Effect of ischemic post-conditioning on spinal cord ischemic-reperfusion injury in rabbits

[Effet du post-conditionnement ischémique sur les lésions d'ischémie-reperfusion de la moelle épinière chez le lapin]

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Objectives: To investigate the potential protective effect of ischemic post-conditioning (Post-con) on ischemia-reperfusion injury of the rabbit spinal cord, and to determine if there is an additive neuroprotective effect when ischemic preconditioning (IPC) and Post-con are combined.

Methods: Forty New Zealand white rabbits were randomly divided into four groups: group Control (C; $n = 10$), aortic occlusion (AOC; for 30 min; group IPC ($n = 10$) three cycles of three-minute AOC plus three-minute reperfusion before the 30-min AOC; group Post-con ($n = 10$), three cycles of three-minute reperfusion plus three-minute AOC immediately upon reperfusion after 30-min AOC; group IPC+Post-con ($n = 10$), where animals were subjected to both IPC and Post-con. At six hours, 24 hr and 48 hr following reperfusion, neurological function was assessed according to Tarlov scores, and at 48 hr, the spinal cords were procured for the histopathologic evaluation, by comparing the number of intact α -motor neurons in the anterior horn.

Results: The median count (and quartiles) of intact α -motor neurons was greatest in group Post-con 73 (69–76) and group IPC+Post-con 29 (22–42) compared to the numbers of viable α -motor neurons in groups C 6 (4–9) and IPC 15 (11–18) ($P < 0.001$). The numbers of animals who developed paraplegia according to Tarlov criteria were 7/10 in groups Post-con and IPC+Post-con, compared to 9/10 animals in each of groups C and IPC.

Conclusions: This laboratory investigation provides histological evidence that Post-con may protect the spinal cord from moderate to severe ischemia reperfusion injury. Ischemic preconditioning conferred no additional benefits in this rabbit model. The results have potential clinical implications for patients undergoing thoracoabdominal aortic reconstructive surgery.

Objectif: Investiguer l'effet protecteur potentiel du post-conditionnement ischémique (Post-con) sur les lésions d'ischémie-reperfusion de la moelle épinière chez le lapin, et déterminer s'il y a un effet neuro-protecteur lors de la combinaison du pré-conditionnement ischémique (PCI) et du Post-con.

Méthode: Quarante lapins blancs de Nouvelle-Zélande ont été répartis en quatre groupes de façon aléatoire : groupe témoin (T ; $n = 10$), occlusion aortique (OCA) de 30 min ; groupe PCI ($n = 10$), trois cycles de reperfusion de trois minutes plus OCA de trois minutes immédiatement à la reperfusion après une OCA de 30 min ; groupe PCI+Post-con ($n = 10$), dans lequel les animaux ont été soumis à un PCI et un Post-con. Six heures, 24 h et 48 h après reperfusion, la fonction neurologique a été évaluée selon le barème de Tarlov, et à 48 h, les moelles épinières étaient soumises à une évaluation histo-pathologique, en comparant le nombre de neurones moteurs- α intacts au niveau de la corne antérieure.

Résultats: Le nombre médian (et les quartiles) de neurones moteurs- α intacts fut plus élevé dans le groupe Post-con 73 (69–76) et le groupe PCI+Post-con 29 (22–42), que le nombre de neurones moteurs- α viables dans les groupes T 6 (4–9) et PCI 15 (11–18) ($P < 0,001$). Le nombre d'animaux ayant développé une paraplégie selon les critères de Tarlov était de 7/10 dans les groupes Post-con et PCI+Post-con, comparé à 9/10 animaux dans chacun des groupes T et PCI.

Conclusions: Cette étude en laboratoire offre des preuves histologiques que le Post-con peut protéger la moelle épinière de lésions d'ischémie-reperfusion modérées à sévères. Le pré-conditionnement ischémique n'a pas apporté de bénéfices supplémentaires dans ce modèle chez le lapin. Ces résultats ont des implications cliniques potentielles pour des patients subissant des chirurgies de reconstruction de l'aorte thoraco-abdominale.

