Learning Impairment and Microtubule-Associated Protein 2 Decrease in Gerbils Under Chronic Cerebral Hypoperfusion

Takashi Kudo, MD, Kunitoshi Tada, MD, PhD, Masatoshi Takeda, MD, PhD, and Tsuyoshi Nishimura, MD, PhD

A coiled stainless steel wire clip was made that allowed us to chronically reduce cerebral blood flow in Mongolian gerbils. After 6 weeks of reduced cerebral blood flow in 15 experimental gerbils, we evaluated their learning ability and found it to be impaired relative to that in 15 control gerbils. Eight weeks after surgery, regional cerebral blood flow in the parietal cortex measured by the hydrogen clearance method in the experimental gerbils was 73–76% of that in the control gerbils. Light microscopy showed minimal histologic changes in the brains of the experimental gerbils. Concentrations of brain proteins analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis showed that among water-soluble brain proteins, the concentrations of cytoskeletal proteins (microtubule-associated protein 2, calspectin, and clathrin) declined in the experimental gerbils. In particular, the concentration of microtubule-associated protein 2 declined significantly. Our findings show that the reduction of cerebral blood flow via carotid stenosis impairs the learning behavior in gerbils, with an associated decrease in the concentration of microtubule-associated protein 2. We believe that Mongolian gerbils with chronically reduced cerebral blood flow are a useful animal model of chronic brain hypoperfusion. (Stroke 1990;21:1205–1209)

Brain function is highly dependent on cerebral blood supply. The effect of transient brain ischemia on learning behavior and neuronal activity has been studied using four-vessel occlusion. Carotid artery occlusion in Mongolian gerbils (Meriones unguiculatus) has been used extensively to study the natural course of infarction, brain edema, and brain metabolism under several pathologic conditions. The effect on behavior of brain ischemia after transient bilateral carotid artery occlusion has also been reported in Mongolian gerbils. We have reported a decrease in the concentrations of brain proteins (microtubule-associated protein 2 [MAP2], calspectin, and clathrin) after 5, 10, and 15 minutes of bilateral carotid artery occlusion in gerbils.

In most studies with gerbils to date, one or both common carotid arteries were permanently or transiently occluded, either by ligation or by the application of various clasps that completely block carotid artery blood flow. There are, however, few clinical situations in which the carotid arteries are totally occluded. Clinical observations using Doppler flow analysis show a decrease in carotid artery blood flow in patients under several pathologic conditions. These observations indicate that chronic reduction of cerebral blood flow is one important factor causing cerebral dysfunction. To establish an animal model of chronically reduced cerebral blood flow, we used newly invented coiled clips to produce bilateral carotid artery stenosis without total occlusion in gerbils. We believe that this model resembles the cerebral hemodynamics that obtain in clinical situations. We report changes in learning behavior, neuropathologic observations, and changes in the concentrations of cytoskeletal proteins in gerbils following chronic reduction of cerebral blood flow by experimental carotid stenosis.

Materials and Methods

Adult male Mongolian gerbils weighing 60–80 g were purchased from Nippon Dobutsu Co. Ltd,
Osaka, Japan. Fifteen gerbils were anesthetized with 50 mg/kg i.p. pentobarbital, and both common carotid arteries were exposed. Stainless steel wire (diameter 0.1 mm) was shaped into a coil (inside diameter 0.2–0.3 mm, one pitch 1.15 mm, length of the coil 2.5 mm) and aseptically placed around each common carotid artery without producing total occlusion. Before and after placing the coiled clip, proximal blood flow velocity in the common carotid artery was measured using a Doppler blood flow meter. After confirming reduction of the proximal blood flow velocity, the gerbils were allowed to recover for 6 weeks. Fifteen control gerbils also had their carotid arteries exposed, but the arteries were only touched with the coiled clip after anesthesia. It was impossible to distinguish experimental gerbils from the controls by their feeding, grooming, or exploring habits.

Six weeks after surgery, the gerbils were tested using the Osaka University Computerized Electronic Maze to evaluate behavior. Each animal was placed on an insulated board 120x180x10 mm, the neutral area, at the center of an electronic platform to which a 0.5 mA electric shock was applied whenever the gerbil stepped out of the neutral area. The amount and pattern of the animal's behavior were detected by a series of photoelectric beam sensors along the edges of the electronic platform and were recorded for later analysis. The apparatus counts any movement of the gerbil that intersects a photoelectric beam (four- and two-footed positions, grooming, rearing, and jumping) as an activity input. The sum of all inputs is the total locomotor activity (TLA) and is expressed as a percentage of the elapsed time. The neutral area stay time (NST) is automatically calculated as the sum of the periods during which two consecutive activity inputs are recorded within the neutral area and is expressed as a percentage of the TLA. The behavioral parameters TLA and NST were measured every minute for 5 minutes.

