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Bicyclol attenuates oxidative stress and neuronal damage following transient forebrain ischemia in mouse cortex and hippocampus

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ABSTRACT

To assess its potential neuroprotective effect against ischemia/reperfusion (IR) injury in mice, bicyclol was administered intragastrically once a day for 3 days. After 6 h of bicyclol pretreatment on the third day, forebrain ischemia was induced for 1 h by bilateral occlusion of the carotid arteries. After different times of reperfusion, the histopathological changes and the levels of mitochondria-generated reactive oxygen species (ROS), malondialdehyde (MDA) and the activity of superoxide dismutase (SOD) in the cortex and hippocampus were measured. We found that extensive neuronal death occurred in the cortex and the CA1 area of the hippocampus at day 7 after IR and that bicyclol significantly attenuated IR-induced neuronal death in a dose-dependent manner. We also found that pretreatment with bicyclol dose dependently decreased the generation of ROS and the MDA content and reduced the compensatory increase in SOD activity in the cortex and hippocampus at 4 h of reperfusion. These results suggest that bicyclol protects the mouse brain against cerebral IR injury by attenuating oxidative stress and lipid peroxidation.

Brief periods of global cerebral ischemia result in delayed neuronal death (DND), which occurs days after the initial ischemic insult in selectively vulnerable regions of the brain, especially in the cortex and the CA1 area of hippocampus. Reactive oxygen species (ROS) are important in initiating the process of cell death [2,13]. Mitochondria play a central role during ischemia/reperfusion (IR)-induced-DND because ROS are generated by mitochondrial electron transport dysfunction during IR [12]. Normally generated ROS during cell metabolism are eliminated by the anti-oxidative system. Production of ROS in mitochondria during IR stress, however, markedly exceeds the capacity of intrinsic cellular scavengers. Excessive ROS not only activate peroxidative enzymes and destroy membrane phospholipids, but also impair mitochondrial function, producing more ROS and inducing the opening of the mitochondrial permeability transition pore and the release of mitochondrial intermembrane proteins. Imbalance between the cellular oxidative and anti-oxidative capacity consequently activates the death signal pathways and leads to DND. Enhancement of anti-oxidant ability is protective against ischemic brain damage [11].

Bicyclol(4,4′-dimethoxy-5,6,5′ 6′-bis(dimethylene-dioxy)-2-hydroxymethyl-2′-methoxy carbonyl biphenyl) is a member of the new generation of anti-hepatitis drug. It has high clinical efficacy in treating chronic hepatitis B patients in China, and is protective against experimental liver injury induced by the chemical toxins ethanol, CCl_4 and acetaminophen [16]. The hepatoprotective mechanisms of bicyclol involve the clearance of ROS, regulation of cytokine expression, and inhibition of apoptosis induced by immunological injury [5]. Recently, it was reported that bicyclol protects the kidney against IR injury, by inhibiting lipid peroxidation, induction of glutathione (GSH) and glutathione S-transferase (GST), and stabilization of the mitochondrial membrane [15]. Little is known, however, about the possible neuroprotective effects of bicyclol.

Therefore, the present study was designed to investigate the effects of bicyclol on DND, ROS generation, lipid peroxidation, and the activity of superoxide dismutase (SOD), an endogenous anti-oxidant enzyme, following transient forebrain ischemia in mouse cortex and hippocampus.

Adult male ICR mice, 26–28 g, were housed at 22 ± 0.5 °C with an alternating 12 h light/dark cycle and free access to food and water. All procedures were performed in accordance with the Ethics Committee for the Use of Experimental Animals in Zhejiang University. Bicyclol at different concentrations in polyethylene glycol-400 (PEG-400), at 10, 25 or 100 mg/kg, was administered intragastrically (i.g.) once a day at the same time for 3 days. Vehicle-treated sham and control mice were treated with PEG-400 in the same manner. Six hours after the last administration, forebrain ischemia was induced by bilateral common carotid artery occlusion for 1 h after the method of Kuang et al. [7] with slight modifications. In brief, mice were fasted for at least 12 h before operation and then

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anaesthetized with chloral hydrate (400 mg/kg, i.p.). Both common carotid arteries were isolated through a midline neck incision. Transient forebrain ischemia was induced by occluding both arteries with aneurysm clips. Perfusion was restored by removing the clips after 60 min ischemia, and the wound was then sutured. For mice in the sham group, the same procedure was performed except for clipping of the common carotid arteries. Body temperature was maintained by a warming plate throughout and after the operation until the mouse recovered from anesthesia.