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PARAPLEGIA following descending thoracic or thoracoabdominal aortic reconstructive surgery remains one of the most devastating complications of this high-risk operation. Although a number of strategies have been used to reduce the risk of spinal cord injury associated with this surgery, including induced hypothermia and cerebrospinal fluid drainage, the therapeutic benefits of these interventions remain uncertain.

Since ischemic preconditioning (IPC) was first reported by Murry *et al.*¹ to delay lethal cell injury in ischemic myocardium, this powerful endogenous neuroprotective mechanism has also been identified in other organs, including the spinal cord.² However, the efficacy of IPC for spinal cord protection remains uncertain,³ a fact which has limited its widespread clinical application.

There are established mechanisms underlying IPC which warrant further evaluation. For example, IPC could reduce the production of oxygen free radicals⁴ that are toxic to the tissues, increase the expression of heat shock proteins that inhibits protein degradation,⁵ and promotes the opening of the K_{ATP} channel⁶ to decrease the inward flux of Ca^{2+} . Some of these mechanisms have also been found to exist in ischemic post-conditioning (Post-con), which may also be protective of ischemic myocardium,⁷ indicating that the reperfusion itself could initiate endogenous cellular protective mechanisms. However, just as its name implies, IPC must be initiated before the ischemic insult. Unfortunately, in most cases, ischemic events are unpredictable. In contrast, Post-con has the potential to be introduced immediately following an ischemic event at the onset of reperfusion, which could result in a potentially beneficial therapeutic benefit. Accordingly, Post-con could have a much broader clinic application compared to IPC.

The present study was designed to assess the effects of Post-con on neurological and histopathological outcomes in a rabbit model of severe spinal cord ischemia, in comparison to the classical IPC. In addition, we also sought to determine a possible additive effect when the two neuroprotective strategies are combined.

Material and methods

Animal care

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Research Committee of Sichuan University. Forty New Zealand white rabbits were studied, each animal weighing between 2.0 and 2.5 kg. The animals were housed with free access to food and water. All

rabbits were neurologically intact before anesthesia and the surgical procedure.

Surgical preparation

The rabbits were fasted overnight before surgery and were anesthetized with ketamine 25 mg·kg⁻¹ *im* and midazolam 1 mg *im*. A 22-G *iv* catheter was then placed in an ear vein for *iv* administration of fluids and drugs. After endotracheal intubation, vecuronium 1 mg *iv* was administered and the rabbits were placed on a ventilator (inspired oxygen fraction, 100%) with an initial tidal volume of 40 mL at a rate of 40 cycles·min⁻¹. Ventilatory parameters were subsequently adjusted to maintain the PaO₂, PaCO₂ and pH values within the normal physiologic range, as confirmed by the arterial blood gas analysis. An ear artery was cannulated with a 22-G catheter to monitor arterial blood pressure (Spacelabs Medical, Inc., Redmond, WA, USA). Heart rate was calculated from the blood pressure wave by the pressure monitor. Hemodynamic parameters were monitored throughout the experiment and were recorded after surgical preparation (baseline), at the mid-point of IPC if any, at the mid-point of the 30-min period of ischemia, at the mid-point of Post-con if any, and 30 min after restored reperfusion, respectively. Each animal's tail was prepared for an arterial pulse oximeter probe, and a rectal thermometer was inserted as a guide to maintaining body temperature around 38°C throughout the operation, with the aid of a heating lamp.

Following a midline laparotomy, the abdominal aorta was carefully exposed. Five minutes before occlusion, heparin 1 mg·kg⁻¹ *iv* was administered. A bulldog clamp was then placed across the aorta, just below the left renal artery, to produce abdominal aortic occlusion (AOC). The clamp remained in place for 30 min while the response was being monitored. Throughout the experiment, lactated Ringer's solution was infused at 10 mL·kg⁻¹·hr⁻¹ and additional doses of midazolam, vecuronium and fentanyl were administered as required to maintain the desired level of anesthesia. After measurements were completed, the aorta was declamped, the laparotomy incision was closed, and animals were weaned from the ventilator and allowed to recover from anesthesia before being returned to their cages for postoperative care.

