Ischemic postconditioning protects the spinal cord from ischemia–reperfusion injury via modulation of redox signaling

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Background: It is well known that ischemic postconditioning reduces ischemic–reperfusion injury, but the underlying mechanism is not fully understood. The current study investigated the role of reactive oxygen species–mediated upregulation of endogenous antioxidant enzymes in the generation of a protective effect induced by ischemic postconditioning against spinal cord reperfusion injury in the rabbit.

Methods: New Zealand White rabbits were randomly allocated to sham, ischemia–reperfusion, and postconditioning groups (3 cycles of 30 seconds of reperfusion and 30 seconds of occlusion during the onset of reperfusion). Spinal cord ischemia was induced by clamping the infrarenal abdominal aorta for 20 minutes in the ischemia–reperfusion and postconditioning groups. Forty-eight hours after reperfusion, the neurologic status of the lower limbs was assessed. Blood samples were collected for analysis of serum neuron-specific enolase levels, and the lumbar spinal cord segments (L5-7) were harvested for histopathologic and antioxidant enzyme activities and mRNA analysis with or without administration of N-2-mercaptopropionylglycine (an effective oxygen free radical scavenger) given at different reperfusion times.

Results: Continuous administration of N-2-mercaptopropionylglycine for 13 minutes, starting at 10 minutes before (but not 10 minutes after) the beginning of reperfusion, attenuated the neuroprotective effect of postconditioning against spinal cord ischemia and reversed the increase in activity of the antioxidant enzymes superoxide dismutase and catalase in spinal cord tissue subjected to ischemic postconditioning.

Conclusions: The results indicate that reactive oxygen species–triggered upregulation of endogenous antioxidant enzyme activities may be involved in the mechanism of neuroprotection of ischemic postconditioning. (J Thorac Cardiovasc Surg 2013;146:688-95)

Despite countless therapeutic strategies and clinical studies to address ischemia–reperfusion (I/R) injury, paraplegia related to the surgical repair of descending and thoracoabdominal aortic aneurysms still occurs. 1, 2 Therefore, novel measures to protect the spinal cord against surgical ischemic injury require further exploration.

After the concept of attenuating reperfusion injury was introduced, 3 ischemic preconditioning (IPC) was first proposed by Murry and colleagues 4 in 1986. IPC had a powerful endogenous protection to enhance the tolerance against ischemia in many organs, such as the heart, brain, and spinal cord. Because the onset of ischemia cannot be predicted and IPC implementation may present a potential risk to patients, IPC is unfeasible in clinical situations. A novel approach termed “postconditioning” (PostC), in which brief repeated cycles of intermittent reperfusion and reocclusion are applied at the onset of reperfusion after a prolonged period of ischemia, significantly reduced myocardial infarct size. 5 PostC has been successfully used in clinical procedures. 6 Our preliminary study demonstrated that PostC, consisting of 3 cycles of 30 seconds of reperfusion and 30 seconds of reocclusion, significantly preserved locomotor function and decreased the paraplegia rate in rabbits subjected to 20 minutes of spinal cord ischemia. Activities of superoxide dismutase (SOD) and catalase in the spinal cord tissue of animals in the PostC group were significantly higher compared with the control group at approximately 30 minutes and 6 hours after reperfusion. 7 However, whether the increase of antioxidant enzyme activities is directly associated with the mechanism of neuroprotection of PostC against reperfusion injury remains unknown.

After an ischemic stroke, the production of reactive oxygen species (ROS) may increase, leading to tissue damage. 8 However, ROS have been paradoxically implicated as an essential signaling component in protective pathways that
mediate the effect of IPC\textsuperscript{9} and pharmacologic PostC.\textsuperscript{10} ROS are obligatory mediators of the signal transduction pathway to upregulate antioxidant enzymes and induce the cardiac protective effects of ischemic PostC.\textsuperscript{11} Oxidative stress related to the enormous generation of ROS may play an important part in reperfusion injury. Excessive ROS production is considered injurious to cell function and mitochondria. On the other hand, low levels of ROS may perform as secondary messengers, modulating signaling pathways by covalent medications of target molecules, which is referred to as “redox signaling.” The current study attempts to provide evidence for the role of ROS in the mechanism of ischemic PostC in a rabbit spinal cord ischemic model.

