

Evaluation of Rapid Ischemic Preconditioning in a Rabbit Model of Spinal Cord Ischemia

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Background: Rapid ischemic preconditioning (IPC) has been shown to reduce cellular injury after subsequent cardiac and cerebral ischemia. However, the data on rapid IPC of the spinal cord is limited. The authors investigated whether pretreatment with sublethal ischemia of spinal cord can attenuate neuronal injury after spinal cord ischemia in rabbits.

Methods: Forty-seven male New Zealand white rabbits were randomly assigned to one of three groups (n = 15 or 16 each). In the IPC(-) group, the infrarenal aorta was occluded for 17 min to produce spinal cord ischemia. In the IPC(+) group, 5 min of aortic occlusion was performed 30 min before 17 min of spinal cord ischemia. In the sham group, the aorta was not occluded. Hind limb motor function was assessed at 3 h, 24 h, 4 days, and 7 days after reperfusion using Tarlov scoring (0 = paraplegia; 4 = normal). Animals were killed for histopathologic evaluation at 24 h or 7 days after reperfusion. The number of normal neurons in the anterior spinal cord (L4-L6) was counted.

Results: Neurologic scores were significantly higher in the IPC(+) group than the IPC(-) group at 3 and 24 h after reperfusion ($P < 0.05$). However, neurologic scores in the IPC(+) group gradually decreased and became similar to those in the IPC(-) group at 4 and 7 days after reperfusion. At 24 h after reperfusion, the numbers of normal neurons were significantly higher in the IPC(+) group than in the IPC(-) group ($P < 0.05$) and were similar between the IPC(+) and sham groups. At 7 days after reperfusion, there was no difference in the number of normal neurons between the IPC(+) and IPC(-) groups.

Conclusion: The results indicate that rapid IPC protects the spinal cord against neuronal damage 24 h but not 7 days after reperfusion in a rabbit model of spinal cord ischemia, suggesting that the efficacy of rapid IPC may be transient.

PARAPLEGIA remains a devastating complication of thoracic and thoracoabdominal aortic operations, with the reported incidence ranging from 5% to 40%.¹⁻⁴ The mechanisms of postoperative paraplegia involve both acute ischemic and delayed reperfusion injury. Several attempts using hypothermia, cerebrospinal fluid drainage, *N*-methyl-D-aspartate receptor antagonists, calcium channel blockers, and free radical scavengers have been made to protect spinal cord against ischemic injury.² However, because their efficacy is limited, a novel therapy to protect against spinal cord ischemia remains a matter of debate.

Ischemic preconditioning (IPC) is a phenomenon whereby a brief episode of sublethal ischemia and reperfusion markedly reduces tissue injury induced by a subsequent prolonged ischemic injury.⁵ An understanding of this phenomenon, which has been designated as *ischemic tolerance*, can contribute to the development of new strategies for preventing ischemic injury but also would provide insight into the factors that may attenuate ischemic tissue damage. There are two types of IPC. Rapid IPC induces ischemic tolerance in minutes to hours after sublethal ischemia, whereas delayed IPC requires days to show ischemic tolerance. Rapid IPC was first described in the myocardium by Murray *et al.*,⁶ and several reports have recently demonstrated the efficacy of rapid IPC in the brain.⁷⁻⁹

The degree of neuronal protection by rapid IPC may be affected by the intensity of sublethal and lethal ischemia and the interval between the sublethal and lethal ischemic injuries. In addition, recent evidence suggests that neuroprotective efficacy of rapid IPC may not be permanent. Perez-Pinzon *et al.*⁶ demonstrated that neuroprotective effect of rapid IPC against neuronal injury after global ischemia was observed 3 days after reperfusion, but its efficacy was no longer apparent at 7 days after reperfusion. Although another report suggested the neuroprotective efficacy of rapid IPC at 7 days after reperfusion in a model of focal cerebral ischemia,⁸ postreperfusion recovery period is one of the important determinants for neuroprotective efficacy of rapid IPC. However, regarding the effects of rapid IPC on spinal cord ischemia, to our knowledge, there were no reports to assess neurologic outcome beyond 2 days after reperfusion. Therefore, in the current study, we evaluated neuroprotective efficacy of rapid IPC in a rabbit model of spinal cord ischemia after a short (24-h) and a relatively long (7-day) recovery period.

