Icariin protects cerebral neural cells from ischemia-reperfusion injury in an *in vitro* model by lowering ROS production and intracellular calcium concentration

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Abstract. Ischemia is one of the major causes of stroke. The present study investigated the protection of cultured neural cells by icariin (ICA) against ischemia-reperfusion (I/R) injury and possible mechanisms underlying the protection. Neural cells were isolated from neonatal rats and cultured *in vitro*. The cells were subjected to oxygen-glucose deprivation and reoxygenation (OGD-R) as an I/R mimic to generate I/R injury, and were post-OGD-R treated with ICA. Following the treatments, cell viability, apoptosis, reactive oxygen species (ROS), lactate dehydrogenase (LDH), superoxide dismutase (SOD) and Ca^{2+} concentration were assessed using Cell Counting Kit-8 assay, flow cytometry, CyQUANT™ LDH Cytotoxicity Assay, H_2DCFDA and SOD colorimetric activity kit. After OGD-R, considerable I/R injury was observed in the neural cells, as indicated by reduced cell viability, increased apoptosis and increased production of ROS and LDH (P<0.05). Cellular Ca^{2+} concentration was also increased, while SOD activity remained unchanged. Post-OGD-R ICA treatments increased cell viability up to 87.1% (P<0.05) and reduced apoptosis as low as 6.6% (P<0.05) in a concentration-dependent manner. The treatments also resulted in fewer ROS (P<0.05), lower extracellular LDH content (440.5 vs. 230.3 U/l; P<0.05) and reduced Ca^{2+} increase (P<0.05). These data suggest that ICA protects the neural cells from I/R injury in an *in vitro* model through antioxidation activity and maintaining cellular Ca^{2+} homeostasis. This function may be explored as a potential therapeutic strategy for ischemia-related diseases after further *in vivo* studies.

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Abbreviations: ICA, icariin; I/R, ischemia-reperfusion; OGD-R, oxygen-glucose deprivation and reoxygenation; ROS, reactive oxygen species; LDH, lactate dehydrogenase; SOD, superoxide dismutase; BBB, blood-brain barrier; IL, interleukin; mPEG, polyethylene glycol monomethyl ether; SD, Sprague Dawley; HBSS, Hanks' balanced salt solution; EBSS, Earle's balanced salt solution; FBS, fetal bovine serum; CCK-8, Cell Counting Kit-8; DMEM, Dulbecco's modified eagle's medium; OD, optical density

Key words: neuroprotection, antioxidant, Ca^{2+} homeostasis, ischemia-reperfusion injury, ischemia, reactive oxygen species

Introduction

Neurological disorders are the leading cause of disability and the second leading cause of death worldwide, and ischemia is one of the major causes of stroke leading to neurological disorders (1,2). Cerebral ischemia causes metabolic and neurochemical alterations and leads to a diverse range of neurological diseases, such as stroke, myocardial infarction, acute heart failure, cerebral dysfunction and selective neuronal loss (3-6). Ischemia-reperfusion (I/R) injury is a pathological event occurring in various disease states. It results in metabolic disorders, excitotoxicity, calcium overload, oxidation stress and inflammatory damage through a number of pathways (7-9) as a result of the sudden reduction of available tissue oxygen and nutrients (10,11).

Following transient forebrain ischemia, neuronal loss may occur causing 'delayed neuronal death' (12), which may also result from neuroinflammatory processes, such as glial activation and increased production and release of inflammatory cytokines (13,14). In addition to delayed neuronal death, ischemia damages the integrity of the blood-brain barrier (BBB) and enhances microglia activation and zinc release, leading to blood and fluid leakage (15,16). The brain is especially sensitive to oxidative stress from reactive oxygen species (ROS) produced as a result of ischemic insult, because neurons have high levels of polyunsaturated fatty acids and low levels of endogenous antioxidant enzymes (17).

Several antioxidant substances have been shown to be able to protect neurons from I/R injury. For example, protocatechuic acid, a major type of benzoic acid that exists in vegetables, fruits and numerous herbal medicines, has been revealed to be a strong anti-oxidant that prevents Parkinson's disease (18). It also has neuroprotective activities on global cerebral ischemia-induced hippocampal neuron death (16) through a combination of the cellular mechanisms of antioxidant

cytoprotection and anti-inflammation (18). The neuroprotective effect has also been demonstrated for stiripentol, which reduces ischemia-induced memory impairment and neuronal death by decreasing astrocyte damage and ameliorating BBB leakage (19). In addition, chlorogenic acid, naturally found in green coffee extracts and tea, has also been revealed to attenuate cognitive impairment, and has a neuroprotective effect against transient forebrain ischemia by increasing the production of superoxide dismutase (SOD)2, interleukin (IL)-4, antioxidant enzymes and anti-inflammatory cytokines (8).

