Neuropathologic Changes in the Gerbil Brain After Chronic Hypoperfusion

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Background and Purpose: An animal model has been developed to elucidate the pathological changes in brain cytoskeletal proteins during chronic hypoperfusion.

Methods: Newly designed coiled clips were placed around both carotid arteries of Mongolian gerbils (n=10) to cause stenosis without occlusion. Those gerbils showing impaired learning ability by the passive avoidance paradigm were killed for neuropathologic study after 12 weeks.

Results: The brains showed ventricular dilatation, cortical atrophy, and rarefaction of the white matter. Immunoreactivity to anti-microtubule-associated protein 2 antibody in the cerebral cortex and the hippocampus was diminished, indicating dendritic changes of neurons. In the thalamic axonal regions, staining with anti-neurofilament 200K protein antibody was increased, suggesting increased amounts of neurofilament proteins or increased phosphorylation of the protein. Increased immunoreactivity to anti-glial fibrillary acidic protein antibody was observed in a wedge-shaped configuration, corresponding to the border zone of perfusion by small vessels.

Conclusions: These findings suggest that changes in the cytoskeletal proteins in dendrites, axons, and glial cells may cause neuronal death under conditions of chronic cerebral hypoperfusion. (Stroke 1993;24:259–265)

KEY WORDS • cerebral ischemia • hypoperfusion • white matter • gerbils

Clinical observations indicate that chronic cerebral hypoperfusion is one of the important factors associated with cerebral dysfunction. Reduced cerebral blood flow (CBF) occurs in multi-infarct dementia, Alzheimer’s disease, and other pathological conditions causing dementia. A suitable animal model is needed to investigate the pathophysiology of cerebral hypoperfusion and to develop potential therapies. We previously described a method of producing cerebral hypoperfusion over 8 weeks by carotid stenosis in Mongolian gerbils using coiled clips. Measurement of regional CBF by the hydrogen clearance technique under anesthesia with 40 mg/kg i.p. pentobarbital showed a 25% reduction in CBF compared with sham-operated animals. Behavioral studies showed that the learning ability of the experimental animals was significantly impaired as measured by the step-down passive avoidance paradigm. Neuropathologic investigations in the previous study, however, revealed few signs of neuronal loss and gliosis. The pyramidal neurons in the hippocampus were preserved, with no apparent neuronal death. There was a tendency for the concentration of microtubule-associated protein 2 (MAP2) to decline in hypoperfused gerbil brain.

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Here we report neuropathologic brain damage due to chronic cerebral hypoperfusion. To accentuate the effect of hypoperfusion, we used a coiled clip with narrower dimensions than the one previously used1 to produce greater stenosis, and we lengthened the period of stenosis from 8 to 12 weeks.

Materials and Methods

The coiled clip (i.d., 0.25 mm; one pitch, 0.70 mm; length of coil, 2.5 mm) was made of stainless steel wire (diameter, 0.1 mm). The new coiled clip’s pitch was reduced from 1.15 mm, with the same inside diameter and total length of the coil as previously reported.1 The pitch was reduced because the present experiment was designed to produce more severe stenosis of the carotid artery than the experiment previously reported; this more severe stenosis could possibly reproduce any brain tissue changes detectable by immunohistochemical staining and electron microscopy (EM).

Surgery was performed in two steps. Adult Mongolian gerbils (n=10) weighing 60–80 g (Nippon Dobutsu Co. Ltd., Osaka, Japan) were anesthetized with 70 mg/kg i.p. pentobarbital (Abbott Laboratories, North Chicago, Ill.). One of the common carotid arteries was exposed, and the coiled clip was aseptically placed around it without occlusion. After 1 week, a second coiled clip was placed around the other common carotid artery. In the control group (n=10), the common carotid arteries were only touched by the clips as part of the sham operation, and a second sham operation was done 1 week later. The animals were kept under conditions of controlled temperature and humidity with free access to food and water.
After 12 weeks, learning ability was studied by using the step-down passive avoidance paradigm. A gerbil was placed on an insulated board 120×180×10 mm (the neutral area) at the center of an electric 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 and recorded for later analysis. The sum of all activities was the total locomotive activity (TLA), and the neutral area stay time was calculated as the sum of the periods during which two consecutive inputs were recorded within the neutral area and was expressed as a percentage of TLA. The behavioral parameters were measured every minute for 5 minutes.

