Short-Term Administration of a New Free Radical Scavenger, Edaravone, Is More Effective Than its Long-Term Administration for the Treatment of Neonatal Hypoxic–Ischemic Encephalopathy

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Background and Purpose—Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a new free radical scavenger that is used for the treatment of adult acute cerebral infarction in Japan. We examined the effect of edaravone on the optimal duration of treatment, the long-term effect on the brain, and the effect on learning and memory disability in a rat model of neonatal hypoxic–ischemic encephalopathy.

Methods—Seven-day-old Wistar rats were subjected to left common carotid artery ligation then 2 hours of hypoxic–ischemic insult or sham operation. Edaravone was administered intraperitoneally (9 mg/kg) after hypoxic–ischemic insult every 24 hours for 2, 5, or 10 consecutive days. The neuroprotective effect of edaravone was evaluated by behavioral test and histological analysis.

Results—Two-day treatment with edaravone significantly gave protection to the learning and memory capability, as well as morphological recovery compared with control rats. Five-day treatment showed morphological improvement but no behavioral improvement. In contrast, 10-day treatment did not show either morphological or behavior improvement.

Conclusions—These findings indicate that edaravone is a promising candidate as a treatment of choice for neonatal hypoxic–ischemic encephalopathy, when its use is limited to the acute phase after hypoxia–ischemia. (Stroke. 2005;36: 2468-2474.)

Key Words: edaravone ■ hypoxia–ischemia ■ learning impairment ■ Levine’s model ■ neonatal rat

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a newly synthesized free radical scavenger used commonly for the treatment of adult acute cerebral infarction. A recent clinical study suggests that it may theoretically be used in treatment of neonatal hypoxic–ischemic (HI) encephalopathy. The fetal–neonate transition involves physiological hyperoxygenation and increased free radical formation. The fetus and neonate have less ability in scavenging free radicals and exerting antioxidant effects; and, lastly, neonatal HI encephalopathy extends beyond the brain to multiple organs, where free radicals play an important role in developing tissue damage. We have reported that edaravone has a neurorescue effect on neonatal rats in a dose-dependent manner after HI. Edaravone (9 mg/kg) significantly decreased the infarct areas compared with the controls after HI. Edaravone (6 mg/kg) showed a significant reduction of infarct areas on 1 day, but not on 7 days after HI, which suggests that multiple rather than single administration of edaravone is required to obtain a beneficial effect after HI. Some neurorescue methods show short-term but not long-term effects. Brief hypothermia after HI produced a significant decrease in cerebral infarction at 1 week but not after 4 weeks in a neonatal HI encephalopathy rat model. This indicates that, in some cases, neuroprotective treatment does not always reduce injury, but only delays injury formation. Recent therapeutic treatments for HI encephalopathy after insult have been evaluated by examining morphology or brain tissue loss, but it remains uncertain whether such findings are accompanied by long-term improvements in functional and morphological outcome. The model by Levine, an animal model of neonatal HI encephalopathy, does not mimic the rat brain damage that we usually encounter in clinical practice. Because of its good survivability, we can observe long-term effects in these model rats. Recent clinical studies show that the characteristic behavioral deficits were observed 18 weeks after HI insult was imposed on 7-day-old rats and that sensorimotor deficits were rarely observed.

The aims of our study were 3-fold: (1) to optimize the times of repeated edaravone injection; (2) to extend the observation to 18 weeks after HI; and (3) to evaluate the long-term effect of
edaravone on learning and memory impairment in a neonatal rat model of HI encephalopathy in terms of clinical application of edaravone in human neonates.

Methods

Animals
Pregnant Wistar rats (Charles River; Shizuoka, Japan) were maintained at temperature of 23 ± 1°C with 50 ± 10% relative humidity in the Experimental Animal Center. Rats had free access to the water, were allowed to deliver spontaneously, and the pups were reared with their dams until the time of experiments. The rats were placed on a restricted food intake (10 to 12 g per day; CE-2; CREA) from week 6 to 15 after HI for the 8-arm radial maze (8-ARM) and choice reaction time (CRT) tasks. The experimental design was approved by the Committee for the Ethics on Animal Experiments at the Faculty of Medicine, University of Miyazaki.

Surgery
Seven-day-old Wistar rats were subjected to defined Levine’s procedure for producing HI brain injury as described previously.2 Rats were anesthetized with ether, and the left carotid artery was sectioned permanently between double ligatures with 4-0 surgical silk. The rats were allowed to recover for 1 to 2 hours and then exposed to 2 hours of hypoxia in a plastic container that was perfused with a mixture of humidified 8% oxygen balanced with nitrogen. The sham-operated group received the same procedure without ligation and hypoxic exposure. The temperature inside the container was kept at 33°C, the usual temperature while huddling with the mother.10 After HI insult, pups were returned to their mothers.

