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Remote ischemic preconditioning protects neurocognitive function of rats following cerebral hypoperfusion

Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

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Summary

Background:

Protection of remote ischemic preconditioning on neurocognitive function caused by bilateral common carotid artery occlusion has been investigated in rats.

Material/Methods:

Thirty-six male Sprague-Dawley rats were divided into 3 groups – control group (Group C, n=12), bilateral carotid arteries occlusion group (Group B, n=12) and remote ischemic precondition group (Group P, n=12). In Group P, remote ischemic preconditioning (RIPC) was performed on the right femoral artery with 3 cycles (10 min) of occlusion/perfusion. After 3 cycles of preconditioning, bilateral carotid arteries were occluded immediately for 60 min. In Group B, ischemic insults were conducted without RIPC. Sham surgeries were performed in Group C. Evaluation of memory and learning capacity was performed on days 5-8 after surgery by Morris water maze testing of spatial learning capacity (n=6 for each group). Apoptosis of cells in the hippocampus region was determined by TUNEL tests and Bcl-2 at this region was determined by ELISA 24 h and 9 days after vessel occlusion (n=6 for each group).

Results:

Neurocognitive tests showed that latency time was significantly longer in Group B than in Group P on day 7 (p=0.016) and day 8 (p=0.036). Moreover, frequency of platform crossings was significant less in group B than in the other 2 groups on day 9. Bcl-2 level was significantly increased in the hippocampal region of rats in Group P on days 1 and 9 after vessel occlusion. TUNEL test showed that apoptosis could be observed at 24 h after occlusion in Group B, but not in Group P and Group C. No apoptosis was observed on day 9.

Conclusions:

Our results suggest that RIPC can protect neurocognitive function of rats after bilateral carotid occlusions, and that Bcl-2 may play an important role in this protective effect.

key words:

remote ischemic preconditioning • neurocognition • water maze • Bcl-2

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BACKGROUND

Postoperative cognitive dysfunction (POCD) is a common complication after surgery [1]. Impairment in neurocognitive and neuropsychologic performance occurs in 30% to 60% of cardiac surgery patients in the first postoperative week (5–8 days) [2]. Most demonstrate mild cognitive impairment at discharge, and a considerable proportion (7–69%, according to different criteria) do not recover within 1–3 months after surgery [3]. Incidence of POCD is also high after non-cardiac surgery. In the International Study of Postoperative Cognitive Dysfunction (ISPOCD) involving 1218 elderly patients undergoing major non-cardiac surgery, incidence of POCD was 25.8% at 1 week and 9.9% at 3 months after surgery [4].

The underlying mechanism of POCD remains unclear, but perioperative hypoperfusion is thought to be one of the most important factors leading to brain damage [5]. Ogasawara et al reported that intraoperative and post-ischemia delayed hypoperfusion in carotid endarterectomy can impair cognition even in the absence of postoperative neurologic deficit [6]. In a recent study, it was demonstrated that even brief to moderate periods of hypoperfusion may lead to a wide spectrum of neurologic injuries, including POCD [7]. Although many methods are employed in clinical practice to prevent or reduce brain damage or/and neuropsychiatric dysfunction, such as hypothermia and some medications, most long-term effects are, unfortunately, uncertain.

Remote ischemic preconditioning (RIPC) is a novel approach for prevention of ischemia/reperfusion (I/R) injury, without direct stress on the target organ. I/R injury of one organ is believed to protect remote organs due to either release of biochemical messengers in circulation or activating nerve pathways, resulting in release of messengers that have a protective effect. The first evidence of RIPC was reported in myocardium by Przyklenk et al. in 1993 [8]. Recently, increasing evidence shows that RIPC can protect the brain from a lethal ischemic episode [9–11], but most reports on RIPC for brain protection have been based on animal models of stroke, and merely revealed the pathological and pathophysiological effects, such as infarct area size and extracellular signal-regulated kinases [11,12]. There have been few reports focusing on cognitive function effects of RIPC.

Because RIPC can provide protection to vital organs or systems through a brief ischemia to non-vital organs without concern about preconditioning site damage, it is more practical for use in clinical practice than is direct preconditioning. Although RIPC is not as effective as direct preconditioning [13], it can result in significant neural protection following ischemia [9]. The present study therefore attempted to determine if RIPC can protect cognitive function during transient hypoperfusion.

