Establishment of a local cooling model against spinal cord ischemia representing prolonged induction of heat shock protein

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Objectives: Paraplegia is one of the serious complications of thoracoabdominal aortic operations. Regional hypothermia protects against spinal cord ischemia although the protective mechanism remains unknown. We attempted to create a simple model of local cooling under transient spinal cord ischemia and evaluated the effect using functional and histologic findings.

Methods: Male domesticated rabbits were divided into 3 groups: control, normothermic group (group N), and local hypothermic group (group H). A balloon catheter was used for spinal cord ischemia by abdominal aortic clamping. A cold pack attached to the lumbar region could lower the regional cord temperature initially. Neurologic function was evaluated by the Johnson score. Cell damage was analyzed by observing motor neurons with the use of hematoxylin and eosin staining, terminal deoxynucleotidyl transferase-mediated deoxy-uracil triphosphate biotin in situ nick end labeling (TUNEL), and immunoreactivity of heat shock protein.

Results: Physiologic estimation showed that local hypothermia improved the functional deficits (group N, 1.3 ± 0.9 ; group H, 4.9 ± 0.3 ; $P = .0020$). Seven days after reperfusion, there was a significant difference in the motor neuron numbers between groups N and H (group N, 7.2 ± 1.9 ; group H, 20.4 ± 3.2 ; $P = .0090$). The number of TUNEL-positive motor neurons was reduced significantly (group N, 7.2 ± 2.4 ; group H, 1.0 ± 0.7 ; $P = .0082$). Heat shock protein immunoreactivity was prolonged up to 2 days after reperfusion in the hypothermic group.

Conclusions: These results suggest that local hypothermia extended the production of heat shock protein in spinal cord motor neurons after reperfusion and inhibited their apoptotic change.

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Received for publication Aug 14, 2000; revisions requested Dec 7, 2000; revisions received Dec 27, 2000; accepted for publication Jan 2, 2001.

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J Thorac Cardiovasc Surg 2001;122:351-7

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0022-5223/2001 \$35.00 + 0 12/1/113935

doi:10.1067/mtc.2001.113935

Spinal cord injury after a successful operation on the thoracic aorta is a serious and unpredictable complication. Various methods of protection, including partial bypass and temporary shunts, have been suggested to prevent this complication.¹⁻⁴ Regardless of the progress with surgical technique or adjuncts, no method has been developed that completely prevents paraplegia.⁵ The reported prevalence of paraplegia ranges from 0.9% to 40% in operations on the thoracic aorta.^{6,7} Ischemia must occur because of permanent exclusion of the essential intercostal arterial blood supply to the spinal cord or by temporal interruption of the spinal cord blood flow.^{8,9} Spinal motor neurons are suggested to be susceptible to ischemia.^{10,11} Hypothermia may prevent lethal neuronal damage or prolong the

Figure 1. Preliminary study on regional hypothermic model. Direct spinal cord temperature of treated group (with cooling pad) representing significantly lower temperature than that of untreated group (without cooling pad) after 15 minutes. Localized hypothermia was achieved in treated group in comparison with rectal and spinal cord temperature (* $P = .0140$, paired t test; † $P = .0090$, Mann-Whitney U test). The vertical bars indicate the standard deviations of the mean.

critical ischemic time. Some vascular operations are clinically managed with whole body hypothermia, but the condition often carries a risk of cardiac dysfunction and coagulopathy.^{12,13} Regional hypothermia has few complications, in contrast, and has been established in experimental models by perfusing vessels supplying the cord with cold blood or crystalloid solution¹⁴ or by perfusing the subarachnoid¹⁵ or the epidural space.^{16,17} However, some of these procedures are too difficult to perform and reproduce. No simple experimental model of regional cooling was available to allow sufficient histologic investigation of hypothermic spinal cord ischemia. On the other hand, the clinical result shows that regional hypothermia reduced the incidence of neurologic deficits.¹⁸ Therefore, we attempted to establish a more simple model of regional cooling during spinal cord ischemia for the purpose of further study.