Drug Administration
Immediately after HI, edaravone was injected IP (9 mg/kg) every 24 hours for 2, 5, or 10 consecutive days (n = 15 per group). The vehicle-treated group (n = 15) and the sham-operated group (n = 12) with or without HI were given IP injection of saline for 2 days.

Behavioral Study

8-ARM Task
The experimental schedule of behavioral study is shown in Figure 1. The modified 8-ARM task (Neuroscience Inc) was performed as reported previously.9 Each test animal was placed in a circular plastic ring on the platform at the middle of the maze. After 1 minute, the ring was lifted, and the test animal was allowed to move freely in the maze. The trial continued until the test animal entered all 8 arms or 10 minutes had elapsed, and it was performed every day for 21 days. Performance of the test animal in each trial was assessed by 3 parameters: the number of correct choices in the initial 8 chosen arms; the number of errors, which was defined as choosing arms that had already been visited; and running time before all 8 of the pellets were eaten. For behavioral analysis, an image motion analyzer, AXIS-30 (Neuroscience Inc), was used to quantify the rats’ task performance.

CRT Task
The CRT task (Neuroscience Inc) was performed as reported previously. In this system, for 1 to 2 weeks before the study, all of the rats were first trained to press either of 2 levers by varying the “correct” lever; a cue lamp was randomly lighted above the correct lever with a continuous reinforcement schedule of a fixed ratio of 1. Trials began with a differential reinforcement of other behavior (DRO) period (random, 2 to 5 s), during which the animals had to refrain from pressing either of the 2 levers. If they repeatedly pressed the levers (> 10 s) during the DRO period, that trial was terminated and was followed by the intertrial interval (ITI) period. During the CRT period (maximum, 10 s), the time between sample presentation with the cue-lamp on and the correct lever pressing was defined as CRT, and the food pellet reinforcement was provided through the pellet dispenser. With additional lever pressing responses, a houselamp was illuminated, and the ITI period (20 s) was begun. One trial took ~30 s, and each test session consisted of 30 trials. The task was performed in a session every day for 30 days. The variables measured were the number of correct responses, the CRT (in seconds) during the correct response, and the number of incorrect lever pressings during the DRO and ITI periods. Collected data were processed using a system program written in BASIC.

Water Maze Task
The modified swimming pool test (Neuroscience Inc) was performed as described previously.9,12 Each rat received 3 trials daily for 5 consecutive days. The pool was divided into 4 quadrants. A trial consisted of placing a rat into the water facing the wall of the pool, at 1 of 3 starting positions, excluding the quadrant containing the platform. The platform was located in a constant position in the middle of its quadrant. During each block of 3 trials, each rat started at each of the 3 starting positions, but the sequence of the positions was selected randomly. In each trial, the latency (swimming time) to escape onto the hidden platform was recorded and the cutoff time was 120 s. At the end of each trial, the rat was returned to its home cage. The ITI time was ~1 minute. Performance of the test animal in each trial was assessed by 3 parameters: swimming time, swimming length, and swimming speed. A personal computer was used for analysis (AXIS-30, Neuroscience Inc). A CCD camera equipped with a personal computer for behavioral analysis in the water maze task was similar to that used in the 8-ARM task.

Quantitative Histological Analysis
After completion of the experiments, on week 18 after the HI insult, animals were anesthetized with pentobarbital (50 mg/kg IP) and

Hypoxia-ischemia:
Seven-day-old Wistar rat Occlusion of left common carotid artery Exposed to 8% Oxygen, 92% Nitrogen for 2 hours

Edaravone, 9 mg/kg, i.p.
1) 2 day edaravone (0, 1 day after hypoxia)
2) 5 day edaravone (0,1-4 day after hypoxia)
3) 10 day edaravone (0,1-9 day after hypoxia)

Sacrifice

8-arm radial maze (7-12th weeks, total 24 days)

7th 10th 11th 12th 15th 16th 18th week

Choice reaction time (10th – 15th weeks, total 30 days)

Water maze (18th week, total 5 days)

Figure 1. Schematic diagram of experimental schedule.
perfused transcardially with saline followed by 4% paraformaldehyde. Brains were removed and sectioned coronally into five 2-mm slices starting at 2, 4, 6, 8, and 10 mm from the anterior pole using a rat brain slicer (Zivic-Miller Laboratory Inc). Both left and right areas were measured (mm$^2$) with National Institutes of Health Image software (version 1.62).

