

Ischemic preconditioning and nicotinamide in spinal cord protection in an experimental model of transient aortic occlusion

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Abstract

Objectives: Spinal cord injury is a devastating complication after aortic surgery. The aim of the present study is to examine the effects of ischemic preconditioning (IPC) and nicotinamide containing perfusate in transient aortic occlusion in the rat. **Methods:** Thirty-two male Sprague–Dawley rats under general anesthesia were randomly assigned to four groups ($n = 8$ in each group). The infrarenal aortas were clamped for 45 min. Groups were as follows: Group 1, undergoing occlusion but receiving no treatment. Group 2, had 5 min of IPC before occlusion. Group 3, received nicotinamide (0.2 ml/l) during the transient occlusion. Group 4, received combined IPC (5 min) and nicotinamide infusion during the transient occlusion. The rats were then allowed for recovery and were tested for their neurological status. All animals were sacrificed at the end of the 48 h and spinal cords also examined histologically. Anti-poly (ADP-ribose) polymerase p85 fragment pAb was used as an immunohistochemical marker for detection of apoptosis. **Results:** In 24 h paraplegia represented as grade 0 and 1 occurred in six animals in Group 1 and two animals in Groups 2 and 3 and one in Group 4. In 48 h six animals in Group 1 and only one animal in Groups 2 and 3 showed a paraplegia. The incidence of neurologic deficit was significantly reduced in animals who had IPC and nicotinamide infusion ($P < 0.05$). At 48 h, combined IPC and nicotinamide showed a significant benefit compared to nicotinamide but not to the IPC alone. Histologic examination of the spinal cords revealed that a neuronal necrosis contributes to acute spinal cord degeneration after a period of aortic occlusion and both nicotinamide and IPC have protective effects against neuronal necrosis. No difference was found among the groups. **Conclusions:** Both IPC and nicotinamide are beneficial in protection against neurological damage in transient aortic occlusion. IPC alone as expected is significantly beneficial both at 24 and 48 h compared to controls. At 24 h combined nicotinamide and IPC show significant benefit compared to only nicotinamide, but this difference is not maintained at 48 h.

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1. Introduction

Descending aortic aneurysm is one of the catastrophic pathologies confronting the surgeons. One of the most common complications after surgery for this disease is known to be paraplegia [1]. The mechanism of the spinal cord injury after operations on the thoracic aorta is highly dependent on oxygen deficiency, edema due to

postinfarction hyperemia and reperfusion injury caused by free radicals in addition to anatomic variables [2–4].

Several investigations have focused on intraoperative techniques that might prevent this neurologic complication. These adjuncts include use of the shunts, hypothermia, cerebrospinal fluid drainage, infusion of free radical scavengers and intrathecal injection of neuroprotective agents [5,6]. However none of these techniques has totally prevented this unpredictable complication.

Ischemic preconditioning can be described as brief non-injurious periods of ischemia followed by reperfusion. It was first mentioned in dogs where series of coronary artery

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occlusions followed by reperfusion, protected the heart from ischemia-reperfusion injury. It was then applied to animal models for spinal cord protection [7]. Previous data showed reduced neurologic injury and improved survival. In the preliminary studies reperfusion interval between ischemic preconditioning (IPC) and subsequent ischemia was several hours or days and therefore quite different than the clinical setting. In recent studies short periods of IPC was used and it was also found to be protective against paraplegia [7–9].

On the other hand it is well known that energy imbalance between supply and demand triggers the onset of ischemia induced cascades. Pharmacological treatment for improving the energy imbalance and attenuating the ischemic damage is of paramount importance in this context [10–12]. Thus any option to improve energy supply and reduce the demand would seem to work well with the tissue at risk. Nicotinamide is an essential precursor of adenine dinucleotide (NAD^+) that protects against decreased production of ATP [13]. Therefore it has the potential to protect against ischemia induced energy imbalance.

The purpose of this study is to compare the effects of IPC and nicotinamide infusion on the spinal cord and to determine if combined IPC and nicotinamide is protective against ischemic injury due to cross clamping of the descending thoracic aorta.