The apoptotic process is partially responsible for ischemic neuronal injuries. The cornu ammonis 1 neuron in the hippocampus is widely known for its susceptibility to ischemia, which is often reported to have some correlation with apoptosis.^{19,20} The spinal motor neuron is most sensitive to ischemia, and apoptosis might have a prominent role in that phenomenon.^{10,11,21} As related in many recent reports, terminal deoxynucleotidyl transferase-mediated deoxy-uracil triphosphate biotin in situ nick end labeling (TUNEL) is most essential evidence of apoptosis.²² We used TUNEL staining to investigate whether the spinal motor neuron deteriorated into apoptosis.

Recent studies have suggested that heat shock protein 70 (Hsp70) is a "molecular chaperone"²³ that may be a useful marker of neuronal injury after cerebral ischemia.^{24,25} Our previous study showed selective induction of Hsp70 in

motor neuron cells, which eventually showed selective delayed neuronal death after transient 15-minute ischemia in the spinal cord.^{10,11}

To evaluate the exact mechanism of preservation of the spinal motor neuron against ischemia in cold circumstances, we attempted to make a simple reproducible model of spinal cord ischemia and analyze cell damage statistically. To observe the overall effect of hypothermia, we used a functional neurologic score to evaluate the degree of ischemic damage to the spinal cord. In addition, a modification of TUNEL staining and Hsp70 immunoreactivity staining were also examined to investigate the protective effect of hypothermia against ischemic neuronal damage. These immunohistochemical investigations can enable us to determine how to reduce ischemic stress during cooling.

Materials and Methods

Forty-seven male domesticated white rabbits (Japan) weighing 2 to 2.5 kg were divided into 3 groups: sham control group, normothermic group (group N), and hypothermic group (group H). All rabbits were allowed free access to food and water before and after the procedure. Anesthesia was induced with intramuscular administration of ketamine at a dose of 50 mg/kg and maintained with 2% halothane inhalation in oxygen. A 5F pediatric balloon-tipped catheter (model 405; Braun, Melsungen, Germany) was inserted through the right femoral artery and advanced 15 cm forward into the abdominal aorta. Our preliminary experiments in 10 animals had already confirmed that the balloon in the distal end of the catheter should be positioned 0.5 to 1.5 cm distal to the left renal artery. The catheter was immediately removed without injection or balloon inflation in the sham control animals. Our previous experiments confirmed that 15 minutes of transient spinal cord ischemia resulted in selective motor neuron death, which might be a part of the apoptotic change.^{10,11} We applied this ischemic model to the

analysis of hypothermic effect. A cold pack was attached to the naked skin of the lumbar region (L1-5) of animals in group H. First, we confirmed that the direct spinal cord temperature could be lowered 3°C to 5°C below systemic temperature with a cooling pad for 15 minutes (Figure 1). There was a significant difference between the 2 temperatures ($P = .0090$). The direct spinal cord temperature was measured by a thermistor catheter at L1 through L2 or L3 laminectomy in the first group. Also, we confirmed the localized cooling effect of this model because a significant difference existed between the temperatures of the spinal cord and rectum ($P = .0140$).

Group N was treated in the same fashion without use of the cooling pad. During this experiment, aortic pressure was continuously monitored both proximal and distal to the balloon. Body temperature was monitored with a rectal thermistor and maintained at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$. After 15 minutes of ischemia, the balloon was deflated and the animals were allowed to recover at ambient temperature. Later they were killed at 8 hours or 1, 2, and 7 days after reperfusion ($n \geq 3$: each group at each point). Immediately after the animals died, the spinal cord was quickly removed with the plunger of a 1-mL syringe.^{26,27} All samples were frozen in powdered dry ice and stored at -80°C until use. Then the spinal cords were cut transversely at about the L2 or L3 level and, finally, mounted on glass slides. In the experiment, rabbits were treated in accordance with the Declaration of Helsinki and the "Guidelines for the Care and Use of Laboratory Animals." Also, both the experimental and animal care protocols were approved by the Animal Care Committee of the Tohoku University School of Medicine.

Neurologic function was estimated before the animals were put to death: 8 hours, 1 day, 2 days, and 7 days after reperfusion. Animals were classified by a 5-point scale devised by Johnson, Kraimer, and Graeber²⁸ as follows: 0 = hind-limb paralysis; 1 = severe paraparesis; 2 = functional movement, no hop; 3 = ataxia, uncoordinated hop; 4 = minimal ataxia; and 5 = normal function. Two individuals without knowledge of the treatment graded neurologic function independently. Statistical analyses of the neurologic score about 7 days after reperfusion of both groups were done with the Mann-Whitney U test. The sections taken 7 days after reperfusion in both groups were stained with hematoxylin and eosin and examined by light microscopy. An observer who was unaware of the animal group or neurologic outcome examined each slide. Statistical analyses for the cell numbers were done with the Mann-Whitney U test.

