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Isolation of Rodent Brain Vessels

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[Abstract] The prevalence of neurodegenerative diseases is increasing worldwide. Cerebrovascular disorders and/or conditions known to affect brain vasculature, such as diabetes, are well-known risk factors for neurodegenerative diseases. Thus, the evaluation of the brain vasculature is of great importance to better understand the mechanisms underlying brain damage. We established a protocol for the isolation of brain vessels from rodents. This is a simple, non-enzymatic isolation protocol that allows us to perform comparative studies in different animal models of disease, helping understand the impact of several pathological conditions on brain vasculature and how those alterations predispose to neurodegenerative conditions.
Keywords: Blood-brain barrier, Cerebrovascular and neurodegenerative disorders, Isolated brain vessels, Non-enzymatic isolation protocol, Rodents

[Background] The brain is highly dependent on a constant supply of oxygen and nutrients that arrive through a vast network of blood vessels. The blood-brain barrier (BBB), mainly composed of microvascular endothelial cells that line cerebral microvessels along with periendothelial structures, which include pericytes, astrocytes and a basement membrane (Saraiva *et al.*, 2016; Librizzi *et al.*, 2017), guarantees the control of an homeostatic environment, necessary to maintain the health of brain cells. Thus, the study of how certain pathologies that can interfere with the integrity of cerebrovasculature is of great importance. Indeed, strong evidence from clinical, imaging, epidemiological and neuropathological studies confirmed over the past two decades that the presence of cerebrovascular disease has a pivotal role in Alzheimer disease (AD) and other dementias associated with aging (Chui *et al.*, 2006; Schneider *et al.*, 2007; Gorelick *et al.*, 2011; Wharton *et al.*, 2011; Yarchoan *et al.*, 2012; Bennett *et al.*, 2013; DeCarli, 2013; Toledo *et al.*, 2013; Yates *et al.*, 2014). Besides the low number of papers dedicated to the study of isolated brain vessels, the isolation protocols used in those studies present some inconsistencies rendering difficult the comparison and interpretation of the published observations. With this protocol, we intend to offer a standardized procedure to help researchers working in this field. This protocol was adapted from a previous protocol described by McNeill *et al.* (1999) and used in our laboratory to isolate total (arterial and venous) brain vessels from rodents (Figures 1 and 4) (Carvalho *et al.*, 2010; Carvalho *et al.*, 2013; Plácido *et al.*, 2017).

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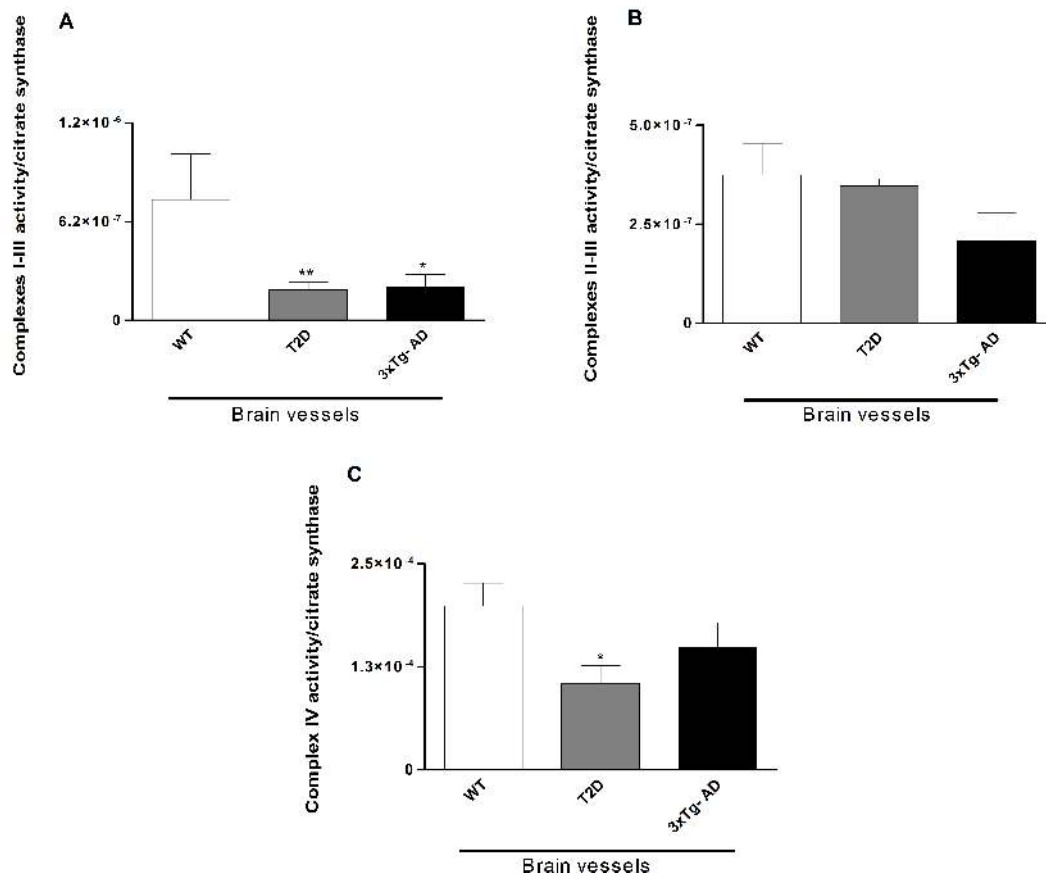