Materials and Methods

The study was approved by the Animal Experiment Committee of Nara Medical University (Kashihara, Nara, Japan). Forty-seven male New Zealand White rabbits weighing 3.1 ± 0.2 kg (mean \pm SD) were used in this study. The rabbits were housed and maintained on a 12-h light-dark cycle with free access to food and water. All animals were neurologically intact before anesthesia and surgical procedure.

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Surgical Preparation

The rabbits were fasted overnight and then anesthetized in a plastic box with 5% isoflurane in oxygen. Then, they were allowed to breathe 2–3.5% isoflurane in 40% oxygen spontaneously with a nonsealing facemask device. An ear vein catheter was inserted for the infusion of saline at rate of $10 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ and drugs. An ear artery was cannulated to monitor proximal mean arterial blood pressure. After infiltration with 1% lidocaine, the right femoral artery was exposed and cannulated 7 cm with SP-55 catheter for monitoring distal mean arterial blood pressure and sampling blood to evaluate blood gases, serum glucose, and hematocrit. A 5-cm left flank incision parallel to the spine was made at the twelfth costal level after preparation of the skin with iodine and infiltration of 1% lidocaine, and then the infrarenal aorta was exposed retroperitoneally. A silicone string 1.5 mm in breadth was carefully placed around the aorta immediately distal to the left renal artery. Then, both ends of the silicone string were passed through an occluder tube for occlusion of the aorta to produce spinal cord ischemia. After completion of surgical preparation, 600 U intravenous heparin was administered.

Experimental Protocol

Animals were randomly assigned to one of three groups: IPC(–) group ($n = 15$), IPC(+) group ($n = 16$), or sham group ($n = 16$). In the IPC(–) group, the infrarenal aorta was occluded for 17 min to produce spinal cord ischemia. In the IPC(+) group, 5 min of aortic occlusion was performed 30 min before 17 min of spinal cord ischemia. In the sham group, the aorta was not occluded. With respect to reperfusion periods, each group was further divided into a 24-h or 7-day recovery group in random order ($n = 7$ or 8 each). Throughout the operative period, paravertebral muscle temperature was monitored by a needle-type thermistor and kept at 38°C by using a water blanket. Proximal and distal mean arterial blood pressure and heart rate were monitored continuously and were recorded after surgical preparation (baseline), at a half-time point of 17 min of ischemia, and 10 min after reperfusion. Arterial pressure of oxygen (PaO_2), arterial pressure of carbon dioxide (PaCO_2), pH, serum glucose, and hematocrit were measured after surgical preparation (baseline). Spinal cord ischemia was produced by pulling the silicone string and clamping the occluder tube. Reperfusion of the spinal cord was made by removing the occluder tube. After the final measurement was made, all vascular catheters were removed and wounds were sutured. Isoflurane was discontinued, and the animals were allowed to recover.

Neurologic Evaluation

At 3 h, 24 h, 4 days, and 7 days after the ischemic injury, the animals were assessed neurologically by blinded observers. Assessment was made using Tarlov

scoring,¹⁰ which consists of a five-point grading scale: 0 = paraplegic with no lower extremity function; 1 = poor lower extremity function, weak antigravity movement only; 2 = some lower extremity motor function with good antigravity strength but inability to draw legs under body or hop; 3 = ability to draw legs under body and hop but not normally; 4 = normal motor function.

Histologic Evaluation

After scoring of neurologic function at 24 h or 7 days, the animals were anesthetized with 5% isoflurane in oxygen in a plastic box followed by administration of 50 mg/kg thiopental intraperitoneally. Transcardiac perfusion and fixation were performed with 1,000 ml heparinized cold saline solution followed by 500 ml buffered formalin, 10%. The lumbar spinal cord was removed and immersed in formalin for 2 days. The L4, L5, and L6 sections were dissected in 5-mm blocks. The blocks of tissues were embedded in paraffin. Transverse sections (3 μm) of each block were sliced for hematoxylin and eosin staining. Normal neuronal cells in the anterior spinal cord (anterior to a line drawn through the central canal perpendicular to the vertebral axis) of L4–L6 sections were counted, and the numbers of normal neuronal cells of each sections were totaled.

