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A Preclinical Controlled Cortical Impact Model for Traumatic Hemorrhage Contusion and Neuroinflammation

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Dear Editors,

Thank you for your consideration of our manuscript “A Preclinical Controlled Cortical Impact Model for Traumatic Hemorrhage Contusion and Neuroinflammation” (JoVE61393R1).

We thank the review editor and reviewers for their comments and suggestions. We revised the manuscript substantially in response to the given suggestions, and we believe the revised manuscript that accompanies this letter addresses the raised concerns. All edits are tracked and the essential steps of the protocol for the video were highlighted in yellow. We provided below a point-by-point response to the comments.

Thank you for your invitation and the opportunity to submit a revised manuscript. We hope that our revisions have made the work acceptable for publication in the *Journal of Visualized Experiments*.

Sincerely,

Che-Feng Chang, PhD

Review Editor:

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached version for revision.

2. Please address all the specific comments marked in the manuscript.

3. Please address all the reviewers' comments.

4. Once done, please ensure that the highlight is no more than 2.75 pages including headings and spacings.

Thank you for your comments. We have made corresponding changes to the provided version for revision. We also ensure that the highlight is less than 2.75 pages including headings and spacings.

Reviewer #1:

The authors have sufficiently revised the manuscript and addressed reviewer comments. The additional clarifications on the model and methods, as well as the addition of the Figure 1 and inset substantially improve the manuscript. The only remaining minor point is to remove the word "revered" in the sentence "The CCI model is revered for its stability and reproducibility in producing injury ranging from mild to severe." (line 202). This seems to be excessive and dramatic language, and also not scientifically founded. After this correction, the manuscript can be accepted for publication.

We appreciate the reviewer’s suggestion. We have corrected the wording.

Reviewer #3:

In this revised version of their paper, authors have improved the quality of the manuscript by following several reviewers' suggestions. However, authors should add information about two minor concern in their manuscript with appropriate references.

Minor concern:

Line 303: other forms of hemorrhage contusions such as traumatic subarachnoid hemorrhage and subdural hemorrhage

I think that CCI protocol containss not only cerebral contusion but also traumatic subarachnoid hemorrhage (tSAH), Subdural hematoma (SDH), and Epidural hematoma (EDH), because both dura mater and arachnoid membrane are injured by device. Please describe this sentence more accurately. tSAH, DSH or EDH is not other type of hemorrhage contusions, but other type of focal injury.

We thank the reviewer for the suggestion. We agree and have made changes to the text.

Comment on #reviewer 2

5. It is surprising that in the day 1 Iba+ image (Fig 2B) there are little to no microglia, as most literature shows a robust and immediate microglia response in and around the impacted area, which leads to further microglia/macrophage proliferation and migration to the injured site in the window of 1-3 days. Can the authors address this discrepancy from other literature?

It depends on what type of antibody was used as an immunohistochemical marker for microglia. In some literature, CD11b positive cells are regarded as microglia/macrophages. However, CD11b antibody is also reactive for immune cells such as neutrophils. Indeed, through raptured BBB, neutrophils accumulate in and around the impacted area at day 1 post injury.

We appreciate this comment. We used Iba1 as a marker to show that neuroinflammation has taken place in the brain after CCI. We entirely agree with the reviewer that the discrepancy in immune cell numbers at day 1 post-CCI between studies could result from the usage of antibody (e.g. CD11b or Iba1) in immunohistochemical analysis. While we will continue to keep this in mind when designing experiments for our future CCI studies, this discussion is out of the scope of the current manuscript.

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TITLE:

A Preclinical Controlled Cortical Impact Model for Traumatic Hemorrhage Contusion and Neuroinflammation

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KEYWORDS:

cerebral contusion, intraparenchymal hemorrhage, innate immunity, microglia, neuroinflammation, iron toxicity

SUMMARY:

Intraparenchymal hemorrhage and neuroinflammation accompanied by cerebral contusion can trigger severe secondary brain injury. This protocol details a mouse controlled cortical impact (CCI) model allowing researchers to study hemorrhage contusion and post-traumatic immune responses and explore potential therapeutics.

ABSTRACT:

Cerebral contusion is a severe medical problem affecting millions of people worldwide each year. There is an urgent need to understand the pathophysiological mechanism and to develop effective therapeutic strategy for this devastating neurological disorder. Intraparenchymal hemorrhage and post-traumatic inflammatory response induced by initial physical impact can aggravate microglia/macrophage activation and neuroinflammation which subsequently worsen brain pathology. We provide here a controlled cortical impact (CCI) protocol that can reproduce experimental cortical contusion in mice by using a pneumatic impactor system to deliver mechanical force with controllable magnitude and velocity onto the dural surface. This preclinical model allows researchers to induce moderately severe focal cerebral contusion in mice and to investigate a wide range of post-traumatic pathological progressions including hemorrhage contusion, microglia/macrophage activation, iron toxicity, axonal injury, as well as short-term and long-term neurobehavioral deficits. The present protocol can be useful for exploring the long-term effects of and potential interventions for cerebral contusion.

INTRODUCTION:

Cerebral contusion is a form of traumatic brain injury that ranks high among the deadliest health issues in modern society[1]. It is primarily caused by accidental events such as traffic accident that results in external forces applying mechanical energy to the head. Traumatic brain injury affects an approximate of 3.5 million people and accounts for 30% of all acute injury-related deaths in the US each year[2]. Patients who survive cerebral contusion oftentimes suffer from long-term consequences including focal motor weakness, sensory dysfunction, and mental illness[1].

