Neuroprotective effect of penehyclidine hydrochloride on focal cerebral ischemia-reperfusion injury

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Abstract

Penehyclidine hydrochloride can promote microcirculation and reduce vascular permeability. However, the role of penehyclidine hydrochloride in cerebral ischemia-reperfusion injury remains unclear. In this study, in vivo middle cerebral artery occlusion models were established in experimental rats, and penehyclidine hydrochloride pretreatment was given via intravenous injection prior to model establishment. Tetrazolium chloride, TUNEL and immunohistochemical staining showed that, penehyclidine hydrochloride pretreatment markedly attenuated neuronal histopathological changes in the cortex, hippocampus and striatum, reduced infarction size, increased the expression level of Bcl-2, decreased the expression level of caspase-3, and inhibited neuronal apoptosis in rats with cerebral ischemia-reperfusion injury. Xanthine oxidase and thiobarbituric acid chromogenic results showed that penehyclidine hydrochloride upregulated the activity of superoxide dismutase and downregulated the concentration of malondialdehyde in the ischemic cerebral cortex and hippocampus, as well as reduced the concentration of extracellular excitatory amino acids in rats with cerebral ischemia-reperfusion injury. In addition, penehyclidine hydrochloride inhibited the expression level of the NR1 subunit in hippocampal nerve cells in vitro following oxygen-glucose deprivation, as detected by PCR. Experimental findings indicate that penehyclidine hydrochloride attenuates neuronal apoptosis and oxidative stress injury after focal cerebral ischemia-reperfusion, thus exerting a neuroprotective effect.

Key Words

neural regeneration; brain injury; penehyclidine hydrochloride; cerebral ischemia-reperfusion injury; ischemic cerebrovascular disease; apoptosis; excitatory amino acid; oxygen free radicals; superoxide dismutase; NMDA receptor; middle cerebral artery occlusion; oxygen-glucose deprivation; photographs-containing paper; neuroregeneration

Research Highlights

(1) Penehyclidine hydrochloride has been used as a novel selective anticholine drug for the clinical treatment of organophosphate poisoning. However, a comprehensive evaluation of its role in cerebral ischemia-reperfusion injury through animal experiments at the gross and molecular level has been absent until now.

(2) In this study, in vitro experiments were designed to explore the effect of penehyclidine hydrochloride on NMDA receptors in hippocampal neurons of rats after oxygen-glucose deprivation, and to observe the mechanism of action underlying this drug on excitatory amino acid receptors.

(3) Our results found that penehyclidine hydrochloride could attenuate neuronal apoptosis and oxidative stress damage following focal cerebral ischemia-reperfusion.
INTRODUCTION

Stroke is one of the most common fatal diseases all over the world, and ischemic stroke accounts for more than 80% of cases. The treatment for ischemic injury is to restore blood flow as soon as possible. However, recent studies show that reperfusion may aggravate ischemic injury and induce death of brain cells, a process termed cerebral ischemia-reperfusion injury. Cerebral ischemia-reperfusion injury may further deteriorate cerebral function and reduce the therapeutic effect of drugs. Therefore, treatments for cerebral ischemia-reperfusion injury have attracted increasing attention and become a major focus of cerebral ischemia research. Excitatory amino acid toxicity, oxidative stress, intracellular calcium overload, as well as inflammation and apoptosis, are involved in the pathological process after cerebral ischemia-reperfusion injury\[1\].

Although a large number of pre-clinical and clinical studies have been performed, the majority of drugs have failed in clinic trials at different stages\[2\]. Because previous stroke trials have demonstrated that drugs that target one or several signal transduction pathways cannot improve clinical outcomes after stroke, multi-pathway drugs have been suggested to overcome this challenge\[3\]. In recent years, many narcotics have been used clinically to reduce damage caused by ischemia-reperfusion\[4-6\], such as scopolamine, which is developed from traditional Chinese medicinal plants, and plays a major role in blocking the muscarinic acetylcholine receptor. Moreover, scopolamine has a variety of biological activities, including antioxidant and cytoprotective actions.