**MATERIALS AND METHODS**

All experiments performed in this study were approved by the Ethics Committee for Animal Experimentation and conducted according to the Guidelines for Animal Experimentation of the Fourth Military Medical University. The study was conducted in Xijing Hospital, Fourth Military Medical University (Xi’an, China).

**Animal Care**

Male New Zealand White rabbits weighing 2.2 to 2.5 kg were obtained from the experimental Animal Center of the Fourth Military Medical University, Xi’an, China, and were housed in an animal center in Xijing Hospital. The experiments described in this study were approved by the Committee of Animal Study of the Fourth Military Medical University. The animals had access to standard rabbit chow and water ad libitum.

**Experimental Protocol**

Part I of the experiment determined the effect of N-2-mercaptopropanoylglycine (MPG) on the induction of ischemic tolerance and antioxidant enzyme activities by PostC. To detect the effect of MPG on the neuroprotection of PostC against spinal cord I/R injury, 84 male New Zealand White rabbits were randomly assigned to 8 groups (n = 6 in each group; Figure 1). Except for the sham group, all animals were subjected to occlusion of the infrarenal abdominal aorta for 20 minutes, followed by reperfusion for 48 hours with or without PostC (3 cycles of 30 seconds of reperfusion and 30 seconds of ischemia). Animals in the vehicle group received an intravenous injection of saline water, and animals in the MPG and PostC + MPG groups received an intravenous injection of MPG dissolved in deionized water (Sigma-Aldrich, St Louis, MO), a potent ROS scavenger, at 0.5 mL·kg\textsuperscript{-1}·min\textsuperscript{-1} with a micro-syringe pump for 13 minutes (started 10 minutes before reperfusion). In the MPG(D) and PostC + MPG(D) groups, MPG was administered 10 minutes after reperfusion for 13 minutes. The dose and administration method of MPG in this experiment were chosen in line with previous research.\textsuperscript{12} At 48 hours after reperfusion, the blood samples were collected for analysis of serum neuron-specific enolase (NSE) levels, the motor function of the hind limbs was scored, and the number of normal motor neurons at the anterior horn of the spinal cord (L5) was counted in a blinded fashion.

Part II of the experiment investigated the effect of MPG on antioxidant enzyme activities (SOD and catalase) after PostC. Thirty male rabbits were randomly divided into 5 groups (n = 6 for each group). All animals were subjected to occlusion of the infrarenal abdominal aorta for 20 minutes, followed by reperfusion for 6 hours. Animals in the Con and PostC groups received a deionized water injection, and animals in the MPG and PostC + MPG groups received MPG (2% [w/v], 0.5 mL·kg\textsuperscript{-1}·min\textsuperscript{-1}) 10 minutes before reperfusion. After the neurologic outcomes were evaluated 6 hours after reperfusion, the spinal cord tissue (L5-7) was harvested and frozen at –80°C to determine SOD and catalase activities by spectrophotometric methods as previously described.\textsuperscript{13,14} mRNA expression levels of the antioxidant enzymes were measured by semiquantitative reverse transcriptase polymerase chain reaction.

**Surgical Preparation**

Animals were neurologically intact before anesthesia and instrumentation. General anesthesia was induced by intravenous injection of sodium pentobarbital (30 mg/kg).\textsuperscript{15} Rabbits did not receive mechanical ventilation support throughout the procedure and were allowed to breathe spontaneously with an oxygen facemask at a flow rate of 2 L/min. Lactated Ringer’s solution was infused intravenously at a rate of 4 mL·kg\textsuperscript{-1}·h\textsuperscript{-1}. In all animals, a 22-gauge catheter was inserted into the ear artery to measure the arterial blood pressure and sample arterial blood. Another catheter was inserted into the left femoral artery to measure femoral blood pressure. Arterial blood pressure and heart rate were monitored continuously using a calibrated pressure transducer connected to an invasive pressure monitor (Colin BP-508 type S; Omron Colin, Tokyo, Japan). Rectal temperature was maintained at 38.5°C ± 0.5°C using a heating blanket and overhead lamp. Arterial blood was sampled at baseline, preischemia, 10 minutes after ischemia, the end of PostC, and 10 minutes after reperfusion to determine the arterial oxygen tension, arterial carbon dioxide tension, pH, and blood glucose. Arterial blood gases were measured using the Rapid Lab 1260 (Bayer HealthCare AG, Leverkusen, Germany).