Bcl-2 is an anti-apoptotic protein that plays an important role in neuroprotection and development of CNS [14]. Bcl-2 combines with other proteins of its family, such as Bax or Bcl-XL (Bcl-2-Bax or Bcl-XL-Bax heterodimers), to stabilizing mitochondrial membranes and prevent mitochondrial release of cytochrome c. As mitochondria may be a gateway to cerebral preconditioning, Bcl-2 might be important for ischemic neurocognitive protection [15].

The present study was performed to investigate the protective effect of RIPC in rats with bilateral common carotid arteries occlusion. Neurocognitive function was assessed by Morris water maze testing and Bcl-2 was determined by ELISA testing in the hippocampus.

MATERIAL AND METHODS

Experimental groups

Male Sprague-Dawley rats weighing 250–310 g were used. They were group housed and maintained on a 12-h light/dark cycle (lights on at 8:00 a.m.) with free access to water and standard rat chow. The room temperature was maintained at 21–23°C with 60% relative humidity. Upon arrival, animals were acclimatized to the animal facility for at least 1 week prior to surgery. All procedures were carried out in accordance with the guidelines set by the Council of Animal Care and were approved by the animal ethics committee of the Second Military Medical University. All rats were randomly divided into 3 groups – control group (Group C, n=12), bilateral carotid arteries occlusion group (Group B, n=12), and remote ischemic preconditioning group (Group P, n=12).

Surgical procedures

Anesthesia was induced by intraperitoneal injection with 3% sodium pentobarbital (30mg/kg), and maintained with one-third of first dosage if necessary. Core body temperatures were monitored with a rectal probe and maintained at 36.2–37.2°C using a heating light during the whole experiment. For Group B, brain ischemia (60 min) was conducted by the bilateral common carotid arteries occlusion method (2VO) as described previously [16]. For Group P, remote ischemic preconditioning was conducted by 3 cycles (10 min) of occlusion/reperfusion on the right femoral artery. Then brain ischemia was conducted immediately, similarly to that in Group B [11]. Sham surgery was performed in Group C without any artery occlusion.

Neurocognitive testing

Evaluation of memory and learning capacity was started at the 5th day after surgery. Morris water maze testing of spatial learning capacity was conducted as described previously [17]. Briefly, the Morris water maze consisted of a large circular black pool (210 cm diameter, 50 cm height, filled to a depth of 30 cm with water at 28±1°C), which was placed in a darkened room, illuminated by dim red light. Within the pool a submerged platform (black, round, 8 cm diameter, 1 cm below surface) was hidden in a fixed location, 55 cm from the edge of the pool. The rat could climb on the platform to escape from the necessity of swimming. The animals were given 3 swimming trials per day on 4 consecutive days with a different starting position in each trial. The rat was given a maximum of 120 s to find the hidden platform and was allowed to stay on it for 30 s. Rats that failed to locate the platform were put onto it by the experimenter for 30 s. After 4 days of training, platform crossings were evaluated while the submerged platform was removed.

Morris maze performance was analyzed for latency to find the platform and for platform crossings, using a video-tracking

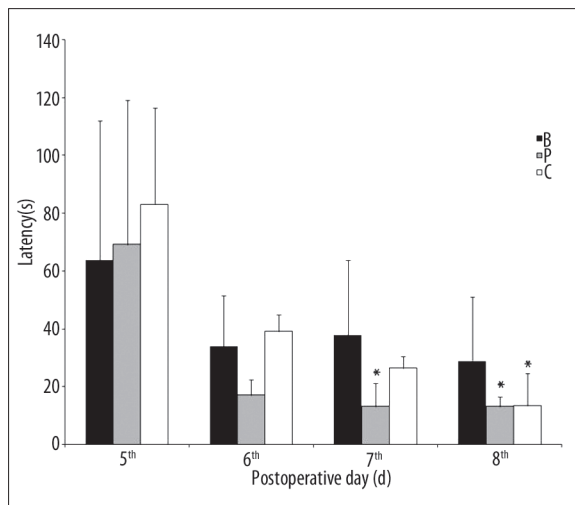


Figure 1. Latency time of Morris water maze. Latency time in each group was not significantly different on day 5 and day 6. But it was significantly different on day 7 ($p=0.016$) and day 8 ($p=0.036$) between Group B and Group P, and on day 8 ($p=0.047$) between Group B and Group C. There were no significant difference between Group C and Group P in any of the four testing days. * $p<0.05$ vs. Group B.

