New method for absolute spinal cord ischemia protection in rabbits

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Objective: This study aims to establish a superior procedure to prevent spinal cord damage after severe spinal cord ischemia during aortic surgery. We examined the synergistic effect of topical hypothermia of the spinal cord combined with radical scavenger infusion into the clamped segment of the aorta to prevent spinal cord damage in an animal model.

Methods: Spinal cord ischemia was induced in rabbits by clamping the aorta between the renal artery and aortic bifurcation for 30 minutes. Rabbits were divided into four groups of 16 each: group I, sham-operated; group II, edaravone (6 mL, 4°C, 1 mg/kg); group III, saline (6 mL, 4°C) with transvertebral cooling pads; group IV, edaravone (6 mL, 4°C, 1 mg/kg) and transvertebral cooling pads. Solutions were injected into the clamped segment of the aorta. Postoperative assessments included the Tarlov score, spinal cord histopathology, and measurement of malondialdehyde levels in the spinal cord tissue.

Results: At 48 hours after reperfusion, the mean Tarlov scores in groups I, II, III, and IV were 4.0, 1.5, 1.9, and 4.0, respectively. The mean number of normal motor neurons was significantly higher in groups I (54.1) and IV (53.7) than in groups II (32.8) and III (36.3; P < .001). The mean malondialdehyde level in groups I (19.8 nmol/mL) and IV (22.6 nmol/mL) was significantly lower than in groups II (64.8 nmol/mL) and III (60.9 nmol/mL; P < .001). At 168 hours after reperfusion, the mean Tarlov scores in groups I, II, III, and IV were 4.0, 1.1, 1.3, and 4.0, respectively. The mean number of normal motor neurons was significantly higher in groups I (52.9) and IV (50.8) than in groups II (22.4) and III (25.9; P < .001). The mean malondialdehyde level in groups I (20.7 nmol/mL) and IV (23.4 nmol/mL) was significantly lower than in groups II (68.9 nmol/mL) and III (61.6 nmol/mL; P < .001).

Conclusion: In a rabbit model with aortic clamping up to 30 minutes, which consistently produces complete paraplegia in rabbits, spinal cord damage was partially reduced by topical cooling with transvertebral cooling pads or the injection of edaravone into the clamped segment of aorta, but was more effectively protected by a combined use of these two strategies. (J Vasc Surg 2011;54:1109-16.)

Clinical Relevance: Spinal cord injury after surgical repair of the thoracic or thoracoabdominal aorta is a disastrous complication. Cold blood infusion into the clamped segment of aorta during thoracoabdominal aortic aneurysm surgery has been used in human operations in our department to detect the presence of a critical Adamkiewicz artery. Edaravone might be used in the same manner, with additional protective effect against spinal cord ischemia-reperfusion injury. This study demonstrated the synergistic effect of topical hypothermia of the spinal cord combined with edaravone infusion into the clamped segment of aorta to prevent spinal cord damage after absolute spinal cord ischemia in an animal model. Further research is needed to investigate the applicability of this technique to clinical settings.

Spinal cord injury after thoracoabdominal aortic aneurysm surgery remains a major cause of morbidity, despite recent improvements in operative techniques, spinal cord monitoring during the operation, and postoperative care. The reported incidence varies from 2.9% to 32%.1,2 For optimal protection of the spinal cord against ischemia-reperfusion injury, various adjunctive procedures have been developed, including cerebrospinal fluid drainage, adequate distal perfusion of the aorta, and pharmacologic agents. Although these have successfully reduced the incidence of postoperative paraplegia, the results are not yet satisfactory.

Mechanisms of ischemia-reperfusion injury of the spinal cord are likely to be multifactorial, among them inadequate blood supply, hypoxic endothelial cell activation, energy failure, excitotoxicity, and oxidative stress. Oxidative stress produces free oxygen radicals, which in turn enhance inflammatory processes such as lipid peroxidation, protein damage, and DNA damage.