Icariin (ICA) is a major active flavonol glucoside component that presents in the medicinal plant *Epimedium grandiflorum,* and has been demonstrated to have activities against neurodegenerative diseases, cardiovascular diseases, osteoporosis, inflammation, oxidative stress, depression and tumors (20,21). It improves carrageenan-induced paw edema by modulating heme oxygenase (HO1)/nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and NF-κB signaling to reduce inflammatory cytokines and increase enzymatic and non-enzymatic antioxidants (21). In a rat model, ICA prevents the production of amyloid β (1-42) and inhibits the synthesis of amyloid precursor protein and β-site APP cleaving enzyme 1 in animal models of Alzheimer's disease (AD) (22). It also alleviates the development of kidney fibrosis by inhibiting IL-1β/transforming growth factor-β-mediated renal fibroblasts in rats (23). Recently, hydrophilic polyethylene glycol monomethyl ether (mPEG) was modified to generate mPEG-ICA nanoparticles with increased protective activity for H9c2 cardiomyocytes under oxygen-glucose deprivation conditions (24). ICA has been demonstrated to have potential neuroprotective activity against Aβ25-35-induced neurotoxicity by balancing intracellular calcium homeostasis in rats (25). It can also mitigate pro-inflammatory responses of microglia in culture and in animal models of cerebral ischemia, depression, Parkinson's disease and multiple sclerosis (22). A previous study demonstrated that ICA protects neurons from endoplasmic reticulum stress-induced apoptosis by suppressing IRE1α-XBP1 signaling pathway *in vitro* (26). However, the effect of ICA on I/R injury of neural cells are largely unclear.

The present study aimed to investigate effect of ICA on neural cells with I/R injury induced by oxygen-glucose deprivation and reoxygenation (OGD-R) and possible mechanisms underlying the protection.

Materials and methods

Animals. A total of five newborn (within 24 h of birth) male Sprague Dawley (SD) rats, weighing 5 g, were purchased from Hunan Silaike Jingda Laboratory Animal Co., Ltd. [license no. SCXK (Hunan) 2020-0104] and were used immediately after arriving for experiments. All animal experiments and animal care protocols were approved by the Institutional Animal Care and Use Committee of Affiliated Zhongshan Hospital, Dalian University (Dalian, China).

Reagents and instruments. Hanks' balanced salt solution (HBSS; cat. no 88284), Earle's balanced salt solution (EBSS; cat. no 14175095), fetal bovine serum (FBS; cat. no 2662002), trypsin inhibitor (cat. no J60982), Infinity Calcium Arsenazo Liquid Stable Reagent (cat. no 265-250), 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; cat. no. D399), CyQUANT™ lactate dehydrogenase (LDH) Cytotoxicity Assay (cat. no. C20302), Invitrogen SOD colorimetric activity kit (cat. no. EIASODC) and ApoDETECT Annexin V-FITC kit (cat. no 331200) were purchased from Thermo Fisher Scientific, Inc.; ICA [cat. no. I1286; purity ≥94% (high performance liquid chromatography)], 0.25% trypsin (cat. no. 9002-07-7), high glucose Dulbecco's Modified Eagle's Medium (DMEM; cat. no. D6429), poly-L-lysine (cat. no. 25988-63-0) and Cell Counting Kit-8 (CCK-8; cat. no. 96992) were purchased from Sigma-Aldrich (Merck KGaA); laminar flow hoods were purchased from Global Lab Supply; CO_2 incubator (NAPCO; Thermo Fisher Scientific, Inc) was obtained from ProVendum SA; flow cytometer (FACS LSR) was purchased from BD Biosciences; ELISA plate reader (VANTAstar) was obtained from BMG Labtech GmbH; FS5 spectrofluorometer was obtained from Edinburgh Instruments Ltd.

Neural cell culture. Neural cells were isolated as previously reported (27). Briefly, newborn (~24 h old) SD rats were euthanized by intraperitoneal injection of sodium pentobarbital (200 mg/kg body weight) followed by decapitation, and were sterilized in 75% alcohol for 5 min. The craniums were cut opened along the midline to isolate the brain tissue. Isolated tissue was washed with HBSS to remove blood, soft meninges and vascular network. The dentate gyrus was then isolated, cut into pieces of 1-2 mm in size after washing three times with HBSS, homogenized, filtered through a 100 mesh filter and digested with equal volume of 0.25% trypsin at 37˚C for 20 min. Digestion was stopped by adding two volumes of trypsin inhibitor. Digested cells were pelleted by centrifugation at 112 x g for 5 min at room temperature and cultured in DMEM medium with 10% FBS in 5% CO_2 at 37˚C for three passages.

Cellular I/R model. The *in vitro* I/R model of neural cells was constructed as previously described using the OGD-R method (28,29). Briefly, cells were cultured in DMEM medium with 10% FBS for 2 days, washed with glucose-free EBSS and cultured in glucose-free EBSS in an anoxic incubator. The incubator was slowly filled with gas containing 95% N_2 and 5% CO_2 to generate an oxygen-free atmosphere to mimic ischemia condition. After culturing at 37˚C for 4 h, the cells were transferred to high-glucose DMEM medium and cultured in 5% CO_2 at 37˚C for 12 h to mimic reperfusion process.

