

White Matter Lesions and Glial Activation in a Novel Mouse Model of Chronic Cerebral Hypoperfusion

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Background and Purpose—Cerebrovascular white matter (WM) lesions are closely associated with cognitive impairment and gait disorders in the elderly. We have successfully established a mouse model of chronic cerebral hypoperfusion that may provide new strategies for the molecular analysis of cerebrovascular WM lesions.

Methods—Adult C57Bl/6 male mice were subjected to bilateral common carotid artery stenosis (BCAS) using external microcoils with varying inner diameters from 0.16 to 0.22 mm. Cerebral blood flow (CBF) in the frontal cortices was measured by laser-Doppler flowmetry at 2 hours and at 1, 3, 7, 14, and 30 days after BCAS. The brains were then removed and examined at 30 days with histological stains and immunohistochemistry for markers of microglia and astroglia.

Results—At 2 hours, the CBF values (ratio to the preoperative value) did not change in the 0.22 mm group but decreased significantly to $77.3 \pm 13.4\%$ in the 0.20 mm group, $67.3 \pm 18.5\%$ in the 0.18 mm group, and $51.4 \pm 11.5\%$ in the 0.16 mm group. At day 1, the CBF began to recover in all groups but remained significantly lower until 14 days in comparison to the control group. In the 0.20 mm and 0.18 mm groups, WM lesions occurred after 14 days without any gray matter involvement. These lesions were the most intense in the corpus callosum adjacent to the lateral ventricle but were mild in the anterior commissure and optic tract. In contrast, 4 of 5 mice developed some gray matter changes in the 0.16 mm group. The proliferation of activated microglia and astroglia was observed in the WM beyond 3 days after BCAS.

Conclusions—WM lesions were successfully induced after chronic cerebral hypoperfusion with relative preservation of the visual pathway. These features in this mouse model are appropriate for cognitive assessment and genetic analysis, and it may provide a powerful tool to understand the pathophysiology of WM lesions. (*Stroke*. 2004;35:2598-2603.)

Key Words: brain ischemia ■ disease models, animal ■ mice ■ microglia ■ white matter

Cerebrovascular white matter (WM) lesions are observed in aging and stroke and constitute the core pathology of Binswanger disease, a form of subcortical vascular dementia. These WM lesions are believed to be responsible for cognitive impairment and are caused by chronic cerebral hypoperfusion.¹ The neuropathological changes in these lesions are characterized by diffuse demyelination, the loss of the axons, and gliosis,^{2,3} but the process leading to these changes remains unclear.

Nonhuman primates appear to represent the best model for the study of WM lesions, because they have well-developed WM and vascular architectures which closely resemble those in human brains. Nevertheless, most experiments studying chronic cerebral hypoperfusion have been performed in rodents because of the ease of handling and higher acceptability from an ethical viewpoint. Chronic cerebral hypoperfusion in the rat and gerbil is induced by the ligation or stenosis of the bilateral common carotid arteries (CCAs). These animals are characterized by pathological changes, such as WM rarefaction,^{4,5} which appear very similar to those in human cerebrovascular WM lesions.

The rat model of chronic cerebral hypoperfusion is accompanied by cognitive impairment and cholinergic deficits⁵⁻⁷ and is used most widely. However, this model also has some drawbacks. For example, the visual pathway is injured by the occlusion of the ophthalmic arteries, and thus may compromise behavioral assessment. Furthermore, genetic studies are hampered because of limited accessibility to molecular technologies using knockout or transgenic animals. We report a mouse model of chronic cerebral hypoperfusion, which is subjected to various degrees of cerebral blood flow (CBF) reduction by narrowing the bilateral CCAs with newly designed microcoils and demonstrates good reproducibility for the WM lesions and glial activation.

Materials and Methods

Animals

All procedures were performed in accordance with the guidelines for animal experimentation from the ethical committee of Kyoto University. Ten-week-old male C57Bl/6 mice (weighing 24 to 29 g; Japan SLC, Hamamatsu) were given free access to food and water ad libitum.

