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**Original Research Article**

# Protective effect of remimazolam on neuronal cells from OGD/R-induced damage: An *in vitro* study

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## Abstract

***Purpose:*** *To investigate the effect of remimazolam (Re) on oxygen glucose deprivation/reoxygenation (OGD/R) model, and to elucidate the mechanism of action.*
***Methods:*** *The neuron cells were treated with OGD/R. MTT assays were performed to confirm The effect of Re on cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, while flow cytometry (FCM) assay was conducted to determine the apoptosis of OGD/R cells. Immunostaining assay and enzyme-linked immunosorbent assay (ELISA) were used to evaluate the effects of Re on mitochondria damage, while immunoblot assay was used unravel its mechanism of action.*
***Results:*** *Remimazolam (Re) significantly enhanced OGD/R-induced neuronal survival ($p < 0.05$). In addition, it significantly inhibited OGD/R-induced neuronal apoptosis, and OGD/R-induced neuron mitochondrial damage. Furthermore, it suppressed MAPK pathway in OGD/R-induced neuron, thus protecting the neuronal cells from OGD/R-induced damage ($p < 0.05$).*
***Conclusion:*** *Remimazolam protects neuronal cells from OGD/R-induced damage by mediating MAPK pathway. Therefore, it is a potential drug for the management of Ischemia-reperfusion injury (IRI). However, in vivo studies are required to buttress this.*

***Keywords:*** *Remimazolam, Oxygen glucose deprivation/reoxygenation (OGD/R), Apoptosis, Mitochondrial damage, MAPK pathway, Ischemia-reperfusion injury*



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## INTRODUCTION

Ischemic stroke is now a leading cause of death worldwide, and a major contributor to disability among the elderly [1]. Oxygen supply is essential for hypoxia/glucose deprivation (OGD) neurons [2]. However, it can also lead to delayed neuronal damage [3]. The process of cerebral IRI involves complex pathological mechanisms, including reactive oxygen species (ROS) production, autophagy activation, mitochondrial membrane potential (MMP) loss, mitochondrial permeability transition pore (MPTP) opening and apoptotic factor release [4]. Mitochondria, as the center of cellular respiration and energy production, plays an important role in the process

of brain IRI [5]. Mitochondrial disorder is considered to be a fundamental factor in IRI [6].

Remimazolam (Re) is a novel benzodiazepine agonist that targets gamma-aminobutyric acid A (GABAa) receptors [7]. It stabilizes hemodynamics while providing rapid anesthesia and recovery, and has a less inhibitory effect on breathing. At the same time, it allows long-term infusion without accumulation, and the metabolites have no pharmacological effect. As a result of these advantages, remazolam has a broad prospect for application in clinical practice. Remazolam reduces the lipopolysaccharide (LPS)-treated inflammatory response by activating peripheral benzodiazepine receptors and inhibiting the macrophage p38 signaling pathway, thus reducing septicaemia-related acute liver injury [8]. Remazolam inhibits glioma cell growth and induces apoptosis [9]. In addition, remazolam also has neuroprotective effects. For example, remazolam, by inhibiting NLRP3 inflammator-dependent cell pyrodeath, effectively ameliorates neurological dysfunction, and reduces infarct size and cortical neuron damage in a rat cerebral ischemia/reperfusion model [10]. Remazolam also delayed neurodegeneration and improved cognitive impairment in older mice by reducing tau phosphorylation [11, 12]. However, the possible effects of Re in the progression of IRI are still unclear. The aim of this study was to investigate the role of Re in the progression of IRI, and to ascertain its suitability as therapeutic agent for the management of IRI.

## EXPERIMENTAL

### Establishment of OGD/R model and treatments

OGD/R model was using mouse neuron cells. Neurons were washed with Hank's balanced salt solution (HBSS), and maintained in glucose-free DMEM in a hypoxic chamber inflated with 94 % $N_2$, 5 % $CO_2$, and 1 % $O_2$ at 37 °C for oxygen and glucose deprivation. For the reoxygenation process, the glucose-deprived medium was removed and complete DMEM was added and cultured under normoxic conditions for 24 h. Control cells were not stimulated with OGD/R. Re (Nanjing Spring & Autumn Biological Engineering, Nanjing, China) was added at concentration of 0, 5, 10, 20, 40, or 80 uM after the initiation of OGD and throughout the hypoxic period.

