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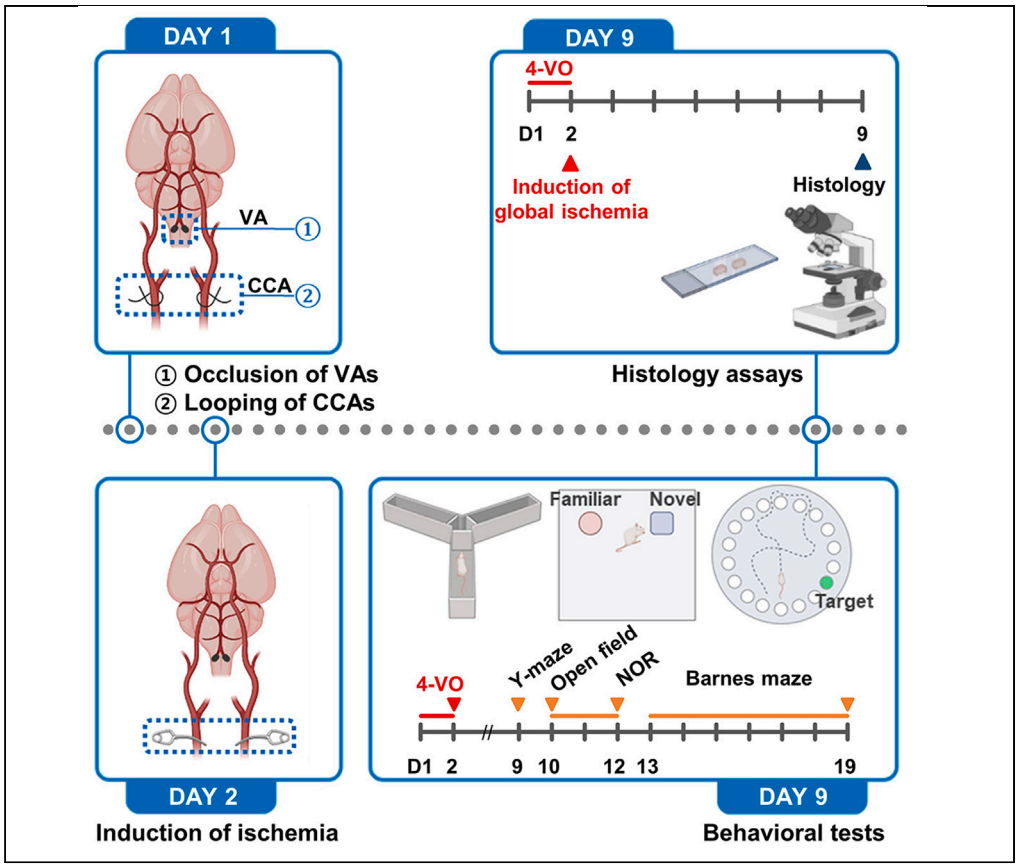
Protocol

Protocol for establishing a global ischemia model using a 4-vessel occlusion in rats



Global cerebral ischemia occurs when blood flow to the entire brain is transiently blocked, which results in delayed neurologic deficits. Here, we present a protocol for performing the four-vessel occlusion rat model to study the neurodegeneration and cognitive deficits associated with global ischemia. We describe steps for carrying out the vertebral and common carotid artery occlusion which enables sufficient blockage of cerebral blood flow. We then detail expected outcomes using histology assays and behavioral tests.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Details of optimized techniques for the vertebral artery occlusion

Step-by-step procedures for transient occlusion of the common carotid arteries

Instructions for scoring the degree of ischemic condition with the pupil dilation

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Protocol for establishing a global ischemia model using a 4-vessel occlusion in rats

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SUMMARY

Global cerebral ischemia occurs when blood flow to the entire brain is transiently blocked, which results in delayed neurologic deficits. Here, we present a protocol for performing the four-vessel occlusion rat model to study the neurodegeneration and cognitive deficits associated with global ischemia. We describe steps for carrying out the vertebral and common carotid artery occlusion which enables sufficient blockage of cerebral blood flow. We then detail expected outcomes using histology assays and behavioral tests.