After 7 days of reperfusion, mice were overdosed with anesthetic and perfused intracardically with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS, pH 7.4). Brains were removed and further fixed for 6 h in 4% paraformaldehyde at 4 °C. Then, brains were dehydrated in gradually increasing concentrations of sucrose in 0.1 M PBS (first 20%, then 30%). Consecutive coronal sections (15 μm) were cut on a microtome (−1.64 mm to −2.75 mm from bregma) and used for Nissl staining. The numbers of morphologically normal neurons in the cortex and CA1 area of hippocampus were counted under high magnification by an investigator who was blind to the experimental conditions.

After 4 h reperfusion, mice were sacrificed and the brains were removed. Then the cortex and hippocampus were isolated bilaterally within 1 min at 0 °C, half for mitochondria isolation, the other half for malondialdehyde (MDA) level and SOD activity measurements. Hippocampal and cortical mitochondria were prepared according to the method described by Hino et al. [5] with slight modifications. In brief, isolated cortex or hippocampus was immersed in ice-cold isolation buffer (320 mM sucrose, 1 mM EDTA, 0.5 mg/ml BSA, 10 mM Tris–HCl buffer, pH 7.4), and homogenized in 3 ml ice-cold isolation buffer using a glass homogenizer. The homogenate was centrifuged at 2000 × *g* for 2 min, and the supernatant was centrifuged at 10,000 × *g* for 10 min. The pellet obtained was resuspended in 1 ml of the same isolation buffer and centrifuged at 10,000 × *g* for another 10 min. The pellet obtained was resuspended in 1 ml of incubation buffer (100 mM KCl, 75 mM mannitol, 25 mM sucrose, 0.05 mM EDTA, 0.5 mg/ml BSA, 15 mM Tris–HCl buffer, pH 7.4) and used for the analysis of mitochondria-generated ROS.

Mitochondria-generated ROS were determined spectrofluorometrically using the membrane-permeable fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) [10]. Briefly, mitochondria (40 μg protein) were incubated in reaction buffer (pH 7.0) containing 250 mM sucrose, 20 mM MOPS, 10 mM Tris, 50 μM EGTA, 0.5 mM Mg^{2+}, 0.1 mM KH_2PO_4, 1.0 mM cyclosporin A and 10 μM H_2DCFDA. Malate and glutamate (2.5 mM each) were added as substrates. After 40 min incubation at 37 °C, the formation of the oxidized fluorescent product dichlorofluorescein (DCF) was monitored with excitation at 488 nm and emission at 525 nm. The results were expressed as arbitrary fluorescence units per mg protein (AFU/mg protein).

MDA level and SOD activity were detected using commercial kits (Jiancheng Bioengineering Institute, Nanjing, China) and 10% tissue homogenate was prepared according to the user manual. Briefly, brain tissue was homogenized in 10 vol (w/v) ice-cold 0.1 M PBS (pH 7.4). Then the homogenate was centrifuged at 4000 × *g* for 10 min and the supernatant was used.

Protein concentration was determined using a Bradford protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China).

All data are expressed as mean ± SD. Statistical significance was determined by one-way ANOVA with Newman–Keuls post-test. Values of $P < 0.05$ were considered significant.

Nissl staining showed that surviving neurons had normal morphology with large cell bodies and clear Nissl bodies, and that damaged neurons were shrunken with condensed nuclei and sparse Nissl bodies. No cell damage was evident in the cortex and CA1 from sham-operated mice. In contrast, few neurons survived in ischemic mice in cortex and CA1 after 7 days of reperfusion. Pretreatment with bicyclol increased the number of surviving neurons in both cortex and CA1 in a dose-dependent manner (Figs. 1 and 2).

Based on the results, we used 25 and 100 mg/kg, the protective concentrations, to determine whether the neural protection by bicyclol was induced by its anti-oxidative effect. We found that ROS levels in mitochondria isolated from cortex and hippocampus increased at 4 h of reperfusion. The elevated ROS levels were decreased by pretreatment with bicyclol in a dose-dependent manner (Fig. 3).

MDA levels in the cortex and hippocampus increased at 4 h of reperfusion after 1 h forebrain ischemia. Treatment with bicyclol markedly decreased the MDA levels in both cortex and hippocampus (Fig. 4). SOD activity also increased at 4 h of reperfusion in cortex and hippocampus. Treatment with bicyclol at 100 mg/kg reduced the increase in SOD activity in both cortex and