ICA treatment. ICA was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 50 mM and stored at -20˚C as a stock solution. It was diluted with DMEM medium with 10% FBS before use. The medium containing 0.1% DMSO served as the control. Immediately after the I/R modelling, cells were adjusted to a density of $1x10^5$ cells/ml with DMEM medium with 10% FBS containing 0 (control), 5, 10 and 15 μM ICA. Subsequently, 200 μl cells were inoculated in the wells of 96-well plates pre-coated with poly-L-lysine and cultured in 5% CO_2 at 37˚C. The concentrations were used based on a previous study on neural stem cells (30). Cells without OGD-R treatments were used as control.

Cell viability assay. Cell viability was assayed using a CCK-8 cell counting kit according to the manufacture's instruction. Briefly, 24 h after reperfusion, 20 μl CCK-8 solution was added to each well of the plates and the plates were incubated in 5% CO_2 at 37˚C for 4 h. The optical density (OD) was read at 460 nm wavelength using an ELISA plate reader. All assays were performed in triplicate in three independent experiments. Cell viability was calculated as $OD_{sample}/OD_{control}$ x100%.

Apoptosis analysis. Apoptosis was assessed using ApoDETECT Annexin V-FITC kit according to the manufacture's instruction. Briefly, cells ($1x10^6$) were harvested by centrifugation at room temperature, washed three times with 1 ml PBS, resuspended in cold binding buffer and incubated with Annexin V-FITC and PI-PE in the dark at room temperature for 10 min. The stained cells were analyzed using FACS LSR flow cytometer using built-in software according to the manufacture's protocols. All assays were performed in triplicate in three independent experiments.

Determination of extracellular LDH level. LDH is a cytosolic enzyme that is released into the cell culture medium upon damage to the plasma membrane (31). LDH level was determined using CyQUANT™ LDH Cytotoxicity Assay according to the manufacture's instruction. Briefly, 24 h after reperfusion, 20 μl aliquots of culture medium were taken and added with the reaction mixture from the kit. After a 10 min incubation at room temperature, the reaction was stopped by adding the stop solution from the kit and the fluorescence was measured with a plate reader by using excitation of 560 nm and emission of 590 nm. All assays were performed in triplicate in three independent experiments. LDH level was calculated as (fluorescence $signal_{sample}$ - fluorescence $signal_{sample\ control}$)/(fluorescence $signal_{standard}$ - fluorescence $signal_{standard\ control}$) x standard concentration X dilution.

ROS determination. For ROS production analysis, cells were incubated with H_2DCFDA diluted in serum-free DMEM to the final concentration of 10 μmol/l. After incubation at 37˚C for 20 min, cells were rinsed three times with serum-free DMEM medium to remove H_2DCFDA and loaded to a cytometer for analysis according to the manufacturer's protocols. All assays were performed in triplicate in three independent experiments.

Measurement of SOD activity. Total SOD activity was measured using an Invitrogen SOD colorimetric activity kit according to the manufacturer's instruction. This kit is designed to quantitatively measure all types of SOD activity, including Cu/Zn, Mn and FeSOD types according to the manufacturer's information. Briefly, 24 h after treatment, cells were washed with cold PBS twice, pelleted by centrifugation at 1,200 x g and room temperature for 5 min, homogenized and lysed using lysis buffer in the kit. The lysates were centrifuged at 1,200 x g at 4˚C for 10 min and the supernatants were used for SOD activity assessment. The optical density at 450 nm was read using a plate reader. All assays were performed in triplicate in three independent experiments.

Ca^{2+} determination. Cells (10^6) were harvested by centrifugation at room temperature at 1,200 x g for 5 min and Ca^{2+} was reacted to Infinity Calcium Arsenazo Liquid Stable Reagent to form a bluish-purple colored complex according to the manufacturer's instructions. The amount of color formed was measured by an increase in absorbance of the reaction mixture at 600 and 660 nm using FS5 spectrofluorometer. All assays were performed in triplicate in three independent experiments.

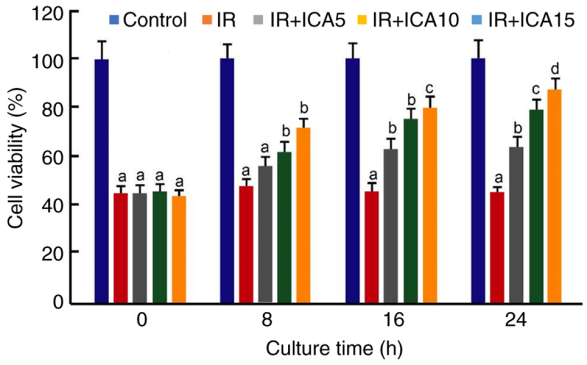

Figure 1. Viability of neural cells after OGD-R and ICA treatment at 0, 5,10 and 15 μM. The cells were subjected to OGD-R and treated with ICA for 0-24 h, harvested and assessed in triplicate in three independent experiments for viability using Cell Counting Kit according to the manufacturer's instructions. [a]P<0.05 vs. control; [b]P<0.05 vs. IR; [c]P<0.05 vs. IR + ICA5; [d]P<0.05 vs. IR + ICA10. OGD-R, oxygen-glucose deprivation and reoxygenation; ICA, icariin.