### Determination of cell viability

The cells, 3 × $10^3$ cells/well, were seeded into 96-well plates, and then MTT was added to each well for cell viability studies. The cells were maintained for another 4 h before their absorbance was measured spectrophotometrically (BioTek Instruments, Inc, Synergy H1, USA) at 490 nm.

### Evaluation of cell apoptosis

The cells were digested into single cells by the use of TE (Thermo, 25300-054, USA) and resuspended in a reaction buffer containing Annexin V and PI for 5 min in dark conditions. Cell apoptosis was analyzed with a flow cytometer (BD Biosciences).

### Immunoblot assay

Cells undergo cell lysis through RIPA (Beyotime, China) and protein concentration was measured by means of BCA assay (Beyotime, China). The proteins were separated by SDS-PAGE, followed by electro-transfer to PVDF membranes. Then the membranes were blocked with 5 % BSA in TBST. Subsequently, the membranes were incubated with primary antibodies of anti-p-ERK1/2 (1:1000, ab131438, Abcam), anti-ERK1/2 (1:1000, ab32537, Abcam), anti-p-c-Jun N-terminal kinase (JNK) 1 (1:500, ab215208, Abcam), anti-JNK1 (1:500, ab110724, Abcam) anti-p-p38 (1:1000, ab178867, Abcam) anti-p38 (1:1000, ab170099, Abcam), and beta-actin (1:3000, ab8226, Abcam). Subsequently, the membranes were cultured with secondary antibodies for 2 h, and were visualized with ECL kit.

### JC-1 staining

To determine mitochondria damage, cells were placed in 12-well plates and kept for 24 h for cell adhesion. The cells were incubated with 2 μM JC-1 (Beyotime, Beijing, China) for 15 min at 37 °C. They were rinsed in phosphate-buffered saline (PBS) for 3 times, and then analyzed by flow cytometry (BD FACSCanto™ II, BD, USA).

### Assessment of ROS

Cellular ROS level was determined using 2′-7′-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich). Then the cells were washed and analyzed in a microplate (BioTek Instruments, Inc, Synergy H1, USA).

### Statistical analysis

Statistical analysis was performed using GraphPad 7.0, and the data are shown as mean ± SD. Statistical comparison was performed

using Student's t-test, and $p < 0.05$ was considered statistically significant.

## RESULTS

### Remazolam increased OGD/R-induced neuronal survival

High concentrations of Re (40 and 80 uM) reduced cell proliferation in neurons (Figure 1 A). However, at lower concentrations, Re had moderate effect on the viability of the neurons ($p < 0.05$; Figure 1 A). Therefore, low concentrations of Re were used in subsequent experiments.

OGD/R induction decreased the viability of the neurons (Figure 1 B). However, Re treatment (5, 10, or 20 uM) significantly reversed the inhibition of neuron viability caused by OGD/R induction ($p < 0.05$; Figure 1 B). Thus, Re increased OGD/R-induced neuronal survival.

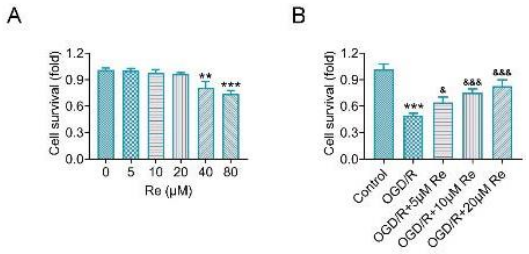


**Figure 1:** Remazolam enhances OGD/R-induced neuronal survival. (A) Cell proliferation of neurons in response to increasing dose of remazolam (0, 5, 10, 20, 40, or 80 uM). (B) Cell proliferation of neurons after various treatments. $^{**}P < 0.01$, $^{***}p < 0.001$, OGD/R *vs* control; $^{\&\&\&}p < 0.001$, OGD/R + remazolam *vs* OGD/R

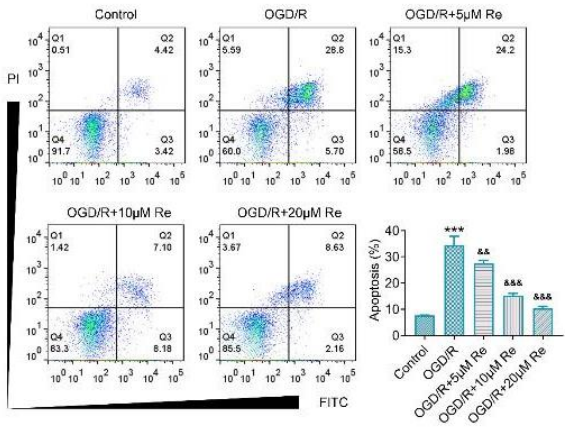


**Figure 2:** Remazolam inhibits OGD/R-induced neuronal apoptosis. $^{***}P < 0.001$, OGD/R vs control; $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$, OGD/R + remazolam vs OGD/R