Eight weeks after surgery, regional cerebral blood flow (rCBF) was measured using the hydrogen clearance method. The gerbils were anesthetized with 40 mg/kg i.p. pentobarbital. Platinum electrodes were placed into the cerebral cortex bilaterally 2 mm lateral to the bregma. The terminals of the platinum electrodes and silver reference electrodes were connected to a computerized tissue blood flow meter (MHG-D1, Unique Medical Co. Ltd., Osaka, Japan), and the electrode current in the absence of H₂ was adjusted to approximately 0 by applying 0.1–0.3 V to the platinum electrode. After delivery of 20% H₂ in air for 1 minute, the hydrogen clearance rate was recorded and rCBF was calculated following the method of Zierler.

The gerbils were perfused with 10% formaldehyde under anesthesia for neuropathologic study after the rCBF measurements. The brain was excised and fixed in 10% formaldehyde, and coronal serial sections were studied using light microscopy after staining with hematoxylin and eosin.

Thirteen experimental and 13 control gerbils were compared in the protein analysis. The frontal cortex, striatum, and hippocampus were dissected on ice.

**Figure 1.** Recordings of proximal blood flow velocity in right (R) and left (L) common carotid arteries of gerbils by Doppler flowmeter with 10-MHz ultrasound before (PRE) and 5 minutes after (POST) placement of coiled stainless steel wire clip. VPK(Max.), maximum blood flow velocity; VMN, mean blood flow velocity; VPK(Min.), minimum blood flow velocity; HRT, heart rate.
and the tissue was homogenized in four volumes of 10 mM phosphate buffer (pH 7.2) and centrifuged at 100,000g for 30 minutes at 4°C. The supernatant was analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis with a 2–16% gradient and a 5% gel for better separation of MAP2 and calscinetin. The bands of water-soluble proteins were identified using antibodies against MAP2, calscpectin, and clathrin following the method described earlier. The amount of protein in each band was estimated by quantitative densitometry after staining with Coomassie brilliant blue. The percentage protein was calculated by dividing the densitometric value of each protein band by that of the total protein obtained on the 2–16% gel.

We compared the behavioral parameters of the two groups using repeated-measures analysis of variance. We compared rCBF and protein concentrations of the two groups using Student's t test. Results are presented as mean±SD.

**Results**

As shown in Figure 1, 5 minutes after placing the coiled clips on the bilateral common carotid arteries mean proximal blood flow velocity was reduced to 73% of that before placement. Three out of 20 experimental gerbils died during the observation period. Eight weeks after implantation, the coiled clip was examined. The desired diameter of the carotid artery was maintained for as long as 8 weeks, with little reactive tissue response.

Both experimental and control gerbils showed time-dependent significant decreases in TLA during every 1-minute period, with no difference between groups (Figure 2, left). In the control gerbils, NST rapidly approached 100% (Figure 2, right); in the experimental gerbils NST increased significantly more slowly (Table 1). There were significant differences during the 2–3 minute (F=7.07, p=0.010) and the 3–4 minute (F=5.65, p=0.020) periods.

The rCBF in the right and left parietal cortex (19.1±5.4 and 17.8±6.4 ml/100 g/min, respectively) of the experimental gerbils was significantly lower than that of the controls (25.1±6.7 and 24.2±5.6 ml/100 g/min, p<0.02 and p<0.01, respectively).

There was no loss of brain weight and no edema, infarction, or hemorrhage in any brain area examined. Light microscopy of the entire cerebrum of the experimental gerbils showed signs of neither neuronal loss nor gliosis. The pyramidal neurons in the hippocampal CA1 region were preserved, with no apparent neuronal death (Figure 3).