For histological evaluation of the brain and carotid arteries, experimental (n=10) and control (n=5) gerbils were anesthetized and then perfused transcardially with isotonic saline followed by 10% formaldehyde in 0.1 M phosphate buffer (pH 7.4). The brain and carotid arteries with the clips were removed and immersed in the same fixative for 1 week until they were embedded in paraffin. The carotid arteries were studied by using hematoxylin and eosin and van Gieson’s stains. Six-micrometer coronal sections of the forebrain were stained with hematoxylin and eosin and with antibodies against MAP2, neurofilament 200K protein (NF200K), and glial fibrillary acidic protein (GFAP). The sections were washed with 0.01 M phosphate buffer/0.9% NaCl (pH 7.2) followed by blocking for 1 hour with 10% normal goat serum in phosphate-buffered saline. The tissue was incubated overnight at 4°C with anti-MAP2 antibody (Amersham Corp., Arlington Heights, Ill.) diluted 1:500, anti-NF200K antibody (Amersham) diluted 1:200, or anti-GFAP antibody (Labsystem) diluted 1:200 with 10% normal goat serum in phosphate-buffered saline. The sections were incubated with biotinylated anti-mouse IgG (Vector Laboratories Inc., Burlingame, Calif.) at a dilution of 1:100 in phosphate-buffered saline for 30 minutes at room temperature, followed by incubation with avidin-biotinylated horse-radish peroxidase complex (Vectastain ABC reagent, Vector) at room temperature for 1 hour. The peroxidase reaction was developed with 2–5 minutes with 40 mg 3,3′-diaminobenzidine tetrahydrochloride and 0.03% hydrogen peroxide in 100 ml phosphate-buffered saline. Cell nuclei were visualized by counterstaining with Mayer’s hematoxylin. Sections were washed three times with phosphate-buffered saline for 5 minutes between steps.

For EM, the gerbils were anesthetized and perfused transcardially with isotonic saline followed by a mixture of 1% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed, and tissue blocks were postfixed in 2% osmium tetroxide, dehydrated in ascending concentrations of alcohol, treated with propylene oxide, and embedded in Epon.

**Results**

The gerbils were studied for passive avoidance learning ability 12 weeks after the initiation of bilateral carotid artery stenosis. The learning ability of chronically hypoperfused gerbils was significantly impaired compared with that of control gerbils (p<0.01, Figure 1).

The carotid arteries were apparently normal by hematoxylin and eosin and van Gieson’s staining. There was no indication of injury to the laminar structural architecture throughout the entire layer of the arteries. The lining of the endothelium was intact, showing no injury or intravascular coagulation.

Various degrees of ischemic brain lesions were observed. Although the number and size of ischemic lesions differed among individual animals, lesions were observed in half of the gerbils (five of 10). The number of animals showing ischemic lesions in the hippocampus, cerebral cortex, basal ganglia, and cerebral white matter were 5, 2, 2, and 5, respectively. The brains of gerbils with and without ischemic lesions were studied by immunohistochemical staining. All animals showed white matter rarefaction and some degree of ventricular dilatation. In the cerebral white matter two types of lesions were observed, one type similar to that seen in the gray matter and the other observed only in the white matter after a long duration of hypoperfusion.

The most severely damaged brain had dilated ventricles, severe cortical atrophy, and rarefaction of the white matter. A typical gerbil brain after 12 weeks of hypoperfusion (Figure 2A) showed patchy areas of neuronal degeneration in the cortex and hippocampus as well as rarefaction of the white matter. A higher magnification of the cortex (Figure 2B) showed patchy neuronal loss and dark neurons. Degenerative neuronal changes were observed in the basal ganglia, hippocampus, and cerebral cortex.

VARING degrees of white matter rarefaction were observed (Figure 2C). Degenerative changes in the white matter were not obvious until after 4 weeks but became increasingly evident by 12 weeks. In contrast, gray matter changes were observed equally throughout the experimental period. Cumulative white matter changes were studied after 1, 4, 8, and 12 weeks of hypoperfusion, and a more detailed description of the white matter changes is reported elsewhere.