system (DigBehv animal behaviors analysis software, Jiliang Software Technology Corporation, Shanghai, China)

TUNEL assay

The brain tissue sample was collected and half cut sagittally on days 1 and 9 after surgery. One half was fixed in 10% buffered formalin. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed using the ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon Billerica, MA, USA) according to the manufacturer's instructions. In brief, sections were incubated in equilibration buffer for 10 minutes and then terminal deoxynucleotidyl transferase and dUTP-digoxigenin were added to the sections and incubated in a 37°C humidified chamber for 1 h. The reaction was then stopped and the slices were washed and incubated with anti-digoxigenin-peroxidase solution, colorized with DAB/H₂O₂, and counter-stained with bis-benzamide. The other half was stored in -80°C refrigerator for ELISA.

Enzyme-linked immunoassay (ELISA)

A total of 1 mg sample was added with 500 µg RIPA lysate (Beyotime, Jiangsu, China), followed by complete homogenization and centrifugation at 12000 rpm for 15 min. The supernatant was used for ELISA with a commercially available ELISA kit (Rapidbio, CA, USA). Briefly, 10 µl supernatant and 40 µl standard dilution were added into the 96-well microplate pre-coated with Bcl-2 antibody (Ab) and incubated for 30 min at 37°C. After washing with washing solution 5 times, 50 µl HRP-conjugate reagent was added into each well and incubated for 30 min at 37°C. Then Chromogen solutions were added, evading the light preservation for 15 min at 37°C. Finally, the reaction was stopped with 50 µl stop solution after washing the plate. The optical density was measured at 450 nm using microplate reader (VMx,

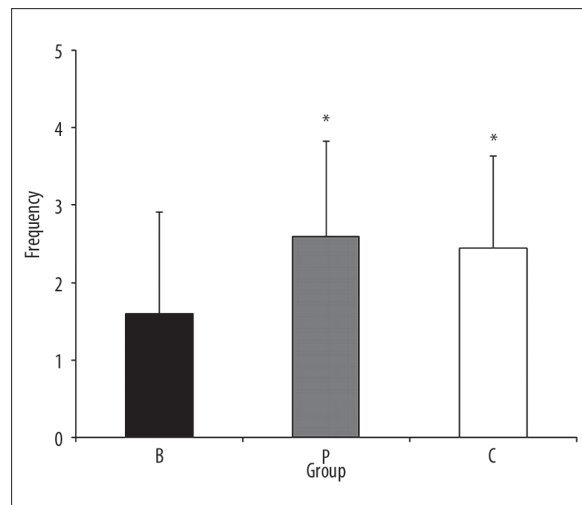


Figure 2. Platform crossings of Morris water maze. There was significant difference between Group B and the other 2 groups in frequency of platform crossings on day 9. * $p<0.05$ vs. Group B

Molecular Devices, Sunnyvale, CA). A Bcl-2 standard curve was generated for quantitation.

Statistical analysis

Results of cognitive tests and Bcl-2 ELISA results were calculated as means \pm standard deviation (S.D.) per group, and analyzed by one-way ANOVA. $P<0.05$ were considered as statistically significant.

RESULTS

Neurocognitive testing

Morris maze latencies and platform crossings are displayed in Figure 1 and 2, respectively. Latency time in each group was similar on days 5 and day 6, but it was significantly longer in Group B than in Group P on day 7 ($p=0.016$) and day 8 ($p=0.036$), and longer in Group B than in Group C on day 8 ($p=0.047$). There were no significant differences between Group C and Group P in any of the 4 testing days (Figure 1). Frequency of platform crossings was significantly lower in Group B than in the other 2 groups on day 9 (Figure 2).

Apoptosis in hippocampal region

TUNEL assay showed that many apoptotic cells were present in Group B on day 1 after vessel occlusion, while there was hardly any apoptosis in the other 2 groups at the same time points (Figure 3). However, no apoptotic cells could be observed on day 9 after surgery (Figure 4).

Bcl-2 enzyme-linked immunoassay (ELISA)

ELISA testing showed that Bcl-2 in the hippocampus was significantly higher in Group P (4.20 ± 0.52 ng/mg) on day 1 after vessel occlusion than in Group B (3.27 ± 0.40 ng/mg, $p=0.02$ compared with Group P) and Group C (3.18 ± 0.37 ng/mg, $p=0.001$ compared with Group P). No difference was observed between Group B and Group C ($p=0.720$).

