A Novel Chronic Cerebral Hypoperfusion Model with Cognitive Impairment and Low Mortality Rate in Rats

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LIST OF ABBREVIATIONS:

VD	*Vascular Dementia*
CBF	*Cerebral Blood Flow*
CCA	*Common Carotid Artery*
CCH	*Chronic Cerebral Hypoperfusion*
rCBF	*Regional Cerebral Blood Flow*
mCCAO	*Modified Common Carotid Artery Occlusion(The New Model)*
WM	*White Matter*
AD	*Alzheimer Disease*
ROS	*Reactive Oxygen Species*
BCCAO	*Bilateral Common Carotid Artery Occlusion*
BCCAS	*Bilateral Common Carotid Artery Stenosis*
LSF	*Laser Speckle Flowmetry*
VA	*Vertebral Artery*
BCM	*Barnes Circular Maze*
CV	*Cresyl Violet*
IHC	*Immunohistochemistry*
LFB	*Luxol Fast Blue*
2VO	*Two Vessel Occlusion*

ABSTRACT

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OBJECTIVE The cognitive deficit of vascular dementia (VD) and vasoocclusive state of moyamoya disease have often been mimicked with bilateral stenosis/occlusion of common/internal carotid artery. However, the cerebral blood flow (CBF) declines abruptly in these models after ligation of common carotid arteries (CCAs), which differs from “chronic” cerebral hypoperfusion. While some modified but time-consuming techniques used staged occlusion of both CCAs, others used microcoils for CCA stenosis that adversely affected the arterial endothelium. Thus, I developed a new chronic cerebral hypoperfusion (CCH) model with cognitive impairment and a low mortality rate in rats.

METHODS I subjected male Sprague–Dawley rats to one-sided occlusion and contralateral side stenosis of the CCA (modified CCA occlusion [mCCAO]) and measured the cortical regional CBF (rCBF) using laser speckle flowmetry. After assigning all rats to either mCCAO or sham operation groups, I assessed their cognitive function and evaluated their cervical/intracranial arteries and the parenchymal injury with magnetic resonance imaging (MRI) after 4 weeks. Then, I histologically evaluated the rat brains. Furthermore, the extended time point at 8 weeks was set with subsequent behavioral and histological evaluation.

RESULTS The mCCAO group revealed a gradual CBF reduction with a low mortality rate (2.3%). I observed white matter (WM) degeneration in the corpus callosum and corpus striatum. While the Cellular density declined in the hippocampus, MRI revealed no cerebral infarctions after mCCAO. Immunohistochemistry revealed upregulated inflammatory cells and angiogenesis in the hippocampus and cerebral cortex. Moreover,

spatial learning and memory impairment were significantly high in the mCCAO group. The 8-week cognitive dysfunction significantly affected hippocampus cell death, unregulated inflammatory cells, and angiogenesis, whereas the WM demonstrated some regeneration approaching sham animals.

CONCLUSIONS My novel CCH model in rats using mCCAO is straightforward, with a low mortality rate, and could potentially investigate VD, moyamoya disease, and CCH, as well as the pathology of angiogenesis.

KEYWORDS carotid artery stenosis, cerebral blood flow, cerebral hypoperfusion, moyamoya disease, vascular dementia, white matter injury.

INTRODUCTION

INTRODUCTION

Stroke is attributed to a sudden shortage of the cerebral blood flow (CBF) to distinct brain regions; however, a moderate but persistent reduction in the regional CBF (rCBF) compromises memory processes and results in the development and progression of dementia.[1,5]

Vascular dementia (VD), an age-related neurodegenerative disease, the second most common dementia after Alzheimer ’s disease (AD), which occurs in about 20% of all dementia patients, is characterized by cognitive impairment primarily caused by the chronic reduction of the CBF.[26,28] Therefore, VD has become a serious threat to the world. According to the survey, the prevalence of VD was more evident not only in the elderly but also in males. In addition to advanced age and sex, the occurrence and development of VD are associated with various diseases, such as hypertension and metabolic syndrome. Smoking and alcohol use were also risk factors for VD.[3,4,26]

Chronic cerebral hypoperfusion (CCH), is one of the known mechanisms of cerebral vascular disorders and diseases that occurs over weeks and affect the cerebral vascular systems and then cause decrease of blood supply to the brain with subsequent events including neuronal energy failure, production of reactive oxygen species, and pro-inflammatory cytokines through activated microglial

cells that finally lead to neurodegeneration, white matter degeneration, and cognitive impairment.[38]

Pathophysiology of CCH and VD has been reported recently, CCH induces progressive cognitive function decline, memory dysfunction in Alzheimer's disease, and VD. CCH causes white matter (WM) degeneration, neuronal degradation of the hippocampal CA1 subregion,[22,24,27,32] and triggers oxidative stress and neuroinflammation in the animal models;[2,30] deprivation of oxygen and glucose caused by ischemia in brain tissue resulted in a serious of homeostasis alterations, which are involved in the pathological changes and cognitive deficits, including generation of reactive oxygen species (ROS), inflammation, mitochondrial and neurotransmitter dysfunction, disturbance of lipid metabolism, and alteration of growth factors, these changes along with reduction in cerebral blood flow, with subsequent diffuse brain lesions leading to spatial learning and memory impairment in the CCH animal model.[3,6,7,17,19,35,39]

Chronic cerebral hypoperfusion (CCH), which is the main event in the pathophysiology of VD in elderly remains poorly understood regarding the mechanism, risk factors, and treatment, and clinically VD has diversity and complexity in cerebrovascular pathologic conditions, risk factors, progression, and severity of the disease. Thus, the chronic cerebral hypoperfusion (CCH)

animal model supposedly mimics VD despite that it is essential to get a better understanding and simulation of the real condition of VD.[31,35,36]

Molecular mechanisms of VD are still unknown, partly because of the lack of an adequate animal model of CCH with the low mortality rate, also in aging and AD, the sequence of the events of cerebral hypoperfusion and neurodegeneration has been a subject of debate. A chronic reduction in CBF was earlier believed to induce the neurodegenerative processes.[3,4]

Animal models are employed to investigate the roles and mechanisms of CCH in cognitive impairment and to evaluate the therapeutic efficacy of potential drugs; vessel occlusion studies aim at creating ischemic or oligemic injuries with various degrees of severity in the brains of experimental animals, initially transient occlusion of middle cerebral artery with reperfusion used to simulate a state of stroke as acute phase of brain damage which is focal and severe. Studies have reported various procedures of a CCH model, for the reproduction of CCH as it occurs in human aging and AD, including single stage bilateral common carotid artery (CCA) occlusion (BCCAO), unilateral CCAO, staged BCCAO, and bilateral CCA stenosis (BCCAS) using microcoils. The most commonly used model is BCCAO, because of the bridging blood supply from posterior communicating arteries, an approximately 50% decrease of frontal

cerebral blood supply can be achieved using this procedure, numerous studies have reported spatial memory deficits in this CCH model after 30 days.[3,4] Such experimental model is widely used to reproduce chronic cerebral hypoperfusion (CCH), characterized by cognitive impairment and white matter (WM) lesions.

Although BCCAO induces neuronal loss and glial cells activation in the cortex and hippocampus besides learning and memory impairments, the abrupt and severe reduction of the CBF during the acute phase after the procedure causes high mortality rates in rats (50–60%), besides the high mortality, severe CBF reduction with significant infarctions that might not effectively represent a VD model.[4,24,35]

Another model was later developed with staged occlusion of both CCAs which allowed gradual development of CCH, but at the cost of the time factor, and it did not alter the high mortality rates seen with the single stage occlusion model. Bilateral CCA stenosis (BCCAS model, which was later developed by placing microcoils or ameroid constrictors around the CCA to induce stenosis, this approach is theoretically better than the two approaches above, but practically, it is more difficult to achieve the same level of cerebral hypoperfusion due to the more challenging technique of BCCAS,[3,4] this model was criticized on the grounds of high costs of the micro-coils.[11,13,20,31,34] The above approaches are

aimed to reduce cerebral perfusion. However, some of the alterations in these animals might be partially caused by other factors associated with or as the consequence of the procedures.[3,4]

To summarize our rationale for the refined model, we studied previous models for CCH, pilot study for some of those models, we identified the major concerns regarding previous models sudden drop of CBF in the BCCAO model after ligation of both CCAs, while some used staged occlusion of both CCAs, but time-consuming technique, others used microcoils for CCA stenosis that adversely affected the arterial endothelium. Thus, I developed a refined chronic cerebral hypoperfusion (CCH) model which passed through pilot study then validation with radiological, histological, and functional outcomes.

The rat is a frequently used species in consequence of the good survival rate, the satisfactory recovery from surgery, the easy and reproducible behavioral testing, the relatively low costs, and ethical acceptance, the rat is a suitable rodent species for this purpose because the complete circle of Willis affords incessant (but reduced) blood flow after the onset of CCH.