2. Materials and methods

2.1. Animal care and surgical technique

We used Sprague–Dawley rats weighing 325–375 g in the experiment. Animal care and all procedures were performed accordingly guide for the care and use of laboratory animals published by the National Institutes of Health (NIH publication 85-23, revised 1985). The protocol was approved by the Animal Research Committee of the Marmara University Hospital. Animals were randomly assigned to four groups ($n = 8$ in each group). In Group 1, abdominal aorta was clamped just distal to the renal artery (45 min). In Group 2 rats, IPC was applied to aorta before 45 min of aortic clamping. In Group 3, nicotinamide containing solution was infused into the aorta with a perfusion pump during 45 min of aortic clamping. In Group 4, IPC and nicotinamide infusion were applied concomitantly.

2.2. Operative procedure

Rats were premedicated with Ketamine 25 mg/kg and Xylazine 5 mg/kg intramuscularly. Animals were then anesthetized with intravenous thiopentalsodium 20 mg/kg and intubated and ventilated with a rodent ventilator (Tidal volume 30 ml and a rate of 35 cycles/min). Intravenous cephalosporin was administrated before skin incision. Temperature probe was inserted into the rectum and the

tail artery was cannulated to measure the arterial blood pressure and heart beat. A midline laparotomy was performed to expose the aorta below the renal artery and above the iliac bifurcation. Temperature was maintained between 36.5 and 37.5°C during the surgical procedure with heating lamp. Arterial blood pressure and heartbeat were continuously monitored throughout the procedure. Heparin was given intravenously (500 U). In Group 1, aorta was clamped just distal to the renal artery and above the iliac bifurcation for 45 min. In Group 2, IPC was applied by clamping the aorta proximally and distally for 5 min before 45 min of aortic clamping. In Group 3, nicotinamide containing solution (0.2 ml/l) was infused into the aorta by a pump (0.5 ml/min) while the aorta was clamped proximally and distally. In Group 4, 5 min of IPC was applied before 45 min of aortic clamping, similar to Group 2 and subsequently nicotinamide infusion was given during the period of 45 min of aortic clamping in the same manner as Group 3.

The tail artery was cannulated to monitor distal aortic pressure to ensure complete aortic occlusion. In all animals aorta was declamped after 45 min and laparotomy was closed by using silk sutures. All animals were weaned from the ventilator and allowed for recovery in their cages.

All animals were sacrificed with a lethal dose of pentobarbital at 48 h. The entire Spinal cords were quickly removed en bloc (within 5–10 min) and immediately frozen in liquid nitrogen for histopathology.

2.3. Neurologic scoring system

The animals were assessed on postoperative 24 and 48 h by a single trained blinded observer by using the Tarlov Criteria: Grade 0, paraplegia with no lower extremity motor function; Grade 1, poor lower extremity motor function; Grade 2, good movement of the hind limbs, but unable to stand; Grade 3, able to stand but unable to walk normally; Grade 4, complete recovery [14]. Hindlimb motor function deficit was also assessed by using the modification of the system reported by Zvara et al. (Table 1) [15].

2.4. Spinal cord histopathologic examination

At 48 h, the animals were anesthetized with pentobarbital (20 mg/kg) and sacrificed. Spinal cords were dissected totally and fixed in buffered formalin for 7 days. Transverse sections were obtained from levels corresponding to the third to fourth cervical segment (normal control) and the fourth to sixth lumbar segment (experimental region of ischemia). The spinal cord tissues were embedded in paraffin and serial transverse sections (4 μm) were obtained from blocks. The slides were stained by hematoxylin eosin, luxol fast blue/PAS and cresyl fast violet methods for histopathologic evaluation. The neuropathologist who was blinded to experimental conditions, performed the histologic assessment by means of light microscopy.

Apoptosis was monitored by an immunohistochemical

Table 1
Determination of Hindlimb motor function

Score	Description
<i>Tarlov Scale Score</i>	
0	Walks normally
1	Walks with mild deficit
2	Supports weight on hind limb; may take one or two steps
3	No weight bearing, but frequent or vigorous movement of hind limb
4	No weight bearing, barely perceptible movement of hind limb
5	No weight bearing, no movement of hind limb
<i>Righting Score</i>	
0	Rat placed on back; immediately turns over (righting)
1	Weak or no righting
<i>Placing Score</i>	
0	Rat grasps ledge with hind limb when suspended in air (placing)
1	Weak or no placing
<i>Inclined Plane Score</i>	
0	Animal grips platform with hind limbs at 45 degree of angle
1	Animal grips platform with hind limbs at a max. angle of 40 degrees
2	Animal grips platform with hind limbs at a max. angle of 35 degrees
3	Animal fails to grip platform with hind limbs at 35 degree angle

