A Mouse Model for Chronic Cerebral Hypoperfusion-induced Cognitive Dysfunction
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ABSTRACT

Background: Chronic cerebral hypoperfusion potentially contributes to the initiation and progression of vascular dementia (VD). Bilateral common carotid artery occlusion (two-vessel occlusion) is the most commonly used animal model to replicate this pathological condition but with high mortality and severe histological cerebral damage. Unilateral common carotid artery occlusion (one-vessel occlusion) was introduced to simulate clinical conditions. Our study was designed to further characterize this model.

Methods: In this study, eight-week old CD-1 mice were subjected to left common carotid artery occlusion (LCCAO). Two weeks after the occlusion, their learning and memory were assessed by Barnes maze and fear conditioning. Histomorphological changes were evaluated by Hematoxylin-Eosin staining. Neuronal and axonal degenerative changes were examined by amino-cupric silver staining.

Results: LCCAO increased the time to find the target box in the Barnes maze test during the 4-day training sessions and one day after the training sessions compared with sham group mice. There was no difference in context-related or tone-related freezing behavior between these two groups. No significant histological neuronal cell damage or degeneration was observed in brain sections of hippocampus and corpus callosum in these two groups.

Conclusions: Our results suggest that LCCAO can be used to mimic the vascular dementia.

Increasing evidence suggests that chronic cerebral hypoperfusion is a common consequence of various cerebral vascular disorders, which contribute to the pathogenesis of vascular dementia (VD) and Alzheimer's disease (1, 2). VD is defined as a progressive neurodegenerative disease characterized by cognitive decline resulting from the disturbance of cerebral blood flow (3). The occurrence of VD is associated with several factors including advanced age and various disease conditions (4, 5). At present, there is no effective therapy for this complex disease (6).

For better understanding the pathophysiology of VD, several animal models have been established. The most widely used experimental model is permanent bilateral common carotid arteries occlusion (two-vessel occlusion, 2-VO) in rodents (7). Permanent 2-VO produces a sudden decrease of cerebral blood flow to both cerebral hemispheres by 30% to 40% of the control level (8), which does not simulate the gradual decline of the ce-
rebral blood flow in patients. Also, there is a 30% to 50% mortality rate in this model (9). Therefore, a new model of chronic cerebral hypoperfusion induced by unilateral common carotid artery occlusion (one-vessel occlusion, 1-VO) was introduced to simulate the chronic cerebral hypoperfusion in which animals developed cognitive decline (10). The chronic cerebral hypoperfusion induced by permanent right common carotid artery occlusion (RCCAO) was applied to both mice and rats which showed chronic mild reduction of cerebral blood flow with significant behavioral deficits. In addition, this cognitive impairment produced by RCCAO parallels with the patho-histological cerebral damage (10, 11).

However, the reproducibility of the 1-VO model presentation has not been examined by various laboratories. In addition, deficits of learning and memory in this model need to be better characterized. Thus, we occluded the left common carotid artery permanently in mice to examine their cognitive changes by Barnes maze and fear conditioning and the neuro-pathological changes by hematoxylin-eosin (HE) staining and silver staining.

MATERIALS AND METHODS

The animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Virginia (Charlottesville, VA, USA). All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications number 80-23) revised in 2011. All efforts were made to minimize the number of animals used and their suffering.

Animal groups and surgery

Eight-week-old male CD-1 adult mice (N=22) weighing 28-32 g from Charles River Laboratories International Inc. (Wilmington, MA, USA) were housed under 12-hour light and 12-hour dark cycle with free access to chow and water. These mice were randomly subjected to either left common carotid artery occlusion (LCCAO) surgery (N=13) or sham surgery (N=9). For anesthesia induction, mice were exposed to 5% isoflurane for 3-5 minutes. Anesthesia was maintained by 2.0% isoflurane. Midline cervical incision was then performed, and the left common carotid artery was carefully isolated from the arterial sheath to avoid damage to vagus nerve. The artery was then doubly ligated with 4-0 nylon suture. Sham surgery was performed by exposing the left common carotid artery without carotid ligation. During anesthesia, anal temperature was strictly maintained at 37 ± 0.2 °C by warming blanket and lamps. The inhaled and exhaled gases were also monitored to maintain normal end-tidal CO₂ concentrations.

