NS-7, a novel $\text{Na}^+/\text{Ca}^{2+}$ channel blocker, prevents neurologic injury after spinal cord ischemia in rabbits

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Objective: We investigated the neuroprotective effect of NS-7 (4-[4-fluorophenyl]-2-methyl-6-[5-piperidinopentyloxy] pyrimidine hydrochloride), a novel $\text{Na}^+/\text{Ca}^{2+}$ channel blocker, on transient spinal cord ischemia in rabbits.

Methods: Spinal cord ischemia was induced in New Zealand white rabbits by means of infrarenal aortic occlusion for 20 minutes. Four experimental groups were enrolled. A sham group (n = 3) underwent the same operation without aortic occlusion. A control group (n = 7) received only saline before occlusion. Group A (n = 8) received NS-7 (1 mg/kg) 15 minutes before ischemia, and group B (n = 8) received NS-7 (1 mg/kg) at the onset of reperfusion. Neurologic function was assessed 24 and 48 hours after the operation with modified Tarlov criteria. Spinal cords were harvested for histopathologic examination and in situ terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL staining). Spinal cord infarction was investigated with 2, 3, 5-triphenyltetrazolium chloride staining.

Results: Tarlov scoring demonstrated marked improvement in both group A and group B compared with the control group at 24 and 48 hours after the operation. Minimal histologic changes were found in lumbar spinal cords of the 2 NS-7-treated groups, whereas severe neuronal necrosis was shown in the control group. TUNEL-positive neurons and the infarct size of lumbar spinal cords were significantly reduced by NS-7 administered both before ischemia and at the onset of reperfusion. No significant difference was noted between group A and group B in terms of spinal cord protection.

Conclusion: These results indicate that NS-7 protects the spinal cord against ischemic injury by preventing both neuronal necrosis and apoptosis.

Paraplegia that results from spinal cord injury remains a major devastating and unpredictable complication after surgical repair of descending and thoracoabdominal aortic aneurysms.¹ The mechanism of spinal cord injury during operations on the thoracic aorta is believed to be related primarily to direct tissue ischemia. Numerous surgical techniques and pharmacologic interventions have been used to protect spinal cords, but the complication still cannot be prevented completely.²

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Supported by the Grand-in-Aid for Scientific Research in Japan Society for the Promotion of Science (no. 15591469).

Received for publication Feb 2, 2004; revisions requested April 28, 2004; accepted for publication May 6, 2004.

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J Thorac Cardiovasc Surg 2005;129:364-71
0022-5223/\$30.00

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doi:10.1016/j.jtcvs.2004.05.006

Although the precise neurochemical sequels after ischemia remain to be clarified, the action of voltage-sensitive Na^+ channels (VSSCs), as well as Ca^{2+} channels, are considered to be involved in the pathogenesis of ischemic neurologic injury.^{3,4} The opening of VSSCs facilitates ischemic glutamate release and intercellular Ca^{2+} overload,^{5,6} which have been considered the major cause of neuronal degeneration after cerebral ischemia.^{7,8} Consistent with these findings, Na^+ and Ca^{2+} channel blockers have been proved to be neuroprotective in models of spinal cord and cerebral ischemia.^{9,10}

NS-7 (4-[4-fluorophenyl]-2-methyl-6-[5-piperidinopentyloxy] pyrimidine hydrochloride; Nippon Shinyaku Co, Kyoto, Japan) is a novel VSSC and Ca^{2+} channel blocker developed as a neuroprotective agent. This compound blocks the function of VSSCs by acting on the neurotoxin receptor site 2 in the brain.¹¹ NS-7 also inhibits Na^+ and Ca^{2+} currents through L- and N-type Ca^{2+} channels in NG108-15 cells¹² and blocks the KCl-induced activation of nitric oxide synthase by closing L- and P/Q-type Ca^{2+} channels in primary cultured neurons.¹³ The cerebroprotective actions of NS-7 have been shown in both in vitro and in vivo models of cerebral ischemia. It protects the cerebral tissue against ischemic insults by reducing the infarct size, improving the disturbance of cerebral energy metabolism, suppressing the cerebral edema, and reversing the behavioral and cognitive dysfunction.¹⁴⁻¹⁸ However, the possible neuroprotective effect of NS-7 on spinal cord ischemia has not yet been examined. Therefore, the aim of this study was to determine whether NS-7 administered before or after aortic occlusion was capable of preserving the motor function and preventing neuronal cell necrosis and apoptosis in a well-characterized rabbit model of spinal cord ischemia.

