and the important signaling gas CO, which is involved in the regulation of gene expression and modulation of protein translation [18]. The NRF2/HO-1 signaling pathway plays an antioxidative role, with NRF2 regulating the expression of protective enzymes. At the same time, HO-1 exerts anti-inflammatory, anti-apoptotic, and antifibrotic effects through heme degradation products in liver-brain disease [19]. A strong correlation between nuclear NRF2/reduced ROS/apoptosis ($r = -0.82$ to -0.89) supports the role of NRF2 in brain stroke and traumatic brain injury, highlighting that ISO exerts neuroprotective effects after CIR through upregulation of Neuroglobin and antioxidant activity [20].

In vitro OGD/R in PCABI rats reduced cell viability, accompanied by increased ROS levels and apoptosis. In contrast, ISO, applied in a dose-dependent manner, suppressed pro-inflammatory cytokine expression, thereby restoring cell viability and reducing ROS and apoptosis. Significantly, ISO further decreases the levels of IL-1β, IL-6, and TNF-α, consistent with the observation that ISO acts as a neuroprotective agent against I/R brain injury by suppressing the inflammatory response and promoting cell recovery through inhibition of TLR4 and C5aR1 expression, promotion of cAMP/PKA activation, and inhibition of I-κB/NF-κB activation and Caspase 3 expression. TLR4 and C5aR1 contributed to inflammation and apoptosis by activating cAMP/PKA/I-κB/NF-κB signaling during cerebral I/R [21]. In addition, ISO suppresses the activation of toll-like receptor 4 (TLR4), nuclear factor-kappa B (NF-κB), and caspase-1; the phosphorylation of ERK1/2, JNK1/2, and p38 mitogen-activated protein kinase (MAPK); and the

secretion of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6, and effectively alleviates hippocampus neuron apoptosis by regulation of cyclic AMP-responsive element-binding protein (CREB), Bax, Bcl-2, and caspase-3 [22]. In addition, the role of the ERK/NRF2 signaling pathway in ISO-mediated treatment in CA/CPR and OGD/R experiments strongly indicates that this pathway is responsible for preventing brain injury.

The study has several limitations; first, the time points used in the *in vivo* and *in vitro* studies are 7 days and 24 hours, respectively, and long-term studies have not been conducted. Secondly, sex differences warrant exploration, as we used only male rats, and specific responses to ischemia may differ by sex. Lastly, future studies are needed to elucidate the mechanisms underlying these interactions and their therapeutic value in enhancing neurological function and inhibiting the progression of brain injury. However, we agree that further replicating the experiments will strengthen the validity of this research.

PCABI remains one of the clinical challenges due to failure of the treatment therapy in targeting molecular mechanisms of brain injury, especially the damage to neurons. Findings from this paper indicate that the therapeutic intervention ISO, which stimulates the ERK/NRF2 pathway, inhibits neural apoptosis, inflammation, and oxidative stress. The neuroprotective effect of ISO in humans and its potential use as supportive therapy for PCABI to enhance neurological performance and survival rates warrants further investigation. Nonetheless, to ensure the safety and efficacy of CA in patients, clinical trials are required to determine the optimal dose.

Conclusion

To conclude, ISO activates the ERK/NRF2 signaling pathway and inhibits neural apoptosis, inflammation, and oxidative stress, thereby conferring neuroprotective effects against PCABI. As reported in this study, 50 μg/ml of ISO is considered the optimal dose to enhance neurological performance, survival, and cell viability in CA/CPR rats and OGD/R neurons. Therefore, ISO could be a promising therapeutic agent protecting the brain against PCABI and warrants further exploration for therapeutic interventions.

