Resveratrol Treatment in Different Time-Attenuated Neuronal Apoptosis After Oxygen and Glucose Deprivation/Reoxygenation via Enhancing the Activation of Nrf-2 Signaling Pathway In Vitro

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Abstract
Recent studies have indicated that resveratrol has protective effects against cerebral ischemia/reperfusion injury. However, the best therapeutic time for resveratrol treatment after acute ischemic stroke remains unknown. We aim to investigate whether resveratrol, administrated at different times after neuronal oxygen and glucose deprivation/reoxygenation (OGD/R) reduced neuronal injury in vitro. There were six experimental groups: normal, model, resveratrol pretreatment, resveratrol post-treatment, resveratrol OGD-treatment, and resveratrol whole-processing group. We found that resveratrol in a concentration-dependent manner decreased the activity of lactate dehydrogenase (LDH) and increased the activity of superoxide dismutase (SOD). Moreover, resveratrol, administrated at different times, increased neuronal viability, reduced neuronal apoptosis, upregulated the protein expressions of Nuclear factor erythroid 2-related factor 2 (Nrf-2), NAD(P)H:quinone oxidoreductase 1 (NQO-1), heme oxygenase 1 (HO-1), and Bcl-2, downregulated the protein expression of Caspase-3, and promoted Nrf-2 to transfer into the nuclei from the cytoplasm. The most effective treatment group was the whole-processing treatment group. These results suggest that resveratrol treatment at different times increased neuronal viability and inhibited neuronal apoptosis in vitro, at least in part, via enhancing the activation of the Nrf-2 signaling pathway.

Keywords
Resveratrol, neuroprotection, oxidative stress, apoptosis, oxygen and glucose deprivation/reoxygenation, Nrf-2, HO-1, NQO-1

Introduction
Resveratrol is found in red wine and the produce of a variety of plant species, including grapes, peanuts, and plums. Recent studies suggested that resveratrol may share many biological properties, including anti-aging, anti-inflammation, antioxidant, anticancer, and anti-apoptosis¹,². The Mediterranean diet, which is rich in resveratrol, can significantly decrease risk of cardiovascular diseases in humans³. It is well-established that pretreatment with resveratrol before ischemic brain damage may reduce neuronal loss and decrease infarct volume of the brain⁴,⁵. Moreover, resveratrol administered during ischemia or 6 h after reperfusion can also effectively decrease infarct volume⁶. These

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results are the basis for clinical application of resveratrol for the prevention and treatment of acute ischemic cerebral stroke. However, the best therapeutic time for administration of resveratrol after acute ischemic cerebral stroke has not been tested. Therefore, it is essential to determine the effect of resveratrol administration at different times before and after acute ischemic cerebral stroke.

The ischemia/reperfusion triggers the generation of abundant reactive oxygen species (ROS), which are associated with increased apoptosis. Antioxidants can protect from ischemia/reperfusion-induced injuries in experimental models by attenuating ROS generation and associated apoptotic pathways. Nuclear factor erythroid 2-related factor 2 (Nrf-2), a transcription factor, regulates the expressions of numerous antioxidant defense genes, such as heme oxygenase-1 (HO-1) and NAD(P)H: quinone acceptor oxidoreductase 1 (NQO-1), and attenuates cellular oxidative stress. HO-1 is a ubiquitous and redox-sensitive inducible stress protein which degrades heme to CO, iron, and biliverdin. Moreover, both HO-1 and its byproducts can inhibit cellular oxidative stress. NQO-1, a widely-distributed FAD-dependent flavoprotein, accelerates obligatory two-electron reductions of nitroaromatics, quinones, azo dyes, and quinoneimines. NQO-1 has highly effective antioxidant functions and a cytoprotective role. Resveratrol, a scavenger of ROS and other free radicals, can reduce markers of oxidative stress such as malondialdehyde and hydroxyl radical levels in the ischemic brain. However, the mechanism of neuroprotection is not well understood. Therefore, we aim to investigate whether and how resveratrol, administered at different times after neuronal oxygen and glucose deprivation/reoxygenation (OGD/R), can reduce neuronal injury in vitro.