A recent theory of paraplegia prevention in thoracoabdominal aortic surgery states that although paraplegia causation is anatomic, which is the interruption of spinal cord supplying the segmental arteries, paraplegia prevention strategies are primarily nonanatomic, deriving from interventions that prolong ischemic tolerance, reduce reperfusion injury, and enhance the collateral perfusion of the spinal cord.2 Adjunctive procedures that prolong the isch-
emic tolerance of the spinal cord (eg, hypothermia, barbiturates, and steroids) and that attenuate reperfusion injury (eg, steroids, hypothermia, and free-radical scavengers) reduce spinal cord injury in thoracoabdominal aortic repair. Spinal cord perfusion is improved by factors that optimize collateral blood flow, such as increasing or maintaining the cardiac index, maintaining a high mean arterial pressure, and initiating cerebrospinal fluid drainage.

Edaravone (3-methyl-1-phenyl-2-pyrazoline-5-one; Mitsubishi Pharma Corp, Osaka, Japan) is a potent free-radical scavenger that has been clinically used to reduce neuronal damage caused by ischemic stroke. Edaravone reduces the amount of reactive oxygen species (ROS) increased by reperfusion after ischemia and prevents impairment of the antioxidant defense system. The antioxidant actions of edaravone protect various cells against damage by ROS by enhancing prostacyclin production, inhibiting lipoxygenase metabolism of arachidonic acid by trapping hydroxyl radicals, inhibiting alloxan-induced lipid peroxidation, and quenching reactive oxygen. We previously reported that the injection of edaravone into the clamped segment of the aorta protects the spinal cord against 15 minutes of aortic clamping.

Hypothermia has also been used as an independent effective adjunctive therapy against ischemic injury. Various techniques for spinal cord cooling have been developed in many experimental and clinical trials. We previously developed a cooling pad that can efficiently cool the spinal cord. However, a longer aortic clamping time of 20 minutes correlates with more severe spinal cord ischemia and the occurrence of delayed-onset paraplegia.

This study aims to establish a superior procedure to prevent spinal cord damage after absolute spinal cord ischemia. We examined the synergistic effect of topical hypothermia, combined with the infusion of a radical scavenger into the clamped segment of aorta, to prevent spinal cord damage during aortic surgery in an animal model.

### MATERIAL AND METHODS

**Animal preparation.** Animal care and all procedures were in compliance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council (National Academy Press, revised 1996). This study protocol was approved by the Committee of Animal Experimentation, Hiroshima University.

**Anesthesia and monitoring.** Japanese White rabbits (weight, 2.5-3.0 kg) were used in this experiment. General anesthesia was induced with an intramuscular injection of ketamine (50 mg/kg). An ear vein catheter was inserted for the intravenous infusion of lactated Ringer solution. Cefazolin (10 mg/kg) was injected through the catheter. After tracheostomy and endotracheal intubation, the rabbits were ventilated mechanically with a fraction of inspired oxygen of 1.0 and isoflurane (1.5%-2.0%) to maintain sufficient anesthesia. Adequate ventilation was monitored by blood gas analysis.

A JMS C3 arterial catheter cutdown tube (JMS Company, Hiroshima, Japan) was inserted into the right axillary artery and the right femoral artery for blood pressure monitoring and blood gas analysis. Rectal temperature was continuously monitored and was kept at 38.0°C ± 0.5°C by using a warming blanket.

**Surgical procedures.** A left-side flank skin incision, parallel to the spine, was made with the rabbit in the right lateral decubitus position. The abdominal aorta was exposed through a retroperitoneal approach and was encircled with vascular tapes at a level distal to the renal artery and at the aortic bifurcation. After intravenous administration of heparin (100 U/kg), the abdominal aorta was clamped at the above levels with vascular clamps and isolated for 30 minutes.

To determine the clamping time that creates highly reproducible spinal cord ischemia, we performed a preliminary investigation in which the abdominal aorta distal to the renal artery and at the aortic bifurcation in 18 rabbits was clamped for 15, 20, and 30 minutes (six animals in each clamping-time group). Only animals in the 30-minute clamping group were completely paraplegic after the operation and during the observation until 168 hours after reperfusion.

