Spinal Cord Protection: Effect of N-Methyl-D-Aspartate Receptor Antagonist MK-801 for Spinal Cord Ischemia in a Rabbit Model

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Summary: Excitatory amino acids (glutamate, aspartate) play an important role in the ischemic cascade leading to cell death. The N-methyl-D-aspartate (NMDA) receptor is an excitatory amino acid (EAA) receptor, and NMDA receptor antagonists have been shown to exert a neuroprotective effect in central nervous system ischemia. The purpose of this study was to investigate the effects of noncompetitive NMDA receptor antagonists MK-801 and to observe the changes in EAAs after spinal cord ischemia in a rabbit model. Spinal cord ischemia was induced by clamping the infra-renal abdominal aorta for 24 min. Group 1 (n=6) received no pharmacologic infusion. Group 2 (n=5) was administered an intra-aortic hypothermic MK-801 (1 mg/kg) solution and group 3 (n=6) was administered an intra-aortic normothermic MK-801 (2 mg/kg) solution immediately after clamping of the abdominal aorta. We evaluated the neurological function at 12, 24 and 48 hrs after spinal cord ischemia. A histopathologic study was carried out 72 hrs after spinal cord ischemia, and the results for groups 1 and 3 were compared. The glutamate and aspartate levels in the blood plasma were compared at pre-ischemia and at 12, 24, and 48 hrs among the groups. The perfusion of a normothermic MK-801 (2 mg/kg) solution significantly reduced the neurological dysfunction and the neuronal damage. There was a significant increase in aspartate at 24 and 48 hrs in group 1, but no such increase in glutamate occurred in groups 1 and 3. In conclusion, these data provide the evidence that therapeutic intervention with MK-801 (2 mg/kg) in the early period of spinal cord ischemia is beneficial in reducing neurological dysfunction and neuronal damage.

Key words rabbit, spinal cord ischemia, noncompetitive NMDA receptor antagonist, MK-801, spinal cord protection

INTRODUCTION

Spinal cord ischemic injury, particularly paraplegia, can occur during operations of the descending and thoracoabdominal aorta and is an extremely serious complication. The complication rate ranges from 5 to 30% [1]. Spinal cord ischemic injury can be caused by a number of factors, including hypoperfusion of spinal cord tissue, thromboembolism of the critical intercostal arteries, and reperfusion injury in the perioperative period [2]. Many surgical and pharmacological attempts have been made to prevent this complication, but as of yet there are no reliable methods for avoiding spinal cord ischemic injury [1,3].

Glutamate and aspartate are an important neurotransmitter in the central nervous system (CNS) and is thought to be an excitatory neurotransmitter [4,5]. Excessive glutamate accumulation in the ischemic condition has been observed to be a neurotoxic agent. Therefore, glutamate is generally said to be an excitotoxic amino acid [6]. The N-methyl-D-aspartate (NMDA) receptor is most likely to be involved in ischemic neuronal injury [7]. After spinal cord ischemia, the activation of NMDA receptors and the subsequent induction of excessive calcium ion influx can lead to neuronal death [6,7]. NMDA receptor

Received for publication December 7, 1999
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antagonists have been shown to reduce spinal cord injury after CNS ischemia in both in vivo and in vitro studies [4-7]. The present study was designed to examine the neuroprotective effects of noncompetitive NMDA receptor antagonist MK-801 [(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate] and to observe the changes in excitatory amino acids (EAAs) in a rabbit model of spinal cord ischemia.

MATERIALS AND METHODS

Seventeen New Zealand white rabbits weighing 2.8-3.2 kg were used in the present study. Some rabbits were eliminated from this study because of technical errors or death from anesthesia. A venous catheter (24 gauge) was placed in a marginal ear vein, and animals were anesthetized with intravenous thiopental sodium (20 mg/kg) and intra-peritoneal pentobarbital (10 mg/kg). Animals were placed in a supine position and allowed to breathe spontaneously without endotrachial intubation or mechanical ventilation. The rectal temperature was monitored continuously and was maintained at above 37 °C with a heating lamp during the operation.