**Spinal Cord Ischemia**

Spinal cord ischemia was induced by infrarenal aortic occlusion as described in our previous studies.\textsuperscript{9,13} Briefly, the rabbits were placed in the supine position after local anesthesia. The abdominal aorta was exposed at the level of the left renal artery through a 3- to 4-cm–long medial incision. Heparin (400 units) was administered 5 minutes before aortic occlusion. Spinal cord ischemia was induced by clamping the aorta with a bulldog clamp just below the renal artery. Occlusion of blood flow lasted 20 minutes. The bulldog clamp was removed, and stabilization of arterial blood pressure was monitored for an additional 20 minutes. The abdominal wall was closed with wound clips. Local infiltration with 0.25% (w/v) bupivacaine hydrochloride around the wound was applied for postoperative analgesia. An antibiotic (40,000 units of gentamicin) was administered intramuscularly immediately after the operation. Animals were placed on a heating pad to maintain body temperature until recovery from anesthesia and then were returned to their home cage. Animals survived for 2 days. Bladder content was compressed manually as required.

**Neurologic Outcome Assessment**

Neurologic status was assessed 6, 24, and 48 hours after reperfusion by a member of the research team who was unaware of the grouping. Modified Tarlov criteria were used: 0 = no voluntary hind-limb function, 1 = only perceptible joint movement, 2 = active movement but unable

**Abbreviations and Acronyms**

- GAPDH = glyceraldehyde 3-phosphate dehydrogenase
- IPC = ischemic preconditioning
- I/R = ischemia–reperfusion
- MPG = N-2-mercaptopropionylglycine
- NSE = neuron-specific enolase
- PostC = postconditioning
- ROS = reactive oxygen species
- SOD = superoxide dismutase
until studied. NSE was measured using chemiluminescent immunometric assays (Roche Applied Science, Indianapolis, Ind; Catalogue no. 12133121122), and the results were expressed as nanograms/milliliter.

**Measurement of Antioxidant Enzyme Activity**

In part II of the experiment, animals were killed 6 hours after reperfusion, and the spinal cords (L5-7) were quickly harvested, weighed, and homogenized. All procedures were performed at 0°C. The homogenate was centrifuged at 3000 × g for 15 minutes at 4°C, and the supernatant was collected and frozen at −80°C for the next assay. Measurements of SOD and catalase activities in tissue homogenates were processed spectrophotometrically according to the specifications of the detection kit provided by the manufacturer (Jian Chen Biologic Institute, Nanjing, Jiangsu, China).

Xanthine–xanthine oxidase was used to generate superoxide flux. The absorbance obtained from the reduction of nitroblue tetrazolium to a blue formazan product by superoxide was determined at 560 nm spectrophotometrically at room temperature. One unit of SOD activity is defined as the amount that reduces the absorbance change by 50%, and the activity was normalized on the basis of total protein content (units/milligram protein).

**Catalase activity in spinal cord tissue was determined by spectrophotometric methods based on the hydrogen peroxide decomposition rate at 240 nm (using an ultraviolet light spectrophotometer, Beckman Coulter Inc, Fullerton, Calif) in the reactive mixture. Catalase activity was given in units/milligram protein.**