Abbreviations

CA/CPR
Cardiac Arrest/Cardiopulmonary Resuscitation
ERK
Extracellular Signal-Regulated Kinase
EPM
Elevated Plus Maze
ISO
Isoquercitrin
KEAP1
Kelch-like ECH-associated protein 1

MCAO
Middle Cerebral Artery Occlusion
NAC
N-acetylcysteine
NDS
Neurological Deficit Scoring
NMDA
N-methyl-D-aspartate
NRF2
Nuclear factor erythroid 2-related factor 2
OFT
Open Field Test
OGD/R
Oxygen-Glucose Deprivation/Reoxygenation
OHCA
Out-of-Hospital Cardiac Arrest
PCABI
post-Cardiac Arrest Brain Injury
ROS
Reactive Oxygen Species
TTM
Targeted Temperature Management.

Declarations

Ethical Approval and Consent to Participate

All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of Hainan Medical University.

Consent for Publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests

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Author Contribution

Pengju Du and Run Lin contributed to data curation, formal analysis, and writing of the original draft. Jieyao Li and Miao Chen contributed to writing, review, and editing. Jieyao Li and Miao Chen conducted conceptualization, methodology, and supervision. Jieyao Li designed the study and supervised the research work. All authors have read and approved the final version of the manuscript for publication.