Barnes Maze

Two weeks after surgery, mice were subjected to Barnes maze as we previously described (12). To test their spatial learning and memory, mice were first placed in the middle of a circular platform with 20 equally spaced holes (SD Instruments, San Diego, CA). One of these holes was connected to a dark chamber called target box. Aversive noise (85 dB) and bright light (200 W) shed on the platform were used to encourage mice to find the target box. They had a spatial acquisition phase that lasted for 4 days with 3 minutes per trial, 2 trials per day and 15 minutes between each trial. Animals then went through the reference memory phase to test the short-term retention on day 5 and long-term retention on day 12. No test or handling was performed from day 5 to day 12. The latency to find the target box during each trial was recorded with the assistance of ANY-Maze video tracking system (SD Instruments).

Fear Conditioning

One day after Barnes maze test, mice were subjected to fear conditioning test as we previously described (12). Each mouse was placed into a test chamber wiped with 70% alcohol and exposed to 3 tone-foot shock pairings (tone: 2000 Hz, 85 dB, 30 seconds; foot shock: 1 mA, 2 seconds) with an intertrial interval of 1 minute in a relatively dark room. The mouse was removed from this test chamber 30 seconds after the conditioning stimuli. The animal was placed back to the same chamber without the tone and shock 24 hours later for 8 minutes. The animal was placed 2 hours later into another test chamber that had different context and smell from the first test chamber in a relatively light room. This
second chamber was wiped with 1% acetic acid. Freezing was recorded for 3 minutes without the tone stimulus. The tone was then turned on for 3 cycles, each cycle for 30 seconds followed by 1-minute inter-cycle interval (4.5 minutes in total). Animal behavior in these two chambers was video recorded. The freezing behavior in the 8 minutes in the first chamber (context-related) and 4.5 minutes in the second chamber (tone-related) was scored in an 8 seconds interval by an observer who was blind to the group assignment. Animals in all groups were sacrificed immediately after fear conditioning test to harvest the brain for the following histo-pathological examinations.

**Histological analysis**
Histo-morphology was assessed after HE staining of brain sections as described before (13). Animals were deeply anesthetized with 5% isoflurane. They were then transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4). The mice were decapitated, and the brains were then immersed in 4% paraformaldehyde for 24 hours, processed for paraffin embedding, and sectioned into 5 μm thick sections on a rotary microtome. Coronal sections consisting of the dorsal hippocampus and corpus callosum were selected and used in HE staining. The paraffin-embedded sections were dewaxed by baking at 55-65 °C for 90 minutes, cleaned with xylene two times with each for 5 minutes, and rehydrated with 100% ethanol twice with each for 5 minutes and 70% ethanol once for 5 minutes. After 5-minute washes in distilled water, the sections were stained in hematoxylin for 2 minutes and then washed two times in running water for 1 minute. The sections were dipped in acid alcohol solution and then washed again using running water for 1 minute. After rinsing each section in ammonia alcohol for 5 seconds, 10 dips were performed in Eosin-Y solution for 30-45 seconds to counterstain the sections. Following two additional rinses in 100% ethanol with each for 1 minute, the sections were dehydrated in graded alcohols, cleared in xylene and coverslipped with Permount. Six coronal brain sections were imaged per animal in the brain area from Bregma -1.06 to Bregma -2.70 based on the mouse brain stereotaxic coordinates.

