

# Post-ischemia common carotid artery occlusion worsens memory loss, but not sensorimotor deficits, in long-term survived stroke mice

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## ABSTRACT

Ischemic stroke in rodents is usually induced by intraluminal occlusion of the middle cerebral artery (MCA) via the external carotid artery (ECA) or the common carotid artery (CCA). The latter route requires permanent CCA occlusion after ischemia, and here, we assess its effects on long-term outcomes. Transient occlusion of MCA and CCA was performed at normal body temperature. After 90 min of ischemia, mice were randomized to permanent CCA occlusion or no occlusion (control group). Body weight, and motor and sensory functions, ie, pole test, adhesive tape removal, and elevated plus maze, were evaluated at 24 h, and at 7 and 28 days after stroke. Infarct volume, apoptosis, and activation of astrocytes and microglia were assessed at 4 weeks by an investigator blinded to groups. The Morris water maze test was performed at 3 weeks in the second experiment. One mouse died at 4 days, and the other mice survived with persistent neurologic deficits. CCA-occluded mice exhibited delayed turn on the pole at 24 h and decreased responses to the von Frey filament, and spent more time on the pole at 7 and 28 days than the control group. Infarction, hemispheric atrophy, glial activation, and apoptotic neuronal death were present in all mice, and no intra-group difference was found. However, CCA-occluded mice had a significantly poorer performance in the Morris water maze compared to the control group, which showed an adverse effect of post-ischemia CCA occlusion on cognition. Thus, the model selection should be well considered in preclinical efficacy studies on stroke-induced vascular dementia and stroke with Alzheimer's disease.

## 1. Introduction

Ischemic stroke is a life-threatening disease in which part of the brain is deprived of its blood supply due to an unexpected blockage in the feeding artery. Currently, the best treatment option is early administration of tissue plasminogen activator (tPA) or endovascular

thrombectomy (Faysel et al., 2019; Bhan et al., 2020; Yang et al., 2020). However, the efficacy is critically impacted by both the status of the patient (eg, hypertension, diabetes, and obesity) and the time lapse to treatment after stroke onset. Further, the survivors must live with various physical and mental disabilities. Thus, novel drugs unique to these populations are urgently needed, and animal models for

**Abbreviations:** AFIM, admission functional independence measure; ANOVA, analysis of variance; CBF, cerebral blood flow; CCA, common carotid artery; CCAO, common carotid artery occlusion; ECA, external carotid artery; GFAP, glial fibrillary acidic protein; HBP, hexosamine biosynthetic pathway; H & E, hematoxylin and eosin; iba1, ionized calcium binding adaptor molecule 1; IQR, interquartile range; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; SD, standard deviation; tPA, tissue plasminogen activator; TTC, 2,3,5-triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; xbp1, spliced X-box binding protein 1.

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preclinical studies should be appropriately selected.

In the first ischemic stroke model, developed by Tamura in 1981 (Tamura et al., 1981), a subtemporal craniectomy was performed to expose the proximal segment of the middle cerebral artery (MCA), which was then occluded between its branches to the rhinal cortex and the lateral striate arteries. In 1986, Koizumi developed a stroke model that required no craniectomy but that used instead an intraluminal approach to occlude the MCA through a small cut on the common carotid artery (CCA) (Koizumi et al., 1986). And in 1989, Longa formalized a reversible MCA occlusion stroke model that inserted a filament through the external carotid artery (ECA) (Zea Longa et al., 1989).

Dissection and ligation of ECA branches requires additional surgical time and skills, but insertion of a filament directly through the CCA simplifies the surgical procedure. Therefore, the Koizumi method is popular (Xiong et al., 2011; Xiong et al., 2013; Han et al., 2009; Wang et al., 2017), even for preclinical drug development (Wang et al., 2017). An acute study that compared these 2 intraluminal occlusion methods reported high mortality in the Koizumi method and more inflammation in the Longa method (Smith et al., 2015). Ischemic damage was detected at 30 min after reperfusion using the Koizumi method, but more reperfusion-related changes were observed using the Longa method (Morris et al., 2016). Interestingly, the long-term effects of isoflurane on focal cerebral ischemia were assessed using each method in different laboratories, and the protective effect of isoflurane was found in the Longa method (Sakai et al., 2007), but not in the Koizumi method (Inoue et al., 2004). Permanent CCA occlusion reduces the collateral blood flow to the ischemic area, and likely contributes to this difference. Studies have demonstrated that unilateral CCA occlusion causes cerebral hypoperfusion (Zhao et al., 2014; Somredngan and Thong-Asa, 2018) and thus, occlusion of bilateral CCAs serves as the model for chronic cerebral hypoperfusion (Yuan et al., 2017; Wang et al., 2016). Some researchers repaired the incision on the CCA to restore blood flow after ischemia (Trotman-Lucas et al., 2017); however, subsequent CCA stenosis was present in most animals (Dittmar et al., 2005). Here, to inform our search for the optimal model for preclinical drug development, we investigated the effect of post-ischemia unilateral CCA occlusion on long-term stroke outcomes.

## 2. Materials and methods

The protocols for the following study were approved (A159-17-06 and A100-20-05) by the Duke University Animal Care and Use Committee. Young male C57Bl6 mice (8–10 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA), and housed at the Duke animal facility with free access to food and water, and a 12-hour light/dark cycle. Room temperature and humidity are well controlled.