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Data Availability

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

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Figures

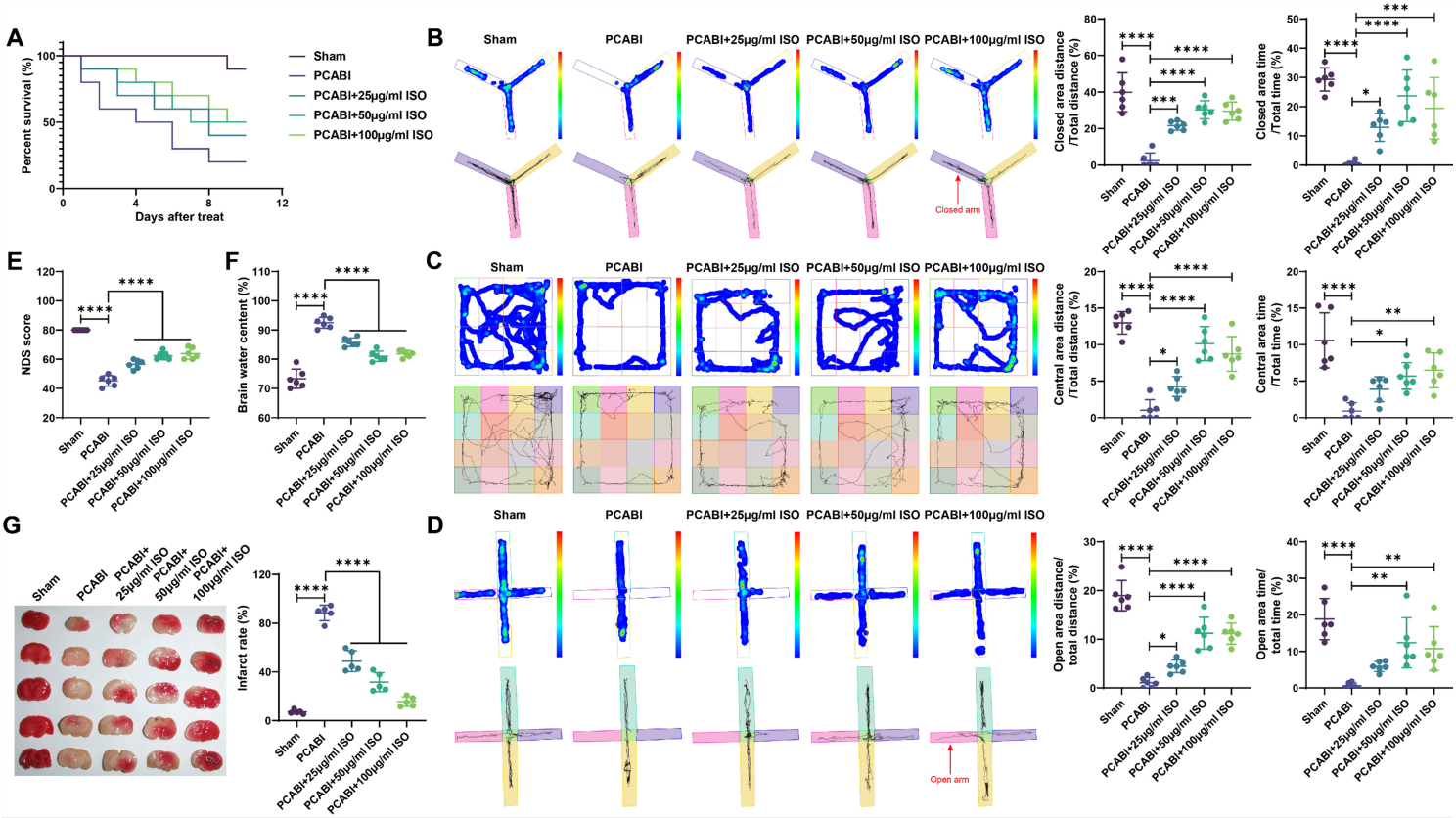


Figure 1

ISO Improves Neurological Function in PCABI Rats. (A) Kaplan-Meier survival curves (%) from different groups. **(B–D)** Behavioral tests (Y-maze, elevated plus maze, open field) among groups. **(E)**Neurological Deficit Scores (NDS) of different groups. **(F)** Brain water content (%) of different groups. **(G)** TTC staining and infarct volume quantification from different groups. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 among the compared groups. The experiments were performed thrice in triplicate (n=3 samples).

