—**Short Communication**—

Maternal Treatment with MCI-186 Does Not Improve Delayed Deterioration of Cellular Bioenergetic State and Mitochondrial Activity Following Transient Intrauterine Ischemia in the Fetal Rat Brain

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Abstract

The mitochondrial respiratory activities and energy metabolism in the fetal rat brain were measured at the end of 30 minutes of intrauterine ischemia and after 2 and 4 hours of recirculation. The transient ischemia was associated with a delayed deterioration of cellular bioenergetic state and mitochondrial activities. The deterioration was not prevented by a free radical scavenger, 3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186), given immediately after recirculation.

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Key words: mitochondrial respiratory activity, energy metabolism, intrauterine ischemia, fetal brain, hydroxyl radical, MCI-186

Recirculation following transient ischemia carries the potential risk of triggering delayed brain damage. This is because recirculation enhances production of reactive oxygen species. The preliminary publication from our laboratory demonstrated that in the cerebral cortex of immature rats, recirculation after transient intrauterine ischemia due to 30 minutes of uterine artery occlusion is accompanied by an initial partial recovery of the cellular bioenergetic state and of mitochondrial function, with delayed deterioration during the first 4 hours of reflow[1,2]. Subsequent studies[3] showed that alpha-phenyl-*N-tert*-butyl-nitrone (PBN), a free radical spin trap, prevented the delayed energy failure. Because clinical studies suggest that such a delayed energy failure is associated with poor neurodevelopment at 1 year of age in the human infant[4], our preliminary results strongly suggest that free radicals play an important role in the development of neonatal neurologic deficit.

MCI-186 (3-methyl-1-phenyl-2-pyrazolin-5-one), a newly synthesized free radical scavenger, has potent free radical quenching action by directly trapping hydroxyl radicals[5]. In addition, a recent study suggests that MCI-186 exhibits some activity in scavenging superoxide anion by measuring cytochrome c reduction[6]. Previous studies have demonstrated that MCI-186 ameliorates ischemic brain damage in several adult animal models[7–9]. In the immature brain, the effect of this drug during ischemia and reperfusion is, however, unclear.

The objective of the present experiments was to explore whether MCI-186 influences the delayed

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Table 1 Changes in energy metabolitesa

	Without MCI-186				With MCI-186		MCI-186 effect (P value)c
	control (n = 6)	30 min Isch (n = 6)	30 min Isch 2-h R (n = 6)	30 min Isch 4-h R (n = 6)	30 min Isch 2-h R (n = 6)	30 min Isch 4-h R (n = 6)	
Non-ischemia uterine horn							
ATPb	2.62 ± 0.26	2.55 ± 0.31	2.59 ± 0.29	2.54 ± 0.21	2.61 ± 0.28	2.60 ± 0.31	0.57
ADPb	0.54 ± 0.07	0.46 ± 0.11	0.50 ± 0.08	0.58 ± 0.09	0.51 ± 0.08	0.57 ± 0.08	0.91
AMPb	0.07 ± 0.04	0.09 ± 0.03	0.08 ± 0.02	0.07 ± 0.02	0.07 ± 0.03	0.08 ± 0.03	0.89
ΣAdnb	3.23 ± 0.28	3.11 ± 0.35	3.17 ± 0.32	3.19 ± 0.27	3.19 ± 0.31	3.25 ± 0.35	0.41
EC	0.89 ± 0.03	0.89 ± 0.02	0.90 ± 0.03	0.89 ± 0.03	0.90 ± 0.03	0.89 ± 0.04	0.94
Ischemia uterine horn							
ATPb	2.54 ± 0.31	0.71 ± 0.38*†	2.19 ± 0.41	1.72 ± 0.25*†	2.28 ± 0.34	1.81 ± 0.23 †	0.41
ADPb	0.52 ± 0.06	0.49 ± 0.30	0.75 ± 0.12 †	0.51 ± 0.14	0.63 ± 0.21	0.54 ± 0.07	0.79
AMPb	0.08 ± 0.03	0.91 ± 0.41*†	0.09 ± 0.04	0.30 ± 0.09*†	0.13 ± 0.06	0.27 ± 0.04 †	0.6
ΣAdnb	3.14 ± 0.33	2.11 ± 0.41*†	3.03 ± 0.46	2.53 ± 0.31*†	3.05 ± 0.37	2.62 ± 0.30 †	0.43
EC	0.89 ± 0.04	0.45 ± 0.09*†	0.84 ± 0.04	0.78 ± 0.03*†	0.85 ± 0.05	0.79 ± 0.04 †	0.67