For complete details on the use and execution of this protocol, please refer to Chung et al. (2022).[1]

BEFORE YOU BEGIN

This 4-VO protocol is used to study molecular mechanisms underlying the pathophysiology of cardiac arrest-induced global cerebral ischemia in rats.[2–6] Importantly, in this model, 'delayed neuronal death' is observed selectively in the hippocampal CA1 pyramidal layer, which is a clinical phenomenon that occurs in humans as well; neurodegeneration is not observed until ~3 days after 4-VO, but robust degeneration with associated cognitive deficits occurs after 7 days.[7–9] Therefore, 4-VO has been used as a well-established animal model of global ischemia to investigate the mechanisms of selective and delayed neuronal death of hippocampal CA1 neurons and hippocampal-based cognitive deficits.

Although this model has been used for a long time, the detailed protocol has not yet been published and it is not easy to follow simply because the surgical process is complicated and tricky. Here, we describe the steps necessary to perform the 4-VO rat model to study the neurodegeneration and cognitive deficits associated with global ischemia. Based on the initial method described by Pulsinelli and Brieley,[10] we have changed a few tools to improve the performance of surgical operations, and optimized surgical procedures but retained the steps of bilateral occlusion of vertebral and common carotid arteries. Performing the protocol, the surgical tools and methods can be further modified and replaced depending on the skills and knowledge of the investigators.

Institutional permissions

All animal care and procedures were approved by the Institutional Animal Care and Use Committee of Creighton University in accordance with National Institutes of Health guidelines.

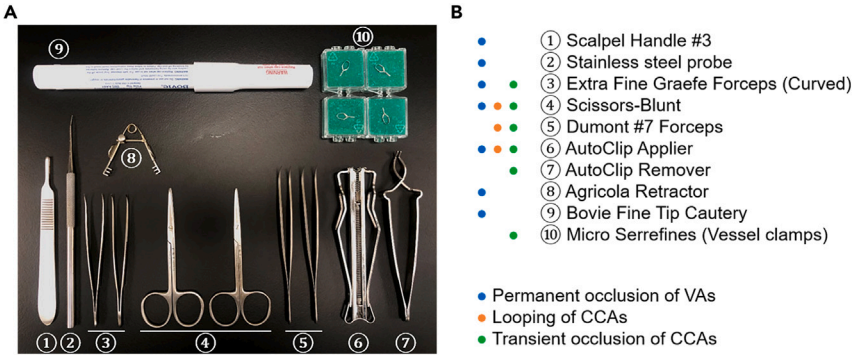

Figure 1. Preparation of surgical instruments

(A) The image shows the main surgical instruments for 4-VO.

(B) The names of the instruments in (A). Color dots denote the instruments needed for each indicated surgery step.

Preparation of surgical instruments

All instruments (Figure 1) should be autoclaved before use. In a series of surgeries, all surgical instruments must be washed with distilled water, rinsed with ethanol, and placed into a glass beads sterilizer for at least 15 s before the next surgery.

Anesthesia

Anesthesia is induced with 5% isoflurane mixed in oxygen at the flow rate of 0.8 L/min in an anesthesia chamber and is maintained with 2–3% isoflurane in the surgical places.

Personnel

Personnel performing the surgery wear personal protective equipment such as a mask, head cover, laboratory coat or gown, and gloves.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
6- to 8-week-old male CD®(Sprague Dawley) rats (200–250 g)	Charles River Laboratories	001CD
Other		
100% cotton gauze sponges	Covidien; Fisher Scientific	22-037-979
Agricola Retractor	Fine Science Tools	17005-04
Betadine surgical scrub	Fisher Scientific	19027132
Black silk braided suture material	Harvard Apparatus; Fisher Scientific	517615
Bovie High Temperature Cauteries Fine Tip	USA Medical and Surgical Supplies	BM-AA01
Dumont #7 Forceps	Fine Science Tools	11271-30
Extra Large Cotton Tipped Swab, Wooden Handle	VWR International	89031-272
EZ Clip Kit	Stoelting	59020
EZ Clips	Stoelting	59027
FB Alcohol Prep Pads	Fisher Scientific	22362750
Flunixin Injection (100 mL)	Patterson Vet	07-890-8177
Graefe Extra Fine Forceps – curved / /serrated	Fine Science Tools	11152-10
Hydrogel	Clear H2O	70-01-5022
Iris forceps (straight)	Fine Science Tools	11064-04
Isoflurane	Patterson Vet	07-893-1389
Lidocaine 5% Ointment	Patterson Vet	07-894-4636
Lidocaine HCl 2% Injection	Patterson Vet	07-892-4325