The primary injury of cerebral contusion is induced by mechanical factors including stretching and tearing forces, leading to immediate parenchymal structure deformation and focal CNS cell death[3]. Hemorrhage contusion is a general term for brain hemorrhages due to vascular tear at the site of head trauma[4]. Specifically, intraparenchymal hemorrhage occurs immediately after a cerebral contusion leading to delayed hematoma formation. Within the hematoma, hemoglobin and free iron released from the lysed red blood cells can further trigger blood-related toxicity[5,6] which cause herniation, brain edema, and intracranial pressure elevation[5,6]. The collaborative functions of neurons (axons), glia, blood vessels, and supportive tissue are also compromised by the mass effect of hematoma[7]. Additionally, persistent and diffuse neuroinflammation with progressive neurodegeneration continue for months and cause secondary damage in the brain[8].

Microglia activation is one of many important pathological features of cerebral contusion[9,10]. After sensing the damage-associated molecular patterns (DAMPs) and leaked blood in the injured tissue, activated microglia trigger neuroinflammation which furthers secondary brain damage[11]. In addition, chemoattractant released from microglia promotes peripheral immune cell infiltration into the traumatic territory resulting in production of reactive oxygen species and pro-inflammatory cytokines. This creates a self-perpetuating pro-inflammatory environment which triggers progressive brain injury[9,12]. Meanwhile, microglia with an alternatively activated phenotype can contribute to tissue homeostatic restoration and brain repair through clearing debris from the injured tissue[13]. Prevention of secondary neuroinflammation by reducing detrimental microglial immune responses has been shown to be particularly useful for promoting brain recovery from cerebral contusion[3,9,10,12].

Several preclinical models have been developed for studying traumatic brain injury including weight-drop model, lateral fluid percussion injury, and blast wave model[14,15]. However, these models each have their weakness including high mortality rate during the procedure, low reproducibility of histological results, and high variability of inflicted injury between laboratories[16,17]. In comparison, the controlled cortical impact (CCI) model is more adequate for studying focal cerebral contusion because of its precise control and high reproducibility[14,15,18,19]. Furthermore, through manipulating the biomechanical deformation parameters such as velocity and depth of impact, the severity of the induced damage can be controlled to produce a wide range of injury magnitudes, allowing researchers to mimic different levels of impairment oftentimes seen in patients[17]. The preclinical model of CCI was first developed in 1896[20]. Since then, CCI has been the broadest applicable model to be modified for the use in primates[21], swine[22], sheep[23], rats[24], and mice[25]. Together these features make CCI one of the most suitable

experimental cerebral contusion models[26].

Our laboratory uses a commercially available pneumatic CCI impact system and tested biomechanical deformation parameters to produce moderately severe focal cerebral contusion that territorializes the primary sensory and motor cortical areas without damaging the hippocampus[27,28]. We and others demonstrated that this CCI procedure can be used to study clinical features of human cerebral contusion including brain tissue loss, neuronal injury, intraparenchymal hemorrhage, neuroinflammation, and sensorimotor deficiency[24,25,27-30]. Here, we detail a standard protocol to perform mouse CCI which allows one to ask questions regarding CCI-induced myelin loss, iron deposition, CNS inflammation, hemorrhagic toxicity and the responses of microglia/macrophages in the aftermath of focal cerebral contusion.

PROTOCOL:

All procedures described in this protocol were conducted under the approval of the Institutional Animal Care and Use Committee at Cheng Hsin General Hospital and National Taiwan University College of Medicine. Eight- to ten-week-old male C57BL/6 wild type mice were used in this protocol.

1. Anesthesia induction

1.1. Anesthetize the mouse with ~4% isoflurane mixed with room air at ~0.2 L/min in an induction chamber connected to the isoflurane vaporizer.

1.2. Ensure the respiratory pattern is smooth. Check the depth of anesthesia by confirming a lack of toe-pinch reflex in the animal.

2. Pre-surgical preparation

2.1. Shave the mouse head with electrical clippers in a caudal to rostral direction. Do not trim the mouse whiskers.

NOTE: Loss of whiskers may influence the accuracy of subsequent behavioral test results.

2.2. Place the mouse onto the stereotaxic frame. Carefully insert the ear bars into the ear canals. Ensure the mouse head is stabilized by both ear bars equally.

2.3. Bring in the nose cone and maintain anesthesia at 1% - 2% isoflurane for the duration of the surgery.

2.4. Apply petroleum jelly to both eyes to prevent drying out during the surgery. Keep the animal on a heating pad to maintain a body temperature of 37 ˚C.

2.5. Disinfect the shaved head with betadine followed by 70% alcohol using sterile cotton swabs. Repeat three times.

3. CCI surgery

3.1. Administer 100 µL of Bupivacaine (0.25%) subcutaneously using a 31 G insulin needle prior to the incision. Gently massage the injection site for better absorption.

NOTE: This local anesthetic provides pain relief directly at the site of surgery.

3.2. Make a longitudinal incision (~1.5 cm) along the midline on the scalp with scissors. Use a hemostat to hold the skin off to the right side and allow the exposed skull to dry for 1 min. Use a sterile cotton swab to clean away any residual blood and tissues on the skull.