**Materials and Methods**

**Primary Culture of Rat Cortical Neurons**

Ten one-day-old Sprague-Dawley rats were purchased from the Department of Animal Experiments and all experimental protocols were carried out with the approval of the Animal Experimental Committee, Chongqing Medical University. Cortical neurons were cultured using a modified method reported by Redmond et al. Briefly, the meninges and blood vessels were removed from the cerebral cortices and then were minced. The tissues were incubated with 0.125% trypsin for 30 min at 37°C, neutralized, and triturated by a Pasteur pipette. Huge particles were eliminated before neurons were seeded on poly-L-lysine precoated plates with DMEM/F12 containing 10% fetal bovine serum (FBS, Gibco, Gaithersburg, MD, USA) at a density of 3 × 10⁵ cells/cm² and cultivated in humidified atmosphere of 95% air/5% CO₂ at 37°C for 24 h. After cell attachment, the medium was changed to a neurobasal medium (Gibco, Gaithersburg, MD, USA) containing 2% B₂₇ (Gibco) and 0.5 mM glutamine, and then the medium was changed every day. Arabinosylcytosine (5 µg/ml) was added on the third day after incubation to prevent the growth of non-neuronal cells. Cells were used for experiments on the seventh day of culture.

**Oxygen and Glucose Deprivation/Reoxygenation**

The OGD/R model in vitro was set up as described previously. After washing the cells twice with D-Hanks solution (Hyclone, Los Angeles, USA), neurons were cultured with D-Hanks solution and placed into an incubator filled with anaerobic gas mixture (95% N₂ and 5% CO₂) at 37°C for 150 min. During reoxygenation, the D-Hanks solution was replaced with neurobasal medium containing 2% B₂₇, and cultures were placed in an incubator under 95% air/5% CO₂ for 24 h.

**Drug Treatment**

To examine the best effect concentration of resveratrol, three groups were studied: (1) normal group (Nor) – neurons were cultured in neuron culture medium without OGD/R; (2) model group (Mod) – cells were treated with neuron medium containing ethanol (volume fraction 1.3%) for 24 h before OGD/R; (3) resveratrol treatment group (Res) – cells were maintained in complete medium containing different concentrations (10 µmol/L, 20 µmol/L, 40 µmol/L, 60 µmol/L, and 80 µmol/L) of resveratrol (purity 99%; Shanxi Ciyuan Biotech, China) for 24 h before OGD/R. The best effect was observed with a concentration of 40 µmol/L resveratrol, and thus 40 µmol/L resveratrol was used for further study.

To determine the best therapeutic time window of resveratrol, six groups were studied: (1) normal (Nor) – cells were cultured in neuron culture medium without OGD/R; (2) model (Mod) – cells were treated with neuron medium containing ethanol (volume fraction 1.3%) for 24 h before OGD/R; (3) resveratrol pretreatment (Pre) – cells were treated with resveratrol for 24 h prior to OGD, followed by 150 min of OGD and 24 h of reoxygenation; (4) resveratrol post-treatment (Post) – cells were treated with resveratrol during 24 h of reoxygenation; (5) resveratrol OGD-treatment (OGD) – neurons were treated with resveratrol during 150 min of OGD and 24 h of reoxygenation; (6) resveratrol whole-processing (WP) – neurons were treated with resveratrol 24 h before and during 150 min of OGD and 24 h of reoxygenation.

**Measurement of LDH Activity**

Neuronal cytotoxicity was determined by the activity of LDH. LDH in the culture medium was detected using a diagnostic kit according to the manufacturer’s instructions (Jiancheng Bioengineering Institute, Nanjing, China). Briefly, 20 µl of supernatant from each well was collected to assay LDH release. The samples were sequentially incubated with a reduced form of buffer solution and 2,4-dinitrophenyhydrazine for 15 min at 37°C (Jiancheng Bioengineering Institute, Nanjing, China), and the reaction was stopped by adding 0.4 mol/L NaOH. The activity of...