**Statistical Analysis**

Results are expressed as means±SEM. Repeated measurement of 2-way ANOVA was applied to each parameter of the 8-ARM task, CRT task, and water maze task. Dunnett test after 1-way ANOVA was used for all of the blocks of each behavioral task and quantitative histological analysis. $P<0.05$ was considered statistically significant.

**Results**

**8-ARM**

In the running time, there was no significant difference among the groups (data not shown). The vehicle group showed a clear decrease in correct choices compared with the sham group, and the edaravone 2-day group significantly increased the number of correct choices compared with the vehicle group ($P<0.0001$), the effect of block ($P<0.001$), and a group×block interaction ($P<0.001$; Figure 2A). The vehicle group significantly decreased the number of correct choices in comparison with the sham group ($P<0.01$), and the edaravone 2-day group significantly increased the number of correct choices in comparison with the vehicle group ($P<0.01$; Figure 2B).

The vehicle group showed a clear increase in errors compared with the sham group, and the edaravone 2-day group significantly recovered to the level of the sham group ($P<0.001$), the effect of block ($P<0.001$), and a group×block interaction ($P<0.001$; Figure 2C). The vehicle group significantly increased errors in comparison with the sham group.

![Figure 2](http://stroke.ahajournals.org/)

Figure 2. Effect of edaravone on spatial learning deficits in the 8-ARM following HI. Each block consists of 3 trials. *$P<0.05$; **$P<0.01$. Values are mean±SEM (n=12 to 15 per group).
(P<0.01), and the edaravone 2-day group significantly decreased errors in comparison with the vehicle group (P<0.01; Figure 2D).

**CRT Task**
The vehicle group showed a clear decrease in the number of correct response compared with the sham group, and the edaravone 2-day group significantly recovered to the level of the sham group (P<0.001), the effect of block (P<0.001), and a group×block interaction (P<0.001; Figure 3A). The vehicle group significantly decreased the correct response in comparison with the sham group (P<0.01; Figure 3B), and the edaravone 2-day group significantly increased the correct response in comparison with the vehicle group (P<0.01; Figure 3B).

In the CRT, the repeated measures of 2-way ANOVA revealed a group difference (P<0.001), effect of block (P<0.001), and a group×block interaction (P<0.001; Figure 3C). The vehicle group significantly prolonged the CRT in comparison with the sham group (P<0.01; Figure 3D), and the edaravone 2-day group had significantly shortened CRT in comparison with the vehicle group (P<0.01; Figure 3D). In the edaravone 10-day group, no statistical significance was observed among the groups.

The vehicle group showed a clear increase in the number of lever pressings during both the DRO and ITI periods compared with the sham group, and the edaravone 2-day group tended to decrease the number of lever pressings (P<0.001), the effect of block (P<0.001), and a group×block interaction (P<0.001; Figure 3E). The vehicle group significantly increased the number of lever pressings in comparison with the sham group (P<0.05; Figure 3F), and the edaravone 2-day group tended to decrease them in comparison with the vehicle group (Figure 3F).

**Figure 3.** Effect of edaravone on attention deficits in the CRT task following HI. Each block consists of 3 trials. *P<0.05; **P<0.01. Values are mean±SEM (n=12 to 15 per group).
Water Maze
The vehicle group showed a clear increase in swimming length compared with the sham group, and the edaravone 2-day group significantly recovered to the level of the sham group \((P<0.001)\), the effect of block \((P<0.001)\), and a group\(\times\)block interaction \((P=0.5731; \text{Figure 4A})\). The vehicle group significantly increased swimming length in comparison with the sham group \((P<0.01; \text{Figure 4B})\), and the edaravone 2-day group significantly decreased swimming length in comparison with the vehicle group \((P<0.01; \text{Figure 4B})\). There were no significant differences in the swimming speed between the groups (Figure 4C and 4D).

Quantitative Histological Analysis
There were no differences of the hemispheric area in each section of the contralateral hemispheres among the groups in week 18 after the HI (data not shown). In each section of the ipsilateral hemisphere, the vehicle-treated group showed a significant decrease in hemispheric area compared with the sham-operated group \((6, 8, \text{and } 10 \text{ mm}: P<0.01; \text{Figure 5})\). Both edaravone 2-day and 5-day treatment groups significantly reversed these decreases compared with the vehicle-treated group \((edaravone 2-day treatment group: 6, 8, \text{and } 10 \text{ mm}: P<0.05; \text{edaravone 5-day treatment group: 6, 8, and } 10 \text{ mm}: P<0.01; \text{Figure 5})\). On the other hand, the edaravone 10-day treatment group showed a significant decrease in all of the sections compared with the vehicle-treated group \((edaravone 10-day treatment group: 6 \text{ and } 10 \text{ mm}: P<0.01; 8 \text{ mm}: P<0.05; \text{Figure 5})\).

