

The neuroprotective effect of apigenin against OGD/R injury in rat hippocampal neurons

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Abstract: To investigate the effects of apigenin on the injury caused by oxygen and glucose deprivation in neurons and the underlying mechanisms, primary cultured rat hippocampal neurons were incubated with apigenin for 90 min before a 2-h oxygen and glucose deprivation followed by a 24-h reperfusion (OGD/R). Subsequently, cell viability, lactate dehydrogenase (LDH) leakage rate, apoptotic rate of neurons and activity of the sodium pump were assessed. In addition, activity of the sodium pump was also examined in the hippocampus of SD rats injected intraperitoneally with apigenin 90 min before a 10-min global cerebral ischemia/24-h reperfusion. The results showed that cell viability and activity of the sodium pump markedly decreased but LDH leakage rate and apoptotic rate significantly increased in OGD/R-treated neurons. However, pretreatment with apigenin (20-50 μmol/L) reversed the changes dose-dependently. Compared to sham controls, activity of the sodium pump was significantly suppressed in global ischemia/reperfusion rats; application of apigenin (200mg/kg) restored the activity of the sodium pump. Furthermore, the neuroprotective effect of apigenin was blocked partly by the sodium pump inhibitor ouabain. Our findings provide the evidence that apigenin has a neuroprotective effect against OGD/R injury and the protective effect may be associated with its ability to improve sodium pump activity.

Keywords: Apigenin, neuron, ischemia, sodium pump.

INTRODUCTION

Acute cerebral ischemia/reperfusion (I/R) causes neuron necrosis, resulting in neurological dysfunction or even death in patients. It is a complicated pathophysiological process, involving at least the following mechanisms: a disturbance of energy metabolism, neurotoxicity induced by excitatory amino acid, peri-infarct depolarization, calcium influx, inflammation and apoptosis. They may overlap and cross-talk with each other (Sims and Muyderman, 2010; Chen *et al.*, 2011; Jin *et al.*, 2010). Among them, disturbance of energy metabolism is a key element in the pathological process of cerebral ischemia/reperfusion injury (Sims and Muyderman, 2010). Under hypoxic-ischemic conditions, activity of mitochondrial oxidative phosphorylation is inhibited and adenosine triphosphate (ATP) synthesis is reduced. The rapid depletion of ATP directly inhibits the activity of Na⁺/K⁺-ATPase. Na⁺/K⁺-ATPase, also called sodium pump, is essential for cell osmotic pressure maintenance, cell volume regulation, and membrane resting potential restoration of excitable cells (Tian *et al.*, 2008).

Apigenin is also named 4',5,7-trihydroxy flavone or 5,7-Dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, a natural flavonoid with low intrinsic toxicity, widely exists in a variety of fruit, vegetables, and herbs. More

and more studies have found that apigenin has a variety of medicinal effects, such as anti-cancer, anti-inflammatory and anti-virus properties (Madunic *et al.*, 2018; Anusha *et al.*, 2017; Balez *et al.*, 2016). Recently, apigenin is found to effectively inhibit the apoptosis and necrosis of rat adrenal pheochromocytoma cells (PC-12) after oxygen and glucose deprivation and reperfusion (OGD/R) injury, suggesting that it has neuroprotective effects (Guo *et al.*, 2014). However, the mechanisms that apigenin protects the neurons against I/R injury are far from clear. The present study aimed to investigate the effects of apigenin on neuronal OGD/R or I/R injury in cultured rat hippocampal neurons and to explore the possible mechanisms, mainly focusing on the activity of the sodium pump.