We randomly divided 64 rabbits into groups I, II, III, and IV, with 16 animals in each group. Each group was further divided into two subgroups of eight animals each: subgroup A was euthanized at 48 hours after reperfusion, and subgroup B was euthanized at 168 hours after reperfusion. The groups underwent the following procedures:

- **Group I:** sham operation in which the abdominal aorta was exposed but was not clamped.
- **Group II:** Edaravone (1 mg/kg) was diluted with cold saline (4°C; total volume, 6 mL) and injected...
allowed to recover from anesthesia and were extubated. The animals were closed, and all catheters were withdrawn. The animals were placed on the pedicle of vertebral arch of the third or fourth lumbar vertebra. After declamping, the abdomen was placed on the peritoneum, lying on the left side of vertebral bodies at the levels of L1 to L5, between the aorta and psoas muscle, during aortic clamping. The transvertebral cooling pad was placed in the abdominal cavity on the peritoneum, lying on the left side of vertebral bodies at the levels of L1 to L5, between the aorta and psoas muscle during aortic clamping (Fig 2). The pad was removed when the temperature of vertebral bone cooled to 25°C and was replaced with a new one when the temperature of vertebral bone raised to 30°C, so that the temperature of vertebral bone was maintained between 25°C and 30°C during aortic clamping (Fig 3).

- **Group III**: Cold saline (4°C, 6 mL) was slowly injected into the clamped segment of the aorta immediately after clamping (Fig 1);
  - **Group IV**: Edaravone (1 mg/kg) was diluted with cold saline (4°C; total volume, 6 mL) and injected. Cooling pads were applied as in group III.

The cooling pads had been cooled at –10°C for at least 30 seconds through an elastic catheter into the clamped segment of the aorta immediately after clamping (Fig 1);

- **Group III**: Cold saline (4°C, 6 mL) was slowly injected into the clamped segment of the aorta immediately after clamping. A transvertebral cooling pad, consisting of 7-×3-×1.5-cm bags made of polyvinyl chloride, packed with 10 mL of gel (98% water, 1% acrylate sodium polymer, and 1% propylene glycol) was placed in the abdominal cavity on the peritoneum, lying on the left side of vertebral bodies at the levels of L1 to L5, between the aorta and psoas muscle during aortic clamping (Fig 2). The pad was removed when the temperature of vertebral bone cooled to 25°C and was replaced with a new one when the temperature of vertebral bone raised to 30°C, so that the temperature of vertebral bone was maintained between 25°C and 30°C during aortic clamping (Fig 3).

- **Group IV**: Edaravone (1 mg/kg) was diluted with cold saline (4°C; total volume, 6 mL) and injected. Cooling pads were applied as in group III.

The cooling pads had been cooled at –10°C for at least 24 hours in a freezer until they were used. The catheter was immediately removed after saline or edaravone was injected, and the puncture site was closed by tying a purse-string suture of 8-0 Prolene (Ethicon Inc, Somerville, NJ).

### Statistical analysis.

All results are expressed as mean ± standard deviation. Statistical significance is assumed at a value of $P < .01$. The Mann-Whitney U test, one-way analysis of variance, and post hoc tests with the Tukey honestly significant difference test were used to identify which group differences accounted for the significant $P$ values. Mean data are presented with the standard deviation.

### RESULTS

**Perioperative data.** No adverse events occurred during the operation. Heart rate, blood pressure, and levels of arterial blood gases were similar in the four groups. Changes in temperature of the pedicle of lumbar vertebrae during hypothermic treatment are shown in Fig 3. All rabbits survived until euthanasia.

