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Neuroprotective effect of diazoxide on brain injury induced by cerebral ischemia/reperfusion during deep hypothermia

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Abstract

Object: The purpose of this study was to determine the effects of diazoxide on apoptosis and the relative mechanisms in a model of brain injury induced by cerebral ischemia/reperfusion (I/R) during deep hypothermia.
Methods: Three-week-old Sprague–Dawley male rats were randomly and equitably divided into sham-operated group, placebo-treated group and diazoxide-treated group respectively. Specific examination of the regional cerebral blood flow (rCBF) was measured in the three groups continuously during the operation by laser Doppler flowmetry. Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) was showed DNA fragmentation. The mRNA expressions of cytochrome *c* and full-length caspase-3 were determined by RT-PCR, while the protein expressions of cytochrome *c* and cleaved caspase-3 were determined by immunohistochemistry at 1 h, 6 h, 24 h, 72 h and 7 days after I/R, respectively. Cytosolic release of cytochrome *c* at 24 h after I/R was also confirmed by Western blot.
Results: rCBF was significantly decreased in both of placebo-treated and diazoxide-treated group just after ischemia in the time interval 0–5 min, and had no obvious changes in all the time intervals during the operation. Diazoxide preconditioning significantly decreased the percentage of TUNEL-positive staining cells. The mRNA expressions of cytochrome *c* and full-length caspase-3 in diazoxide-treated group were significantly decreased. In addition, diazoxide provided a significant reduction in the protein expressions of cytochrome *c* and cleaved caspase-3.
Conclusion: These results suggested that the neuroprotective effects of diazoxide against cerebral I/R injury during deep hypothermia correlated with the reduction of DNA fragmentation, prevention of mitochondrial cytochrome *c* release and inhibition of caspase-3 activation.

Keywords: Diazoxide; Apoptosis; Cerebral ischemia; Reperfusion; Cytochrome *c*; Caspase-3; Deep hypothermic low flow

1. Introduction

Deep hypothermic low flow (DHLF) and deep hypothermic circulatory arrest (DHCA) cardiopulmonary bypass are often used during repairs of complex congenital cardiac defects. Compared with DHCA, DHLF can improve more oxygen metabolism of brain, but in fact, it also experiences a process of cerebral ischemia/reperfusion (I/R) injury. Although deep hypothermia could attenuate the brain injury in I/R process, many patients undergoing DHLF still have neurodevelopmental abnormalities that are manifested in days or even years after recovery from the surgical procedure. These abnormalities range from subtle subclinical radiographic findings to overt cognitive and functional impairment, including seizures, choreoathetosis, mental retardation and so on [1–3].

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Apoptosis contributes to I/R injury in the brain. Recently, diazoxide, a specific opener of mitochondrial ATP-sensitive potassium (mitoKATP) channels, had been proposed as the site of neuroprotective action. Several reports have shown that diazoxide protects the brain against ischemic injury. For instance, pretreatment with the agent could reduce infarct volume after middle cerebral artery occlusion in rats and mice [4,5]. Further in vivo experiments have provided evidence that diazoxide preserves hypercapnia-induced arteriolar vasodilation after global cerebral ischemia in piglets [6]. The mechanism behind these neuroprotective properties appears to be a selective opening of mitoKATP because diazoxide effect can be abolished by the mitoKATP blocker 5-hydroxydecanoate (5-HD) [7–9]. Recent evidences suggest that diazoxide can inhibit apoptosis by preserving mitochondrial membrane potential and stabilizing mitochondrial integrity in cardiomyocytes and neurons [10–12]. However, the precise mechanisms of this protection are not well established. In the present study, we explored the potential molecular mechanisms for diazoxide to reduce brain injury by using a deep hypothermic low cerebral blood flow model. We investigated the effect of diazoxide on the changes of cytochrome *c*, caspase-3 and subsequent DNA fragmentation after cerebral I/R during deep hypothermia in rats.

2. Materials and methods

2.1. Animal model preparation

We used male, three-week-old Sprague–Dawley rats (weight = 52 ± 3 g), which were obtained from the Experimental Animals Center of Nanjing Medical University. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University. All surgical procedures were carried out under sterile conditions. Foods were withdrawn from all rats 12 h before surgery. The environmental temperature was controlled at 18–20 °C by air conditioning.