### 2.1. Focal cerebral ischemia model

Destruction of carotid artery branches affected the outcome in ischemic stroke studies (Trotman-Lucas et al., 2017; Dittmar et al., 2003; Chen et al., 2008). To avoid this adverse effect, a transcranial proximal middle cerebral artery occlusion (MCAO) model was used in this study, as previously described (Jiang et al., 2017). Mice were fasted with free access to water the night before surgery. Briefly, mice were anesthetized with 2% isoflurane in 30% oxygen balanced with nitrogen, intubated orally, and ventilated. Rectal temperature was maintained at  $37.0\text{ }^{\circ}\text{C} \pm 0.2\text{ }^{\circ}\text{C}$  using a heating lamp and blanket during surgery and ischemia. Mice were placed in the supine position, and a neck incision was made to expose the right CCA, which was temporarily ligated using a soft silk suture. Mice were then placed in the left lateral position, and a small skin incision was made between the eye and ear. The lowest part of the temporal muscle was slightly cut using a high temperature loop to expose the zygomatic arch, and a 3-mm segment of the arch was removed. With the help of 4 small needle retractors, the skull base and trigeminal nerve branch were clearly exposed. The proximal MCA and

its cortical branch were visible through the transparent skull. A small window ( $1 \times 2\text{ mm}$ ) was then gently drilled to the surface of the MCA, and the thinned skull was peeled off using the tip of fine forceps. A segment of the proximal MCA was lifted with an 8–0 needle (ETHILON, San Lorenzo, Puerto Rico), and blood flow was blocked by fastening the MCA under the needle using a single strand of 4–0 black silk suture. The needle was withdrawn at the end of 90 min ischemia to restore blood flow through the MCA, and the silk suture was removed. The muscle and skin layers were then closed separately.

### 2.2. Group assignment

Following the MCAO procedure, mice were randomly assigned to post-ischemia unilateral CCA permanent occlusion (CCAO) or no CCA occlusion (Control) ( $n = 10/\text{group}$ ). The silk suture on the right CCA was removed in the control group. The skin incision was sutured, and antibiotic and pain relief ointment were applied to the surface. Mice were returned to their home cages after recovering from the anesthesia, and were survived for 28 days.

A second experiment was performed as above, and included CCAO ( $n = 6$ ), control ( $n = 6$ ), and sham ( $n = 3$ ) groups. A Laser Doppler probe was glued on the temporal skull for cerebral blood flow monitoring before, during, and 10 min after ischemia. At 3 weeks after stroke, the Morris water maze test was performed, and brain infarct volume was measured after TTC staining.

### 2.3. Outcome measurements

#### 2.3.1. Body weight

Mice were weighed daily during the first week after stroke, and once weekly thereafter. Soft food was provided daily only for the first week.

#### 2.3.2. Neuroscore

Neurologic deficits were assessed at 24 h, and at 7 and 28 days after ischemia using the scoring system we previously reported (Taninishi et al., 2016), which includes spontaneous activity, body symmetry, gait, front limb symmetry, circling spontaneously or while holding tail, beam walking, vertical screen climbing, body proprioception, and vibrissae touch and tactile response. This scoring system has 14 categories with 0–4 points each. The score given to each animal at the completion of the testing was the sum of the points for all categories: 0 was the minimum score (best), and 48 was the maximum score (worst). The observer was blinded to group assignment.

#### 2.3.3. Von Frey filament

Response of the left front paw to filament stimulation is decreased after right MCAO. This response was examined prior to stroke, and at 24 h, and 7 and 28 days post ischemia. Bottomless plexiglass boxes ( $10 \times 10 \times 12\text{ cm}$ ) with a flip-top cover were placed on the screen. Mice were introduced to the boxes 15 min before testing. A series of touches with a plastic filament varying in force from 0.008 to 26 g (Touch Test Sensory Evaluators, North Coast Medical, Inc, Morgan Hill, CA, USA) was then applied to the front paw through the screen grids, and the force of the filament was recorded when mice had a quick paw withdrawal at the touch of the filament.

#### 2.3.4. Pole test

A vertical steel pole (60 cm) with a rough surface was used. Mice were trained prior to surgery, and tested at 24 h, and at 7 and 28 days post ischemia. Mice were placed at the top of the pole with head up, and the activities were monitored. The time lapse to make a complete turn and the time lapse to descend the pole were recorded.

#### 2.3.5. Adhesive tape removal

Mice were trained on tape placement and removal before testing. At 28 days post ischemia, 2 researchers worked together to perform the

test. One held the mouse, and attached a patch of tape firmly to the front paw. Mice were gently released into an empty mouse cage. The other researcher monitored mouse movement, and recorded the time lapse for the mouse to touch the paw and remove the tape. Both left and right front paws were tested.

#### 2.3.6. Elevated plus maze

This test is a measure of post-injury anxiety, and was performed at 28 days post ischemia. The plus-shaped apparatus has 2 open arms and 2 closed arms elevated 50 cm above the floor. Each mouse was placed at the intersection of the 4 arms heading toward the open arm, and was observed for 5 min. The time lapse for the mouse to enter the open and closed arms, as well as the time spent in the open and closed arms were recorded.

#### 2.3.7. Morris water maze

A 7.5-cm diameter platform was hidden under the water in a 105-cm diameter round pool, and 4 signs of different size and color were posted on the wall. The water was kept at 22°C, and made opaque using dry milk powder. Mice were placed on the platform for 15 s before testing. Then, mice were gently induced into the water, and the location of the platform was rotated clockwise for each trial. If unable to find the platform after 90 s, the mouse was assisted to the platform and left there for 30 s on the first trial and 15 s on subsequent trials in order to become oriented. Four trials were conducted each day for 5 consecutive days. The inter-trial interval was 30 min, and during this interval, mice were placed in a heated cage. After 4 trials on day 5, the platform was removed, and one 60-second trial was performed to evaluate their memory for the location of the platform. Mice were induced into the pool from the east zone. The time spent in the west zone (platform zone), and the speed and distance traveled were recorded for each mouse.

#### 2.3.8. Histologic analysis

After all function tests, mice were anesthetized with isoflurane and intubated. Intra-cardiac perfusion was performed with heparinized saline followed by 10% formalin. Brains were harvested, imaged, and paraffin-embedded. A series of tissue sections was cut at a thickness of 5  $\mu\text{m}$ , and stained using hematoxylin and eosin (H&E), luxol fast blue (Luxol fast blue, Sigma-Aldrich, St. Louis, MO, USA), and TUNEL (In situ apoptosis detection kit, catalog #4812–30-K, Trevigen, Gaithersburg, MD, USA), and immunohistochemistry of NeuN (1:500 monoclonal mouse anti-NeuN antibody; Millipore, Burlington, MA, USA), GFAP (1:500 polyclonal rabbit anti-gial fibrillary acidic protein; Dako, Carpinteria, CA, USA), and Iba-1 (1:500 rabbit anti-iba1; catalog #019–19741, Wako, Richard, VA, USA) was performed. Infarct size, brain atrophy, and color density of corpus callosum were measured using an M2 turnkey system image analyzer. Numbers of activated astrocytes and microglia, and apoptotic neuronal cells were assessed manually under light microscopy. The spleen was also harvested and weighed.