**Neurologic outcome.** Neurologic function in group IV-B was better than in groups II-B and III-B in all intervals (Fig 4). The mean Tarlov scores in groups II-B, III-B, and IV-B were, respectively, 2.8 ± 0.7, 3.4 ± 0.7, and 4.0 ± 0.7 at 8 hours after reperfusion; 1.8 ± 0.9, 2.2 ± 0.9, and 4.0 ± 0.0 at 24 hours; 1.5 ± 0.9, 1.9 ± 0.6, and 4.0 ± 0.0 at 48 hours; and 1.1 ± 0.8, 1.3 ± 1.0, and 4.0 ± 0.0 at 168 hours. The Tarlov score in sham-operated group was 4.0 ± 0.0 at all intervals. The scores of groups I-B and IV-B were significantly higher than those in II-B at 8 hours after reperfusion ($P < .01$) and at the other intervals after reperfusion ($P < .001$). The scores of groups I-B and IV-B were significantly higher than those of III-B at 24, 48, and 168 hours after reperfusion ($P < .001$). The scores between

**Assessment of neurologic function.** The neurologic function of the hind limbs of the animals in subgroup B was assessed at 8, 24, 48, and 168 hours after reperfusion by an observer blinded to the protocol used for each animal. The motor function of the hind limbs was graded by using the Tarlov score, in which 0 indicates no movement of the hind limbs, 1 indicates perceptible movement of the joints of hind limbs, 2 indicates good movement of the joints but an inability to stand, 3 indicates the ability to stand and walk, and 4 indicates a complete recovery (the ability to hop).
groups I-B and IV-B and also between II-B and III-B were not significantly different at all intervals after reperfusion.

**Histopathologic findings.** The numbers of normal motor neurons in the anterior horn of the spinal cord in a slice of specimen at 48 hours after reperfusion by group were I-A, 54.1 ± 3.2; II-A, 32.8 ± 4.3; III-A, 36.3 ± 3.9; and IV-A, 53.7 ± 3.1 (Fig 5). Significantly more normal motor neurons were found in groups I-A and IV-A than in II-A and III-A (P < .001; Fig 6). The difference in the number of normal motor neurons between groups I-A and IV-A and between II-A and III-A was not significant. The numbers of normal motor neurons at 168 hours after reperfusion by group were I-B, 52.9 ± 2.4; II-B, 22.4 ± 5.5; III-B, 25.9 ± 6.3; and IV-B, 50.8 ± 2.6. Groups I-B and IV-B had a significantly higher number of normal motor neurons than II-B and III-B (P < .001). The difference in the number of normal motor neurons between groups I-B and IV-B and between II-B and III-B was not significant. The difference between group II-A and II-B and between III-A and III-B was significant (P = .001).

**Malondialdehyde measurement.** Malondialdehyde levels at 48 hours after reperfusion by group were I-A, 19.8 ± 5.9; II-A, 64.8 ± 5.2; III-A, 60.9 ± 7.1; and IV-A, 22.6 ± 5.9 nmol/mL. The levels of malondialdehyde in groups I-A and IV-A were significantly lower than in II-A and III-A (P < .001; Fig 7). The malondialdehyde levels between group I-A and IV-A and between II-A and III-A were not significantly different. Malondialdehyde levels at 168 hours after reperfusion by group were I-B, 20.7 ± 5.2; II-B, 68.9 ± 6.5; III-B, 61.6 ± 7.3; and IV-B, 23.4 ± 5.1 nmol/mL. The malondialdehyde levels in groups I-B and IV-B were significantly lower than in groups II-B and III-B (P < .001). The malondialdehyde level between groups I-B and IV-B and between groups II-B and III-B was not significantly different. The difference between subgroups within each group was not significant.

**DISCUSSION**

Paraplegia after surgical repair of descending thoracic and thoracoabdominal aortic aneurysm is closely related to the duration and severity of ischemia and to reperfusion injury. Many modalities have been developed to minimize the risk of neuronal damage, including hypothermia, cerebrospinal fluid drainage, distal perfusion of the aorta, and pharmacologic agents such as steroids, barbiturates, or free-radical scavengers.1,11,14,15 We considered that edaravone and hypothermia could be promising for spinal cord protection and evaluated a combined use of these two strategies in a rabbit model.