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Low Profile Anesthesia Masks for SomnoSuite (small, for mouse)	Kent Scientific	SOMNO-0801
Stainless Steel Micro Serrefines (Clips) 13 mm (Stainless Steel, Straight)	Fine Science Tools	18055-04
Optixcare Eye Lubricant + Hyaluron	Patterson Vet	07-893-2780
Scalpel Blades - #11	Fine Science Tools	10011-00
Scalpel Handle - #3	Fine Science Tools	91003-12
Fine Scissors – Straight / Blunt-Blunt / 11.510 cm	Fine Science Tools	14060-1114078-10
Scissors – Straight / Sharp-Sharp /10.5 cm	Fine Science Tools	91402-10
Stainless Steel Probe, Angled needle tip	Electron Microscopy Sciences	78326-07
Stereo Zoom Boom Microscope 7×-45×	Richter Optica Microscopes (VWR)	S6-BMSQ
Sterile saline solution	Aspen Veterinary Resources	46066-807-25
Triple Antibiotics Topical Ointment	Patterson Vet	07-893-9936
V-1 Tabletop Laboratory Animal Anesthesia System	VetEquip	901806
Vaporizer – Funnel-Fill	VetEquip	011103

MATERIALS AND EQUIPMENT

All resources, materials, and equipment are listed in the key resources table.

STEP-BY-STEP METHOD DETAILS

Timing: 2 days (overall)

The 4-VO model in this protocol involves a 2-day procedure inducing transient forebrain ischemia by permanent coagulation of the vertebral arteries and transient occlusion (10 min) of the common carotid arteries.

Stage I - Permanent occlusion of vertebral arteries (VAs): DAY 1

Timing: 20 min

1. Anesthetize the rat with 5% isoflurane mixed in oxygen at the flow rate of 0.8 L/min in the anesthesia chamber.
2. Using a hair clipper, shave the incision regions on the rat, which includes the back of the head and the front of the neck.
3. Transfer the rat to a stereotaxic apparatus, and fix the rat's head with ear bars (Figure 2A) (troubleshooting 1).

   Note: Adjust the anesthesia machine between 2% to 3% isoflurane for maintenance depending on the rat's condition; while maintaining 2% isoflurane is recommended during the surgery process, increase it to 3% at the moment when the rat seems not fully anesthetized and then reduce back to 2% once the rat becomes stable.

4. Apply the eye lubricant ointment to the rat's eyes.
5. Apply Betadine to the back of the head, and wipe with alcohol pads (repeat).
6. Make an incision up to 2 cm from the base of the skull using a scalpel blade (Figure 2B).
7. Separate the paraspinal muscles from the midline with scissors and find the base of the skull and alar foramen.
8. Expose the left and right alar foramen of the first cervical vertebra (C1) by scrapping the muscles and connective tissues from the alar foramen with forceps (Figure 2C).
9. Separate the skins and muscles to the left and right with a retractor (Figure 2D).