**TTC staining and infarct size measurement** Mice were anesthetized with 5% isoflurane and decapitated. Brains were harvested and immersed in ice-cold saline for 20 min. Brains were then cut into slices 1 mm thick, and transferred into a 2% TTC solution. After the brain slices turned red, they were removed from solution, and stored in 10% formalin. The next day, the brain slices were imaged with a video camera controlled by an image analyzer (M2 Turnkey System, Imaging Research, Inc., St. Catharines, Ontario, Canada). The image of each slice was stored as a 1024×1024 matrix of calibrated pixel units. The digitized image was then displayed on a video screen. With the observer blinded to the experimental condition, the infarct border was outlined using an operator-controlled cursor. The area ( $\text{mm}^2$ ) was determined automatically by counting the pixels within the outlined regions of interest. The volumes ( $\text{mm}^3$ ) were computed as running sums of interest area multiplied by the thickness. The infarct volume was calculated as the brain volume of the left hemisphere minus the remaining brain volume of the

right hemisphere.

#### 2.4. Statistical analysis

Most of the data were expressed as mean  $\pm$  SD, and analyzed using unpaired student *t*-test or two-way ANOVA with repeated measures for behavioral performance except for neurologic score, which is nonparametric, expressed as median  $\pm$  IQR, and analyzed using the Mann-Whitney U test. All statistical analyses were performed using Prism 6 software (GraphPad Software, Inc, San Diego CA, USA), and a *P* value  $<$  0.05 was considered statistically significant.

### 3. Results

One mouse in the CCAO group died 4 days after ischemia, but no hemorrhage was found. All other mice recovered from injury, and were survived for 28 days. The CCAO group had a lower body weight at 7 days (26.9 g  $\pm$  1.4 g) compared to the control group (27.5 g  $\pm$  2.4 g, *P* = 0.54). The CCAO mice gradually gained body weight, and reached the weight of the control group by the end of the experiment (Fig. 1). In the second experiment, all mice survived for 3 weeks.

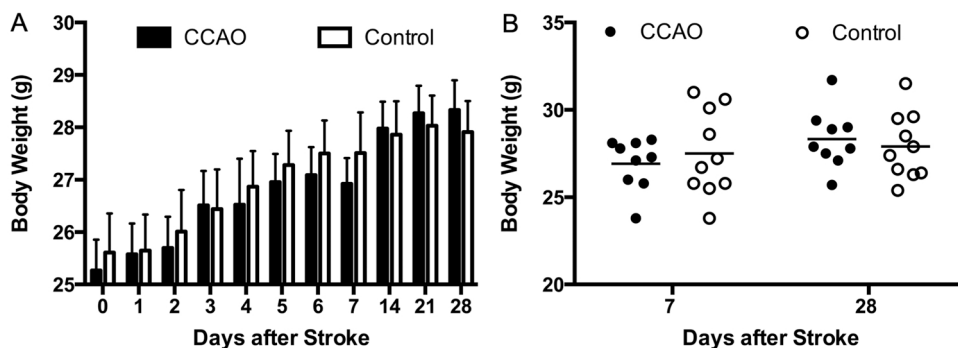
Cerebral blood flow (CBF) was monitored before, during, and at 10 min after ischemia in the second experiment, using a Laser Doppler fiber probe glued on the temporal skull. In both the CCAO and control group, CBF was reduced to  $<$  20% of baseline during ischemia (Fig. 2). CBF recovered slowly in CCAO mice. At 10 min after reperfusion, CBF in CCAO mice was 47%  $\pm$  10% vs 80%  $\pm$  16% in controls (*P*  $<$  0.01).

Based on our comprehensive neurologic examination, neurologic deficits were found in all stroke mice. The neurologic score was highest at 24 h post ischemia and then, improved over time (Fig. 3A). The score was slightly higher in the CCAO group at 24 h post ischemia compared to controls (14  $\pm$  3 vs 12  $\pm$  3, *P* = 0.37), but matched the control group at 7 and 28 days.

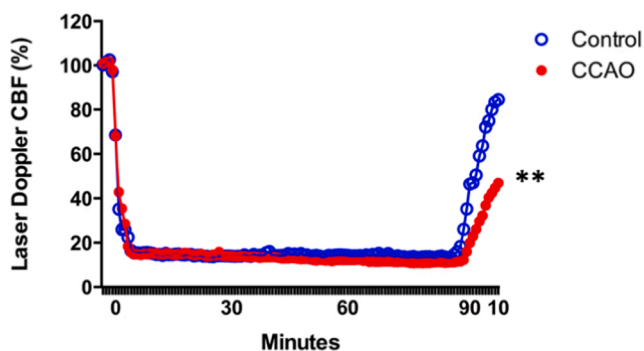
The Von Frey filament test is a quantitative measure of sensory loss in stroke mice, with greater filament forces needed to elicit paw withdrawal representing more severe sensory loss. The filament forces needed to elicit a response from the left paw were significantly greater in both groups on days 1, 7, and 28 after ischemia vs day 0 (Fig. 3B, *P*  $<$  0.01). Stroke did not affect right paw function and therefore, the filament forces needed to elicit a response from the right paw were significantly smaller compared to the left paw (*P*  $<$  0.01). The CCAO group required a greater force than the control group on days 1, 7, and 28, but no significant inter-group differences were detected (*P* = 0.25, 0.37, and 0.20, respectively).