MATERIALS AND METHODS

Primary hippocampal neuron culture

Primarily cultured hippocampal neurons were prepared from newborn Sprague-Dawley rats (Experimental Animal Center of Zhejiang University, China). Animal experiments were carried out in accordance with the guidelines established by the institution of Zhejiang University. After being sterilized in 70% ethanol, the hippocampal tissues were isolated and digested with trypsin for 15 min at 37°C. Tissues were transferred to a

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new tube, and 1ml DMEM medium containing 10% fetal bovine serum were added to terminate the digesting process. The supernatant was abandoned. DMEM medium was added to the tube and tapped 10-15 times gently to dissolve. Suspension was collected after the larger tissue being settled down. The trituration cycle was repeated for 3 times. Suspension was centrifuged at the speed of 1000rpm/min for 5 min, the supernatant was abandoned and the cell sediment was suspended by gently tapping several times with neurobasal medium (Gibco, Carlsbad, Calif, USA) containing 2% B27 (Gibco, Carlsbad, Calif, USA), 1% penicillin-streptomycin (Sigma, St. Louis, MO, USA), 0.5mmol/L L-glutamine (Sigma, St. Louis, MO, USA) and 0.2 mmol/L L-glutamic acid (Gibco, Carlsbad, Calif, USA). Neurons were seeded onto a 24-well plate (Nunclon, Roskilde, Denmark) or coverslip coated with Poly-L-Lysine (Sigma, St. Louis, MO, USA) and maintained in a 5% CO₂ incubator at 37°C for 12-15 days. Three days after neuron plating, cellular proliferation was inhibited by adding 10 μmol/L Cytosine-D-arabioside (Ara-C, Sigma, St. Louis, MO, USA). One-half of the medium was changed every three days by neurobasal medium containing 2% B27, 1% penicillin-streptomycin and 0.5 mmol/L L-glutamine.

Oxygen and glucose deprivation and reperfusion

The original glucose-containing culture medium in the OGD/R group was replaced with glucose-free medium (NaCl 137mmol/L, KCl 5.4mmol/L, CaCl₂ 1.8mmol/L, MgSO₄ 0.8mmol/L, HEPES 10mmol/L) pretreated with 95% N₂ and 5% CO₂. Afterwards, neurons were transferred into an anaerobic chamber and flushed with 95% N₂ and 5% CO₂ for 20 min, then kept in hypoxia atmosphere at 37°C for 1, 2, or 4-h, respectively. Medium were then replaced with normal neurobasal medium and neurons were cultured in 5% CO₂ at 37°C for 24-h. Medium in control group were replaced with normal fresh neurobasal medium and neurons were incubated in 5% CO₂ at 37°C. Neurons in the treatment groups were incubated with different concentrations of apigenin (Sigma, St. Louis, MO, USA) or ouabain (Sigma, St. Louis, MO, USA) 90 min before OGD/R.

Global cerebral ischemia and reperfusion

Male SD rats, weighed 200-250g, were randomly assigned into three groups: sham, ischemia/reperfusion (I/R), I/R + apigenin. As described previously, a global cerebral ischemia model was established by Pulsinelli's four-vessel occlusion (Pulsinelli *et al.*, 1983). Under anesthesia with sodium pentobarbital (50mg/kg body weight, Sigma-Aldrich, St. Louis, MO, USA), cervical posterior midline was incised, and the bilateral vertebral arteries were permanently occluded by thermocoagulation. Then bilateral common carotid arteries were exposed and tied loosely with sutures. Ischemia was induced by occluding both carotid arteries with aneurysm clips for 10 min after the animals woke up from anesthesia. Then the

clips were loosened to initiate reperfusion for 24-h. The spasmodic rats were excluded from our study. The same surgical procedure, except for the occlusion, was performed on the sham group. Rats in the I/R+apigenin group were injected intraperitoneally with apigenin (200mg/kg, dissolved in normal saline containing 0.5% sodium carboxymethyl cellulose) 90 min before carotid arteries occlusion, while the rats in the sham and I/R groups were injected with normal saline containing 0.5% sodium carboxymethyl cellulose.