A median laparotomy was performed. We then injected 150 units/kg of the heparin intravenously for systemic anticoagulation. Spinal cord ischemia was induced by clamping the infra renal abdominal aorta, the bilateral common iliac artery, and the posterior mesenteric artery for 24 min (Fig. 1). The rabbits were divided into three groups: in group 1 (n=6), control rabbits underwent no preventive method; group 2 (n=5), rabbits received intra-aortic perfusion by hypothermic saline at 23 °C with MK-801 (1 mg/kg) in 25 ml saline at a constant rate of 60 ml/min immediately after clamping; group 3 (n=6), rabbits received intra-aortic perfusion by normothermic saline at 39 °C with MK-801 (2 mg/kg) by the same method (Table 1). In groups 2 and 3, a 24-gauge catheter was inserted on the proximal side of the left common iliac artery in order

![Fig. 1. Experimental model. Group 2 and 3 rabbits were administered an intra-aortic MK-801 solution immediately after clamping of the infra-renal abdominal aorta. In order to perfuse the lumbar arteries, we clamped the PMA, the rt. CIA, and the distal side of lt. CIA during perfusion. PMA: posterior mesenteric artery; CIA: common iliac artery](image)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
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<tbody>
<tr>
<td>Body weight (kg)</td>
<td>3.03 ± 0.08</td>
<td>2.96 ± 0.15</td>
<td>2.93 ± 0.10</td>
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<tr>
<td>Infusion temperature (°C)</td>
<td>23.0</td>
<td>NS</td>
<td>39.0</td>
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NS: not significant

There were no significant differences in the preoperative body weight among 3 groups.

Group 1 (n=6): The control rabbits received no pharmacological administration.

Group 2 (n=5): The rabbits received MK-801 (1 mg/kg) solution which was dissolved in hypothermic saline at 23 °C.

Group 3 (n=6): The rabbits received MK-801 (2 mg/kg) solution which was dissolved in normothermic saline at 39 °C.

The rectal temperature in the rabbits was indicated at 39-40 °C in the early period of the operation. We determined the MK-801 dissolved in saline solution at 39 °C for normothermic MK-801 solution.
to administer the MK-801 solution (Fig. 1). After declamping, the 24-gauge catheter was removed, and the arteriotomy was closed with 7-0 polypropylene, after which the laparotomy incision was closed. The rabbits were then returned to their cages and allowed free access to water and food. The neurological status was scored by a Modified Tarlov scale (Table 2) at 12, 24, and 48 hrs after spinal cord ischemia. Rabbits were also categorized as paraplegic (grade 0), severely paraparesic (grade 1-2), slightly paraparesic (grade 3-4), or normal (grade 5).

All animals received humane care and treatment in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication 85-23, revised 1985) and Guidelines for Animal Experiment, Kurume University School of Medicine.

**Histopathologic study**

After 72 hrs, all animals were anesthetized again, and their spinal cords were fixed with 10% formaldehyde solution (total 100 ml) perfusing through the abdominal aorta. The lumbosacral cords were removed and stored in 10% formaldehyde solution for 3 days. After alcohol dehydration and paraffin embedding, sections were cut 5 μm thick and stained with hematoxylin and eosin.

We selected six sections at the L-6 level from each rabbit and counted the number of surviving neurons on both sides of the ventral horn per section. For the histopathologic grading of neuronal damage, we counted the mean number of surviving neurons within the range of a rectangular area (Fig. 2). Spinal cord tissues from three normal rabbits were sectioned and used to compare with the number of motor neurons in the ventral horn in groups 1 and 3.

**Excitatory amino acid analysis**

We compared the time course of changes in the blood plasma levels of amino acids (glutamate, aspartate) at the pre-ischemic period and following 12, 24, and 48 hrs after spinal cord ischemia in both groups 1 and 3. Blood plasma samples were separated for electrochemical detection using a high-speed amino acid analyzer by the ninhydrin method (HITACHI L-8500 Amino Acid Analyzer).

**Statistical analysis**

For the statistical assessment of neurological changes, we used the Mann-Whitney U test analysis of variance to determine the significant differences among the 3 groups. The numbers of surviving neurons and the excitatory amino acid levels in the two groups were analyzed by an unpaired Students t test. A p value of <0.05 was considered significant.

**RESULTS**

All animals survived for 72 hrs after spinal cord ischemia. In all three groups, the preoperative status, body weights (Table 1), intraoperative rectal temperatures (Fig. 3), and doses of anesthetic agents were not significantly different.