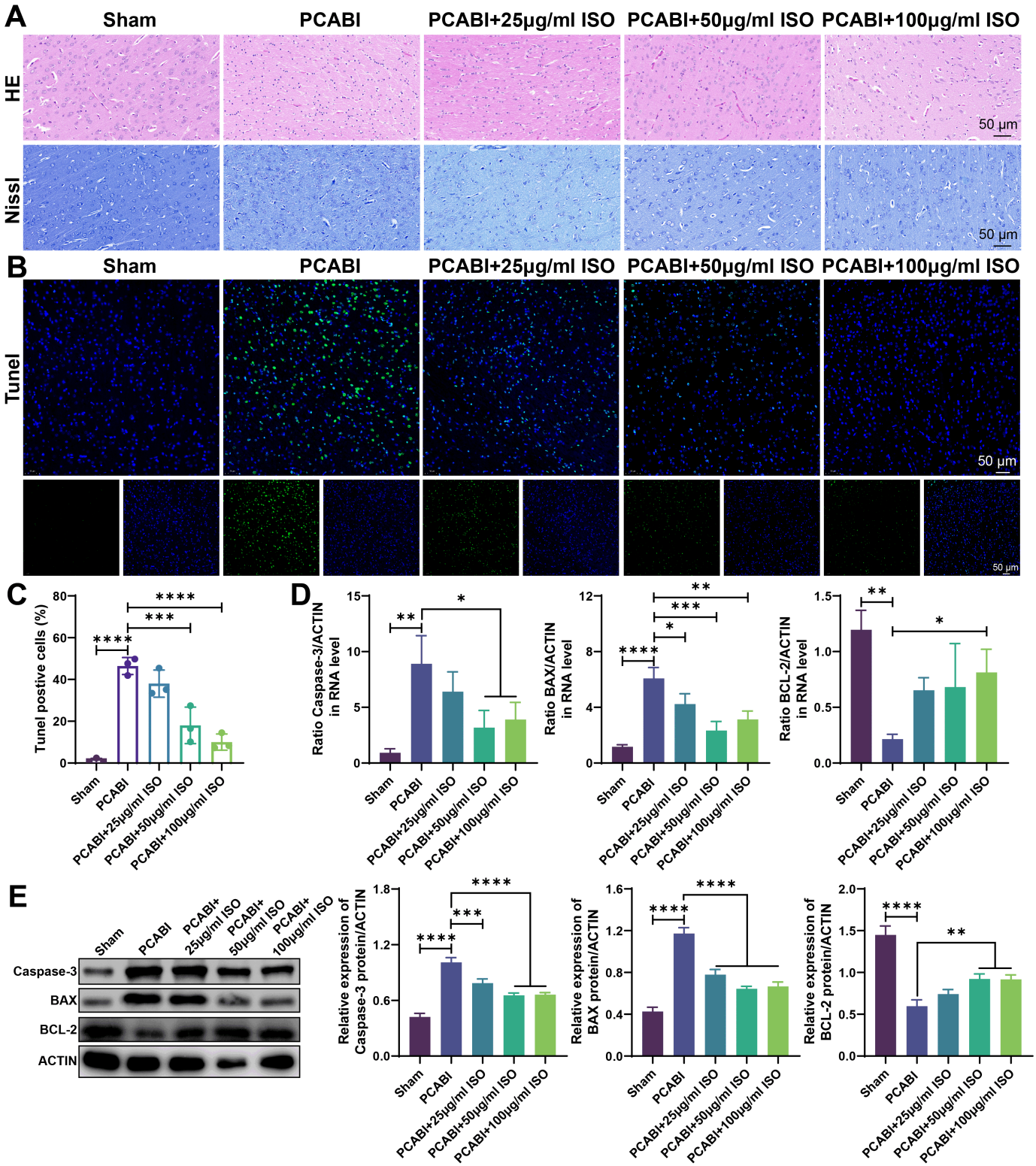


Figure 2

ISO Reduces Neuronal Apoptosis in PCABI Rats. (A) H&E and Nissl staining of brain tissue from different groups. **(B, C)** TUNEL staining indicating reduced apoptotic neurons in different groups. **(D)** Quantitative analysis of mRNA expression of apoptosis-related genes (Caspase-3, Bax, Bcl-2) by qRT-PCR. **(E)** Western blot detection and quantification of relative expression levels of Caspase-3, Bax, and

Bcl-2. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 among the compared groups. The experiments were performed in triplicate (n =3). Scale bar = 50 μm.