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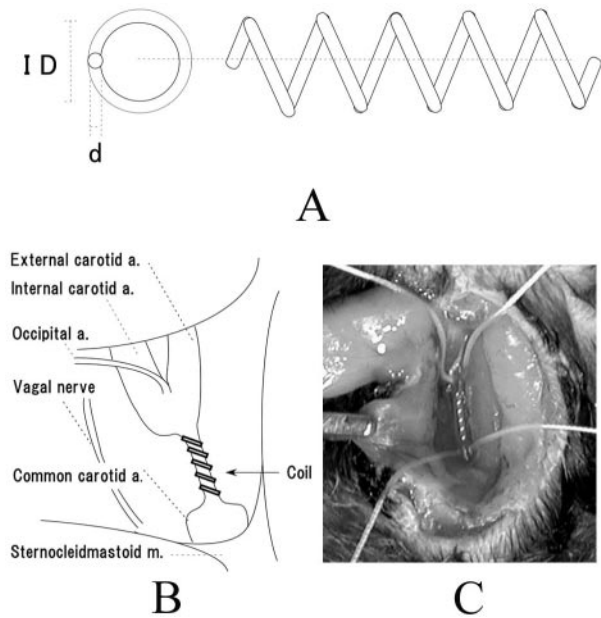


Figure 1. The microcoils (A) and the procedure for BCAS (B and C). A microcoil made of piano wire (d; wire diameter of 0.08 mm) with an inner diameter of 0.18 mm, pitch 0.50 mm, and total length 2.5 mm was twined by rotating it around the CCA just below the level of the carotid bifurcation.

Coil Preparation and CBF Measurement

Four types of microcoils were constructed in collaboration with Sawane Spring Co. They were made of piano wire with varying inner diameters (ID) from 0.16 mm to 0.22 mm (Figure 1A).

Under deep anesthesia with sodium pentobarbital (50 mg/kg, intraperitoneal), the skin overlying the right skull was reflected. A plastic guide cannula (outer diameter 3 mm, ID 2 mm, and length 4 mm) for a laser-Doppler flowmetry probe was fixed perpendicularly to the skull at 1 mm posterior and 2.5 mm lateral to the bregma using dental resin. The CBF was recorded by placing a 2.0 mm straight probe (OmegaFLO-N1, Neuroscience Inc) through the guide cannula. The baseline CBF recordings were obtained just before and at 2 hours and 1, 3, 7, 14, and 30 days after the surgery. The CBF values were expressed as a percentage of the baseline value.

Surgical Procedure of Bilateral Common Carotid Artery Stenosis

After a recovery period of 3 days, the mice were anesthetized with sodium pentobarbital. Through a midline cervical incision, both CCAs were exposed and freed from their sheaths. Two 4–0 silk sutures were placed around the distal and proximal parts of the right CCA. Then, the artery was gently lifted by these sutures and placed between the loops of the microcoil just below the carotid bifurcation. The microcoil was twined by rotating it around the CCA. After 30 minutes, another microcoil of the same size was twined around the left CCA (Figure 1B and 1C). The rectal temperature was maintained between 36.5°C and 37.5°C. The cessation of CBF for >1 minute was avoided. The diameters of the CCAs were measured under direct inspection with the operation microscope (Model OMK2, Olympus Optical Co Ltd) just before applying the microcoils.

The experimental animals were divided into 4 groups which received 0.22 (n=8), 0.20 (n=12), 0.18 (n=13), or 0.16 mm microcoils (n=20). Those in the control group (n=7) were given a sham operation, during which their CCAs were exposed. These animals were euthanized at 30 days after bilateral common carotid artery stenosis (BCAS). The body weight and mean arterial blood pressure (MABP), measured by the tail-cuff plethysmographic method, were monitored until euthanized.

Another set of animals was subjected to BCAS with either 0.20 or 0.18 mm microcoils and was euthanized immediately after BCAS or after 3, 7, 14, or 30 days to determine the temporal profile of the histological changes. Seven animals were examined in the 0.20 and 0.18 mm microcoil groups, and 5 sham-operated animals in the control group were prepared for each postoperative period.

