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Effect of the free radical scavenger, 3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186), on hypoxia-ischemia-induced brain injury in neonatal rats

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Abstract

The free radical scavenger 3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186), which has been approved in Japan for use in patients with cerebral infarction, was used to treat ischemic-hypoxic brain damage in neonatal rats. Seven-day-old rat pups were subjected to a modified Levine procedure, then given either vehicle or MCI-186 (at one of three dosage levels: 3, 6, or 9 mg/kg), and the extent of brain damage was evaluated either 24 h or 7 days later. The administration of MCI-186 significantly attenuated damage, in a dose-dependent manner. These results indicate that MCI-186 is a promising candidate for the treatment of neonatal hypoxic-ischemic encephalopathy.

Keywords: Neonatal rat; Hypoxia-ischemia; Oxidative stress; Free radical scavenger; 3-Methyl-1-phenyl-2-pyrazolin-5-one

There is a growing body of literature concerning the generation of free radicals and perinatal hypoxic-ischemic brain damage, for which a standard regimen of medications has not been established [3,5,8]. The role of oxidative stress seems more important in the pathogenesis of asphyxia-induced brain damage in the neonatal brain than in the adult brain. All newborn infants experience a great increase in oxygenation at birth relative to that experienced the intrauterine environment, which contributes to the production of reactive oxygen species [4]. The systems that protect against oxidative stress are less effective in the neonate than in the adult. For example, the concentrations of serum transferrin, ceruloplasmin, glutathione peroxidase, and superoxide dismutase are lower than the corresponding adult values [2]. The newborn infant has a relative deficiency of brain superoxide dismutase and glutathione peroxidase [9].

A newly synthesized free radical scavenger, 3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186), inhibits not only hydroxyl radicals but also iron-induced peroxidative injuries [22]. Protective effects conferred by MCI-186 against transient or permanent cerebral ischemia have been reported in adult rats [1,13,14,21,23]. In April 2001, after clinical trials [6,16], MCI-186 was endorsed by the Ministry of Heath, Labor and Welfare in Japan and became commercially available for the treatment of acute cerebral infarction due to thrombosis or embolism.

It is reasonable to expect that MCI-186 will have greater beneficial effects on neonatal brain damage than on adult damage. The purpose of this study was to evaluate the effects of MCI-186 in a rat model of neonatal hypoxic-ischemic encephalopathy.

This study was approved by the Animal Research Committee of Miyazaki Medical College. Pregnant Wistar rats were purchased from Japan Charles River (Shizuoka, Japan).

On postnatal day 7, littermates were assigned to one of four groups: MCI-186 3 mg/kg (MCI-3 mg; $n = 28$), MCI-186 6 mg/kg (MCI-6 mg; $n = 28$), MCI-186 9 mg/kg (MCI-9 mg; $n = 28$), and vehicle (normal saline; $n = 84$). Each pup was subjected to a modified Levine procedure for producing hypoxic-ischemic injury [11,15]. Briefly, pups were anesthetized with ether and the left carotid artery was sectioned between double ligatures with 4-0 surgical silk. The pups were allowed to recover for 1–2 h and were then exposed to 2 h of hypoxia in a plastic container that was perfused with a mixture of humidified 8% oxygen balanced with nitrogen. The temperature inside the container was maintained at 33°C, the usual temperature to which rat

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pups are exposed when huddling with the mother [12]. Immediately after removal from the hypoxic chamber, pups were treated with 100-μl intraperitoneal injections of vehicle or 3, 6, or 9 mg/kg MCI-186 dissolved in normal saline. The injection was repeated 30 min after the first one. In each experiment, the same number of vehicle-treated control animals and MCI-186-treated animals were prepared, because the histological results can vary between littermates and can be affected by the experimental conditions during the modified Levine procedure [11].

Brain damage was evaluated 24 h and 7 days after the application of hypoxic-ischemic insult. Brain damage at 24 h after recovery was considered to be necrosis-derived, and that observed at 7 days after recovery was attributed to a combination of necrosis and apoptosis as pathogenesis.