marker; anti-poly (ADP-ribose) polymerase (PARP) p85 fragment pAb; which is a polyclonal antibody directed against the 85 kDa caspase-cleaved fragment (p85) of human PARP. Paraffin-embedded blocks were cut at 4 μ m sections, deparaffinized, and rehydrated. Anti-PARP p85 fragment pAb was used as the primary antibody (anti-PARP p85 fragment pAb; Promega Corporation, WI; dilution 1:100). Streptavidin-horseradish peroxidase was performed followed by 3-3'-diaminobenzidine. All slides were counterstained with hematoxylin. Appropriate slides were used as positive and negative controls. A neuropathologist undertook evaluation of the slides without knowing the groups. In the process of examining the immunoreactivity within the neurons, the following grading scheme was applied: 0 = no staining, 1+ = <25%, 2+ = 25–50%, 3+ = 50–75%, and 4+ = >75% positive nuclei.

2.5. Statistical methods

Data are expressed as the mean \pm standard error of the mean (SEM). For statistical assessment of changes in post-operative neurologic status, we used the Kruskal–Wallis

analysis of variance to determine significant differences among the four groups and the Mann–Whitney *U*-test to compare Groups 2 through 4 with Group 1.

3. Results

Mortality for the 45 min of occlusion was 12% (four of 32 animals). We lost three animals in nicotinamide group due to hemorrhage. One animal died in control group after intubation. All animals showed some degree of paraplegia after immediate recovery. In 24 h paraplegia represented as grade 0 and 1 occurred in 75% of Group 1 and 25% of Groups 2 and 3 and 12% of Group 4. In 48 h paraplegia occurred in six animals in Group 1 representing 75% of group whereas none of the animals in Group 4 showed paraplegia. One animal in Groups 2 and 3 showed a paraplegia representing 12% of groups. Neurologic deficit stated as mean neurological score is presented in Fig. 1. The incidence of neurologic deficit was significantly reduced in animals who had IPC and nicotinamide infusion. At 48 h, combined IPC and nicotinamide showed a significant benefit compared to nicotinamide but not to the IPC alone (Table 2).

The histopathologic alterations were restricted to neurons (Fig. 2). Cells with pronounced cytoplasmic eosinophilia and loss of definition of cell membranes in hematoxylin and eosin (H + E) stained sections were considered to be chromatolytic. No Nissl substance could be seen in the cytoplasm. Another type of cellular damage observed in neurons was loss of nuclear hematoxylin staining with shrunken amphibolic/pink cytoplasm. The neurons with these alterations were also considered to be chromatolytic [16,17].

In the control group the ischemic/necrotic neurons were observed in Rexed's laminae 1–10 [17]; whereas in nicotinamide group and IPC group the ischemic/necrotic neurons were observed more frequently in the intermediate zone of the gray matter (Rexed's laminae 7) and the dorsal horns (laminae 3–5). The large neurons in the ventral horn (Rexed's laminae 8 and 9) were relatively spared in these two groups. The distribution of necrotic neurons in the dorsal horn and intermediate zone (Rexed's laminae 3–5 and 7) were more frequent in Group 2.

In addition to the necrotic neurons described in H + E stained sections, neurons with morphologically suggestive of apoptosis were seen with anti-PARP immunostaining. The positive immunoreactivity was observed within the cytoplasm and nuclei of the neurons in the gray matter. The apoptotic neuronal counts of the gray matter was statistically significant between Groups 2 and 4 (IPC, 3.6 ± 0.5 versus Combined IPC + Nicotinamide, 2.3 ± 0.6 , $P < 0.05$) (Fig. 3).