Histochemical Evaluation of WM Lesions and Glial Activation

After BCAS, the mice were deeply anesthetized with sodium pentobarbital and perfused transcardially with 0.01 mol/L PBS, then with a fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 mol/L phosphate buffer (pH 7.4), and stored in 20% sucrose in 0.1 mol/L PBS (pH 7.4). The brains were embedded in paraffin and sliced into 2 μm-thick coronal sections. Klüver-Barrera (KB) and hematoxylin and eosin stains were used to observe any histological changes. The severity of the WM lesions was graded as normal (grade 0), disarrangement of the nerve fibers (grade 1), the formation of marked vacuoles (grade 2), and the disappearance of myelinated fibers (grade 3) as described elsewhere.⁵

For immunohistochemistry, the rest of the coronal blocks were cut into serial sections (20 μm thick) in a cryostat and incubated overnight with a rabbit anti–glial fibrillary acidic protein (GFAP) antibody (diluted 1: 10 000; DAKO, Denmark) or a rat anti-mouse major histocompatibility complex (MHC) class II antigen antibody (diluted 1:10 000; BD Biosciences, Pharmingen, San Diego, Calif.). Subsequently, these sections were treated with the appropriate biotinylated secondary antibodies (diluted 1:200; Vector Laboratories, Burlingame, Calif) and were visualized with 0.01% diaminobenzidine tetrahydrochloride and 0.005% H₂O₂ in 50 mmol/L Tris HCl (pH 7.6). The WM lesions were evaluated in 5 regions: the optic tract, internal capsule, fiber bundles of the caudoputamen, corpus callosum, and anterior commissure. We counted the numerical density of the glial cell nuclei with immunopositive perikarya in a 0.125 mm² area in 4 regions: the optic tract, internal capsule, fiber bundles of the caudoputamen, and corpus callosum in 7 animals from each group.

Statistical Analysis

All data were presented as mean±SD. One factorial ANOVA was used to compare the physiological parameters. The body weight, MABP, and CBF values were analyzed by repeated measure ANOVA followed by a post hoc Dunnett test. A Mann–Whitney U test was used to compare the severity of WM lesions between the groups. The nonparametric Spearman rank correlation was used to test for the relationship between the CBF and WM lesions. P<0.05 was considered to be statistically significant.

Results

Mortality Rates and Neurological Deficits in the Experimental Groups

All procedures for BCAS were accomplished within 15 minutes (except an interval for 30 minutes). The preoperative body weight and diameter of the distal CCA did not differ significantly among the experimental groups (data not shown). MABP of the surviving mice did not change significantly at any postoperative intervals until 30 days, compared with the sham-operated controls. The mortality rates were 13% (1/8) in the 0.22 mm group, 17% (2/12) in the 0.20 mm group, and 15% (2/13) in the 0.18 mm group (Figure 2A). In contrast, 75% (15/20) in the 0.16 mm group died within 14 days after the surgery, most of which was found to have cerebral infarctions. In the sham-operated controls, all of the mice survived until euthanized. In the second set of experiments to clarify the time course, 18% (6/34) in the 0.20 mm

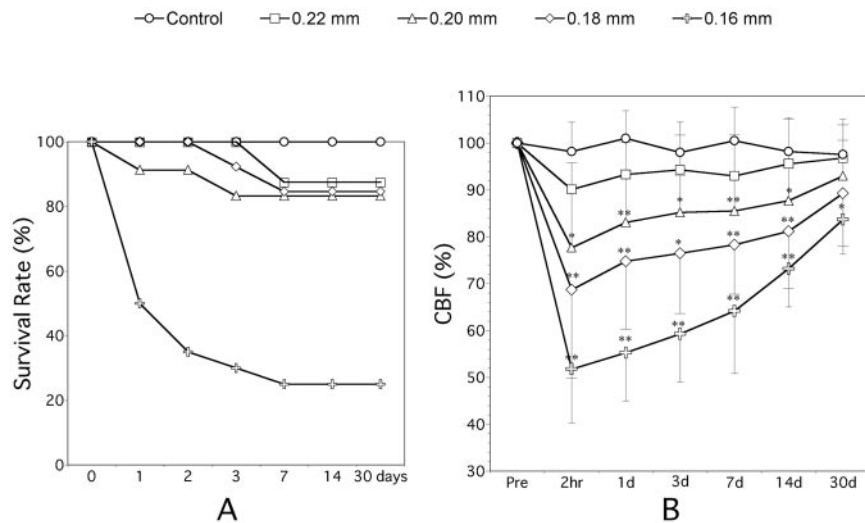


Figure 2. Survival rates (A) and CBF values (B) in each group after BCAS. The data represent mean \pm SD expressed as a percentage of the preoperative value (B). * $P < 0.05$, ** $P < 0.01$ compared with the control by Dunnett multiple comparison test.

group and 13% (4/32) in the 0.18 mm group died after the surgery.