Animals (14 MCI-186 and vehicle-treated sets in each different dosage group) were anesthetized and killed 24 h after ischemic-hypoxic insult, and the brains were removed. The brains were cut into five coronal slices (2 mm in thickness) using a rat brain slicer (Zivic-Miller Lab., Inc., Allison Park, PA). To determine the infarction area, each section was stained with 2,3,5-triphenyltetrazolium chloride (TTC) solution (Wako, Osaka, Japan). Infarcted areas do not stain with TTC [10]. The size of the infarcted area was measured in three sections: 2–4, 4–6, and 6–8 mm from the front, using an NIH computerized-image analysis system. We did not analyze sections taken 0–2 or 8–10 mm from the front, because the data were insufficient. The infarcted area was defined as the area unstained by TTC divided by the hemispheric area of the brain on the side contralateral to the ligated carotid artery, expressed as a percentage.

One week after hypoxic-ischemic insult, animals (14 MCI-186 and vehicle-treated sets in each different dosage group) were anesthetized and killed, and the brain was removed and fixed in ethanol-acetic acid (19:1) for 24 h. Coronal brain slices cut 2 mm and 6 mm anterior to the interaural line were embedded in paraffin. Sections (5–8 μm thick) for light microscopy were cut and stained with hematoxylin and eosin. Hypoxic-ischemic damage induced in the parietal and frontal cortices, in the CA1, CA3, and the dentate gyrus of the hippocampus, and in the striatum and the thalamus was evaluated microscopically in coded sections by different investigators, according to an arbitrary scale of severity, as described previously [15]: none, no damage; mild, damage to 25% or less of the surface area in a single section; moderate, damage to more than 25% but less than 50%; and severe, damage to more than 50% of the area.

Non-parametric statistics were used for statistical analyses because the data were not normally distributed. To compare the MCI-186 and vehicle groups, the Mann–Whitney test was used following analysis of variance. A value of $P < 0.05$ was considered statistically significant.

Two and four animals died in the vehicle- and MCI-186-treated groups, respectively, during surgery, hypoxic stress, or the recovery phase. The infarction areas evaluated by TTC staining are shown in Fig. 1. There were no differences in the infarction areas of the vehicle- and MCI-3 mg-treated groups. The infarction areas of the MCI-6 mg-treated group were smaller ($P < 0.05$) than those of the vehicle-treated group at slices 4–6 mm (18.5 ± 4.0 versus 35.1 ± 6.3%, respectively) and 6–8 mm (20.9 ± 5.3 versus 38.7 ± 6.9, respectively), but not at slice 2–4 mm. In the MCI-9 mg-treated group, the infarction areas were smaller ($P < 0.01$) than those of the vehicle-treated group at all slice levels: at 2–4 mm, 13.0 ± 4.9 versus 34.5 ± 5.0%, respectively; at 4–6 mm, 14.0 ± 6.3 versus 38.9 ± 4.5%, respectively; and at 6–8 mm, 13.1 ± 4.9 versus 37.7 ± 5.5%, respectively.

Table 1 shows the number of animals, with the corresponding grade of damage, 7 days after ischemic-hypoxic