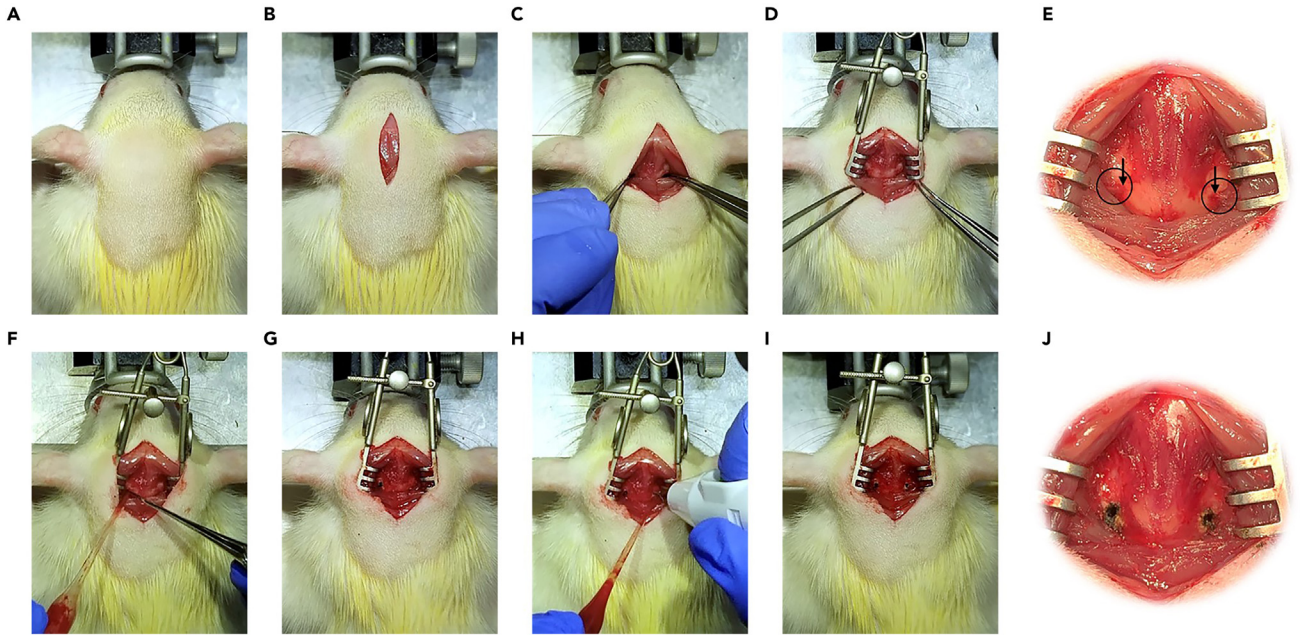

Figure 2. Procedures of permanent occlusion of VAs

(A) The rat's head was fixed in the stereotaxic apparatus.
(B) An incision was made vertically on the back head of the rat.
(C) The paraspinal muscles were separated.
(D) The alar foramen of the first cervical vertebra (C1) was exposed with a retractor.
(E) Enlarged images in (D). The black arrows indicate the alar foramen of the first cervical vertebra.
(F) Bleeding was induced by making a puncture in the left VA. Simultaneously, blood was aspirated by the glass pipette connected to the bench-top vacuum system.
(G) The left VA was cauterized by electrocoagulation.
(H) Bleeding was induced by making a puncture in the right VA and cauterized by a Bovie cautery.
(I) The cauterization of both VAs was confirmed (no bleeding from the VAs).
(J) Enlarged images in (I).

10. Insert a stainless-steel probe vertically through the inside of the foramen and induce bleeding by making a puncture in the VA (Figures 2E and 2F) (troubleshooting 2).

    Alternatives: A syringe with a needle (26 Gauge) can be used instead of a stainless-steel probe.

11. When bleeding occurs from the artery, aspirate blood to clear the surgical region with a glass pipette connected to a benchtop vacuum valve and cauterize the VA with a Bovie cautery (Figures 2F and 2G).
12. To confirm that the artery is completely occluded, insert a stainless-steel probe a few times to the cauterized spot and ensure there is no bleeding from the cauterized artery (troubleshooting 3).

    Note: Perform steps 10–12 on one side of the VAs and then the contralateral side (Figures 2H and 2I). These steps are the most critical and challenging part to block blood flow from the VAs. If the VAs are not completely occluded, the results of the global ischemia will be variable and lack robustness.

13. After cauterization of both VAs (Figure 2J), close the wound with a clip applier.

    Alternatives: A suture can be used instead of wound clips.

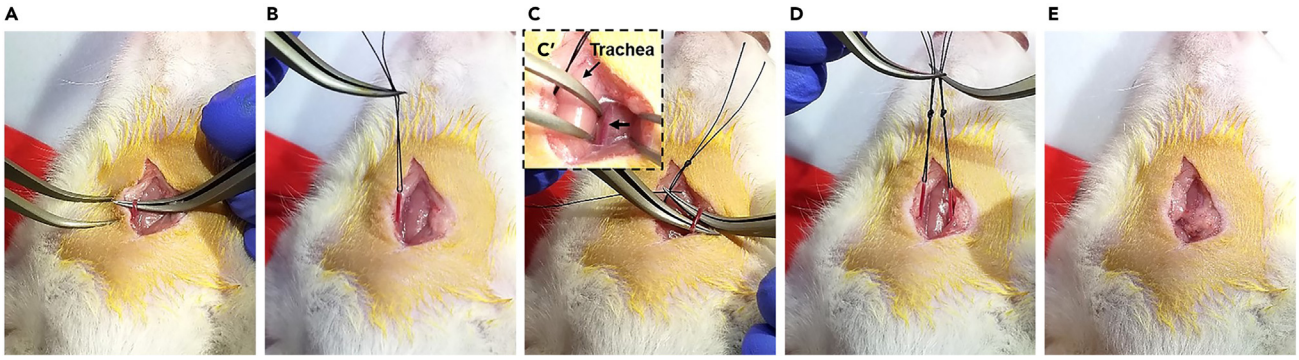

Figure 3. The procedure of looping of bilateral CCAs

(A) The right CCA was isolated.