[a] Values are mean ± SD, Isch = ischemia, R = recirculation.
[b] mmol/kg wet tissue, ΣAdn; the sum of adenine nucleotides. EC; energy charge = ([ATP] + 0.5 [ADP]/ΣAdn.
[c] MCI-186 effect is evaluated by two-factor ANOVA.
* Against control, one-factor ANOVA followed by Scheffé's F test ($p < 0.05$).
† Against non-ischemia uterine horn, unpaired t test ($p < 0.05$).

deterioration of the cellular bioenergetic state and cerebral mitochondrial function after transient intrauterine ischemia in fetal rats.

This study was approved by the Ethics Committee for Animal Experimentation at our university. Six groups of pregnant animals (n = 36) were studied. One group was used as a control (n = 6), in which procedures were identical to those in the experimental groups except that uterine artery occlusion was not induced. In the other groups of animals, uterine artery occlusion was induced for 30 minutes. At the end of 30 minutes of ischemia the fetuses of one group of pregnant rats (n = 6) were delivered by cesarean section; the other animals were allowed recovery periods of either 2 h (n = 6 with saline; n = 6 with MCI-186), or 4 h (n = 6 with saline; n = 6 with MCI-186) of recirculation. MCI-186 (3 mg/m*l* saline/kg pregnant rat body weight) or vehicle (saline) was injected into the maternal tail vein immediately after the start of reperfusion. MCI-186 was kindly given to us by Tokyo Mitsubishi Chemical Industries Ltd. (Tokyo, Japan).

Ten-week-old pregnant Wistar rats (Sankyo Lab Service Tokyo, Japan) weighing 250～300 g were used. At 20 days of gestation (term 21.5 days), the animals were intubated and artificially ventilated on 1.0% to 1.5% halothane after overnight fasting. Thirty minutes of uterine artery occlusion in the right uterine horn was induced according to the technique of Tanaka et al.[10]. For each experiment the fetuses in the right uterine horn served as the ischemia group and those in the left horn as the non-ischemia group. After the operation, the animals were extubated and allowed to wake up. After predetermined times of recovery (see above) the animals were re-anesthetized, tracheotomized, and artificially ventilated. After the physiological parameters had been stabilized for at least 5 minutes, the fetuses were delivered by cesarean section. To keep sampling conditions stable, two fetuses were sampled from the middle portion of each uterine horn. In each uterine horn, one fetus, used to measure mitochondrial respiratory function, was decapitated immediately after birth and another fetus, used to measure cerebral energy metabolism, was immersed into liquid nitrogen.

After freezing, brain tissue was chiseled out during intermittent irrigation of the head and the brain with liquid nitrogen for the measurement of cerebral energy metabolism. The forebrain weighing 100～200 mg was removed from the frozen brain, extracted at −20℃ with 1.0 m*l* HCl-methanol (0.1

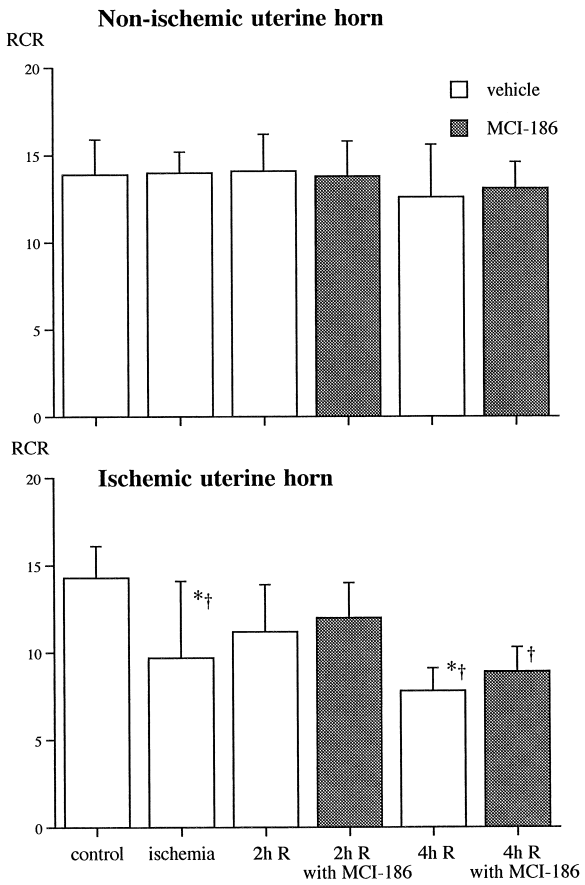

Fig. 1 Changes in respiratory control ratio (RCR) of fetal brain tissue in each group after 30 min of transient intrauterine ischemia or 1, 2, or 4 h of recirculation. Bar indicates the mean value and standard deviation in each group. Significant differences were found among the experimental groups (*$p < 0.001$; against control, one-way ANOVA followed by Scheffé F test; and † $p < 0.05$; against non-ischemia uterine horn, unpaired t test).