![FIGURE 1. Plot of learning ability of gerbils after 12 weeks of chronic cerebral hypoperfusion evaluated by passive avoidance paradigm. Percent of time spent on neutral area (NST) was calculated for 5 minutes. Hypoperfused gerbils (n=10, ○) showed significantly impaired learning compared with sham-operated gerbils (n=10, △). Difference was significant by Student’s t test (p<0.01) at 1–2 minutes and at 2–3 minutes.](image-url)
The brains were immunostained with antibodies against MAP2, NF200K, and GFAP. Areas where neurons were preserved as indicated by hematoxylin and eosin staining were studied by immunostaining. Anti-MAP2 antibody immunostaining in control (Figure 3A) and hypoperfused (Figure 3B) cortices were compared. Control cortices were positively stained by anti-MAP2 antibody, reflecting the dendritic processes of neurons. Immunoreactivity to anti-MAP2 antibody was significantly diminished in hypoperfused cortices (Figure 3B). Decreased immunoreactivity was observed in larger areas of the cortex than in areas of patchy neuronal loss. Decreased immunoreactivity was more clearly shown in the hippocampus of hypoperfused gerbils (Figure 3C) than in the hippocampus of control gerbils (Figure 3D). It was previously shown that the decrease in anti-MAP2 antibody immunoreactivity is one of the earliest changes observed in gerbil brain after transient ischemia.

Anti-NF200K antibody immunostaining in the thalamus of control (Figure 4A) and hypoperfused (Figure 4B) gerbils revealed increased staining in the latter. Increased immunoreactivity to anti-NF200K antibody was observed in almost all cortical areas, most clearly in the thalamus.

Anti-GFAP antibody immunostaining revealed increased immunoreactivity in several cortical areas in hypoperfused gerbils (Figure 5), while no staining was observed in the brains of control animals. The distribution of increased immunoreactivity to anti-GFAP antibody was wedge-shaped (Figure 5).

EM showed swelling and degeneration of apical dendrites as well as proliferation of glial filaments in the cortex (Figure 6A). Myelin sheaths were degenerated and glial fiber proliferation was observed in astrocytes (Figure 6B). Glial fiber proliferation was observed in the perivascular areas, and the stratified proliferation of endoplasmic reticulum was also noticed (Figure 6C).

**Discussion**

The neuropathology of chronic cerebral hypoperfusion has not been previously reported because of the lack of a suitable animal model. We have developed such a model in gerbils whose carotid arteries were stenosed to produce a CBF of about 75% of normal. In the present study we produced cerebral hypoperfusion of greater severity than previously reported and studied the neuropathologic alterations after 12 weeks of chronic brain hypoperfusion.

Varying degrees of cortical atrophy, ventricular dilatation, and rarefaction of the white matter with or without obvious ischemic foci were seen. Areas of patchy neuronal loss were demonstrated in cerebral cortical layers and the hippocampus, frequently accompanied by gliosis. In addition to gray matter damage, degenerative changes in the white matter were also seen. These changes were not seen before 4 weeks of hypoperfusion, but their frequency increased over 12 weeks. The time-related changes in the white matter are described elsewhere. The incremental progression of degenerative white matter changes indicates that the neuropathology of chronic cerebral hypoperfusion is different from that of acute ischemia. Immunohistochemical studies indicate that changes in cytoskeletal proteins in the gerbil brain occur during chronic hypoperfusion. Anti-MAP2 antibody immunoreactivity was markedly diminished in hypoperfused cortex, even in areas where little neuronal loss was observed. Loss of immunoreactivity to anti-MAP2 antibody is one of the earliest events occurring during cerebral ischemia in gerbils and rats. Changes in anti-MAP2 antibody immunoreactivity in the hippocampus after transient ischemia, however, may not be directly related to delayed neuronal death. Since MAP2 is localized in neuronal dendrites, the results suggest that dendritic changes in cortical neurons occur during chronic hypoperfusion, which is supported by EM observations of swelling and degeneration of apical dendrites in cortical neurons.