LDH was calculated from the absorbance at 450 nm and background absorbance from culture medium that was not used for any cell cultures. The results were expressed as units per milligram of protein (U/mg protein). Experiments were repeated three times.

**Measurement of SOD Activity**

For assay of SOD activity, the cells were lysed with RIPA lysis buffer and the cell suspension was centrifuged at 4°C according to the manufacturer’s instructions (Jiangchong Bioengineering Institute). This method employs xanthine and xanthine oxidase to generate superoxide radicals that react with p-iodonitrotetrazolium violet (INT) to form a red formazan dye. The absorbance of each standard and sample was read at 450 nm using a 96-well microplate reader (Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China) and background absorbance from culture medium that was not used for any cell cultures. SOD activity was expressed as U/mg protein. Experiments were repeated three times.

**Immunocytochemistry**

Cells were rinsed twice with cold phosphate-buffered saline (PBS, Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China) and fixed with 4% paraformaldehyde at room temperature for 15 min. Subsequently, cells were washed three times (5 min each) in PBS, permeated by incubating in 0.05% Triton X-100 for 10 min (Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China), washed again, and blocked with 10% normal goat serum blocking solution for 1 h at 37°C (Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China). Then, cells were incubated overnight at 4°C with antibody anti-Nrf-2 (1:100; Santa Cruz Biotechnology, CA, USA). Cells were then washed three times with PBS (5 min each) before incubation with FITC-conjugated goat anti-rabbit IgG (1:50; Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd., Beijing, China) for 1 h in humidified chambers at 37°C. The cell nucleus was stained with PI (Beyotime Institute of Biotechnology, Jiangsu, China). The slides were washed three times with PBS and mounted in PBS/glycerol (50:50). In the negative control group, the primary antibody was omitted while the other steps remained the same; no fluorescence signals were detected. Images were captured on a Leica TCS SP2 confocal laser imaging system (Leica Microsystems, Wetzlar, Germany). All immunocytochemical experiments were repeated three times.

**TUNEL Staining**

The terminal deoxynucleotidyl transferase dUTP-mediated nick end labeling (TUNEL) assay (Beyotime Institute of Biotechnology, Jiangsu, China) was used to assess neuronal cell death. Briefly, cortical neurons were seeded on PLL-coated glass slides at a density of 3 × 10^5 cells/cm² and treated as described above. After treatment, cells were fixed in 4% p-formaldehyde for 15 min, and subsequently washed with PBS. Then, cells were incubated for 1 h at 37°C with fluorescein. Slides were rinsed briefly with PBS, air dried, and then mounted in antifluorescein fading medium. Slides were analyzed under an IX70 fluorescence microscope (Olympus, Tokyo, Japan). The percentage of apoptotic cells was determined by counting the number of nuclear-condensed cells versus total cells in each experimental condition to determine the ratio. The experiment was repeated three times.

**Measurement of Cell Viability**

Cell viability was measured with the MTT assay (Gibco). Briefly, primary cortical neurons were seeded in 96-well plates and subjected to various treatments as described above. MTT solution (5 mg/ml) was added to each culture well, and the cells were incubated for 4 h at 37°C. The medium was carefully aspirated, and 200 µl DMSO per well was added to dissolve the blue formazan products. The values of absorbance at 490 nm were measured using a microplate reader. The results of the absorbance of the test wells were expressed as cells alive. Cell survival rates were expressed as percentages of the value of cells without any treatment. The experiment was repeated three times.

**Western Blotting Analysis**

Total proteins and nuclear proteins were extracted. The samples were treated with 5 × SDS-PAGE sample loading buffer prior to heating at 100°C for 10 min. Equal amounts of protein from different groups were electrophoresed through sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE), and was then transferred onto PVDF membranes. After blocking with 5% nonfat dry milk in TBS-T buffer for 2 h, the membranes were incubated with the desired primary antibodies at 4°C overnight. The primary antibodies used in this study were as follows: Nrf-2, NQO-1 (1:1000; Abcam, Hong Kong, China), HO-1, Bcl-2, Caspase-3, and anti-β-actin (1:500; Santa Cruz Biotechnology, CA, USA). They were then treated with an AP-conjugated secondary antibody (1:100; Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd.) and immunoreactive bands were visualized using the enhanced chemiluminescence method. The films were scanned with an imaging densitometer (Bio-Rad, Hercules, USA), and the results were quantified using Quantity One 1-D analysis software. Quantities of each product were normalized by dividing the average gray level of the signal by that of the corresponding β-actin amplicon. Experiments were repeated three times.