MTT assays

Cell viability of neurons was monitored using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Neurons were incubated with 0.5g/L MTT for 4-h. Then the solution was removed and replaced with DMSO to dissolve the MTT formazan crystals. Absorbance of each sample was measured in a microplate reader at 490nm. Cell viability in the control group was defined as 100%. Cell viability was calculated as follows: cell viability = absorbance in each group/absorbance in the control group × 100%.

Lactate dehydrogenase (LDH) leakage rate

Cellular damage was evaluated by measuring the LDH leakage rate using a LDH assay kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). LDH was found in the cytoplasm of normal cells, and is released into the medium only after the cell membrane was damaged. In order to quantify the LDH leakage rate, medium was removed following reperfusion and LDH activity in the extra cellular solution was measured, while LDH activity in the intracellular solution was measured when cells were lysed. The absorbance was recorded at 440nm using a microplate reader and the leakage rate was calculated as follows: LDH activity in extracellular solution/ (LDH activity in the extracellular solution+LDH activity in the intracellular solution)×100 %.

Hoechst 33258 staining

Cells were fixed for 10 min and stained with Hoechst 33258 (Beyotime, Jiangsu, China) for 30 min at room temperature in the dark. Morphology of the cells' nuclei was observed under a fluorescence microscope at 350nm. Apoptotic cells are characterized by morphological changes such as the condensation of nuclear chromatin or fragmentation. Apoptotic rate was calculated as the ratio of apoptotic cell number to the total cell number.

Sodium pump activity

Hippocampal neurons or tissues were homogenized in 20 volumes of ice-cold 10mM Tris-HCl buffer, followed by a 30-min centrifugation at 12,000rpm to obtain the membrane pellet. The total protein concentration was measured by using Coomassie Brilliant Blue, and the activity of the sodium pump in the supernatant was measured according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, Jiangsu,

China). In brief, the reaction was initiated by adding 50 μL of ATP solution after 100 μg of sample protein was added to the reaction mixture. The reaction baseline was established by measuring the change of its absorbance ($\Delta\text{A}/\text{min}$) at 660nm in an ultraviolet spectrophotometer. Subsequently, the absorbance was measured again after addition of 50 μL of ouabain. Sodium pump activity was calculated as follows: $\Delta\text{A}/\text{min}$ before the addition of ouabain - $\Delta\text{A}/\text{min}$ after the addition of ouabain. The final sodium pump activity was expressed as U/mg of protein.

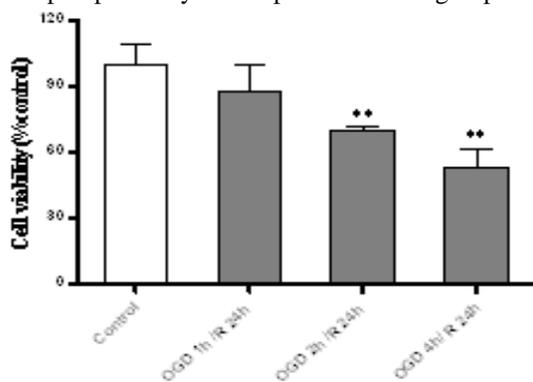


Fig. 1: Effect of different OGD/R models on cell viability in hippocampal neurons. Cell viability is determined by MTT assay after OGD treatment for 1, 2 or 4h, respectively and reperfusion for 24 h. The viability of control group was defined as 100%. ** $P < 0.01$ vs. control, $n = 8$.

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS 17.0 (SPSS, Chicago, IL, USA). Data is expressed as mean \pm standard deviation (SD). Comparisons between groups were analyzed by one-way analysis of variance followed by Scheffe's post hoc test. P -value less than 0.05 was considered significant.

RESULTS

Establishment of neuron OGD/R model

To induce mild to moderate insult to neurons, 1-, 2-, and 4-h OGD were induced in neurons and followed with 24-h reperfusion, and cell viability was assessed by MTT assay. As shown in fig. 1, cell viability was significantly decreased after 2-h and 4-h OGD followed by 24-h reperfusion when compared with the control group. The results showed that the OGD/R could induce seriously damage on neurons, and the cell viability decreased with the prolongation of the deprivation time. Combined with morphological results, we chose the protocol of 2-h OGD and 24-h reperfusion to induce a mild to moderate insult in hippocampal neurons.