The caudal portion of the rabbit spinal cord receives blood exclusively from the infrarenal abdominal aorta and thus is susceptible to ischemic injury when this segment is clamped. In other animals frequently used as study models of spinal cord ischemia, including cat, dog, pig, and mon-
The thoracic aorta mainly supplies the caudal portion of the spinal cord; thus, producing a spinal cord ischemic injury requires occlusion of the thoracic aorta. Even though the rabbit’s anatomy is different from humans, rabbits are often used as experimental models of spinal cord ischemia because of the model’s highly reproducible ischemic lesion and simple procedure.

Oxidative stress leads to massive production of free oxygen radicals, which enhance inflammatory processes, such as lipid peroxidation, protein damage, and DNA damage. Unstable radicals are potent initiators of protein degradation and lipid peroxidation, which in turn can lead to cell membrane dysfunction and cell death. Free oxygen radical-mediated lipid peroxidation is a self-perpetuating process that can spread to the circumferential undamaged neuronal tissue, leading to further collapse of microcirculation and to irreversible damage to myelin and axons.

Edaravone has been used in patients with acute cerebral infarction since April 2001 in Japan because of its protective effect against ischemia-reperfusion injury. It not only improves functional outcomes in acute stroke injury patients but also prevents brain edema after ischemia-reperfusion injury. Edaravone salvages the boundary zone of the infarct by scavenging reactive oxygen species, especially in the neurons during permanent focal cerebral ischemia. The putative mechanism underlying the antioxidant action of edaravone is as follows:

- An electron transfer from an edaravone anion to a peroxy radical yields an edaravone radical and peroxy anion.
- This reaction breaks the oxidation chain of lipids.
- The edaravone peroxy radical transforms to 4,5-dione by elimination of a hydrogen atom and one electron.

Fig 5. Histopathologic findings of the spinal cord are shown at 48 hours (subgroup A) and at 168 hours (subgroup B) after reperfusion (hematoxylin and eosin staining; original magnification, ×200). I-A and I-B, sham-operated rabbits; II-A and II-B, edaravone-treated rabbits; III-A and III-B, rabbits treated with saline and transvertebral cooling pad; IV-A and IV-B, rabbits treated with edaravone and transvertebral cooling pad. Arrows, normal motor neurons; arrowheads, ischemic neurons (the cell body was shrunken; pyknotic nucleus; the cytoplasm became eosinophilic, with loss of Nissl granules).
Finally, 2-oxo-3-(phenylhydrazono)-butanoic acid is produced by the hydrolysis of 4,5-dione.23 Edaravone is thought to exist near the cell membrane or, perhaps, on the cell membrane. It has a low molecular weight (174.2), is both lipid-soluble and water-soluble, and has good cell membrane permeability.23 Edaravone is metabolized to its glucuronide and sulfate conjugates in the liver and is excreted rapidly in the urine.24 Side effects are occasionally observed during edaravone treatment, including acute renal failure, liver dysfunction, acute allergic reaction, disseminated intravascular coagulation, thrombocytopenia, leukocytopenia, and renal dysfunction.25

We previously reported that an edaravone injection into the clamped aortic segment protects the spinal cord against 15 minutes of aortic clamping.7 However, longer aortic clamping time (≥20 minutes) correlates with more severe spinal cord ischemia and the occurrence of delayed-onset paraplegia.14 As aortic clamping time was extended to 30 minutes in the current study, the intra-aortic injection of edaravone showed preservation of neurologic function (average Tarlov score, 1.1-2.8) and an elevated malondialdehyde level in the spinal cord. Because malondialdehyde is indicative of oxidative stress lipid peroxidation in the spinal myelin, these results suggest that the neuronal damage was related to an involvement of free oxygen radicals in neuronal injury. Thus, edaravone is effective in reducing ischemic injury of the spinal cord, but the effect was inadequate for the prolonged ischemic insult.