4. Discussion

Brief ischemic episodes followed by periods of

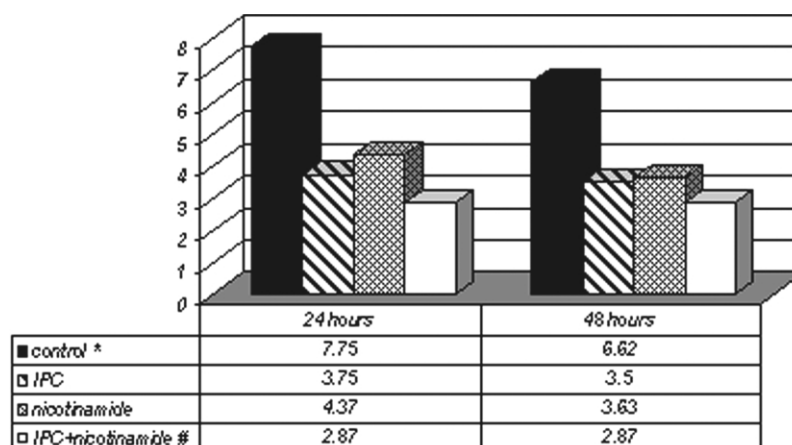


Fig. 1. Mean Neurologic scores of animals (the lower the better) showed a significant difference between control and treatment groups ($*P < 0.05$). Combined IPC and Nicotinamide infusion leads to a significant difference compared to Nicotinamide alone at 24 h ($\#P < 0.05$). Treatment with IPC leads to small reductions both at 24 and 48 h, but it is not statistically significant over Nicotinamide.

reperfusion increase the resistance to further ischemic damage. This response is called ischemic preconditioning. Ischemic preconditioning is a powerful protective mechanism against ischemic injury and has both immediate and delayed effects.

The occlusion of the infrarenal aorta for 45 min leads to almost complete paraplegia. To assess the neuroprotective effect of IPC and nicotinamide, the motor function of the hindlimb was scored and the spinal cords of control and treated animals were analyzed histopathologically to determine the necrotic and apoptotic cell deaths.

The analysis of the neurologic status demonstrates that the tolerance of the spinal cord to ischemia-reperfusion injury was significantly improved both by IPC and nicotinamide infusion. The best protection was obtained when IPC and nicotinamide infusion administered together. In early periods (24 h) IPC and nicotinamide infusions significantly protected the spinal cord as reflected by decline in hindlimb motor function scores and higher neurologic status grade in Tarlov Scale. In 48 h, neurological status was superior in IPC group compared to nicotinamide group reflecting that IPC was better. Combined IPC and nicotinamide group was equally effective in both 24 and 48 h

reflecting a better protection with the two combined among the four groups.

Injury to the gray matter of the spinal cord with neuronal death has generally been considered important issue in the pathology of spinal cord injury. Histological examination of the spinal cords revealed that neuronal necrosis contributes to acute spinal cord degeneration after a period of aortic occlusion and nicotinamide have protective effect against neuronal necrosis. One possible explanation for the effect of nicotinamide on spinal cord is, it might prevent the expression of i-NOS [18]. Unexpectedly IPC was found to be ineffective in histopathology. Previous studies demonstrated that neuronal cell death suggestive of apoptosis might occur after spinal cord injury [19]. Apoptosis might be apparent in this setting. The necrotic cells in light microscopy might be apoptotic, and terminal damage might not be occurred. Our results demonstrate that death of spinal cord cells in both gray and white matter following transient aortic occlusion involves apoptotic demise of cells in the cord. We observed lower concentration of apoptotic neurons in combined IPC and nicotinamide group compared to nicotinamide and IPC alone.

This finding suggests that preoperative IPC treatment

Table 2
Tarlov Scale for rats at 24 and 48 h

Tarlov Scale	24 h				48 h			
	Control	IPC	Vit.B	IPC + Vit.B	Control	IPC	Vit.B	IPC + Vit.B
Grade 0	4	0	0	0	4	0	0	0
Grade 1	2	2	2	1	2	1	1	0
Grade 2	1	2	1	1	1	2	2	2
Grade 3	1	3	2	3	1	3	3	3
Grade 4	0	1	3	3	0	2	2	3

Tarlov Scale: Grade 0, paraplegia with no lower extremity motor function; Grade 1, poor lower extremity motor function; Grade 2, good movement of the hind limbs, but unable to stand; Grade 3, able to stand but unable to walk normally; and Grade 4, complete recovery.