(B) A knot was made by looping the right CCA loosely using a 4–0 silk suture.

(C) The left CCA was isolated. (C′) The left CCA is next to the trachea.

(D) A knot was made by looping the left CCA loosely using a 4–0 silk suture.

(E) The knotted sutures were covered with tissues and skin.

Looping of bilateral common carotid arteries (CCAs): Immediately after the permanent occlusion of VAs

Timing: 10 min

14. Transfer the operated rat onto a surgical board with a heating pad and continue anesthesia.
15. Apply the front neck with Betadine, and wipe with alcohol pads (repeat).
16. Make a midline vertical skin incision of about 1.5 cm.
17. Separate the fat, connective tissues, and sternocleidomastoid muscle around the trachea.
18. Under the microscope, isolate the CCA from the nerves and veins surrounding them (Figures 3A and 3C).
19. Pass one end of the silk suture underneath the CCA and make a knot as in Figures 3B–3D. Put the knotted sutures inside the skin (Figure 3E).
20. Close the incision region with clips and clean it.
21. Follow the post-operative care as described below.

Note: Rats may be fasted overnight in order to maximize the condition of glucose deprivation.

Stage II - Transient occlusion of CCAs: DAY 2

Timing: 15 min

22. Anesthetize the rat in an anesthesia chamber.
23. Transfer the rat to the surgical board.
24. Insert the rectal temperature probe to monitor the rat's body temperature.
25. Remove wound clips from the incision region of the front of the neck and open the wound.
26. Find the knotted sutures placed around each CCA. Clean each CCA with the forceps to avoid any unknown debris being clasped together with CCA in the next step.
27. Clasp bilateral CCAs using two vessel clamps per each CCA (troubleshooting 4 and 5).

Alternatives: The vessel clamp can be used once per CCA.

Note: Remove the rat from the nose cone and anesthesia. Do not leave the animal under the anesthetic condition; immediately keep the rat far enough away from the anesthesia

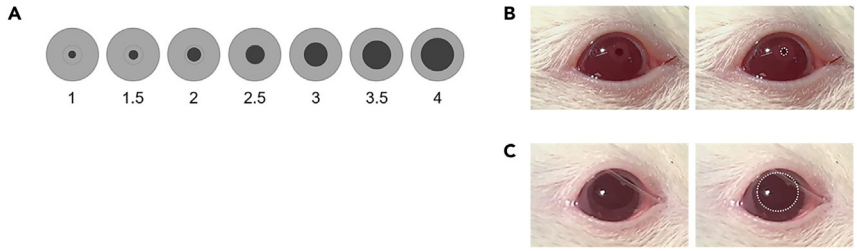

Figure 4. The pupil's dilation during 4-VO
(A) Scores according to the pupil's dilation size.
(B and C) The rat's pupil dilation (white dotted line) is of a score of 1 (B) and a score of 4 (C).

equipment. In this way, you will be able to discern that the rat is unconscious as a result of the 4-VO and not by anesthesia.

Note: Count for 10 minutes and measure the pupil's dilation score every minute (must lose righting reflex after CCAs occlusion if the animal undergoes global ischemic condition, Figure 4).

⚠ CRITICAL: It is possible that respiratory failure occurs within ~3 seconds after clipping of CCAs (Details of the symptoms are shown in Methods video S1) in some animals. This phenomenon usually occurs for 1 min and the animal starts breathing again after but remains unconscious for 10 min. The pupil dilation reaches score 4 at the moment that breathing is stopped and slowly gets back to score 1–2 during the remaining time. This condition can be considered as severe ischemia.

28. After 10 min, remove the vessel clamps, close the incision region with a clip applier, and clean it.
29. Follow the post-operative care as described below.

Post-operative procedures and care

Timing: 15 min

This step is performed every time after surgery is finished.

30. Apply lidocaine ointment to reduce pain in the incision regions and administrate flunixin meglumine (2.2 mg/kg), the non-steroidal anti-inflammatory analgesic agent to the operated rat.

Alternatives: Carprofen, ketoprofen, and meloxicam (non-steroidal anti-inflammatory drugs, NSAIDs) can be used instead of flunixin. These agents may have the duration of analgesic action up to 24 hours. Therefore, they can be injected once daily for three days.