**Reverse Transcriptase Polymerase Chain Reaction**

The total RNA was extracted from spinal cord tissues by Trizol (Invitrogen Technology, Carlsbad, Calif) according to the manufacturer’s directions. Gene-specific primers for Cu,Zn-SOD (forward: 5'-GTGTCGCCGTTCTGAAAGGCGA-3' and reverse: 5'-CATTCCACCTTGGCCCAAGTC-3'), catalase (forward: 5'-AGGTTGGTCTCCAACTACTAC-3' and reverse: 5'-GGCTTGAGGATGTTACTGTC-3'), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward: 5'-GCATACATGAGGAGTTTCCA-3' and reverse: 5'-GCTAAGCAGTGTTGGTGAGAG-3') were adopted from the study by Cigremis and colleagues. Equal amounts of RNA (500 ng) from each sample were reverse transcribed in a volume of 10 μL to produce cDNA using the Takara RNA PCR Kit (AMV) version 3.0 (TaKaRa Bio Inc, Otsu, Japan). The reaction conditions were as follows: initial denaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, annealing at 60°C (Cu,Zn-SOD and GAPDH) or 45°C (catalase) for 30 seconds, and extension at 72°C for 30 seconds. There were 32 polymerase chain reaction cycles. Standard curves plotting the threshold amplification cycle number values against input quantity for each gene were constructed using 5-fold serial dilutions of the reverse transcriptase product. GAPDH served as an endogenous internal standard control. Detection based on SYBR Green I (Applied Biosystems, Foster City, Calif) was carried out on a real-time polymerase chain reaction instrument (ABI PRISM 7300; Applied Biosystems) with a thermal cycler.

**Statistical Analysis**

All data, excluding the neurologic function score, are expressed as the mean ± standard error of the mean. The neurologic function scores are expressed as the median (minimum–maximum value). The physiologic variables, serum NSE level, antioxidant enzyme activities, and mRNA in the spinal cord were analyzed using 1-way analysis of variance followed by the Student–Newman–Keuls test for multiple comparisons. The scores of hind-limb motor function and the number of normal neurons in the anterior spinal cord were analyzed with the nonparametric Kruskal–Wallis test followed by the Mann–Whitney U test with Bonferroni correction. All statistical analyses were performed using the Statistical Package for the Social Sciences version 13.0 (SPSS Inc, Chicago, Ill).
RESULTS

Physiologic Variables
There were no differences in relevant physiologic parameters among the groups regardless of treatment patterns. The value of distal blood pressure decreased to 7 to 11 mm Hg after the abdominal artery was clamped. Ten minutes after the beginning of reperfusion, the value was restored to approximately preischemic levels (Table 1). During the experiment, the rectal temperature was maintained between 38°C and 39°C by an overhead lamp.

Neurobehavioral Manifestation
All animals survived until the final neurologic evaluation at 48 hours after reperfusion. The neurologic function scores after reperfusion are shown in Figure 2, C. All animals in the Con group developed paraplegia in the hind limb (Tarlov score ≤ 2) 48 hours after reperfusion, whereas 4 animals in the PostC group had preserved motor function (Tarlov score ≥ 3). The PostC group showed significantly higher hind-limb motor function scores than the Con group (P = .002 vs the Con group). Nevertheless, the neurologic function outcome in the PostC + MPG group was worse than in the PostC group 48 hours after reperfusion (P = .004 PostC vs PostC + MPG). There were no significant differences between the PostC and the PostC + MPG(D) groups (P = .699).

Histopathologic Outcomes
The histopathologic results at 48 hours after reperfusion are shown in Figure 2, A and B. Compared with the Con group, there were more normal neurons observed in the anterior spinal cord of the animals in the PostC group (P < .001 vs Con) and PostC + MPG(D) group (P < .001 vs Con). However, there was no significant difference in the numbers of normal neurons between the Con group and the PostC + MPG group (P = .382).

Serum Neuron-Specific Enolase Levels
There was a significant increase in serum NSE levels in the control animals compared with the sham animals. The NSE levels obtained in the PostC (P = .026 vs Con; Figure 3) and PostC + MPG(D) (P = .036 vs Con; Figure 3) groups were significantly lower than in the Con group, and there was no significant difference compared with the sham group. There was no difference in serum NSE levels between the PostC and PostC + MPG(D) groups (P = .885).

