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Original Article

Impact of ischemic preconditioning on ischemia-reperfusion injury of the rat sciatic nerve

Shuanghai Dong, Yun Cao, Haoqing Li, Jiwei Tian, Chengqing Yi, Weilin Sang

Department of Osteology, Shanghai First People's Hospital, No. 100 Haining Road, Shanghai 200081, China

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Abstract: The aim of this study was to assess the preventive effects of ischemic preconditioning (IPC) on ischemia-reperfusion (IR) injury in the sciatic nerve of the rat hind limb. This study included two experiments. For Experiment 1, 40 Sprague-Dawley (SD) rats were randomly divided into 4 equal groups that received different IPC treatments prior to IR. Serum concentrations of aspartate aminotransferase (AST), lactate dehydrogenase (LDH), malondialdehyde (MDA), and superoxide dismutase (SOD) were assessed following reperfusion. Furthermore, we tested the electrophysiological response and ultrastructural changes in the ipsilateral sciatic nerve after IR. After determining the best IPC protocol for protection, we performed a second experiment with 30 SD rats randomly divided into 3 equal groups. Each group underwent 1, 2, or 3 IPC cycles before prolonged ischemia and reperfusion. The same analyses as in Experiment 1 were performed. In Experiment 1, the AST, LDH, and MDA concentrations were decreased in all IPC groups compared with the control group. Concentration of these enzymes showed decreases with increasing IPC cycle number in Experiment 2; however, the difference between 2 and 3 cycles of IPC did not reach significance. Conversely, SOD activity increased in the rapid and delayed groups, and with increasing cycles of IPC. The electrophysiological test showed a decrease in amplitude and increase in conduction velocity with increasing IPC cycles. Moreover, ultrastructural damage decreased with increasing IPC cycles. IPC protected against IR injury in the peripheral nerves. This effect was positively correlated with the number of IPC cycles.

Keywords: Ischemic preconditioning, ischemia-reperfusion, sciatic nerve, rat

Introduction

Ischemic preconditioning (IPC) refers to a phenomenon that can delay or reduce tissue damage in a subsequent ischemia-reperfusion (IR) injury through transient repeated ischemia. Murry et al. first discovered this phenomenon in 1986 by using a canine myocardial ischemia model [1]. Following this, the protective effects of IPC in myocardial ischemia have been confirmed in many studies. IPC was first applied in skeletal muscle experiments in the 1990s. IPC was found to be more protective in skeletal muscle than the myocardium, improving the ischemic tolerance of skeletal muscle and reducing the area of muscle necrosis [2]. Subsequently, many experiments have confirmed that IPC improves ischemic tolerance in animal organs and tissues [3-12]. In recent years, orthopedic studies have concentrated on the protective effects of IPC in IR injury of limb skeletal muscles [13, 14]. Peripheral nerve injury has been associated with toxic free radicals [15] or reductions in TNF-α level [16]; however, the mechanism underlying the protective effect of IPC in IR injury is not fully understood. In this study, we established an experimental model that temporarily blocked the iliac artery of Sprague-Dawley (SD) rats to observe the impact of IPC on IR injury of the rat hind limb sciatic nerve. We sought to explore whether IPC is protective in peripheral nerves and the optimal mode of treatment.

Materials and methods

Animals

Seventy male SD rats were used in this study, weighing 200-300 g. They were housed in a cleanroom of ISO 2 standard. In the first experiment, 40 rats (n=10 per group) were randomly divided into four groups: control, rapid effect, delayed effect, and superposed effect. In the

second experiment, 30 rats (n=10 per group) were randomly divided into three groups: 1-, 2-, and 3-cycle. The protocol for each group was as follows: the control group, ischemia for 3 h followed by reperfusion for 2 h; rapid effect group, ischemia for 10 min, reperfusion for 10 min, and ischemia for 3 h followed by reperfusion for 2 h; delayed effect group, ischemia for 10 min, reperfusion for 10 min followed by ischemia for 3 h, then reperfusion for 2 h after 24 h; superposed effect group, ischemia for 10 min and reperfusion for 10 min, followed by ischemia for 10 min, reperfusion for 10 min, ischemia for 3 h, and reperfusion for 2 h after 24 h. A cycle was defined as a 10 min ischemia-reperfusion period. All experimental animals were purchased from the Department of Animal Experiments of Fudan University. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai First People's Hospital.

