**TITLE:**

Sub-Acute Cerebral Microhemorrhages Induced by Lipopolysaccharide Injection in Rats

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**KEYWORDS:**

Blood-brain barrier, cerebral microhemorrhages, lipopolysaccharide, neuroinflammation, rats, protocol

**SUMMARY:**

We present a protocol to induce and detect CMHs caused by LPS injection in Sprague-Dawley rats, which may be utilized in future research investigations on the pathogenesis of CMHs.

**ABSTRACT:**

Cerebral microhemorrhages (CMHs) are common in aged patients and are correlated to various neuropsychiatric disorders. The etiology of CMHs is complex, and neuroinflammation is often observed as a co-occurrence. Here, we describe a sub-acute CMH rat model induced by lipopolysaccharide (LPS) injection, as well as a method for detecting CMHs. Systemic LPS injection is relatively simple, economical, and cost-effective. One major advantage of LPS injection is its stability to induce inflammation. CHMs caused by LPS injection could be detected by gross observation, hematoxylin and eosin (HE) staining, Perl’s Prussian staining, Evans blue (EB) double-labeling, and magnetic resonance imaging-susceptibility weighted imaging (MRI-SWI) technology. Finally, other methods of developing CMH animal models, including their advantages and/or disadvantages, are also discussed in this report.

**INTRODUCTION:**

Classical cerebral microhemorrhages (CMHs) refer to tiny perivascular deposits of blood degradation products such as hemosiderin from red blood cells in the brain1. According to the Rotterdam Scan Study, CMHs could be found in nearly 17.8% of persons aged 60-69 years and 38.3% in those over 80 years2. The prevalence of CMHs in the elderly is relatively high, and a correlation between the accumulation of CMHs and cognitive and neuropsychiatric dysfunction has been established3,4. Several animal models of CMHs have recently been reported, including rodent models induced by type IV collagenase stereotaxic injection5, APP transgenic6, β-N-methylamino-L-alanine exposure7, and hypertension8, with CMHs induced by systemic inflammation as one of the most well-accepted choices. Fisher *et al.*9 first used LPS derived from *Salmonella typhimurium* to develop an acute CMH mouse model. Subsequently, the same group reported the development of a sub-acute CMH mouse model using the same approach2.

LPS is considered as a standardized inflammatory stimulus *via* intraperitoneal injection. Previous studies have confirmed that LPS injection could cause neuroinflammation as reflected by large amounts of microglia and astrocyte activation in animal models2,10. Furthermore, a positive correlation between activation of neuroinflammation activation and the number of CMHs has been established2,10. Based on these previous studies, we were prompted to develop a CMH rat model by intraperitoneal injection of LPS.

Advances in detection technologies have resulted in an increase in the number of research investigations on CMHs. The most widely acknowledged methods of detecting CMHs include the detection of red blood cells by hematoxylin and eosin (HE) staining, ferric iron detection by Prussian Blue staining9, detection of Evans blue (EB) deposition by immunofluorescence imaging, and 7.0 Tesla magnetic resonance imaging-susceptibility weighted imaging (MRI-SWI)10. The present study aims to develop a method of screening for CMHs.

**PROTOCOL:**

All methods described here have been approved by the Animal Care and Use Committee (ACUC) of the PLA Army General Hospital.

1. **Materials**
   1. Preparation of LPS injection
      1. Add 25 mL of distilled water to 25 mg of LPS powder derived from *S. typhimurium* to a final concentration of 1 mg/mL. Store the injection in a sterile tube at 4 °C.

CAUTION: LPS is toxic.

* + 1. Prepare a 2% EB solution in normal 0.9% saline solution to keep EB injection at working concentration.

1. **Animals**
   1. Administer LPS to male Sprague-Dawley (SD) rats, aged 10 weeks (average weight: 280 ± 20 g) by intraperitoneal injection.

CAUTION: If rats are purchased from another organization, then the adaptive phase should not be less than 7 days.