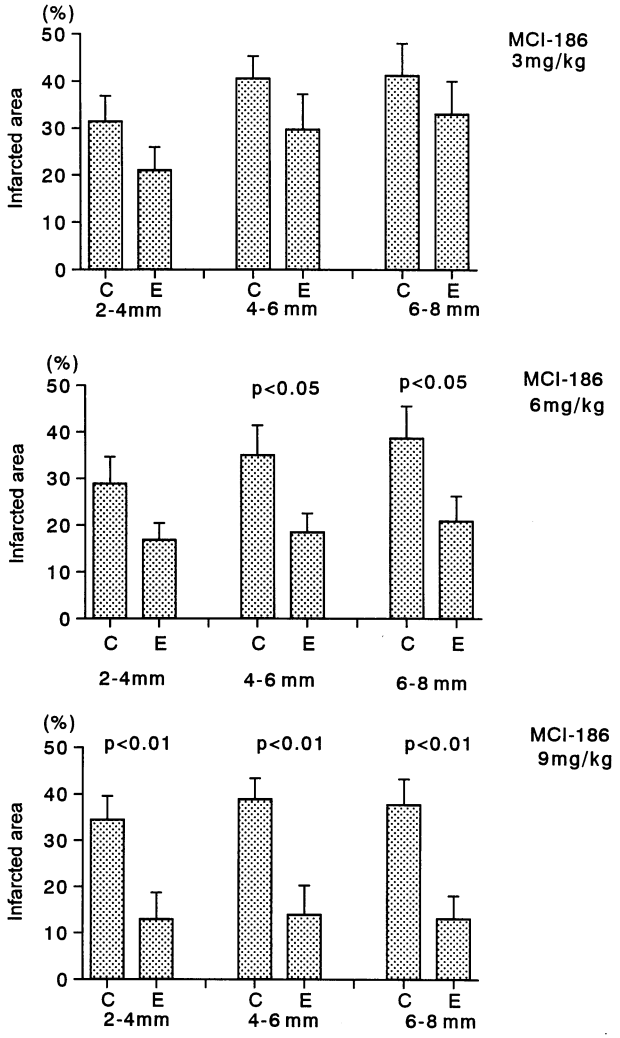

Fig. 1. Effect of MCI-186 on infarcted area 24 h after hypoxic-ischemic insult in 7-day-old rats. Results are given as means ± SEM. The size of the infarcted area was measured in three sections: 2–4, 4–6, and 6–8 mm from the front of the brain. The infarcted area was defined as the area unstained by TTC divided by the hemispheric area of the brain on the side contralateral to the ligated carotid artery, expressed as a percentage. The number of animals in each bar was 12–14. C, vehicle-treated control; and E, MCI-186-treated group.

Table 1
Number of animals with histological grading of damage following ischemic/hypoxic insults[a]

	None	Mild	Moderate	Severe	None	Mild	Moderate	Severe
	Vehicle ($n=14$)				3 mg/kg MCI-186 ($n=14$)			
Cortex	1	2	0	11	4	0	1	9
Hippocampus CA1	1	2	0	11	3	1	1	9
Hippocampus CA3	1	0	0	13	3	0	0	11
Hippocampus dentate	3	2	2	7	6	2	0	6
Thalamas	3	1	2	8	4	2	3	5
Striatum	2	1	1	10	4	0	4	6
	Vehicle ($n=14$)				6 mg/kg MCI-186 ($n=14$)			
Cortex	3	1	0	10	7	0	1	6
Hippocampus CA1	3	1	0	10	7	1	1	5
Hippocampus CA3*	2	1	0	11	7	0	1	6
Hippocampus dentate	4	2	0	8	9	1	1	3
Thalamas	4	3	0	7	8	0	1	5
Striatum	4	0	1	9	7	1	0	6
	Vehicle ($n=14$)				9 mg/kg MCI-186 ($n=14$)			
Cortex*	3	1	0	10	7	2	1	4
Hippocampus CA1*	3	1	0	10	7	1	2	4
Hippocampus CA3*	2	1	0	11	8	0	1	5
Hippocampus dentate*	4	2	2	6	10	2	0	2
Thalamas*	4	2	2	6	9	3	1	1
Striatum*	3	1	1	9	8	2	2	2

[a] None, no damage; mild, 25% or less of surface area on a single section; moderate, more than 25% but less than 50%; severe, more than 50%. The incidence of damage was decreased ($P<0.05$) in the MCI-186 treated groups (6 and 9 mg/kg) represented by asterisks.

insult. Histologically, the great majority of vehicle-treated animals demonstrated massive infarction that involved more than 50% of the cerebral cortex of the injured hemisphere. Complete necrosis with tissue breakdown and cavitation was the usual outcome in the cortex. The incidence of the various grades of damage in each part of the cerebrum did not differ between the vehicle- and MCI-3 mg-treated groups. In the MCI-6 mg-treated group, the CA3 part of the hippocampus had less severe histological damage than in the vehicle-treated group ($P < 0.05$). However, damage levels in the other parts of the cerebrum did not differ between groups. The incidence of damage was significantly reduced ($P < 0.05$) in the MCI-9 mg-treated group in all the parts of the brain evaluated.