Electrophoresis showed neither addition nor loss of the water-soluble protein bands in the experimental gerbils compared with the controls. Differences in the amount of protein in the bands, however, were

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FIGURE 3. Histologic observation in hippocampal CA1 region of experimental gerbil shows no apparent neuronal loss after 8 weeks of reduced regional cerebral blood flow. Hematoxylin and eosin stain, ×73.

noticed. There was a tendency for the concentrations of MAP2, calspectin, and clathrin to decline in all brain areas of the experimental gerbils. The concentration of MAP2 in particular declined significantly in the frontal cortex and striatum of the experimental gerbils compared with the controls (by 84.1% and 82.6%, respectively; *p*<0.05) (Table 2).

**Discussion**

We undertook this study to establish a more suitable animal model of brain hypoperfusion. The newly invented coiled stainless steel wire clip successfully reduced rCBF as confirmed by Doppler flow analysis immediately after bilateral application of the coiled clip. Although a few gerbils died during the observation period, most experimental gerbils showed no abnormal behavior and no gross neurologic deficits. The morphologic observation 8 weeks after placing the coiled clips revealed little granulation around the operated area and maintenance of the desired degree of carotid stenosis. Thus, the newly invented coiled stainless steel wire clip successfully produced carotid stenosis for up to 8 weeks. rCBF in the experimental gerbils was reduced to 73–75% of that in the controls, indicating chronic reduction of rCBF for 8 weeks.

Behavioral evaluation 6 weeks after surgery was designed to detect differences in overall locomotor activity and learning behavior in the experimental gerbils. Gerbils with reduced rCBF showed no decline in TLA, suggesting no ischemia-induced abnormal activity. However, NST of the experimental gerbils differed significantly from that of the controls. Reduced NST may indicate impaired learning behavior. Several studies have reported disturbances of learning and memory after transient forebrain ischemia.2-4 Bothe et al15 reported that mild cerebral ischemia induced by permanent unilateral carotid artery ligation impaired learning behavior in gerbils. In our study, rCBF was chronically reduced by bilateral carotid artery stenosis instead of by selective ligation of one carotid artery. Our results demonstrate a learning deficit in gerbils with chronically reduced rCBF.

Light microscopy of the brain tissue 8 weeks after surgery showed little loss of neurons and little gliosis in any region studied, including the hippocampal CA1 region. Our previous study showed decreases in the amounts of MAP2, calspectin, and clathrin in the frontal cortex, striatum, and hippocampus following

| Table 2. Differences in Concentrations of MAP2, Calspectin, and Clathrin After 8 Weeks of Reduced Regional Cerebral Blood Flow in Gerbils |
|-----------------|---|---|---|---|---|---|---|
| Brain area      | MAP2 | Calspectin | Clathrin |
| Cortex          | 0.82±0.15 | 0.69±0.16* | 0.91±0.21 | 0.80±0.065 | 0.89±0.19 | 0.82±0.19 |
| Striatum        | 0.46±0.12 | 0.38±0.079* | 0.66±0.20 | 0.55±0.15 | 0.82±0.32 | 0.77±0.12 |
| Hippocampus     | 0.77±0.18 | 0.73±0.18 | 0.85±0.14 | 0.78±0.12 | 0.81±0.23 | 0.74±0.14 |

MAP2, microtubule-associated protein 2; C, control group (n=13); E, experimental group (n=13). Data are mean±SD.

*p*<0.05 different from C by Student’s *t* test.
transient cerebral ischemia induced by bilateral carotid artery occlusion in gerbils.\textsuperscript{10,11} In our present study chronic reduction of rCBF produced a tendency for the concentrations of these same proteins to decline in several brain areas; MAP2 declined significantly in the frontal cortex and striatum. This supports the hypothesis that a similar ischemic pathologic process results from both chronically reduced rCBF and acute ischemia.

MAP2 has been shown to stimulate microtubule assembly\textsuperscript{16,17} and to interact with other cytoskeletal proteins such as neurofilaments\textsuperscript{18,19} and actin filaments.\textsuperscript{17,20} MAP2 may modulate the interaction between microtubules and other filaments.\textsuperscript{21} It is very likely that a decrease in the concentration of MAP2 causes abnormal interactions of cytoskeletal proteins and/or the abnormal assembly of microtubules. This in turn may cause neuronal dysfunction, resulting in impaired learning behavior. Though the cellular mechanism by which the concentration of MAP2 selectively declines in specific areas of the chronically hypoperfused brain remains to be clarified, our present results indicate that the loss of MAP2 is closely related to neuronal dysfunction in the hypoperfused brain. We believe that gerbils with chronically reduced rCBF can be a useful animal model to help us understand the pathology of chronic brain hypoperfusion.

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