Materials and Methods

Animal Care and Surgical Procedure

Twenty-six male New Zealand White rabbits weighing 2.2 to 3.0 kg were used in this experiment. The animal protocol was approved by the Ethics Review Committee for Animal Experimentation of Hamamatsu University School of Medicine and in accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals."

Surgical preparation was conducted according to the method described by our department previously.¹⁹ A 24-gauge venous catheter was placed in the marginal ear vein for drug administration. The rabbits were anesthetized with intravenous sodium pentobarbital (25 mg/kg) and allowed to breathe spontaneously. Lidocaine (0.5%) was administered at the site of the skin incision as local anesthesia. The left common carotid artery was cannulated with a 24-gauge catheter for monitoring the arterial pressure. Core body temperature was continuously monitored with a rectal probe and was maintained at 38.5°C with the aid of a heating lamp. The infrarenal abdominal aorta was exposed through a transperitoneal approach. After systemic heparinization (200 U/kg), spinal cord

TABLE 1. Semiquantitative scoring for histologic assessment of spinal cord injury

Histologic assessment	Score
Healthy	0
Perineural edema or scattered 1-cell necrosis	1
Unilateral necrosis of central medial portion of anterior horn	2
Bilateral necrosis of central medial portion of anterior horn	3
Unilateral necrosis of entire anterior horn	4
Bilateral necrosis of entire anterior horn	5

ischemia was induced by occluding the abdominal aorta with snares for 20 minutes just distal to the renal arteries and just above the aortic bifurcation. Then the snares were released, and the flank was closed in 2 layers.

All animals were killed by means of a lethal injection of pentobarbital (200 mg/kg) 48 hours after the operation. Spinal cords were quickly harvested and sectioned from the low thoracic level to the low lumbar level into the following 4 segments: low thoracic (T10–T12), upper lumbar (L1–L2), mid lumbar (L3–L4), and low lumbar (L5–L6).

Experimental Protocol

NS-7 was dissolved in 0.9% saline. The rabbits were assigned randomly to 1 of the 4 groups. The sham group ($n = 3$) underwent the same operation without aortic occlusion. The control group ($n = 7$) received saline (1 mL) intravenously 15 minutes before aortic occlusion. NS-7 (1 mg/kg) was administered intravenously as a bolus dose 15 minutes before aortic occlusion in group A ($n = 8$) or just after releasing the snares (at the onset of reperfusion) in group B ($n = 8$).

Neurologic Assessment

Hind-limb motor function was scored 24 and 48 hours after the operation by the modified Tarlov scale²⁰: 0, no movement; 1, slight movement; 2, sit with assistance; 3, sit alone; 4, weak hop; 5, normal hop. The neurologic function was graded by an observer without knowledge of the treatment.

TTC Staining

To measure the infarct size of spinal cords, 2, 3, 5-triphenyltetrazolium chloride (TTC; Sigma Chemical, St Louis, Mo) staining was used, which can turn the viable tissue brick red and the necrotic tissue pale. Spinal cords (mid lumbar segment) were quickly removed, and transverse sections of about 2 mm in thickness were cut. Slices were then incubated for 30 minutes in 1% TTC at 37°C. After fixation in a 10% formalin solution, the TTC-stained sections were photographed. The area of infarction was calculated with National Institutes of Health Image software. The infarct size was expressed as a percentage of the area of the whole gray matter.

Histopathologic Examination

The spinal cords were fixed in 10% formalin and embedded in paraffin. Two transverse sections (4 μm) of each segment were cut

TABLE 2. Physiologic parameters

	Sham (n = 3)	Control (n = 7)	Group A (n = 8)	Group B (n = 8)
Weight (kg)	2.4 ± 0.1	2.4 ± 0.1	2.6 ± 0.2	2.5 ± 0.2
Rectal temperature (°C)				
Baseline	38.7 ± 0.6	38.7 ± 0.4	38.4 ± 0.7	38.5 ± 0.4
Ischemia, 10 min		38.3 ± 0.5	38.1 ± 0.4	38.4 ± 0.5
Reperfusion, 10 min		38.5 ± 0.3	38.2 ± 0.2	38.6 ± 0.5
MAP (mm Hg)				
Baseline	83 ± 8	86 ± 5	83 ± 5	91 ± 6
Ischemia, 10 min		82 ± 3	85 ± 5	86 ± 7
Reperfusion, 10 min		79 ± 12	78 ± 10	83 ± 8

Values are expressed as means ± SD. There were no significant differences in these physiologic parameters among the 4 groups at any time point. MAP, Mean arterial blood pressure.

