Original Article
Quercetin improves rat brain function after ischemia by regulating DJ-1/Nrf2

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Abstract: Nuclear transcription factor NF-E2 related factor 2 (Nrf2) is an important nuclear transcription factor in anti-oxidative stress. DJ-1 is a multifunctional protein widely distributed in various tissues. It plays a role in enhancing Nrf2 stability and promoting Nrf2 protein expression. Quer is a flavonoid that provides antioxidant and neuroprotective effects. In this study, a rat brain I-R injury model was established to detect caspase-3 activity, malondialdehyde (MDA) levels, and superoxide dismutase (SOD) levels. Modified nerve injury severity (mNSS) scores were used to evaluate neurological function. DJ-1 and Nrf2 expression was compared. Rat Rn-c neurons were cultured in vitro and divided into three groups, including the control group, IR group, and I-R+Quer group. Cell apoptosis and reactive oxygen species (ROS) were detected using flow cytometry. MDA content and caspase-3 activity levels were significantly elevated, SOD activity was obviously reduced, and mNSS scores were increased in brain tissues of the I-R model rats. DJ-1 and Nrf2 expression was apparently upregulated in the IR model. MDA content, caspase-3 activity, and mNSS scores were significantly decreased, while SOD activity, DJ-1, and Nrf2 expression levels were obviously elevated in the I-R+Quer treatment group. IR-treatment induced Rn-c cell apoptosis and intracellular ROS production. It also upregulated DJ-1 and Nrf2 expression. Quer treatment further enhanced DJ-1 and Nrf2 levels, attenuated ROS production, and reduced apoptotic rates in H9C2 cells under IR treatment. Quer attenuates brain cell apoptosis and neuronal damage after I-R by increasing DJ-1 levels, enhancing DJ-1/Nrf2 pathways, and elevating anti-oxidation levels.

Keywords: IR injury, DJ-1, Nrf2, oxidation, apoptosis, brain

Introduction

Ischemic cerebral infarction (ICI), also known as ischemic stroke, is a common cerebral vascular disease (CVD) [1-3]. ICI is characterized by a rapid onset, critical illness, death, and disability. Thus, it poses a serious threat to human life. Ischemia-reperfusion (I-R) injury is brain tissue damage caused by reperfusion of blood after ICI, occurring mainly in the treatment stage of ICI [4, 5].

Nuclear factor NF-E2-related factor (Nrf2)/antioxidant response element (ARE) is the most important endogenous anti-oxidative stress (OS) signaling pathway. Activation of Nrf2 plays a role in reducing OS response and alleviating

brain IR damage [6, 7]. DJ-1 is a regulatory protein widely distributed in various tissues, playing an important role in various biological effects, such as anti-OS, reduction of apoptosis, and promotion of cell growth and survival [8-10]. Many studies have confirmed that DJ-1 plays a role in enhancing Nrf2 stability, as well as promoting Nrf2 protein expression and activation. Anti-OS effects of DJ-1 depend on regulation of Nrf2 [10, 11].

Quercetin (Quer) is a natural plant-derived flavonoid. In recent years, many studies have found that Quer delays Alzheimer's disease [12, 13], reduces cerebral ischemic injury [14], improves cognitive and motor function [15], and decreases neuronal apoptosis [14, 16]. It is unclear

whether Quer affects the activity of DJ-1/Nrf2 pathways and alleviates I-R injuries. The current study established a rat model of cerebral I-R injury, investigating the roles of Quer in regulating DJ-1/Nrf2 pathway activity and alleviating rat brain I-R injuries.

Materials and methods

Main reagents and materials

Healthy adult male Sprague-Dawley rats (6 weeks old, weighing 200-220 g) were purchased from Beijing Weitong Lihua Experimental Animal Co., Ltd. Rat brain nerve RN-c cell line was purchased from Shanghai Zibo Bio. DMEM medium and fetal bovine Serum (FBS) were purchased from Gibco. Rabbit anti-rat Nrf2, DJ-1, and β-actin were purchased from Abacm. HRP-conjugated secondary antibody was purchased from Sangon. PrimeScript™ RT reagent Kit was purchased from Takara. MDA, SOD, ROS, and Caspase-3 enzyme activity detection kits were purchased from Beyotime. Annexin V-FITC/PI apoptosis detection kit was purchased from Solarbio.