Statistical analysis. All data were expressed as means ± standard error obtained from three independent experiments. Statistical comparisons among the groups were assessed using a one-way ANOVA with post-hoc Tukey honest significant difference test. P<0.05 was considered to indicate a statistically significant difference.

Results

ICA increases the cell viability after I/R. First, the effect of ICA on the viability of cultured neural cells after I/R was investigated. CCK-8 cell viability assays showed that after reperfusion, the viability of cells after OGD-R was significantly reduced as compared with the viability of control cells, indicating that the OGD-R generates significant cellular injury. On the other hand, post-OGD-R treatments with ICA increased the viability as the duration and concentration of exposure to ICA increased; however, the increases were insignificant between the low ICA concentration (ICA 5) and IR model before 16 h exposure (P>0.05; Fig. 1). At 24 h after reperfusion, the viabilities increased to 63.1, 79.5 and 87.1%, respectively, at the concentrations of 5, 10 and 15 μM. The increases in the ICA treated group were statistically significant between the concentrations at 24 h after culture (P<0.05; Fig. 1), suggesting that ICA protects neural cells from I/R injury, although the highest cell viability after treatment with 15 μM ICA was still lower compared with that of control at 24 h after culture (P<0.05; Fig. 1). Since exposure to ICA for 24 h generated significant improvement of cell viability after I/R, the cells at this time point were used for subsequent analysis.

ICA reduces apoptosis after I/R. Apoptosis is one of the major mechanisms leading to cell death. To investigate if apoptosis

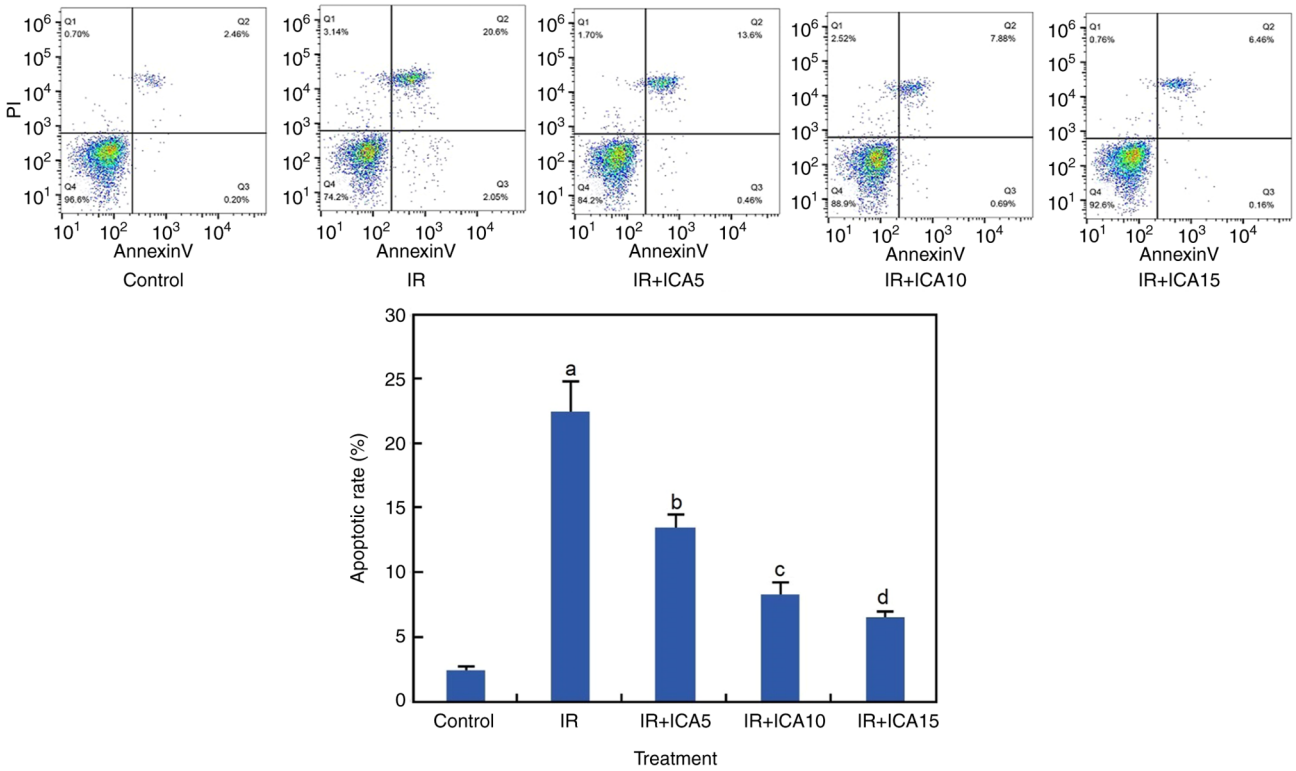

Figure 2. Apoptosis of neural cells after OGD-R and ICA treatment at 0, 5,10 and 15 μM. The cells were subjected to OGD-R and treated with ICA for 24 h, harvested and assessed in triplicate in three independent experiments for apoptosis using ApoDETECT Annexin V-FITC Kit according to the manufacturer's instructions. [a]P<0.05 vs. control; [b]P<0.05 vs. IR; [c]P<0.05 vs. IR + ICA5; [d]P<0.05 vs. IR + ICA10. OGD-R, oxygen-glucose deprivation and reoxygenation; ICA, icariin.

was induced after OGD-R induced I/R damage, apoptosis was assessed using Annexin V-FITC staining method. At 24 h after I/R, significant increases in apoptotic rates were observed in the OGD-R treated neural cells as compared with untreated cells (P<0.05; Fig. 2). However, apoptosis was significantly reduced to 14.1, 8.4 and 6.6% when the OGD-R-treated cells were exposed to 5, 10 and 15 μM ICA, respectively (P<0.05; Fig. 2).