3.3. Check that the mouse head is level in the horizontal plane.

3.3.1. Identify anatomical landmarks Bregma and Lambda and mark both locations with a pencil.

3.3.2. Ensure that the head of the animal is level in the rostral-caudal direction. Do this by measuring the Z coordinates of both Bregma and Lambda using a 31 G insulin needle attached to the stereotaxic frame.

NOTE: Adjust the ear bar vertically if necessary.

3.3.3. Perform for the horizontal positioning of the animal head by following the same procedure of checking the Z coordinates at the midline along with two corresponding locations on the left and right side of the midline and adjust the ear bars if needed.

NOTE: A level and stable placement of the animal head is crucial for the reproducibility and reliability of the CCI model.

3.4. Use the same 31 G insulin needle to identify the craniectomy site. Set the XY origin to Bregma and laterally move the needle 3 mm to the right. Mark this position as the site of craniectomy and draw a circle 4 mm in diameter on the skull with a pencil.

3.5. Use a high-speed micro drill with a trephine (4 mm diameter) to cut along the pencil-outlined circle to create a 4 mm diameter open hole. Use a speed setting of 20,000 rpm. Avoid applying excess pressure.

NOTE: Perform this step quickly (usually within 30 s to 1 min) to prevent any thermal damage to the brain. Applying excess pressure while drilling may lead to accidental penetration that could compress and injure the brain surface.

3.6. Carefully remove the bone flap with tweezer and temporally store it in ice cold normal saline. Gently rinse the hole with normal saline before applying pressure on the brain surface with the cotton swab tip to stop bleeding.

3.7. Set the 2.5 mm diameter rounded impactor tip on the CCI device to an angle of 22.5˚. Zero the impact tip to the dural surface. Set the impact parameters on the control box to a velocity of 4 m/s and a deformation depth of 2 mm. Retract the metal tip.

NOTE: Zeroing the tip while it is statically and slightly pressed against the dural surface in the full stroke position improves the accuracy of the zero point and the reproducibility of the injury level.

3.8. Discharge the piston to generate impaction on the brain. Place a sterile cotton swab onto the injured area to stop bleeding.

3.9. Place the bone flap back to the mouse brain and secure with dental cement. Close the scalp with tissue adhesive (e.g., 3M Vetbond).

4. Postoperative recovery

4.1. Place the mouse in a clean recovery cage with bedding under the heat lamp until full recovery.

4.2. Provide moistened chow food and subcutaneously administer ketoprofen (5 mg/kg) for two consecutive days after surgery.

4.3. Perform the above procedures except steps 3.7 and 3.8 for sham control animals.

5. Mouse euthanasia

5.1. Euthanize mice on the day of study by isoflurane overdose and then decapitation.

NOTE: Several strategies can be used to euthanize the experimental animals prior to the sample collection.

5.2. Collect brain samples for histological analysis.

REPRESENTATIVE RESULTS:

Illustration of stereotactic placement and craniotomy procedure.

The CCI model is known for its stability and reproducibility in producing injury ranging from mild to severe[18]. Proper stereotactic technique and craniotomy procedure are major determinants in producing stable and reproducible CCI-induced brain injury (**Figure 1A**,**B**). An ideal craniotomy procedure would cause minimal histological injury in the sham-operated brain[27,28] as shown (**Figure 1C**).

CCI induces long-term brain damage, blood-related toxicity, and neuroinflammation.

To evaluate brain damage, axonal loss, hemorrhagic pathology, and microglia/macrophages activation induced by CCI, we performed a battery of histological analysis including Nissl

(neurons), luxol fast blue (myelin), Ly76 (erythrocytes), Iba1 (microglia/macrophages), and Perls (ferric iron) staining on injured brain slices from acute to chronic phases of CCI[25, 27-32]. Changes in brain damage (**Figure 2A**) and corpus callosum (axon) loss (**Figure 2B**) were observed at days 1, 3, 7, and 28 after CCI. In addition, unilateral brain atrophy on the contusion side was seen at day 28 post-CCI (**Figure 2**). A successful CCI procedure produces focal injury between Bregma +1.42 mm and -1.34 mm at the coronal level[27,30]. Injury severity peaks around days 3 to 5 post-CCI the core of which centers approximately around Bregma +0.02 mm at the coronal level along with corpus callosum and striatal injuries[25,28,31,32]. Neuroinflammation was revealed by Iba1-positive activated microglia/macrophages accumulation around the border of the brain contusion area where the intraparenchymal hemorrhage was also detectable by the Ly76-positive staining from days 1 to 7 post-CCI (**Figure 3B**). A mixture of red blood cells (RBCs) with ruptured and intact cell morphology was observed at day 7 post-CCI (**Figure 3B**) suggesting the RBCs were lysed at this stage. This phenomenon is consistent with the observation that ferric iron deposition was detectable in the contusion region from days 7 to 28 post-CCI (**Figure 3C**). As CCI also induces corpus callosum and striatal injuries, activated microglia/macrophages are observed in the striatum far away from the contusion site (**Figure 3D**) from days 3 to 28 post-CCI. These observations indicate that the CCI method can reproduce the pathological features closely resembling human cerebral contusion including neuronal/axonal injury, intraparenchymal hemorrhage, and CNS inflammation.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative images of stereotactic and craniotomy procedures and histology of sham-operated brains. (**A**) The mouse head was levelly and stably placed on the stereotactic frame. Notice the readings on the horizontal and vertical scales of the ear bars are the same. (**B**) The bone flap was removed after craniotomy (**left**) and representative images of brain surface after proper (**middle**) and faulty (**right**) craniotomy are shown. (**C**) Representative images of cresyl violet (**left**) and luxol fast blue (**right**)stained sham-operated brain sections at day 3.