Apigenin protected neurons against OGD/R injury

After OGD/R treatment, cell viability was significantly decreased, while LDH leakage rate was significantly

increased. Pretreatment with apigenin (20, 50, 100 $\mu\text{mol/L}$) significantly improved the survival of neurons and reversed the increase of LDH leakage rate after OGD/R. In particular, the protective effect of apigenin was dose-dependent and approximately reached its maximum at a concentration of 50 $\mu\text{mol/L}$, whereas the apigenin concentration below 10 $\mu\text{mol/L}$ showed no significant improvement on neuronal injury (fig. 2A, 2B). Compared with the control group, apoptotic rate of neurons increased significantly after OGD/R treatment (fig. 2C, 2D). Pretreatment with apigenin dose-dependently reversed the neuronal damage. Apoptotic rate of neurons reduced to (9.03 ± 1.17) % when pretreated with apigenin at a concentration of 50 $\mu\text{mol/L}$ and reached its maximum effect (fig. 2E, 2F).

Ouabain partially blocked the neuroprotective effect of apigenin

In order to explore the mechanism of neuroprotective effect of apigenin on OGD/R injury, neurons were co-incubated with apigenin and sodium pump inhibitor ouabain before OGD/R, cell viability and LDH leakage rate were assessed subsequently. We found that the neuroprotective effect of apigenin (50 $\mu\text{mol/L}$) was partially blocked by ouabain (100 $\mu\text{mol/L}$), resulting in a marked worsening in cell viability and LDH leakage rate (fig. 3A, 3B), which suggested that the enhancement of sodium pump activity might be a major mechanism of the neuroprotective effect of apigenin.

Apigenin enhanced the activity of the sodium pump in hippocampal neurons after OGD/R or I/R injury

Next, we studied the direct effect of apigenin on the activity of the sodium pump in hippocampal neurons *in vitro* or *in vivo*. In cultured hippocampal neurons, OGD/R treatment significantly decreased the activity of the sodium pump, while pretreatment with apigenin (50 $\mu\text{mol/L}$) significantly improved the activity of the sodium pump after OGD/R (fig. 4A). We further established rat global cerebral ischemia/reperfusion models by 10-min four-vessel occlusion followed by 24-h reperfusion. The activity of the sodium pump in hippocampus was significantly lower than that in the sham group, but it was obviously reversed after pretreatment with apigenin (200 mg/kg) administered intraperitoneally before I/R (fig. 4B). The results confirmed that apigenin enhance the activity of the sodium pump in hippocampal neurons.

Apigenin failed to protect neurons against glutamate induced excitotoxicity

To further investigate the neuroprotective mechanism of apigenin, effects of apigenin on glutamate-mediated excitotoxicity were studied in cultured neurons. Hippocampal neurons were incubated with glutamate (100 $\mu\text{mol/L}$) for 10 min and changed into normal culture solution for 24 h.