ICA reduces extracellular LDH activity. LDH is released from cells when cell membrane is damaged as a result of various cytotoxicities. The extracellular LDH level in the culture medium of the cells was measured 24 h after OGD-R and ICA treatments using CyQUANT LDH cytotoxicity assay. The results showed that 24 h after OGD-R treatment, the extracellular LDH activity was significantly increased as compared with control (440.5 vs. 230.3 U/l) but decreased after being exposed to ICA at the three ICA concentrations (P<0.05; Fig. 3).

ICA reduces ROS production but not total SOD activity. ROS induction is a common consequence of I/R, and the production of ROS after OGD-R and ICA treatment was measured using H_2DCFDA as a fluorescent probe. The results showed that the ROS level was significantly increased after the cells were subjected to OGD-R as compared with control and was significantly decreased after ICA treatments at the three concentrations (P<0.05; Fig. 4A). To examine the effect of ICA on OGD/R-induced oxidative stress, the SOD activity was also

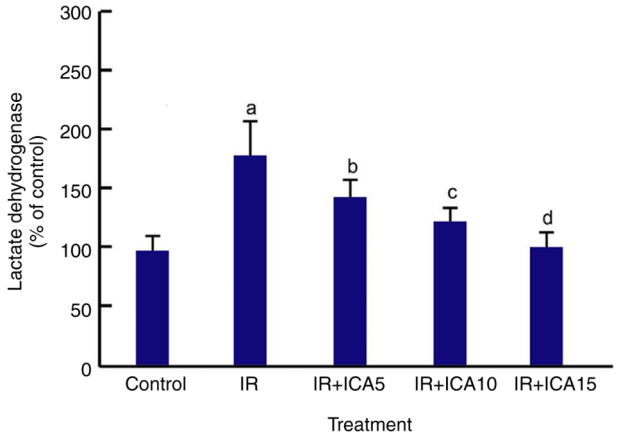

Figure 3. Extracellular LDH activity in neural cells after OGD-R and ICA treatment at 0, 5,10 and 15 μM. The cells were subjected to OGD-R and treated with ICA for 24 h and assessed in triplicate in three independent experiments for extracellular LDH activity using CyQUANT™ LDH cytotoxicity assay kit according to the manufacturer's instructions. [a]P<0.05 vs. control; [b]P<0.05 vs. IR; [c]P<0.05 vs. IR + ICA5; [d]P<0.05 vs. IR + ICA10. LDH, lactate dehydrogenase; OGD-R, oxygen-glucose deprivation and reoxygenation; ICA, icariin.

examined. It was revealed that the SOD activity did not change significantly after these treatments (P<0.05; Fig. 4B).

ICA reduces cytosolic Ca^{2+} level. One of the constant early responses to hypoxia in almost all cell types is an increase