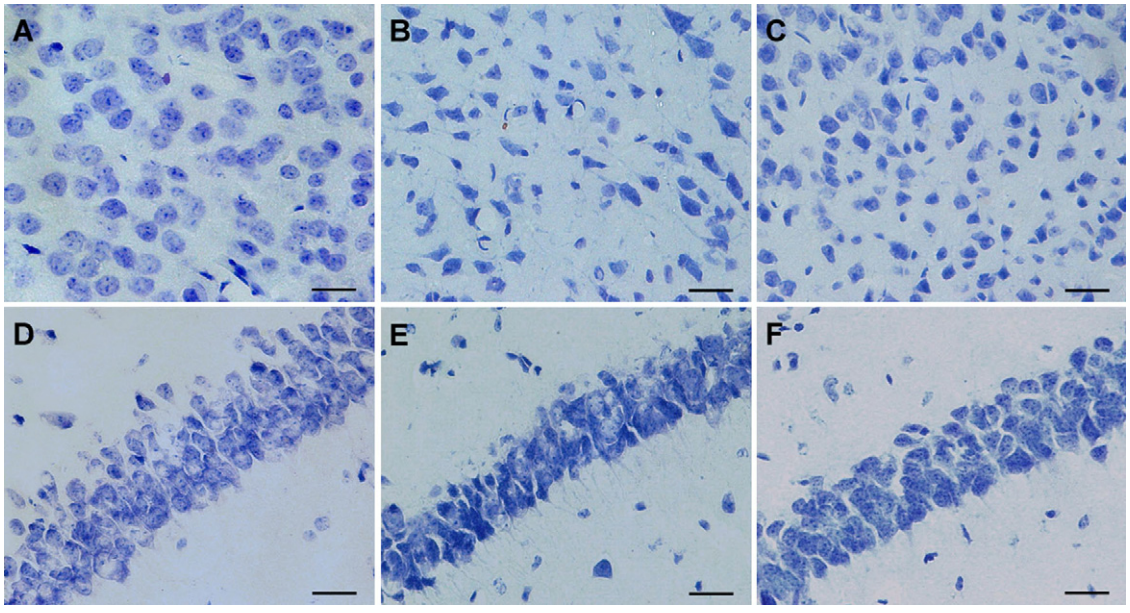

Fig. 1. Representative micrographs of Nissl-stained cells in the cortex and hippocampus (CA1) from vehicle-treated sham group (A and D); vehicle-treated control group (B and E); and 100 mg/kg bicyclol-treated group (C and F) at 7 days of reperfusion. Scale bar = 50 μm.

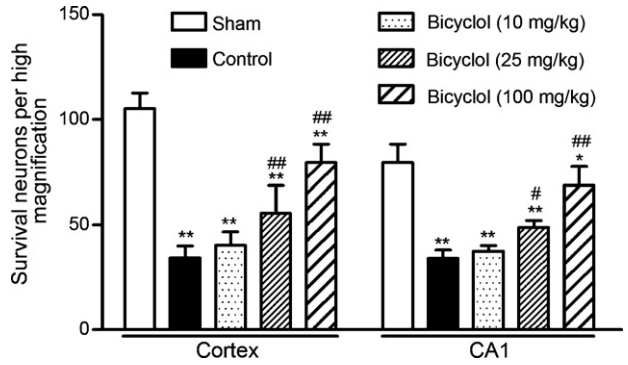

Fig. 2. Influence of bicyclol on numbers of survival of neurons in cortex and hippocampus (CA1) in mice subjected to 7 days of reperfusion after 1 h forebrain ischemia. Data are mean ± SD ($n = 4$ each). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vehicle-treated sham group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with vehicle-treated control group.

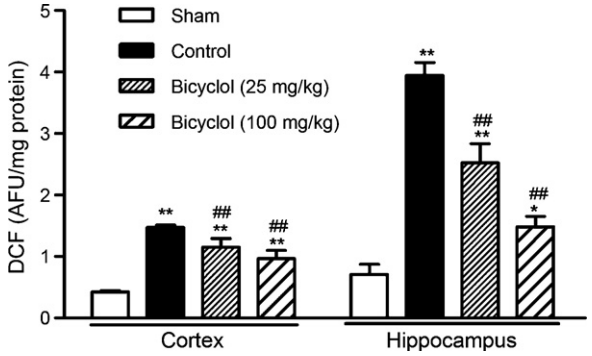

Fig. 3. Effect of bicyclol on ROS generation in mitochondria isolated from cortex and hippocampus of mice at 4 h after 1 h forebrain ischemia. ROS levels were assessed by dichloroflurescein (DCF) assay. Data are mean ± SD ($n = 6$ each). $^{**}P < 0.01$ compared with vehicle-treated sham group, $^{\#\#}P < 0.01$ compared with vehicle-treated control group.

hippocampus while no significant change was observed in SOD activity in mice pretreated with 25 mg/kg bicyclol compared with vehicle-treated controls (Fig. 5).

