Epidural Cooling Minimizes Spinal Cord Injury after Aortic Cross-clamping through Induction of Nitric Oxide Synthase

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**Background:** By using a U-shaped lumen catheter, the authors examined the effects of epidural cooling on spinal cord injury after aortic cross-clamping (ACC), with a focus on changes of spinal cord blood flow and expression of inducible nitric oxide synthase.

**Methods:** Sixteen pigs were randomized into two groups: Control group (n = 8) or Cooling group (n = 8). In the latter, epidural cooling started at 30 min (baseline) before 45 min of ACC and persisted for the next 30 min of reperfusion period. Spinal cord blood flow and somatosensory-evoked potentials were assessed during peri-ACC period. At 48 h, we evaluated hind limb function by using Tarlov score and expression of inducible nitric oxide synthase on spinal cord using immunohistochemistry.

**Results:** After ACC, spinal cord blood flow dropped to a similar extent in both groups. During the reperfusion period, spinal cord blood flow increased up to 113% (103–124%), median (interquartile range), level transiently and decreased to 32% (22–47%) level versus baseline in the Control group, whereas it increased and remained at 92% (86–97%) level in the Cooling group. Simultaneously, somatosensory-evoked potentials showed that onset of loss time was delayed and recovery time was shortened in the Cooling group. Tarlov scores in the Cooling group were significantly higher and accompanied by normal-appearing motor neurons and significantly greater expression of inducible nitric oxide synthase on spinal cord versus the Control group.

**Conclusions:** This study shows that epidural cooling during ACC minimized the risk of spinal cord injury, possibly by preventing delayed hyperperfusion and upregulating inducible nitric oxide synthase expression.

ALTHOUGH the outcome of surgical repair for thoracoabdominal aortic aneurysm has been improved as a result of a marked advance of perioperative care and surgical techniques, paraplegia, occurring with high incidence between 6 and 16%, is not yet resolved as the most severe complication.1,2 Although the underlying mechanisms remain to be fully determined, several factors, such as interruption of blood flow and/or increased cerebrospinal fluid pressure, have been proposed.3 Clinically available protection of spinal cord consists of two approaches, i.e., preservation of regional blood supply and reduction of oxygen demand during aortic cross-clamping (ACC). The former can be achieved with intercostal implantation, left heart bypass grafting, or cerebrospinal fluid drainage, whereas the latter with various pharmacologic agents and hypothermia.4–5 Hypothermia is one of the most promising ways; however, given at systemic level detrimental complications such as coagulation abnormality, cardiac arrhythmia and pulmonary dysfunction could be unmasked.6,7

Cambria et al. demonstrated that regional hypothermia of spinal cord by infusion of iced saline into epidural space reduced spinal cord injury without systemic complications.8 However, another concern has been raised to question whether this approach increases cerebrospinal fluid pressure associated with further deterioration of spinal cord perfusion. We, therefore, developed a newly designed epidural cooling catheter with closed perfusion circuit and demonstrated protective effects against spinal cord injury without elevating cerebrospinal fluid pressure.9 Other investigators also described as a preliminary report that regional cooling with a closed perfusion system could be a good approach to prevent spinal cord injury during aortic surgery.10 We, therefore, designed the current study to examine the effects of epidural cooling with focus on the changes of blood flow and somatosensory-evoked potentials (SEP) of spinal cord during peri-ACC periods, accompanied by evaluation of neurologic outcomes in a porcine model. In spinal cord injury induced by ischemia, activation of inducible nitric oxide synthase (iNOS) and subsequent nitric oxide production has been appreciated as a marker and therapeutic target.11,12 Whereas two time periods of moderate to excessive nitric oxide discharge after spinal cord ischemia have been defined, i.e., immediately after injury and several hours to days later, previous studies indicated that iNOS expression was mainly responsible for the latter.13–15 In the current study, we therefore assessed histologic alterations concurrent with iNOS expression on spinal cord at 48 h after ischemic insults.