Figure 2: Brain tissue damage and myelin loss in CCI-induced cerebral contusion. (**A**) Representative images of cresyl violet-stained brain sections after CCI at days 1, 3, 7, and 28. The dotted line indicates the contusion area. Scale bar = 1 mm. (**B**) Representative luxol fast blue-stained CCI brain sections at days 1,3,7, and 28. The residual myelin at corpus callosum are outlined by dotted line. Scale bar = 1 mm.

Figure 3: CCI-induced intraparenchymal hemorrhage and neuroinflammation. (**A**) Representative cresyl violet stained CCI brain section shows boxed regions selected for Iba1/Ly76 (red), Perls (yellow), and Iba1 (green) imaging. (**B**) Representative immunostaining of activated microglia/macrophage marker Iba1 (green), RBC marker Ly76 (red), and nuclear marker DAPI (blue). The dotted line indicates the border of contusion region. Scale bar = 100 μm in the full images and 50 μm in the inset images. (**C**) Representative Perls staining show iron deposition in the contusion region at days 1, 3, 7, and 28 post-CCI. Scale bar = 100 μm. (**D**) Representative images of activated microglia/macrophage marker Iba1 (green) expression at days 1, 3, 7, and 28 post-CCI. Scale bar = 100 μm in the full images and 50 μm in the inset images.

DISCUSSION:

The CCI protocol produces highly reproducible mechanical injury to the brain for cerebral contusion research. The following steps are crucial for generating consistent brain injury in animals using this CCI protocol.

First, the mouse head should be stably mounted on the stereotaxic frame and the anatomical landmarks Bregma and Lambda always in the same horizontal plane. Unsteady or unlevel head placement oftentimes result in varied injury levels between animals. To ensure the animal head is safely secured, use a pair of forceps to gently nudge the mouse head laterally. The head needs to be laterally immobile and any movement is indicative of an incorrect placement of the ear bars. Last, gently suspend the mouse body by lifting its tail to confirm that the head is firmly fixed on the stereotaxic frame.

Next, avoid damaging the dura mater when drilling into the skull by applying even pressure along the circular path as mechanical damage to the dura mater can cause unnecessary injury to the brain. Use great care to inspect the depth of drilling by gently tapping the bone window with a pair of fine forceps. In addition, heat generated during prolonged drilling process also induces cortical injury. In our protocol we use trephine rather than the round tip drill bit seen in most other CCI protocols[33,34] to shorten the duration of craniotomy. Ice cold saline can be applied onto the skull during the drilling step for additional protection against heat. After drilling, the bone flap must be gently lifted without damaging the brain tissue underneath. Keeping a consistent size of the drilled bone window between animals is crucial for ensuring a consistent level of intracranial pressure and degree of brain deformation.

Furthermore, the impactor tip must be properly positioned at the center of the bone window and slightly pressed against the dural surface in order to generate consistent brain damage. Depth variance of tip zeroing on the dural surface can affect the extent of CCI injury.

Lastly, the bone flap should be repositioned and well secured after impaction. In fact, removing the bone flap prior to the mechanical impact is not relevant to human cerebral contusion. Therefore, repositioning bone flap immediately after the CCI helps maintain a consistent intracranial pressure in the closed head between animals during the subsequent disease progression.

High reproducibility, low mortality, and ease of controlling severity are among the many strengths of using a CCI model[14-16,18,34]. The requirement of anesthesia and craniotomy during the procedure are the major limitations of CCI model. Meanwhile, the neurologic deficits are usually resolved within days to weeks in rodents whereas human patients' deficits can persist for months. However, no single animal model can mimic all clinical features. The CCI model is still highly reliable and oftentimes the preferred method to study short- and long-term pathophysiological events and to search for potential therapeutics for cerebral contusion[14,15]. Here we described the application of a CCI model to study hemorrhage contusion and secondary CNS inflammation after cerebral contusion. This protocol demonstrates a moderately severe CCI that produces focal brain injury in the primary sensory and motor cortical areas without

damaging the hippocampus[27,28]. Depending on the research purpose, velocity and depth of impact, size of impactor tip, and position of the impaction can all be modified to create various severity of injury in different brain regions[35].

Although we have applied this protocol in studying traumatic intraparenchymal hemorrhage, this CCI model can also be used to study other types of focal injury such as traumatic subarachnoid hemorrhage (tSAH), subdural hemorrhage (SDH), and epidural hemorrhage (EDH)[1,7]. While adult animal was used for the representative study, this CCI protocol with modification can also be applied to neonates to study the effect of cerebral contusion in the immature brain based on the goal of study[36,37]. A CCI model can not only facilitate study of single cerebral contusion but also repetitive mechanical cerebral impact that is relevant to people at high risk of multiple brain injuries such as military personnel[19]. Finally, we suggest that researchers perform pilot experiment to fine-tune the biomechanical parameters in the CCI model to better meet their research goal.