Immunostaining with anti-NF200K antibody increased in the thalamic areas of hypoperfused gerbils. NF200K is primarily localized in axons, suggesting involvement of neuronal axons. Since the antibody used in this experiment reacts with the phosphorylated epitope on NF200K, the results indicate either overphosphorylation of NF200K or increased total amounts of NF200K. Abnormal phosphorylation of neurofilament proteins occurs in Alzheimer’s disease, aluminum intoxication, and aging. Chronic hypoperfusion may also induce overphosphorylation of neurofilament proteins, but further study is needed to resolve this issue.

Reactive astroglia were present in many areas of hypoperfused brain by EM and immunohistochemistry. Reactive astroglia were well stained by anti-GFAP antibody in a wedge-shaped configuration in cortical layers. The distribution of the increased numbers of glial fibers corresponds to the border zone of small
vessels in the brain. Combined with the results of EM, we speculate that chronically reduced CBF through capillaries triggers astrocyte proliferation.

The observed changes in cytoskeletal proteins are not unique to the present model. It is well documented that neurofilament accumulates in neuronal perikarya of the lower brain stem nuclei in aluminum intoxication. We recently reported decreased immunoreactivity to anti-MAP2 antibody in rabbit brain in chronic aluminum intoxication and speculated that dislocation of MAP2 is a phenomenon accompanying the production of experimental neurofibrillary changes in the chronically aluminum-intoxicated rabbit brain. Even though the etiologic triggers are quite different between these two experimental models, similarities in the changes in immunoreactivity suggest that the decrease in anti-

FIGURE 3. Photomicrographs. Anti-microtubule-associated protein 2 (MAP2) antibody clearly stains dendrites of cortical neurons in cortex from control gerbil (A), but staining was diminished in cortex from hypoperfused gerbil (B); x86. Anti-MAP2 staining of hippocampus of control gerbil (C) was significantly greater than staining in hippocampus of hypoperfused gerbil (D); x86.
MAP2 antibody immunoreactivity and the increase in anti-NF200K antibody immunoreactivity represent the chronic state of dysfunction in which neurons are exposed to severe stress, eventually causing neuronal degeneration.

It remains to be clarified which protein changes in the brain occur first after hypoperfusion. Since loss of anti-MAP2 antibody immunoreactivity is observed early after an acute ischemic insult without an immediate increase in neurofilament immunogenicity, we speculate that loss of anti-MAP2 antibody immunoreactivity is the initial event. It is only after prolonged hypoperfusion that an increase in anti-NF200K antibody immunoreactivity occurs.

Glial cells have many functions, including acting as buffers for ions released by neurons during electrical

**FIGURE 4.** Photomicrographs. Immunostaining with anti-neurofilament 200K protein antibody in thalamus of control gerbil (A) was less than staining in thalamus of hypoperfused gerbils (B and C); ×80.

**FIGURE 5.** Photomicrograph of immunohistochemical staining with anti-glial fibrillary acidic protein antibody in cortex of hypoperfused gerbil. Increased immunoreactivity is distributed in wedged-shape configuration; ×34.
activity,\textsuperscript{15} reuptake of excitatory amino acids,\textsuperscript{16} storage of glutamine synthetase\textsuperscript{17} and glycogen,\textsuperscript{18} and release of neurotrophic factors that support neuronal function and survival.\textsuperscript{19} The protective influence of astrocytes on neurons under anoxic conditions has been suggested.\textsuperscript{20} We speculate that astrocytes surrounding capillaries increase in reactivity during chronic cerebral hypoperfusion to increase neuronal support. Glial cell partici-

pation in response to chronic hypoperfusion deserves further investigation.

Our findings show that the bilateral carotid artery coiled clip stenosis model can be used to elucidate the pathological processes occurring in chronic cerebral hypoperfusion and that changes in the cytoskeletal proteins of dendrites, axons, and astrocytes are observed before neuronal death in this model.

References

16. Drejor J, Larsson OM, Schousboe A: Characterization of l-gluta-
mate uptake into and release from astrocytes and neurons cultured from different brain regions. Exp Brain Res 1982;47:259–269
17. Noremburg MD, Martinez-Hernandes A: Fine structural localiza-