TABLE 3. Neurologic score at 24 and 48 hours after the operation

Tarlov score	Sham (n = 3)		Control (n = 7)		Group A (n = 8)		Group B (n = 8)	
	24 h	48 h	24 h	48 h	24 h*	48 h†	24 h*	48 h†
0	0	0	5	6	0	0	0	0
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	1	0	1
3	0	0	1	0	2	2	2	2
4	0	0	1	1	3	4	1	1
5	3	3	0	0	3	1	5	4

* $P < .01$ compared with the control group of 24 hours (Mann-Whitney U test).

† $P < .01$ compared with the control group of 48 hours (Mann-Whitney U test).

and stained with hematoxylin and eosin. Histologic damage was graded by using semiquantitative scoring (Table 1).²¹ An investigator who was unaware of the animal group and neurologic outcome examined each slide.

TUNEL Staining

Paraffin-embedded sections (mid lumbar segment) were used for in situ terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL staining) with Apop Tag (Intergen Co, New York, NY), according to the protocols recommended by the manufacturer. In brief, after being deparaffinized, the sections were treated with proteinase and 0.3% H_2O_2 . Then the sections were incubated for 60 minutes with terminal deoxynucleotidyl transferase enzyme at 37°C. The color was developed with a DAB/ H_2O_2 solution. The sections were counterstained in methyl green. The number of motor neurons that were positive in TUNEL staining was counted in 3 sections, and the values were averaged.^{22,23}

Statistical Analysis

The data are presented as means ± SD. Statistical analyses of neurologic scores and histologic scores were performed with Mann-Whitney U tests. The physiologic data were processed by means of analysis of variance for repeated measures. The numbers of TUNEL-positive neurons and the infarct size of spinal cords were evaluated by 1-way analysis of variance, followed by the Dunnett test when significant differences were identified.

Results

Physiologic Parameters

All the animals survived the entire observation period. Body weight, rectal temperature, and mean arterial blood pressure values at baseline, 10 minutes after ischemia, and 10 minutes after reperfusion are shown in Table 2. There were no significant differences in the weights of the animals. Rectal temperature and mean arterial blood pressure were not significantly different among the 4 groups at each time point.

Neurologic Assessment

Neurologic assessment results are summarized in Table 3. All the rabbits in the sham group remained normal (Tarlov score of 5) throughout the observation period. Twenty minutes of infrarenal aortic occlusion resulted in severe lower-extremity neurologic deficits in the control group. NS-7 treatment (group A and group B) remarkably enhanced the recovery of motor function in the hind limbs both 24 hours and 48 hours after the operation ($P < .01$ for group A and B vs the control group at both time points). Compared with group A, better Tarlov scores were obtained with animals that received NS-7 at the onset of reperfusion (group B), but the differences were not significant.

Figure 1. Representative photographs of the lumbar spinal cord sections stained with TTC. Viable tissue is brick red, whereas infarcted tissue is pale. White matter is also pale. The specimen from the sham group (A) shows no infarction in the gray matter, whereas almost the entire anterior horns show infarction in the section from the control group (B). Minimal gray matter infarction is shown in rabbits that received NS-7 before ischemia (C) or at the onset of reperfusion (D).

Infarct Size of Spinal Cord

Photographs of TTC staining of the spinal cords (mid lumbar level) are shown in Figure 1. No spinal cord infarction was detected in the rabbits that underwent a sham operation. Forty-eight hours after the spinal cord ischemia, the average size of the infarction in the control group was $54.7\% \pm 19.8\%$ (Figure 2). NS-7 was effective in reducing the infarct size when it was injected before ischemia ($14.0\% \pm 14.3\%$, $P < .05$) or at the onset of reperfusion ($12.1\% \pm 12.8\%$, $P < .05$). Overall, the results show a good correlation with the average neurologic scores at 48 hours.