1. **LPS Injections**
   1. Inject LPS intraperitoneally into each rat at a dose of 1 mg/kg, and return the rats to their home cage.

Note: Anesthesia is not necessary. Sucking back the LPS during injection may be necessary to confirm that LPS is not injected into the blood vessels.

* 1. Six hours later, inject the same dose of LPS injection into the rats.
  2. Sixteen hours later inject the same dose of LPS into the rats.

CAUTION: Injecting SD rats with LPS at a dose of 1 mg/kg may result in a 5% mortality rate. The mortality rate could further increase in younger or older rats or pregnant female rats.

* 1. Return the rats to their cages after LPS injection and provide *ad libitum* access to food and drink.

Note: Rats will exhibit a distinct systemic inflammatory response, and thus it is essential that the cages remain clean throughout the experiment.

1. **Sample Collection**
   1. **EB injection**
      1. Seven days after the first injection, anesthetize the rat by intraperitoneally injecting 10% chloral hydrate (1 mL/300 g).
      2. Wait 1-2 min until the rats do not show corneal reflex responses. Perform a 5-8 mm-deep cardiac puncture on each rat; the puncturing point should be 5 mm to the left margin of sternum at the 3rd and 4th intercostals space.
      3. Wait until blood recovery is observed, and then inject EB at a dose of 0.2 mL/100 g body weight into the left heart ventricle.

**CAUTION:** Cardiac puncture and EB injection should be carefully performed. EB might be injected into thoracic cavity or pericardium instead of the left heart ventricle.

* + - 1. Suck back with an empty tube and replace this volume with an EB tube before injection to help reduce the failure rate (*e.g*., injection to rib cage by error).
    1. Keep the rat in a supine position for 10 min.

Note: If sample is not used to detect EB leakage in immunofluorescence imaging, then this step may be omitted.

* 1. **Gross observation**
     1. Perform cardiac perfusions using ice-cold 1 M phosphate buffer saline (PBS) to clear the cerebral vasculature and brains.
        1. Using a scalpel, make an abdominal incision across the entire length of the diaphragm.
        2. Cut through ribs just left of the rib cage midline.
        3. Open up the thoracic cavity. Use clamps to expose the heart and to facilitate drainage of blood and other fluids.
        4. While steadily holding the heart with forceps (the heart should still be beating), directly insert a needle into the protrusion of the left ventricle to extend this to about 5 mm. Secure the needle at that position by clamping near the point of entry.

CAUTION: Do not excessively extend the needle, as it can pierce the interior wall and compromise the circulation of solutions.

* + - 1. Release the valve to allow the flow ice-cold PBS solution (200-300 mL) at a slow and steady rate at around 20 mL/min using a perfusion pump. If the animals require fixation instead of gross observation, then use the same dose of ice-cold 0.9% saline solution.
      2. Using sharp scissors, make an incision in the atrium, ensuring that the solution continuously flows. If the fluid does not flow freely or is coming from the animal’s nostrils or mouth, reposition the needle.
    1. Screen for CMHs by gross observation.

Note: If the sample is not used in calculating the number of CMHs by gross observation, then this step may be omitted.

* 1. Fixation
     1. Perform cardiac perfusions using ice-cold 0.9% saline solution (200-300 mL) to clear the cerebral vasculature and brains.
     2. Perform cardiac perfusions using ice-cold 4% paraformaldehyde for fixation.
     3. Decapitate the rat, isolate the brain, and immerse the brain in 20% sucrose solution for at least 6 h.
     4. Change the solution into 30% sucrose and fix for another 6 h**.**
  2. Prepare 10-mm-thick brain tissues sections using a cryostat.

1. **HE Staining**

Note: This procedure is performed using a HE Staining Kit.