Antioxidant Activities
As in Part 1, the rabbits in the PostC group had significantly higher hind-limb motor function scores than the rabbits in the Con group 6 hours after reperfusion (P = .04; Figure 4, A). A higher activity of SOD (P = .009 vs Con)

### TABLE 1. Physiologic parameters for experiment 1 (n = 6)

<table>
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<tr>
<th></th>
<th>MAP (mm Hg)</th>
<th>HR (beats/min)</th>
<th>T (°C)</th>
<th>BG (mmol/L)</th>
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<td>89 ± 1</td>
<td>91 ± 1</td>
<td>261 ± 5</td>
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<td>6.2 ± 0.1</td>
<td>7.37 ± 0.02</td>
<td>102 ± 1</td>
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<tr>
<td>PostC</td>
<td>90 ± 1</td>
<td>91 ± 2</td>
<td>259 ± 5</td>
<td>38.6 ± 0.1</td>
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<td>7.36 ± 0.01</td>
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<td>257 ± 5</td>
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<td>PostC + MPG</td>
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<td>I/R + (D)MPG</td>
<td>88 ± 2</td>
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<td>91 ± 1</td>
<td>256 ± 4</td>
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All values are presented as the mean ± standard error of the mean. All animals underwent 20 minutes of ischemia and 48 hours of reperfusion. The ischemic PostC group received 3 cycles of 30 seconds of reperfusion and 30 seconds of occlusion during the onset of reperfusion. The I/R + MPG and PostC + MPG groups were treated with MPG (2% [w/v], 0.5 mL·kg⁻¹·min⁻¹) administered intravenously 10 minutes before reperfusion. I/R + (D)MPG and PostC + (D)MPG groups were treated with MPG 10 minutes after reperfusion. Hemodynamic data: MAP and HR. Gasometric data: pH, Pao2, Paco2, BG, and T. MAP: Mean arterial pressure; HR: heart rate; T: rectal temperature; BG: blood glucose; Pao2: arterial oxygen tension; Paco2: arterial carbon dioxide tension; I/R: ischemia–reperfusion; PostC, postconditioning; MPG: N-2-mercaptopropionylglycine.
and catalase \( (P < .001 \text{ vs } \text{Con}) \) was detected in the spinal cord in the PostC group compared with the Con group 6 hours after spinal cord I/R. Nevertheless, no significant difference was observed between the PostC + MPG and Con groups \( (\text{SOD } P = .425 \text{ vs } \text{Con}; \text{catalase } P = .072 \text{ vs } \text{Con}) \). In addition, there was no significant difference observed in the 2 antioxidant activities of the spinal cord among the Con, MPG, and PostC + MPG groups \( (\text{Figure 4, B and C}) \).

**Gene Expressions of Cu,Zn-SOD and Catalase**

The mRNA expressions of Cu,Zn-SOD and catalase in the spinal cord were also measured. Expression of Cu,Zn-SOD and catalase mRNA in the sham group was significantly higher than in the other 4 groups \( (\text{Figure 4}) \). Cu,Zn-SOD mRNA expression was notably higher in the PostC group than in the Con group \( (P = .005 \text{ vs } \text{Con}, \text{Figure 4, D}) \), indicating that PostC increased the level of Cu,Zn-SOD mRNA expression. However, no significant difference was observed between the PostC + MPG and Con groups \( (P = .365 \text{ vs } \text{Con}, \text{Figure 4, D}) \), demonstrating that MPG reversed the effect of PostC on Cu,Zn-SOD mRNA expression. Meanwhile, there was no considerable difference in catalase mRNA expression among the Con, MPG, PostC, and PostC + MPG groups \( (\text{Figure 4, E}) \).

**DISCUSSION**

The current study found that ischemic PostC induced a neuroprotective effect in injured spinal cord, accompanied
by an increase in activities of the major antioxidant enzymes SOD and catalase, and gene expression of Cu,ZnSOD in spinal cord tissue, as well as a decrease of the serum biomarker NSE. Moreover, the protective effect and most of the biochemical alteration are attenuated if ROS is eliminated by its scavenger MPG administered 10 minutes before reperfusion, indicating that an antioxidant mechanism is associated with the mechanism of production of neuroprotection of ischemic PostC.