In the 0.22, 0.20, and 0.18 mm groups, all of the animals regained consciousness within a few hours but occasionally showed transient ptosis. None of them showed any apparent motor weakness. In contrast, in the 0.16 mm group, 35% (7/20) did not regain consciousness, 30% (6/20) showed rolling or circling movements lasting 2 to 6 hours after awakening, and 20% (4/20) showed severe akinesia with a squatting posture.

The body weight decreased after the surgery but recovered to the baseline by \approx day 7 in the control, 0.22, 0.20, and 0.18 mm groups. Although the mice in the 0.22, 0.20, and 0.18 mm groups tended to have a lower body weight than in the control group, no significant difference was noted at any postoperative interval. In contrast, the mice in the 0.16 mm group showed a significantly lower body weight at all postoperative intervals, as compared with the control group.

Changes of CBF in the Experimental Groups

Figure 2B shows the mean CBF values in the surviving mice; 7 of 7 in the control group, 7 of 8 in the 0.22 mm group, 10 of 12 in the 0.20 mm group, 11 of 13 in the 0.18 mm group, and 5 of 20 in the 0.16 mm group. The CBF values in the nonsurviving mice were excluded, because these mice showed extremely low CBF values caused by systemic hypotension.

In the control group, the mean CBF after the sham operation varied from 96.0% to 103.2%, without any significant changes between any time intervals (1 factorial ANOVA, $P > 0.2$). In contrast, the CBF values decreased significantly from the preoperative baseline after the surgery in the 0.20, 0.18, and 0.16 mm groups. At 2 hours, there was a significant reduction of the CBF values to $77.3 \pm 13.4\%$ (mean \pm SD) in the 0.20 mm group, $67.3 \pm 18.5\%$ in the 0.18 mm group, and $51.4 \pm 11.5\%$ in the 0.16 mm group. On day 1, the CBF values began to recover but remained significantly lower in all groups until 14 days, as compared with the control group. At 30 days, the CBF values were still decreased in the 0.16 mm group. Intergroup differences in the

CBF values were detected between the 0.16 mm and other groups, but there were no differences among the 0.22, 0.20, and 0.18 mm groups.

Histological Changes at 30 Days After BCAS

There were no infarctions or hemorrhage in any gray matter regions in the 0.22, 0.20, and 0.18 mm groups. In contrast, 4 of the 5 surviving mice with 0.16 mm microcoils showed focal histological changes such as ischemic neurons or foci of glial cell infiltrates in the cerebral cortex, hippocampus, or basal ganglia. There were microinfarcts in the parietal cortices in 3 of 5 mice (Figure 3A and 3B), neuronal loss in the CA1 subfield of the hippocampus in 4 of 5 mice (Figure 3C and 3D), and patchy necrotic lesions in the caudoputamen in 3 of 5 mice.

In the 0.20 and 0.18 mm groups, the staining intensity of the myelinated fibers was reduced, and the integrity of the myelin was compromised in the corpus callosum (Figure 3E and 3G), caudoputamen (Figure 3F and 3H), internal capsule, and optic tract. The remaining fibers were disorganized, and vacuoles were frequently observed in the neuropil (Figure 3G and 3H). With respect to their distribution, WM lesions were the most intense in the medial part of the corpus callosum adjacent to the lateral ventricles; were moderate in the paramedian part of the corpus callosum, fiber bundles of the caudoputamen, and the internal capsule; and were less severe in the anterior commissure and the optic tract (Table 1). There was a significant negative correlation between the CBF at any time point after BCAS and the grading scores of WM lesions at 30 days (Spearman rank correlation $r = -0.69$ to -0.90 , $P < 0.001$). Thus, the lower CBF appeared to result in the more severe WM injuries.