**Statistical Analysis**

All statistical analyses were performed using the commercially available software SPSS 17.0 for Windows. Data are
expressed as mean ± standard deviation (SD). The one-way analysis of variance (ANOVA) and Tukey’s post-hoc test were employed to test differences among experimental groups. A P value <0.05 was defined as statistically significant. Each experiment was repeated three times.

Results

Resveratrol Enhances Neuronal Viability and Inhibits Neuronal Apoptosis After OGD/R Injury In Vitro

LDH and SOD activity assay at 24 h after OGD/R injury was used to determine the optimal concentration of resveratrol treatment on neuronal injury following OGD/R in vitro. As shown in Fig. 1, the activity of SOD was significantly higher in the resveratrol groups than in the normal and model groups, and peaked in the 40 μmol/L resveratrol group (P < 0.05) (Fig. 1A). The LDH activity was significantly decreased in the resveratrol groups compared to the model group and the 40 μmol/L resveratrol decreased the OGD/R injury of neurons most effectively, so the most favorable concentration of resveratrol was 40 μmol/L. Therefore, 40 μmol/L resveratrol was selected for further experiments.

MTT assay at 24 h after OGD/R injury showed that neuronal viability was markedly decreased in the model and resveratrol groups compared to the normal group (set 100%) (P < 0.05). However, in the resveratrol groups (Pre, Post, OGD, and WP), the viability was increased to 60.8 ± 2.1%, 44.6 ± 1.8%, 54.8 ± 1.6%, and 73.8 ± 2.7%, respectively, compared with the model group (39.2 ± 1.2%) (P < 0.05) (Fig. 2C). The best effective group was the WP group.

Fig. 1. Resveratrol increased SOD activity, decreased LDH activity, and enhanced neuronal viability at 24 h after OGD/R injury. (A) SOD activity was significantly higher in the resveratrol groups than those in the normal and model groups, and peaked in the 40 μmol/L resveratrol group. (B) LDH activity was significantly decreased in the resveratrol groups compared to the model group and lowest in the 40 μmol/L resveratrol group. *P < 0.05 vs. Nor group; "P < 0.05 vs. Mod group; "P < 0.05 vs. 40 μmol/L Res group. (C) Cell viability was significantly increased in the resveratrol groups, especially for the WP and Pre groups, compared to the Mod group. *P < 0.05 vs. Nor group; "P < 0.05 vs. Mod group; "P < 0.01 vs. WP group, "P < 0.05 vs. Pre group.
Further, we studied the effect of resveratrol on apoptosis at 24 h after OGD/R injury. TUNEL showed that TUNEL-positive cells were increased significantly in the model (37.0 ± 6.4%) and resveratrol groups (Pre, Post, OGD, and WP groups, 17.0 ± 8.7%, 29.9 ± 4.0%, 27.2 ± 4.0%, and 13.5 ± 4.1%, respectively) compared to those in the normal group (2.8 ± 0.7%). However, the TUNEL-positive cells were decreased significantly in the resveratrol group (Fig. 2A-M) (P < 0.05). Moreover, western blot analysis at 24 h after OGD/R injury showed that anti-apoptosis protein Bcl-2 increased significantly in the model and resveratrol groups compared to the normal group. However, resveratrol decreased significantly the protein expression of Caspase-3 compared to the model group, and was highest in the WP resveratrol group (Fig. 2N, O) (P < 0.05). Caspase-3, a sensitive indicator of apoptosis, increased significantly in the model and resveratrol groups compared to the normal group. However, resveratrol decreased significantly the protein expression of Caspase-3 compared to the model group, and was lowest in the WP resveratrol group (Fig. 2N, P) (P < 0.05). These results indicate that resveratrol inhibited the apoptosis of neurons after OGD/R injury in vitro.