Histopathologic Examination

No sign of spinal cord damage was observed in the hematoxylin and eosin–stained sections at any level in the sham-operated rabbits. The low thoracic and upper lumbar regions of the spinal cords from the other 3 groups showed no or only minimal injury. Although minor histologic changes occurred more frequently in the control group, the differences in histologic scores did not reach statistical significance. Examination of the mid lumbar and low lumbar spinal cords of the control group revealed severe neuronal damage, as evidenced by eosinophilic neuronal degeneration, vacuolization, and frank necrosis (Fig 3, B). However,

Figure 2. Infarct size of the lumbar spinal cord (mid lumbar level). * $P < .05$ compared with the control group.

the rabbits treated with NS-7 (group A and group B) showed only slight changes in the spinal cords at these 2 levels, and the average histologic scores were significantly lower than those of the control group (Figure 4).

TUNEL Staining

Cells with double-strand breaks in DNA, which are suggestive of apoptosis, are detected by means of TUNEL staining. The spinal cords from the rabbits in the sham group showed

Figure 3. Histologic sections of lumbar spinal cords (mid lumbar). (Original magnification 200 $\times$.) No histologic changes are shown in the ventral horns of a sham-operated rabbit (A). The specimen from a control animal (B) exhibits eosinophilic neuronal degeneration (arrows), vacuolization, and frank necrosis, whereas animals that received NS-7 before ischemia (C) or at the onset of reperfusion (D) show minimal evidence of cellular damage.

Figure 4. Mean histologic scores of ischemic injury at different levels of the spinal cord. LT, Low thoracic; UL, upper lumbar; ML, mid lumbar; LL, low lumbar. * $P < .05$, # $P < .01$ compared with the control group (Mann-Whitney U tests).

no evidence of TUNEL staining. Samples from the control group stained strongly positive, with numerous TUNEL-positive motor neurons scattered in the ventral gray matter (Figure 5, B). NS-7 injected 15 minutes before ischemia or at the onset of reperfusion strongly reduced the total number of apoptotic motor neurons compared with that seen in the control group ($P < .05$, Figure 6). No significant difference in the total number of apoptotic motor neurons was found between the 2 NS-7-treated groups.

Discussion

The present study provides evidence for the first time that NS-7, a novel $\text{Na}^+/\text{Ca}^{2+}$ channel blocker, reduces the neurologic injury caused by spinal cord ischemia. In this study occlusion of the infrarenal aorta for 20 minutes resulted in severe neurologic deficits at both 24 and 48 hours after ischemia.²⁴ Significantly better neurologic outcomes were induced by a single injection of NS-7. The treatment with NS-7 prevented both neuronal necrosis and apoptosis of the ischemic spinal cord. Potential neuroprotective effects were also observed, even when NS-7 was given after spinal cord ischemia.

As a novel neuroprotective agent, the important and direct effect of NS-7 is to block the function of VSSCs and Ca^{2+} channels. Evidence has accumulated that excessive glutamate release and subsequent increase of intercellular Ca^{2+} concentration involved in the pathogenesis of ischemic neurologic injury contribute to the activation of the voltage-gated Na^+ and Ca^{2+} channels. During cerebral ischemia, depletion of cellular adenosine triphosphate and the resultant dysfunction of Na^+/K^+ adenosine triphosphatase causes an increase in the intracellular Na^+ concentration, which in turn depolarizes the neuronal membrane, thereby activating voltage-gated Na^+ and Ca^{2+} channels.²⁵ The opening of the Na^+ channel augments the accumulation

Figure 5. TUNEL staining of sections of lumbar spinal cords from rabbits in the sham (A), control (B), and the 2 NS-7-treated groups (C, treatment before ischemia; D, treatment at the onset of reperfusion). (Original magnification 200 $\times$.) Arrows show the TUNEL-positive motor neuron cells.

of intracellular Na^+ , which in turn operates the glutamate transporter in the reverse direction and leads to a massive glutamate release.^{6,26} The rundown of the Na^+ gradient also causes an excessive Ca^{2+} entry through the reversed $\text{Na}^+/\text{Ca}^{2+}$ exchanger.⁵ The resultant breakdown of intracellular Ca^{2+} homeostasis activates a variety of intracellular Ca^{2+} -dependent enzymes, including the protease calpain, nitric oxide synthase, and phospholipases, and causes cell death.²⁷