**Materials and Methods**

This experimental protocol was approved by the Animal Care Committee of Saitama Cardiovascular and Respiratory Center, Kumagaya, Saitama, Japan.
Animal Model and Preparatory Surgery
Male domestic pigs (40-50 kg) were studied after a 3- to 7-day period of acclimatization in our laboratory. The animals were initially anesthetized with intramuscular ketamine (10 mg/kg), and the trachea was intubated. With isoflurane anesthesia in oxygen, respiratory rate and tidal volume were adjusted to maintain normocapnia. Under the right lateral decubitus position, a cooling-warming blanket was applied to maintain rectal temperature at approximately 36°C throughout the study periods. An epidural cooling catheter (Unitika, Tokyo, Japan), which was developed by our group, was inserted at L2 level via laminectomy and removal of ligamentum fluvum. The catheter has 16-gauge outer diameter and 35-cm length and a closed countercurrent lumen, allowing fluid to circulate the inside. The tip of catheter directed into cephalad of epidural space was placed at Th6 level, which was confirmed for each animal by using an x-ray examination. The other side of this catheter was connected to an external roller pump and heat exchanger (MERA modular type and CP-4; Senko Medical Instrument, Tokyo, Japan), which enabled cooled-saline to circulate inside the catheter (fig. 1). Epidural cooling catheter, cooling unit, and circulating pump made up the circuit. Distilled water was not infused into epidural space directly but was circulated as a coolant within the U-shaped lumen of epidural catheter and extracorporeal circuit. This system and an epidural catheter with closed countercurrent lumen could provide regional cord cooling without increasing intrathecal pressure as described previously. Bipolar electrodes for monitoring of SEP were placed in epidural space at L4/5 level for stimulation and at Th5/6 level for record. To continuously monitor epidural and subarachnoidal temperatures, thermistor probes were placed on dorsal dura and in subarachnoid space at L3 level. Arterial catheters were inserted in right axillary artery (proximal to clamp site) and right femoral artery to measure arterial blood pressure (distal to clamp site), respectively. Heart rate and rectal temperature were continuously monitored throughout the study periods.

Study Protocol
After the placement of epidural cooling and monitoring catheters, 16 domestic pigs were randomly allocated into two groups by using a computer-generated random number. Control group (n = 8) received placement of an epidural catheter alone, Cooling group (n = 8) received regional cooling by continuous cord cooling system with an epidural cooling catheter (fig. 2). The animals then underwent thoracotomy at the fourth and seventh intercostals under sterile conditions. After surgical preparation for ACC, heparin sulfate (60 U/kg) was injected to provide systemic anticoagulation. In Cooling group, epidural cooling by circulating cooled saline (4°C) at a rate of 75-90 ml/min was started 30 min before ACC and continued until 30 min after ACC (105 min for total epidural cooling time). All animals underwent segmental ACC for 45 min between the distal site to left subclavian artery and the proximal site to the diaphragm. Complete ACC was confirmed by the loss of femoral artery pressure (distal blood pressure) during ACC. Transient hypertension detected at the right axillary artery after ACC was regulated with intermittent injection of intravenous nicardipine hydrochloride. Blood pressure after declamping was maintained with fluid resuscitation and intravenous injection of phenylephrine hydrochloride. After a 3-h examination period, all catheters and monitors were removed and wounds were closed. When emergence from anesthesia was achieved, the trachea was extubated, and the animal was returned to the cage for subsequent examination of neurologic outcome in hind limb for 48 h.

Measurements of Blood Flow and SEP of Spinal Cord
Blood flow to spinal cord (SCBF) was continuously measured by using laser-Doppler flowmetry (Model LIBF-III; Biomedical Science, Tokyo, Japan) adjusted for high-flow measurement (12 kHz, gain 1 or 3) and a time constant of 3.0 s. The signal was continuously recorded on a 2-channel strip-card recorder and referred to as SCBF. The tip of laser-Doppler probe was implanted on the epidural surface at L2 level, because porcine spinal