THE AIM OF THE WORK

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This study aimed to overcome the highlighted issues of the currently used CCH models regarding high mortality and high variability of model with BCCAO, long procedure and double times anesthesia with staged model, and injury of endothelium with microcoils stenosis model; and attempted to develop a straightforward and safe model and with an improved survival rate in rats. I illustrated a modified CCA occlusion (mCCAO) model, with one-sided occlusion of the CCA and simultaneous stenosis of the contralateral CCA with a well-controlled outer-diameter needle. Modified technique used to be easy, reproducible, and economic; the most highlighted point of our model is improved survival rate and using the thining technique for minimally invasive measurement of rCBF with LSF, and then validation of the chronic hypoperfusion model was investigated in relation to the main and well established pathophysiologic events of CCH to confirm validity of our refined model to be used in future researches along mean cortical blood flow measurements, radiological validation of macrovessels changes, percentage of cell death, glial activation, WM degeneration assesssment, all with the functional behavioral outcome.

METHODS

METHODS

Animals

All animals in this study were treated per the Code of Ethics of the World Medical Association and Tohoku University guidelines based on the International Guiding Principles for Biomedical Research Involving Animals. Furthermore, animal protocols were approved by Tohoku University's Administrative Panel on Laboratory Animal Care.

I randomly assigned 62 male adult Sprague-Dawley rats (age: 8–10 weeks; 260–300 g) to the 4 weeks mCCAO group (n=31), Sham group (n=19); and the extended time point separately set at 8 weeks mCCAO group of rats (n=12) under same protocols and procedures. All rats were maintained on a 12-h light/dark cycle at a constant temperature of 22±1°C and were housed in plastic cages (4 rats/cage) with free access to food and water. The number of animals not same in all experiments on basis of limited availability of MRI experiment to large group of animals also using the BCM had limited availability to choice of study group number; the time chart (Fig. 1A) presents the protocol of each group.

mCCAO Model

A pilot study included 6 animals for BCCAO showed 50% mortality, while another 6 animals used for staged CCAO showed 33.3% mortality with observed hazard of two timed anesthesia to rats. Thus we advance with our refined model

that showed 100% survival in the pilot group of 6 animals as explained all over our study in the following sections.

All rats were anesthetized with 1.5% isoflurane and maintained with 1% room air as a carrier gas through a face mask. I controlled the rectal temperature at 37°C during surgery using a homeothermic blanket. After making a midline ventral neck skin incision, both CCAs were carefully exposed from their sheaths. While the left CCA was permanently occluded using a 6-0 silk suture, the right CCA was banded, tight to stop distal blood flow completely, with a 6−0 silk suture tied around a blunt 29-G needle, which was then quickly withdrawn (Fig. 1B). In this study, this needle technique preserved portions of the blood flow facilitating various degrees of artery stenosis using different gauge needles, 29-G needle successfully induced safe and satisfactory stenosis for CCH (data not shown). Although this model induced one-sided occlusion and contralateral side stenosis in the early stage. Furthermore, sham-operated controls (n=19) received similar surgical procedures without carotid artery ligation. I recorded the survival rates of each group during the experiment.

Physiological Parameters

I assessed the systolic (SBP) and diastolic blood pressure (DBP) and pulse rate (PR) under anesthesia before and just after CCA occlusion and

stenosis using a rat tail-cuff blood pressure monitor (Softron Tokyo BP-98AL V3.02).

CBF Measurement

I used 9 rats from the mCCAO and 6 from the sham-operated rats for the CBF measurement. The rCBF of the frontal and parietal cortices after surgery were determined using laser speckle flowmetry (LSF; Omega Zone, Omegawave, Tokyo, Japan) as described previously[13] with some modifications. Briefly, I made midline scalp incision under general anesthesia with 1.5% isoflurane, followed by thinning of the skull using a microdrill until only a small translucent sheet of bone remained over the intact dura (Fig. 1C). Then, I set a circular region of interest (ROI; diameter: 2 mm) at the point (3.5 mm posterior and 3 mm lateral to the bregma) for assessment of the rCBF. The rCBF was recorded just before surgery and 1 h, 3, 7, 14, 21, and 28 days after the surgery. Notably, the mean blood flow values of both sides were expressed as a percentage of the baseline value.

The rCBF measured through a thinned skull, as described above, compared to the open window measurement (total removal of the bone), and reported no differences in the rCBF throughout the 4 weeks of the observational period (data not shown). Thus, the skull-thinning method was selected because of less-invasive and time-saving aspects.

Magnetic Resonance Imaging

I performed magnetic resonance imaging (MRI) on 6 mCCAO and 4 sham rats 4 weeks after the surgery using a dedicated small animal scanner at 7 T (PharmaScan, Bruker BioSpin). In addition, time-of-flight (TOF) magnetic resonance angiography (MRA) with a three-dimensional (3D) fast low-angle shot method validated occlusion or stenosis of CCAs and collateral blood flow changes. Scan parameters were as follows: echo time, 2.5 ms; repetition time, 15 ms, matrix=256×128; slice thickness, 0.16 mm; and scan duration, 13 min and 32 s average for all scans. Coronal T2-weighted image (T2WI) was acquired to eliminate cerebral infarction with an echo time of 33 ms, repetition time of 2000 ms, matrix=256×256, slice thickness of 2 mm, number of slices at 10, and scan duration at 8 min and 32 s average**.**

During all *in vivo* assessments, rats were anesthetized by inhalation of 2.5 - 3.0% isoflurane in a nitrous oxide/oxygen carrier gas mixture through a nose cone. The ambient temperature was maintained at 37°C by warm air, and the respiration was monitored during the entire experiment. I analyzed images for quantitative assessments by NIH- Image J software version 1.41 (Rasband, W.S., Image J, US National Institutes of Health, Bethesda, MD; https://imagej.nih.gov/ij/, 1997-2016).[29] VA length was measured from the plane of VAs intracranial entry point to the plane of CCA bifurcation; VA diameter averaged 3 measurements from intracranial segment of VAs. Proximal CCA

diameter was set as the unified scale for size corrections in semi-quantitative measurements; the CCA stenosis percentage was measured at the maximum stenotic segment, then all values were analyzed as a percentage of shams.

Behavioral Assessment using Barnes Circular Maze

After 28 days of surgery, I assessed 9 mCCAO and 6 sham-operated rats for cognitive impairment by the Barnes circular maze (BCM). Furthermore, 12 rats from the extended study group were assessed 8 weeks after the surgery. The BCM is a dry-land maze test for the assessment of spatial learning and memory and assesses cognitive deficit after mCCAO, enabling the evaluation of the latency to locate the escape cage during the training days.[8,10,39]

I performed the Barnes task on “dry land,” a gray acrylic platform (122 cm in diameter), with 16 equidistant holes around the perimeter (O' Hara & Co., Tokyo, Japan). An overhead camera assessed the performance of rats. In 4-day training, two trials were performed per day with the maximum trial length of 300 s; each trial began with the start box positioned at the center of the maze and a rat placed inside it for 30 s and then permitted to explore the maze freely. After reaching the target and before being returned to its home cage, the rat was left in the escape box for 30 s. If the rat did not enter the escape box within 300 s, the experimenter gently picked and placed it inside the target box for 30 s before return to its home cage. I assessed the latency (time to reach the target box), and the number of wrong holes stayed at by rats and compared between

sham animals and the two groups (4 and 8 weeks) of mCCAO. Furthermore, I analyzed the average speed (cm/s) moved by rats in each trial and compared between all groups of animals to exclude motor dysfunction.

Histological Analysis of Hippocampal and WM Injury

After 28–33 days of the surgery, anesthetized rats were perfused with saline and, subsequently, with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH7.4) to evaluate hippocampal and WM injury. I removed brains and postfixed in the same fixative at 4°C overnight, followed by storing in 10, 20, and 30% sucrose in PBS until the tissues sank. Frozen blocks were made with liquid nitrogen after embedding into the optimum cutting temperature (OCT) compound and subsequently cut into 10-μm sections with a microtome-cryostat.

I stained all sections with 1% cresyl violet (CV) to evaluate the hippocampal neuronal injury, which in the pyramidal cell layer of the hippocampal CA1 subregion was detected under a light microscope. Then, I counted the neurons in the late stages of degeneration that demonstrated a shrunken morphology and dark staining. Next, the numbers of injured neurons in three randomly selected sections were averaged under 20× original magnifications.

All sections were stained with Luxol fast blue (LFB) followed by CV staining, as previously described, to assess the WM injury.[16,33] A new time point was added at 1 week after the model with the same procedures of obtaining

sections as mentioned above, and then I obtained 3 sections/ rat and assessed three randomly selected fields of the corpus callosum and striatum under light microscopy at the same exposure level. NIH-Image J was used to quantify the LFB staining. The areas covered by the LFB stain were expressed as a percentage of the total area of the WM assessed.

Immunohistochemistry

After blocking of non-specific bindings with 10% bovine serum albumin for 2 h at room temperature, I incubated sections overnight with the following antibodies: rabbit anti cleaved caspase-3 (Asp175) antibody (#9661; Cell Signaling Technology) to detect cell death; rabbit anti-CD34 antibody [EP373Y] (ab81289, Abcam) to detect angiogenesis; anti-GFAP antibody (astrocyte; Mouse mAb #3670; Cell Signaling Technology) to detect astrocytes; and rabbit anti-Iba-1 antibody (019-19741; Wako, Japan) to detect microglias, I exposed all sections to appropriate biotinylated secondary antibodies (1:200; Vector Laboratories) and visualized with 0.01% diaminobenzidine tetrahydrochloride and 0.005% H_2O_2 in 50 mmol/L Tris-HCl (pH=7.6). then, two ROIs (100 μm^2) were randomly chosen in the middle segment of the hippocampal CA1 subregion and frontoparietal cortex of both right (stenosis) and left (occlusion) sides. I calculated the numbers of immunopositive cells in each ROI and averaged the results to obtain the mean density of each cell type.