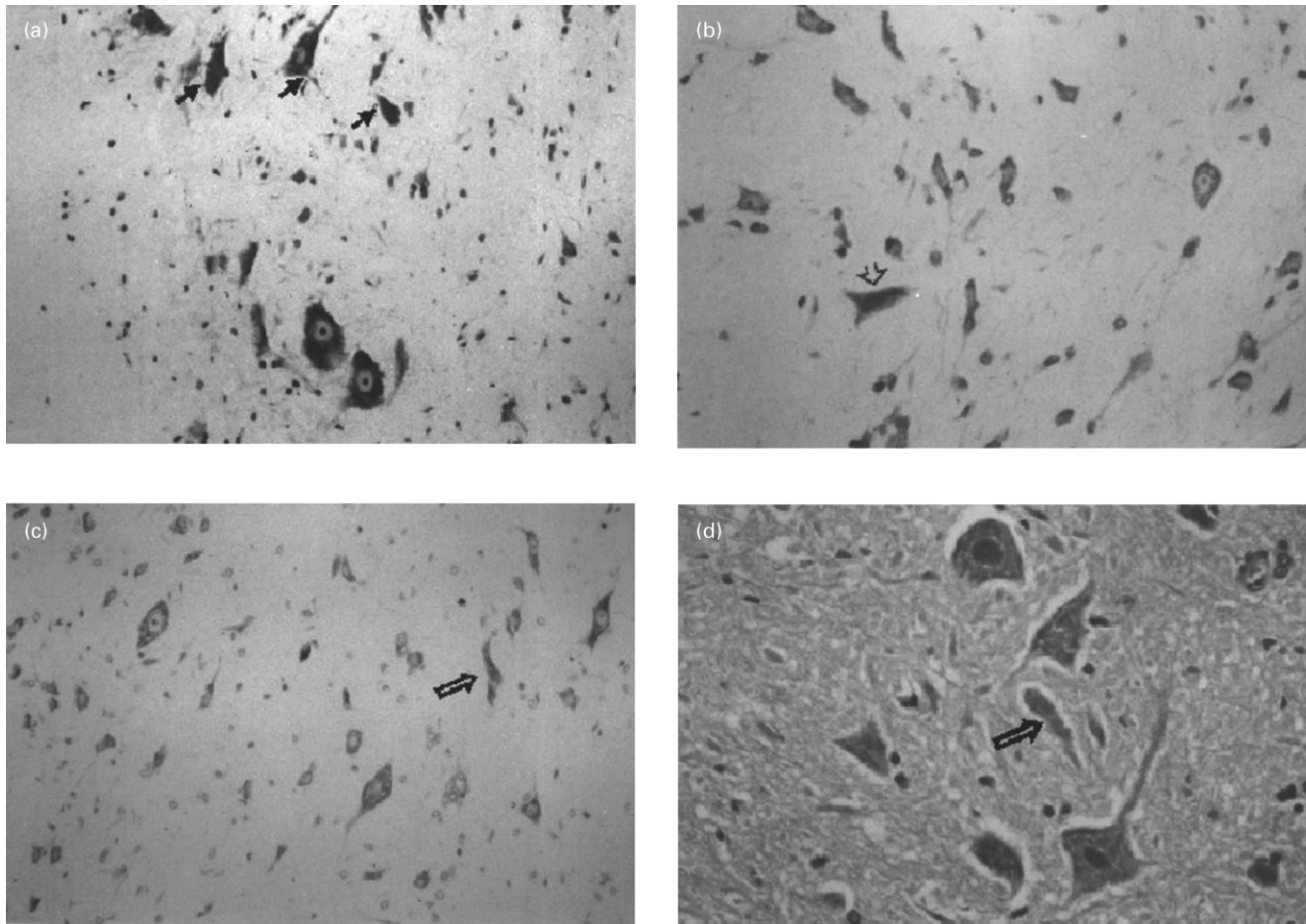


Fig. 2. Photomicrographs of section of lumbar cords of experimental animals. A through D, Groups 1–4. Arrows indicate chromatolytic neurons in spinal gray matter (hematoxylin-eosin $\times 200$).

reduced the number of apoptotic neurons within the gray matter of the lumbar spinal cord. However the exact mechanism for reduced apoptotic cell death by IPC was not focused in this study.

Another unexpected observation was that the sections obtained from the cervical spinal cords of the three groups planned as control groups, showed the same degree of necrotic damage in the same regions of the gray matter