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Maas, A. I. R. et al. Traumatic brain injury: integrated approaches to improve prevention, clinical care, and research. *The Lancet Neurology.* **16** (12), 987-1048 (2017).
2. Taylor, C. A., Bell, J. M., Breiding, M. J., Xu, L. Traumatic Brain Injury-Related Emergency Department Visits, Hospitalizations, and Deaths - United States, 2007 and 2013. *Morbidity and Mortality Weekly Report Surveillance Summaries.* **66** (9), 1-16 (2017).
3. Pearn, M. L. et al. Pathophysiology Associated with Traumatic Brain Injury: Current Treatments and Potential Novel Therapeutics. *Cellular and Molecular Neurobiology.* **37** (4), 571-585 (2017).
4. Nyanzu, M. et al. Improving on Laboratory Traumatic Brain Injury Models to Achieve Better Results. *International Journal of Medical Sciences.* **14** (5), 494-505 (2017).
5. Zhao, M. et al. Iron-induced neuronal damage in a rat model of post-traumatic stress disorder. *Neuroscience.* **330**, 90-99 (2016).
6. Cepeda, S. et al. Contrecoup Traumatic Intracerebral Hemorrhage: A Geometric Study of the Impact Site and Association with Hemorrhagic Progression. *Journal of Neurotrauma.* **33** (11), 1034-1046 (2016).
7. Robicsek, S. A., Bhattacharya, A., Rabai, F., Shukla, K., Dore, S. Blood-Related Toxicity after Traumatic Brain Injury: Potential Targets for Neuroprotection. *Molecular Neurobiology.* **57** (1), 159-178 (2020).
8. Morganti-Kossmann, M. C., Semple, B. D., Hellewell, S. C., Bye, N., Ziebell, J. M. The complexity of neuroinflammation consequent to traumatic brain injury: from research evidence

to potential treatments. *Acta Neuropathologica.* **137** (5), 731-755 (2019).
9. Ramlackhansingh, A. F. et al. Inflammation after trauma: microglial activation and traumatic brain injury. *Annals of Neurology.* **70** (3), 374-383 (2011).
10. Wang, G. H. et al. Microglia/macrophage polarization dynamics in white matter after traumatic brain injury. *Journal of Cerebral Blood Flow & Metabolism.* **33** (12), 1864-1874 (2013).
11. Karve, I. P., Taylor, J. M., Crack, P. J. The contribution of astrocytes and microglia to traumatic brain injury. *British Journal of Pharmacology.* **173** (4), 692-702 (2016).
12. Huber-Lang, M., Lambris, J. D., Ward, P. A. Innate immune responses to trauma. *Nature Immunology.* **19** (4), 327-341 (2018).
13. Russo, M. V., McGavern, D. B. Inflammatory neuroprotection following traumatic brain injury. *Science.* **353** (6301), 783-785 (2016).
14. Xiong, Y., Mahmood, A., Chopp, M. Animal models of traumatic brain injury. *Nature Reviews Neuroscience.* **14** (2), 128-142 (2013).
15. Johnson, V. E., Meaney, D. F., Cullen, D. K., Smith, D. H. Animal models of traumatic brain injury. *Handbook of Clinical Neurology.* **127**, 115-128 (2015).
16. Albert-Weissenberger, C., Siren, A. L. Experimental traumatic brain injury. *Experimental & Translational Stroke Medicine.* **2** (1), 16 (2010).
17. Ma, X., Aravind, A., Pfister, B. J., Chandra, N., Haorah, J. Animal Models of Traumatic Brain Injury and Assessment of Injury Severity. *Molecular Neurobiology.* **56** (8), 5332-5345 (2019).
18. Osier, N. D., Korpon, J. R., Dixon, C. E. in Brain Neurotrauma: Molecular, Neuropsychological, and Rehabilitation Aspects. *Frontiers in Neuroengineering (ed F. H. Kobeissy)* (2015).
19. Osier, N. D., Dixon, C. E. The Controlled Cortical Impact Model: Applications, Considerations for Researchers, and Future Directions. *Frontiers in Neurology.* **7**, 134 (2016).
20. Kramer, S. P. VI. A Contribution to the Theory of Cerebral Concussion. *Annals of Surgery.* **23** (2), 163-173 (1896).
21. King, C. et al. Brain temperature profiles during epidural cooling with the ChillerPad in a monkey model of traumatic brain injury. *Journal of Neurotrauma.* **27** (10), 1895-1903 (2010).
22. Costine, B. A. et al. Neuron-specific enolase, but not S100B or myelin basic protein, increases in peripheral blood corresponding to lesion volume after cortical impact in piglets. *Journal of Neurotrauma.* **29** (17), 2689-2695 (2012).
23. Anderson, R. W., Brown, C. J., Blumbergs, P. C., McLean, A. J., Jones, N. R. Impact mechanics and axonal injury in a sheep model. *Journal of Neurotrauma.* **20** (10), 961-974 (2003).
24. Chen, S., Pickard, J. D., Harris, N. G. Time course of cellular pathology after controlled cortical impact injury. *Experimental Neurology.* **182** (1), 87-102 (2003).
25. Lee, H. F., Lin, J. S., Chang, C. F. Acute Kahweol Treatment Attenuates Traumatic Brain Injury Neuroinflammation and Functional Deficits. *Nutrients.* **11** (10) e2301 (2019).
26. Dixon, C. E., Clifton, G. L., Lighthall, J. W., Yaghmai, A. A., Hayes, R. L. A controlled cortical impact model of traumatic brain injury in the rat. *Journal of Neuroscience Methods.* **39** (3), 253-262 (1991).
27. Hung, T. H. et al. Deletion or inhibition of soluble epoxide hydrolase protects against brain damage and reduces microglia-mediated neuroinflammation in traumatic brain injury. *Oncotarget.* **8** (61), 103236-103260 (2017).
28. Wu, C. H. et al. Post-injury treatment with 7,8-dihydroxyflavone, a TrkB receptor agonist,