![Fig. 1. Schematic drawing of continuous cord cooling system and epidural cooling catheter with a closed countercurrent lumen.](http://anesthesiology.pubs.asahq.org/pdfaccess.ashx?url=/data/journals/jasa/931088/ on 08/18/2017)
cord extends at L6 and preservation of spinal cord under physiologic state is consequential. In our pilot study, we measured both direct and transepidual spinal cord blood flow simultaneously by using two independent laser-Doppler probes placed on dorsal lumbar spinal cord and epidural space at 2 mm apart, and we found that the change of direct measurement was correlated well with indirect approach, i.e., transepidual measurements of spinal cord blood flow. Characteristics of translucent dura with few vessels in pigs accompanied by capability of laser-Doppler to measure up to 1 mm in depth allowed us to measure accurate and real-time alterations of SCBF.

We simultaneously examined SEP directly derived from spinal cord. Stimulation was given with 0.2-ms pulse duration and 3- to 5-m A current with a rate of 5.0 Hz (Neurapack; Nihon Kohden, Tokyo, Japan). Potentials were recorded on a time base of 3 ms after passing through a bandpass filter of 50 to 1500 Hz. Each record represented an average of 50 repetitions. A baseline SEP was recorded before the initiation of epidural cooling, and the recording was repeated at 60-s intervals. Signal amplitude and latency of each SEP were recorded and compared to the baseline.

**Neurologic and Histologic Assessments**

Hind limb motor function was evaluated at 24 and 48 h after the ACC by using a modified Tarlov score (0 = complete paralysis, 1 = minimal movement, 2 = standings alone but unable to walk, 3 = weak walking, 4 = full recovery with normal walking). After neurologic examination at 48 h, the animals were sacrificed with an overdose injection of sodium pentobarbital. Spinal cord was removed immediately and fixed in 10% formaldehyde solution. Spinal cord at L4 level was sliced into sections, and each section was stained with hematoxylin and eosin. Ten slices per animal were examined for histologic assessment. A pathologist who was unaware of the study protocol and neurologic outcomes of the animals examined each slice by light microscopy and counted the total number of motor neurons (larger than 50 μm in diameter) with normal appearance. Simultaneously, histologic alterations to reflect inflammatory responses of ischemic spinal cord injury such as proliferation of microglia cells at perivascular area and formation of hydropic vacuolation were assessed as described previously.

**Immunohistochemical Staining of Spinal Cord**

Spinal cord fixed with 10% formalin and embedded with paraffin were sectioned into 4 μm. After de-waxing with xylene and rehydrating with methanol, these sections were heat-treated in 10 mM citrate buffer (pH 6.0) at 105°C for 10 min and then washed with Tris-buffered saline three times for 5 min. Specimen was blocked with Image-iT FX (Tokyo, Japan) for 30 min. After washing, iNOS (Epitope-specific rabbit antibody, LVC; Lab Vision Corp., Fremont, CA) and normal rabbit immunoglobulin (control antibody, Vector) were incubated for 60 min at room temperature. After washing with Tris-buffered saline-Tween 20, each section was treated with Alexa Fluor 488-conjugated goat anti-rabbit antibody (Invitrogen; Carlsbad, CA). After washing with Tris-buffered saline-Tween 20, the sections were counterstained and mounted with an antifade reagent (ProLong Gold®, Invitrogen) and 4′,6-diamidino-2-phenylindole.

The level of iNOS expression on spinal cord was determined by digital imaging after immunofluorescence. Images were taken by using epifluorescent microscopy with Applied Imaging Ariol® SL-50 system (Genetix, Hampshire, United Kingdom) equipped with magnification of 40× objective. The slide which has maximum signal to noise ratio in caudal extremity in spinal cord were manually selected. In selected slides, the optimal exposure time of acquisition was set with a highly sensitive charge-coupled device camera. Then, the images of iNOS expressed were obtained from all specimens with the same acquisition setting and were depicted by pseudo color merging with image processing software ImageJ (National Institutes of Health, Bethesda, MD). Color-merged Red-Green-Blue images were split into each 8-bit gray channel with ImageJ. Motor neuron in each image was selected as measurement region of interest. Signals of iNOS were extracted from background noise subtraction with threshold adjustment in binary images. Target average intensities were measured with ImageJ measurement and were indicated as histogram.