Figure 1. Evaluation of the activity of the mitochondrial enzymatic complexes of mice brain vessels. Mitochondrial complexes I-III (A), II-III (B) and IV (C) were determined in vessels isolated from the brains of 11-month-old male wild type (WT; C57BL6/129S), type 2 diabetes-like mice (WT mice exposed to 20% sucrose solution during 7 months) and triple transgenic mice for Alzheimer disease (3xTg-AD, B6;129-Psen1 Tg(APPSwe,tauP301L)1Lfa/Mmjax). A significant decrease in the activity of mitochondrial complexes I-III was observed in brain vessels isolated from 3xTg-AD and type 2 diabetes-like mice. Also, a significant decrease in the activity of complex IV was observed in brain vessels isolated from 3xTg-AD mice. Data shown represent mean ± SEM from 6-8 pools of n = 3. Statistical significance: $^*P < 0.05$; $^{**}P < 0.01$ when compared with WT mice. Statistical significance was determined using the paired Student's *t*-test and Kruskal-Wallis test for multiple comparisons, followed by the posthoc Dunn test (GraphPad Prism 5). These graphs have been previously published in Journal of Alzheimer's Disease (DOI: 10.3233/JAD-130005) with permission from IOS Press.

Materials and Reagents

1. 50 ml Oak Ridge polysulfone centrifuge tubes w/screw caps (Thermo Fisher Scientific, catalog number: 3115-0050)
2. Eppendorf microtubes 1.5 ml (VWR, catalog number: 700-5239)
3. PIPETMAN TIPS Diamond–ECOPACK™ D1000 (Gilson, catalog number: F161670)

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4. PIPETMAN TIPS Diamond–ECOPACK™ D200 (Gilson, Catalog number: F161930)
5. PIPETMAN TIPS Diamond–ECOPACK™ D10 (Gilson, catalog number: F161630)
6. Stainless steel surgical blades (Swann Morton, catalog number: 0308)
7. Rodent brain
   Note: This protocol has only been tested with brains from male young and mature (3- and 12-month-old) Wistar rats and wild type, type 2 diabetes-like and triple transgenic for Alzheimer disease (3xTg-AD) mice (11-month-old). Nevertheless, we believe that this protocol can be applied to different strains, ages and sex, though the amount of obtained sample can be a limiting factor.
8. Ice
9. Distilled water
10. Isoflurane (Lab. Vitória, Portugal)
11. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S7653)
12. Sodium phosphate dibasic (Na_2HPO_4) (Sigma-Aldrich, catalog number: S7907)
13. Dextran from *Leuconostoc mesenteroides* (Sigma-Aldrich, catalog number: 31398)
14. Phosphate buffer (0.01 M) (see Recipes)
15. Dextran (16%) (see Recipes)

Equipment

1. 50 ml Fisherbrand™ reusable glass low-form Griffin beakers (Fisher Scientific, catalog number: FB10050)
2. Bone cutting forceps (Aesculap, catalog number: FO611R)
3. Centrifuge (Refrigerated Centrifuge) (Sigma Laborzentrifugen, model: SIGMA 3-16K)
4. Curved fine tip forceps (Aesculap, catalog number: FB401R)
5. Heidolph mechanical overhead stirrers, Brinkmann (Heidolph Instruments, model: RZR 1)
6. Laboratory bottles, narrow mouth, with screw cap (VWR, catalog number: 215-1594)
7. Micropipette PIPETMAN L (Light) type P200L (Gilson, catalog number: FA10005)
8. Micropipette PIPETMAN L (Light) type P1000L (Gilson, catalog number: FA10006)
9. Nickel/SS Lab spatulas with 1.63” Flat Rounded Ends (Cole-Parmer, catalog number: EW-06369-05)
10. Petri dish with cover 60 x 15 mm (Corning, catalog number: 70165-60)
11. Potter-Elvehjem with PTFE pestle and glass tube (DWK Life Sciences, Kimble®, catalog number: 886000-0023)
12. Precision scale (Mettler-Toledo International, model: AE240)
13. Precision balance PLE-N (KERN, model: PLE-N)
14. Scalpel handle (Aesculap, catalog number: BB084R stainless)
15. Soft hair brush, 3 mm dia. (CONTROLS, catalog number: 86-D1672)
16. Swing-out rotor (Sigma Laborzentrifugen, catalog number: 11133)

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Procedure

All the isolation protocol steps must be performed at 4 °C (always maintain solutions, homogenates and pellets on ice).