Systemic hypothermia has been applied to protect the spinal cord in surgery on the thoracoabdominal aorta. However, it leads to deterioration of platelet function and can be responsible for increased amount of bleeding during surgery. Thus, we previously devised a transvertebral cooling pad to provide regional cooling of the spinal cord through the anterolateral vertebrae and, preferentially, the anterior horn of spinal cord. It effectively protected the spinal cord in this study, but its protective effect was not adequate when the ischemic time was prolonged.

Neuronal injury caused by spinal cord ischemia occurs in two phases: immediate and delayed neuronal injury.26 Immediate injury is often caused by a severe ischemic insult and rapidly necrotized neuronal cells. The delayed injury is
caused by apoptosis or necrosis. These studies also reported that disruption of the infrarenal abdominal aortic blood flow for 15 minutes caused immediate neurological injury by 2 days and delayed paraplegia by 7 days. In our study, a number of motor neurons in group II-B and III-B that were able to survive up to 48 hours died during the next 120 hours. Bolli et al. showed that potent oxidant radicals are produced within the first few minutes of reflow and are crucial in the development of reperfusion injury. To our surprise, sufficient spinal cord protection was obtained by one dose of edaravone just after aortic clamping and regional cooling of the spinal cord during the aortic clamping. Inhibition of spinal cord injury at an early period after aortic clamping seems to be responsible for its long-lasting effect to prevent the occurrence of immediate and delayed neuronal death. This inhibition of spinal cord injury did not appear in groups II and III because neurologic function and histologic assessment at 48 and 168 hours after reperfusion showed the progression of neuronal injury. Because edaravone has such a short half-life—30 minutes in the circulation and 4 hours in the brain tissue—it seemed that the cells in groups II and III that eventually died after delayed phase might be the cells that were injured during the early phase and were unable to survive 

Christie et al. reported that malondialdehyde levels in spinal cord tissue increased twice after acute spinal cord injury and remained at high levels for several days. Our study showed that malondialdehyde levels, as a marker of oxidative stress and free radical–mediated damage, were not significantly different between the assessments at 48 and 168 hours. These results suggested that the neuronal death was not caused by apoptosis, although this could not be clearly evidenced in our study without apoptosis assessments.

Sueda et al. reported cold blood infusion into the clamped segment of aorta under motor-evoked potentials monitoring for detecting the presence of the critical artery of Adamkiewicz. However, the cooling effect on the spinal cord was transient. This study has demonstrated a synergistic protective effect by a combined use of edaravone and continuous regional cooling with a cooling pad in neurologic assessment at four intervals as well as in the number of motor neurons and malondialdehyde levels at 48 hours and 168 hours. An integrated use of these two strategies appears to be advantageous to spinal cord protection, but further investigations must be performed to know the efficacy and safety of the drug in the clinical setting.

This study has some limitations. First, it might be difficult to apply the cooling pad on the vertebral bone in the operative field in clinical cases due to the presence of aneurysm. A potential solution is to place the cooling pad after the aneurysm is opened.

Second, it is not clear how edaravone injected into the aorta was delivered to the spinal cord tissue, and it may also be difficult to elucidate this point in clinical cases. However, the effect of the cold edaravone solution was apparent in the current study. In vivo monitoring of reactive oxygen species production in the spinal cord, measurement of systemic levels of edaravone after an intra-aortic injection of edaravone, or the upregulation of the antioxidant defense system would provide more information.

Third, thiobarbituric acid reacts not only with aldehyde end products of lipid peroxidation such as malondialdehyde but also with nonlipid thiobarbituric acid–reactive substances. But the concentration of such potentially misleading substances in tissue extracts is low under normal conditions, and an error in measurement is unlikely.

CONCLUSIONS

Long-term aortic clamping (30 minutes) consistently causes complete paraplegia in rabbits. Spinal cord damage was partially reduced by topical cooling or edaravone injection into the aorta, but the spinal cord was adequately protected by the combined use of both strategies, probably by blocking two mechanisms of ischemic injury synergistically. However, further investigation is mandatory for the clinical application of this method.
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