Penehyclidine hydrochloride or 3-(2’-phenyl-2’-cyclopentyl-2’-hydroxyl-ethoxy), is a new anti-cholinergic drug derived from scopolamine, having both anti-muscarinic and anti-nicotinic activities, and retains effective central and peripheral anticholinergic activities. Clinical data showed that penehyclidine hydrochloride is effective in treatment for soman poisoning and pulmonary dysfunction of chronic obstructive pulmonary disease\[7-8\]. More importantly, penehyclidine hydrochloride can improve microcirculation, depress microvascular permeability, attenuate the release of lysome and inhibit lipid peroxidation, and it is also reported to have protective effects against organ injury\[9-11\], and is used widely in the clinic as an antagonist of organic phosphorus poisoning at present in China\[12-13\]. However, the role and mechanism associated with penehyclidine hydrochloride in cerebral ischemia-reperfusion injury is not clear.

This study was performed to explore the protective effects and the possible mechanisms of penehyclidine hydrochloride on neuronal injury in rats following focal cerebral ischemia-reperfusion, in a broader attempt to guide further studies on ischemic prevention and treatment.

RESULTS

Quantitative analysis of experimental animals

A total of 24 Sprague-Dawley male rats were divided randomly into four groups with 6 rats in each: sham-surgery group (only the right middle cerebral artery was isolated but not occluded), model group (middle cerebral artery occlusion + intravenous injection of normal saline 20 minutes before surgery), penehyclidine hydrochloride group (middle cerebral artery occlusion + intravenous injection of penehyclidine hydrochloride 20 minutes before surgery), and Scopolamine group (Sco; middle cerebral artery occlusion + intravenous injection of scopolamine 20 minutes before surgery), with 24-hours reperfusion after middle cerebral artery occlusion for 2 hours. All rats were killed and the cerebral cortex, hippocampus and striatum were isolated for the following experiments. All 24 rats were included in the final analysis without any loss.

Effect of penehyclidine hydrochloride on infraction volume and morphological changes in neuronal cells induced by cerebral ischemia-reperfusion injury

Brain tissues were sliced at a section thickness of 2 mm and were incubated in 2% (v/v) of 2,3,5-triphenyl-tetrazolium chloride solution. Intact mitochondria were stained with 2,3,5-triphenyl-tetrazolium chloride, the dark red area represented the living tissue, whereas infarction areas that in the parietotemporal cortex, hippocampus and striatum remained unstained (gray)\[14\]. As shown in Figure 1A, the volume of the gray region (infarcted tissue) was significantly reduced in the penehyclidine hydrochloride group as compared with the other injury group. As shown in Figure 1B, the model group showed obvious vacuoles, ampliative gaps between neurons, and more dead neurons, while the penehyclidine hydrochloride group showed an greater number of surviving neurons and less cell plasma condensation, as well as less dead neurons, as revealed by hematoxylin-eosin staining (Figure 1).
Penehyclidine hydrochloride inhibited neuronal apoptosis in rats with cerebral ischemia-reperfusion injury

The number of positive cells was evaluated by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) and immunohistochemistry. Compared with the model group, the number of TUNEL-positive cells was reduced in the penehyclidine hydrochloride group ($P < 0.001$), which indicated that penehyclidine hydrochloride could protect nerve cells against focal cerebral ischemia-reperfusion injury, and the effect was associated with its anti-apoptotic action (Figure 2A). Immunohistochemical staining results showed that Bcl-2 expression in the cytoplasm was higher in the PHC group than that in the model group ($P < 0.001$), while that of caspase-3 were decreased ($P < 0.001$). This result is evidence that penehyclidine hydrochloride can prevent apoptosis through the regulation of apoptosis-related genes, which is one of the possible neuroprotective mechanisms underlying penehyclidine hydrochloride following focal cerebral ischemia-reperfusion.

Effect of penehyclidine hydrochloride on the expression of superoxide dismutase, malondialdehyde and excitatory amino acids in rats with cerebral ischemia-reperfusion injury

The change of oxygen radicals and excitatory amino acids in brain tissue was determined to investigate whether penehyclidine hydrochloride could play a protective role in neurons by influencing the content of oxygen free radicals and excitatory amino acids. Compared with the model group, penehyclidine hydrochloride was shown to upregulate the sensitivity of superoxide dismutase and downregulated the concentration of malondialdehyde in ischemia cerebral tissue. In addition, glutamic acid, aspartate, glycine and gamma-aminobutyric acid in brain tissue decreased in the penehyclidine hydrochloride group when compared with the model group ($P < 0.05$; Figure 3).