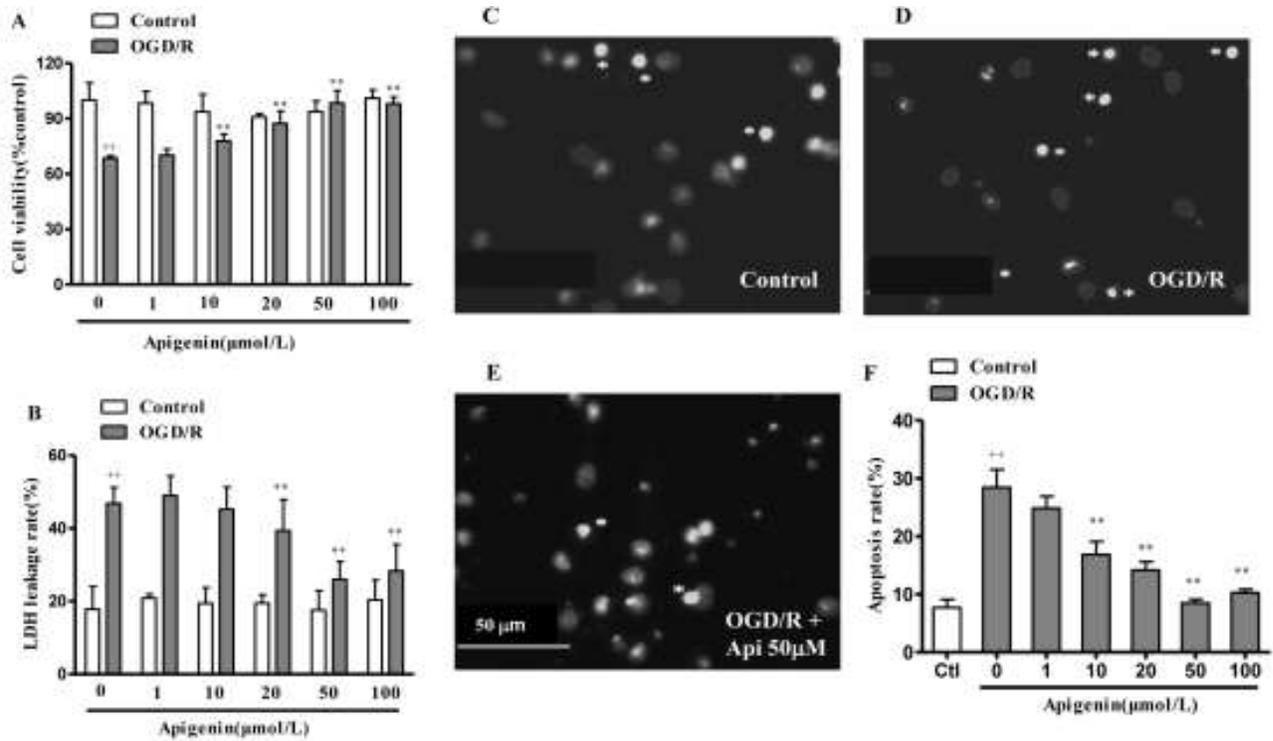
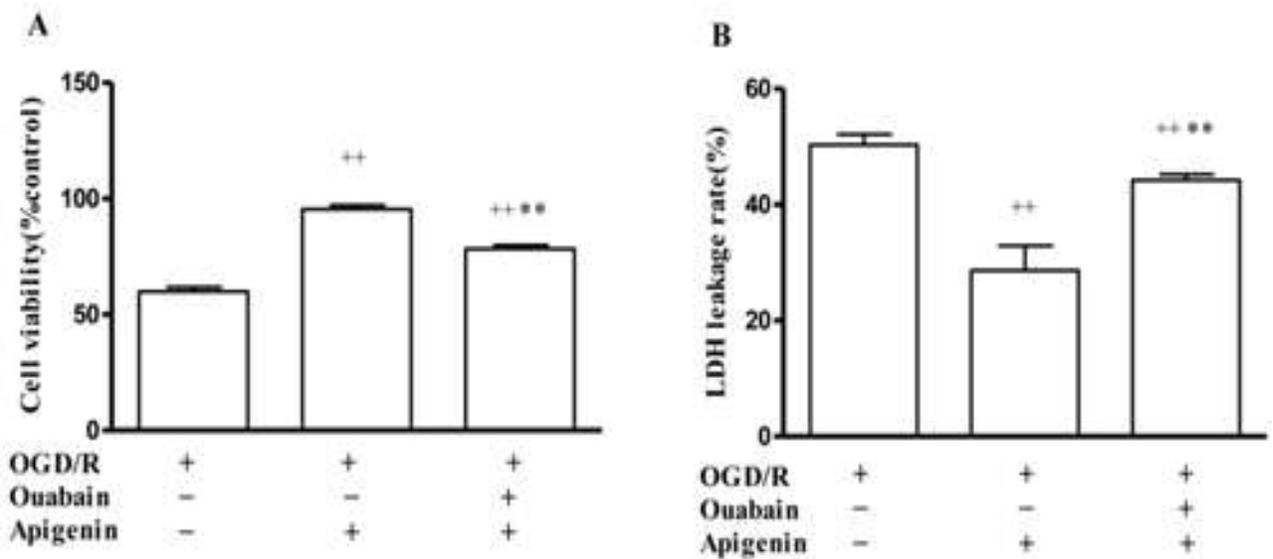