M HCl) and then at 0℃ with 4.0 m*l* (0.3 M) perchloric acid, as described elsewhere[1]. The homogenate was centrifuged at 3,000 rpm for 10 min. The supernatant was neutralized with KOH-imidazole (1.5 M KOH, 0.4 M imidazole, 0.3 M KCl). To obtain a complete precipitation of potassium perchlorate, the neutralized supernatant was left in ice water for one hour, and then centrifuged again at 3,000 rpm for 10 min. Fluorometric enzymatic techniques of Lowry et al.[11] were used to measure ATP, ADP, and AMP, under the experimental conditions described.

For the measurement of mitochondrial respiratory activity, the forebrain mitochondria were isolated according to the procedure of Sciamanna et al.[12]. Mitochondrial respiration was measured polarographically with an oxygen microelectrode (Presearch LTD, Hertfordshire, UK) in a closed magnetically stirred chamber of 770 μL capacity, at 28℃ (YSI LTD, Hampshire, UK)[1–3]. Samples (usually 80～100 μL) were added to the reaction buffer (usually 0.65～0.7 mL) containing 100 mM KCl, 75 mM mannitol, 25 mM sucrose, 5 mM Tris-phosphate, 0.05 mM potassium-EDTA, and 10 mM Tris (hydroxymethyl) aminomethane (Trizma Base®, Sigma) (pH 7.4). Substrates consisting of 10 μL of 0.5 M glutamate and 0.5 M malate (neutralized with KOH) were also added. Stimulated (+ADP) respiration was initiated by the addition of 0.1 M ADP (typically, 0.3 μL followed by 5 μL). Non-stimulated (−ADP) respiration was measured from tracings obtained after the ADP added to stimulate respiration was depleted and the rate had declined to a constant value. The respiratory control ratio (RCR), which reflects the coupling of respiration to ATP synthesis, was calculated as the ratio of stimulated to non-stimulated respiration.

In the ischemic uterine horn, the ATP concentration and the sum of adenine nucleotides (ΣAdn) with vehicle treatment decreased to 26% and 55% of non-ischemic controls after 30 minutes of uterine artery occlusion, respectively, but AMP concentrations rose. Recirculation for 2 hours led to a recovery of energy state, but continued reflow (4 hours) was associated with a delayed deterioration of high-energy phosphates (**Table 1**). As shown in **Fig. 1**, in the ischemic uterine horn, the RCR values decreased slightly after 30 minutes of ischemia (not significant). After 2 hours of recirculation, mitochondrial activities were close to the non-ischemic control levels. However, recirculation for 4 hours was associated with a delayed deterioration of the RCR values. The delayed deterioration of both high-energy phosphates and mitochondrial activities was not prevented by MCI-186, given immediately after the start of reperfusion (**Fig. 1**).

Because MCI-186 was demonstrated to cross the placental and blood-brain barriers[13], we speculated that the drug may reduce perinatal problems from fetal hypoxia. In the present circumstances, however, MCI-186 had no protective effect on the

delayed deterioration of both high-energy phosphates and mitochondrial activities following transient intrauterine ischemia in the immature rats. The results are in contrast to those of previous reports in various different experimental models of the adult animals that indicated a beneficial effect of MCI-186 in attenuating brain injury following cerebral ischemia and reperfusion[7–9].

Although there are obvious differences among these studies, including animal species, the methods of inducing ischemia, and the age of animals, the conflicting results may be attributable to differences in the role of hydroxyl radicals and/or superoxide anion in the development of brain damage at an early time period of recirculation between adult and immature animals. However, to our knowledge, the generation of hydroxyl radicals and/or superoxide anion of the present model has not yet been measured. To finally determine the effects of MCI-186 on neurologic outcome following prenatal hypoxic-ischemic events, additional studies with direct measurement of free radical generation will be necessary.

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