Rat brain I-R model establishment

Rats were anesthetized by intraperitoneal injections of 10% chloral hydrate and placed on the operating table in the supine position. After skin disinfection, the skin of the neck was cut at the intersection of the internal carotid artery. The plug line was slowly inserted from the small mouth to the internal carotid artery, about 18 mm in depth, with obvious resistance. This indicated that the suture had caused middle cerebral artery embolization. Next, the plug line was slowly pulled out after 2 hours, restoring the blood supply and establishing the rat brain IR injury model. Sham-operated group rats were only exposed to the right common carotid artery, internal carotid artery, and external carotid artery, with no plug lines inserted. The rats were sacrificed at 24 hours after surgery. Brain tissues were collected to detect expression of DJ-1 and Nrf2. Brain homogenates were prepared to detect MDA content and SOD enzyme activity levels.

Quer treatment

I-R injury rats were divided into two groups. The I-R+Quer group was treated with 50 mg/Kg Quer intraperitoneal injections once a day for 7 continuous days, while the IR group was treated with 50 μL/Kg normal saline intraperitoneal injections once a day for 7 continuous days.

mNSS scoring

At 24 hours after model establishment, modified neurological severity scores (mNSS) were used to evaluate motor, sensory, balance, and reflex function. The total score was 18 and the normal rat score was 0. Higher scores indicate more serious neurological damage.

Caspase-3 activity detection

According to kit instructions, the pNA standard product was diluted in a concentration gradient to prepare standard products with different concentrations. Absorbance was measured at 405 nm to make a standard curve. Tissues were smashed to prepare the homogenate. The homogenate was added with Caspase lysis buffer on ice for 20 minutes and centrifuged at 10,000 g and 4°C for 20 minutes. The supernatant was taken to a new 1.5 mL centrifuge tube and quantified using the BCA kit. Next, 65 μl assay buffer, 25 μl lysate supernatant, and 10 μl Ac-DEVD-pNA (2 mM) were added to a 96-well plate and incubated for 2 hours at 37°C. When the color change was obvious, the plate was measured at 405 nm on a microplate reader (Awareness, USA). Relative enzyme activity was calculated based on A405, with the experimental group/A405 and the control group × 100%.

MDA and SOD detection

Rat cerebral homogenate was prepared and quantified using the BCA method. MDA and SOD contents were tested in accordance with instructions, assessing oxidative stress conditions and antioxidant capacity.

RN-c cell I-R treatment

Rat Rn-c neurons were cultured in vitro and divided into three groups, including the control group, IR group, and I-R+Quer group. For I-R treatment, the cells were cultured in low glucose serum free DMEM to simulate ischemic conditions. Next, the cells were maintained in an incubator with 5% CO_2 and 95% N_2 to simulate hypoxic conditions (FORMA3131, Thermo,

Table 1. mNSS scores between the two groups

Group	mNSS score
Sham	0
I-R	12.67±2.13*

*$P < 0.05$, compared with the Sham group.

USA). The cells were changed to a routine medium after 12 hours and further cultured under normal conditions for 12 hours.

ROS detection

Cells were washed in PBS and incubated in 0.1% DCFH-DA probe at 37°C for 20 minutes. Afterward, they were washed by serum-free DMEM medium. The cells were then tested using a Beckman FC500MCL flow cytometer.

Cell apoptosis detection

The cells were washed twice in PBS and digested by trypsin. Next, the cells were resuspended in 500 µL binding solution and added with 5 µL of Annexin V-FITC and 5 µL of PI solution in sequence. They were incubated, void of light, for 15 minutes. Finally, the cells were tested using a flow cytometer.

qRT-PCR

Total RNA was extracted using TRIzol Reagent. PrimeScript™ RT reagent kit was used to reverse transcribe RNA to cDNA for PCR reactions. Moreover, the qPCR reaction system contained 5.0 µL 2 × SYBR Green Mixture, 0.5 µL forward primer (5 µm/L), 0.5µL reverse primer (5 µm/L), 1.0 µL cDNA, and ddH$_2$O. Reverse transcription conditions were 50°C for 15 minutes and 85°C for 5 minutes. Furthermore, qPCR reaction conditions were: pre-denatured at 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute using the Bio-Rad CFX96 Real-Time PCR Detection System.