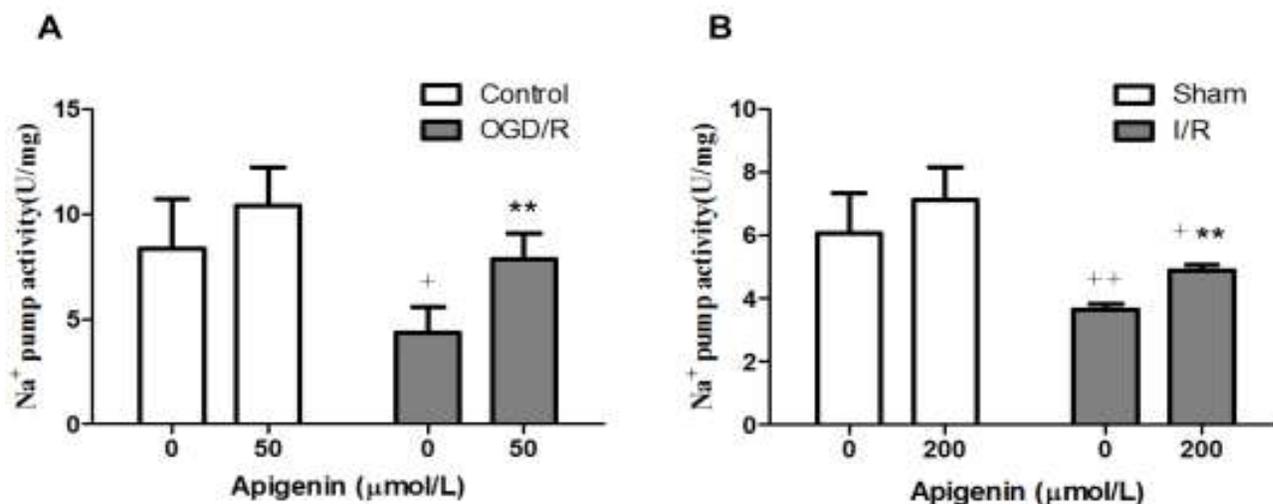