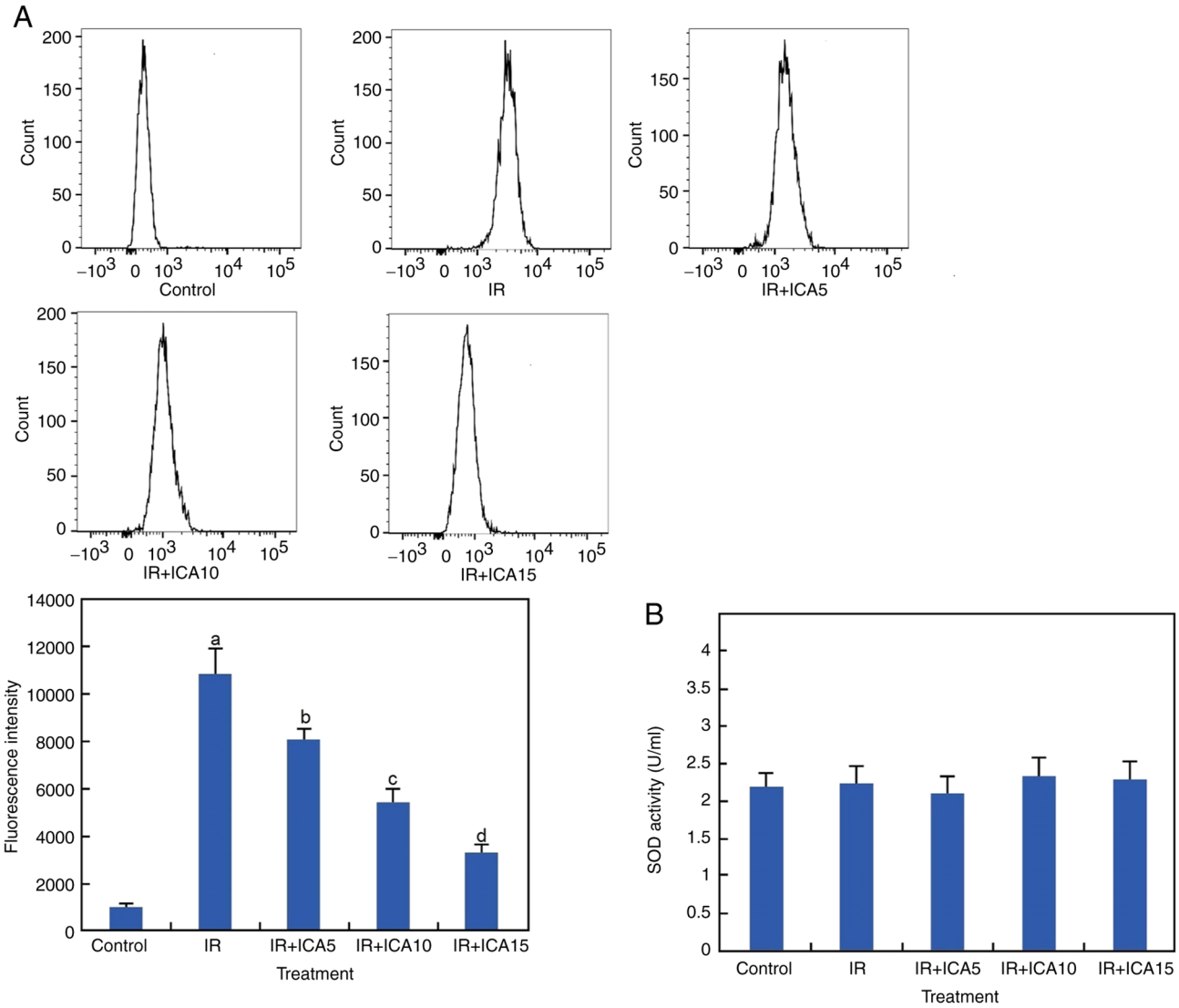

Figure 4. (A) Reactive oxygen species production and (B) SOD activity in neural cells after OGD-R and ICA treatment at 0, 5,10 and 15 μM. The cells were subjected to OGD-R and treated with ICA for 24 h, harvested and assessed in triplicate in three independent experiments for ROS production using fluorescent probe H_2DCFDA and SOD colorimetric activity kit according to the manufacturer's instructions. (A) Upper panel=fluorescence intensity; lower panel=statistical analysis of ROS production; (B) SOD activity. [a]P<0.05 vs. control; [b]P<0.05 vs. IR; [c]P<0.05 vs. IR + ICA5; [d]P<0.05 vs. IR + ICA10. OGD-R, oxygen-glucose deprivation and reoxygenation; ICA, icariin; ROS, reactive oxygen species; SOD, superoxide dismutase.

in intracellular Ca^{2+} due to the activation of various plasma membrane Ca^{2+} ion channels (32). Analysis using a dual wavelength fluorescence spectrophotometer showed that the cytosolic Ca^{2+} level increased significantly after OGD-R treatment, and reduced after the cells were exposed to ICA in a dose-dependent manner (P<0.05; Fig. 5).

Discussion

Using cultured neural cells, the present study revealed that OGD-R could mimic I/R to reduce cell viability, induce apoptosis, increase LDH release and ROS production. These cellular damages were alleviated after the cells were exposed to ICA in a dose-dependent manner. This suggested that ICA has protective activity and may be explored clinically for its therapeutic functions in I/R-related diseases and complications after the protective activity is validated with *in vivo* models.

ICA is a prenylated flavonol glycoside of the *Epimedium* herb and has been shown to have various pharmacological activities against neurodegenerative diseases, cardiovascular diseases, osteoporosis, inflammation, oxidative stress depression and cancer (20,33,34). It reduces I/R-induced gap junctional intercellular communication injury by regulating the synthesis of gap junctional protein connexin 43 (35). Since ICA regulates the expression of sirtuin 1 to inhibit the synthesis of amyloid-β protein and improves other amyloid-β cascade pathogenesis related to AD, it is considered to be candidate therapeutic agent for AD and other neurodegenerative diseases (36). In addition, due to its anti-inflammatory and anti-oxidant properties, it is recognized as a potential natural compound to slow the progression of CNS disorders, such as neurodegenerative diseases (37).