The pole test assesses the ability of mice to climb on the upper part of a vertical pole, turn around when it reaches the top, and walk down to the floor. Latency for stroke mice to complete this action on the pole was longer on day 1 post ischemia vs day 0 prior to injury. Latency to turn around was significantly longer in CCAO mice on day 1 vs day 0 (Fig. 3C, *P* = 0.008), while the turn time was only slightly longer in the control group (*P* = 0.07). Latency to walk down the pole was slightly longer in both groups on day 1 post ischemia vs day 0 prior to injury (*P* = 0.48 in CCAO mice and 0.41 in controls). However, on day 7 post ischemia, control mice had recovered better, and latency to walk down the pole was significantly shorter compared to CCAO mice (*P* = 0.04). By the end of 4 weeks of recovery, latency to walk down the pole was significantly shorter in both groups (day 28 vs day 1, *P* = 0.04 in CCAO mice and 0.0005 in controls); however, the difference between groups was not significant on day 28 (8.2  $\pm$  3.4 s in CCAO mice vs 6.6  $\pm$  1.9 s in controls, *P* = 0.22).

Adhesive tape removal is a test that evaluates paw sensory and motor functions by measuring latency to feel a small square of adhesive tape on the paw and to remove it. After ischemic stroke in the right hemisphere, latency to remove the tape on the left paw at 28 days post stroke was significantly longer compared to the right paw (*P* = 0.006 in the control group, Fig. 3D). Some mice in the CCAO group did not learn well in the



**Fig. 1.** Body weight changes after ischemia. (A) Body weight during 28 days of recovery. (B) Body weight at 7 and 28 days. CCAO mice had a slight loss in body weight within the first week after stroke (CCAO group: n = 9; controls: n = 10).

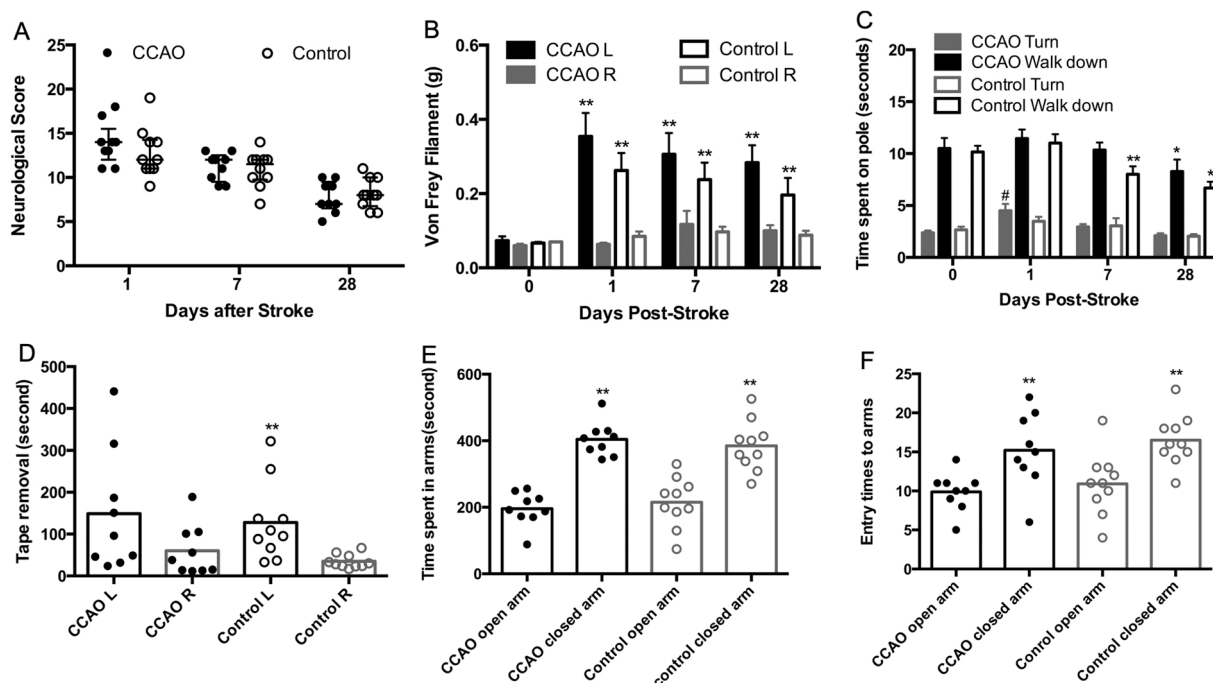


**Fig. 2.** Regional cerebral blood flow (CBF) measured by Laser Doppler before, during, and after surgery. In CCAO (n = 6) and control (n = 6) groups, CBF was < 20% of baseline during ischemia, and recovered after reperfusion. CBF recovery was significantly slower in CCAO mice. \*\* P < 0.01 vs control mice.

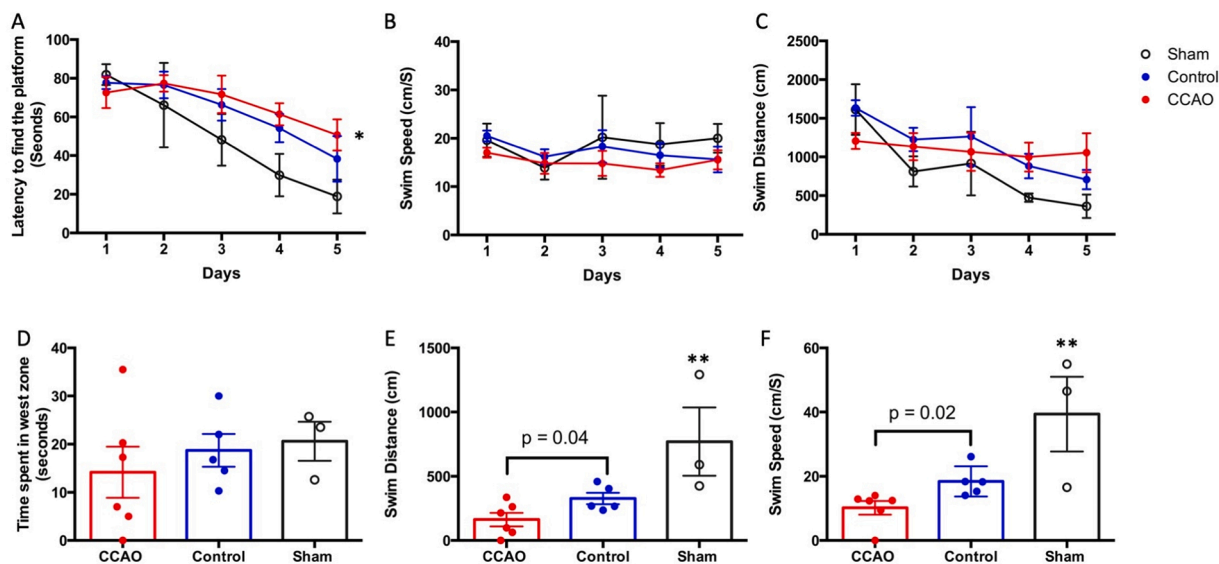
training trials, and spent more time removing the tape on the right healthy paw. Latency to remove the tape on the left paw was longer, but was not significantly different from the right paw (P = 0.108 in the CCAO group). An inter-group difference in left paw performance was absent at 28 days (P = 0.7).