Effects of penehyclidine hydrochloride on the expression of NR1 mRNA in cultured hippocampal cells

By measuring the expression of NMDA receptor NR1 subunit mRNA in hippocampal neurons after oxygen-glucose deprivation and reperfusion, we explored the change of index and the role of penehyclidine hydrochloride at different time points in a broader attempt.
to provide experimental evidence for the development of hydrochloric acid penehyclidine.

**DISCUSSION**

At present, clinical measures to protect neurons from ischemic damage mainly focuses on the following aspects, such as addressing the energy depletion, acidosis, excessive oxygen free radical production, increase in intracellular calcium, enhanced excitatory amino acids and inhibitory amino acids, changes in cytokines, rising levels of inflammatory cytokines and upregulation of apoptosis-related genes. However, the mechanism of damage is unclear and requires further study.

Penehyclidine hydrochloride is a new anti-cholinergic drug derived from hyoscyamine, which has no or poor M2 receptor-associated cardiovascular side effects owing to selectively blocking M1 M3 receptors and N receptors. Moreover, penehyclidine hydrochloride has been reported to have a good protective effect against both soman-induced seizures, especially over the non-cholinergic phase, and against pentylenetetrazo- induced convulsions.
and NMDA-induced mortality in mice. It has been well-established that increased acetylcholinesterase expression or activity is detected in apoptotic cells after apoptotic stimuli in vitro and in vivo. In addition, elevated acetylcholinesterase activity was reported after middle cerebral arterial occlusion. In this study, penehyclidine hydrochloride was able to protect against apoptosis, and we propose that penehyclidine hydrochloride may have anti-apoptotic properties similar to acetylcholinesterase inhibitors in the treatment of middle cerebral arterial occlusion injury.

Cell apoptosis, also known as programmed cell death, has obvious morphological and biochemical characteristics. Programmed cell death is carried out in a regulated process and leads to characteristic cell changes and death. These changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. Programmed cell death has fundamental functions during both plant and multicellular animal tissue development. Inhibiting apoptosis has been confirmed to be the most effective way to delayed neuronal death after cerebral ischemia reperfusion. Neuronal apoptosis has been divided into caspase-dependent and caspase-independent processed and mitochondria play an important role in both pathways. After cerebral ischemia-reperfusion, a series of transformations may increase the permeability of the inner mitochondrial membrane, resulting in failure of energy metabolism, calcium imbalance, mitochondrial transmembrane potential change and the generation of oxygen free radicals. Apoptosis initiation factors are released from the mitochondrion, which triggers the cell death cascade. Despite the increased apoptosis following middle cerebral arterial occlusion, the importance and regulatory mechanisms of apoptosis have not been fully elucidated.

Of the apoptosis-related gene products, the caspase family plays a significant role in the incidence of apoptosis, which mediate apoptosis involved in two important pathways: the extrinsic pathway and the intrinsic pathway. Caspase-3, a terminal effector protein, plays a crucial role in the occurrence of apoptosis mediated by the above two pathways, and causes a subsequent cascade that ultimately leads to apoptosis. The role of Bcl-2 in the progress of apoptosis has been evaluated in several in vivo and in vitro systems. The Bcl-2 oncogene inhibits apoptosis by affecting several cell pathways. One possibility is that Bcl-2 plays a role in desensitizing thymocytes to apoptosis. In contrast, activation of caspase-3 results in apoptosis via chromosome condensation, cell membrane blebbing and DNA fragmentation. Caspase-3 can be activated by cell death signals via three ways: mitochondrial (related to bax and Bcl-2), death receptors (FAS, FAS-L), or the endoplasmic reticulum. Furthermore, caspase-3 can cleave Bcl-2 protein in vivo, which promotes the release of cytochrome C and activation of caspase-3 to strengthen the apoptotic effect as a positive feedback loop.