protects against experimental traumatic brain injury via PI3K/Akt signaling. *PLoS One.* **9** (11), e113397 (2014).
29. Chen, S. F., Su, W. S., Wu, C. H., Lan, T. H., Yang, F. Y. Transcranial Ultrasound Stimulation Improves Long-Term Functional Outcomes and Protects Against Brain Damage in Traumatic Brain Injury. *Molecular Neurobiology.* **55** (8), 7079-7089 (2018).
30. Su, W. S., Wu, C. H., Chen, S. F., Yang, F. Y. Low-intensity pulsed ultrasound improves behavioral and histological outcomes after experimental traumatic brain injury. *Scientific Reports.* **7** (1), 15524 (2017).
31. Chen, S. F. et al. Salidroside improves behavioral and histological outcomes and reduces apoptosis via PI3K/Akt signaling after experimental traumatic brain injury. *PLoS One.* **7** (9), e45763 (2012).
32. Chen, C. C. et al. Berberine protects against neuronal damage via suppression of glia-mediated inflammation in traumatic brain injury. *PLoS One.* **9** (12), e115694 (2014).
33. Furmanski, O., Nieves, M. D., Doughty, M. L. Controlled Cortical Impact Model of Mouse Brain Injury with Therapeutic Transplantation of Human Induced Pluripotent Stem Cell-Derived Neural Cells. *Journal of Visualized experiments.* (149), e59561 (2019).
34. Romine, J., Gao, X., Chen, J. Controlled cortical impact model for traumatic brain injury. *Journal of Visualized experiments.* (90), e51781 (2014).
35. Saatman, K. E., Feeko, K. J., Pape, R. L., Raghupathi, R. Differential behavioral and histopathological responses to graded cortical impact injury in mice. *Journal of Neurotrauma.* **23** (8), 1241-1253 (2006).
36. Robertson, C. L. et al. Cerebral glucose metabolism in an immature rat model of pediatric traumatic brain injury. *Journal of Neurotrauma.* **30** (24), 2066-2072 (2013).
37. Adelson, P. D., Fellows-Mayle, W., Kochanek, P. M., Dixon, C. E. Morris water maze function and histologic characterization of two age-at-injury experimental models of controlled cortical impact in the immature rat. *Child's Nervous System.* **29** (1), 43-53 (2013).