Discussion
The central findings of this study were that 2-day administration of edaravone relieved hemispheric volume loss induced by HI stress at postnatal day 7, and behavioral disabilities returned to the control levels in the 3 different learning and memory tasks. In contrast, 5-day administration of edaravone relieved hemispheric volume loss but did not affect learning and memory disabilities. Ten-day treatment did not protect hemispheric volume loss or behavior impairment.

10-Day Treatment Did Not Improve Both Morphology and Behavior
These results explicitly contrast with the recommended regimen of edaravone for treatment of adult acute cerebral infarction, which is 30 mg 12 hourly for 14 days. This regimen came from the concept that free radicals were produced persistently in the brain for 2 to 3 weeks after infarction, where white blood cell migration followed initial radical production because of the reperfusion of the brain.\(^{13}\) Clinical trials showed that the clinical signs and symptoms significantly improved in 69% of patients treated with edaravone for \(\geq 13\) days, whereas improvement was observed in only 24% of patients treated for <13 days.\(^{14}\) Therefore, our results in the developing brain suggest to clinicians that the standard adult regimen of edaravone may not be applicable to neonatal HI encephalopathy.

Although the mechanisms in the developing brain for the adverse effects after long-term use of edaravone are unknown, one possible explanation is that edaravone itself may
produce some reactive oxygen species. Some antioxidants, including vitamin A, vitamin E, quercetin, epigallocatechin gallate, and green tea catechins, can induce hydrogen peroxide generation and produce subsequent damage to isolated and cellular DNA in spite of antioxidants. Some antioxidants may also have prooxidant properties. Another possible explanation may be that free radicals may play important roles in the subacute phase after HI stress, and suppression of their production might have adverse effects both behaviorally and histologically. When the acute phase is over and the regenerative process starts, the removal of degenerative tissue by white blood cells is very important. Suppression of free radicals, which play important roles in white blood function, by edaravone may lead to adverse effects.

Two-Day Treatment Improved Morphology and Behavior
Edaravone for 2 days gave full protection to the long-lasting learning and memory capability, as well as morphological recovery. Until now, several mechanisms been postulated to explain the underlying neuroprotective and neurorescue effects of edaravone, mainly in the mature brain. In vitro, edaravone scavenges hydroxy-peroxide and lipid peroxide. In vivo, it also suppresses production of hydroperoxicatetraenoic acid, one of the lipoxygenase metabolites, in vascular endothelial cells after cerebral embolization. Edaravone was effective in treating adult rat brain edema caused by focal ischemia, by inhibiting both nonenzymatic free radical reactions and lipoxygenase activity. In a rat model for neonatal HI encephalopathy, pretreatment with edaravone suppresses both necrosis and apoptosis. We reported that edaravone inhibited lipid peroxidation and suppressed the NO metabolite level after HI stress. To estimate the in vivo intracerebral antioxidant ability in the same HI model, we performed temporal electron paramagnetic resonance imaging and found that the antioxidant ability was decreased in the contralateral cerebral hemisphere by edaravone. This study explicitly showed that the neurorescue effect of edaravone 2-day treatment was persistent up to 18 weeks after HI and was accompanied by preservation of learning and memory capability.
Five-Day Treatment Improved Histology But Not Behavior

Administration of edaravone for 5 days showed ambivalent results: improvement of cerebral hemispheric areas but no improvement in the 3 learning and memory tasks. One explanation for this discrepancy could be that even animals without significant brain damage have fewer cells in the cortex, striatum, and hippocampus, as shown by immunohistochemistry.26 Because we did not perform qualitative histology to characterize the various cellular subtypes of the brain parenchyma, subtle injury to neuronal cells involved in behavioral learning cannot be excluded.27 Future studies should include both morphological study and neurochemical studies, such as neurotrophic factors and neurotransmitters.

In conclusion, 3 different treatment courses of edaravone have different effects on brain histology and behavior through 18 weeks of observation. Unexpectedly, 2-day treatment with edaravone showed the best effect, and 10-day treatment was worst. These results indicate that edaravone is a promising candidate as a treatment of choice for neonatal HI encephalopathy, when its use is limited to the acute phase after HI.

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