The excitatory amino acid theory has received much attention in recent years. Excitatory amino acids, such as glutamate, are important and ubiquitous excitatory neurotransmitters in the mammalian central nervous system and are involved in the mediation of physiological functions such as learning, memory, synaptic plasticity and cardiovascular regulation. Moreover, excitatory amino acid receptors are known to mediate synaptic excitation throughout the central nervous system. The role of glutamatergic neurotransmission in the central regulation of cardiovascular function is emerging. Excessive accumulation of excitatory amino acids can trigger cerebral ischemic injury and play an important role in the pathophysiology of cerebral ischemia. In addition, excitatory amino acids may activate intracellular signal transduction pathways and trigger inflammatory gene expression second to the ischemia. Therefore, regulating excitatory amino acid levels is the initial goal for the treatment of cerebral ischemia. Recent studies indicate that cerebral ischemia-reperfusion injury of brain cells can trigger damage involving at least four different mechanisms: energy failure, excitotoxicity, peri-infarct depolarization, and inflammation and programmed cell death. This ischemic injury cascade may be initiated by energy barriers and excitatory amino acids. In this study, penehyclidine hydrochloride reduced the concentration of extracellular excitatory amino acids during cerebral ischemia-reperfusion injury in brain cells.

The endogenous excitatory amino acids mediate their excitatory actions via multiple synaptic transmitter receptors. According to their selectivity for different agonists, amino acid receptors can generally be divided into N-methyl-D-aspartate type, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, and other metabolic receptors. NMDA receptors are the most important and the most studied receptor. The elevated concentration of glutamate stimulates the over-activation of NMDA receptors, and a transient increase in Ca2+ is produced due to influx of calcium from the extracellular environment. Excessive intracellular Ca2+ can activate phospholipase A, and biofilm phospholipid degradation can generate arachidonic acid, prostaglandins, thromboxanes and leukotrienes, resulting in...
in an inflammatory reaction and the generation of oxygen free radicals causing cell damage. In short, abnormally high levels of excitatory amino acids can play a key role in the pathological process of cerebral ischemic neuronal damage. Therefore, inhibition of Glu excitotoxicity in ischemia-reperfusion injury has a defensive role in the prevention and treatment of cerebrovascular disease.

Receptor regulation is one of the most important mechanisms to stabilize the internal environment. Excitatory amino acids must activate excitatory amino acid receptors on postsynaptic membranes. NMDA receptors are ligand-gated Ca$^{2+}$ channels and are highly permeable to Ca$^{2+}$, which plays an essential role in excitation-depression. Thus, the NMDA receptor family is attracting much attention in cerebral ischemia-reperfusion injury. NMDA receptors, the major ionotropic glutamate receptors, are highly expressed in the central nervous system and play a key role in excitatory synaptic transmission. In addition, they are involved in many physiological and pathological processes. NMDA receptors are composed of NR1, NR2A, NR2B, NR2C, or NR2D subunits, and the different subunits combine with different NMDA receptor subtypes with distinct functional features. Overactivation of NMDA receptors can lead to excitotoxicity, which is associated with the pathogenesis of neurodegenerative diseases of the central nervous system. The NR1 subunit of the NMDA receptor is the basic functional unit and is closely related to cerebrovascular disease. In this study, hippocampal cells expressing NMDA receptors were selected to study the variation of NMDA receptors after oxygen-glucose deprivation and reperfusion. Reverse transcription-PCR was used to detect the mRNA expression levels of the NR1 subunit. Compared with the control group, NR1 subunit mRNA expression was significantly increased in the experiment group.

Penehyclidine hydrochloride was able to reduce the expression of NR1 after reperfusion for 6–24 hours, which confirmed that penehyclidine hydrochloride plays a role in the activity of the NMDA receptor.

Oxygen is an essential for the maintenance of metabolism and survival. The various causes of hypoxic injury clinically require further investigation. The high demand for oxygen and low tolerance to hypoxia of brain tissue is the root cause of cerebral hypoxic-ischemic injury. A large number of experiments have shown that oxygen free radicals are involved in the pathological process of neuron death after ischemia-reperfusion injury. In hypoxic-ischemic encephalopathy, lipid peroxidation induced by oxygen free radicals is an extremely important factor in pathogenesis and damage. Ischemia-generated free radicals are considered to be an important basis of ischemic damage resulting in cell membrane structure damage and lipid peroxidation. Excessive reactive oxygen species can cause damage to macromolecules in cells, including lipids, proteins, and nucleic acids, culminating in neuronal dysfunction and depression. There is an intrinsic antioxidant defense system in cells for scavenging reactive oxygen species to prevent cellular damage. Superoxide dismutase, one of the most important antioxidant enzymes, is the endogenous oxygen free radical scavenger that protects cells from oxidative damage, and its activity may reflect the ability to eliminate free radicals in cells. The use of exogenous superoxide dismutase has been evaluated against various reactive oxygen species-mediated brain injuries, especially those associated with ischemia/reperfusion. In animals with superoxide dismutase overexpression, the infarct volume of cerebral ischemia-reperfusion injury was significantly reduced.