Our previous experiments demonstrated that implementation of three 30/30-second cycles of ischemic PostC at the onset of reperfusion in a rabbit model with 20 minutes of ischemia reduced spinal cord injury. However, the cellular mechanism that leads to the induction of spinal cord protection is not fully understood.

The results from the present study indicate that the mechanism underlying the protective effect involves intracellular redox signaling. First, we measured the activity of endogenous antioxidant enzymes. The results showed that PostC induced an increase in the activities of the major antioxidant enzymes SOD and catalase in tissue. Although it is well known that a burst of ROS contributes to reperfusion injury, the ROS scavenger MPG administered before reperfusion restrains the spinal cord protection of ischemic PostC, demonstrating that the generation of ROS by PostC is critical for its effect. Antioxidant enzyme activities are highly associated with the ROS generation during PostC (particularly during early reperfusion). Next, we demonstrated that a PostC-induced appropriate increase of ROS upregulates the endogenous antioxidant enzyme activities. The results suggest that redox signaling involves a mechanism of a neuroprotective effect induced by PostC against spinal cord ischemia.

As one of the pivotal enzymes in the antioxidant enzyme system of the body, SOD plays a key part in the body’s response to oxidative stress damage. There are two types of SOD: Cu,Zn-SOD and Mn-SOD, and Cu,Zn-SOD forms 80% of total SOD. Therefore, we measured the mRNA expression of Cu,Zn-SOD and another antioxidant enzyme, catalase. The results showed that PostC induced an increase in the expression of Cu,Zn-SOD mRNA in tissue, and this change could be reversed by MPG (a ROS scavenger) given before reperfusion, indicating that PostC increased the generation of ROS and in turn stimulated the expression of Cu,Zn-SOD mRNA. However, in the present study, although PostC increased the activity of catalase, no alteration was found in its mRNA expression in the PostC group compared with the Con group. This was consistent with several other studies. Cigremis and colleagues found that oxidative stress in the small intestine of diabetic rats resulted in elevated catalase and SOD activities, whereas their mRNA expressions were unchanged. We speculated that PostC induced a neuroprotective effect mainly through SOD upregulation to reduce oxidative stress injury after reperfusion.

During ischemia, a burst of ROS production may lead to tissue damage and even subsequent cell death. However, the function of ROS in the protective pathway is more subtle during the reperfusion phase. The current study found that MPG inhibited the neuroprotective effect against spinal cord ischemia if given before PostC, indicating that ROS production during early reperfusion is an important step toward limiting I/R injury. This is in line with previous studies demonstrating that ROS release can be protective. This is also in line with the observation that N-acetyl cysteine (a type of ROS scavenger) administered during the initial 3 minutes of reperfusion, but not delayed N-acetyl cysteine, altered the protective effect of PostC against heart ischemia.

In the clinic, spinal cord ischemia may be one complication of aortic reconstructive procedures or spinal cord surgery for tumor or vascular malformation. If spinal cord ischemia (eg, occlusion of aorta or hypoperfusion) occurred during the surgical procedure, controlled reperfusion (PostC) might be used before to reduce final reperfusion injury. However, the safety and effectiveness of the clinical application of PostC in patients require further study.

The results from the present study are in agreement with our earlier observation, which showed that spinal cord protection induced by sevoflurane PostC was via ROS-mediated SOD and catalase activity. Likewise, Liu showed that PostC protected CA1 neuronal injury and increased SOD and catalase activity in a brain I/R model.
Study Limitations
The current study has some limitations. Previous studies have demonstrated that cardiomyocyte protection induced by PostC is associated with ROS release from mitochondria after opening of the mK<sub>ATP</sub> channel.<sup>20,22</sup> Whether the mK<sub>ATP</sub> channel and other mediators or effectors mediating the protective effect of PostC in myocytes could also participate in the induction of ischemic tolerance by PostC in the spinal cord remains to be clarified.

CONCLUSIONS
Overall, our data support the idea that PostC can protect neurons from injury by upregulating the endogenous antioxidants SOD and catalase. This effect can be blocked by a ROS scavenger administered at an early stage of PostC. Additional investigation is required to explore the precise role of ROS and its pathways in ischemic PostC after injury.

References