Temporal Profile of WM Lesions and Glial Activation

In the second set of experiments with 0.20 mm or 0.18 mm microcoils, the temporal profile of the WM lesions was examined. WM lesions were not detected in any regions of the brain after 3 and 7 days of BCAS. After 14 days, the WM

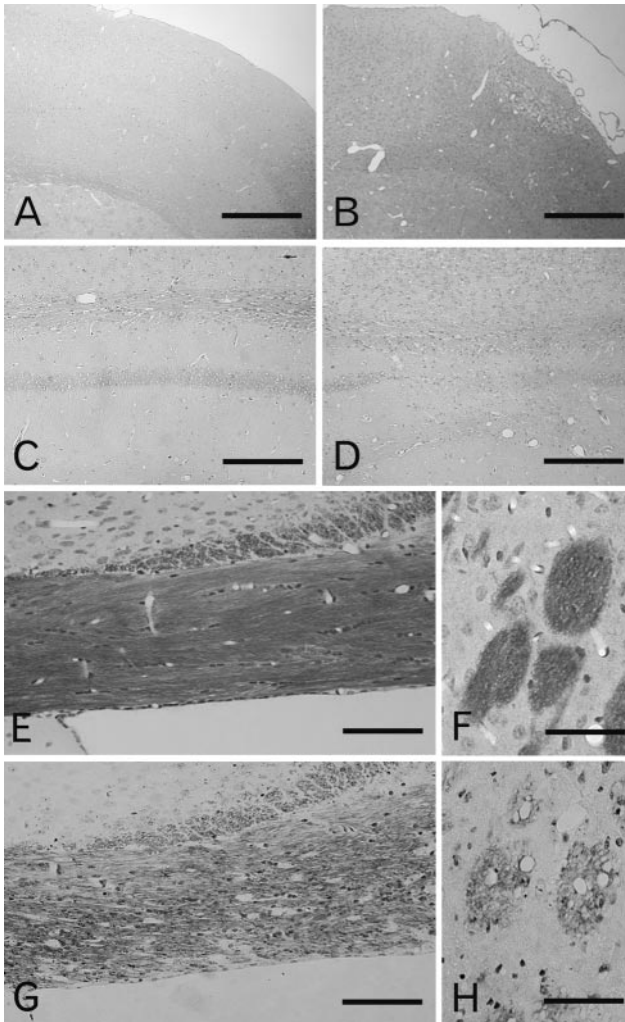


Figure 3. Photomicrographs of the hematoxylin and eosin (A through D) and KB staining (E through H) in the parietal cortex (A and B), CA1 subfield of the hippocampus (C and D), the corpus callosum (E and F), and the fiber bundles of the caudoputamen (G and H). Neuronal necrosis was observed in the 0.16 mm group (B and D) in comparison with the 0.18 mm group (A and C). There was rarefaction of the myelin and vacuole formation in the 0.18 mm group (G and H). These changes were not found in the control group 30 days after a sham operation (E and F). Scale bar, 500 μ m (A and B), 250 μ m (C and D), 100 μ m (E and G), and 50 μ m (F and H).

lesions were evaluated as grade 0 or 1 in the medial part of the corpus callosum, caudoputamen, and the internal capsule, but after 30 days, severe rarefaction occurred in these regions

(Figures 4A, 4D, 4G, and 5A). In contrast, the WM lesions in the optic tract did not emerge until 30 days (Figure 5A).

In each region of the WM, the numerical densities of the microglia/macrophages immunolabeled for MHC class II antigen increased significantly from 7 to 30 days after BCAS (Figures 4B, 4E, 4H, and 5B), whereas astroglia immunolabeled for GFAP increased from 14 to 30 days (Figures 4C, 4F, 4I, and 5C). The regions with intense glial activation corresponded to those with a greater loss of WM myelin.

Discussion

By using 0.20 or 0.18 mm microcoils, BCAS successfully induced moderate cerebral hypoperfusion and cumulative WM lesions but without any histological abnormalities in the gray matter. Postmortem study showed that the implanted microcoil maintained its diameter and the CCAs remained patent with wall thickening (Figure I available online only at <http://www.strokeaha.org>). Although there was no significant difference in the severity of WM lesions and the CBF values between the 0.20 and 0.18 mm groups, ID 0.18 mm microcoil is recommended because of its high reproducibility.