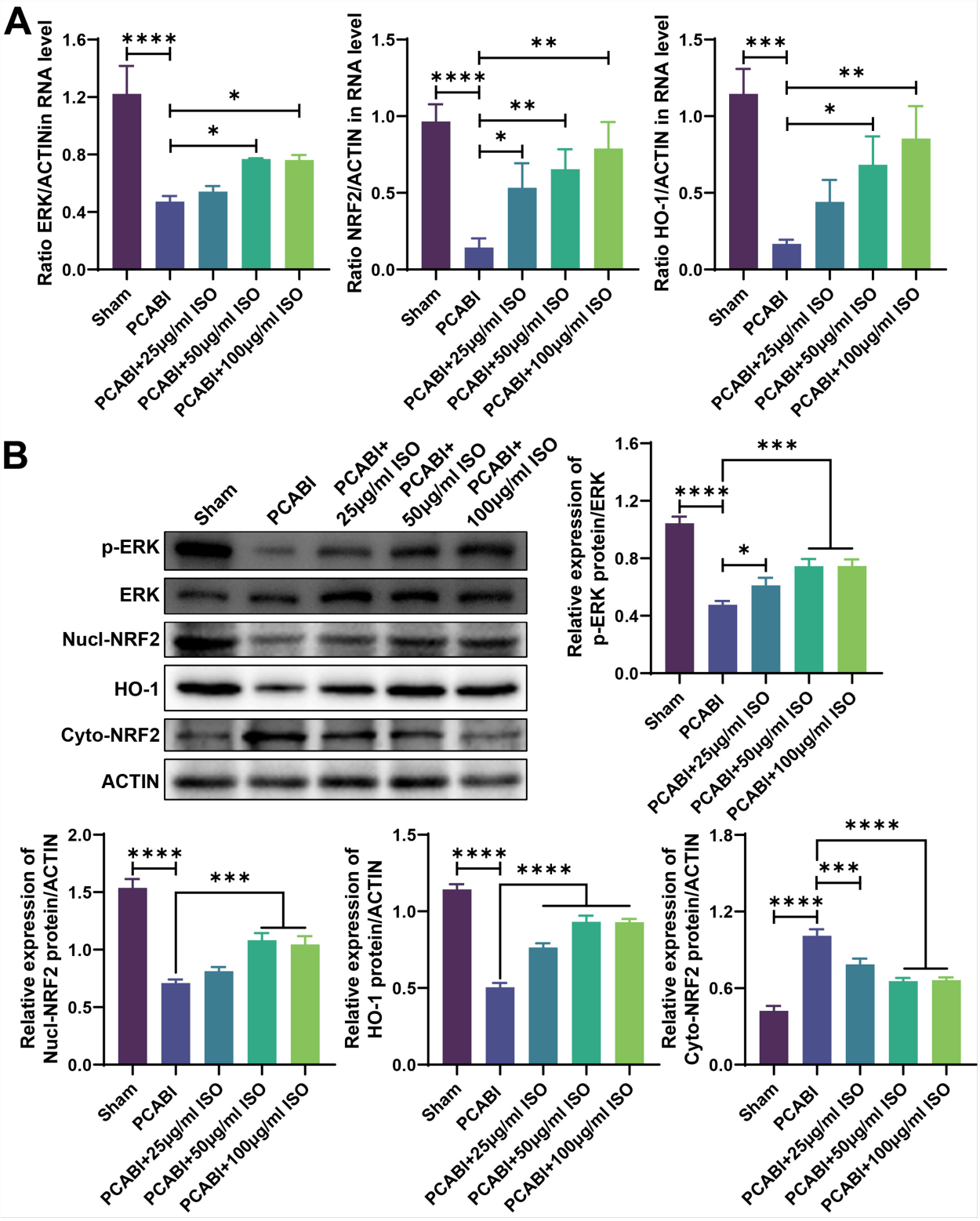


Figure 3

ISO Activates ERK/NRF2 Pathway in PCABI Rats. (A) qRT-PCR detection of ERK, NRF2, and HO-1 mRNA expression among different groups. **(B)**Western blot detection of p-ERK, nuclear NRF2, and HO-1, and

cytosolic NRF2 expression in different groups. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 among the compared groups. The experiments were performed in triplicate (n =3).