**Silver staining**
Neuronal and axonal degeneration were examined by using a modified amino-cupric silver staining (14). Briefly, mice were deeply anesthetized with 5% isoflurane, and then perfused transcardially with 4% paraformaldehyde in 0.1 M cacodylate buffer. The brain was removed and stored in the same fixative solution for 24 hours at 4 °C. The brain was embedded in 0.5% gelatin solution and cut into 50 μm thick sections by vibrotome. The brain sections were washed five times with distilled water for 5 minutes each in a staining tray, and then were transferred into cupric-silver solution in a dish. This dish was wrapped and placed on a hot plate at 40 °C for 1 hour and then left in the hood at room temperature overnight. On the next day, the staining tray was shaken in a dish with 100% acetone for 30 to 90 seconds and then was quick transferred to a dish with silver diamine incubation solution. The incubation lasted for 35 minutes. The sections were then transferred to the reducing agent solution for 5 minutes, and then transferred to ferricyanide bleaching solution for another 10 minutes with continuous gentle agitation. When dark straw color appeared on the slice, the sections were washed in distilled water two times with each for 5 minutes and then were stabilized in 2% sodium thiosulfate solution for 1 minute with light agitation. After differentiation and stabilization, the sections were rinsed again in distilled water for 2 to 5 minutes, and then mounted on gelatin-treated glass slides. The slices were then rinsed with distilled water and dehydrated in graded ethanol solution. After cleaning in pure xylene, the slices were cover-slipped with Permount before the xylene dried out. Four coronal brain sections were imaged per animal in the brain area from Bregma -1.06 to Bregma -2.70 based on the mouse brain stereotaxic coordinates.

**Statistical analysis**
Results are presented as means ± S.D. Data from the training sessions of Barnes maze were analyzed by two-way repeated measures analysis of variance followed by Tukey’s test. The other data were analyzed by one-way analysis of variance followed by Tukey’s test or by Student’s t
test as appropriate. Differences were considered significant at a \( P<0.05 \). All statistical analyses were performed with SigmaStat (Systat Software, Inc., Point Richmond, CA, USA).

**RESULTS**

One mouse died in the LCCAO group on day 1 after surgery. No animals died in the sham group during the surgery and the following observation period.

LCCAO induced learning and memory impairment

Mice in both LCCAO and sham surgery groups took less time on the fourth training day than on the first training day to identify the target box in the Barnes maze test. LCCAO was a significant factor to affect the mouse performance in these training sessions \( [F(1, 19)=11.398, P=0.003] \). Mice in the LCCAO group took a longer time than mice in the sham group to find the target hole on day 1 after the training ses-
sions. However, this effect did not reach statistical significance on day 8 after LCCAO initiation ($P=0.149$). The context- and tone-related freezing behavior in the LCCAO group of mice was not different from that of sham surgery group (Figure 1).

**Neuronal death and whiter matter damage was not observed after LCCAO**

There were no morphological changes of neuronal death and white matter damage in the left hippocampus and corpus callosum after LCCAO. No histological difference was observed in these regions between LCCAO group mice and sham mice (Figure 2 and 3).

**Neuronal and axonal degeneration was not observed after LCCAO**

Degeneration of neuron and axon was not observed in the left hippocampus and corpus callosum after LCCAO (Figure 4).

**DISCUSSION**

In this study, a mouse model of chronic cerebral hypoperfusion induced by LCCAO was established. Our results revealed that LCCAO conducted in 8-week-old mice developed significant spatial learning and memory deficits during the training sessions and one day after the training sessions, suggesting significant spatial learning and memory impairment. However, there was no impaired performance of mice in fear conditioning test that evaluates the non-spatial working memory. These results suggest that only certain forms of learning and memory are affected in this model.

However, in the histo-pathological examination, not only neuronal cell damage but also neuronal and axonal degeneration were not observed in the selected brain regions of ipsilateral hemisphere including hippocampus and corpus callosum.

We decided to start learning and memory tests 2 weeks after LCCAO. These tests take about 2 weeks to complete. Two weeks in mouse life may
be equivalent to months in human life. In addition, unilateral common carotid ligation induces a sharp decrease in the ipsilateral cerebral blood flow with the peak reduction at 2 hour after the ligation in mice. The decreased cerebral blood flow lasts for at least 28 days. Accompanying these changes in cerebral blood flow, there are an increase in proinflammation cytokines and a decrease in inhibitory cytokines in the brain. These acute changes occurred within 3 days after the ligation of unilateral common carotid artery (10). Thus, acute brain changes may have been over within 2 weeks after LCCAO and testing learning and memory after this time may represent a change at a delayed phase.