Statistical Analysis

I performed comparisons among multiple groups with one or two-way analysis of variance (ANOVA). Comparisons between two groups were attained with the Student's unpaired *t*-test, using Graph-Pad Prism version 5.03. Then the latency to escape box and the number of stays at wrong holes of the BCM were assessed with a two-way mixed-design ANOVA with independent measures Bonferroni posttests on groups (sham vs. 4-weeks mCCAO; sham vs. 8-week mCCAO; 4-weeks mCCAO vs. 8-week mCCAO) and repeated measures on time using Graph-Pad Prism version 5.03. Data were expressed as mean ± SD and $P<0.05$ was considered statistically significant.

RESULTS

RESULTS

Physiological Parameters and Mortality Rate

Our modified model that showed that only 1 out of 43 (2.32%) rats in the mCCAO group died during the 8-week study period. All 19 animals in the sham-operated group survived until euthanasia indicating that the mCCAO animal model exhibited low mortality. No significant differences were observed in the physiological parameters between the mCCAO and sham-operated groups (SBP, P=0.4; DBP, P=0.35; PR, P=0.38; Table 1).

Moderate Reduction and Gradual Recovery of rCBF after mCCAO

The mean blood flow values of both sides of the baseline were 63%±1.6% (occlusion side) and 73%±2.1% (stenosis side), 1 h after surgery. Low rCBF values lasted till the second week (3 days, 60±2.7% [occlusion side] and 71±1.8% [stenosis side]; 1 week, 60±3% and 73±2.5% respectively). Then the rCBF increased (2 weeks, 78±1.2% and 82±3.1%; 3 weeks, 83±1.1% and 84±3.4%; 4 weeks, 94±1.2% and 96±1.4%, respectively, time difference P<0.001, F=2.5; Fig. 1D and E). These results suggested that mCCAO induced moderate rCBF reduction for 1-2 weeks that possibly affected microcirculation in the brain cortex and gradually recovered up to 4 weeks. Furthermore, no significant difference was observed in the rCBF between occlusion and stenosis sides over different time-points (group difference, P>0.05)

Development of Collateral Circulation after mCCAO with No Focal Cerebral Infarctions

In the sham group, while I clearly observed bilateral CCAs, vertebral arteries (VAs) were relatively faint and narrow. Conversely, 4 weeks after mCCAO, despite not observing the the signal of CCA at the occlusion side, exhibited a signal defect at the stenosis side, while VAs comprised a thickened wall, increased diameter and tortuosity in the mCCAO group compared to the shams, suggesting well-developed collateral circulations toward the anterior circulation (Fig. 2A). Data quantification demonstrated significant CCA stenosis (83.2±3.2%, *P*=0.004). In the mCCAO group 4 weeks after the surgery, the VA length and area increased as percentages of the shams (VA length, 158.1±3.5%, *P*=0.013; VA area, 159.8±3.5%, *P*=0.009; Fig. 2B). T2WI revealed no high-intensity lesion in the parenchyma, demonstrating no cerebral infarction after mCCAO (Fig. 2C). These results revealed that mCCAO caused moderate ischemia without cerebral infarction with the development of collateral circulation through the vertebrobasilar arteries.

Cognitive Dysfunction after mCCAO

During training days 1 and 4, example paths were demonstrated by representative tracking plots, and complex paths and hesitating attitude of rats

presented 4 and 8 weeks after mCCAO were compared to sham-operated rats (Fig. 3A). The latency of escape to goal in mCCAO rats was significantly longer than the control in the 4-days training (day 1: sham, 181.8±83.1 seconds; 4-week mCCAO, 262.3±48.7 s, day 4: sham, 15.3±4.5 s; 4-week mCCAO, 150.6±91.7 s; group difference, P<0.01; 8-week mCCAO, day 1: 275.8±57.7 s, day 4: 172.5±66.6 s; group difference, P<0.001, F=16.97, DFn=2, 24; time difference, P<0.01, F=44.3, DFn=3, 72, Fig. 3B). The latency of 8-week mCCAO rats was significantly longer than the 4-week mCCAO on days 2 and 3 (day 2: 4-week mCCAO, 192.1±66.0 seconds; 8-week mCCAO, 265.7±62.5 s; P<0.05, day 3: 4-week mCCAO, 141.6±94.1 s; 8-week mCCAO, 239.2±89.9; P<0.05, Fig. 3B), indicating more impaired performance of 8-week mCCAO rats for spatial learning and using different search strategies. Besides, the number of times rats stayed at wrong holes increased at all days of training with 4-week, and 8-week mCCAO rats compared independently to sham animals (day 1: sham, 34±9.3 holes; 4-week mCCAO, 42.2±4.6, day 4: sham, 5.5±4.1 holes; 4-week mCCAO, 21.3±6.8; group difference, P<0.01; 8-week mCCAO, day 1: 47.2±19.8, day 4: 20.1±6.5; group difference, P<0.05, $F=17.21$, DFn=2, 24; time difference, P<0.01, $F=39.7$, DFn=3, 72, Fig. 3C). The assessment of the average movement speed per rat in each trial session revealed no significant difference between 4-week, 8-week mCCAO rats and sham rats (Fig. 3D), signifying spatial learning disabilities in mCCAO rats rather than motor dysfunction or inactive rats. Compared to shams,

both 4-week and 8-week mCCAO rats exhibited a significant long latency to detect the escape box and had significantly more numbers of stays at wrong holes. Moreover, latency to detect the escape box was longer on days 2 and 3 in the 8-weeks rats compared to 4-weeks rats, suggesting mCCAO induced cognitive impairment, and it may be progressively deteriorated until 8 weeks after the surgery.

WM Injury after mCCAO

The myelin density of the corpus callosum and striatum significantly decreased 1 week after mCCAO. At 4 weeks, the myelin density likely increased compared to 1-week time-point, but was significantly lower than that of shams (corpus callosum: sham, 8.2 μm^2; 1 week, 5.4 μm^2; 4 weeks, 5.9 μm^2; P=0.002, F=1.7; striatum: sham, 17.4 μm^2; 1 week, 8.4 μm^2; 4 weeks, 13.1 μm^2; P=0.001, F=2.1). Eight weeks after mCCAO, the myelin density of the corpus callosum and striatum likely reversed toward sham values (corpus callosum: 7.6$\pm$0.4 μm^2; P=0.006, F=0.5; striatum: 17.3$\pm$0.3 μm^2; P=0.09, F=1.9; Fig.4 A and B). Furthermore, the WM injury, especially in the striatum, reversed after 8 weeks in mCCAO rats.

Hippocampal Neuronal Injury after mCCAO

The cellular density of the hippocampal CA1 subregion with CV staining decreased in the 4-week and 8-week mCCAO rats compared to the shams (sham, 1.3±0.2/100 μm^2; 4-week mCCAO, 11.5±0.4/100 μm^2; P<0.001, F=3.5; 8-week mCCAO, 8.5±1.3/100 μm^2; P<0.001, F=1.8, Fig. 5A). In addition, immunohistochemistry of cleaved caspase-3, marker for apoptotic cell death, revealed an increase in cleaved caspase-3-positive cells in the hippocampal CA1 subregion 4 and 8 weeks after mCCAO (sham, 2.5%±1.6%; 4-week mCCAO, 29.5%±1.2%; P<0.001, F=1.1; 8-week mCCAO, 23%±0.9%; P<0.001, F=2.7 Fig. 5B). These results suggested that CCH induced by mCCAO causes neuronal cell death in the hippocampal CA1 subregion.

Glial Activation after mCCAO

Both GFAP and Iba-1 increased in the hippocampal CA1 subregion and cerebral cortex in mCCAO rats. Compared to shams, 4 and 8 weeks after mCCAO, the density of GFAP-positive cells (CA1: sham, 1.6±0.1/100 μm^2; 4-week mCCAO, 8.9±0.2/100 μm^2; P=0.001, F=2.1; 8-week mCCAO, 5.9±1.4/100 μm^2; P=0.001, F=4.7; cortex: sham, 1.8/100 μm^2±0.2; 4-week mCCAO, 6.7±0.4/100 μm^2; P=0.001, F=5.8; 8-week mCCAO, 6.5±1.6/100 μm^2; P=0.001, F=6.4. Fig. 6A) and Iba-1-positive cells (CA1: sham, 1.4±0.1/100 μm^2; 4-week mCCAO, 4.6±0.3/100 μm^2; P=0.002, F=3.8; 8-week mCCAO, 6.1±0.8/100 μm^2;

P=0.001, *F*=1.4; cortex: sham, 1.7±0.2/100 μm^2; 4-week mCCAO, 5.7±0.2/100 μm^2; *P*=0.002, *F*=1.6; 8-week mCCAO, 4.5±0.9/100 μm^2; *P*=0.002, *F*=3.3. Fig. 6B) significantly increased in the hippocampal CA1 subregion and the cerebral cortex. Reactive astrocyte and microglia were activated after mCCAO and contributed to neuroinflammation.