**Statistical Analysis**

Data were presented as median and interquartile range unless otherwise specified, and Mann–Whitney U-test was applied by using a statistical software package of SPSS/15.0J for Windows (SPSS Inc., Chicago, IL). In all cases, two-tailed P value less than 0.05 was considered significant.

**Results**

All animals in both study groups survived for 48-h study periods, and no animal was excluded from the analyses.

**Changes of Epidural and Subarachnoidal Temperatures and SCBF during Peri-ACC Periods**

Rectal temperatures in both the Control and Cooling groups remained around 36°C (36.1 ± 0.4°C and 36.2 ± 0.4°C, respectively; mean ± SD) throughout the study periods. In the Control group, both epidural and subarachnoidal temperatures were kept within comparable ranges with the ranges of rectal temperatures (fig. 3A).
On the other hand, epidural temperature in the Cooling group dropped immediately after initiating epidural cooling and kept approximately 24–26°C ranges during ACC. Simultaneously, subarachnoidal temperature in the Cooling group reached 26°C level and stayed between 26 and 28°C. After the end of epidural cooling, both temperatures in the Cooling group increased gradually up to the baseline level.

Figure 3B illustrates the changes of SCBF during peri-aortic cross-clamping periods in Control and Cooling groups. Values are mean (SD). Note that SCBF in both groups were kept at approximately 20% level during aortic cross-clamping versus baseline, and that SCBF in the Cooling group increased and remained at 92% level throughout the study period.

Table 1. Loss and Recovery of Spinal Cord Somatosensory-evoked Potentials in the Control and Cooling Groups

|                          | Control Group | Cooling Group | P Value |
|--------------------------|---------------|---------------|---------|
| Onset of SEP loss, min   | 12.9 (9.6–13.7)| 26.5 (22.3–30.0)| 0.0008  |
| Total duration of SEP loss, min | 41.9 (37.2–43.8) | 21.3 (20.4–25.9) | 0.0008  |
| Recovery time of SEP, min | 6.9 (5.7–9.9)  | 3.2 (2.4–4.2)  | 0.0023  |

Data were expressed as median (interquartile range). Mann-Whitney U-test was used to compare the difference between the groups. SEP = spinal cord somatosensory-evoked potentials.

versus the baseline. On the contrary, SCBF after de-clamping in the Cooling group increased gradually and remained over 92% (86–97%) level throughout the reperfusion period.

SEP of Spinal Cord and Neurologic Outcome

No animal showed the abortion of SEP waveform as a result of epidural cooling itself. During epidural cooling before ACC, latency of SEP was prolonged, but its amplitude remained unchanged. Onset time of SEP loss after ACC was significantly longer in the Cooling versus the Control group, whereas total duration of SEP loss in the Cooling group was significantly shorter versus the Control group (table 1). In addition, recovery time of SEP after de-clamping was significantly shorter in the Cooling group versus Control group.

Figure 4 illustrates the data of neurologic outcome by scoring hindlimb function at 24 and 48 h after ACC. The animals in the Cooling group showed significantly better Tarlov scores at 24 and 48 h versus the Control group (Mann-Whitney U-test P = 0.0003 and 0.0004, respectively). Besides, the total number of intact motor neu-
rons per each section was significantly greater in the Cooling versus the Control group (123 [117–126] vs. 66 [52–75]: Mann–Whitney U-test $P = 0.0001$), indicating that almost half of motor neurons were lost in the Control group.

**Histopathological Alterations and Expression of iNOS in Spinal Cord**

Figures 5A and 5B show representative microphotographs of anterior horn of spinal cord in both the Control and Cooling groups. Nearly normal appearance of motor neurons with Nissl granules and glia cells were found in the Cooling group (panel B), whereas severe necrosis of motor neurons accompanied by a moderate level of hydropic vacuolation and apparent gliosis, *i.e.*, proliferation of astrocytes and microglia cells, and vascularization was observed in the Control group (panel A).