Western blotting

Cells and tissues were lysed by RIPA. Next, 40 µg proteins were separated by 12% SDS-PAGE gel and 4% concentrated gel. Proteins were then transferred to PVDF membranes at 300 mA for 90 minutes. Next, the membrane was blocked with 5% skim milk at room temperature for 60 minutes and incubated in a primary antibody (DJ-1, Nrf2, and β-actin at 1:1000, 1:600, 1:8000, respectively) at 4°C overnight. Afterward, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG (H+L) secondary antibody (1:15000) at room temperature for 60 min. Finally, it was detected by ECL chemiluminescence.

Statistical analysis

Data analyses were performed using SPSS 18.0 software. Measurement data are expressed as mean ± standard deviation and were compared by t-test or one-way ANOVA. Bonferroni's method was adopted for post-hoc testing. $P < 0.05$ indicates statistical significance.

Results

Brain function decreased in I-R model rats

Rats in the Sham group exhibited good mental states, rapid activity, and no neurological deficits, with mNSS scores of 0 points. In contrast, the IR group showed hemiplegia, physical coordination dysfunction, unstable standing, and unresponsiveness at 24 hours after modeling, with abnormally elevated mNSS scores (**Table 1**).

Brain OS upregulated and caspase-3 activity enhanced in I-R model rats

Results showed that MDA content significantly increased (**Figure 1A**) and SOD enzyme activity was obviously decreased (**Figure 1B**) in brain tissues of I-R model rats. Caspase-3 activity in the brain tissues of I-R model rats was significantly enhanced, compared with the Sham group, indicating an increase of apoptosis in brain tissues (**Figure 1C**).

Quer upregulated DJ-1 and Nrf2 expression and improved neurologic function in I-R rat brain tissues

Intraperitoneal injections of Quer significantly reduced MDA content in brain tissues of I-R model rats (**Figure 2A**). Quer intraperitoneal injections obviously enhanced SOD activity in brain tissues of I-R model rats (**Figure 2B**). Caspase-3 enzyme activity in the Quer treatment group was markedly lower than that of the
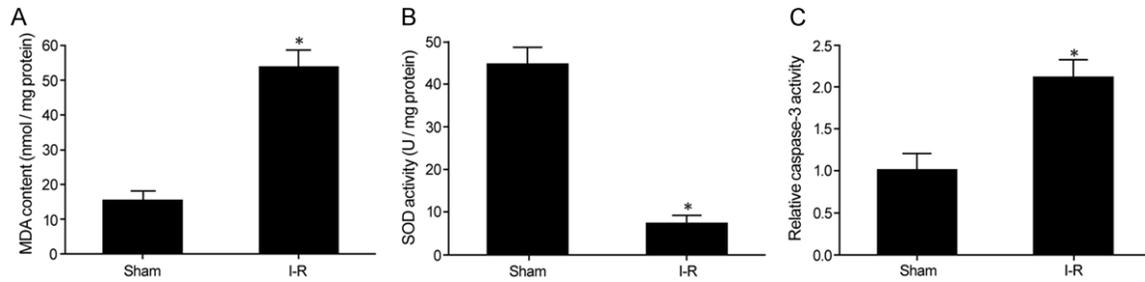
Figure 1. Brain OS upregulated and caspase-3 activity enhanced in I-R model rats. (A) MDA content in the rat brain tissue; (B) SOD enzyme activity in rat brain tissue; (C) Caspase-3 activity in rat brain tissue; *$P < 0.05$, compared with the Sham group.
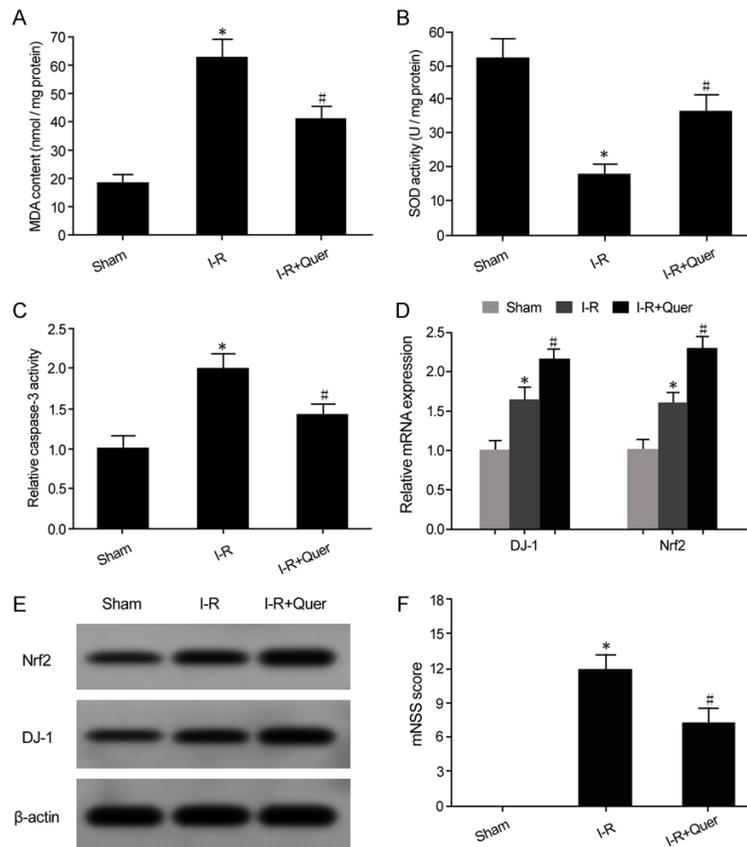