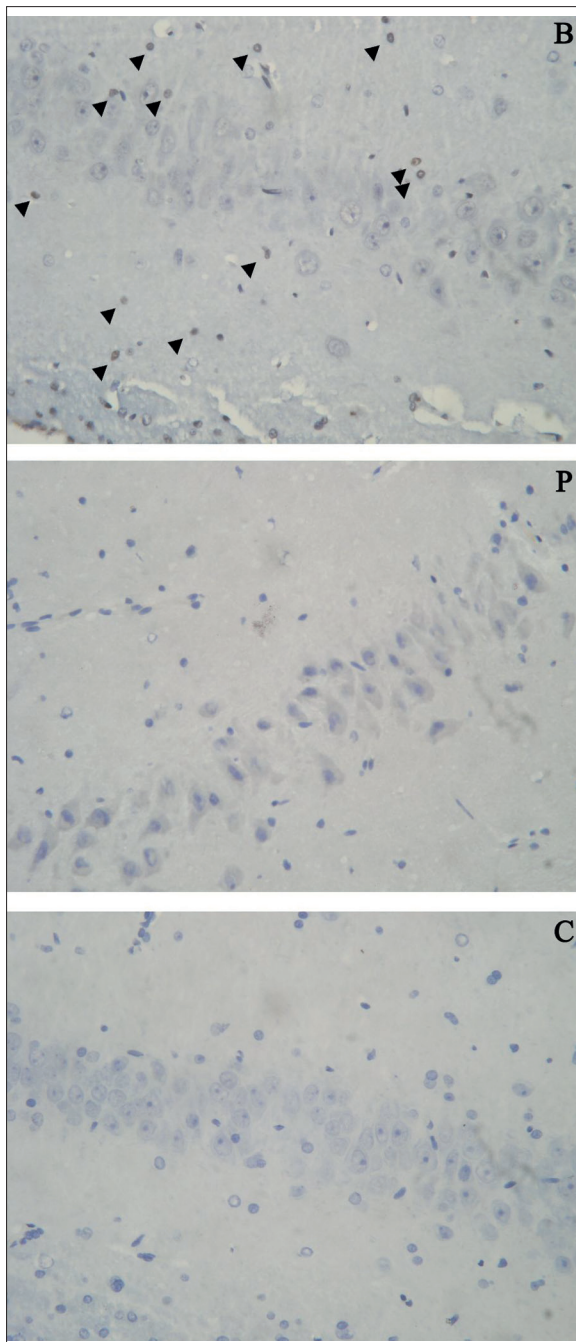


Figure 3. TUNEL assay in hippocampal CA1 region at 24 h after vessel occlusion.

Similarly, on day 9 Bcl-2 levels in the hippocampus significantly increased in Group P (4.09 ± 0.61 ng/mg) compared with Group B (2.45 ± 0.53 ng/mg, $p < 0.001$ compared with Group P) and Group C (3.15 ± 0.65 ng/mg, $p = 0.017$ compared with Group P). There was no significant difference between Group B and Group C ($p = 0.063$) (Figure 5).

DISCUSSION

POCD not only results in poor prognosis and increased mortality, but also reduce the activities of daily living (ADL), which increase the burden of care on the patient himself