**Neurological function**

We showed the neurological changes following spinal cord ischemia in all animals in Fig. 4 (A, B, C, G, H, I, L, O, P, Q, R, S, T, U, V, W, X, Y, Z, K, M, N, K, L, J, I, H, G, F, E, D, C, B, A).
C). There was no change in neurological function between 48 and 72 hrs after spinal cord ischemia in any of the rabbits. In group 1, 5 of 6 rabbits had paraplegia (Tarlov scale of 0), while 1 of 6 rabbits exhibited severe paraparesis (Tarlov scale of 1) at 48 hrs after spinal cord ischemia. In group 2, 3 of 5 rabbits had paraplegia (Tarlov scale of 0), and 2 of 5 rabbits exhibited severe paraparesis (Tarlov scale of 1-2) at 48 hrs. In groups 1 and 2, 1 of 6 rabbits (group 1) had delayed-on set paraplegia and the other 10 rabbits developed progressive paraplegia or severe paraparesis from which they were not able to recover. There were no significant differences between groups 1 and 2 at any period after spinal cord ischemia (12h: p=0.2245, 24h: p=0.1274, 48h: p=0.4094). In group 3 (n=6), 5 of 6 animals recovered fully (Tarlov scale of 5), 1 of 6 animals had slight paraparesis (Tarlov scale of 4) at 48 hrs after spinal cord ischemia. None of the rabbits in group 3 showed paraplegia at 48 hrs. In group 3, until 12 hrs after spinal cord ischemia, 5 of 6 rabbits developed either slight or severe paraparesis (Tarlov scale of 1-2-3), while 1 of 6 rabbits were intact. At 24 hrs after spinal cord ischemia, the other 4 of 5 rabbits had fully recovered, with a Tarlov scale of 4-5. Therefore, there were no significant differences in neurological status between groups 1 and 3 at 12 hrs after spinal cord ischemia (p>0.9999); at 24 and 48 hrs, however, significant differences were observed (24h: p=0.0032, 48h: p=0.0019).

**Fig. 4A, B, C.** The neurological function was scored by Modified Tarlov scale at 12, 24, and 48 hrs after spinal cord ischemia. There were significant differences in the neurological function at 24 and 48 hrs after spinal cord ischemia between group 1 and 3.
Fig. 5A, B. Histopathology (1): Group 1. Hematoxylin-eosin stained. (A)×20; (B)×100.

Fig. 6A, B. Histopathology (2): Group 3. Hematoxylin-eosin stained. (A)×20; (B)×100.

Fig. 7A, B. Histopathology (3): normal. Hematoxylin-eosin stained. (A)×20; (B)×100.
Histopathologic study

The histopathologic changes in the spinal cord were primarily limited to the gray matter, and in group 1 these areas showed a significant loss of neurons (Fig. 5). The most severe changes were found in the segments L-5 to L-6. Edema, and vacuolization were remarkable in the anterior horn. The cells were round, and the surrounding white matter was edematous in the rabbits with complete paraplegia. On the other hand, in group 3 rabbits, motor neurons in the anterior horn were preserved well (Fig. 6), similar to the spinal cord tissue of normal rabbits (Fig. 7).

An average of 72±12 neurons per section was found in the normal spinal cord tissue (Fig. 8). The mean numbers of surviving neurons at L-6 were 23±6 neurons per section in group 1 (p<0.001 versus average), and 52±11 neurons per section in group 3 (p<0.001 versus group 1) (Fig. 8). A statistical difference in the mean number of surviving neurons between group 3 and normal rabbits (p=0.0017) was found.

EAA analysis

The results of the blood plasma analysis for EAAs are shown in Table 3 (A, B). In group 1, there were significant differences in the elevation of aspartate between the levels observed at 12 and 48 hrs (p=0.0124), and at 24 and 48 hrs (p=0.0132). There were no significant differences, however, in the elevations of glutamate at any period. The glutamate and aspartate levels were not significantly elevated between 24 and 48 hrs in group 3.