Figures 6A and 6B illustrate the representative microscopic images of positive immunostaining iNOS expression on spinal cord in both study groups. The extent of iNOS expression and the number of motor neurons were apparently augmented in the Cooling group versus the Control group. Figure 6C demonstrates histogram of iNOS expression of spinal cord in both study groups quantitatively assessed using ImageJ. The expression of iNOS was significantly augmented in the Cooling group versus the Control group (Mann–Whitney U-test $P = 0.012$).

**Discussion**

By using a porcine model of ACC, we demonstrated that epidural cooling catheter with closed circuit lumen preserved regional hypothermia of spinal cord approximately at 26°C and completely prevented the development of paraplegia at 48 h. We also showed that epidural cooling prevented reactive hyperemia and delayed hypoperfusion of SCBF after ACC, accompanied by apparent reduction of histologic alterations such as necrosis of motor neurons, hydropic vacuolation, and proliferation of microglia cells and microvessels. Simultaneously, regional epidural cooling during ACC augmented the induction of iNOS in motor neurons at 48 h after ischemic insult, which could be associated with the improvement of neurologic outcomes. Although its protective property against delayed-onset paraplegia remains to be determined, the current study shed light on clinical application of this continuous cord cooling system in patients undergoing thoracoabdominal surgery.

Because of its similarities of anatomical characteristics and hemodynamic responses to human as well as its reproducibility of paraplegia, the porcine model has been widely used for examination of spinal cord injury and validation of protective strategy during ACC. In the current study, ACC induced abrupt reduction of SCBF irrespectively from epidural cooling, whereas approximately 20% level of baseline SCBF was preserved during ACC (fig. 3B), indicating that SCBF was in part supplied by collateral vessels and that paraplegia observed in the Control group was not fully attributed to complete block of blood flow during ACC. Although our approach for blood flow measurement does not directly reflect the ventral side of spinal cord, where vulnerable motor neurons are rich, previous study demonstrated that there were no differences in blood flow among different areas of spinal cord during insults. In addition, some may argue that motor-evoked potentials rather than SEP, mediated through posterior and lateral column of spinal cord, could be more reasonable for the assessment of ischemic spinal cord. Due to its lesser sensitivity to...
volatile anesthetics, more feasibility in clinical settings, and compatibility with motor-evoked potentials to predict paralysis during ACC, however, we used SEP during spinal cord ischemia in this study. We then found that total time of SEP loss could predict a risk of paraplegia more reliably than the amplitude of recovery wave. On the other hand, the time to loss of SEP observed in the Cooling group was twice as much versus the Control group, indicating that functional activity of neuronal cells assessed by SEP was not always dependent on the amount of blood supply. Previous study also showed that the time to loss of SEP and the time required for SEP recovery reflected neurologic outcomes of motor function.

As a postischemic common phenomenon, reactive hyperemia, defined as transient augmentation of blood supply to tissues after ischemic insults, could be the major trigger to evoke tissue edema and organ dysfunction through excessive discharges of oxidative species and other mediators. Indeed, paraplegia is directly correlated with the extent of reactive hyperemia, which response found in our study appeared to be too small compared to previous reports. Barone et al. demonstrated that eight times greater extent of hyperemia versus baseline induced apparent edema of spinal cord and paraplegia and that 200% level of augmented blood flow was not high enough to evoke apparent paraplegia after spinal cord ischemia. On the contrary, other studies demonstrated that delayed hypoperfusion per se was more consequential to modulate the size of cerebral infarction and to develop structural alterations of spinal cord, including hydropic vacuolation and proliferation of microglia cells and microvessels, accompanied by swollen motor neurons as observed in the Control group (fig. 5A). Collectively, prevention of delayed hypoperfusion could be a prevailing component of epidural cooling properties to protect spinal cord from ischemic injury.