The elevated plus maze is a test of anxiety, and injured mice prefer to stay in closed arms. In this study, we found that both the times to enter the closed arms and the time spent in the closed arms were significantly increased in both the CCAO and control groups compared to the open arms (Fig. 3E, the times to enter the closed arms vs open arms, P = 0.009 and 0.002 in CCAO and control groups, respectively, Fig. 3E; the time spent in the closed arms vs open arms, P < 0.0001 in both groups, Fig. 3F). No significant inter-group difference was detected in the entry times (P = 0.502) and the time spent in the closed arms (P = 0.521).

In the second experiment, stroke-induced cognitive deficits were assessed 3 weeks after surgery, using the Morris water maze. CCAO mice learned more slowly, and latency to find the hidden platform was significantly longer compared to sham mice (50.72 ± 19.87 s vs 18.84 ± 15.06 s, P < 0.01, Fig. 4A). Control mice had a long latency (38.33 ± 26.42 s); however, there was no significant difference compared to



**Fig. 3.** Neurologic functional deficits after ischemia. (A) Neurologic score (median ± IQR). (B) Von Frey filament test, \*\* P < 0.01 vs CCAO R or control R (C) Pole test, #P < 0.01 vs CCAO turn on day 0; \* P < 0.05 vs walk down on day 1; \*\* P < 0.01 vs CCAO walk down on day 7. (D) Adhesive tape removal test, \*\* P < 0.01 vs control R. (E and F). Elevated plus maze, \*\* P < 0.05 vs open arms.



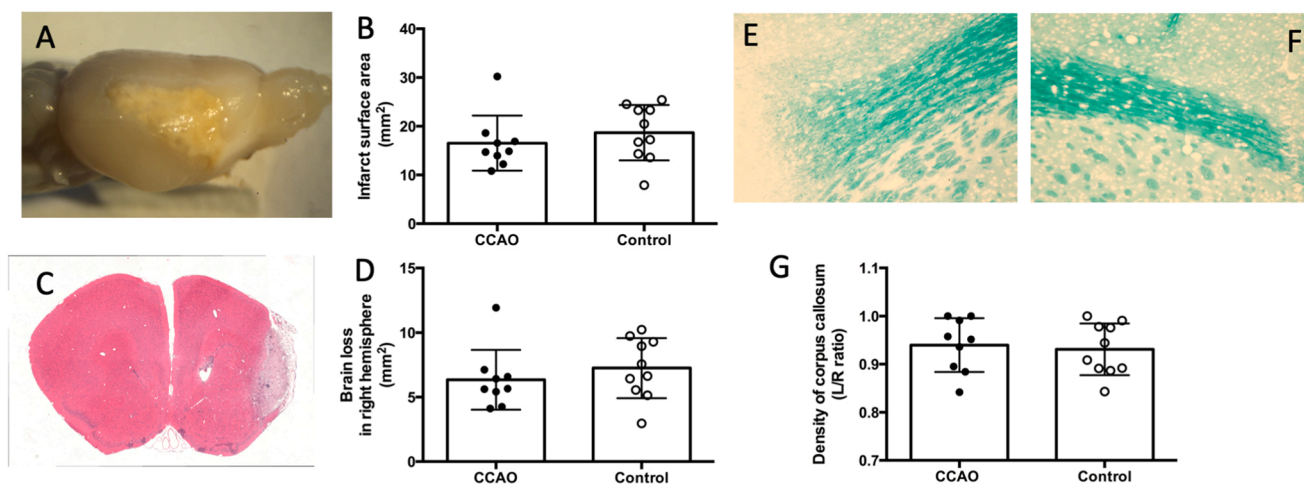
**Fig. 4.** Using Morris water maze to detect cognitive deficits after stroke. 15 mice were enrolled (CCAO n = 6, control n = 6, and sham n = 3). A-C, 5 days of water maze with platform in the west zone. D-F, last trial with platform removed. Time, speed, and travel distance were recorded. CCAO group had a poor performance, even compared to the stroke control group. \*  $P < 0.05$  vs sham group, \*\*  $P < 0.01$  vs CCAO or control group.

CCAO or sham groups. The swim distance had a similar pattern. CCAO mice traveled a long distance to find the platform ( $1054 \pm 618$  cm), but no inter-group difference was detected (control group  $706 \pm 279$  cm and sham group  $362 \pm 260$  cm,  $P > 0.05$ , Fig. 4C). In the end, the platform was removed, and memory was further tested. CCAO mice spent less time searching in the west zone where the platform had been located. One mouse in the CCAO group was slow to search, so the time spent searching was not different from the other 2 groups (CCAO  $14 \pm 12$  s, control  $18 \pm 8$  s, and sham  $21 \pm 8$  s, Fig. 4D). However, there were significant differences in speed and distance traveled. The CCAO group had a poor performance compared to control or sham groups (swim speed: CCAO  $10 \pm 5$  cm/s, control  $18 \pm 4$  cm/s, sham  $39 \pm 20$  cm/s;  $P = 0.02$  CCAO vs control,  $P < 0.01$  sham vs CCAO or control, Fig. 4E; travel distance: CCAO  $162 \pm 129$  cm, control  $327 \pm 98$  cm, sham  $769 \pm 460$  cm;  $P = 0.04$  CCAO vs control,  $P < 0.01$  sham vs CCAO or control, Fig. 4F).