In a rat model of chronic cerebral hypoperfusion, the CBF is reduced to between 25% to 87% of the baseline values immediately after ligation of the bilateral CCAs. Between 14 and 30 days postligation, the CBF values remain at the ranges between 66% to 77%.^{8,9} The CBF of the WM is lower than that of the gray matter but changes in parallel.⁸ This model is most widely used in studies of chronic cerebral hypoperfusion, but damage to the visual pathway and the occasional involvement of the cerebral cortices may compromise cognitive assessment.⁷ On the contrary, the mouse model shows no damage to the gray matter structures and little damage to the visual pathway, because BCAS in mice induces a milder decrease in the CBF than in the rat model and maintains a residual blood flow within the CCAs and its branches, such as the ophthalmic arteries (Figure II available online only at <http://www.strokeaha.org>). As for the temporal profile, this mouse model exhibits WM lesions only after 14 days. This feature differs from the rat model in which the WM lesions appear at 3 to 7 days after the surgery.⁵ Delayed time course in this mouse model may be attributed to a relatively mild decrease of the CBF, although there may be species difference in the vulnerability of the myelin.

Among a variety of mouse strains, we selected the C57Bl/6 strain because it has been widely used as a wild type control against genetically modulated mice. In addition, C57Bl/6 is

Severity of the WM Lesions in Each Group After 30 Days of BCAS

	Corpus Callosum (medial)	Corpus Callosum (paramedian)	Caudoputamen	Internal Capsule	Anterior Commissure	Optic Tract
Control	0±0	0±0	0±0	0±0	0±0	0±0
0.22 mm	0.3±0.5	0.1±0.4	0.3±0.5	0.1±0.4	0±0	0±0
0.20 mm	1.0±0.8*	0.7±0.5*	0.6±0.5*	0.6±0.5*	0.4±0.5	0.3±0.5
0.18 mm	1.5±0.8†	0.9±0.7†	0.8±0.4†	0.7±0.5*	0.5±0.5*	0.4±0.5
0.16 mm	1.8±0.4†	1.4±0.9*	1.4±0.5†	0.6±0.5*	0.8±0.5*	0.6±0.5*

Tissue damage was graded 0 to 3 (see Materials and Methods). Values are expressed as mean±SD.

*P<0.05.

†P<0.01 compared with the sham-operated control.

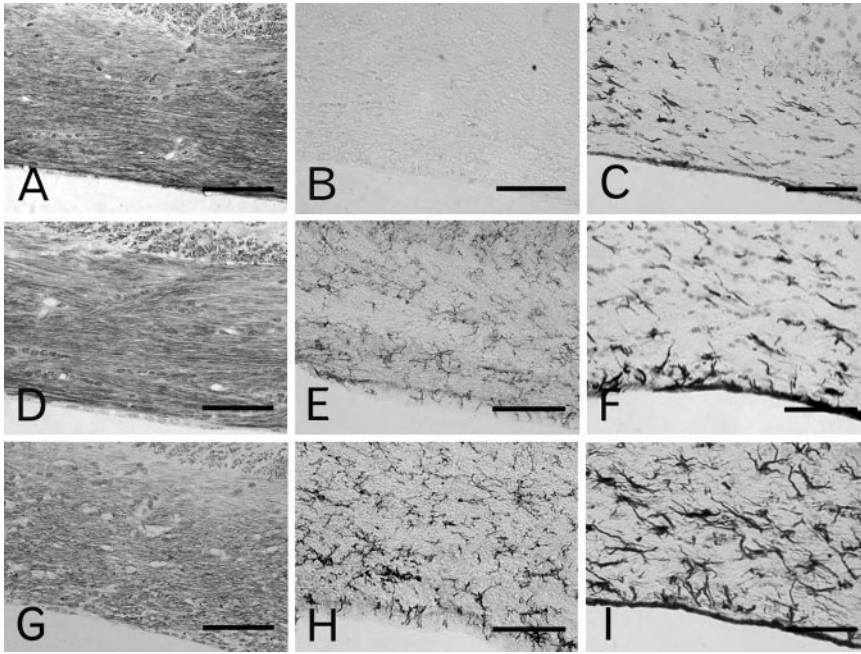


Figure 4. Photomicrographs of the KB staining (A, D, and G), immunohistochemistry for MHC class II antigen (B, E, and H), and GFAP (C, F, and I) in the medial parts of the corpus callosum of mice subjected to BCAS with the 0.18 mm microcoils. The animals were euthanized immediately after the surgery (A, B, and C) or after 7 (D, E, and F) and 30 days (G, H, and I). Scale bar, 60 μ m.

the most vulnerable to cerebral ischemia among 7 mouse strains^{10,11} with a poorly-developed posterior communicating artery.^{12,13} It has been hypothesized that the gradual recovery of the CBF is caused by collateral blood supply, such as through the posterior communicating artery. Therefore, a poorly-developed posterior communicating artery is suitable

for maintaining chronic cerebral hypoperfusion over an extended period.