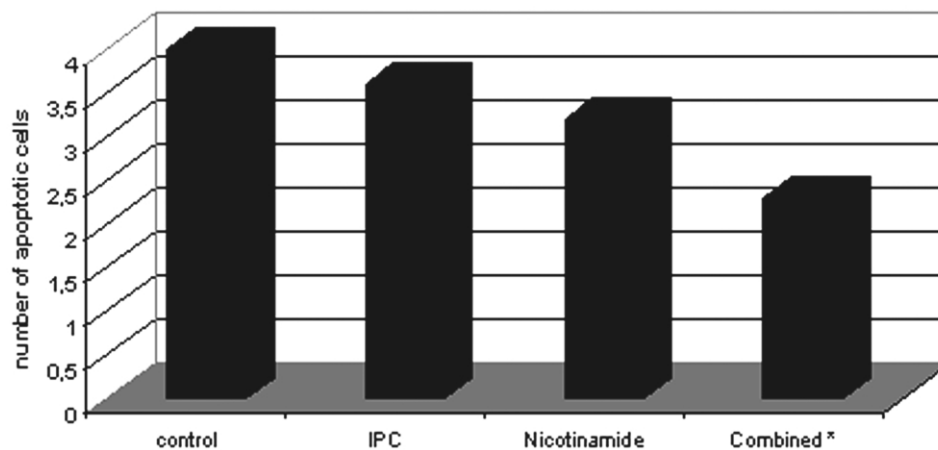


Fig. 3. Number of apoptotic neurons in the gray matter of the lumbar spinal cord in control, IPC, nicotinamide and combined IPC and nicotinamide groups (* $P < 0.05$ compared with IPC).

observed in lumbar sections. However increased cerebrospinal fluid pressure proximal to cross clamp, so called stagnant hypoxia might be responsible from this finding.

Several models of paraplegia secondary to aortic ischemia have been used in experimental models [14,20]. Surgery with big animals like dogs and pigs is expensive and time consuming. Several reports indicate that rat or rabbit models of spinal cord ischemia should be considered in this type of experiments. Small animal models are particularly attractive for use in rapid screening of neuroprotective strategies [21]. Poor collateral circulation to the lumbar cord is another advantage of these animals. In such animals abdominal aorta occlusion distal to the renal arteries and above the iliac bifurcation is sufficient to mimic the clinical situation. In our rat model, abdominal aorta was occluded both proximally and distally and distal pressure was measured below the iliac bifurcation.

Despite the fact that several studies have shown IPC to have a protective effect on the spinal cord there is still some doubt as to IPC duration. Zvara et al. reported that protective effect of IPC occurred after 3 min of ischemia and 30 min of reperfusion interval [8]. Abraham et al. reported a protective effect of IPC after 2 and 5 min of ischemia and 48 h of reperfusion interval [9]. Comparisons between the results of different studies are difficult as owing to the differences in species, experimental protocols and outcome measures. However one important thing is that, ischemia must be present at least 5 min to elicit the phenomenon [21,22]. We applied a short period of IPC in order to be similar to application in clinical situations.

There are several limitations to this study. First of all the blood supply of the rat spinal cord is not the same as in humans. Thus it may not necessarily be applicable to humans. Second, this study did not address to the molecular basis of apoptotic cell death. Therefore further studies are needed to clarify these results.

Results from our study provides evidence that death of spinal cord cells following transient aortic occlusion involves neuronal apoptosis as well as necrosis. They also support the contention that IPC and nicotinamide together may be beneficial in spinal cord injury by inhibiting apoptotic process. Understanding the molecular basis of apoptotic pathway and combinations of treatment modalities acting on different pathophysiologic cascades may further reduce the neurologic complications in aortic surgery.