The edge of the infarct area was clearly identified in all stroke mice at 28 days. The CCAO group had a slightly smaller stroke surface area

compared to controls ( $16.5 \pm 5.6$  mm<sup>2</sup> vs  $18.7 \pm 5.6$  mm<sup>2</sup>, respectively,  $P = 0.42$ , Fig. 5A, B). A series of tissue sections was cut from the paraffin-embedded brain samples, and brain atrophy was calculated from the difference between normal areas in the left vs right brain hemispheres. Brain loss was less in the CCAO group compared to controls ( $6.3 \pm 2.3$  mm<sup>2</sup> vs  $7.2 \pm 2.3$  mm<sup>2</sup>, respectively,  $P = 0.41$ , Fig. 5C, D). Ischemic tissue does not stain well, and the color intensity of the corpus callosum was lighter on the ischemic side. The color ratio of left to right corpus callosum was  $93.9 \pm 0.7\%$  in CCAO mice vs  $93.1 \pm 0.1\%$  in controls ( $P = 0.73$ , Fig. 5E, F, and G). The post-stroke immune response decreases spleen size, which serves as an additional outcome parameter. The average spleen weight was  $0.092 \pm 0.015$  g in the CCAO group and  $0.083 \pm 0.09$  g in the control group ( $P = 0.17$ ).

In the second experiment, brains were harvested 3 weeks after surgery, and stained with TTC. Brain volumes of the left hemisphere and the remaining part of the right hemisphere were measured, and the infarct volume was calculated. The damaged brain tissue was absorbed at this time. There was no infarct volume difference between CCAO and control



**Fig. 5.** Post-ischemia brain histology. (A) Representative CCAO brain with scar tissue on the right hemisphere. (B) Surface area of the infarct tissue. (C) Representative CCAO coronal section of the infarcted brain. (D) Brain loss was calculated from area difference between left and right hemispheres. (E) Representative CCAO brain corpus callosum next to infarct (100X). (F) Representative CCAO brain corpus callosum contralateral to ischemia (100X). (G) Color density ratio of left to right corpus callosum. No inter-group difference was found in these observations.

groups (CCAO  $35.72 \pm 10.98 \text{ mm}^3$  and control  $32.86 \pm 4.57 \text{ mm}^3$ ,  $P > 0.05$ , Fig. 6).

In addition to brain histopathology, inflammatory responses were assessed in these sections as subordinate evidence for the influence of CCAO on stroke outcomes. Immunohistochemical staining of GFAP and Iba-1 was performed, and the positive cells were counted in ischemic and non-ischemic hemispheres. In the ischemic hemisphere, there were  $42 \pm 7$  GFAP-positive cells in the CCAO group vs  $39 \pm 7$  in the control group ( $P = 0.34$ , Fig. 7A, B), and  $31 \pm 10$  Iba-1-positive cells in the CCAO group vs  $32 \pm 9$  in the control group ( $P = 0.72$ , Fig. 7C, D). The count for both GFAP- and Iba-1-positive cells was significantly higher in the ischemic vs non-ischemic hemisphere ( $P < 0.01$ ).

The main concern about filament insertion through the CCA vs post-ischemia permanent CCAO is the possibility of additional apoptotic neuronal cell death induced after CCAO, which would very likely abolish the benefit of acute treatment. TUNEL staining was used to address this concern, and the number of TUNEL-positive cells in the area next to the infarct was counted. There were  $110 \pm 50$  TUNEL-positive cells in the CCAO group vs  $95 \pm 56$  in the control group ( $P = 0.54$ , Fig. 7E, F). There was no significant difference between the 2 groups.

#### 4. Discussion

Intraluminal occlusion of the MCA has been a standard model for stroke studies in mice and rats for decades. The Koizumi and Longa MCAO methods are both widely used in laboratories around the world. Filament insertion through the CCA shortens the surgical preparation time, and is easy for beginners to learn. However, there is concern that permanent occlusion of the CCA after ischemia, which is required by the Koizumi method, may affect stroke outcome.

In 2015, Smith et al. were the first to compare the 2 MCAO methods. They subjected mice to 30 min MCAO followed by 24 h or one week reperfusion (Smith et al., 2015). The mortality at 24 h post stroke was 44% in the Koizumi group and 26% in the Longa group. The neurologic score in the Koizumi group was similar to the score in the Longa group at 24 h but interestingly, was significantly lower than the score in the Longa group at one week due to reperfusion-related inflammation in the Longa group. The Longa group had a small infarct volume at 24 h that

was significantly larger at one week compared to the Koizumi group. In 2016, Morris et al. reported their comparison of both methods. They subjected mice to 60 min MCAO and 24 h reperfusion (Morris et al., 2016). Although the post-ischemic reperfusion pattern was different, the total lesion volume and survival rate were not significantly different between the Koizumi and Longa methods at 4 h after reperfusion. However, neither of these studies compared long-term effects of these 2 MCAO methods.

Preconditioning with isoflurane produces dose-dependent neuroprotection in rat focal cerebral ischemia (Xiong et al., 2003). Isoflurane also provides neuroprotection in neonatal hypoxic ischemic brain injury (Burchell et al., 2013). To determine the extent to which isoflurane provides a long-term protective effect, 2 research groups performed similar experiments in rats. Inoue et al. subjected rats to 60 min focal cerebral ischemia using the Koizumi method, and assessed the infarct volume and neurologic function 14 days after reperfusion. The isoflurane group was not different from the vehicle group, but the combination of isoflurane with caspase inhibitor z-VAD-fmk did provide protection (Inoue et al., 2004). Using the Longa method, Sakai et al. (2007) subjected rats to 50 min focal cerebral ischemia, and assessed neurologic function and infarct volume at 14 days after reperfusion. Neurologic deficits and infarct volume were both significantly reduced after isoflurane treatment. Sakai et al. also showed that the infarct volume was larger in rats with permanent CCA occlusion compared to rats without post-ischemia CCA occlusion, even though neurologic function was not affected. This result is opposite to what Smith's group reported (Smith et al., 2015). It seems that different MCAO methods potentially result in different long-term outcomes, and this should be carefully considered when pursuing preclinical development of new compounds.