Statistical Analysis

Physiologic variables were analyzed using one-way analysis of variance, and when significant differences were identified, the Student-Neumann-Keuls test was performed for intergroup comparisons. Hind limb motor function was analyzed using nonparametric method (Kruskal-Wallis test) followed by the Mann-Whitney U test. The numbers of normal neuronal cells in the anterior spinal cord was analyzed by one-way analysis of variance, and when significant differences were identified, the Student-Neumann-Keuls test was performed for intergroup comparisons. A P value less than 0.05 was considered statistically significant. The physiologic data and the numbers of the normal neuronal cells are expressed as mean \pm SD, and the neurologic function scores are expressed as medians with interquartile ranges in parentheses.

Results

There were no differences in the weight of the animals among the groups. Table 1 shows the blood gas data and glucose concentration at baseline. There were no significant differences in pH, PaCO_2 , hematocrit, and glucose concentration among the groups. PaCO_2 values at 7 days after reperfusion in the IPC(+) group were significantly higher compared with those in the IPC(–) group. Changes in hemodynamic parameters and temperature during the experimental period are shown in table 2.

Table 1. Physiologic Variables at Baseline

	IPC(-)	IPC(+)	Sham
pH			
24 h	7.41 ± 0.06	7.40 ± 0.06	7.45 ± 0.05
7 days	7.45 ± 0.05	7.41 ± 0.04	7.43 ± 0.01
Paco ₂ , mmHg			
24 h	33 ± 6	35 ± 5	36 ± 6
7 days	35 ± 9	39 ± 7	33 ± 6
Pao ₂ , mmHg			
24 h	231 ± 47	248 ± 67	267 ± 109
7 days	274 ± 35	219 ± 31*	222 ± 54
Hematocrit, %			
24 h	41 ± 5	40 ± 2	39 ± 5
7 days	39 ± 4	41 ± 3	38 ± 4
Glucose, mg/dl			
24 h	178 ± 54	155 ± 55	144 ± 34
7 days	164 ± 47	131 ± 13	163 ± 42

Data are expressed as mean ± SD. Ischemic preconditioning (IPC)(-) rabbits received 17 min of spinal cord ischemia. IPC(+) rabbits received 5 min of sublethal ischemia 30 min before 17 min of spinal cord ischemia. Sham rabbits received sham operation.

* $P < 0.05$ vs. IPC(-).

Paco₂ = arterial partial pressure of carbon dioxide; Pao₂ = arterial partial pressure of oxygen.

Mean arterial pressure at baseline and after reperfusion were similar among the three groups. Distal mean arterial pressure during ischemia was similar between the IPC(-) and IPC(+) groups. There were no significant differences in heart rate and paravertebral temperature among the groups.

Neurologic Outcome

The hind limb motor functions at each time point of reperfusion are shown in table 3. All animals in sham group were neurologically normal at 3 h, 24 h, 4 days, and 7 days after reperfusion. Neurologic scores in the IPC(+) group and IPC(-) group were significantly lower than those in the sham group 3 h, 24 h, 4 days, and 7 days after reperfusion, while neurologic scores in the IPC(+) group were higher than in the IPC(-) group at 3 and 24 h after reperfusion. Neurologic scores in the IPC(+) group gradually decreased and became similar to those in the IPC(-) group at 4 and 7 days after reperfusion. Individual neurologic scores at the final assessments (24 h or 7 days) are shown in figure 1.

Histopathologic Outcome

Histopathology of the lumbar spinal cord at 24 h after reperfusion was examined in eight animals of each group, and that at 7 days after reperfusion was examined in seven animals in the IPC(-) group, and eight animals in the IPC(+) group, and eight animals in the sham group. Individual numbers of normal neurons at the final assessment (24 h or 7 days) are shown in figure 1. At 24 h after reperfusion, the number of normal neurons in the anterior spinal cord in the IPC(+) group was significantly higher than in the IPC(-) group ($P < 0.05$) and

was similar to the sham group (fig. 2A). However, at 7 days after reperfusion, the number of normal neurons in the IPC(+) group was significantly less than in the sham group ($P < 0.05$), and there was no significant difference in the number of normal neurons between the IPC(+) group and the IPC(-) group (fig. 2B).