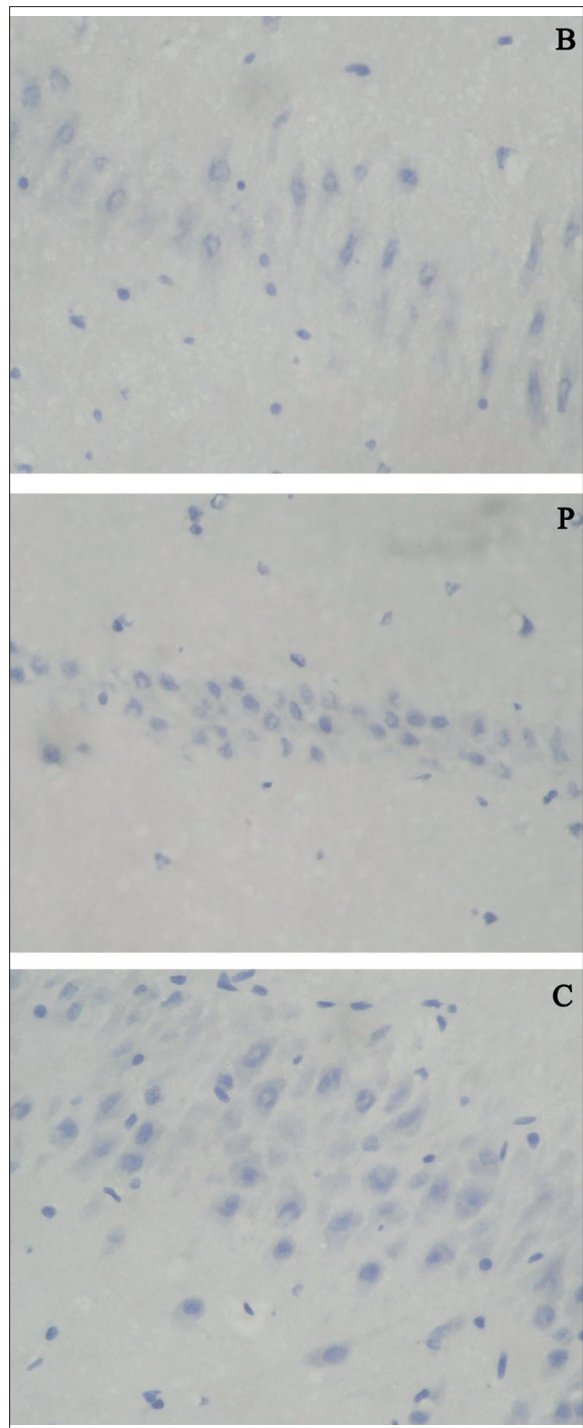


Figure 4. TUNEL assay in hippocampal CA1 region at Day 9 after vessel occlusion.

and his family, as well as society. Although earlier studies reported that neurodegenerative disorders such as AD and Parkinson's disease might be accelerated by anesthesia and surgery [18,19], there is still a debate whether POCD patients can be considered as AD patients and whether POCD patients undergo AD-related neuropathogenesis. Clinicians are endeavoring to prevent POCD, but there are still many problems relating to the preventive therapies through surgical or medical management. Clinical concerns include

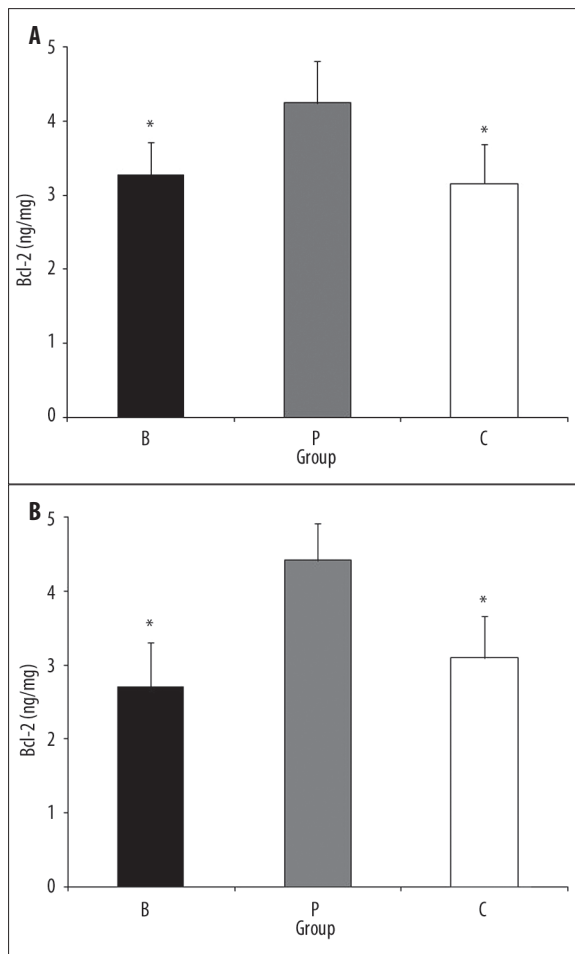


Figure 5. Enzyme-linked immunoassay (ELISA) of Bcl-2 in hippocampal region of rats. ELISA results demonstrated that Bcl-2 levels were significantly increased in Group P. The levels of Bcl-2 was 2.45 ± 0.53 ng/mg in Group B, 3.15 ± 0.65 ng/mg in Group C and 4.09 ± 0.61 ng/mg in Group P, respectively. Bcl-2 level in Group P was significantly higher than in Group B ($p < 0.001$) and in Group C ($p = 0.017$). There was no significant difference between Bcl-2 levels in Group B and Group C ($p = 0.063$). * $p < 0.05$ vs. Group P.