31. Place the operated rat in an empty cage under a heating lamp and monitor it every 5 min until recovered from the anesthesia. Return the rat to the home cage (troubleshooting 6).
32. Put hydrogels and feed on the cage bottom.

EXPECTED OUTCOMES

The 4-VO model leads to delayed and selective neuronal death and cognitive impairment.[1,11] To measure cell viability and cognitive function, histological assays and behavioral tests were performed at 7 days after 4-VO (Figure 5A). The number of neurons was significantly reduced in the hippocampal CA1 pyramidal layer after 4-VO, but not in the CA3 or dentate gyrus region as shown by Toluidine Blue staining. Consistent with this, the results from the Fluoro-Jade (FJ) C staining which

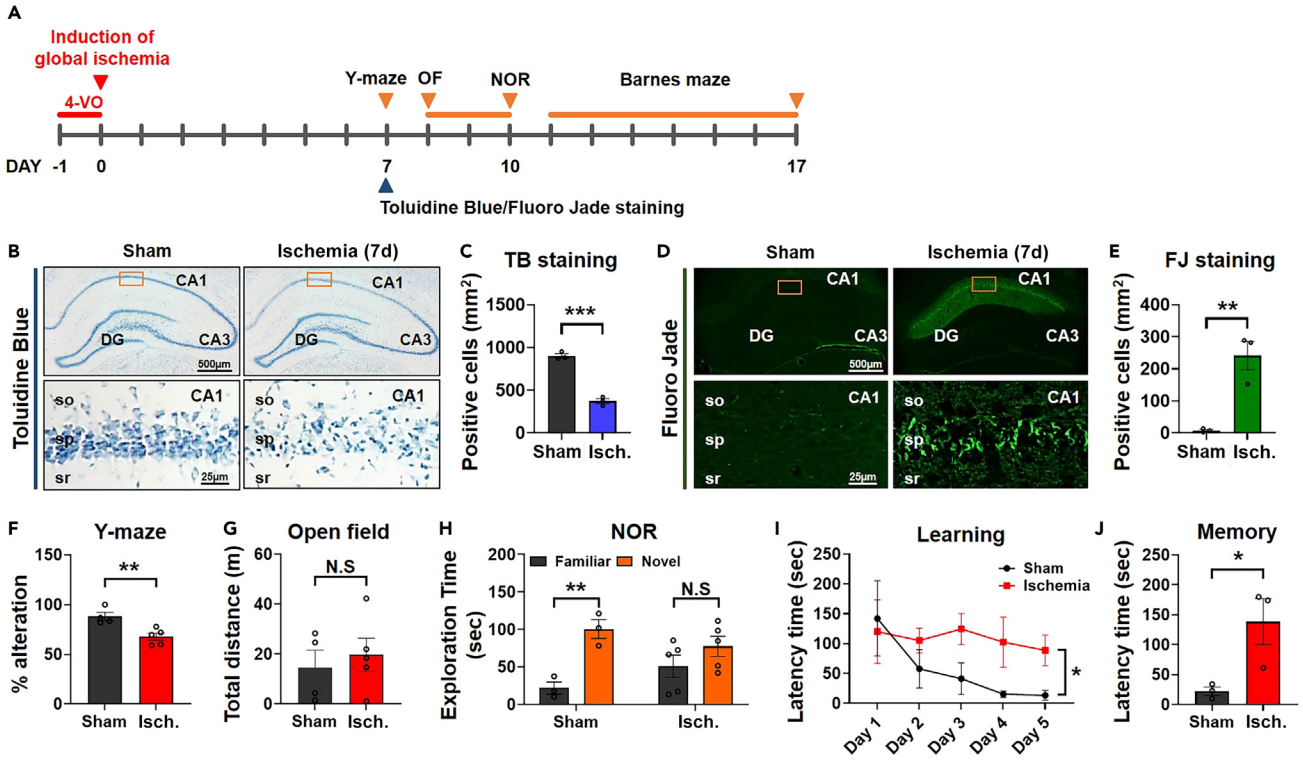

Figure 5. Global cerebral ischemia induces neuronal cell death and cognitive deficits in the rat 4-VO model

(A) Schematic diagram of the experimental procedure.

(B) Representative images of Toluidine Blue (TB) staining in hippocampal CA1 region 7 days after 4-VO. so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum.