Experimental protocol

Animals were randomly assigned to one of four groups (Figure 1) according to a computer-generated random number schedule: 1) control (C, *n* = 10): the aorta was occluded for 30 min; 2) ischemic preconditioning (IPC, *n* = 10): before the 30 min AOC, the aorta was

FIGURE 1 Experimental protocol: [control (C, $n = 10$)]; the aorta was reversibly occluded for 30 min; ischemic preconditioning (IPC, $n = 10$): before the 30 min period of aortic occlusion (AOC), the aorta was occluded for three minutes followed by three minutes of reperfusion, repeated for three cycles; ischemic post-conditioning (Post-con, $n = 10$): after the 30 min AOC, reperfusion was initiated for three minutes followed by three minutes of reocclusion, repeated for three cycles; Ischemic preconditioning plus post-conditioning (IPC+Post-con, $n = 10$): the above IPC and Post-con protocols were combined.

occluded for three minutes followed by three minutes of reperfusion, repeated for three cycles; 3) ischemic post-conditioning (Post-con, $n = 10$): after the 30 min AOC, reperfusion was initiated for three minutes followed by three minutes of re-occlusion, repeated for three cycles; 4) ischemic preconditioning plus post-conditioning (IPC+Post-con, $n = 10$): the above IPC and Post-con protocols were combined.

Hemodynamics

The mean arterial pressure and heart rate were monitored continuously throughout the experiment, via the ear artery, as well as the arterial pulse oxygen saturation (S_pO_2) from the tail. Hemodynamic data were collected at baseline, at the mid-point of the 30 min period of ischemia, at the mid-point of IPC and Post-con if any, and after 30 min of reperfusion.

Neurological evaluation

At six hours, 24 hr and 48 hr following reperfusion, the hind limb motor function was assessed by a trained observer. Assessment was done according to the modified Tarlov criteria:⁸ 0 = paraplegia with no lower-extremity movement; 1 = poor lower-extremity movement but unresistant to gravity; 2 = good lower-extremity motor function with resistance to gravity, but unable to draw legs or hop; 3 = ability to draw leg

FIGURE 2 Representative photomicrographs of spinal cord sections stained with hematoxylin and eosin. A number of viable α -motor neuron cells (arrow) were preserved in the ischemic preconditioning plus post-conditioning group (IPC+Post-con), whereas prominent vacuolization and eosinophilic red neurons (arrow head) were observed in the Control group (C) and group IPC (original magnification, $\times 200$).

and hop but not normally; 4 = normal lower-extremity motor function. Animals with Tarlov grades 0–2 were rated as being paraplegic, while grades 3–4 were considered non-paraplegic.

Histopathologic studies

Forty-eight hours after reperfusion, all animals were sacrificed and their spinal cord were immediately harvested and immersed in formalin for later histopathologic examination. Each segment of the spinal cord below L-5 (including L-5) was dissected in a 5-mm block. The blocks of tissues were embedded in paraffin. Transverse sections of each block were sliced for hematoxylin and eosin staining. One section from each block was evaluated by an experienced pathologist, who was unaware of the experimental conditions. Viable α -motor neurons in the ventral horns were counted, and the results from all the sections of each spinal cord were aggregated to give a final result for each animal. A viable α -motor neuron was defined according to the following criteria: fine granular cytoplasm with Nissl body and a soma diameter of 30–60 μ m. Neurons with eosinophilic cytoplasm were considered to be either dead or necrotic (red neurons).