Immunostaining against Hsp70 in rabbit spinal cord sections was performed by the avidin-biotin-peroxidase complex method¹⁰ with the use of a kit (PK-6120; Vector Laboratories, Burlingame, Calif). The sections were fixed for 10 minutes in ice-cold acetone, air dried, and rinsed in 0.01 mol/L phosphate buffer containing 0.15 mol/L NaCl (pH 7.4). After being blocked with 10% normal horse serum for 2 hours, the slides were washed and incubated overnight at 4°C with a mouse monoclonal antibody against the stress-inducible species of the Hsp70 family (RPNII97, which was originally designated as clone C92; Amersham Pharmacia Biotech Inc, Piscataway, NJ) diluted in buffer (1:100) containing 10% normal horse serum and 0.3% Triton X-100 octylphenoxypolyethoxyethanol (Union Carbide Corporation, Danbury, Conn). Endogenous peroxidase was blocked for 20 minutes with 0.3% H_2O_2 and 10% methanol. Then

sections were washed and incubated for 3 hours with biotinylated antimouse immunoglobulin G (1:200) in the buffer, followed by incubation for 30 minutes with the avidin-biotin-peroxidase complex. Staining was developed with 3,3'-diaminobenzidine tetrahydrochloride (0.5 mg/mL in 50 mmol/L Tris-HCl buffer, pH 7.4) in the presence of 0.02% H_2O_2 . Specificity of the antibody has been noted elsewhere.^{10,11} Staining was categorized into 4 grades in the following manner: no staining (–), slight staining (±), moderate staining (+), or dense staining (2+).

To detect DNA fragmentation in nuclei of the cells, the modified TUNEL reaction was applied to the cryosections according to previous methods by means of a kit (4810-30-K; Trevigen, Inc, Gaithersburg, Md). Nuclei of tissue sections were stripped of proteins by incubation with 20 $\mu\text{g/mL}$ proteinase K for 10 minutes. After being treated with 0.3% of H_2O_2 in distilled water for 5 minutes, they were incubated with terminal deoxynucleotidyl transferase (TdT) and biotinylated deoxy-uracil triphosphate (dUTP) in TdT buffer in a humidified chamber at 37°C for 120 minutes. Further incubation with peroxidase-conjugated streptavidin was carried out for 30 minutes at room temperature. The slices were colored with 3'-diamino-benzidine/ H_2O_2 solution and then counterstained with methyl green.

Data Analysis

The results were expressed as the mean $\pm$ the standard deviation of the mean. The Mann-Whitney U test or paired t test was used for comparison of nominal data.

Results

All rabbits survived until being put to death. When the balloon was inflated in the abdominal aorta, systemic blood pressure increased within 10 mm Hg and then returned promptly. The arterial pressure distal to the inflated balloon fell to 10 mm Hg and no pulsation was recorded. On balloon deflation, systemic blood pressure decreased about 10 mm Hg below the normal level and then returned to the normal level within 5 minutes (data not shown). Spinal cord ischemia was achieved by the inflation of the balloon so as to obstruct blood flow to the spinal cord as described.¹⁰ The cooling pad method did not make any differences in blood pressure and recovery time from anesthesia except in the direct temperature of the spinal cord, which was supposed to be 3°C to 5°C lower than that of group N after 15 minutes (Figure 1).

Neurologic Assessment

Neurologic results are summarized in Table 1. All sham-operated controls ($n = 5$) had normal neurologic function (score of 5). Both group N ($n = 21$) and group H ($n = 21$) were divided into their individual reperfusion times (8 hours, 1 day, 2 days, and 7 days). In group N at 8 hours, 1 day, and 2 days after the procedure ($n = 11$), all rabbits had severe paraparesis or no hop. In group N at 7 days after the procedure ($n = 10$), all rabbits had still worse scores than before, scoring less than 3 points. All group H rabbits after

TABLE 1. Neurologic functional scores on the seventh day after the procedure

Animal	Normothermic ischemia	Hypothermic ischemia
1	0	4
2	0	5
3	1	5
4	1	5
5	1	5
6	1	5
7	2	5
8	2	5
9	2	5
10	3	5
Mean $\pm$ SD	1.3 $\pm$ 0.9	4.9 $\pm$ 0.3*

* $P = .0020$ compared with the scores during normothermic ischemia: Mann-Whitney U test.