<table>
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<tr>
<th>TABLE 3.</th>
<th>(A) The changes of aspartate levels at 12, 24, and 48 hours after spinal cord ischemia</th>
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<tr>
<td></td>
<td>12h</td>
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<tr>
<td>Group 1</td>
<td>0.665 ± 0.162</td>
</tr>
<tr>
<td>Group 3</td>
<td>1.935 ± 1.123</td>
</tr>
<tr>
<td>NS: not significant, *: p=0.0124 versus 12h, p=0.0132 versus 24h</td>
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|           | 12h     | 24h     | 48h     |
|-----------|-------------------------------------------------|
| Group 1   | 0.965 ± 0.361 | NS      | 1.046 ± 0.434 | NS      | 1.220 ± 0.441 |
| Group 3   | 3.969 ± 1.805 | NS      | 3.313 ± 2.407 |
| NS: not significant |

In group 1, there were significant differences in the elevation of aspartate between the levels observed at 12 and 48 hrs (p=0.0124), and at 24 and 48 hrs (p=0.0132). But the glutamate levels were not significantly elevated in group 1.

Kurume Medical Journal Vol. 47, No. 1, 2000
DISCUSSION

Distal aortic perfusion and hypothermia as adjunct methods for descending and thoracoabdominal aortic aneurysm repair are theoretically effective for preventing paraplegia or paraparesis [9]. However, the risk of neurological dysfunction after descending and thoracoabdominal aortic replacement has ranged from 5% to 30%. Some patients awake from anesthesia with acute-onset paraplegia, while others develop delayed-onset paraplegia over the following 1 to 5 days [1,3,10]. It has therefore been thought that postoperative neurological dysfunction occurs for multifactorial reasons during the perioperative period. It has also been suggested that a variety of pharmacologic methods, including the use of calcium channel blockers, superoxide dismutase, allopurinol, mannitol, dantrolene, and ketamin, have been evaluated for the prevention of spinal cord ischemia [9,11,12]. However, none of these agents have been particularly effective in comparison to most hypothermia and perfusion techniques [9,11,12].

In the present study, a model of borderline ischemia, which existed between acute and delayed onset paraplegia, was produced in the rabbit. Based on the results of our pilot study, a clamping period of 24 min was used to reproduce the borderline ischemic period because some rabbits and others presented acute-onset paraplegia and delayed-onset paraplegia individually under these conditions. Moreover, we considered that the borderline ischemic model would well represent the effects of the drug and relationship between EAA and delayed neuronal death.

Glutamate and aspartate are both abundant in CNS system [5,13]. Although a protective mechanism suppresses the neurotoxicity of EAA under physiologic circumstances, hypoxic energy failure disturbs ion homeostasis in the context of glutamate accumulation during the ischemic period [13-15]. There are three subtypes of glutamate receptors: NMDA, quisqualate, and kainate [6]. Activation of the NMDA receptors is the primary route of calcium entry and has been reported to be the primary course of extracellular calcium influx, which leads to irreversible neuronal cell injury [7,16]. There is evidence that the excessive accumulations of glutamate express the neurotoxicity after CNS ischemia and lead to delayed neuronal death [7,14]. As previously detailed, NMDA receptor antagonists have been shown to be neuroprotective for CNS ischemia and are supposed to prevent neurotoxic intracellular calcium increases [4,16-18].

The present study demonstrates that the administration of the noncompetitive NMDA receptor antagonist MK-801 (2 mg/kg) immediately after 24 min of spinal cord ischemia in rabbits significantly improved the neurological findings and limited the neuronal damage. In fact, our experimental results indicated that there was significant neurological improvement, with a Modified Tarlov scale 4 to 5, at 24 and 48 hrs between groups 1 and 3. However, there were no significant differences between group 1 and 3 at 12 hrs after reperfusion. The rabbits receiving MK-801 (2 mg/kg) appeared to be paraplegic at 12 hrs after reperfusion, as the higher dose of MK-801 has profound sedative effects because the higher dose administration of MK-801 has profound sedative effects [17]. It was therefore difficult to score the neurological findings precisely. Kochhar et al. have observed a 75% increase in the duration of ischemia required to produce paraplegia in rabbits receiving the systemic administration of MK-801 (1 mg/kg) 5 min after the induction of spinal cord ischemia in the rabbit model [17]. Yum and Faden, Martinez-Arizala and colleagues have shown that delayed systemic administration with MK-801 (1 mg/kg) 5 min after reperfusion is effective in reducing neurological dysfunction in 25 min spinal cord ischemia models [6,19]. Hypothermia reduces intracranial pressure, which in turn can limit edema formation, in addition hypothermia is effective in reducing both acute and delayed neuronal injury [20]. However, in group 2 (MK-801 1 mg/kg dissolved in hypothermic saline), there was no neurological improvement, which is contrary to the results of these previous studies. MK-801 (1 mg/kg) did not have sufficient spinal cord protective capabilities in our spinal cord ischemia model. Differences in the methods of drug administration, however, may help to explain these results. It has been shown that higher doses of MK-801 can cause side effects in some animals that can interfere with neuroprotection [17,21]. However, at the dose used in our study, we found that MK-801 (2 mg/kg) was beneficial in our model for decreasing the effects of spinal cord ischemia.