* 1. Wash the slides in distilled water.
  2. Stain in hematoxylin solution for 8 min. Wash in running tap water for 5 min.
  3. Differentiate in 1% acid alcohol for 30 s. Wash in running tap water for 1 min.
  4. Stainin 0.2% ammonia water (Bluing) or saturated lithium carbonate solution for 30 s to 1 min. Wash in running tap water for 5 min.
  5. Rinse in 95% alcohol (10 dips).
  6. Counterstain with eosin solution for 30 s to 1 min.
  7. Dehydrate through 95% alcohol, two changes of absolute alcohol, for 5 min each.
  8. Clear in xylene for 30 s.
  9. Mount using Liu’s method9.
  10. Analyze using a brightfield fluorescence microscope.

Note: Red blood cells released from blood vessels, which are the components of CMHs, appear in red-orange under HE staining.

1. **Perl Prussian Blue Staining**

Note: This procedure is performed using a Perls Staining Kit.

* 1. Wash slides with distilled water.
  2. Stain in reaction solution with equal parts mixture of ferrocyanide and hydrochloric acid for 10 min.
  3. Dehydrate, clear, mount, and analyze the slides as described in steps5.7-5.10.

1. **EB double-staining**

Note: This procedure follows step 4.1.3.

* 1. Wash slides with PBS three times for 5 min each.
  2. Incubate slides with 4',6-diamidino-2-phenylindole (DAPI) solution for 15 min at room temperature.
  3. Wash the slides with PBS solution three times for 5 min each, and mount slides with PBS-glycerol solution.
  4. Analyze images on a fluorescence microscope. EB deposition is indicated by red fluorescence; nuclei are indicated by blue fluorescence.

1. **MRI-SWI**
   1. PerformMRI on a 7-T magnet equipped with an actively shielded gradient system (16 cm inner diameter).
   2. Seven days after treatment, anesthetize the rats by facemask inhalation of 1.5%-2.0% isoflurane using an isoflurane anesthesia system.
   3. Apply vet ointment onto the eyes of the rat to prevent dryness while under anesthesia.
   4. Keep the rats in prone position and perform an MRI-SWI scan.
   5. Obtain SWI weighted scans using a fast-spin echo sequence using the following parameters: echo time (TE) 8 ms, repetition time (TR) 40 ms, flip angle=12°, field of view (FOV) 35 mm × 35 mm, acquisition matrix 384 × 384, to acquire 1-mm-thick slices.
   6. Identify CMHs in MRI-SWI according to Greenberg *et al.*11, which included the following criteria: (1) a diameter of ≤10 mm, and (2) circular, isolated, and low-signal intensity spots.
   7. At the end of the experiment, euthanize the rats using the overdose carbon dioxide (at a flow of 6 L/min) method.

**REPRESENTATIVE RESULTS:**

CMHs can be detected using various approaches. The most well-accepted methods include the following: (1) gross observation and assessment of surface CMHs (shown in **Figure 1**, upper panel); (2) HE staining for the detection of red blood cells (shown in **Figure 2A**, upper panel) or Prussian staining detecting ferric iron derived from lysis of red blood cells (**Figure 2A**, lower panel); (3) EB doubled staining for the detection of EB deposition derived from BBB leakage (**Figure 3**, left panel); (4) MRI-SWI detection of CMH hypointense signals (**Figure 4**, left panel).

**Figure 1. Gross observation of surface CMHs.** Upper panel (A): Rat model; Lower panel (B): Control animal. Red arrows signify surface CMHs. Modified and reused with permission10.

**Figure 2. HE staining and Prussian staining images.** (A) Reflected rat model. Red blood cells were found outside the capillaries in upper panel, and ferric iron points derived from lysis of red blood cells were detected in lower panel; (B) Reflected control animal. Neither red blood cells nor ferric iron points were found. Scale bars in left panel of (A) and (B): 100 µm. Scale bars in left panel of (A) and (B): 50 µm. Modified and reused with permission10.

**Figure 3. Double labeled EB deposition fluorescence imaging.** Left panel: Rat model. Amount of EB molecules leaked from injured BBB were detected (in red); Right panel: Control animal. No EB molecules were detected. DAPI in blue was used as mount medium. Scale bars: 100 µm. Modified and reused with permission10.