Necrosis is one of the main pathophysiologic changes of ischemia-induced neurologic injury. In vivo NS-7 has been demonstrated to reduce the size of cerebral infarction significantly after permanent middle cerebral artery occlusion,¹⁵⁻¹⁷ and a delayed injection of NS-7, at 6 to 12 hours after ischemia, is still effective in reducing the infarct size.¹⁵ In a transient cerebral ischemia model, a single injection of NS-7 also produces a marked reduction of cerebral infarction, even when it is used at the onset of reperfusion.¹⁸ In the present study TTC staining showed that the infarct size of the spinal cord was markedly reduced by NS-7 treatment. Furthermore, a delayed administration of NS-7, such as after ischemia, was also effective, similar to its cerebral protective effects. Persistent activation of cyclic adenosine monophosphate response element binding protein (CREB) has recently been reported to be crucial for neuronal survival. In another transient cerebral ischemia model, NS-7 administrated after recircula-

Figure 6. Number of TUNEL-positive motor neurons of spinal cords (mid lumbar level) 48 hours after ischemia. * $P < .05$ compared with the control group.

tion was found to retain a normal histologic appearance in the inner border zone of the ischemic area, with a moderate but sustained increase in CREB phosphorylation. The upstream cascades not mediated through voltage-gated Na^+ and Ca^{2+} channels seem to moderately activate CREB phosphorylation during postischemic recirculation, which might contribute to the neuroprotection of NS-7, even when it is used after reperfusion.¹⁷ Moreover, the infarct size was grossly proportional to the neurologic score. TTC staining has been commonly used

to detect myocardial infarction,²⁸ as well as cerebral infarction.¹⁵ To our knowledge, this is the first time this method has been applied to spinal cords. The results demonstrated that TTC staining is effective in the assessment of ischemia-induced spinal cord injury. The histopathologic examination of the spinal cord also confirmed these observations. Control rabbits exhibited frank necrosis within the mid and low lumbar regions of the spinal cord, whereas NS-7-treated animals demonstrated less histologic damage.

Neuronal cell death induced by ischemia has been revealed to occur also through apoptosis. As programmed cell death, apoptosis is defined morphologically and biochemically by cellular shrinkage, chromatin condensation, and internucleosomal fragment of DNA.²⁹ Apoptosis has been demonstrated to be an important mode of neuron death in the ischemic spinal cord.^{22,23,29} Influx of Ca^{2+} into the cell has been shown to play an important role in the initiation of apoptotic cell death. On the other hand, agents that block Ca^{2+} entry have been demonstrated to inhibit apoptosis.³⁰ In this study apoptotic cell death was detected on the basis of positive TUNEL staining, with the brown nucleus in a granular pattern. Numerous apoptotic motor neurons were observed in the spinal cords of the control animals. The total number of TUNEL-positive motor neurons was reduced significantly after NS-7 treatment. The results showed that, in addition to its antinecrosis effect, NS-7 also prevented neuron apoptosis.

Some previous $\text{Na}^+/\text{Ca}^{2+}$ channel blockers have been reported to be neuroprotective, but serious adverse cardiovascular actions, such as hypotension and cardiac arrhythmia, have limited the clinical use of these agents.¹⁷ A definite advantage of NS-7 is freedom from these side effects.¹⁷ NS-7 preferentially distributes to the membrane-enriched synaptosomal fraction of the brain rather than the heart and has a much higher affinity for brain VSSCs than cardiac VSSCs, indicating that it might exert less adverse effects on cardiac function.¹¹ As shown in the present study, NS-7 administration did not induce hypotension. In any case, the possibility that mechanisms other than $\text{Na}^+/\text{Ca}^{2+}$ channel blockade contribute to the neuroprotective actions of NS-7 cannot be ruled out.

In conclusion, the administration of NS-7 resulted in a significant improvement in the neurologic outcome after transient spinal cord ischemia. NS-7 protected the spinal cord against ischemic insults by preventing neuron necrosis and apoptosis. This study suggests that NS-7 possesses a potential value in the prevention of neurologic injury after thoracic aneurysm surgery.

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