Angiogenesis after mCCAO

After 4 and 8 weeks of mCCAO, CD34-positive cells (positive microvessels) were upregulated in the cerebral cortex (sham, 1.3±0.3/100 μm^2; 4-week mCCAO, 6.5±0.3/100 μm^2; *P*=0.004, *F*=1.6; 8-week mCCAO, 5.2±0.7/100 μm^2; *P*=0.005, *F*=1.9. Fig. 7), indicating that CCH caused by mCCAO triggered angiogenesis and microcirculation changes in the cerebral cortex.

Summary of extended time point changes

No significant differences were observed between 4 and 8 weeks mCCAO rats regarding neuronal cell death, glial activation, and angiogenesis. After 8 weeks of mCCAO; despite the reversal of the WM injury, cognitive dysfunction remained and may be deteriorated in part. No significant differences were noted between right and left sides of rat brains at 4 and 8 weeks of mCCAO.

DISCUSSION

DISCUSSION

My modified CCH model was quick and straightforward with a low mortality rate. In addition, the rCBF moderately declined before gradually recovering up to 4 weeks with the development of collateral circulation from vertebrobasilar arteries. My mCCAO caused the white matter injury and hippocampal cell death with glial activation and angiogenesis. These results along with cognitive impairment are, perhaps, identical to the VD and the pathophysiology of moyamoya disease as a cerebrovascular chronic occlusive disease with microcirculation changes that were partly elucidated in my model by the activation of microvessels and the rCBF changes in line with previous studies.[15,37] Reportedly, the most prevalent treatment for moyamoya disease, indirect bypass (e.g. encephalo-myo-synangiosis) could be applied using my model using the temporal muscle of rat over a hypoperfused brain of CCH, this treatment could be further improved combined with gene or stem cell therapy.[9,12] As my mCCAO model can be easily combined with indirect bypass or cell therapies, thus updates of molecular biology and genetic research in the CCH field can help to provide some explanations of moyamoya disease pathophysiology.

A pilot study was done to develop different types of CCH model, however I could observe the drawbacks mentioned in previous reports about those models,

the bilateral occlusion model caused a high mortality rate as 50 to 60 percent of animals, survived animals had variable degree of injury from severe injury with infarction to mild form of hypoperfusion. Furthermore, the staged model with an interval of 1 week caused higher mortality than my modified model also with a variable degree of injury, in addition, exposure of animals to anesthesia twice per week warrant a higher risk of instability of results and longer time of the procedure. My model represents a better modification as single staged surgery, stable results, a similar degree of injury among all animals aiming to reach a high grade of standardization, and validated most of previously reported parameters of cerebral hypoperfusion in rats all with a clearly improved survival rate.

Replication, reproducibility, and external validation of my model could be warranted on basis of simple technique using the same size of needle and same suture for performing the model. In addition, the stenosed side of CCA supposed to have progressive slower blood flow that may finally reach to complete occlusion in later time; despite I don't have detailed follow of the conversion of stenosis into occlusion, indirectly proven by histological assessment, rCBF measurements, and MRI study that generally the model behaves like bilateral occlusion rather than unilateral occlusion, which explained the continue of cognitive deficit and neuronal cell death up to 8 weeks after the model, similar to previous reports of classic model of bilateral occlusion, and against the results of

previous reports of unilateral occlusion which represented short timed and ipsilateral only neuronal injury and non significant nor persistent cognitive deficit.[37]

My model represents a clinically relevant and feasible features in the chronic stage without the problems of the acute stage in 2VO model, a novel technique of model production that can be used for both acute and chronic hypoperfusion studies, new minimally invasive modification of measuring cortical rCBF with thinning of skull before application of LSF were performed, with validation of neuronal injury, WM injury, glial activation, and angiogenesis, beside the behavioral pattern tested with the dry land maze BCM instead of usually used water maze as a further modification. Importantly, the wide scale of variation between animals as reported before in the 2VO model as mild, moderate, to severe injury; further subdivide animals that make a difficulty to perform a large study with caring to such variations.[5,38] Our model represents the high survival 97.7%, stable to standardized results among all survived animals.

The reaction of glial cells response to CCH is evident also in the hippocampus and cerebral cortex; a number of studies have been made of the presence and time course of reactive astrocytosis in chronic cerebral hypoperfusion with the help of GFAP immunocytochemistry, Ischemic insults

impose rapid microglial activation, which participates in the defense of the nervous tissue, although microglia may also transform into cytotoxic cells.[4] In my model, two types of glial cells (astrocytes and microglia) were co-activated at 4 and 8 weeks.

MRI demonstrated no areas of infarction on the T2 mapping after mCCAO, as is reported to occur after BCCAO because of the sudden decrease in the rCBF.[5,16,23,29,31] Perhaps, one-sided stenosis of my model could keep the minimal CBF in the early stage as it might be thrombosed in the chronic phase, possibly attributing to the development of the collateral circulation from the vertebrobasilar artery, and inducing a moderate global hypoperfusion state elucidating equivalent changes in both sides of the brain regarding the histological evaluation, unlike previously reported unilateral occlusion of CCA.[37] This is particularly important and focal areas of infarction might skew the results away from VD pathology, and their inconsistent occurrence would introduce errors in the data that would be hard to explain.

The highlights of my model are low mortality rate less that 3% and easy procedure. Obviously, it needs to use large numbers of animals with high mortality model. Reportedly, the mortality rate of the standard BCCAO model was 50% in Sprague-Dawley rats.[8,11,13,16,39] Despite extending the experimental

period, staged occlusion of bilateral CCA exhibited high mortality exceeding 20%.[8,13,16,39] Although, the use of microcoils to develop stenosis in mice reported more stable results than CCAO,[8,13,39] the risk of the endothelial injury and cost of microcoils warrant consideration. In this study, I assessed the CBF by LSF using the skull thinning technique with microdrill compared to the previous complete open window of the skull.[13] My technique offers an easier procedure for serially following LSF and avoiding the dura matter injury.

The compensation of the CBF was strongly correlated with the formation of collateral circulation, it has been well established that aging and dementia are accompanied by a reduced CBF, the degree of cerebral hypoperfusion observed in clinical studies ranges from 94% in the parietal and temporal cortices of elderly individuals as compared with younger volunteers to 73% in the same regions and the hippocampus of AD patients as compared with age-matched, healthy controls,[3,4] the greatest reduction in CBF (to 35–45% of the control level) was recorded in the cortical and WM areas, the CBF in the hippocampus decreasing to a lesser extent (up to 60% of the control), the CBF values had already started to gradually recover at 1 week, but were still significantly lower than the control values 4 weeks after surgery of BCCAO. In addition, previous reports measured CBF up to no more than 4 weeks with laser Doppler flowmetry, they reported a 68% reduction of rCBF 1-3 days after surgery, that elevated to only 48%, 41%

after 1, 2 weeks respectively. With larger sized animal even lower reduction of CBF reported as 38% in the 1st few hours after surgery.[4]

In my model, the CBF demonstrated moderate progressive reduction over the first week, plateaued at the second week, and then started recovering from the third to fourth week, a very close pattern of CBF changes to the mentioned above previous reports; however, this recovery did not affect the histological and behavioral sequel of CCH that was tested at 4 and 8 weeks after the surgery. In addition, we could not identify the real mechanism of events occurred later at 8 weeks after mCCAO whether related to the acute injury or related to chronic oligaemic state after the model, however this issue still a debate in previous studies as well.[4,5,38]

Possible mechanisms for recovering the CBF in CCH brains could be associated with the biomedical regulation of the CBF, recruitment of non-perfused capillaries, angiogenesis, and dilation of vertebrobasilar arteries; regarding macrovascular changes, compensatory blood flow may be provided through artery dilation, the recruitment of non perfused capillaries (if there are such in the brain), and angiogenesis. Biochemical regulation of the CBF may also play a significant part in the adaptation; experimental evidence has been acquired on the enlargement of the arteries at the base of the brain. The posterior vessels contributing to or emanating from the circle of Willis (e.g. the

basilar artery, the posterior cerebral artery, and the posterior communicating artery) considerably increased.[8,11] On the other hand, regarding microvascular changes, the specific compensatory mechanisms are still uncertain, an increased capillary diameter, neovascularization, and an enhanced signal for vascular endothelial growth factor were noted in the cortex and hippocampus after 4 weeks of BCCAO.[4] However, the findings were not confirmed at 13 weeks, discrepancy between these results may indicate the occurrence of dynamic, microvascular remodeling following the changes in CBF: growth factors may induce angiogenesis at lower perfusion rates (at early time points after BCCAO onset), while the capillary network may readjust to its original density as the CBF normalizes.[4]

In this study, I observed two compensation mechanisms as follows: (a) the macrovascular level, the dilation of vertebrobasilar arteries was observed by 3D-TOF MRA with increased tortuosity and diameter of both VAs; and (b) the microvascular level, angiogenesis demonstrated by IHC with CD34-positive cells, was observed at 4 and 8 weeks after surgery. The tortuosity (length) and diameter (area) of both vertebral arteries were clearly increased at 4 weeks after CCH. It has been reported that VAs, basilar artery, proximal segments of the posterior cerebral arteries, and posterior communicating artery gradually enlarge starting 4 days to 6 months after one-step BCCAO model, the initial arterial

dilation is probably flow-induced, then vascular remodeling and arteriogenesis take place, as indicated by the appearance of extracranial collaterals emanating from the vertebral arteries and by the tortuosity of the basilar and vertebral arteries[11] In line with previous studies, capillary densities in both frontal and parietal cortices increased to approximately 1.3-fold of the control level at 28 days after the surgery.[11,14] Based on my results, CBF starts to be recovered to the pre-occlusion level at 3 weeks after surgery, while angiogenesis evident from 4 weeks. Supporting the theory proposed that early compensation occurs by VA dilatation and collateral blood flow while late compensation is induced by angiogenesis.