Malondialdehyde, reporting the degree of cell injury, is one of the stable metabolites generated from the lipid peroxidation reaction of oxygen free radicals and biofilm polyunsaturated fatty acids. Malondialdehyde levels are proportional to lipid peroxidation and oxidative stress. Tissue malondialdehyde levels may indicate the content of oxygen free radicals. Twenty-four hour reperfusion reduced the activity of superoxide dismutase and increased the content of malondialdehyde, which confirmed the presence of free radicals following cerebral ischemia-reperfusion injury. Results in this study showed that penehyclidine hydrochloride could upregulate the sensitivity of superoxide dismutase and downregulate the content of malondialdehyde in ischemic cerebral tissue. These data indicated that penehyclidine hydrochloride reduced the toxicity of free radicals and played an antioxidative role, resulting in the protection of nerve cells during the process of ischemia-reperfusion injury.

In summary, penehyclidine hydrochloride not only altered the apoptotic gene expression of Bcl-2 and caspase-3 to suppress nerve cell apoptosis, it also reduced free radical toxicity by regulating superoxide dismutase and the concentration of malondialdehyde. In addition, penehyclidine hydrochloride protected hippocampal neurons from injury by inhibiting excitotoxicity and NMDA receptor overexpression following focal cerebral ischemia-reperfusion injury. The present study demonstrated that penehyclidine hydrochloride provides a protective effect against cerebral ischemia-reperfusion injury and may be a promising therapeutic drug.

**MATERIALS AND METHODS**
Design
A randomized, controlled animal experiment.

Time and setting
The experiments were completed at the First Affiliated Hospital of China Medical University and Yantai Yuhuangding Hospital Central Laboratory from October 2009 to January 2012.

Materials
A total of 24 normal male Sprague-Dawley rats, weighing 250–300 g, aged 10 weeks, and of specific free grade, were provided by the Experimental Animal Center of China Medical University, China (license No. SCXK (Liao) 2008-0005). All animal experiments were performed in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China.

Penehyclidine hydrochloride (C₂₀H₂₉NO₂•HCl) was provided by Lisite Pharmaceutical Co., Ltd., Chendu City, Sichuan Province, China (batch No. 060502-1). The molecular formula is as follows:

\[
\text{H} \cdot \text{HCl}
\]

Penehyclidine hydrochloride is a new anticholinergic drug, which can cross the blood-brain barrier and has both anti-muscarinic (M receptor) and anti-nicotinic (N receptor) activities, and retains potent central and peripheral anticholinergic activities. Penehyclidine hydrochloride has been used widely clinically as an antagonist of organic phosphorus and soman poisoning in China. Penehyclidine hydrochloride has little or no effect on M2 receptor subtypes, which has no significant effect on heart rate.

Methods

Establishment of middle cerebral arterial occlusion models in vivo
Middle cerebral arterial occlusion was induced by extracranial vascular occlusion of the in Sprague-Dawley rats (body weight of 250–300 g), according to previously reported procedures with slight modification. Sprague-Dawley rats were anesthetized using 10% (v/v) chloral hydrate and 4% (w/v) formaldehyde (200 mL), and then decapitated at a given time. Brain samples, including the cortex, hippocampus and striatum, were post-fixed in 4% (w/v) formaldehyde for 2 hours, dehydrated in alcohol, hyalinized by dimethylbenzene, embedded in paraffin, sectioned at a thickness of 4 μm, adhered to superfrost plus slides, and finally stored at room temperature. A standard TUNEL method was employed to detect the fragmented nuclear DNA associated with apoptosis on paraffin sections. After standard deparaffinization, hydration, incubation with proteinase K, and