### Remazolam inhibited OGD/R-induced neuronal apoptosis

OGD/R increased apoptosis neurons (Figure 2). However, treatment with Re (5, 10, or 20 uM) significantly reversed the promotion of neuron apoptosis caused by OGD/R induction ($p < 0.05$; Figure 2).

### Remazolam inhibited OGD/ R-induced neuron mitochondrial damage

OGD/R treatment decreased the level of JC-1 aggregates in the neurons, suggesting damage to the mitochondria (Figure 3 A, B). However, increased JC-1 aggregates were found in Re-treated cells, indicating a reduction in the membrane potential and the inhibition of mitochondrial damage ($p < 0.05$; Figure 3 A and B). OGD/R treatment increased the ROS levels of the neurons, but Re reversed the increase ($p < 0.05$; Figure 3 C).

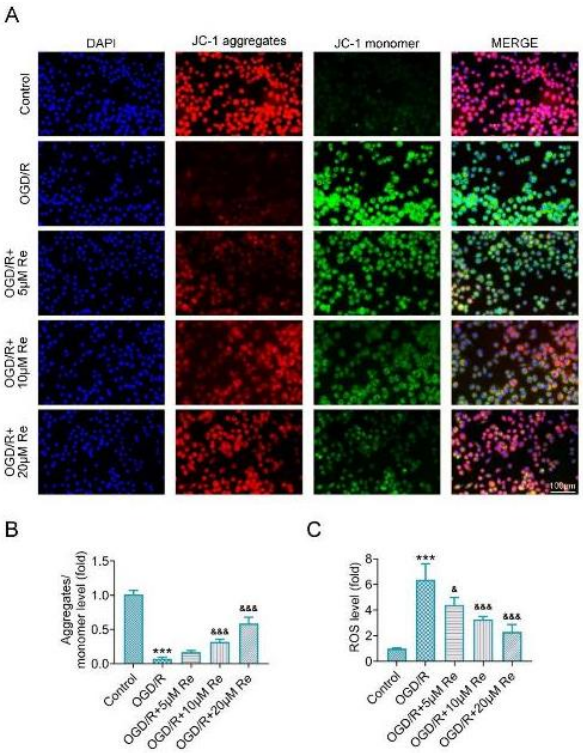


**Figure 3:** Remazolam inhibited OGD/R-induced neuron mitochondrial damage. (A) JC-1 staining in neurons. (B) Levels of JC-I aggregates and monomers. (C) ROS levels $^{***}P < 0.001$, OGD/R vs control; $^{\&}p < 0.05$, $^{\&\&\&}p < 0.001$, OGD/R + remazolam vs OGD/R

### Remazolam suppressed MAPK pathway in OGD/R-induced neurons

The levels of pERK1/2, p-JNK and p-p38 suggested the activation of the MAPK pathway in

neurons (Figure 4). The results further revealed that Re treatment reversed the increase of the phosphorylation levels of ERK1/2, JNK, and p38 in neurons caused by OGD/R treatment ($p$ < 0.05; Figure 4). Thus, Re suppressed MAPK pathway in OGD/R-induced neurons.

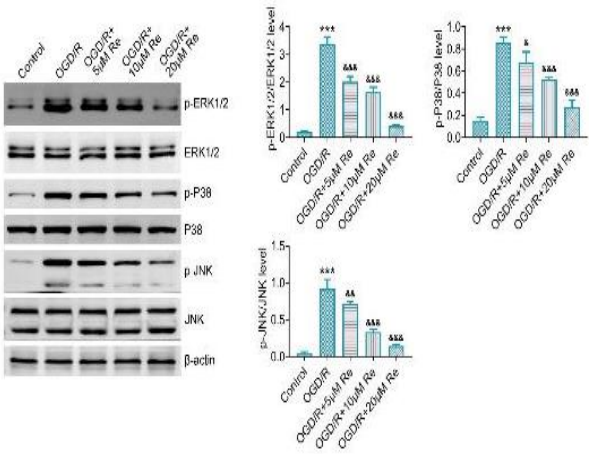


**Figure 4:** Remazolam restained the MAPK pathway in OGD/ R-induced neurons. Expression and phosphorylation levels of JNK, p38, and ERK1/2 in neurons. ***$P$ < 0.001, OGD/R vs control; $^{\&}p$ < 0.05, $^{\&\&}p$ < 0.01, $^{\&\&\&}p$ < 0.001, OGD/R + remazolam vs OGD/R