The results of our study clearly show that MCI-186 confers protection against hypoxia-ischemia-induced brain damage in neonatal rats in a dose-dependent manner.

Some antioxidants have been reported to have neuroprotective or neuronal rescue effects in animal models of neonatal hypoxic-ischemic brain damage. Allopurinol, a xanthine oxidase inhibitor, reduces the histological damage in neonatal rats when applied before hypoxic-ischemic insult, and its beneficial effects are still evident when the inhibitor is applied after the insult [18]. Deferoxamine, an iron chelator, also demonstrates neuroprotective and rescue effects [7,17]. The antioxidative effects of vitamins C and E are well known, but they are not associated with any histological improvement in the brain damage caused by hypoxia-ischemia in animal experiments. No clinical human trials have been carried out with these or other antioxidants.

In the MCI-6 mg-treated group, significant improvement to neuronal damage was observed 24 h after hypoxic-ischemic insult. However, 7 days after the insult, only the CA3 of the hippocampus showed an improvement. One explanation for these findings is that a second injection within 30 min of recovery did not exert enough antioxidative effect to extend beyond 24 h after the insult. It is important to evaluate whether the effects of repeated injections, which is the current standard clinical practice for the treatment of adult cerebral infarction in Japan [16], will be effective in neonatal patients.

There are good theoretical reasons for using MCI-186 as the drug of choice in treating neonatal hypoxic-ischemic encephalopathy. First, no significant adverse effects have been reported since the inception of its clinical trials for acute cerebral infarction in Japan [16,20]. Second, as described earlier, the neonate is thought to be susceptible to oxidative stress because it is exposed to physiological hyperoxygenation relative to the intrauterine environment and because of its relatively deficient antioxidative system [2,4,9]. Third, unlike adult cerebral infarction, neonatal infarction involves other organs, such as the heart, kidney, and lung, as well as the brain, and oxidative stress plays important roles in the pathogenesis of these organs [19].

These facts, taken together, suggest that MCI-186 might prove to be an effective and potent protective agent against perinatal hypoxic-ischemic encephalopathy.