My results have shown that CCH induced a compensatory mechanism of CBF and blood vessels in the cerebral cortex. However, this compensation could not prevent neuronal death. For several reasons, the favored brain region for the study of CCH induced neurodegeneration is the hippocampus; the hippocampus is the area that displays the most characteristic neuropathological damage in VD, highly implicated in spatial learning and memory as assessed by the Morris water maze, and neuronal injury in the hippocampus and impaired spatial learning can be related, additionally, the hippocampus (and particularly its CA1 subfield) is one of the brain regions most sensitive to ischemia.[3,4,6,29] At 4 and 8 weeks, neuronal death in the cerebral cortex and hippocampus and the following

cognitive impairment were evident in this study. Several studies reported the occurrence of degeneration of different cell types in the CCH, pyramidal cells of the CA1 subregion of hippocampus(the same region I studied in my model) showed 26% cell death at 8 weeks after the BCCAO model,[4,7,8,29,31] which was further supported in this study because the percentage of damaged neurons in the CA1 subregion increased to around 30% at 4-week mCCAO and 23% at 8-week mCCAO.

The type of cell death in our study represent 2 types of neuronal degeneration; necrotic cells of the acute ischemic phase demonstrated by the CV histological examination, and the apoptotic cells of chronic oligaemic phase validated by IHC of cleaved caspace 3 antibody, Thus my model represent the 2 stages of cell death that were difficult to discriminate clearly so lack of explanation of different percentage of cell death at 4 and 8 weeks after surgery as a real lower percentage of cell death at 8 weeks or just a variation between different kinds of cell death. The cognitive impairment assessment by BCM in my model further validated that CCH induces spatial learning and memory impairment.[7,8,13,18,21,32,34,35,39]

In my study, the WM injury 1 week after mCCAO, as revealed by LFB staining, was higher than that of 4 weeks but was reversed at 8 weeks, suggesting that mCCAO causes the WM injury regeneration, contrary to the

neuronal injury, which was not reported previously earlier than 8 weeks, and could be an open gate for studies of neuroprotection.[19,25,31] This finding might contribute to the development of cognitive impairment of the CCH along with the neuronal injury in the CA1 region of hippocampus, cerebral WM lesions that accompany human aging and dementia have received increasing attention as WM injury visualized with clinical imaging techniques has been found to coincide with cognitive and psychiatric disorders in the elderly and AD patients, CCH model has emerged as a suitable approach to study the relationship between cerebrovascular insufficiency and WM lesions.[4,13,16,22,27,32]

An increasing number of studies have demonstrated that BCCAO rats cover longer swim paths or display longer escape latencies in the Morris water maze, it has been firmly established that experimental cerebral hypoperfusion compromises spatial learning in rats but not yet answered whether the learning impairment develops exclusively due to the sudden drop in blood flow in the acute phase or worsens in the chronic phase of BCCAO.[3,4,15,25] In my results I extended the study to 8 weeks added to original 4weeks study that showed persistent cognitive impairment for spatial learning and memory till 8 weeks regarding the latency time to reach target box and number of trial errors selecting the correct hole in the BCM study, I further support the BCM over the classic

water maze for being less irritating and cause less fear and anxiety to rats with the aim of eliminating distracting parameters from the real cognitive insult.

CCH caused by vascular structural lesions, cerebral hemodynamic changes, or increased blood viscosity is common in the elderly and often contributes to memory impairment, neurodegeneration, and sporadic AD Because CCH rarely exists alone and is usually accompanied by other brain pathologies, it is challenging to dissect the exact role of CCH in neurodegeneration and sporadic AD. Many studies have shown an active and even causative role of CCH in VD-like brain pathology and neurodegeneration. Future research should focus on the major molecular mechanisms by which CCH causes or promotes cognitive impairment and neurodegeneration.[3,4]

This study has some limitations. First, I did not completely establish irreversibility of the cognitive function and the detailed associated behavioral functions along with further changes at cellular and subcellular levels. Second, although I wished I could subject all animals in each group to all experiments, technical difficulties and equipment availability of MRI, and BCM prevented this, so large group of animals were used which was further subdivided to different groups. In addition, we have to mention that the numbers of animals were not predefined; therefore we could limit our study as negative results may depend on

small sample size. Additionally, we have to mention another limitation for subjective variability of degree of tightening the suture around the needle; though, we tried to unify suture type and size, needle size, and the degree of tightness further external validation for future research is required. Finally, LSF only measured the cortical CBF, and I could not evaluate deep brain microcirculation changes, and CBF changes. Thus, the lack of precise evaluation of microcirculatory changes other than angiogenesis requires more advanced imaging tools. Overall, further mechanistic evaluations and extensive treatment trials with better equipment are warranted in the future studies.

SUMMARY & CONCLUSIONS

SUMMARY

Background The occurrence of a stroke is attributed to a sudden shortage of the cerebral blood flow (CBF) to distinct brain regions; however, a moderate but persistent reduction in the regional CBF (rCBF) compromises memory processes and results in the development and progression of dementia. Vascular dementia (VD) is characterized by cognitive impairment primarily caused by the chronic reduction of the CBF. Thus, the chronic cerebral hypoperfusion (CCH) animal model supposedly mimics VD. Studies have reported various procedures of a CCH model, including single stage bilateral common carotid artery (CCA) occlusion (BCCAO). However, the cerebral blood flow (CBF) declines abruptly in these models after ligation of common carotid arteries (CCAs), which differs from “chronic” cerebral hypoperfusion. While some modified but time-consuming techniques used staged occlusion of both CCAs, others used microcoils for CCA stenosis that adversely affected the arterial endothelium. Thus, I developed a new chronic cerebral hypoperfusion (CCH) model with cognitive impairment and a low mortality rate in rats. This study aims to overcome the highlighted issues of the currently used CCH models and attempts to develop a straightforward and safe model and with an improved survival rate in rats. I illustrate a modified CCA occlusion (mCCAO) model, with one-sided occlusion of the CCA and simultaneous stenosis of the contralateral CCA with a well-controlled outer-diameter needle.

METHODS I subjected male Sprague–Dawley rats to one-sided occlusion and contralateral side stenosis of the CCA (modified CCA occlusion [mCCAO]) and

measured the cortical regional CBF (rCBF) using laser speckle flowmetry. After assigning all rats to either mCCAO or sham operation groups, I assessed their cognitive function by the Barnes circular maze and evaluated their cervical/intracranial arteries and the parenchymal injury with magnetic resonance imaging (MRI) after 4 weeks. Then, I histologically evaluated the rat brains. Furthermore, the extended time point at 8 weeks was set with subsequent behavioral and histological evaluation.

RESULTS The mCCAO group revealed a gradual CBF reduction with a low mortality rate (2.3%). I observed white matter (WM) degeneration in the corpus callosum and corpus striatum. While the Cellular density declined in the hippocampus, MRI revealed no cerebral infarctions after mCCAO. Immunohistochemistry revealed upregulated inflammatory cells and angiogenesis in the hippocampus and cerebral cortex. Moreover, spatial learning and memory impairment were significantly high in the mCCAO group. The 8-week cognitive dysfunction significantly affected hippocampus cell death, unregulated inflammatory cells, and angiogenesis, whereas the WM demonstrated some regeneration approaching sham animals.

DISCUSSION At 4 and 8 weeks, neuronal death in the cerebral cortex and hippocampus and the following cognitive impairment were evident in this study. In my model, two types of glial cells (astrocytes and microglia) were co-activated at 4 and 8 weeks. Of note, MRI demonstrated no areas of infarction on the T2 mapping after mCCAO, as is reported to occur after BCCAO because of the sudden decrease in the rCBF. In this study, I observed two compensation mechanisms for cerebral blood flow

reduction as follows: (a) the macrovascular level, the dilation of vertebrobasilar arteries was observed by 3D-TOF MRA with increased tortuosity and diameter of both VAs; and (b) the microvascular level, angiogenesis demonstrated by IHC with CD34-positive cells, was observed at 4 and 8 weeks after the surgery. In my study, the WM injury 1 week after mCCAO, as revealed by LFB staining, was higher than that of 4 weeks but was reversed at 8 weeks, suggesting that mCCAO causes the WM injury that regenerates faster than the neuronal injury, which was not reported previously earlier than 8 weeks. This study has some limitations. First, I did not completely establish irreversibility of the cognitive function and the detailed associated behavioral functions along with further changes at cellular and subcellular levels. Second, although I wished I could subject all animals in each group to all experiments, technical difficulties and equipment availability of MRI, and BCM prevented this. Finally, LSF only measured the cortical CBF, and I could not evaluate deep brain microcirculation changes, and CBF changes. Thus, the lack of precise evaluation of microcirculatory changes other than angiogenesis requires more advanced imaging tools. Overall, further mechanistic evaluations and extensive treatment trials with better equipment are warranted in the future studies.