Discussion

The results of the current study show that preconditioning with 5 min of spinal cord ischemia 30 min before subsequent lethal ischemia protected the spinal cord

Table 2. Hemodynamic Parameters and Temperature during the Experimental Period

	IPC(-)	IPC(+)	Sham
Proximal-mean arterial pressure, mmHg			
Baseline			
24 h	69 ± 9	64 ± 10	70 ± 8
7 days	56 ± 1	59 ± 6	64 ± 11
During ischemia			
24 h	56 ± 10	64 ± 9	
7 days	62 ± 4	59 ± 8	
Reperfusion			
24 h	54 ± 9	61 ± 5	68 ± 11
7 days	60 ± 7	56 ± 8	58 ± 5
Distal mean arterial pressure, mmHg			
Baseline			
24 h	66 ± 7	68 ± 10	71 ± 9
7 days	60 ± 2	63 ± 7	70 ± 11
During ischemia			
24 h	11 ± 1	13 ± 2	
7 days	12 ± 1	10 ± 2	
Reperfusion			
24 h	59 ± 11	64 ± 7	72 ± 13
7 days	63 ± 8	60 ± 5	63 ± 6
Heart rate, beats/min			
Baseline			
24 h	306 ± 23	284 ± 35	289 ± 30
7 days	283 ± 25	292 ± 42	289 ± 30
During ischemia			
24 h	300 ± 18	284 ± 41	
7 days	288 ± 22	300 ± 31	
Reperfusion			
24 h	283 ± 30	289 ± 32	295 ± 13
7 days	289 ± 16	305 ± 21	311 ± 16
Paravertebral temperature, °C			
Baseline			
24 h	38.5 ± 0.9	38.0 ± 0.2	38.4 ± 0.7
7 days	37.9 ± 0.2	38.0 ± 0.3	38.1 ± 0.4
During ischemia			
24 h	37.6 ± 0.3	37.9 ± 0.2	
7 days	37.7 ± 0.4	37.9 ± 0.3	
Reperfusion			
24 h	38.2 ± 0.3	38.0 ± 0.1	38.0 ± 0.2
7 days	38.2 ± 0.2	38.2 ± 0.1	38.1 ± 0.2

Data are expressed as mean ± SD. There were no significant differences among the three groups. Ischemic preconditioning (IPC)(-) rabbits received 17 min of spinal cord ischemia. IPC(+) rabbits received 5 min of sublethal ischemia 30 min before 17 min of spinal cord ischemia. Sham rabbits received sham operation.

Table 3. Hind Limb Motor Function

	IPC(-)	IPC(+)	Sham
3 h	2 (0-2) (n = 15)	3 (3-4)* (n = 16)	4 (4-4)* (n = 16)
24 h	1 (0-2) (n = 15)	2.5 (1.5-4)* (n = 16)	4 (4-4)* (n = 16)
4 days	0 (0-1) (n = 7)	1 (1-2) (n = 8)	4 (4-4)* (n = 8)
7 days	0 (0-0) (n = 7)	0 (0-1.5) (n = 8)	4 (4-4)* (n = 8)

Data are expressed as median with interquartile range in parentheses. Hind limb motor function was assessed using Tarlov scoring (0 = paraplegic with lower extremity function; 1 = poor lower extremity function, weak antigravity movement only; 2 = some lower extremity motor function with good antigravity strength but inability to draw legs under body or hop; 3 = ability to draw legs under body and hop but not normally; 4 = normal motor function). Ischemic preconditioning (IPC(-)) rabbits received 17 min of spinal cord ischemia. IPC(+) rabbits received 5 min of sublethal ischemia 30 min before 17 min of spinal cord ischemia. Sham rabbits received sham operation.

* $P < 0.05$ vs. IPC(-).