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Figure 1

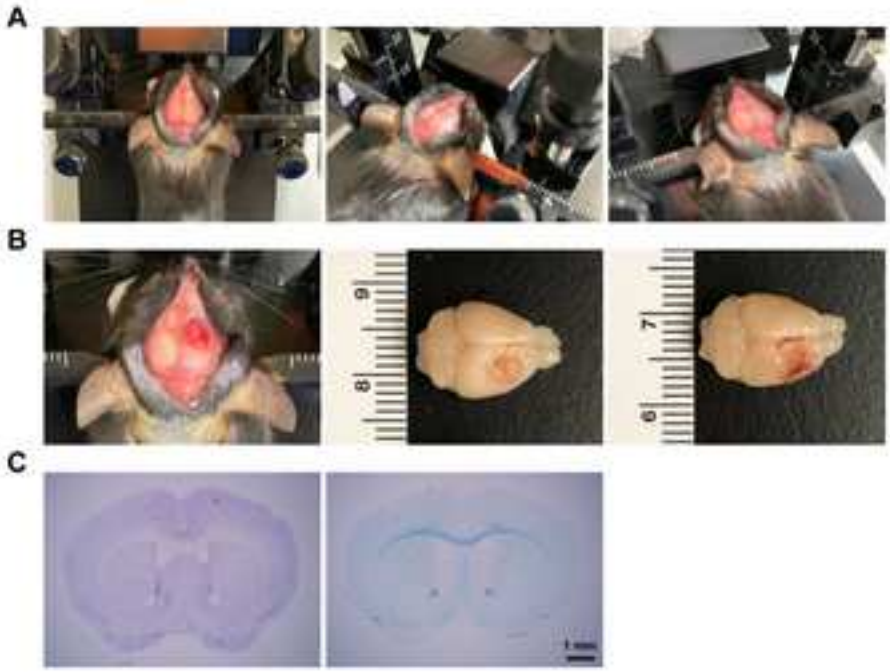


Figure 2

Click here to access/download;Figure;JoVE figure2_revision-02.tif

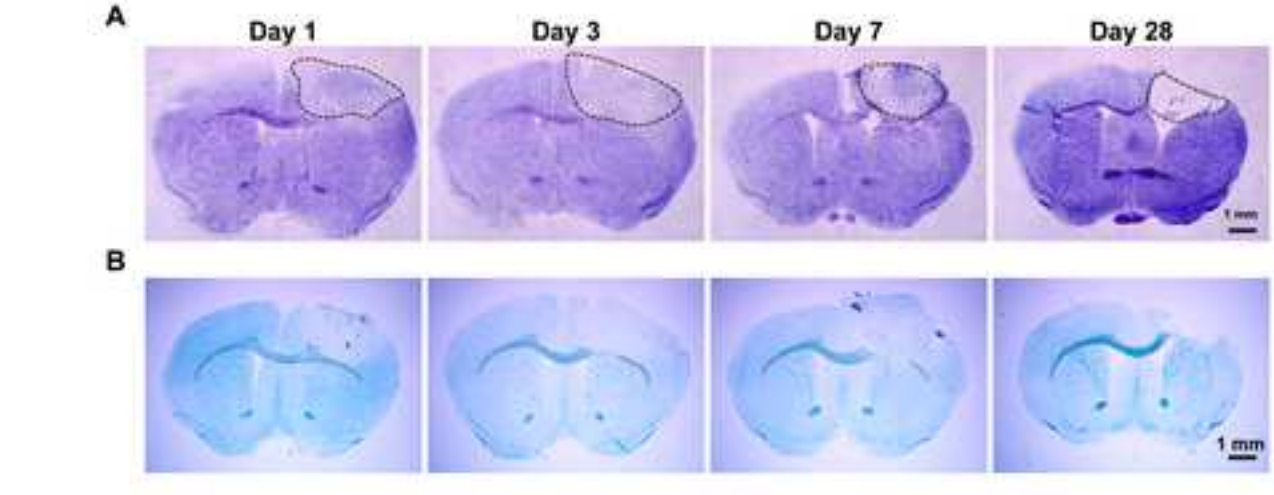
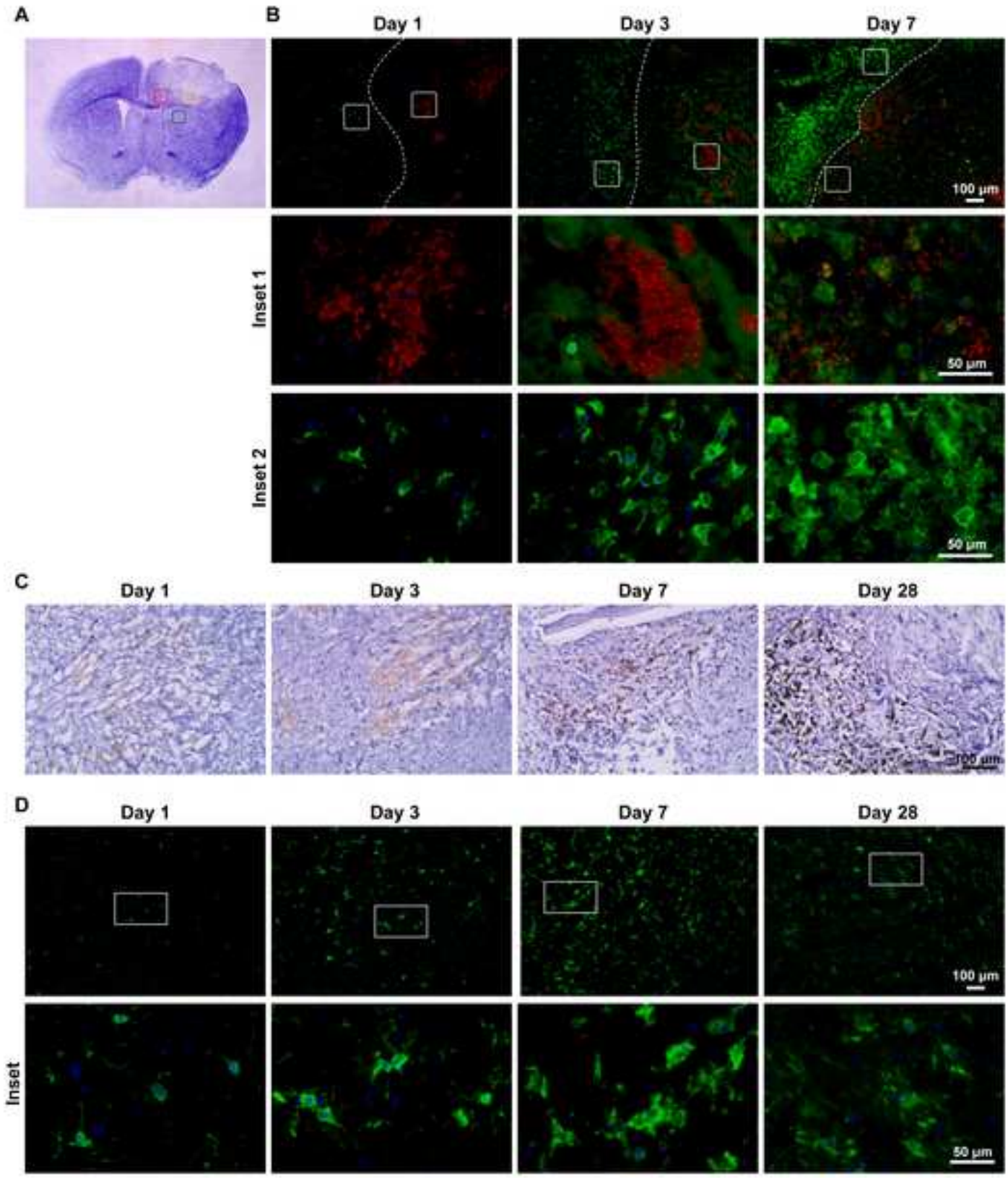