Determination of infarction volume in the rat brain
Brain tissues were stored at –70°C for 8 minutes and sliced at a thickness of 2 mm. Slices were incubated in 2% (v/v) 2,3,5-triphenyl-tetrazolium chloride solution for 30 minutes at 37 °C. Samples were then washed in distilled water for 30 seconds and photographed. OSIRIS soft 4.19 (http://www.seekbio.com/soft/176.html) was used to measure the infarct area in each sample, and the percentage of the hemispheric infarction volume was calculated. To minimize errors in the estimation of infarction volume, three measurements were needed on each slice for calculation of the volume of infarction. The infarct volume (V) was calculated using the following equation:

\[ V = t(A_1 + A_2 + \ldots + A_n) \]

where t is the thickness of the brain tissues (mm), A is the area of the infracted hemisphere slice (mm²), and n is the number of the brain tissues.

Analysis of neuronal apoptosis by TUNEL
To detect cell apoptosis, TUNEL staining was performed according to the protocol of the TUNEL Detection System (In Site Cell Apoptosis Detection Kit I, POD, MK1020; BOSTER, Wuhan, Hubei Province, China). Three rats in each group were deeply anesthetized using 10% (v/v) chloral hydrate (300 mg/kg), transcardially perfused with sodium chloride and 4% (w/v) formaldehyde (200 mL), and then decapitated at a given time. Brain samples, including the cortex, hippocampus and striatum, were post-fixed in 4% (w/v) formaldehyde for 2 hours, dehydrated in alcohol, hydrolyzed by dimethylbenzene, embedded in paraffin, sectioned at a thickness of 4 μm, adhered to superfrost plus slides, and finally stored at room temperature. A standard TUNEL method was employed to detect the fragmented nuclear DNA associated with apoptosis on paraffin sections. After standard deparaffinization, hydration, incubation with proteinase K, and
blocking of endogenous peroxidase, tissue sections were incubated with terminal deoxynucleotidyl transferase and digoxigenin-dUTP (TUNEL reaction mixture) at 37°C for 60 minutes and then with peroxidase converter antibody at 37°C for 30 minutes. For negative controls, some slides were incubated with label solution that did not contain terminal deoxynucleotidyl transferase. Under the optical microscope (Olympus, Tokyo, Japan), the nucleus of apoptotic cells appeared yellow. Positive cells were counted and the mean number in five random views was recorded.

**Immunohistochemical staining for Bcl-2 and caspase-3 in nerve cells**

Formalin-fixed and paraffin-embedded sections (5 mm thickness) were first dewaxed in xylene and rehydrated with a graded ethanol series. Endogenous peroxidase was inactivated by incubation in 3% (v/v) H₂O₂ for 10 minutes at room temperature. Sections were placed in citrate buffer (0.01 M, pH 6.0) for antigen retrieval by microwave heating. Nonspecific binding was blocked by incubation in goat serum (1:10 dilution) for 30 minutes at room temperature, and then sections were incubated at 4°C overnight with rabbit anti-Bcl-2 and -caspase-3 polyclonal antibodies (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After three washes in phosphate buffered saline for 15 minutes, the slides were incubated with the appropriate secondary antibody at room temperature for 30 minutes. Slides were incubated in horseradish peroxidase-labeled anti-rabbit IgG (1:200; DAKO, Copenhagen, Denmark) at room temperature for 20 minutes and developed with 3,3′-diaminobenzidine (Sigma, St Louis, USA). The sections were finally counter stained with hematoxylin. For negative-control purposes, the same procedure was used on tissue sections in which 1% (v/v) bovine serum albumin in PBS was substituted for the primary antibody. Under the optical microscope (Olympus, Tokyo, Japan), brown particles or patches in the cytoplasm indicated positive staining. Ten fields per section were selected in the cortex and striatum. The numbers of positive cells in the grid were counted and a mean value was recorded.