Animal modeling

The animals were fasted 24 h before the experiment but provided with water *ad libitum*. Rats were anesthetized with 0.5% sodium pentobarbital (30 ml/kg^{-1}, i.p.). The skin of the left groin was cut, and the subcutaneous tissue and muscles were bluntly dissected to expose the left external iliac artery. The dissection extended upwards against the artery to expose the left common iliac artery and a non-damaging microsurgical clip was used to block its blood flow [17]. Reperfusion was performed by loosening the clip to restore blood flow at the designated experimental time point for each ischemic group.

Determination of serum enzymes

Blood (1.8 ml) was obtained from the femoral vein ipsilateral to the IR injury 2 h after reperfusion. The blood was stored in an anticoagulant tube and left at room temperature for 2 h. Following this, samples were centrifuged (Eppendorf, Hamburg, Germany) at 3500 rpm for 5 min, and the serum was removed to measure enzyme concentrations. LDH and AST concentrations were measured using an automatic biochemical analyzer. MDA concentration and SOD activity were measured using the thiobarbituric acid and xanthine oxidase methods, respectively.

Electrophysiology

Three rats from each group were randomly selected for electrophysiological testing of the left sciatic nerve to be conducted at the Affiliated Huashan Hospital of Fudan University 2 h after the final reperfusion. We used a reporter myoelectric nerve-evoking potentiometer (Esaote Biomedica, Indianapolis, Italy) to test the sciatic nerve distal to the ischemic block. Following exposure of the anterior pathway, a 1.6-cm silicone tube was placed around the sciatic nerve. The intraoperative stimulating electrodes were formed into a groove with the stainless steel wire. The pole distance was 3 mm, the poles were 15-20 mm in length, and the interpole resistance was > 100 KΩ. Electrical stimulation was performed once the electrodes were against the sciatic nerve at both the proximal and distal ends of the silicone tube. The receiving electrode was the homo-core needle electrode, which was inserted inside the gastrocnemius of the detection side, while the monopolar needle was the grounding electrode. The latency (Lat), amplitude (Amp), and conduction velocity (NCV) of the sciatic nerve were recorded. The detecting electrodes were manufactured by the electromyogram (EMG) room, Huashan Hospital, Fudan University. All tests were performed by the same physician.

Observation of ultrastructural changes

One rat from each group was randomly selected and conventional sampling was performed 2 h after the reperfusion. The sampling position was 2 cm above the left knee. The specimen was fixed in 2.5% glutaraldehyde, embedded in pure resin, and thinly sliced. Double staining with 3% uranyl acetate-lead citrate was performed, and the sections were visualized using a Philips CM120 TEM (Philips, Amsterdam, Netherlands). The magnifications used were 4801.5× and 16500×. Embedding, sectioning, and staining were performed in the Electron Microscopy Laboratory, School of Medicine, Fudan University.

Statistical analysis

Quantitative data were expressed as mean ± standard deviation ($\bar{x}$ ± S). Stata 7.0 was used

Table 1. Changes of serum GOT, LDH, MDA and SOD of each group in Part I experiment

Group	GOT (IU/L)	LDH (IU/L)	MDA (nmol/L)	SOD (U/ml)
Superposition	199.800±53.043	407.300±103.872	2.591±0.517	259.060±7.183
Delay	224.400±45.749	437.400±172.514	3.463±0.811	228.020±3.306
Rapid	231.500±46.481	439.400±169.237	3.388±0.376	227.350±13.647
Control	297.600±62.957	687.000±239.607	5.769±0.636	206.580±3.731

Table 2. EMG changes of each group in Part I experiment

Group	Incubation period (ms)		Amplitude (mv)		Conduction velocity (m/s)
	Distal	Proximal	Distal	Proximal	
Superposition	1.12±0.06	1.34±0.03	4.45±0.21	4.80±0.14	80.00±0.00
Delay	1.48±0.11	1.84±0.11	1.60±0.14	1.20±0.00	44.40±0.00
Rapid	1.52±0.17	1.86±0.14	6.80±0.99	4.20±0.28	47.20±3.96
Control	2.06±0.31	2.46±0.25	3.20±1.41	2.30±0.99	28.75±10.82

to perform analysis of variance (ANOVA) and pairwise comparisons. $P<0.05$ was considered statistically significant.