In the intraluminal filament stroke model, a silicone-coated nylon filament is inserted into the internal carotid artery and advanced to block the opening of the MCA. Because of several factors such as animal age, filament size, and artery vibration, the success of blocking the blood flow through the MCA varies in individual animals. Surgical preparation is perhaps the most important factor affecting stroke outcome. For example, Chen et al. (2008) reported that blocking pterygopalatine arterial blood flow decreases infarct volume variability. And Dittmar et al. (2003) found that when the ECA is cut, restoration of body weight

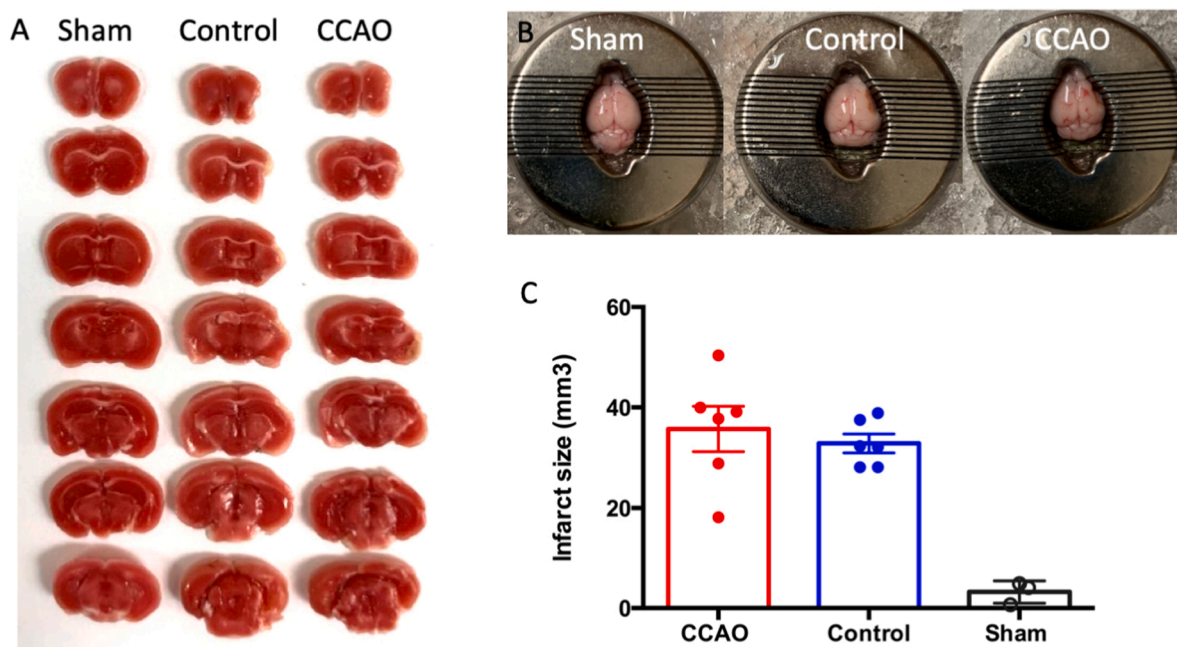
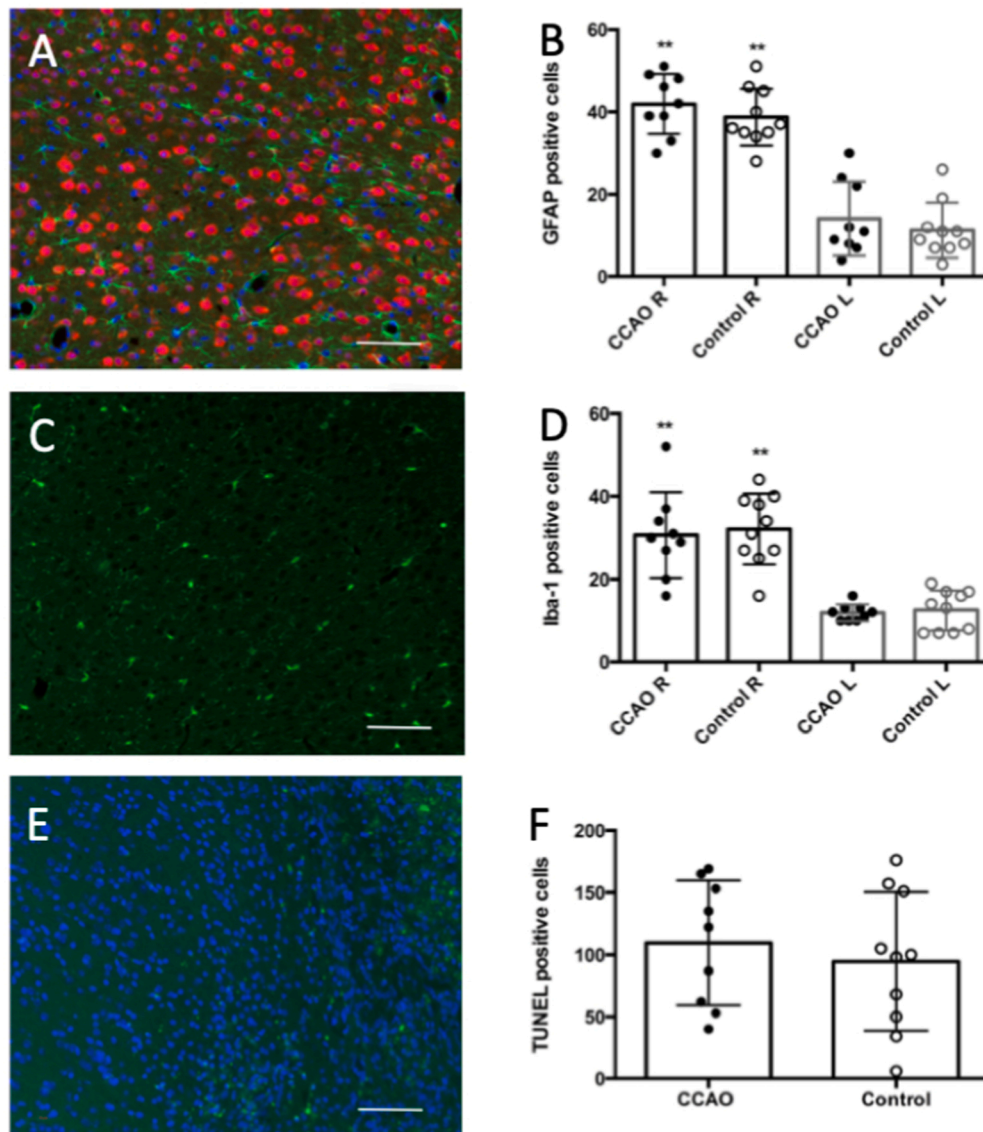


Fig. 6. Brain TTC staining and infarct volume measurement. (A) Representative images from 3 groups. (B) Top view of representative brains from 3 groups. (C) Infarct volumes. No infarct volume difference was found between CCAO and control ( $n = 6/\text{group}$ ,  $P > 0.05$ ).