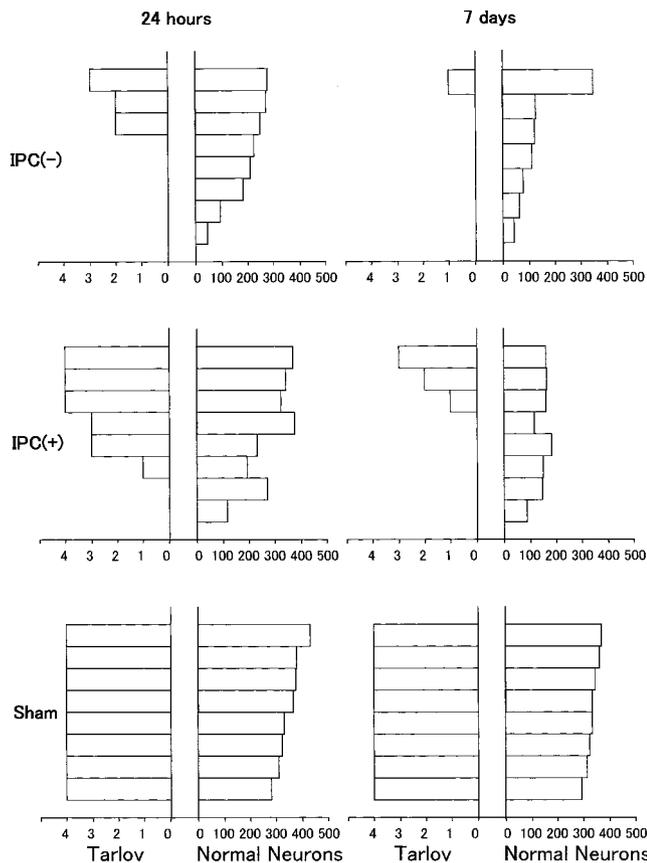


Fig. 1. Histograms of neurologic score and number of normal neurons per animal in the six experimental groups. Hind limb motor function was assessed using Tarlov scoring (0 = paraplegic with no lower extremity function; 1 = poor lower extremity function, weak antigravity movement only; 2 = some lower extremity motor function with good antigravity strength but inability to draw legs under body or hop; 3 = ability to draw legs under body and hop but not normally; 4 = normal motor function). Animals in the ischemic preconditioning (IPC(-)) group received 17 min of spinal cord ischemia, and those in the IPC(+) group received 5 min of sublethal ischemia 30 min before 17 min of spinal cord ischemia. In the sham group, the aorta was not occluded.

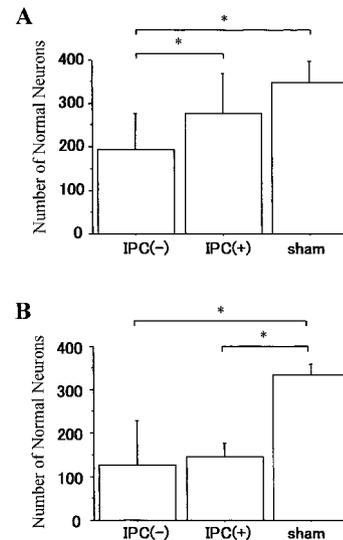


Fig. 2. Number of normal neurons at 24 h (A) and 7 days (B) after 17 min of spinal cord ischemia. Animals in the ischemic preconditioning (IPC(-)) group received 17 min of spinal cord ischemia, and those in the IPC(+) group received 5 min of sublethal ischemia 30 min before 17 min of spinal cord ischemia. In the sham group, the aorta was not occluded. Data are presented as mean \pm SD. * $P < 0.05$ versus IPC(-)

against ischemic neuronal damage at 24 h after reperfusion in rabbits. However, this protective efficacy of preconditioning was no longer apparent at 7 days after reperfusion. These results suggest that efficacy of rapid IPC on spinal cord may be transient.

There have been a few reports regarding the rapid IPC on spinal cord. Zvara *et al.*¹¹ investigated the effects of IPC induced by 3 min of ischemia with a 30-min reperfusion interval on spinal cord damage by a subsequent 12 min of ischemia in a rat model of spinal cord ischemia and demonstrated that IPC reduced neurologic injury at 24 and 48 h after reperfusion. Sirin *et al.*¹² also investigated the protective effect of IPC with 5 min of ischemia and a 25-min reperfusion interval on spinal cord injury mediated by a subsequent 20 min of spinal cord ischemia in rabbits and demonstrated that IPC reduced the spinal cord injury and improved neurologic outcome 48 h after reperfusion. Similar results regarding rapid IPC on the brain have been demonstrated in a model of global and focal cerebral ischemia. Perez-Pinzon *et al.*⁷ demonstrated that IPC with 2 min of ischemia and a 30-min reperfusion interval reduced brain damage from 10 min of ischemia at 3 days after reperfusion in a rat model of global ischemia. Stagliano *et al.*⁸ have shown that one or three 5-min episodes of ischemia given 30 min before lethal ischemia reduced infarct volume at 24 h after 60 min or permanent ischemia in a mouse model of focal cerebral ischemia. These results are compatible with the results in the current study, in which IPC with a 30-min reperfusion interval reduced neuronal injury in rabbits subjected to spinal cord ischemia at 24 h after reperfusion. These indicate that rapid IPC can ameliorate the

injury of the brain and spinal cord especially at an early time point (up to 3 days) after ischemic injuries.