Figure 3

Click here to access/download;Figure;JoVE figure3_revision-03.tif



Materials

Click here to access/download;Table of Materials;Materials_F.xlsx

Name of Material/ Equipment	Company	Catalog Number
4mm Short Trephine Drill	Salvin Dental Specialties, Inc.	TREPH-SHORT-4
anti-Iba1 antibody	Wako chemicals	#019-19741
anti-Ly76 antibody	abcam	ab91113
carboxylate cement	3M	70201136010
cortical contusion injury impactor	Custom Design & Fabrication, Inc.	S/N 49-2004-C, eCCI Model 6.3
cresyl violet acetate	Sigma-Aldrich	C5042
DAB staining kit	Vector	SK-4105
goat anti-rabbit IgG secondary antibody, Alexa Fluor 488	Invitrogen	A11034
goat anti-rat IgG secondary antibody, Alexa Fluor 594	Invitrogen	A11007
Mayer's Hematoxylin	ScyTek	HMM500
tweezers	fine science tools	11252-20 NO. 5
isoflurane	Panion & BF Biotech Inc.	
lithium carbonate	Sigma-Aldrich	62470
steriotexic frame	stoelting	
scissors	fine science tools	14068-12
solvent blue 38	Sigma-Aldrich	S3382

Comments/Description
CCI device (S/N 49-2004-C, eCCI Model 6.3)

Rebuttal Letter

Click here to access/download;Rebuttal Letter;Cover letter_R1_F.docx

May 22, 2020

Dear Editors,

Thank you for your consideration of our manuscript “A Preclinical Controlled Cortical Impact Model for Traumatic Hemorrhage Contusion and Neuroinflammation” (JoVE61393R1).

We thank the review editor and reviewers for their comments and suggestions. We revised the manuscript substantially in response to the given suggestions, and we believe the revised manuscript that accompanies this letter addresses the raised concerns. All edits are tracked and the essential steps of the protocol for the video were highlighted in yellow. We provided below a point-by-point response to the comments.

Thank you for your invitation and the opportunity to submit a revised manuscript. We hope that our revisions have made the work acceptable for publication in the *Journal of Visualized Experiments*.

Sincerely,

Che-Feng Chang, PhD

Review Editor:

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached version for revision.

2. Please address all the specific comments marked in the manuscript.

3. Please address all the reviewers' comments.

4. Once done, please ensure that the highlight is no more than 2.75 pages including headings and spacings.

Thank you for your comments. We have made corresponding changes to the provided version for revision. We also ensure that the highlight is less than 2.75 pages including headings and spacings.

Reviewer #1:

The authors have sufficiently revised the manuscript and addressed reviewer comments. The additional clarifications on the model and methods, as well as the addition of the Figure 1 and inset substantially improve the manuscript. The only remaining minor point is to remove the word "revered" in the sentence "The CCI model is revered for its stability and reproducibility in producing injury ranging from mild to severe." (line 202). This seems to be excessive and dramatic language, and also not scientifically founded. After this correction, the manuscript can be accepted for publication.

We appreciate the reviewer’s suggestion. We have corrected the wording.

Reviewer #3:

In this revised version of their paper, authors have improved the quality of the manuscript by following several reviewers' suggestions. However, authors should add information about two minor concern in their manuscript with appropriate references.

Minor concern:

Line 303: other forms of hemorrhage contusions such as traumatic subarachnoid hemorrhage and subdural hemorrhage

I think that CCI protocol containss not only cerebral contusion but also traumatic subarachnoid hemorrhage (tSAH), Subdural hematoma (SDH), and Epidural hematoma (EDH), because both dura mater and arachnoid membrane are injured by device. Please describe this sentence more accurately. tSAH, DSH or EDH is not other type of hemorrhage contusions, but other type of focal injury.

We thank the reviewer for the suggestion. We agree and have made changes to the text.

Comment on #reviewer 2

5. It is surprising that in the day 1 Iba+ image (Fig 2B) there are little to no microglia, as most literature shows a robust and immediate microglia response in and around the impacted area, which leads to further microglia/macrophage proliferation and migration to the injured site in the window of 1-3 days. Can the authors address this discrepancy from other literature?

It depends on what type of antibody was used as an immunohistochemical marker for microglia. In some literature, CD11b positive cells are regarded as microglia/macrophages. However, CD11b antibody is also reactive for immune cells such as neutrophils. Indeed, through raptured BBB, neutrophils accumulate in and around the impacted area at day 1 post injury.

We appreciate this comment. We used Iba1 as a marker to show that neuroinflammation has taken place in the brain after CCI. We entirely agree with the reviewer that the discrepancy in immune cell numbers at day 1 post-CCI between studies could result from the usage of antibody (e.g. CD11b or Iba1) in immunohistochemical analysis. While we will continue to keep this in mind when designing experiments for our future CCI studies, this discussion is out of the scope of the current manuscript.