**Detection of excitatory amino acid concentrations in the rat brain**

Rats were sacrificed by an overdose of chloral hydrate after 24 hours of reperfusion. Both sides of the cerebral cortex and hippocampus were separately weighed and stored at −70°C. The samples were homogenized in 0.9% (w/v) saline solution using a homogenizer. The homogenate was then centrifuged at 100 × g for 10 minutes at 4°C. The supernatant obtained was used for assays of malondialdehyde content and superoxide dismutase activity. Malondialdehyde content was determined by the thiobarbituric acid method, whereas superoxide dismutase activity was evaluated according to the kit instructions (Jiancheng Institute of Biological Products, Nanjing, Jiangsu Province, China).

**Neurons in primary culture and establishment of oxygen-glucose deprivation and reperfusion models in vitro**

Primary cultures of the hippocampus were prepared from 1-day-old Wistar rats based on previously reported articles. Briefly, the skin and skull were removed and brain tissue was exposed completely. The bilateral hippocampus were bluntly separated and repeatedly washed with D-Hank’s buffer with 10 μg/mL gentamycin to remove blood vessels and meninges. The dissected pieces were digested in trypsin (0.125% (w/v)) for 20–25 minutes at 37°C, neutralized with trypsin inhibitor, and washed three times with D-Hank’s buffer. Dissociated cell suspensions of 10⁶/L density were transferred into Dulbecco’s Modified Eagle Medium buffer with 15% (v/v) serum at 37°C in a humidified atmosphere of 5% (v/v) CO₂ in air. Fresh medium was replaced after overnight incubation to remove dead cells and cytarabine was added into the medium on day 3. Half medium changes were performed after overnight incubation, and medium was replaced twice per week there after. After 8–12 days, oxygen-glucose deprivation and reperfusion was performed. Neuronal cells was incubated with 100 μL Hank’s buffer without glucose at 37°C in 24-well culture plates for 2.5 hours and maintained in a 95% (v/v) N₂ and 5% (v/v) CO₂ incubator. After Oxygen levels in the medium of cultured
cells was recovered and the cells were randomly divided into six groups with treatment of different penehyclidine hydrochloride concentrations 20 minutes before oxygen-glucose deprivation/reperfusion: control group with normal Hank’s buffer, OGD/R model group, high dose penehyclidine hydrochloride group (1.6 μM), middle dose penehyclidine hydrochloride group (0.4 μM), low dose penehyclidine hydrochloride group (0.1 μM) and MK-801-positive group (0.1 μM), and incubated for 1, 6, 12, 24 hours of after reoxygenation.

**NMDA-NR mRNA expression in primary cultured hippocampal neurons by reverse transcription-PCR**

Total RNA was directly extracted from primary cultured cells using Trizol reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). The absorbance at 260 and 280 nm was measured regularly. The quality of total RNA was examined by running it on a 1% (w/v) agarose/denaturing formaldehyde gel. Samples with a ratio of absorbance at 260 and 280 nm of 1.8–2.0 and a 28s/18s of approximately 2 were qualified for the next round. Three micrograms of total RNA was reverse-transcribed by M-MuLV reverse transcriptase (MBI, Vilnius, Lithuania) with oligo(dT)16 as a primer. Using equal amounts of cDNA, PCR amplification was performed with 20 nM of each primer, 25 mM Mg2+ and 1 U Taq polymerase with the following NMDA-NR primers (20 mM each) and glyceraldehyde-3-phosphate dehydrogenase primers (20 mM each; Table 1). All experiments were performed in triplicate. The mRNA level of NMDA-NR or GAPDH was assessed by measuring the density of the PCR generated amplicon on the gel by densitometric analysis. The final expression level was the average value of the three amplicons. Relative NMDA-NR expression is given as NMDA-NR/ GAPDH absorbance ratio.

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<th>Table 1 Sequence of NMDA-NR1 and GAPDH primers</th>
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<tr>
<td><strong>Gene</strong></td>
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<td>NMDA-NR</td>
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**Statistical analysis**

Statistical analysis was performed using SPSS 13.0 software (SPSS, Chicago, IL, USA). Data are expressed as the mean ± SD. Statistical comparison was performed by one-way analysis of variance followed by Fisher least significant difference test. Group differences resulting in values of less than 0.05 were considered to be statistically significant.

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**Ethical approval:** The experimental procedures were approved by the Animal Use and Care Advisory Committee of Experimental Center of China Medical University, and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). Rats in our experiments were provided by the Experimental Animal Center of China Medical University.

**Author statements:** The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application disputations.

**REFERENCES**


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