Among the animal models of chronic cerebral hypoperfusion, permanent 2-VO has been widely used but has a high mortality rate and reduced blood flow to both cerebral hemispheres suddenly, which does not match the situations in patients (7-9). A study performed on adult C57BL/6J mice with permanent RCCAO showed that 1-VO induced non-spatial memory deficits detected by objective recognition test, but these mice did not have spatial memory deficits detected by T maze spontaneous alternation test. These two behavioral assessments were used to demonstrate the frontal- subcortical circuits related pathological changes in the brain (10). Another previous study showed progressive deficit in spatial learning and memory that was peaked 2 months after right common carotid artery was occluded in young rats. This impaired spatial learning and memory detected by radial arm and water maze test were recovered 4 months after surgery. However, the impairment detected by Morris water maze reappeared when these rats grew to 18 months old (11). In our study, spatial learning and memory deficit detected by Barnes maze test was shown in mice at 2 weeks after LCCAO. However, both context- related learning and memory and tone- related freezing behavior were not affected in mice with LCCAO. Fear conditioning tests non- spatial, amygdala- and hippocampus-dependent learning and memory (15). Together, these results suggest that some forms of learning and memory may not be significantly impaired and that at least two learning and memory test paradigms that test different forms of learning and memory shall be used to detect the learning and memory impairment

Previous studies have suggested that learning and memory deficit in 1-VO model parallels with its pathological changes, especially ischemic hippocampal and white matter damage under chronic cerebral hypoperfusion (10, 11). Hippocampal injury is often studied under chronic cerebral hypoperfusion, because hippocampus is highly implicated in learning and memory (16). White matter lesions had received increasing attentions because its injury has been found to correlate with cognitive disorder in clinical patients (17). In animal models, 2-VO in rats displayed characteristic neurodegeneration in hippocampus (18); whereas no apparent damage was found in the hippocampus of mice with both RCCAO and LCCAO (10, 19). Several studies have identified that corpus callosum exhibits typical ischemia-related histo-pathologic changes as the consequence of 2-VO in mice and rats (20, 21). In RCCAO model, there was similar white matter damage in the corpus callosum both in rats and mice (10, 11). In our study, consistent with the previous mouse study (19), no hippocampal neuronal damage was observed in sections with HE staining following LCCAO. Silver staining is used to visualize degenerating neurons and their processes in various animal models (14, 22). In our study, there was no typical dark staining of degenerating axons in hippocampus. Thus, our results provide the evidence that LCCAO does not cause significant white matter lesions.

Our study did not show significant cerebral structural damage. Two possible mechanisms may contribute to this phenomenon. One is the development of collateral arteries and autoregulatory vasodilatation in the ipsilateral hemisphere to maintain blood supply at the occluded side. Another mechanism is that repeated training in the learning and memory tests might stimulate brain to reduce brain injury (23- 25). All three previous studies that found to have neuropathological changes after RCCAO were performed with using C57BL/6J mice (an inbred strain) (10, 26, 27); whereas CD- 1 adult mice (an outbred strain) were used in our study. Currently, there are no studies demonstrating that the side of common carotid artery (right or left) that is ligated will determine whether the white matter damage occurs or not. It seems that differ-
ent mouse strains used may be one of the reasons to explain why we did not see white matter damage but previous studies have shown brain injury after unilateral common carotid artery ligation. Thus, it still needs further study to clarify why there was no white matter damage in mice underwent LCCAO compared with that in mice underwent RCCAO. In addition, it has been reported that the mortality rate of RCCAO in rats is as low as 5% to 10% (11). A previous study and our current study demonstrated that the mortality rate of unilateral common carotid artery occlusion in mice is even lower than that in rats (10). Thus, it may be useful to further develop the mouse model to determine the effects of chronic cerebral hypoperfusion on brain function.

In summary, consistent with previous RCCAO, the chronic cerebral hypoperfusion induced by LCCAO in mice resulted in significant spatial learning and memory deficit but without histo-pathological changes in the brain. These results indicate that this model can be used to study VD-related learning and memory changes.

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