(C) Quantification of TB-positive cells in CA1 region. ***$p < 0.001$ *vs.* sham group (Student's t-test, n = 3 per group, 4–6 sections per animal, mean ± SEM).

(D) Representative images of Fluoro-Jade (FJ) staining in hippocampal CA1 region 7 days after 4-VO.

(E) Quantification of FJ positive cells in CA1 region. **$p < 0.01$, ***$p < 0.001$ *vs.* sham group (Student's t-test, n = 3 per group, 4–6 sections per animal, mean ± SEM).

(F) Percentage of spontaneous alteration (% alteration) by Y-maze. **$p < 0.01$ *vs.* sham group (Student's t-test, n = 3–5 per each group, mean ± SEM).

(G) Summary data of open field test. N.S, not significant (Student's t-test, n = 3–5 per each group, mean ± SEM).

(H) Summary data of novel object recognition test. **$p < 0.01$ *vs.* sham group (Student's t-test, n = 3–5 per each group, mean ± SEM).

(I and J) Summary data of spatial learning and memory test using Barnes maze.

(I) Average latency time to find escape hole on days 1–5 during the learning test. *$p < 0.05$ *vs.* sham group (two-way ANOVA, Tukey's test with multiple comparisons, n = 3 per each group, mean ± SEM).

(J) Summary data of latency time to find the escape hole relocated (the maze was moved at 180°) during the memory test. *$p < 0.05$ *vs.* sham group (Student's t-test, n = 3 per each group, mean ± SEM).

stains degenerating neurons showed that neuronal death was significantly increased in the hippocampal CA1 regions, but not in the CA3 or dentate gyrus (Figures 5B–5E).

As a working memory test, we first performed the Y maze task. Rats subjected to the 4-VO showed reduced explorative behavior as seen by less spontaneous alteration (Figure 5F). In the open-field test evaluating locomotor activity, there was no significant difference between sham and 4-VO rats (Figure 5G). This result indicates that the locomotor ability is not affected by 4-VO-induced global ischemia. We next performed the Novel object recognition test to evaluate cognition and recognition memory.[12–14] While sham rats spent more time exploring novel than familiar objects, 4-VO rats had no significant difference in the time spent between the novel and familiar objects (Figure 5H). Finally, we performed the Barnes maze test which is a well-established animal behavioral test for spatial learning and memory.[15] To test learning, rats were given two learning trials a day for five days. Whereas sham-operated rats showed reduced latency time to enter the escape hole

over the learning trials for five days, 4-VO rats did not improve (Figure 5I), suggesting that ischemic rats have deficits in learning. To test spatial memory, animals were given a day after the last learning test and then subjected to the Barnes maze test after rotating the escape hole 180° with visual cues. The 4-VO rats showed increased latency time to find the escape hole compared with the sham rats (Figure 5J) indicating memory deficits in ischemic rats. Taken together, these data indicate that global ischemic brain injury by 4- VO induces neuronal death selectively in hippocampal CA1 and cognitive deficits.

LIMITATIONS

One limitation is that the success rate of the 4-VO model varies depending on the strain of the rat, as the initial study by Pulsinelli and Brierley also described differences in the animal's unresponsive state (success of inducing ischemic condition) among the strains.[10,16] In addition, we also agree with the statement that rats from the same strain, but different suppliers or different batches may vary in their response to 4-VO according to our experience using this protocol over the last 15 years. This "failing" of inducing global ischemic condition may occur due to the blood source from the anterior spinal and collateral arteries within the cervical and paravertebral muscles, which still can supply cerebral blood flow to the brain even after the 4-VO itself is well operated. Nevertheless, Wistar and Sprague-Dawley rats, the two strains typically used for this animal model, show effective results with ~70% induction rate. The survival rate of these operated rats is ~80%–90% due to either severe ischemic condition or death during or post operation.

Another limitation is that visual impairment by 4-VO could affect behavioral tests in long-term studies (>2–6 months). Degeneration of the optic tract can occur in a few months after 4-VO,[17] which causes visual impairment. Therefore, the feasibility of vision-dependent cognitive tests such as the Morris water maze or Barnes maze for long-term studies is unclear. For accurate cognitive behavioral assessment, vision-independent cognitive tests such as Fear conditioning or Passive avoidance task could be considered for long-term studies. In a short-term study, however, there was no significant damage in the optic tract and retina, and no impairment in visual function at 28 days after 4-VO[18] indicating that the cognitive behavioral tests during this period are not affected by vision.