Fig. 2: Effect of apigenin on cell viability, LDH leakage rate and apoptotic rate in hippocampal neurons treated by OGD/R. Cultured hippocampal neurons were treated by OGD for 2 h followed by reperfusion for 24 h. Different concentrations of apigenin were incubated with the cells 90 min before OGD treatment. (A) Cell viability is determined by MTT assay and the viability of control group was defined as 100%. (B) LDH leakage rate is calculated as LDH activity in extra cellular solution/LDH activity in extra cellular and intracellular solution $\times 100\%$. (C-E) After staining with Hoechst 33258, the apoptotic neurons (labeled with *) show the morphological changes in nuclei. (F) Apoptotic rate is calculated by dividing the number of apoptotic neurons by the total number of neurons. $^{++}P < 0.01$ vs. control cells without apigenin. $^{**}P < 0.01$ vs. OGD/R-treated cells without apigenin. $n=8$.



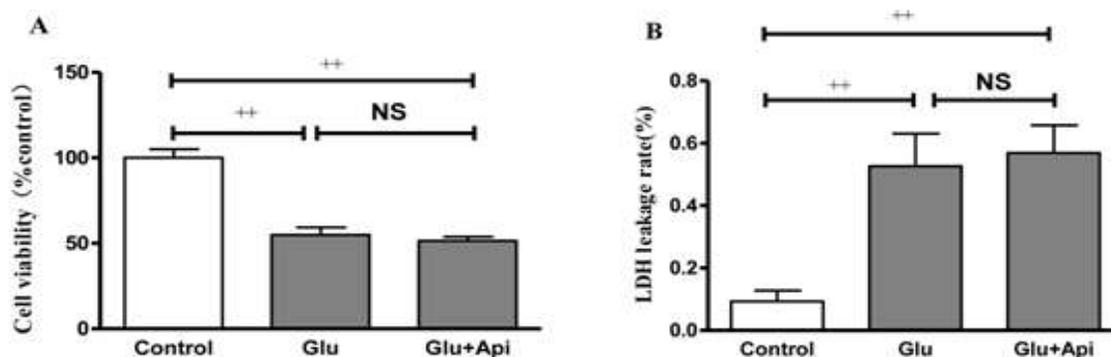
(A) Cell viability is determined by MTT assay. (B) LDH leakage rate is calculated as LDH activity in extracellular solution/LDH activity in extracellular and intracellular solution $\times 100\%$. $^{++}P < 0.01$ vs. OGD/R-treated alone. $^{***}P < 0.01$ vs. OGD/R-treated cells with apigenin. $n=8$.

Fig. 3: Effect of ouabain on the neuroprotective effect of apigenin in hippocampal neurons subjected to OGD/R.



(A) The hippocampal neurons were pretreatment with apigenin (50μmol/L) before OGD/R treatment, which significantly improved the activity of the sodium pump. ⁺ $P < 0.05$ vs. Control, ^{**} $P < 0.01$ vs. OGD/R without apigenin, $n = 8$. (B) The SD rats were subjected to global cerebral ischemia (I) by four-vessel occlusion for 10 min followed by reperfusion (R) for 24 h. Application of apigenin (200 mg/kg) in advance increased sodium pump activity in I/R treated rat. ⁺⁺ $P < 0.01$, ⁺ $P < 0.05$ vs. sham, ^{**} $P < 0.01$ vs. I/R without apigenin, $n = 6$.

Fig. 4: Effect of apigenin on sodium pump activity after OGD/R or I/R treatment.



(A) Cell viability was determined by MTT assay and the viability of control group was defined as 100%. (B) LDH leakage rate was calculated as LDH activity in extracellular solution/LDH activity in extracellular and intracellular solution $\times 100\%$. ⁺⁺ $P < 0.01$ vs. control cells. NS stands for not significant, $n = 8$.

Fig.5: Effects of apigenin on glutamate induced excitotoxicity in hippocampal neurons.

Cell viability was decreased and LDH leakage rate was increased, compared with the control cells. However, 90 min of apigenin (50μmol/L) incubation did not reverse the decrease of cell viability and the increase of LDH leakage rate induced by glutamate (fig. 5A, 5B). The results suggested that the inhibitory effect on excitotoxicity of glutamate was not involved in the neuroprotective activity of apigenin against OGD/R damage.