Figure 2. Quer upregulated DJ-1 and Nrf2 expression and improved neurologic function in I-R rat brain tissues. (A) MDA content in the rat brain tissue; (B) SOD enzyme activity in rat brain tissue; (C) Caspase-3 activity in rat brain tissue; (D) qRT-PCR detection of mRNA expression; (E) Western blot detection of protein expression; (F) mNSS scores of neuro function. *$P < 0.05$, compared with the Sham group; #$P < 0.05$, compared with the I-R group.

were further upregulated (**Figure 2D**). Western blotting demonstrated that, compared with the Sham group, DJ-1 and Nrf2 protein levels in brain tissues of the IR group were markedly increased. Compared with the I-R group, DJ-1 and Nrf2 protein expression in brain tissues of the Quer treatment group was further enhanced (**Figure 2E**). Neurological function assessment showed that, compared with the I-R group, neurological functions, such as hemiplegia, physical coordination dysfunction, unstable standing, and unresponsiveness, were significantly improved in the Quer treatment group. Thus, mNSS scores were markedly reduced (**Figure 2F**).

Quer alleviated Rn-c cell apoptosis and ROS production induced by I-R by upregulating DJ-1 and Nrf2

In this study, qRT-PCR showed that, compared with the control group, D-1 and Nrf2 mRNA expression was significantly increased in the IR treatment group. Quer treatment further elevated D-1 and Nrf2 mRNA levels under IR treatment (**Figure 3A**, **3B**). Western blotting demonstrated that, compared with the control group, D-1 and Nrf2 protein levels were obviously upregulated in the IR treatment group. Quer intervention further enhanced D-1 and