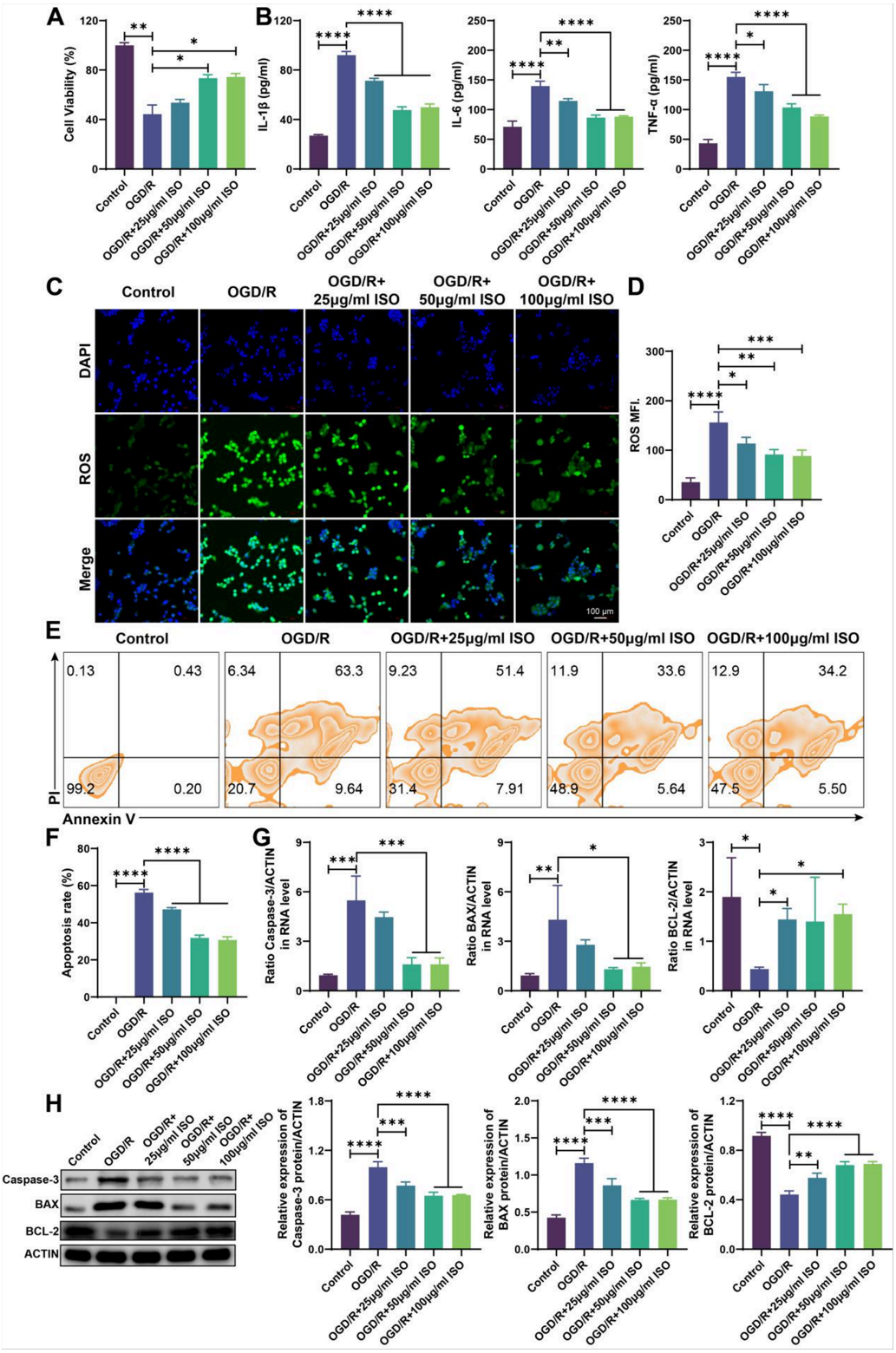


Figure 4

ISO Suppresses Apoptosis in OGD/R Neurons. (A) CCK-8 assay for detecting cell viability (%) under different treatment conditions. **(B)**ELISA detection of IL-1β, IL-6, and TNF-α expression. **(C, D)** ROS

staining and ROS MFI of different groups to evaluate the oxidative stress. **(E, F)**Flow cytometry analysis to detect apoptosis rates (%) across groups. **(G)** RT-PCR detection of Caspase-3, Bax, and Bcl-2 gene expression in brain tissue from different groups. **(H)** Western blotting detection of Caspase-3, Bax, and Bcl-2 proteins in brain tissue from different groups. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 among the compared groups. The experiments were performed in triplicate (n =3). Scale bar = 100 μm.