Another important finding of this study is that epidural cooling augmented the expression of iNOS on spinal cord versus the Control group. Spinal cord injury after ACC is basically caused by both primary injury associated with severe reduction of SCBF and secondary injury associated with free radical production, intracellular cal-

Fig. 6. Immunohistochemical staining of spinal cord for inducible nitric oxide synthase (iNOS) (magnification ×40) and histogram of average intensity of iNOS expression in ventral gray matter. (A) A representative picture of the Control group. Positive staining for iNOS is green. Note that iNOS expression was found in proliferated glia cells (arrow) and that motor neurons were obviously swollen without iNOS expression (asterisk). (B) A representative picture of the Cooling group. Positive staining for iNOS is green. Note that iNOS was expressed in motor neurons (asterisk), which have normal architecture, and that no proliferation of glia cells was found. (C) Histogram of average intensity of iNOS expression in ventral gray matter of spinal cord. Average intensity in the Cooling group was significantly augmented versus the Control group (P = 0.01275).
cium mobilization, and/or excessive nitric oxide production.\textsuperscript{12,22} To protect spinal cord from ischemic injury, therefore, it is crucial to reestablish normal SCBF as quickly as possible and to simultaneously prevent the progression of secondary cascades. However, since a marked expression of iNOS was unveiled more than 24 h after ischemic insults in central nervous system,\textsuperscript{11,39,40} prevention of reactive hyperemic response and delayed hypoperfusion during the early phase after ischemic insults observed in the Cooling group cannot be accounted for by iNOS activation, but possibly by other types of nitric oxide synthase, such as neuronal or endothelial.\textsuperscript{14} On the other hand, a marked iNOS expression with subsequent nitric oxide discharges \textit{in situ} could be associated with the prevention of secondary injury in the Cooling group. Previous study demonstrated that protective effects of volatile anesthetics on spinal cord ischemia were mediated by nuclear factor-κB-dependent pathway, \textit{i.e.}, secondary pro-inflammatory pathway.\textsuperscript{41} Another report using a mesenteric ischemia-reperfusion model indicated that topical hypothermia prevented both nuclear factor-κB activation and iNOS expression, accompanied by significant reduction of increased intestinal permeability and histologic injury.\textsuperscript{42} On the basis of the clear difference of neurologic outcomes, augmented iNOS expression in the Cooling group (fig. 6A) is likely to provide its protective properties on ischemic spinal cord, possibly through the inhibition of secondary cascade rather than the immediate restoration of blood flow.

Although there is ongoing controversy regarding the roles of nitric oxide in ischemia-reperfusion injury,\textsuperscript{14} double-sward action of nitric oxide depends on the cell type from which nitric oxide is released, the type of nitric oxide synthase, the time period after ischemia, or the severity of ischemic insult.\textsuperscript{43} Previous study showed that cerebral infarct size and degree of motor deficit produced by artery occlusion were smaller in iNOS knockout mouse.\textsuperscript{39} In addition, expression of iNOS in microglial cells observed in the Control group was shown to initiate neurotoxic process and subsequent neuronal death, possibly through increased susceptibility to glutamate.\textsuperscript{14,44} Others showed that induction of iNOS in motor neurons in ventral horn appeared to be protective despite depending on the amount of nitric oxide discharges.\textsuperscript{11} Although it remains to be fully clarified, either iNOS expression in intact motor neurons or inhibition of glia-derived nitric oxide production could serve to protect spinal cord from ischemic insults, subsequently improving neurologic outcomes in the Cooling group. Previous study demonstrated that expression of iNOS after ischemic injury in central nervous system stimulated neurogenesis,\textsuperscript{46} indicating that iNOS expression might not be the results of acute inflammatory response against ischemia-reperfusion but consequential process to restore injured neurons. Nevertheless, further investigation should be warranted to clarify the roles of different types of nitric oxide synthase at the early and late phases of spinal cord injury by using a specific inhibitor.\textsuperscript{39}

In conclusion, regional epidural cooling during peri-ACC period minimized both functional and structural alterations of spinal cord and thereby improved neurologic outcome possibly through prevention of delayed hypoperfusion and augmentation of iNOS expression.

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