CONCLUSIONS

My new CCH model by mCCAO in rats is straightforward and with a low mortality rate and could be potentially used to investigate VD, moyamoya disease, CCH pathophysiology and treatment options, and cognitive function as well as reviewing angiogenesis, and neuroinflammation.

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TABLES & FIGURES

Table 1: Physiological parameters

Systolic blood pressure (SBP), Diastolic blood pressure (DBP), and Pulse rates (PR) measure using rat tail cuff monitor under anesthesia just pre and immediate post occlusion/stenosis of Common Carotid Arteries (CCAs)

	SBP (mmHg) Mean ± SD	DBP (mmHg) Mean ± SD	PR (beat/minute) Mean ± SD
Pre occlusion/ stenosis of CCA	145.8 ± 2.9	77.7 ± 1.8	445.2 ± 3.5
Post occlusion/ stenosis of CCA	147.8 ± 4.8	79.3 ± 3.8	447.2 ± 4.0
P value	*P*=0.4, *F*=2.76, DFn=5	*P*=0.35, *F*=4.65, DFn=5	*P*=0.38, *F*=1.28, DFn=5

Figure 1

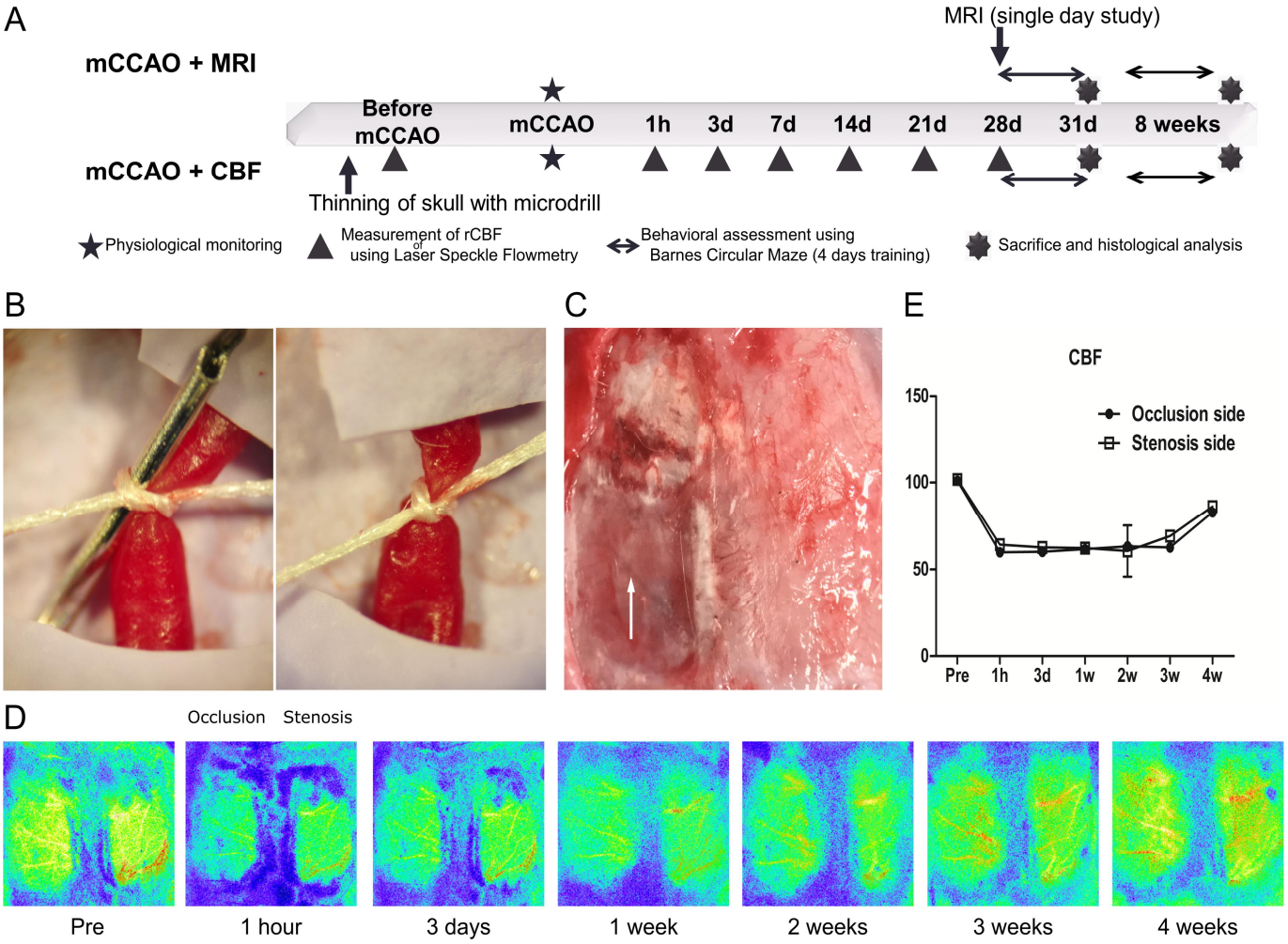

Fig. 1. Summary of the mCCAO model. **A,** time chart shows two protocols and the sequence of experiments conducted. **B,** induction of severe CCA stenosis by the needle-suture technique. **C,** a photograph of the thinned skull from surgical microscope eyepiece; the left half of the thinned skull with visible cortical vessel (arrow) compared with the right half of the normal skull, which is also thinned before measuring the mean blood flow. **D and E,** the temporal profile of the rCBF after mCCAO. The rCBF color map measured by LSF (**D**) and a graph of the rCBF value compared to the baseline (**E**). The rCBF significantly decreases immediately after the surgery; it decreases further a little more at 3 days and then plateaued till the second week. The rCBF increases again at 3 weeks, then reaches a near normal value at 4 weeks after mCCAO. No significant differences in the rCBF between stenosis and occlusion sides (time difference $P<0.001$; group difference, $P>0.05$).