TABLE I Hemodynamic values

	Group	Baseline	IPC	30-min ischemia	Post-con	Restored reperfusion
HR (beats·min ⁻¹)	C	258 ± 14	–	277 ± 16	–	272 ± 13
	IPC	240 ± 17	260 ± 17	238 ± 15	–	280 ± 17
	Post-con	260 ± 15	–	263 ± 11	268 ± 13	278 ± 17
	IPC+Post-con	286 ± 15	288 ± 16	277 ± 13	279 ± 13	276 ± 14
MAP (mmHg)	C	94 ± 6	–	99 ± 9	–	100 ± 8
	IPC	99 ± 6	83 ± 9	94 ± 7	–	98 ± 10
	Post-con	98 ± 14	–	99 ± 7	83 ± 5	96 ± 5
	IPC+Post-con	94 ± 8	80 ± 8	89 ± 8	84 ± 7	95 ± 6
T (° C)	C	37.8 ± 0.2	–	38.0 ± 0.2	–	37.9 ± 0.2
	IPC	38.0 ± 0.5	37.8 ± 0.4	38.0 ± 0.3	–	38.1 ± 0.4
	Post-con	37.9 ± 0.4	–	38.2 ± 0.3	38.1 ± 0.2	38.3 ± 0.4
	IPC+Post-con	38.1 ± 0.3	38.0 ± 0.3	37.9 ± 0.1	37.7 ± 0.4	38.0 ± 0.3

All values are expressed as mean ± SD. There were no significant differences in hemodynamic variables amongst the four groups at corresponding time periods. Statistical analysis was by means of one-way analysis of variance. C = control; IPC = ischemic pre-conditioning; HR = heart rate, MAP = mean arterial pressure, Post-con = ischemic post-conditioning; T = rectal temperature, (–) = results not recorded for the group and time period indicated.

Statistical analysis

Hemodynamic data were compared using repeated measures analysis of variance (ANOVA) to test for group-time interactions, and when significant differences were identified, the Student-Neumann-Keuls test was applied post-hoc. Tarlov scores were compared *via* the Kruskal-Wallis test, followed by the Mann-Whitney U test where indicated. The incidence of paraplegia was compared by the Mantel Haensel test. Hemodynamic data are presented as means ± SD, Tarlov scores and the numbers of viable neuron are expressed as medians (and 25% and 75% quartiles), and the incidence of paraplegia is presented in absolute numbers. Data were analyzed using SPSS software (version 11.5, SPSS Inc, Chicago, IL, USA), and a *P* value < 0.05 was considered statistically significant.

Results

Hemodynamics

Mean arterial pressure and heart rate were similar amongst the four groups at corresponding time periods (Table I). Throughout the duration of AOC, the pulsatile wave of S_pO₂ disappeared in all animals, confirming completeness of the AOC. Soon after aortic cross clamp release, S_pO₂ values returned to 100% with corresponding reappearance of normal oximetric waveforms, confirming reperfusion (data not shown). No differences in body temperature were observed, and anesthetic doses and volumes of infused fluid were similar in the four groups.

Neurological outcome

Neurological outcome data are summarized in Table II. After six, 24 and 48 hr, no significant differences in neurological outcomes were observed between the four groups. Seven of ten animals (70%) in groups Post-con and IPC+Post-con developed paraplegia according to the Tarlov grading, compared to nine of ten animals (90%) in group C.

Histopathological data

The numbers of viable α motor neurons were greatest in group Post-con [73 (69–76)] in comparison with animals in group C [6 (4–9)], group IPC [15 (11–18)], and group IPC+Post-con [29 (22–42)] (*P* < 0.001, Post-con *vs* all other groups). The numbers of viable α motor neurons were also greater in group IPC+Post-con *vs* C (*P* < 0.001), whereas IPC alone demonstrated no histological evidence of spinal cord protection (*P* > 0.05 *vs* C).

Discussion

This study provides histopathological evidence that Post-con may protect the spinal cord against ischemia reperfusion injury, providing further evidence in support of a potential benefit of post-ischemic conditioning.⁹ The underlying mechanisms by which Post-con may protect the spinal cord are unknown. What has been learned from cardiac studies is that post-conditioning can reduce the production of reactive oxygen species (ROS) and the inflammation caused by ROS.