[1] Abe, K., Yuki, S. and Kogure, K., Strong attenuation of ischemic and postischemic brain edema in rats by a novel free radical scavenger, Stroke, 19 (1988) 480–485.
[2] Buonocore, G., Perrone, S. and Bracci, R., Free radicals and brain damage in the newborn, Biol. Neonate, 79 (2001) 180–186.
[3] Espinoza, M.I. and Parer, J.T., Mechanisms of asphyxial brain damage, and possible pharmacologic interventions, in the fetus, Am. J. Obstet. Gynecol., 164 (1991) 1582–1589.
[4] Evans, P.J., Evans, R., Kovar, I.Z., Holton, A.F. and Halliwell, B., Bleomycin-detectable iron in the plasma of premature and full-term neonates, FEBS Lett., 303 (1992) 210–212.
[5] Hasegawa, K., Yoshioka, H., Sawada, T. and Nishikawa, H., Lipid peroxidation in neonatal mouse brain subjected to two different types of hypoxia, Brain Dev., 13 (1991) 101–103.
[6] Houkin, K., Nakayama, N., Kamada, K., Noujou, T., Abe, H. and Kashiwaba, T., Neuroprotective effect of the free radical scavenger MCI-186 in patients with cerebral infarction: clinical evaluation using magnetic resonance imaging and spectroscopy, J. Stroke Cerebrovasc. Dis., 7 (1998) 315–322.
[7] Hurn, P.D., Koehler, R.C., Blizzard, K.K. and Traystman, R.J., Deferoxamine reduces early metabolic failure associated with severe cerebral ischemic acidosis in dogs, Stroke, 26 (1995) 688–694.
[8] Ikeda, T., Choi, B.H., Yee, S., Murata, Y. and Quilligan, E.J., Oxidative stress, brain white matter damage and intrauterine asphyxia in fetal lambs, Int. J. Dev. Neurosci., 17 (1999) 1–14.
[9] Inder, T.E., Graham, P., Sanderson, K. and Taylor, B.J., Lipid peroxidation as a measure of oxygen free radical damage in the very low birth weight infant, Arch. Dis. Child Fetal Neonatal Ed., 70 (1994) F107–F111.
[10] Isayama, K., Pitts, L.H., Nishimura, M.C., Hurn, P.D., Koehler, R.C., Blizzard, K.K. and Traystman, R.J., Evaluation of 2,3,5-triphenyltetrazolium chloride staining to delineate rat brain infarcts, Stroke, 22 (1991) 1394–1398.
[11] Levine, S., Anoxic-ischemic encephalopathy in rats, Am. J. Pathol., 36 (1960) 1–17.
[12] Mortola, J.P. and Dotta, A., Effects of hypoxia and ambient temperature on gaseous metabolism of newborn rats, Am. J. Physiol., 263 (1992) R267–R272.
[13] Nishi, H., Watanabe, T., Sakurai, H., Yuki, S. and Ishibashi, A., Effect of MCI-186 on brain edema in rats, Stroke, 20 (1989) 1236–1240.
[14] Oishi, R., Itoh, Y., Nishibori, M., Watanabe, T., Nishi, H. and Saeki, K., Effect of MCI-186 on ischemia-induced changes in monoamine metabolism in rat brain, Stroke, 20 (1989) 1557–1564.
[15] Ota, A., Ikeda, T., Ikenoue, T. and Toshimori, K., Sequence of neuronal responses assessed by immunohistochemistry in the newborn rat brain after hypoxia-ischemia, Am. J. Obstet. Gynecol., 177 (1997) 519–526.
[16] Otomo, E., Tohgi, H., Kogure, K., Hirai, S., Terashi, A., Gotoh, F., Tazaki, Y., Ito, E., Sawada, T., Kobayashi, S., Fujishima, M. and Nakashima, M., Clinical efficacy of a free radical scavenger, MCI-186, on acute cerebral infarction, Ther. Res., 19 (1998) 1311–1332.
[17] Palmer, C., Roberts, R.L. and Bero, C., Deferoxamine posttreatment reduces ischemic brain injury in neonatal rats, Stroke, 25 (1994) 1039–1045.
[18] Palmer, C., Towfighi, J., Roberts, R.L. and Heitjan, D.F., Allopurinol administered after inducing hypoxia-ischemia reduces brain injury in 7-day-old rats, Pediatr. Res., 33 (1993) 405–411.
[19] Saugstad, O.D., Hurn, P.D., Koehler, R.C., Blizzard, K.K. and Traystman, R.J., Update on oxygen radical disease in neonatology, Curr. Opin. Obstet. Gynecol., 13 (2001) 147–153.
[20] Tabrizchi, R., Hurn, P.D., Koehler, R.C., Blizzard, K.K. and Traystman, R.J., Edaravone Mitsubishi-Tokyo, Curr. Opin. Investig. Drugs, 1 (2000) 347–354.
[21] Watanabe, T. and Egawa, M., Effects of an antistroke agent MCI-186 on cerebral arachidonate cascade, J. Pharmacol. Exp. Ther., 271 (1994) 1624–1629.
[22] Watanabe, T., Morita, I., Nishi, H. and Murota, S., Preventive effect of MCI-186 on 15-HPETE-induced vascular endothelial cell injury in vitro, Prostaglandins Leukot. Essent. Fatty Acids, 33 (1988) 81–87.
[23] Watanabe, T., Yuki, S., Egawa, M. and Nishi, H., Protective effects of MCI-186 on cerebral ischemia: possible involvement of free radical scavenging and antioxidant actions, J. Pharmacol. Exp. Ther., 268 (1994) 1597–1604.