Results

Serum enzymes

The serum concentrations of AST, LDH, and MDA were significantly decreased in the delay and rapid, and 1-, 2-, and 3-cycle groups, when compared with the control group, whereas there were no significant differences between the delay and rapid group. In addition, enzyme concentrations in the 3- and 2-cycle groups were significantly different compared with those in the 1-cycle group, with no significant differences between 3- and 2-cycle groups. Conversely, SOD activity was increased in the delay and rapid group and in the 1-, 2-, and 3-cycle groups compared with the control group ($P<0.05$). SOD activity was strongly increased in the 1-, 2-, and 3-cycle groups, while there was no significant difference between the delay and rapid group. In addition, there was a significant increase in SOD activity in 3- and 2-cycle groups when compared with the 1-cycle group, with no difference between the 3- and 2-cycle groups (**Table 1**).

Electrophysiology

Three rats from each group were randomly selected for EMG testing of the left sciatic nerve. The superposition group performed significantly better than the other groups in Experiment 1; they exhibited the shortest incubation period, widest amplitude, and fastest nerve conduction velocity. The control group showed a significantly prolonged the incubation period and slower nerve conduction velocity when compared with the other groups. There were no significant differences between the delay and rapid groups. In Experiment 2, we found no significant differences in the IR-induced electrophysiological changes between the 2- and 3-cycle groups, although these groups showed better results than the 1-cycle group. The amplitude and nerve conduction velocity of the 1-cycle group was significantly narrower and slower than those of the 2- and 3-cycle groups (**Table 2**).

Ultrastructural changes

Electron microscopy revealed a neat layer structure of the myelinated nerve fiber in sections from the 2-cycle group (**Figure 1A**). In addition, the axonal structure was integrated and Schwann cell organelles were rich with no obvious damage.

The layer structure of the myelinated nerve fiber was loose and curly, like curly hair, in the delay group (**Figure 1B**). Certain layers were melted, with swollen mitochondria and broken cristae in partial Schwann cells. Small vacuole-like structures were present and the axon was squeezed by the loose myelin.

The incisures among the layer structure of the myelinated nerve fiber were widened in the rapid group (**Figure 1C**). The layers of partial Schwann cells were melted, forming a marrow-