TROUBLESHOOTING

Problem 1

Unstable anesthesia during occlusion of VAs (step 3).

When the rat's head is fixed to a stereotaxic apparatus, the head should be angled down at about 30° to the horizontal plane for a clear view of the bilateral alar foramen (see the potential solution for the problem 3 below). At this time, it may be difficult to establish a connection between the stereotaxic frame nose cone and the rat's nose entirely. This can cause unstable anesthesia.

Potential solution

- The stereotaxic frame nose cone mask should be installed lower down with a proper angle to fit the rat's nose.
- Use the flexible tubing nose cone to connect to the rat's nose.

Problem 2

Cerebrospinal fluid leak during the VA puncturing (step 10).

When making a puncture with a stainless-steel probe or a needle in the VA through the inside of the foramen, puncturing too deep or a wrong spot (not the foramen) may cause a cerebrospinal fluid leak. This can induce extra neurological damage and symptoms not caused by global ischemia.

Potential solution

- Avoid puncturing deep into the spinal cord.
- Puncturing angled a bit downward of the foramen inside can be helpful to avoid this problem.

Problem 3

The difficulty of confirming complete occlusion of the VAs by electrocauterization (step 12).

The VAs are invisible because they are inside the alar foramen tunnel. Therefore, it is difficult to visually confirm whether the VAs are completely electrocauterized. Incomplete occlusion of VAs may lead to sub-optimal induction of a global ischemic condition or excessive hemorrhage which can induce mortality during or after stage I of the surgery.

Potential solution

- The position of the rat's head in the stereotaxic frame should be with the head angled down at approximately 30° to the horizontal plane. This can provide a clear view of the bilateral alar foramen, the horizontal position of alar wings to the table, and easy access to the alar foramen from the dorsal neck incision.[19]
- Cauterize the bleeding VAs as quickly as possible after puncturing them. Over time, the pressure of bleeding markedly decreases, and it will be difficult to determine whether cauterization is completed; additionally, excessive bleeding can occur which can lead to rat mortality.
- Make sure that bleeding is completely stopped by repeating electrocauterizing and poking with the stainless-steel probe or needle (2–3 times).

Problem 4

High mortality during the transient occlusion of CCAs (step 27).

When severe global ischemia occurs, the animal may stop respirating. After ~1 min, breathing resumes, though the animal remains fully unconscious for the 10-min period as described in step 27 and showed in Methods video S1. However, in some cases, when the CCAs are clasped, rats could die as a result of excessive brainstem ischemia and respiratory arrest.

Potential solution

- After breathing resumed, overall condition of the rat should be monitored during the occlusion of CCAs with the clamps. If breathing is unstable, weak, or stopped, remove one clamp out of two at the left CCA, the artery arising directly from the heart artery (aorta arch). This can prevent animals' death while still keeping them under an ischemic condition.

Problem 5

When the rat wakes up from the unconscious state during transient occlusion of the CCAs (step 27).

Potential solution

- This could occur when the VAs are not completely occluded. To reduce this, refer to problem 1.
- When you have rats that wake up during CCAs occlusion, exclude them from the 4-VO animal model group for further studies or data analysis. In particular, exclude the rat if the total pupil dilation score is less than 17 or the rat wakes up earlier than 7 min in total 10 min occlusion. You may still use the rat that has a pupil dilation score over 17 and wakes up less than 3 min before the endpoint.

Problem 6

Continuous bleeding from the head's wound (steps 30–31) during the post-operative care.

Potential solution

- Clean and stem the bleeding; apply pressure gently to the wound with a sterile gauze.
- If the bleeding still continues, reopen the wound and find the bleeding region. If the blood comes from the connective tissues or muscles, stem the bleeding with a sterile gauze. If the blood comes from the alar foramen, re-cauterize the VA with a Bovie cautery as described in steps 11 and 12.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jee-Yeon Hwang, jeeyeonhwang@creighton.edu.

Materials availability

This study did not generate new reagents.

Data and code availability

This article includes all datasets generated or analyzed during this study.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102630.

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AUTHOR CONTRIBUTIONS

H.K. and J.-Y.H. designed the research. H.K. and R.U. performed the research. H.K. and J.-Y.H. analyzed data. H.K., R.U., F.P., T.J.-M., D.O., and J.-Y.H. interpreted the data and wrote the paper.

DECLARATION OF INTERESTS

F.P. and D.O. are employees of Sanofi.

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