TABLE 2. Numbers of large motor neurons in the ventral gray matter on the seventh day after the procedure

Animal	Sham	Normothermic ischemia	Hypothermic ischemia
1	21	5	20
2	24	6	17
3	24	7	22
4	25	8	25
5	27	10	18
Mean $\pm$ SD	24.2 $\pm$ 2.2	7.2 $\pm$ 1.9*	20.4 $\pm$ 3.2†

* $P = .0088$ compared with the sham control group: Mann-Whitney U test.

† $P = .0090$ compared with the normothermic ischemia group: Mann-Whitney U test.

TABLE 3. Immunoreactivity for heat shock protein 70 in the spinal motor neuron under hypothermia and normothermia

	Time course			
	8 Hours	1 Day	2 Days	7 Days
Sham control	— — — — —			
Group N	++/++/++	— — — — —	— — — — —	— — — — —
Group H	++/++/++	+++	+++++	— — — — —

Group N, Normothermic ischemia group; group H, hypothermic ischemia group; —, negative immunoreactivity; +/—, subtle positive reaction; +, positive reaction; ++, strongly enhanced reaction.

the procedure had almost normal function. There was a significant difference in physiologic function between group N and group H 7 days after the procedure by the Mann-Whitney U test ($P = .0020$ 7 days after the procedure).

Histologic Assessment

The results of counting viable motor neurons are summarized in Table 2. In sham-operated control animals, the spinal cord was intact, with many large motor neurons in the anterior horn (not shown). However, in the spinal cord 7 days after blood flow restoration of group N, more than 70% of motor neurons in Rexed's laminae VII, VIII, and IX were lost, and some glial cells showed necrotic change. On the other hand, there was less evidence of necrotic motor neurons indicating eosinophilic change 7 days after reperfusion in group H, nor was any glial change found in that group. The number of viable motor neurons of group N was significantly smaller than that of group H ($P = .0090$) (Table 2).

Histochemical Study

The results of the histochemical study are shown in Table 3. Representative photographs for immunoreactive heat shock protein in sections of spinal cords are shown in Figure 2. The spinal cord of sham-operated animals did not show heat shock protein immunoreactivity in any cells (Figure 2, A).

In group N, high immunoreactivity was shown in the 8-hour reperfusion group (Figure 2, B) and gradually reduced in proportion to the reperfusion time course seen in Table 3. Two more days after reperfusion, group N had no heat shock protein immunoreactivity (Figure 2, D). In group H, prolonged enhancement with heat shock protein immunoreactivity was detected up to 2 days after reperfusion, as shown in Figure 2, C and E. Moderate staining was noticed both 1 day and 2 days after reperfusion in group H.

TUNEL Study

The cells that displayed morphologic features of apoptotic cells, including cell shrinkage and nuclei condensation, were intensely labeled by the TUNEL method in its fragment of DNA in the nuclei. The cells with a morphologically necrotic pattern were also weakly and diffusely stained in the cytoplasm, although the staining was not so intense as for other apoptotic cells. In quantitative analysis, apoptotic cells displaying morphologic features and intense labeling were counted. Figure 3, A, shows the TUNEL-positive cells of group N 2 days after reperfusion. The motor neurons are most densely stained on the anterior horn of all groups, and scattered stained neurons, which fell in necrosis, are also shown. Group H had less dense staining and a reduced number of TUNEL-positive motor neurons than observed in

Figure 2. No intensity of Hsp70 immunoreaction was shown in the sham control group (A). The highest immunohistochemical intensity was indicated at 8 hours of reperfusion after normothermic ischemia (B). Proportionally lower intensities were found with reperfusion (D, 1 day after reperfusion at normothermic ischemia). Within the 2 days after reperfusion, heat shock protein immunohistochemical reaction continued in the hypothermic ischemia group (C, 8 hours after reperfusion and E, 2 days after reperfusion) (bar = 100 mm).

group N (Figure 3, B). Table 4 shows that significantly more TUNEL-positive motor neurons were counted in the ventral gray matter of group N than of group H ($P = .0082$).