The results of our histopathologic study confirmed the ability of MK-801 to limit neuronal damage in the anterior horn. Although normal animals had neuronal cells in the anterior horns, rabbits with paraplegia showed total destruction of the anterior horn cells with vacuolization. In the tissue samples of MK-801-treated rabbits, the motor neurons were well preserved.

There were significant differences among the
three groups according to the number of surviving neurons per section. We observed a significant loss of surviving neurons in the ventral horns in group 1 (p < 0.001 versus group 3). There were significantly fewer surviving neurons in group 3 than in the normal group (p=0.0017). In contrast, according to Modified Tarlov scale, the neurological scores in the MK-801 (2 mg/kg) -treated group did not significantly differ from those of the normal rabbits. These results suggested that MK-801 reduce the extensive neuronal damage without remarkable neurological dysfunction by a partial block of mechanism described by Olney’s theory, in which one CNS neuron excites another CNS neuron to death [5].

The elevation of glutamate and aspartate levels after spinal cord ischemia has been measured in the rat using microdialysis [13] and in a clinical study using cerebrospinal fluid samples [22]. In these studies, marked elevations in glutamate and aspartate above basal levels after severe spinal cord injury have been observed [13,22]. Under physiological circumstances, most glutamate is located intracellularly, and released glutamate is rapidly taken up by astrocytes and neurons, leaving low extracellular concentrations of the transmitter [14,15]. It appears likely that an inhibition of the reuptake systems due to severe ischemia contributes to extracellular glutamate accumulations [13]. Since trauma to the spinal cord causes a rapid disturbance in the blood-spinal cord barrier, leakage of glutamate from the blood may well occur after opening of the blood-brain barrier, as has been reported [23,24]. Some of these amino acids may be derived from cells of the injured cord, and leakage of amino acids from the blood due to an opening of the blood-spinal cord barrier is also possible [13]. In our study, we did not observe any changes in glutamate levels, although we did observe changes in aspartate levels in group 1. There were significant differences in the increase rate of aspartate between 12 and 48 hrs (p=0.0124) and between 24 and 48 hrs (p=0.0132). The increase rates of glutamate and aspartate in group 3, which were observed to result in no neurological injury, showed no significant differences statistically. The increases in aspartate levels in the post ischemic period could possibly be related to the neurological outcome. It has been suggested that there is a significant glutamate release after moderate and severe spinal cord ischemic injury that results in extensive edema; no glutamate release, however, has been noted after mild injury, although this degree of compression still produces vasogenic edema [25]. In our experimental model, the grade of spinal cord ischemia was mild and moderate and other factors might be related to the neurological dysfunction occurring after mild injury.

In conclusion, we found that the noncompetitive NMDA receptor antagonist MK-801 (2 mg/kg) produced a beneficial effect in the rabbit spinal cord ischemia model when administered through an isolated aorta in the early ischemic period. MK-801 can reduce the neuronal ischemic damage and increase the tolerance of the spinal cord to ischemia. We observed an elevation of aspartate, but not glutamate levels in our rabbit model of spinal cord ischemia. The elevation of aspartate in the post ischemic period is possibly related to the postoperative neurological outcome. The cause of neurological dysfunction in the post ischemic period is multifactorial. We now believe that neurological dysfunction is improved not only by pharmacological support but also by a reduction of risk factors based on the combination of several protective methods.

ACKNOWLEDGMENTS: The author would like to thank Prof. S. Aoyagi and also Dr. H. Akashi, Dr. T. Fujino and Dr. A. Tanaka of the Department of Surgery, and Dr. T. Aoki of the Department of Pathology, Kurume University School of Medicine for their critical advice and suggestions.

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