The rats were anesthetized by intraperitoneal injection of ketamine chlorhydrate (100 mg/kg). The initial dose was rarely sufficient. An additional supplement was often given shortly before intubation. Electrocardiogram monitor, bronchial intubation and mechanical ventilation were performed. Respiratory rate: 65 times/min, airway pressure peak value: 9.0 cmH_2O. The femoral artery was cannulated to record blood pressure. The rectal temperature was monitored during the surgical procedure by a rectal probe of thermometer. Through a midline cervical incision, the bilateral common carotid arteries (CCAs) were carefully exposed and isolated from the surrounding connective tissues and nerve fibers. Then, the rectal temperature of rats was reduced to 21 ± 0.5 °C slowly within about half an hour by ice bag and keep constant (The animal would be put on the ice bag again to control the temperature change if the temperature would increase.). At this time the rats were submitted to the simultaneous bilateral clamping of CCAs for 120 min using bulldog clamps and then reopened. For our experience, we usually add a supplemented ketamine with a half-dose at about 1 h after ischemia. The rats then were placed in the neonatal radiant warmer to rewarm the rectal temperature slowly to 34 ± 0.5 °C. Rectal temperature, main artery blood pressure, heart rate, arterial blood gas and saturation of arterial blood oxygen were continuously monitored during the surgical procedure. Sham-operated group were received the same operation but without occlusion of the CCAs. The animals were then sacrificed at 1 h, 6 h, 24 h, 72 h and 7 days (d) after reperfusion.

2.2. Experimental protocol

Three-week-old Sprague–Dawley male rats were randomly and equitably divided into sham-operated group, placebo-treated group and diazoxide-treated group, respectively. Each group was redistributed into five subgroups: 1 h, 6 h, 24 h, 72 h and 7 d after reperfusion. In the sham-operated group and placebo-treated group, the rats were only received 0.25 ml dimethyl sulphoxide (DMSO) (Sigma, USA), and in the diazoxide-treated group, the rats were treated with diazoxide (Sigma, USA), which was dissolved in 0.25 ml DMSO. Diazoxide (5 mg/kg) or the same volume of DMSO was administered intraperitoneally 30 min before occlusion of the CCAs.

2.3. Regional cerebral blood flow (rCBF) was measured by laser Doppler flowmetry (LDF)

To assess our deep hypothermic cerebral I/R model, rCBF was measured by LDF as previously described [13,14]. Briefly, the rats (n = 6 for each group) were anesthetized with ketamine chlorhydrate (100 mg/Kg). A 1-mm diameter burr hole was drilled 1 mm posterior to bregma and 2 mm from the

Table 1
Hemodynamic parameters of rat model

	Reducing the temperature [(25 ± 2.7)min]			Ischemia (120 min)			Rewarming after ischemia [(31 ± 3.6)min]		
Re.T (°C)	34	28	24	21	21	21	24	28	34
HR (/min)	235 ± 11.4	169 ± 9.6	125 ± 14.4	76 ± 12.3	74 ± 8.7	73 ± 10.5	110 ± 10.1	164 ± 7.8	228 ± 15.2
MAP (mmHg)	87 ± 6.7	73 ± 7.5	68 ± 5.1	55 ± 6.4	47 ± 4.8	50 ± 5.4	65 ± 8.3	72 ± 5.8	89 ± 4.5
SaO_2 (%)	97.7 ± 1.2	98.0 ± 0.9	98.7 ± 0.5	95.7 ± 1.9	95.3 ± 1.6	94.2 ± 1.5	97.5 ± 1.5	98.7 ± 0.5	98.3 ± 0.8
pH	7.41 ± 0.05	7.35 ± 0.03	7.25 ± 0.08	7.27 ± 0.08	7.24 ± 0.11	7.29 ± 0.06	7.38 ± 0.03	7.35 ± 0.05	7.39 ± 0.04
HCO_3^- (mmol/L)	23.4 ± 2.4	24.1 ± 1.6	21.8 ± 1.4	20.7 ± 2.1	19.6 ± 1.6	21.3 ± 3.3	22.5 ± 0.9	23.8 ± 2.5	23.4 ± 1.7

Re.T: rectal temperature; MAP: main artery blood pressure; SaO_2: saturation of arterial blood oxygen.