The present study investigated the protective activity of ICA using cultured neural cells following OGD-R treatment. As a well-established method, OGD-R (I/R mimic) has been used

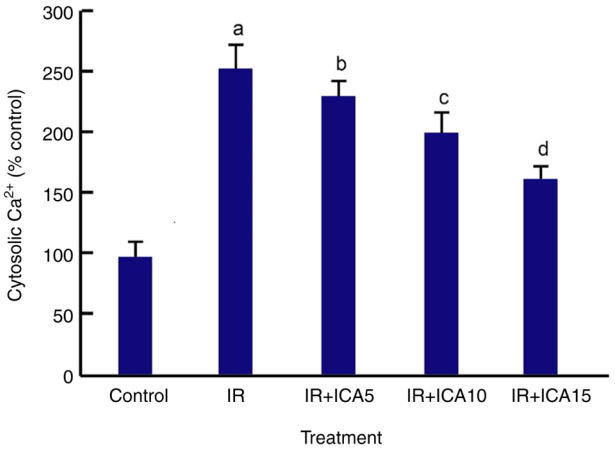

Figure 5. Cytosolic Ca^{2+} level in neural cells after OGD-R and ICA treatment at 0, 5, 10 and 15 μM. The cells were subjected to OGD-R and treated with ICA for 24 h, harvested and assessed in triplicate in three independent experiments for Ca^{2+} level by reacting to the Arsenazo Reagent according to the manufacturer's instructions. [a]P<0.05 vs. control; [b]P<0.05 vs. IR; [c]P<0.05 vs. IR + ICA5; [d]P<0.05 vs. IR + ICA10. OGD-R, oxygen-glucose deprivation and reoxygenation; ICA, icariin.

to generate an *in vitro* cellular model of I/R injury (38,39). The present results confirmed that generated injury in the cultured neural cells. After the OGD-R treatment, cell viability was significantly reduced and apoptosis was increased, and there was increased production of ROS. These results are consistent with previous studies showing that OGD-R induces oxidative stress in astrocytes as a result of increased ROS production, reduces cell viability (39) and increases LDH release and apoptosis in neuroblastoma cells (40).

Several molecules have been shown to protect neural cells from I/R injury, mainly by reducing oxidative stress, apoptosis and autophagy. The protection might be achieved by blocking NF-κB signaling and activating Nrf2/HO1, Akt and mTOR/p70S6K/4E-BP-1 pathways (40), downregulating the CaMKKβ/AMPK/mTOR signaling pathway (41) or by inhibiting the TLR2/4 signaling pathways (42). The present study revealed that post-OGD-R ICA treatment increased cell viability and reduced apoptosis, suggesting that ICA reduced OGD-R-induced cytotoxicity. As a consequence, less LDH was released, suggesting that the cells had more intact plasma membranes after I/R as compared with untreated cells, because I/R damage is especially associated with increased LDH activity (43). However, it remains to be investigated how ICA regulates the expression of apoptosis- and LDH-related genes to regulate the apoptosis pathways. Whether ICA modulates aforementioned pathways such as the Nrf2/HO1, Akt and mTOR/p70S6K/4E-BP-1 pathways and the CaMKKβ/AMPK/mTOR signaling pathway to reduce I/R injury warrants further investigation. Among them, Nrf2 signaling is of particular interest due to its role in stress response and cellular protection (44,45).

Recent studies show that brain injury may occur after transient or permanent focal cerebral ischemia as a result of complex series of pathophysiological events, such as ROS injury caused by oxidative stress, ion balance disorder, excitotoxicity, apoptosis and inflammation and brain edema (46,47). As a consequence, clinical management of stroke still faces various challenges (48). Although therapeutic approaches including mechanical or thrombolytic reperfusion, arteriogenesis, pharmacological neuroprotection, ischemic preconditioning and regeneration have been attempted, an improved method of differentiation between hemodynamic and molecular factors contributing to the manifestation of ischemic injury is considered to be important for therapeutic interventions (49). At the molecular level, increased neutrophil-derived neurovascular matrix metalloproteinase-9 activity is an important mechanism underlying the exacerbation of ischemic brain injury by systemic inflammation that contributes to the poor clinical outcome in stroke patients (50).

Brain tissue has high concentrations of unsaturated fatty acids and consumes large amount of oxygen. It is therefore sensitive to oxidative stress injury (51). During cerebral I/R, excessive production of ROS, if not scavenged sufficiently or timely, would result in lipid peroxidation and damage the membrane structure of nerve cells (52,53). It will also aggravate brain tissue damage associated with hypoxia/reoxygenation-induced apoptosis in cultured forebrain neurons, suggesting that oxidative stress might be responsible for hypoxia-induced neurotoxicity (54). On other hand, increased synthesis of intracellular SOD attenuates the hypoxia-reoxygenation injury by scavenging intracellular-free superoxide anion and protecting mitochondria from damage (55). Hypoxic postconditioning has also been attempted to reduce the cell loss since a hypoxic postconditioning containing three cycles of 5 min of reoxygenation and 5 min of rehypoxia applied before 6 h of reoxygenation reduces ROS generation, cardiomyocyte death and mitochondrial Ca^{2+} overload (56). However, SOD activity was not changed after ICA treatment, implying that other mechanisms including lipid synthesis *de novo* and prevention of lipid oxidation may be involved in the reduced cell death, likely including reduced production of ROS (57,58), which is main cause of lipid peroxidation (59).