Discussion

We have demonstrated the hypothermic preservation of rabbit spinal cord motor neuron against transient ischemia. We have already demonstrated delayed selective motor neuron death in lumbar lesions of the rabbit spinal cord with a reproducible model, and the death might be an apoptotic change.¹¹ In this experiment, we established a simple, local cooling model and investigated the relief of ischemic damage to spinal cord motor neurons under regional hypothermia using immunoreactivity of the heat shock protein and TUNEL methods. Direct temperature of the spinal cord was measured only in the preliminary group, but there was a significant difference on the functional scores between both groups. Mild hypothermia has been shown to confer protection against histopathologic²⁹⁻³¹ and metabolic derangements that follow ischemic injury.^{32,33} A persistent depression of protein synthesis in vulnerable brain regions, reported to be seen after 48 hours of normothermic ischemia, is ameliorated by mild hypothermia.³⁴ This protection is sometimes explained as nitric oxide synthase inhi-

Figure 3. Typical granular pattern (TUNEL-positive cell) shown 2 days after reperfusion in the normothermic ischemia group (A). No granular pattern cell was observed in the hypothermic ischemia group (B) (bar = 100 mm).

bition.³⁵ Neuronal death is also reported as a result of increased amounts of some neurotoxic amino acids. Inhibiting release of neurotransmitter amino acids, like glutamate, might prevent ischemic stress. Production of these amino acids is suppressed in accordance with regression of temperature. In our model, mild hypothermia is maintained, and the direct temperature of the spinal cord of rabbits is 3°C to 5°C colder than the control temperature during ischemia (Figure 1). Mild hypothermia, even 2°C colder than normal, indicates enough neuroprotective effects on neurons in many reports.²⁹⁻³¹

Two major forms of cell death, necrosis and apoptosis, have been distinguished morphologically.^{36,37} The determination of apoptosis is also additionally supported by positive TUNEL staining,³⁸ which has been used in some studies as a sole criterion for apoptosis. We showed a lower number of TUNEL-positive motor neurons in group H than in group N. These results indicate that apoptosis occurs in motor neuron during normothermia but might be reduced during hypothermia. There are many cascade reactions by biosynthetic enzymes through apoptosis. Hypothermia might inhibit these reactions, but further research is needed to verify that hypothesis.

Heat shock proteins are a set of proteins that are expressed at increased levels in cells subjected to a variety of stresses such as hyperthermia,³⁹ trauma,³⁴ and ischemia.^{24,25} Recent studies have suggested that Hsp70 messenger RNA molecules should be useful markers of neuronal injury after cere-

TABLE 4. Numbers of TUNEL-positive large motor neurons

Animal	Normothermic ischemia		Hypothermic ischemia	
	2 Days	7 Days	2 Days	7 Days
1	4	0	0	0
2	6	0	1	0
3	7	0	1	0
4	9	1	1	0
5	10	1	2	0
Mean $\pm$ SD	7.2 $\pm$ 2.4	0.4 $\pm$ 0.5	1.0 $\pm$ 0.7*	0

* $P = .0082$ compared with 2 days after reperfusion during normothermia; Mann-Whitney U test.

bral ischemia. The selective induction of Hsp70 mRNA and protein in motor neuron cells may indicate a stress response that occurs in the spinal cord after 15 minutes of transient ischemia. Our hypothermic model showed more prolonged heat shock protein immunoreactivity than our normothermic model. Kumar and associates⁴⁰ reported that mild hypothermia did not make heat shock protein induction or synthesis increase in gerbil brain ischemia. They established the hypothermic model by placing the gerbil on a slurry of ice in cold water. There is a significant difference between their study and ours because they presented a preischemic and intransischemic hypothermic model, whereas we presented the hypothermic model only during ischemia. In our model, therefore, spinal cord neurons would be directly subjected to ischemic stress at first during normothermia and would be gradually relieved during regional mild hypothermia. Elongation of heat shock protein induction has not been reported elsewhere. Stress response might be prolonged in our hypothermic model.

This is the first quantitative study in which surviving spinal cord motor neurons after ischemia during mild hypothermia have been investigated immunohistologically. We established a simple model of local cooling and demonstrated that mild hypothermia reduced cell damage through both suppression of apoptosis and ease of stress response. This result is compatible with a less severe neurologic deficit after hypothermic surgical operations.

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