However, the long-term efficacy of rapid IPC for brain damage remains controversial. Perez-Pinzon *et al.*⁷ demonstrated that rapid IPC with 2 min of ischemia protected rats against ischemic neuronal damage after 3 days but not 7 days of reperfusion after 10 min of global cerebral ischemia. Kato *et al.*¹³ reported that 2 min of sublethal ischemia with a short interval did not affect neuronal damage 7 days after a subsequent 3 min of bilateral carotid occlusion in gerbils. By contrast, Nakamura *et al.*⁹ indicated that the efficacy of IPC with 30 min of ischemia and a 30-min reperfusion interval lasted for 7 days in a rat model of middle cerebral artery occlusion. To our knowledge, this is the first report to evaluate clearly the short-term and relatively long-term efficacy of rapid IPC on spinal cord ischemia. The results indicate that rapid IPC may delay but not prevent spinal cord damage after subsequent spinal cord ischemia. Although the protective efficacy of rapid IPC may not be permanent, this efficacy is probably beneficial because it can prolong the therapeutic window after ischemic injury.

Mechanisms by which rapid IPC can protect the spinal cord at an early point after ischemic injury are unknown. However, possible mechanisms are as follows. First, adenosine and adenosine triphosphate-sensitive potassium channels may be involved in the acquisition of ischemic tolerance by rapid IPC. Nakamura *et al.*⁹ reported that rapid tolerance to focal cerebral ischemia in rats was attenuated by adenosine A1 receptor antagonist. A contribution of adenosine triphosphate-sensitive potassium channels in the acquisition of ischemic tolerance has been repeatedly demonstrated in the myocardium.¹⁴ Caparrelli *et al.*¹⁵ demonstrated that administration of a potent mitochondrial adenosine triphosphate-sensitive potassium channel opener, diazoxide, improved neurologic injury in a model of spinal cord ischemia. Second, rapid IPC may modulate inflammatory process after ischemia. Perez-Pinzon *et al.*¹⁶ recently reported that rapid IPC reduced microglial activation after subsequent cerebral ischemia, suggesting that beneficial effects of IPC may involve an antiinflammatory process. Finally, rapid IPC may affect spinal cord blood flow and catecholamine concentration. Fan *et al.*¹⁷ reported that rapid IPC might enhance the tolerance of the spinal cord by increasing spinal cord blood flow and decreasing norepinephrine concentration after lethal ischemia. Further study would be required to clarify the mechanisms of beneficial effects of rapid IPC on the spinal cord.

There are methodologic limitations to merit comments. First, because the efficacy of rapid IPC can vary depending on the severity of lethal ischemia, the results in the current study might differ if we selected milder lethal ischemia. Perez-Pinzon *et al.*⁷ demonstrated that rapid IPC was not effective 7 days after a 10-min test ischemia. However, when the test ischemia was reduced

to 7 min, a clear trend of neuroprotection by IPC was observed at 7 days after reperfusion. Second, the method for preconditioning might affect the results. In the current study, we used single, brief (5-min) ischemia 30 min before lethal ischemia for IPC. Based on the results of the previous reports^{11,12} and our preliminary study, we selected a 30-min reperfusion interval. However, with a shorter or longer reperfusion interval, the protective efficacy of IPC might differ 7 days after reperfusion. Furthermore, previous reports suggested that the efficacy of rapid IPC was stronger when brief ischemia was repeated three times.⁸ Repetitive IPC might have protected the spinal cord against neuronal damage after 7 days following reperfusion. Finally, because sample sizes in the current study may be small for long-term follow-up, further study with more sample sizes may be required. However, it is unlikely that increasing number can detect any differences in motor function and the number of normal neurons between the IPC(+) and IPC(-) groups 7 days after reperfusion.

In conclusion, we investigated the neuroprotective efficacy of rapid IPC in a rabbit model of spinal cord ischemia after a short (24-h) and a relatively long (7-day) recovery period. The results indicate that rapid IPC with brief ischemia protected against ischemic neuronal injury 24 h after subsequent lethal ischemia. This ischemic tolerance was induced within 30 min. However, the protective efficacy of rapid IPC did not last for 7 days after lethal ischemia. Although these findings suggest that rapid IPC can prolong the therapeutic window for ischemic injury, further strategies on the prevention or treatment for spinal cord damages are required.

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