## DISCUSSION

In cerebral ischemia, recovery of blood supply often fails after a certain period, with the result that not only are brain functions not restored, but leads to a more severe brain dysfunction, called the cerebral ischemia-reperfusion injury [12]. Neuronal metabolic disorders and mitochondrial dysfunction are the main mechanisms of IRI [6]. A series of pathophysiological changes occurs in brain cell metabolism during cerebral ischemia-reperfusion. The mechanism of the injury is complex, and is mainly related to excessive production of oxygen free radicals, intracellular calcium overload, neurotoxic effect of excitatory amino acids and abnormal gene expression [13]. After cerebral ischemia-reperfusion, the mitochondrial damage of brain tissue is obvious, and mainly manifests as increased mitochondrial fragments and abnormal mitochondrial function [14]. Targeting mitochondria is an effective means to treat IRI. Interestingly, in the present study, remazolam (Re) inhibited MAPK pathway, increased the survival of OGD/R-induced neuronal cells, and inhibited apoptosis and mitochondrial damage. Therefore, Re is a promising therapeutic agent for IRI.

Remazolam mainly acts on the inhibitory neurotransmitter GABA receptor in the brain, enhances the activity of the GABA receptor containing the gamma sub-unit, and produces central inhibitory effects by opening the Cl-channel. It also increases Cl- inflow and membrane potential, enables nerve membrane hyperpolarization, inhibits neuronal activity, and alleviates neuronal excitability [15]. Furthermore, Re reduces body activity, causes sedation, amnesia, etc. Previous clinical studies have reported that the peak time for remazolam blood concentration is about 1 min, which is shorter than midazolam and similar to propofol, and the mean retention time is only 1/7 of midazolam [16]. In the present study, remazolam increased OGD/R-induced neuronal survival, inhibited OGD/R-induced neuronal cell apoptosis and mitigated mitochondrial damage in neuronal cells. Therefore, Re slowed the progression of IRI in OGD/R model.

Remazolam also inhibited the growth of glioma cells and the induced apoptosis in this work. It not only suppressed neuronal growth and apoptosis but also exhibited neuroprotective effects in that by inhibiting NLRP3 inflammatory-dependent cell pyrodeath, ameliorated neurological dysfunction, reduced infarct size, mitigated cortical neuron damage in the rat cerebral ischemia/reperfusion model. In animal models of OGD/R, it alleviated mitochondrial damage and afforded neuroprotection. In addition, Re delayed neurodegeneration and improved cognitive impairment in elderly mice by reducing tau phosphorylation.

Mitogen-activated protein kinases (MAPKs) are a type of serine/threonine protein kinases [17]. Recent studies have found that the MAPK signaling pathway is related to nerve injury [18]. MAPK pathway is rapidly phosphorylated and activated in the area of nerve injury in order to reduce the impact of nerve injury on the body. This pathway also promotes the apoptosis of nerve cells and aggravates the occurrence of nerve injury [18]. It enhances nerve cell survival increasing insulin expression in neurons [18]. In present study, Re inhibited IRI progression mainly by regulating MAPK pathway. Therefore, this pathway may be a potential target for IRI management.

## CONCLUSION

Remazolam increases OGD/R-induced neuronal cell survival, inhibits apoptosis and alleviates mitochondrial damage by inhibiting MAPK pathway. Therefore, Re has potentials to be developed as a therapeutic agent for the treatment of IRI.

## DECLARATIONS


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None provided.


### *Ethical approval*

None provided.

### *Availability of data and materials*

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

### *Conflict of Interest*

No conflict of interest associated with this work.

### *Contribution of Authors*

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Xianwen Wan, Haixia Gong, Hongxia Leng and Yulian Zhang designed the study and carried them out, supervised the data collection, analyzed and interpreted the data, prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript for publication.



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