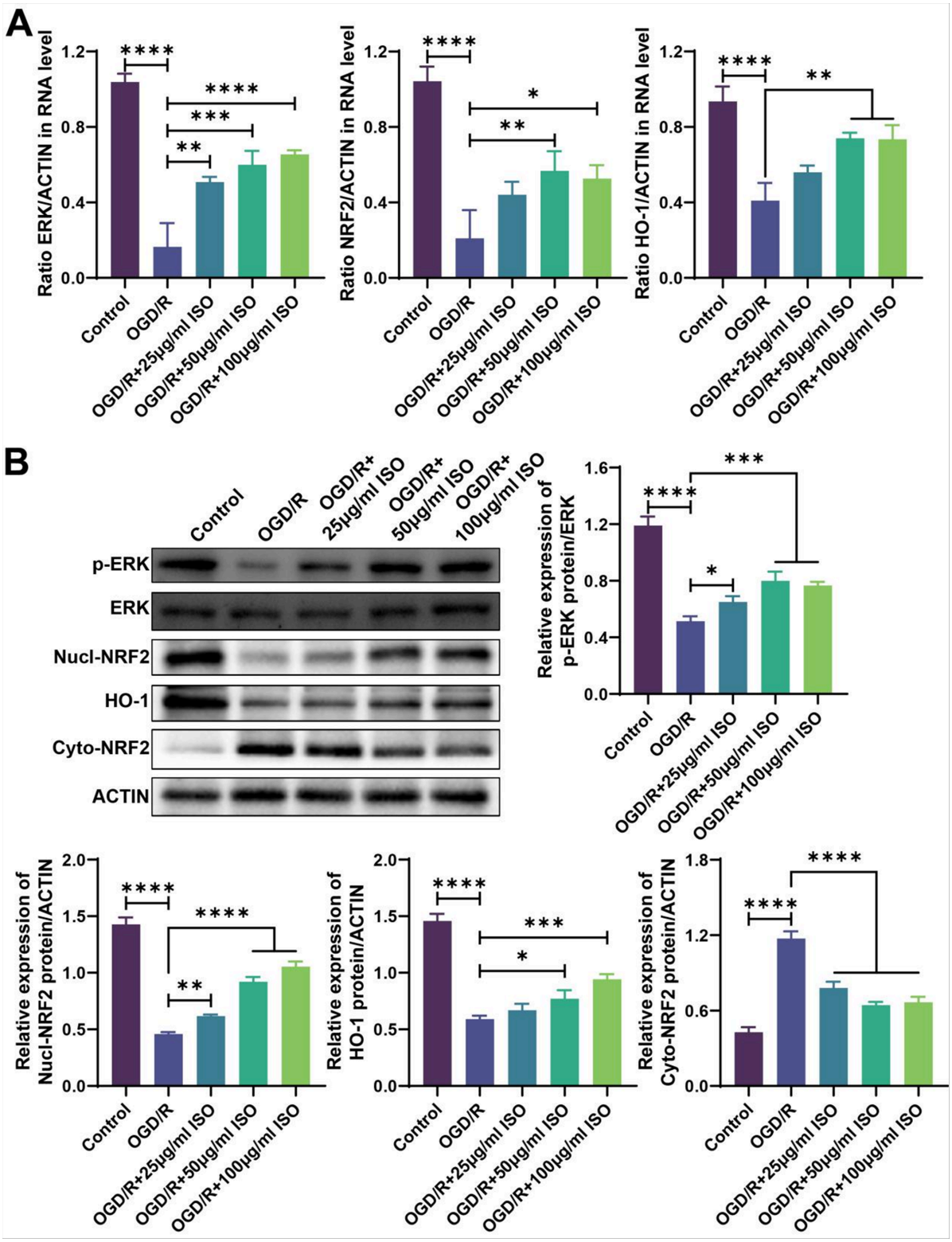


Figure 5

ISO Activates ERK/NRF2 Pathway in OGD/R Neurons. (A) Quantitative analysis of ERK, NRF2, and HO-1 mRNA expression by qRT-PCR. **(B)** Western blot detection of p-ERK, nuclear NRF2, and HO-1, and cytosolic NRF2 expression. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 among the compared groups. The experiments were performed in triplicate (n =3).

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