I-R group (**Figure 2C**). Moreover, qRT-PCR revealed that DJ-1 and Nrf2 mRNA expression in brain tissues of the IR group was apparently higher than that of the Sham group. Compared with the I-R group, DJ-1 and Nrf2 mRNA levels in brain tissues of the Quer treatment group
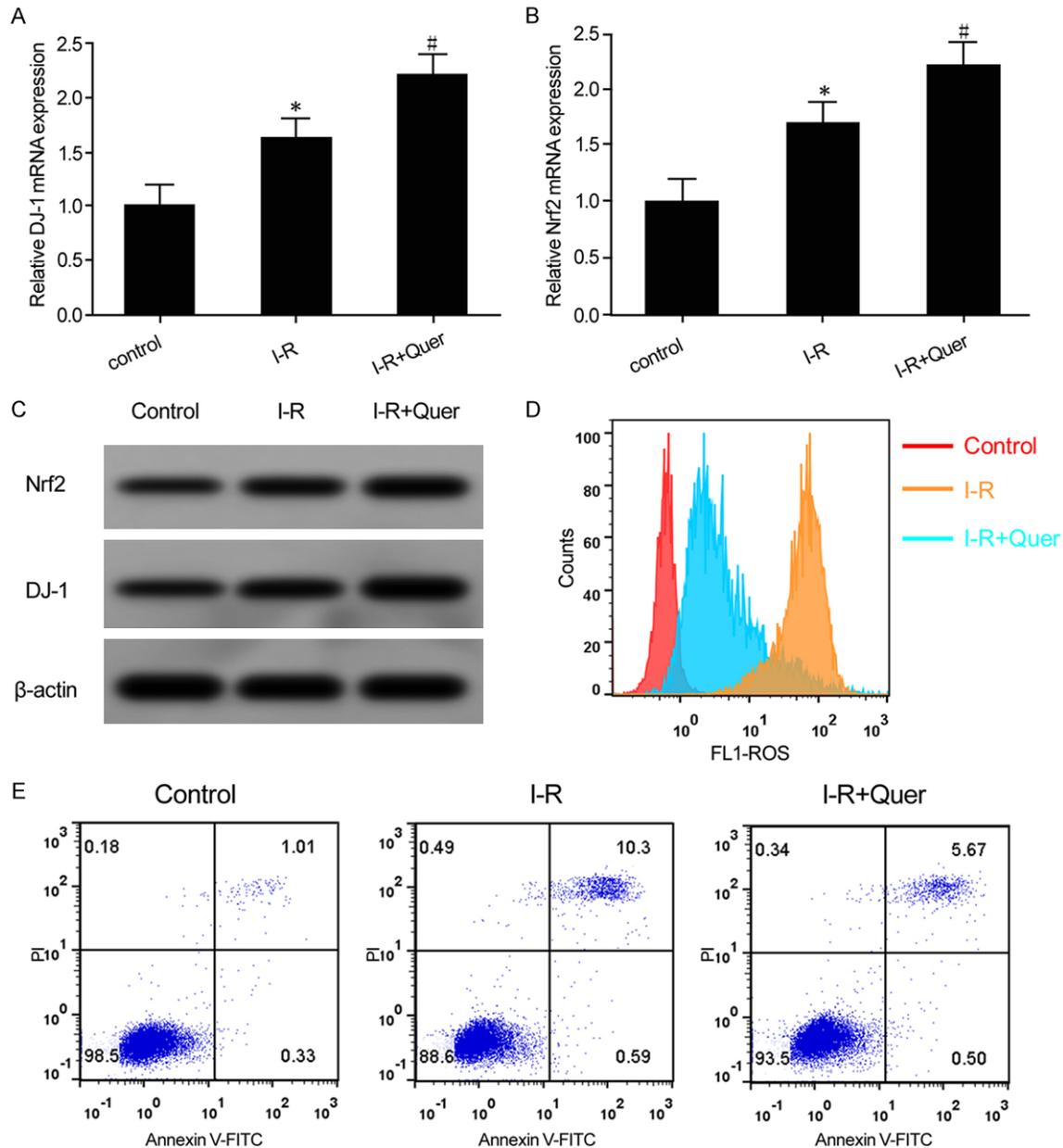
Figure 3. Quer alleviated Rn-c cell apoptosis and ROS production induced by I-R by upregulating DJ-1 and Nrf2. (A) qRT-PCR detection of DJ-1 mRNA expression; (B) qRT-PCR detection of Nrf2 mRNA expression; (C) Western blot detection of D-1 and Nrf2 protein expression; (D) Flow cytometry detection of ROS content; (E) Flow cytometry detection of cell apoptosis. *P < 0.05, compared with the Sham group; #P < 0.05, compared with the I-R group.

Nrf2 protein levels under IR conditions (**Figure 3C**). Flow cytometry revealed that, compared with the control group, ROS content was markedly increased (**Figure 3D**), while apoptosis was apparently enhanced in the IR treatment group (**Figure 3E**). Rn-C-cell intracellular ROS production and apoptosis were significantly attenuated after treatment with Quer (**Figure 3D**, **3E**).

Discussion

Nrf2/ARE is the most important endogenous anti-OS signaling pathway discovered. When OS levels are within the physiological normal range, Nrf2 and Keap1 are coupled and bound to cytoplasmic actin and anchored in the cytoplasm, maintaining a state of constant degradation and low expression of non-transcribed

activity. Obvious oxidative stress changes the structure of Keap. At this time, Nrf2 dissociates from Keap1, enters the nucleus, binds to ARE, and upregulates HO-1, γ-GCS, SOD, glutathione transferase (Glutathione), S-Transferase (GST), and nicotinamide adenine dinucleotide (phosphoric acid) [NAD (P)H], achieving the goal of reducing OS levels and alleviating OS damage [17-20]. Activation of Nrf2 plays a role in reducing OS response and alleviating brain I-R injuries [6, 7].

DJ-1/PARK7 (Parkinson gene 7) is located at chromosome 1p36.2-36.3, with a length of 24 kb. It encodes a protein with a molecular weight of 21 kD, consisting of 189 amino acids [21]. Numerous studies have confirmed that DJ-1 plays a role in enhancing Nrf2 stability and promoting Nrf2 protein expression and activation. Anti-OS effects of DJ-1 depend on regulation of Nrf2 [10, 11].