To further probe the mechanism underlying observed ICA-mediated neuroprotection, ROS production was assessed. ROS is a major by-product of aerobic metabolism and is often increased after the reperfusion process after prolonged ischemia (5). ROS induce apoptosis by oxidizing the inhibitor of apoptosis signal-regulating kinase and upregulating the expression of FasL, a well-known and well-characterized death-inducing ligand, or they can bind to the tumor necrosis factor receptor to activate caspase (60,61). In the present study, a significant increase in ROS production was observed in the neural cells after OGD-R treatment, suggesting that overproduction of ROS is likely a cause of cell damage. On the other hand, ROS generation was reduced by ICA treatment in the OGD-R treatment cells, suggesting that ICA inhibits the production of ROS or facilitates the clearance of ROS and functions as an antioxidant. As a flavone compound, ICA is likely to have antioxidant activity through the scavenging of free radicals, or by chelating metal ions or by inhibiting the enzymatic systems responsible for producing free radicals (62). In a recent study, ICA was demonstrated to have radical scavenging activities using a 2,2'-diphenyl-1-picrylhydrazyl radical scavenging assay (63). It is hypothesized that the phenol functional groups in ICA could undergo H-atom abstraction for stable and delocalized radical species (63).

SOD is one of the most important antioxidant enzymes that scavenges ROS and eliminates oxidative stress caused by excessive ROS (64,65). The activity of SOD was also assessed after OGD-R and ICA treatments. However, no change in SOD content was observed after these treatments, suggesting that SOD does not contribute to reduce OGD-R-induced ROS production in the neutral cells. However, the result is in contrast with a previous study, where SOD activity was diminished in neurons when ICA was applied during I/R process (66). The difference in SOD expression between the studies may be due to difference in ICA treatment methods, although the exact reason that SOD did not change after ICA treatment in the present study is not clear. Therefore, ICA may exert its antioxidation activity via other mechanisms. For example, ICA may regulate Nrf2 to generate antioxidant response, as observed in porcine oocyte (57) and further studies are needed to investigate whether ICA activates Nrf2 signaling to generate antioxidant activity. In addition, ICA was shown to activate AMPK-SIRT3 signaling pathway, and it may mitigate the antioxidant activity via mitochondrial ROS homeostasis by AMPK-SIRT3 signaling pathway (58).

Ca^{2+} is one of the most important secondary messengers in cells with complex biological functions, including regulating the release of neurotransmitters and neuron excitability (67). Maintenance of intracellular Ca^{2+} homeostasis is achieved through the integrated and coordinated function of Ca^{2+} transport molecules and Ca^{2+} buffers, mainly in the endoplasmic reticulum and mitochondria (68) since increase in intracellular Ca^{2+} is often cytotoxic (69). Increasing evidence indicates that $Ca2^{+}$ overload is a pathological factor associated with AD (70) and brain ischemia (68). There are 3 channels related to Ca^{2+} intake and release on endoplasmic reticulum: Ryanodine receptor (RyR), inositol 1,4,5-trisphosphate receptor (IP3R) and sarco (endo) plasmic reticulum calcium-ATPases that pumps Ca^{2+} from the cytoplasm to the endoplasmic reticulum. Under normal circumstances, Ca^{2+} in the endoplasmic reticulum cavity is released to the cytoplasm mainly through RyR and IP3R and pumped into the endoplasmic reticulum from the cytoplasm to achieve dynamic equilibrium (71,72). However, this homeostasis can be disrupted during the I/R process, leading to calcium overload and increased concentration of Ca^{2+} in the cytoplasm, thus causing cell damage (73). To examine if the change in Ca^{2+} concentration is involved in ICA-mediated protection of the neural cells, cytosolic Ca^{2+} level was determined after OGD-R and ICA treatment. Increased Ca^{2+} was observed after I/R as compared with untreated cells. On the other hand, ICA treatment reduced the increase, suggesting that ICA may influence the release or intake of Ca^{2+} to balance Ca^{2+} after OGD-R. This is consistent with the results from the previous studies showing that ICA reduces the calcium content to protect MC3T3-E1 cells from hydrogen peroxide-induced damage (74). Several mechanisms are likely to underly this regulation although the exact mechanisms remain unclear. ICA may bind with IP3R in the endoplasmic reticulum to antagonize IP3R-mediated calcium release from ER calcium pool (75), or block the calcium channels to reduce Ca^{2+} being pumped into cytoplasm (76). Further studies are needed to elucidate the molecular mechanisms underlying ICA-mediated Ca^{2+} homeostasis.

Taken together, data from the present study with an *in vitro* model show that ICA protects neural cells from I/R injury and that this protection activity is likely achieved through its antioxidation activity and ability to maintaining cellular Ca^{2+} homeostasis. Further studies are needed to further elucidate the molecular mechanisms underlying the neuroprotection activity, including *de novo* lipid synthesis and lipid oxidation, which were not assessed in the present study. Studies with *in vivo* I/R model are needed to further validate the protective activity and develop approaches for potential clinical use of ICA as a therapeutic strategy for ischemia-related diseases.

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Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KN and RG contributed to project conceptualization, investigation and data analysis. KN and RG also performed data collection, analysis, methodology development and investigation All authors have read and approved the final manuscript. KN and RG confirm the authenticity of all the raw data.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Affiliated Zhongshan Hospital, Dalian University (Dalian, China; approval no. HSH221D). All methods were performed in accordance with the relevant guidelines and regulations.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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