**Figure 4. MRI-SWI images.** Left panel (A): Rat model. Black arrows signify hypointense signals of CMHs; Right panel (B): Control animal. No hypointense signals were found. Modified and reused with permission10.

**DISCUSSION:**

Research studies on CMHs have increased in the past few years. However, the mechanism of CMHs remains unclear, prompting scientists to establish animal models that simulate this particular condition. For example, Hoffmann *et al.* developed a hypoxia-induced CMH mouse model that shows that CMHs are caused by hypoxia and disruption of cerebrovascular autoregulation12. Reuter *et al.*5 established a CMH model in APP23-transgenic mice, which showed that cerebral amyloid angiopathy (CAA) plays a major role in the etiology of CMHs. Fisher *et al.* reported a CMH mouse model that was induced by LPS injection9, which revealed that CMHs are caused by inflammation2,13,14. Similarly, we have successfully developed a sub-acute CMH model in SD rats using LPS injection and have found that nitric oxide synthase (NOS), especially neural NOS and endothelial NOS, are involved in the etiology of CMHs, which result in inflammation10.

The critical step of our protocol is the LPS injection, which has been extensively used in developing murine models of neuropsychiatric disorders such as depression15, schizophrenia16, Parkinson’s disease17, Alzheimer’s disease18, and Prion disease19. To our knowledge, our use of a single dose (1 mg/kg) is much higher than that was applied in other studies15-19. This might be a plausible reason for the mortality in the current study. LPS dose modification for CMH induction may be an interesting research topic, as different doses or injection schedule have been shown to influence the number, size, distribution, and progression of CMHs2. A previous study has shown that the administration of LPS to mice at a dose of 3 mg/kg induces acute CMHs2.

Although the development of different animal models has facilitated research investigations on CMHs, we have to admit that those animal models do not simulate the process of clinical CMHs. For example, in our rat model, as well as Fisher’s mice model, CMHs were observed in the cerebellum, although most were observed in lobar regions (mainly CAA-related) and deep- or infra-tentorial locations (mainly hypertension-related)20,21. We have no explanation for this discrepancy in distribution, although both Fisher and our team attribute this observation to the vulnerability of cerebellar blood vessels to inflammation.

In most clinical cases, CMHs result from more than one etiological factor, although inflammation plays an important role in its etiology. Studies focusing on multiple factors that induce CMHs, instead of pure inflammation-induced CMHs, can thus be more helpful in simulating this condition. The LPS injection model could be used with other underlying factors to investigate the mechanism of CMHs. For example, Fisher *et al.* have conducted a preliminary, yet interesting step with aging mice injected with LPS, and demonstrated that aging is a key factor that could make the brain more susceptible to inflammation-induced CMHs14. In our opinion, the significance of the present model is its compatibility with other factors in animal models for aging14, trauma22, as well as chronic diseases such as hypercholesterolemia23, or transgenic models such as hypertension24 because of the simplicity, time-effectiveness, and stability of this protocol.

Patients with CMHs show cognitive declines, neuropsychiatric manifestations, and vertigo, which are associated with the distribution of CMHs. One limitation of the present protocol is that after LPS injection, we could not rule out the effects of peripheral inflammation on the behavior of the rats, although a decrease in social behavior and burrowing (intrinsic natural activity of rodent reflecting the impairment of daily activities) were observed. Further studies on ways to improve our method of generating a CMH animal model, *e.g.,* method of injecting LPS or schedule of observation, are warranted.

Nevertheless, this simple, cost-effective, and stable CMH murine model induced by LPS injection may be utilized by researchers in elucidating the etiology of CMHs.

**ACKNOWLEDGMENTS:**

We thank Teacher Jian Feng Lei and colleagues from Capital Medical University for guidance during MRI. We also thank Jing Zeng from the Department of Neurology, the Second People’s Hospital of Yichang for providing technical support.

**DISCLOSURES:**

The authors have nothing to disclose.

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