DISCUSSION

Currently, more and more attention in the field of drug

discovery has been paid to the neuroprotection of flavonoids extracted from traditional medicinal herbs (Wu *et al.*, 2010). The anti-oxidant activity of flavonoids would be a strong basis for the neuroprotective activity against I/R injury. The structural and biological study in apigenin has revealed that its various medicinal effects are related to its strong anti-oxidant activity (Wang *et al.*, 2017; Dajas *et al.*, 2003b; Jeyabal *et al.*, 2005), which protects organs like heart and liver against ischemia/reperfusion injury (Hu *et al.*, 2015; Tsalkidou *et al.*, 2014). However, the clinical application of anti-oxidants, e.g. vitamin C or vitamin E, has not achieved satisfactory results (Dajas *et al.*, 2003a). Interestingly,

apigenin has a better cytoprotective effect against lipid peroxidation (Jeyabal *et al.*, 2005; Psotova *et al.*, 2004) compared with quercetin, which shows a stronger anti-oxidant capacity *in vitro*. We speculated that apigenin has other complex mechanisms in addition to its anti-oxidant activity to protect cells against ischemic injury. Therefore, the possible mechanism of apigenin against I/R injury is proposed other than its anti-oxidant effect.

When cerebral ischemia occurs, due to the lack of oxygen and glucose supply and the damage to mitochondria from free radicals, the amount of ATP produced by mitochondria is significantly decreased, which leads to the inhibition of ATP dependent sodium pump activity (Petrushanko *et al.*, 2007). Inhibition of the activity of the sodium pump, in turn, interferes with nucleotides metabolism, resulting in a large amount of adenosine (Pimentel *et al.*, 2013) and further aggravation of neuronal damage. Thus, inhibition of sodium pump activity aggravates subsequent neuronal damage after cerebral ischemia (Pierre *et al.*, 2007; Inserte, 2007).

To elucidate the protective effect of apigenin on neurons against OGD/R or I/R injury and to explore the new mechanisms, this study compared the effects of apigenin at different concentrations on cell viability, LDH leakage rate and apoptotic rate in rat hippocampal neurons subjected to OGD/R treatment. The results showed that apigenin increased cell viability and decreased LDH leakage rate and apoptotic rate dose-dependently. We also found that this neuroprotective effect of apigenin was abolished after the activity of the sodium pump was blocked specifically by ouabain, suggesting that apigenin protected neurons against OGD/R injury by enhancing the sodium pump activity. To further illustrate the neuroprotective effect of apigenin, we also measured the activity of the sodium pump in the hippocampus of a rat global cerebral ischemia/reperfusion model and found a significant reduction in sodium pump activity in hippocampus. Consistently, pretreatment of apigenin partially reversed the activity of the sodium pump. This result showed that apigenin has a definite promoting effect on sodium pump activity in neurons, which might be a new mechanism against cerebral I/R injury.

Glutamate is the major excitatory amino acid transmitter in the adult central nervous system. Numerous studies have shown that glutamate excitotoxicity plays a central role in the pathological process of neuronal injury after cerebral ischemia/reperfusion. Excess glutamates activate NMDA receptor mediated Ca^{2+} influx, resulting in intracellular Ca^{2+} overload, which causes cell apoptosis and necrosis (Butcher *et al.*, 1990; Lai *et al.*, 2014). Glutamates also participate in a variety of metabolic processes in the brain. Excess glutamates block the tricarboxylic acid cycle and reduce ATP synthesis, further aggravate the neuronal injury. Here, we found that

glutamates caused a reduction in neuronal viability and an increase in LDH leakage rate, but apigenin pretreatment did not reverse the neuronal injury caused by toxic glutamate exposure. Thus, the neuroprotective effect of apigenin on OGD/R treated neurons may not be induced by inhibiting the toxicity of glutamate.

CONCLUSION

In this study we demonstrated that apigenin protected neurons against OGD/R injury through enhancing sodium pump activity on cell membrane, but not through inhibiting excitatory amino acid toxicity. At present, enhancement of sodium pump activity has been believed to be one of the most important mechanisms in ischemic preconditioning (Tian *et al.*, 2008; Pierre *et al.*, 2007; Inserte, 2007). Pretreatment with apigenin before cerebral I/R might be equivalent to the effect of ischemic preconditioning. Therefore, the present study may bring new insights into future research and treatment of cerebral ischemia/reperfusion injury.

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Conflict of Interest

The authors declare no conflict of interest.

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