References

- [1] Svensson LG, Crawford ES, Hess KR, Coselli JS, Safi HJ. Experience with 1509 patients undergoing thoracoabdominal aortic operations. *J Vasc Surg* 1993;17:350–70.
- [2] Wadoux F, Arndt CF, Metzger H, Hartmann M, Wadoux R, Borst HG. Direct measurements of oxygen tension on the spinal cord surface of pigs after occlusion of the descending aorta. *J Thorac Cardiovasc Surg* 1985;89:787–94.
- [3] Barone GW, Joob AW, Flanagan TL, Dunn CE, Kron IL. The effect of hyperemia on spinal cord function after temporary thoracic aortic occlusion. *J Vasc Surg* 1998;8:535–40.
- [4] Lim KH, Connolly M, Rose D, Siegman F, Jacobowitz I, Acinapura A, Cunningham Jr JN. Prevention of reperfusion injury of the spinal cord: use of recombinant superoxide dismutase. *Ann Thorac Surg* 1986;42:282–6.
- [5] Salzano RP, Ellison LH, Altonji PF, Richter J, Deckers PJ. Regional deep hypothermia of the spinal cord protects against ischemic injury during thoracic aortic cross-clamping. *Ann Thorac Surg* 1994;57:65–71.
- [6] Svensson LG, Von Ritter CM, Groeneveld HT, Rickards ES, Hunter SJ, Robinson MF, Hinder RA. Cross-clamping of the thoracic aorta: influence of aortic shunts, papaverine, calcium channel blocker, allopurinol, and superoxide dismutase on spinal cord blood flow and paraplegia in baboons. *Ann Thorac Surg* 1986;204:38–47.
- [7] Matsuyama K, Chiba Y, Ihaya A, Kimura T, Tanigawa N, Muraoka R. Effect of spinal cord preconditioning on paraplegia during cross-clamping of the thoracic aorta. *Ann Thorac Surg* 1997;63:1315–20.
- [8] Zvara DA, Colonna DM, Deal DD, Vernon JC, Gowda M, Lundnell JC. Ischemic preconditioning reduces neurologic injury in a rat model of spinal cord ischemia. *Ann Thorac Surg* 1999;68:874–80.
- [9] Abraham SV, Swain JA, Forgash AJ, Williams BL, Musulin MM. Ischemic preconditioning protects against paraplegia after transient aortic occlusion in the rat. *Ann Thorac Surg* 2000;69:475–9.
- [10] Rokkas CK, Helfrich LR, Lobner DC, Choi DW, Kouchoukos NT. Dextrorphan inhibits the release of excitatory amino acids during spinal cord ischemia. *Ann Thorac Surg* 1994;58:312–9.
- [11] Lang-Ladzunski L, Heurteaux C, Mignon A, Mantz J, Widmann C, Desmonts JM, Ladzunski M. Ischemic spinal cord injury induced by aortic cross-clamping: prevention by riluzole. *Eur J Cardiothorac Surg* 2000;18:174–81.
- [12] Lang-Ladzunski L, Bachet J. Pharmacological spinal cord protection with magnesium during replacement of the thoracic and thoraco-abdominal aorta. *Ann Thorac Surg* 2003;72:2180–1.
- [13] Ayoub IA, Lee JE, Ogilvy CS, Beal FM, Maynard KI. Nicotinamide reduces infarction up to 2 h after the onset of permanent focal cerebral ischemia in Wistar rats. *Neurosci Lett* 1999;259:21–4.
- [14] Tarlov IM. Spinal cord compression: mechanism of paralysis and treatment. Springfield, IL: Charles C.Thomas; 1957. p. 147.
- [15] Garcia JH, Kamijyo Y. Cerebral infarct evaluation of histopathological changes after occlusion of a middle cerebral artery in primates. *J Neuropathol Exp Neurol* 1974;33:408–21.
- [16] Rexed B. The cytoarchitectonic organisation of the spinal cord in the cat. *J Comp Neurol* 1952;96:415–85.
- [17] Kroll K, Schrader J, Piper HM, Henrich M. Release of adenosine and cyclic AMP from coronary endothelium in isolated guinea pig hearts: relation to coronary flow. *Circ Res* 1987;60:659–65.
- [18] Kato H, Kanellopoulos GK, Matsuo S, Wu YJ, Jacquin MF, Hsu CY, Choi DW, Kouchoukos NT. Protection of rat spinal cord from ischemia with dextrorphan and cyclohexemide: effects on necrosis and apoptosis. *J Thorac Cardiovasc Surg* 1997;114:609.
- [19] Zhou Y, Zhao YN, Yang EB, Ling EA, Wang Y, Hassouna MM, Mack P. Induction of neuronal and inducible nitric oxide synthase in the motoneurons of spinal cord following transient abdominal aorta occlusion in rats. *J Surg Res* 1999;87(2):185–93.
- [20] Lang-Ladzunski L, Matsushita K, Hirt L, Waeber C, Vonsattel JPG, Moskowitz MA. Spinal cord ischemia development of a model in the mouse. *Stroke* 2000;31:208–13.
- [21] Van Winkle DM, Thornton JD, Downey DM, Downey JM. The natural history of preconditioning: cardioprotection depends on duration of transient ischemia and time to subsequent ischemia. *Coron Artery Dis* 1991;2:613–9.
- [22] Fan T, Wang CC, Wang FM, Cheng F, Qiao H, Liu SL, Guo W, Xiang FY. Experimental study of ischemic preconditioning to spinal cord ischemia. *Surg Neurol* 1999;52(3):299–305.