**Resveratrol Strengthens Activation of the Nrf-2 Signaling Pathway After OGD/R Injury In Vitro**

Immunocytochemistry showed that Nrf-2 mainly existed in the cytoplasm of neurons in the normal group (Fig. 3A). After OGD/R, few cells were positive for Nrf-2 in the nucleus in the model group (Fig. 3B). In the resveratrol groups, Nrf-2 was mainly located in the nucleus after OGD/R (Fig. 3C). Western blot analysis showed that the protein levels of Nrf-2 in the nucleus, and NQO-1 and HO-1 in the cytoplasm, were significantly increased in the model and resveratrol groups compared to those in the normal group (P < 0.05). It increased significantly in the resveratrol groups compared to the model group, and was highest in the WP group (P < 0.05) (Fig. 3D). These results show that resveratrol strengthened activation of the Nrf-2 signaling pathway after OGD/R injury in vitro.

**Discussion**

This study showed that resveratrol in a concentration-dependent manner increased the activity of SOD and decreased the activity of LDH. The most effective concentration of resveratrol was 40 μmol/L. LDH, an endocellular enzyme, is a marker for cell damage. SOD plays a crucial role in maintaining intracellular reduction-oxidation balance and establishing the mechanisms of cellular defenses augmented by oxidative stress. The activity of SOD indirectly reflects the body’s free-radical scavenging ability. Therefore, our results showed that resveratrol in a concentration-dependent manner reduced neuronal OGD/R injury.

After stroke, thrombolysis is the first choice of treatment for ischemic stroke within 4.5 hours of symptom onset. However, because the majority of patients arrive in the hospital at later time points after symptom onset, only 2–3% of stroke patients receive thrombolytic therapy. Therefore, we administrated resveratrol at different time points during OGD/R injury to explore the therapeutic time of resveratrol. The present results showed that resveratrol, administrated at different times after OGD/R injury, increased neuronal viability, reduced neuronal apoptosis, upregulated the protein expression of Bcl-2, and downregulated the protein expression of Caspase-3. The most effective treatment group was the WP treatment group. Deng and Mi reported that resveratrol pretreatment decreased Aβ25-35 caused neurotoxicity and enhanced neuronal viability. Sinha et al. reported that resveratrol administered during ischemia or 6 h after reperfusion can effectively decrease infarct volume. Consequently, resveratrol may have preventive and therapeutic potential for ischemic stroke in vivo. Moreover, it may be more beneficial to patients for immediate and whole-range usage of resveratrol after ischemic stroke.

The numbers of apoptotic cells determines the severity of the final injury. Therefore, anti-apoptosis is a therapeutic strategy in ischemic stroke. Bcl-2, an important endogenous anti-apoptotic gene, can inhibit mitochondrial factors from being released by mitochondria, inhibit apoptosis, and increase cell viability. Caspase-3, a sensitive indicator of apoptosis, is the key enzyme of caspase cascade, and execution. Activated caspase-3 cleaves nDNA repair enzymes, which leads to nDNA damage and apoptosis. The present study showed that resveratrol treatment at different times significantly reduced apoptosis of neurons, increased the expression of Bcl-2, and decreased the expression of Caspase-3. Zhang et al. reported that resveratrol inhibited oxyhemoglobin-induced neuronal apoptosis and reduced early brain injury after experimental subarachnoid hemorrhage. Our prior studies also showed that resveratrol reduced neuronal apoptosis after OGD/R injury. Therefore, in accordance with previous reports, we also demonstrated that resveratrol had anti-apoptotic ability in vitro.