**Fig. 7.** Astrocyte and microglial activation, and apoptotic neuronal death after ischemia. (A) Representative CCAO brain with double immunohistochemical staining of NeuN (Red) and GFAP (Green). (B) Number of GFAP-positive cells in each hemisphere of both groups,  $** P < 0.01$  vs left hemisphere. (C) Representative CCAO brain with immunohistochemical staining of Iba-1 (Green). (D) Number of Iba-1-positive cells in each hemisphere of both groups,  $** P < 0.01$  vs left hemisphere. (E) Representative CCAO brain with TUNEL staining (Green). (F) Number of TUNEL-positive cells. There was no inter-group difference in these observations. Bar = 100  $\mu\text{m}$ .

is delayed, and recovery of motor function is poorer. Thus, in this study, we used the transcranial MCA occlusion model, and preserved all carotid arteries and branches. The effect of post-ischemia permanent CCAO on long-term stroke outcomes was precisely compared. Neurologic function and histologic outcome are the most critical parameters assessed in translational studies for drug development. We found no significant difference in functional deficits and infarct volume between CCAO and control groups. Reperfusion increases inflammation, which potentially increases apoptosis. This has been demonstrated in the acute post-ischemic phase (Smith et al., 2015). In this study, however, we detected no significant difference in the number of GFAP-, Iba-1, and TUNEL-positive cells between groups.

In the second experiment, stroke-induced cognitive deficits were assessed using the Morris water maze. The results demonstrated that the CCAO group had a poor performance, worse than the control group, indicating that CCA occlusion truly affects cognitive functions even though the infarct volumes were not significantly different between the 2 groups. This may be related to axon degeneration. The color intensity of the corpus callosum may not be sufficient to detect this change. A more comprehensive investigation of white matter damage is needed.

After permanent CCA occlusion, blood flow to the MCA territory comes from the anterior and posterior communicating arteries. Using

the Laser Doppler technique, Morris et al. (2016) showed that blood flow returned to 70%–80% of baseline following ischemia induced by the Koizumi method. Although such blood flow recovery may be sufficient for essential activities of the brain, future studies should compare brain oxygen consumption and tissue metabolism between the 2 MCAO methods for further clarification.

The limitation of this study is that we did not have data from aged stroke mice due to insufficient funding. Age-related behavioral changes have been reported in normal C57BL/6 J mice (Shoji et al., 2016; Shoji and Miyakawa, 2019). General health and neuromuscular strength decline significantly in mice with advancing age. For example, young mice perform better on the wire hanging test, rotarod, open field test, light/dark transition test, social interaction test, startle response/pre-pulse inhibition test, elevated plus maze, and Barnes maze (Shoji et al., 2016). In stroke patients, a strong relationship between increasing age and poorer outcome was found in all patients with an admission functional independence measure (AFIM) score < 40, but not in those with an AFIM of 40–80 or > 80 (Black-Schaffer and Winston, 2004). An animal study showed that neurologic deficits are more severe in 16-month-old mice at 24 h post stroke compared to 2–3-month-old mice (Liu et al., 2009).

Aging also alters stroke-induced inflammatory and immunologic

responses, which influences stroke outcome (Ritzel et al., 2018). In 18–20-month-old stroke mice, circulating leukocytes and leukocytes infiltrating the ischemic brain were markedly increased, and higher levels of reactive oxygen species and extracellular matrix-degrading enzymes (ie, MMP-9), as well as hemorrhagic transformation were also observed (Ritzel et al., 2018).

Stroke induces activation of cellular pathways such as the unfolded protein response. A recent study reported that acute stroke outcome is improved in mice with neuron-specific overexpression of xbp1 via activation of the xbp1s/HBP/O-GlcNAc axis (Wang et al., 2021). This pathway is impaired in the aged brain; however, boosting O-GlcNAcylation with thiamet-G provided long-term improvement in neurologic recovery (Wang et al., 2021).

Post-stroke white matter damage is also age-dependent, and increased inflammation, greater secondary white matter atrophy, and worse behavioral outcomes were found in 24-month-old stroke mice (Rosenzweig and Carmichael, 2013). The importance of aging on ischemic stroke outcomes in preclinical animal models is further emphasized in a recent translational perspective paper (Candelario-Jalil and Paul, 2021).

In conclusion, unilateral CCA occlusion post ischemia partially affects the long-term outcome in young mice, especially cognitive function. These effects may be more profound in aged animals. Use of the Koizumi method in preclinical stroke studies is practical when only sensorimotor deficits and infarct volume are compared. However, it is critical to note that the effect of permanent CCA occlusion (the Koizumi method) on cognitive function may abolish the efficacy of new therapeutic candidates that target stroke-induced vascular dementia or stroke with Alzheimer's disease.

#### CRedit authorship contribution statement

**Zhong Yang:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Xuan Li:** Conceptualization, Methodology, Software, Validation, Investigation, Writing – original draft, Writing – review & editing. **Zhipeng Cao:** Methodology, Validation, Investigation, Writing – review & editing. **Peng Wang:** Methodology, Validation, Investigation, Writing – review & editing. **David S. Warner:** Conceptualization, Formal analysis, Resources, Writing – review & editing. **Huaxin Sheng:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition.

#### Conflict of interest

The authors have no conflicts of interest to declare.

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