The duration of post-conditioning ischemia is critical. Kin *et al.*¹⁰ demonstrated that the protective effect

TABLE II Tarlov functional scores and frequency of paraplegia ($n = 10$ per group)

Time	Group	Tarlov score	Paraplegia (n)	P value (Chi-square test vs group C)
6 hr	C	0 (0-0.25)	9	–
	IPC	1 (0-1)	9	1.000
	Post-con	1 (0.38-4)	7	0.264
	IPC+Post-con	1(0-4)	7	0.264
24 hr	C	0 (0-0.25)	9	–
	IPC	1 (0-1)	9	1.000
	Post-con	1 (0-3.25)	7	0.264
	IPC+Post-con	1 (0-4)	7	0.264
48 hr	C	0 (0-0.25)	9	–
	IPC	1 (0-1)	9	1.000
	Post-con	0 (0-3.25)	7	0.264
	IPC+Post-con	0.25 (0-4)	7	0.264

Data are expressed as median (25% and 75% quartiles). There were no significant differences between groups with respect to Tarlov scores and the corresponding incidence of paraplegia (Tarlov scores 0 – 2). Statistical analysis was by means of Kruskal-Wallis test for Tarlov scores and Mantel Haensel test for incidence of paraplegia.

Tarlov criteria: 0 = paraplegic with no lower-extremity movement; 1 = poor lower-extremity movement but un-resistant to gravity; 2 = good lower-extremity motor function with resistance to gravity but unable to draw legs or hop; 3 = ability to draw leg and hop but not normally; 4 = normal lower-extremity motor function. C = control; IPC = ischemic pre-conditioning; Post-con = ischemic post-conditioning.

of Post-con would be lost if the first reperfusion event lasted longer than one minute, because of a significant burst of oxygen-derived free radicals generated within the first few minutes of reperfusion. However, compared to Kin's study (in a rat model examining the myocardial response to three cycles of one-minute reperfusion and one minute of ischemia), the reperfusion in our study was longer (involving the spinal cord in rabbits, with three cycles of three-minute reperfusion and three minutes of ischemia), although histological evidence of a protective effect still existed. Furthermore, in Jiang *et al.*'s study,⁹ the neuroprotective effect persisted, even though the first episode of reperfusion lasted as long as five minutes.

There are several possible explanations to account for the variances between these studies. First, xanthine oxidase in rats¹¹ is considerably more active than in the rabbit, resulting in greater free oxygen radical production, and potentially greater exposure to tissue injury in rats compared to rabbits. In addition, the heart is metabolically more active than the spinal

cord. As more oxygen free radicals are generated in tissues of higher metabolic activity, the myocardium may be more susceptible to injury by the toxic free radicals. Lips *et al.*¹² have also reported that blocking of the N-methyl-D-aspartate (NMDA) receptor may protect the ischemic spinal cord against ischemia reperfusion injury, although the potential role of the NMDA receptor in Post-con has not been evaluated. Considering the differences between species and tissues, the mechanism of the potential Post-con spinal cord neuroprotection observed in rabbits warrants further investigation.

In group Post-con, histological evidence of neuroprotection did not translate into a significant reduction in the incidence of paraplegia according to Tarlov scores. One possible reason is that the injury severity induced by the protocol in this rabbit model exceeded a duration such that Post-con could only improve the injury to a degree that was just between the threshold of histopathologic change and functional change. It has been demonstrated previously that cellular function is compromised well before the morphologic changes of cell death.¹³ Compared to Sakurai *et al.*'s¹⁴ study in which the 15-min ischemia produced only mild injury in the C group (there were no animals with Tarlov scores of 0 or 1 in this group), our 30-min period of ischemia caused more severe injury (in the C group, nine of ten animals had paraplegia scores of 0 according to Tarlov's criteria, data not shown). We chose this duration of ischemia to provide greater potential clinical relevance. As pointed out by Svensson *et al.*¹⁵ mild paraparesis is likely to resolve and many patients can regain their preoperative motor function. Ueno *et al.*¹⁶ also reported that the correlation between histological findings and functional outcomes are variable. Some authors¹⁷ report that the protective effect of IPC could be transient, disappearing within seven days following reperfusion. However, Jiang *et al.*⁹ have demonstrated that the histopathologic status in their Post-con group was better than that observed in the C group after a ten-day period of reperfusion.