During the OGD/R, abundant ROS may causes oxidative stress damage of neuronal lipids, proteins, and DNA, leading to neuronal dysfunction and death. Moreover, neurons are very vulnerable to ROS. Therefore, it is key to antioxidant treatment of neuronal OGD/R injury. Cell defense mechanisms that may protect cells from the destructive consequences of ROS include superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase. SOD plays a crucial role in maintaining intracellular reduction-oxidation balance and establishing the mechanisms of cellular defenses augmented by oxidative stress. The level of SOD indirectly reflects the body’s free-radical scavenging ability. In the present study, resveratrol significantly increased the activity of SOD after OGD/R injury. Movahed et al. reported that resveratrol induced SOD synthesis and protected adult cardiomyocytes against oxidative stress-mediated cell injury. Wang et al. reported that resveratrol increased SOD activity and protected neurons in a rat model of...
cerebral ischemia. Our results, together with previous reports, indicated that resveratrol may decrease oxidative stress.

The Nuclear factor erythroid 2-related factor 2/antioxidant response element (Nrf-2/ARE) signaling pathway has been increasingly considered as a key contributor to the...
cellular response to neuronal injury in vitro and in vivo\textsuperscript{28,29}. Nrf-2 is involved in the regulation of a number of ROS-detoxifying enzymes. Upon activation, Nrf-2 liberates from Keap-1 and translocates to the nucleus, where it combines with ARE and activates ARE-dependent transcription of phase II and antioxidant defense enzymes, including HO-1, NQO-1, and $\gamma$-glutamylcysteine synthetase (GCLC), the rate-limiting enzyme for glutathione synthesis, to attenuate cellular oxidative stress\textsuperscript{8}. In the present study we demonstrated that Nrf-2 partly translocates to the nuclei, and the protein expression of Nrf-2 in nuclei and HO-1 and NQO-1 in cytoplasm are slightly upregulated after OGD/R injury. The result showed that the Nrf-2/ARE signaling pathway was activated after OGD/R injury. After resveratrol treatment, Nrf-2 significantly translocated to the nuclei, the expressions of Nrf-2 in nuclei, and NQO-1 and HO-1 in
cytoplasm, were significantly upregulated. These results suggest that resveratrol enhanced activation of the Nrf-2/ARE signaling pathway during OGD/R injury. HO-1 plays a crucial role in the endogenous defense against oxidative stress in gene-knockout mice because cells from HO-1–/– mice have increased susceptibility to oxidative insults\textsuperscript{30}. NQO-1 serves as a neuroprotective guard to eliminate ROS and prevents cellular injury in brain ischemia and various neurodegenerative diseases such as Alzheimer’s Disease, Parkinson’s Disease, and multiple sclerosis\textsuperscript{31}. Ungvari et al. reported that resveratrol may induce NQO-1, HO-1, and GCLC in an Nrf2-dependent manner for cultured endothelial cells\textsuperscript{8}. Sakata et al. reported that resveratrol selectively increased HO-1 in a concentration- and time-dependent manner in cultured mouse cortical neurons and prevented neurons from free-radical or excitotoxicity damage\textsuperscript{32}. Our results, together with previous reports\textsuperscript{32,33}, suggest that the Nrf-2/ARE signaling pathway plays a vital role in neuroprotection of resveratrol treatment the damage from neuronal oxidative injury.

We found that resveratrol may reduce neuronal damage and suppressed oxidative responses and apoptosis when administrated at different times after OGD/R injury. Our findings may shed light on the therapeutic potential of resveratrol in ischemic cerebral damage.

Taken together, the present study showed that resveratrol, administrated at different times after OGD/R injury, had a neuroprotective effect on OGD/R injury neurons via enhancing, at least in part, activation of the Nrf-2/ARE signaling pathway. In the future, we will determine whether the Nrf-2/ARE signaling pathway mediates resveratrol to promote neurite outgrowth or axonal remodeling of neurons after stroke in vivo and in vitro.

**Ethical Approval**

This study was approved by the First Affiliated Hospital of Chongqing Medical University ethics committee. The research plan submitted by this project conforms to the ethical principles of experimental animal research and the Helsinki declaration. The feeding and death methods of animals conform to the requirements of scientific research ethics, give the maximum protection to the safety of animals, do not bring unnecessary injury to the animals, and have scientific basis for the research and design.

**Statement of Human and Animal Rights**

Statement of Human and Animal Rights is not applicable for this article.

**Statement of Informed Consent**

Statement of Informed Consent is not applicable for this article.

**Declaration of Conflicting Interests**

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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