indefinite protective effects, procedure- or medication-related adverse effects, etc. Among the various neural protection measures, RIPC is novel. Many studies have suggested that RIPC has a definite protective effect. This novel protection can promote both immediate and delayed protection, and the latter may provide promising long-term effects on neural damages, which is deficient in many current therapies. Unfortunately, most of previous studies were focused on animal models of stroke and complicated biochemical mechanisms for neurologic protection. Hypoperfusion is important to POCD during the perioperative period, but stroke is not. To the best of our knowledge, there is no published study on the effect of RIPC on cognitive function for mild hypoperfusion as we usually confront it in clinical practice.

The transient 2-vessel occlusion (2VO) model was used in our study. Four-vessel occlusion (4VO) and middle cerebral artery (MCA) model or permanent 2VO of rats may not be suitable for cognitive function evaluation. Other neurologic

functions might be impaired, such as psychomotor and optical functions [20], which influence neurocognitive evaluation results. Although all of them are commonly used for brain ischemic research, they are not appropriate for evaluation of neurocognitive function in hypoperfusion. A previous study suggested that transient 2VO induced reduction in cerebral blood flow by 50% and produced persistent cognitive function changes [21]. Our study reached a similar conclusion about 2VO on cognitive function changes. After a 2-day training, 2VO rats had significantly longer escape latency and lower frequency of platform crossings than did sham operation rats. There was no significant difference in escape latency and platform crossings between RIPC rats and sham operation rats during the experiment, but they were significantly different between RIPC and 2VO, thus we inferred that RIPC induced the reduction of cognitive function decline in 2VO.

It was interesting that apoptosis, obvious after vessel occlusion, was blunted after RIPC, but it was recovered at day 9. Brain tissue of the hippocampal region was collected for Bcl-2 ELISA. Bcl-2 in the region was elevated in Group P, while no change in Bcl-2 was found between the other 2 groups in the hippocampal region. Thus, RIPC resulted in Bcl-2 elevation in the hippocampal region. Bcl-2 is an anti-apoptotic protein that locates in the outer mitochondrial membranes and the membranes of the endoplasmic reticulum. Bcl-2 overexpression is known to block the release of cytochrome c and thereby to inhibit cell apoptosis. It was reported that 2 minutes of 2VO in gerbils produced an increase in Bcl-2 at 30 h and peaked at 96 h, suggesting that up-regulation of Bcl-2 was involved in ischemic preconditioning [22]. In another study, ischemic preconditioning for 20 min with transient focal ischemia attenuated infarction volume [23]. Western blot analysis from caudate-putamen showed an increase of Bcl-2 expression 3-7 days after preconditioning. The transcription factors and many other factors stimulate overexpression of Bcl-2, including cAMP response element binding protein (CREB), which is the Bcl-2 promoter containing a cAMP-response-element (CRE). CREB is regulated by N-methyl-D-aspartic acid (NMDA) receptor activation, and phosphorylated in the penumbral region of the preconditioned rats [24]. Recent research has revealed the importance of activation of cAMP-CREB-Bcl-2 signaling in the survival of newborn neurons in adult hippocampus undergoing apoptosis after ischemia [25,26]. Therefore, Bcl-2 plays an important role in preconditioned rats for ischemic protection. Unlike apoptosis assay, Bcl-2 level in remote ischemic preconditioning was still higher than the other 2 groups on the 9th day. The explanation might be that ischemia induced both early and late increases in the levels of anti-apoptotic Bcl-2 in hippocampal region, which was involved in a much delayed recovery process.

CONCLUSIONS

In summary, RIPC ameliorates 2VO ischemic damages to spatial learning capacity of rats according to the latency time on day 4 and platform crossing on day 5 in Morris water maze testing. Although there is no significant change in the histological examination, Bcl-2 level in the hippocampal region of rats is elevated, which suggests the apoptotic pathway is activated by RIPC against damages to neurocognitive function. Thus RIPC may prevent injury of 2VO ischemia

by activating anti-apoptotic activity of the Bcl-2 pathway and may be a promising novel prevention for POCD.

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