There have been very few reports to date examining the effects of IPC on the spinal cord. Zvara *et al.*¹⁸ demonstrated that IPC induced by three minutes of ischemia reduced neurological injury resulting from a subsequent 30-min period of ischemia. Sirin *et al.*¹⁹ also investigated the effects of IPC with five minutes of ischemia on spinal cord injury mediated by a 20-min ischemic insult, and demonstrated that IPC reduces the extent of spinal cord injury. In the present study, a lack of response to IPC with three cycles may explain the lack of protection by IPC, due to

the frequency of preconditioning. Iliodromitis *et al.*²⁰ showed that six to eight cycles of brief ischemia result in a loss of efficacy of IPC in rabbit myocardium, but four cycles were still protective in their study. Ischemic preconditioning and Post-con have some overlapping elements in their signal pathways, which has been confirmed in the myocardium.²¹ The effect of Post-con was also dampened in group IPC+ Post-con, suggesting there may be an interaction between the two ischemic conditionings. However, it remains unclear as to why an increasing number of preconditioning cycles may dampen the protective phenomenon. Interestingly, the preconditioning itself would induce no harm to the tissues, since the results in the IPC group were not significantly different from the results in group C, an observation similar to that observed by Iliodromitis *et al.*²⁰

Another possible explanation for the absence of neuroprotection in the IPC group of our study is that the reperfusion interval between IPC and the subsequent ischemic event was only three minutes, much shorter than that of the other “protective” IPC protocols.^{18,22} Recently, Contreras *et al.*²³ demonstrated the importance of the reperfusion interval by monitoring the somatosensory evoked potentials. Further experiments using different cycles and durations of IPC and reperfusion interval are required to address the observed between-study variances.

In our study, transient hypotension was observed in all animals after releasing the aortic clamp, however, mean arterial pressure returned spontaneously to baseline values within one minute without requiring further intervention. Other investigators have reported similar hemodynamic responses.^{12,24} In contrast to Toupoulis *et al.*'s study,² hypotension should not have been a confounding variable in the interpretation of our data.

There are several methodological limitations of this study which merit comment. Given the fact that the burst of toxic free radicals peaks at four to seven minutes during reperfusion in some models,²⁵ and most reports evaluating post-conditioning responses adopt three cycles, we used a protocol of three cycles of three-minute reperfusion following three-minute ischemia in group Post-con. For the convenience of symmetric comparison between the two conditionings, we also used three cycles of three-minute ischemia for preconditioning, which may not be an optimal protocol. Further studies are needed to determine the optimum number of cycles and cycle duration for both IPC and Post-con. Further, while the study design was able to control a number of variables, as a preliminary laboratory investigation of

spinal cord histological responses to post-ischemic conditioning it was not powered to detect differences in functional outcomes between groups. While the numbers of paraplegic animals were numerically less in groups Post-con and IPC+Post-con, the study was not powered to detect between-groups differences in Tarlov scores. The data from this study will be useful to design larger experiments to specifically address the duration and frequency of post-conditioning cycles and the effects on functional outcomes.

In conclusion, this laboratory investigation provides histological evidence that Post-con may protect the spinal cord from moderate to severe ischemia reperfusion injury. Ischemic preconditioning conferred no additional benefits in this rabbit model. The results have potential clinical implications for patients undergoing thoracoabdominal aortic reconstructive surgery, suggesting that further studies are well warranted.

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