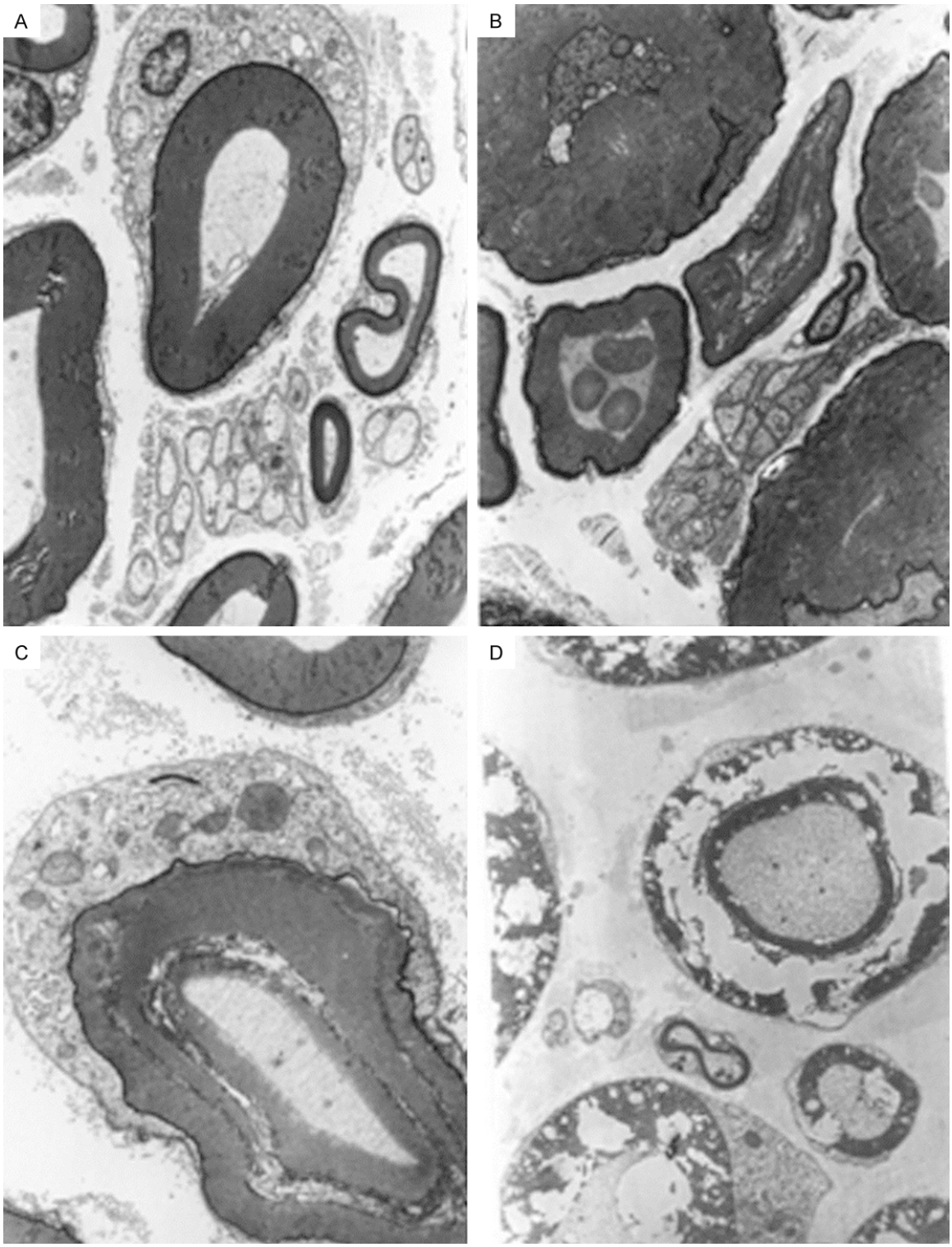

Figure 1. Ultrastructural changes in rat sciatic nerve (A. The 2-cycle superposition group; B. The delayed effect group; C. The rapid effect group; D. The control group).

like substance that contained endosomes. The axonal structure was still intact and the morphology of most Schwann cells was not abnormal.

The majority of the layer structure of the myelinated nerve fiber was melted in the control group (**Figure 1D**); vacuolization was visible, and part of the axon was compressed by loose myelin. Partial organelles existed inside the Schwann cells; however, the membrane of partial Schwann cells was ruptured.

In Experiment 2, the 3-cycle group retained a neat layer structure in the myelinated nerve fiber. The axonal structure was integrated and Schwann cell organelles were rich with no obvious damage. Similar patterns were found in the 2-cycle group; however in the 1-cycle group, while the layer structure of the myelinated nerve fiber was neat, partial Schwann cells exhibited small vacuole-like structures. Other structures were normal.

Discussion

The results showed that concentrations of AST, LDH, and MDA were decreased in the rapid and delay groups when compared with the control group. These concentrations decreased further with increasing IPC cycles, although there was no significant difference between the 2- and 3-cycle groups. Conversely, SOD activity showed the opposite pattern. AST and LDH are both increased with damage to the liver or heart, and MDA and SOD have been shown to increase and decrease respectively with oxidation stress; therefore, we have found that IPC is protective in IR-induced sciatic nerve injury. The electrophysiological results showed that the incubation period, amplitude, nerve conduction velocity decreased with more IPC cycles. In addition, there was less ultrastructural damage with more IPC cycles. Therefore, we have confirmed the protective effect of IPC on IR by assessing the electrophysiology and ultrastructure of the sciatic nerve.

Tissue injury and inflammation in various organs following ischemia and reperfusion has been recognized for many years [18]. Myocardial infarction, stroke, and peripheral vascular disease continue to be the most frequent causes of debilitating disease and death [19]. Ischemia-induced tissue damage occurs both when tissue perfusion is inadequate but also, importantly, when tissue perfusion is recovered. This is because a large amount of oxygen free radicals are generated, which induce peroxidation of the lipids in ischemic tissue membranes. This breaks the peptide chains among the proteins and enzymes and causes nucleic acids cross-linkages. Subsequently, the membranes are damaged and their permeability is altered. When membrane permeability increases, liquids permeate into the space of the endoneurial capillary endothelial cells. This produces lacuna compartment syndrome, which further impacts local tissue nutrition and formation of a vicious cycle of compression, edema, nutritional dysfunction, and further edema. This induces nerve fiber demyelination and Wallerian degeneration. Axons rupture, amount of fibrous connective tissue is increased, and the neural stem shrinks back, thereby causing dysfunction of nerve conduction [17]. In Experiment I of this study, we found serum enzyme level, electrophysiological, and ultrastructural changes in the control group, which had the IR injury alone; this suggested the existence of sciatic nerve dysfunction.