Figure 2

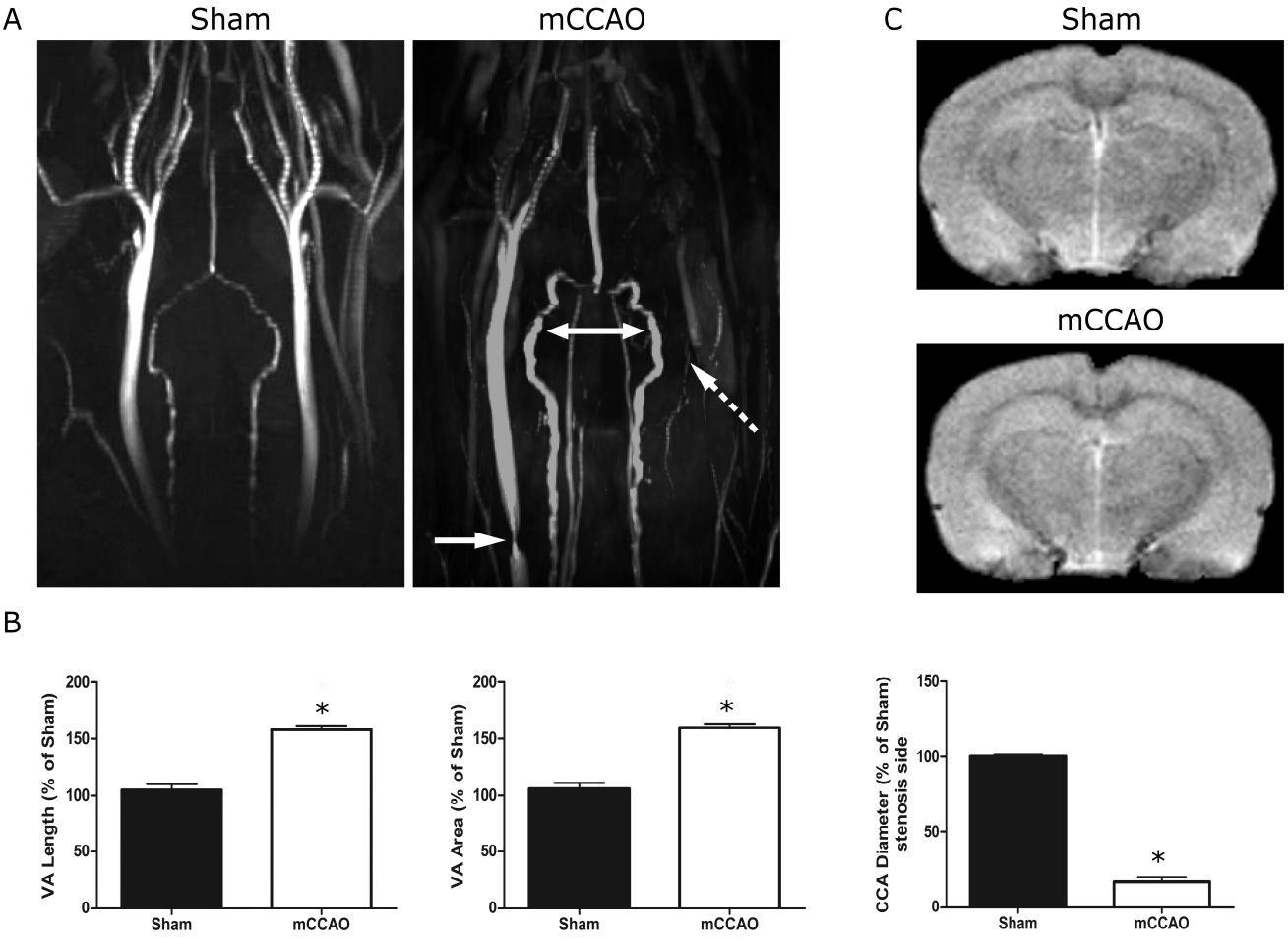

Fig. 2. MRI 4 weeks after the mCCAO. **A**, the maximum intensity projection of the 3D TOF MRA. In sham-operated rats, bilateral CCAs are clearly visible, whereas VAs are faint and narrow. Conversely, the CCA signal at the occlusion side is not visualized (dotted arrow), whereas CCA of the stenosis side revealed a filling defect (arrow) in the mCCAO group. VAs (double-headed arrow) have thickened wall, increased diameter and tortuosity in the mCCAO group compared to shams. **B**, quantification revealed a significant CCA stenosis (83.2%±3.2%, *P*=0.004; the VA length and area increased in mCCAO rats 4 weeks after the surgery as percentages of shams (*P*=0.013, and *P*=0.009, respectively). C**,** coronal T2WI at 4 weeks after mCCAO. No brain infarction in the mCCAO group and shams.

Figure 3

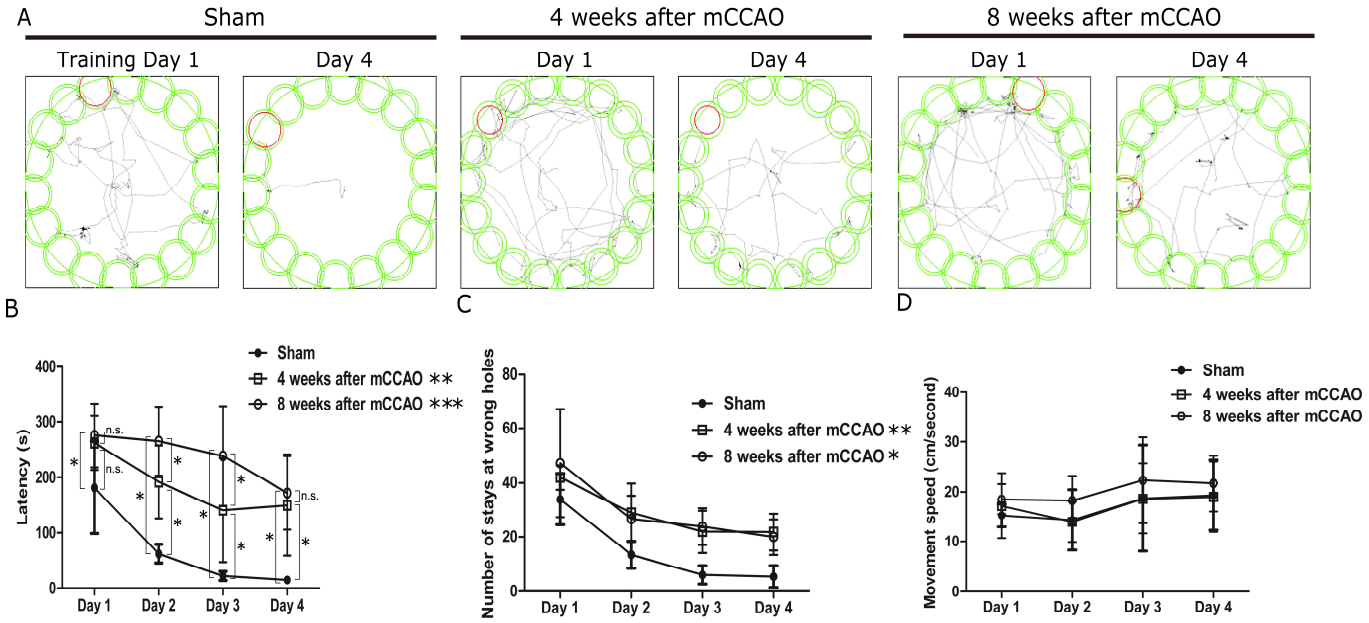

Fig. 3. Cognitive dysfunction after mCCAO evaluated using the BCM. **A**, a diagram of path recorded during animal trials to detect the target box comparing the first and fourth days of training on the BCM demonstrates more complex and longer path to reach the target in the 4 and 8 weeks mCCAO groups compared to shams. **B**, time latency to find the target box revealing the latency is significantly longer for both operation and time at all training days in the mCCAO group (*P<0.05). Multiple comparisons by Bonferroni revealed the latency of the 8-week mCCAO rats was significantly longer than sham-operated rats on day 1 (*P<0.05), the latency of 8-week mCCAO and 4-week mCCAO rats was significantly longer than sham-operated rats on day 2 (*P<0.05 each), the latency of 8-week mCCAO rats was significantly longer than 4-week mCCAO on day 2 (*P<0.05), the latency of 8-week mCCAO and 4-week mCCAO rats was significantly longer than sham-operated rats on day 3 (*P<0.05 each), the latency of 8-week mCCAO rats was significantly longer than 4-week mCCAO on day 3 (*P<0.05), and the latency of 8-week mCCAO and 4-week mCCAO rats were significantly longer than sham-operated rats on day 4 (*P<0.05 each). **C**, the number of times that rats stayed at wrong holes demonstrating significantly increased number for both operation and time at all training days in mCCAO rats (*P<0.05). **D**, the average movement speed; no significant difference between mCCAO and sham rats indicates exclusive spatial learning and memory impairment.

Figure 4

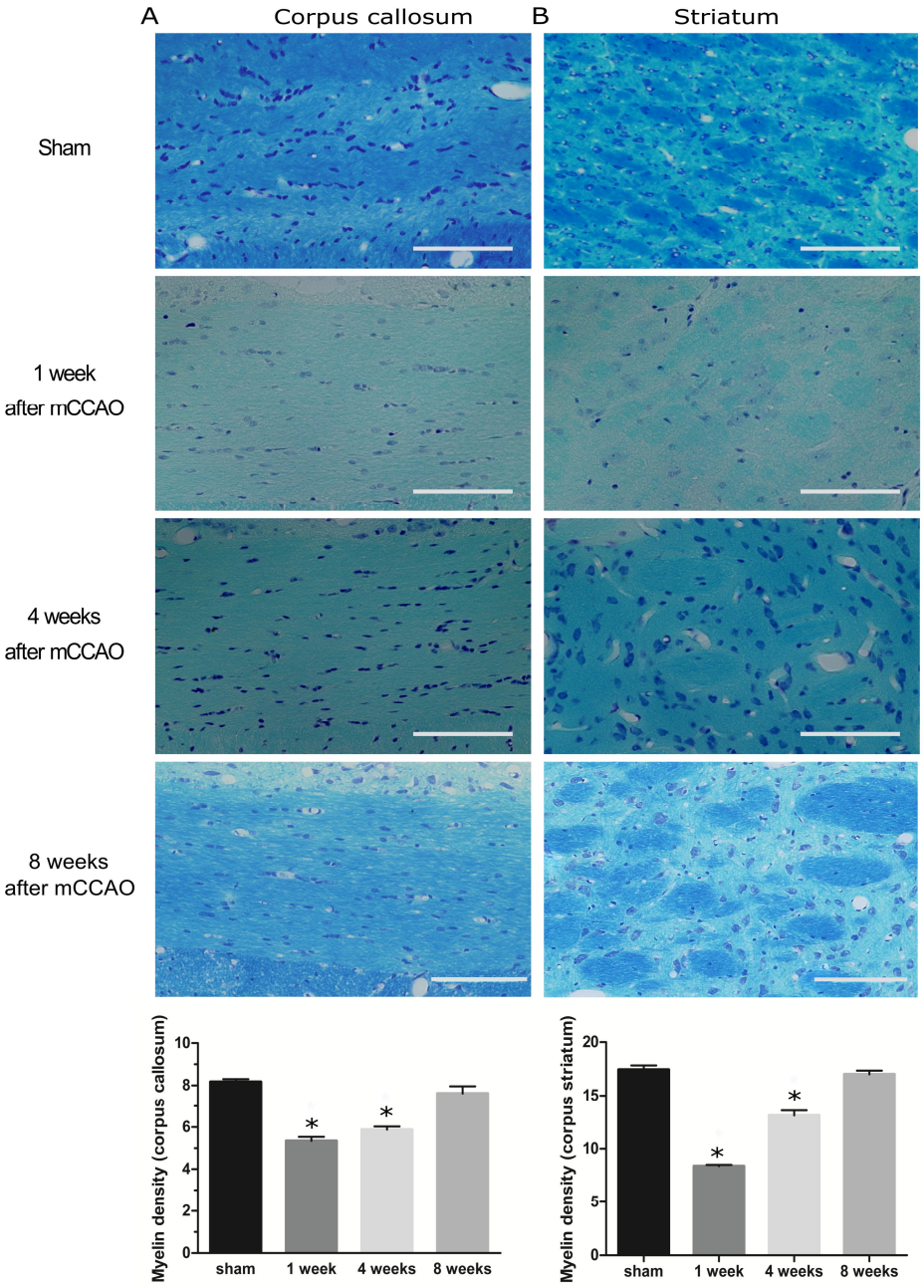

Fig. 4. WM degeneration after mCCAO. LFB + CV staining of the mid corpus callosum (**A**) and striatum (**B**) 1, 4, and 8 weeks after mCCAO demonstrates significantly less LFB staining density in the mCCAO group at 1 week compared to shams (**P*=0.002). Four weeks after mCCAO, the LFB staining density tends to increase compared to the 1-week time-point but is still low compared to shams (**P*=0.001). Eight weeks after mCCAO, the LFB staining density tends to reverse to sham values (*P*=0.09). Original magnification 20x; scale bar=200 μm.