Vascular changes and a subsequent hypoperfusion have been implicated as major causes of cerebrovascular WM lesions.¹ This study directly demonstrated that chronic cerebral hypoperfusion induced WM lesions, the severity of

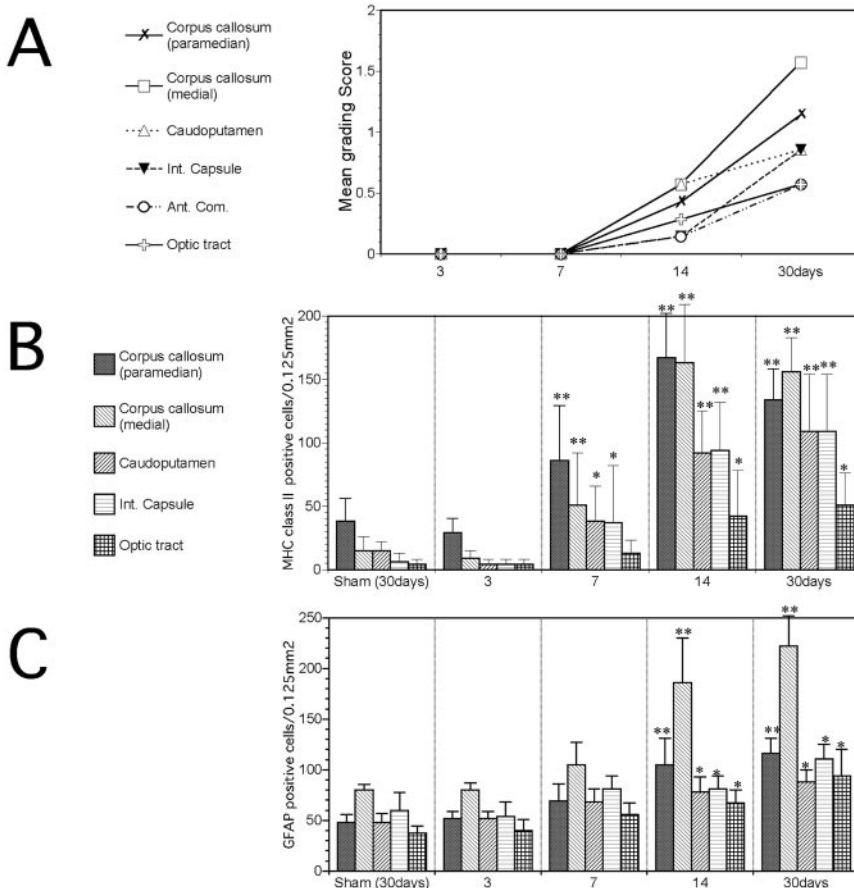


Figure 5. Temporal profiles of the WM lesions (A) and numerical densities of the activated microglia (B) and astroglia (C) in the WM of mice after BCAS with 0.18 mm microcoils. * $P < 0.05$, ** $P < 0.01$ compared with control mice at 30 days after a sham operation (Mann-Whitney U test).

which was closely correlated with the degree of cerebral hypoperfusion. In addition, WM lesions in experimental chronic cerebral hypoperfusion share common features with those in subcortical vascular dementia involving blood-brain barrier disruption,^{14,15} the activation of matrix metalloproteinase^{16,17} and glial cells,⁵ and oligodendroglial apoptosis.^{9,18,19} The therapeutic strategies in subcortical vascular dementia have been limited, but recently the usefulness of calcium antagonists and acetylcholine esterase inhibitors have been demonstrated.²⁰ This model is easily applicable to transgenic or knockout mice and, therefore, may become a new tool to investigate the molecular pathology and to design therapeutic drugs for WM lesions. However, this model should be applied exclusively to C57B/6 strain, because the CBF in the other strains may have a greater variability after BCAS.

In conclusion, a mouse model of chronic cerebral hypoperfusion was established with relative preservation of the visual pathway and the gray matter. This model may become a powerful tool in studies of WM lesions and subcortical vascular dementia.

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