We demonstrated for the first time that pretreatment with bicyclol protected the brain from ischemia/reperfusion damage in a model of transient forebrain ischemia in mice. Our study showed that mice intragastrically pretreated with bicyclol notably decreased the neuronal cell death in a dose-dependent manner in both cortex and hippocampus, which have been reported to be the most vulnerable areas to brain ischemia [1], at 7 days of reperfusion following 1 h of forebrain ischemia.

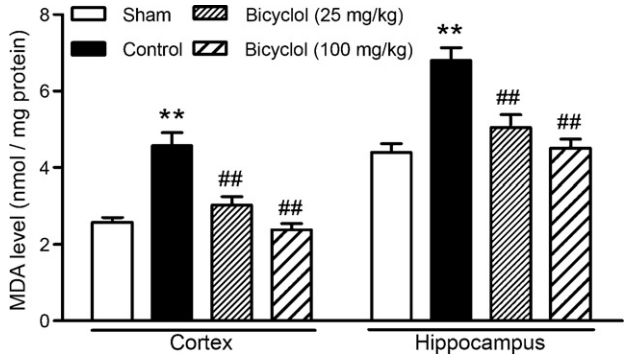

Fig. 4. Effect of bicyclol on malondialdehyde (MDA) level in mouse cortex and hippocampus at 4 h following 1 h forebrain ischemia. Data are mean ± SD ($n = 6$ each). $^{**}P < 0.01$ compared with vehicle-treated sham group, $^{\#\#}P < 0.01$ compared with vehicle-treated control group.

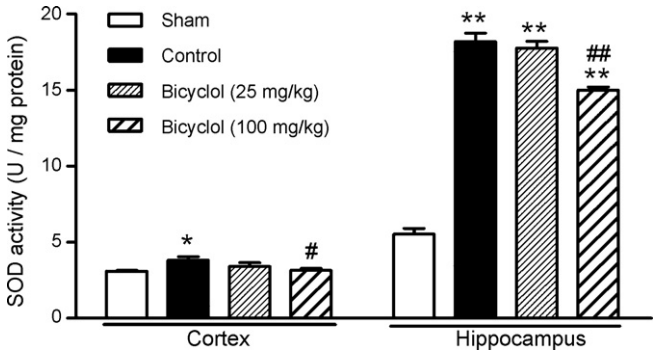

Fig. 5. Effect of bicyclol on superoxide dismutase (SOD) level in mouse cortex and hippocampus at 4 h following 1 h forebrain ischemia. Data are mean ± SD ($n = 6$ each). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vehicle-treated sham group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with vehicle-treated control group.

Recent studies have confirmed the pivotal role of oxidative stress in the pathogenesis of cerebral IR-induced-DND [2,13]. Transient cerebral ischemia, followed by 1–24 h of reperfusion, significantly increases the generation of ROS such as superoxide radicals, hydroxyl radicals and hydrogen peroxide [4,6]. The excessive production of ROS can lead to oxidative damage to membrane lipids, proteins, and DNA, and disturb mitochondrial function, inducing the opening of the mitochondrial permeability transition pore and the release of mitochondrial intermembrane proteins to activate apoptotic pathways [2]. It has been shown that mitochondria are the major source of ROS generated during IR [12]. To evaluate the change of oxidative stress in mouse brains in this study, we measured the levels of ROS generation in isolated mitochondria, as assessed by DCF generation, and the MDA content, a product of lipid peroxidation, in cortex and hippocampus. Our results showed that ROS production and the level of MDA in the cortex and hippocampus increased at 4 h after transient global cerebral ischemia. Pretreatment with bicyclol decreased ROS generation and MDA content, indicating that the neuroprotection conferred by bicyclol is due to its anti-oxidative effect of attenuating oxidative stress and lipid peroxidation. Several studies have shown that bicyclol can decrease the levels of hydroxyl radicals and superoxide anion in CCl_4 stimulated neutrophil as detected by electron spin resonance [9], and increase the GSH content and the activity of GST, both endogenous anti-oxidants, and enhance membrane stability and membrane fluidity in rat liver and kidney [15,16]. So the anti-oxidative effect of bicyclol here may be attributed to its multiple capacity. SOD is a major member of the anti-oxidant defense system and specifically detoxifies superoxide radicals to hydrogen peroxide, which is then scavenged by peroxisomal catalase. Here, we found that the activity of SOD increased in both cortex and hippocampus at 4 h of reperfusion, similar to previous studies [3,8]. The increase in SOD activity may be another sign for the increased oxidative stress [14]. Bicyclol might be involved in the decreased elevation of SOD activity via scavenging ROS.

Taking all these findings together, bicyclol protects the mouse brain against cerebral IR injury. The neuroprotection shown by bicyclol may be attributed to attenuating oxidative stress and lipid peroxidation.

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