Figure 5

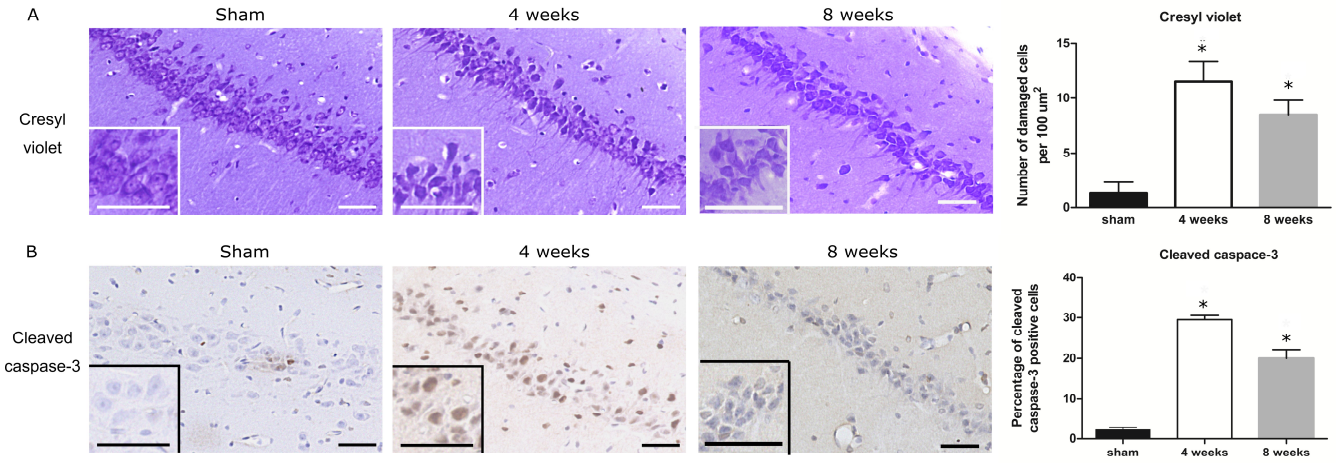

Fig. 5. Morphological changes in the neuronal cells in the hippocampal CA1 subregion. **A**, CV staining of the hippocampal CA1 area. Damaged cells (pyknotic nucleus, dark shrunk cytoplasm) significantly increased in the hippocampal CA1 subregion of the mCCAO group compared to shams (**P*<0.001). Scale bar=50 μm. **B,** immunohistochemistry of the cleaved caspase-3, 4 and 8 weeks after mCCAO; cleaved caspase-3-positive cells increased in the hippocampal CA1 subregion 4 and 8 weeks after mCCAO (**P*<0.001). Original magnification 20x; scale bar=50 μm.

Figure 6

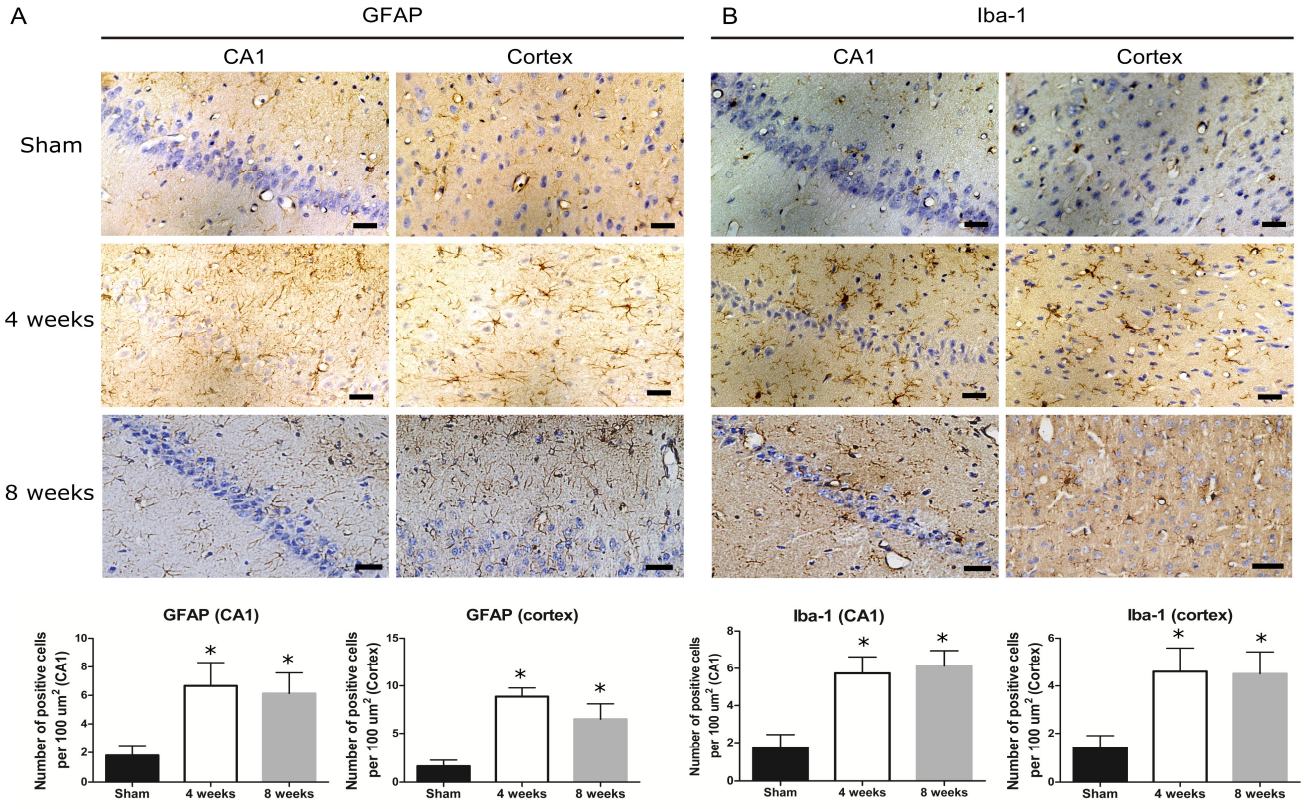

Fig. 6. A, Glial activation in the parietal cortex and the hippocampal CA1 subregion. GFAP (**A**, **P*=0.001) and Iba-1 (**B**, **P*=0.002) -positive cells significantly increased in the bilateral parietal cortex, and the hippocampal CA1 subregion 4 and 8 weeks after mCCAO compared to shams. Original magnification 20x; scale bar=100 μm.

Figure 7

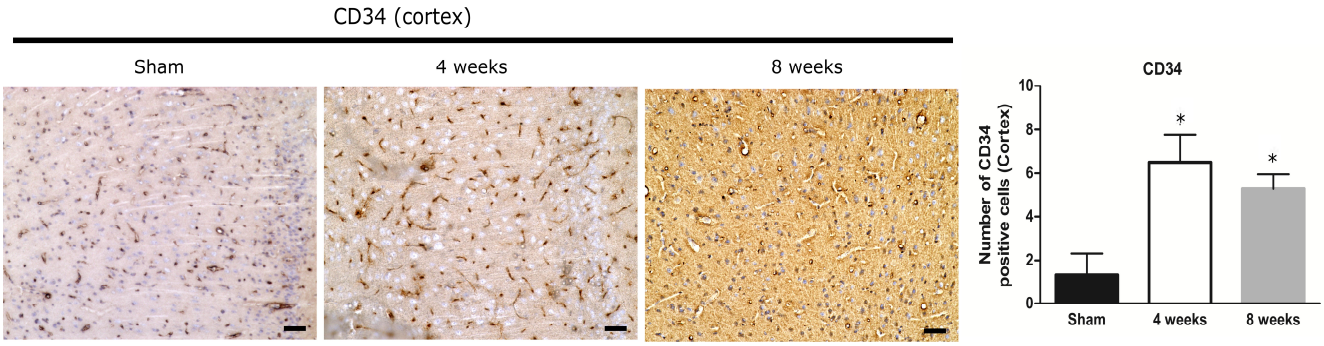

Fig. 7. Immunohistochemistry of the CD34. CD34-positive microvessels significantly increased in the parietal cortex 4 and 8 weeks after mCCAO compared to the shams (*P=0.004). Original magnification 20x; scale bar=100 μm.