Quer is a natural plant-derived flavonoid with the chemical name of 3,3',4',5,7-pentahydroxy-flavone and molecular formula of $C_{15}H_{10}O_7$. Quer is widely found in a variety of fruits, vegetables, grains, and medicinal plants, including tea, apples, onions, grapes, quercetin, hyperoside, red dates, dodder, mulberry, and ginkgo leaves. In recent years, it was shown that Quer provides various neuroprotective effects, such as delaying Alzheimer's disease [12, 13], alleviating cerebral ischemic injury [14], and improving neurocognition and motor function [15]. However, remains unclear whether Quer mitigates I-R damage by affecting DJ-1/Nrf2 pathways. The current study established a rat model of cerebral I-R injury and treated rats with Quer, investigating the role of Quer in regulating DJ-1/Nrf2 pathway activity and alleviating rat brain IR injuries.

Compared with the Sham group, the I-R model group showed neurological impairment, such as limb coordination and movement disorder, unresponsiveness, and hemiplegia, indicating successful modeling. Compared with the Sham group, MDA content and caspase-3 activity levels were significantly increased, while SOD activity was obviously reduced in brain tissues of the IR model group. This indicated that neurological function impairment was related to OS elevation and increasing apoptosis. DJ-1 and Nrf2 levels in the I-R model group were significantly higher than those in the Sham group.

Results indicated that the IR model initiated anti-OS emergency mechanisms by upregulating DJ-1 and Nrf2. However, the actual anti-OS ability in the body remained insufficient, leading to high MDA contents and reduced SOD activities in the brain. Intraperitoneal injections of Quer before I-R modeling significantly reduced MDA content and caspase-3 activity. They obviously elevated antioxidant enzyme SOD activity in rat brain tissues. Compared with the I-R group, DJ-1 and Nrf2 expression was apparently increased in brain tissues of the Quer treatment group. Intraperitoneal injections of Quer improved cranial nerve function, alleviated I-R injuries after cerebral ischemia, and reduced mNSS scores by elevating DJ-1 and Nrf2 expression, thus enhancing anti-OS mechanisms and abilities. Pei et al. [22] reported that Quer can suppress the apoptosis of brain tissue induced by IR damage and OS by inhibiting ASK1/JNK3/caspase-3 pathways, improving brain function. Yao et al. [23] revealed that Quer can reduce brain cell apoptosis, attenuate I-R injuries after brain tissue ischemia, and improve rat brain function by activating BDNF-TrkB-PI3K/Akt pathways. Ahmad et al. [24] found that Quer treatment can significantly reduce infarct size, improve neurological function, and decrease neuron loss and apoptosis in rats after ischemia. The current study found that Quer has a significant impact in the reduction and treatment of I-R injuries after brain ischemia, confirming the neuroprotective effects of Quer. Present results are in accord with those of Yao [23] and Ahmad [24].

Using *in vitro* studies, it was found that IR treatment significantly induced Rn-c cell apoptosis and elevated DJ-1 and Nrf2 expression. Quer treatment further upregulated DJ-1 and Nrf2 levels, reduced ROS production, and decreased apoptosis in Rn-c cells. Thus, Quer has neuroprotective effects against IR injury and apoptosis after cerebral ischemia. Upregulating DJ-1 and Nrf2 expression and initiation of the anti-oxidant emergency response system are important mechanisms for Quer in exerting neuroprotective effects. It was found that Quer plays a role in activating Nrf2 and protecting nerve cells. Shi et al. [25] found that Quer upregulates Nrf2 expression, activates Nrf2/ARE antioxidant mechanisms, enhances ganglion cell damage, reduces OS, and alleviates cell apoptosis induced by high glucose. Shi et al. [26]

demonstrated that Quer can protect cells from high glucose-induced cell damage by activating Nrf2/ARE antioxidation, reducing ROS production, and alleviating apoptosis in ganglion cells. However, whether Quer exerts anti-oxidative damage and apoptosis through regulating Nrf2 by DJ-1 has not yet been reported. Present results suggest that Quer plays a role in upregulating DJ-1 expression, activating DJ-1/Nrf2 pathways, enhancing neuronal antioxidant damage, and reducing neuronal apoptosis after I-R.

Conclusion

Quer attenuates brain cell apoptosis and neuronal damage after I-R by increasing DJ-1 levels, enhancing DJ-1/Nrf2 pathways, and elevating anti-oxidation levels.

Disclosure of conflict of interest

None.

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