Necrosis of the cardiac and skeletal muscle, kidney, and nervous system releases LDH. In addition, necrosis of skeletal muscle cells releases AST into the blood. Therefore, the serum levels of LDH and AST are used as evidence of IR injuries in limb skeletal muscle and neural function [20-22]. It is widely recognized that oxygen free radicals play a major role in IR injury. MDA is the final product of lipid peroxidation; therefore, its content could reflect the velocity and intensity of lipid peroxidation. The endogenous free radical scavenging system consists of superoxide dismutase (SOD) and peroxidase (CAT) *in vivo*; therefore, SOD activity could reflect the level of anti-oxidants. Levels of MDA are often associated with the activity of SOD; SOD activity is thought to indirectly reflect the body's ability to eliminate oxygen free radicals, while MDA levels indirectly reflect how the severity of the damage from free radicals. We found that the serum AST, LDH, and MDA levels in each IPC-treated group were lower than in the control group, while the SOD activity was higher. Interestingly serum AST, LDH, and MDA levels were significantly lower, and SOD activity was significantly higher, in the superposed group compared with all other groups. Furthermore, while serum AST, LDH, and MDA levels were not different between the 2- and 3-cycle groups, they were significantly lower than in the one-cycle group. In addition, SOD activity was significantly higher than in the 2-

and 3-cycle groups compared with the one-cycle group.

Biochemical parameters cannot fully reflect changes in rat limb functions [23]; electrophysiological examination is an important objective indicator in the peripheral nerve injury diagnosis, intraoperative monitoring, and postoperative recovery judgment. Pedowitz et al. [24] found that 1 day after the application of a 350 mmHg tourniquet, action potential was restored, but structural and functional abnormalities of nerve persisted. Therefore, he hypothesized that electrophysiological examination was one of the most sensitive indicators of nerve injury. The conduction velocity of the motor nerve reflects both the conduction of the neural stem and the function of the neuromuscular junction. In this study, we used electrophysiological measures as important observational indicators. In the first experiment, we found that sciatic nerve function of all the IPC-treated groups was better than the control group, with the superposed group exhibiting the best function. Nerve conduction velocity in the superposed group was significantly better than the other groups. In the second of experiment, we found that while there was no significant difference in nerve conduction velocity between the 2- and 3-cycle groups, both showed significantly better velocity than the 1-cycle group. This indicated that the IPC was protective effects against sciatic nerve IR injury and superposed treatment was most effective.

In this study, we also observed the ultrastructure of each experimental group using electron microscopy. In Experiment 1, we found that the majority of the layer structure of the myelinated nerve was dissolved, with vacuolization in the control group. In addition, the axon was squeezed and some Schwann cell membranes were ruptured. There were signs of damage to the small myelinated nerves in the delay and rapid effect groups; however, the damage was lesser than that in the control group. The ultrastructure of the superposed group was almost normal, strongly suggesting that IPC improved the tolerance of the sciatic nerve to IR injury, with the superposed treatment providing the best protection. In Experiment 2, we observed that the myelinated nerve fibers of the three groups were close to the normal, with the 2- and 3-cycle group showing slightly better fiber structure than the 1-cycle group. Therefore, we speculated that the protective effect of IPC could be positively proportional to the pretreatment frequency within a certain range.

We have conducted a two-part study using continuous observation and assessment with three major outcomes. Firstly, the protective effect of IPC can be found in the peripheral nerves, in addition to cardiac and skeletal muscle as shown in previous studies. Secondly, we confirmed the presence of the immediate and delayed protective effects of IPC described by the previous studies [25] and found no significant difference between these two. Thirdly, we found that superposing the immediate and delayed effect was more protective than the immediate or delayed effect. Furthermore, we explored the relationship between the protective effect of IPC and the number of times it was administered. We found a small, but non-significant difference between 2 and 3 cycles of pretreatment; however, the protection effects were better for both these treatments compared with 1 cycle of pretreatment. This suggests that the protective effect of IPC might be positively proportional to the number of pretreatment cycles within a certain range.

Disclosure of conflict of interest

None.

Address correspondence to: Jiwei Tian, Department of Osteology, Shanghai First People's Hospital, No. 100 Haining Road, Shanghai 200081, China. Tel: +86 21 37798591; Fax: +86 21 37798833; E-mail: jiweitiancn@163.com

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