1. Euthanize rodents by cervical displacement (mice) and decapitation (rats and mice). All procedures are approved by the Federation of Laboratory Animal Science Associations (FELASA).
2. Perform a midline incision, posterior to anterior, along the scalp to reveal the skull (Video 1).

Video 1. Brain removal, dissection and homogenization

3. Cut the cranium carefully from the neck to the nose through the lateral cranial sutures. Two additional cuts can be performed, in the occipital hole to the ears direction, to facilitate the access to the brain (Figure 2A, Video 1).

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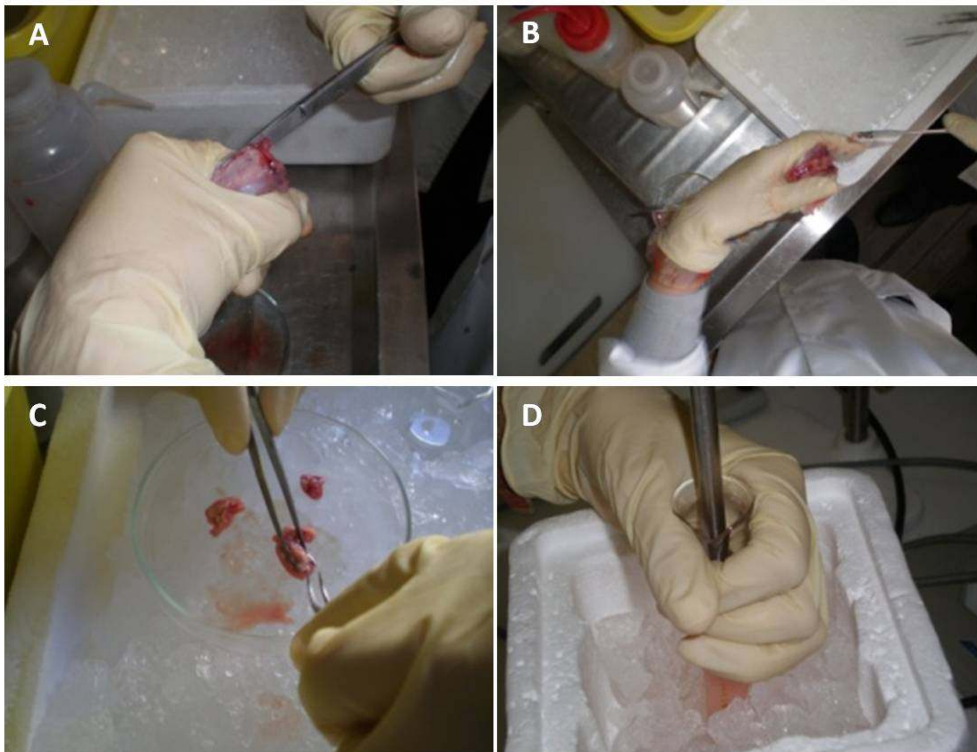

Figure 2. Illustrative images of brain dissection and homogenization. Cut the cranium carefully from the neck to the nose through the lateral cranial sutures (A). Remove the intact brain from the cranial box using a small spatula (B). Then, remove the cerebellum, olfactory bulbs and white matter using fine dissection forceps and keep the brain cortices (C). Finally, homogenize brain tissue, at 4 °C, in 10 ml PBS using a Potter-Elvehjem with PTFE pestle and glass tube, at 800 rpm/min (D).

4. Remove the intact brain from the cranial box using a small spatula (Figure 2B, Video 1).
5. Remove the cerebellum, olfactory bulbs and white matter using fine dissection forceps; keep the brain cortices (Figure 2C, Video 1, see Note 1).
6. Perform a quick wash (approximately 30 sec) in non-sterile PBS (Video 1; see Recipes).
7. Homogenize fresh brain tissue, at 4 °C, in 10 ml PBS using a Potter-Elvehjem with PTFE pestle and glass tube, at 800 rpm/min (Figure 3, Video 1).

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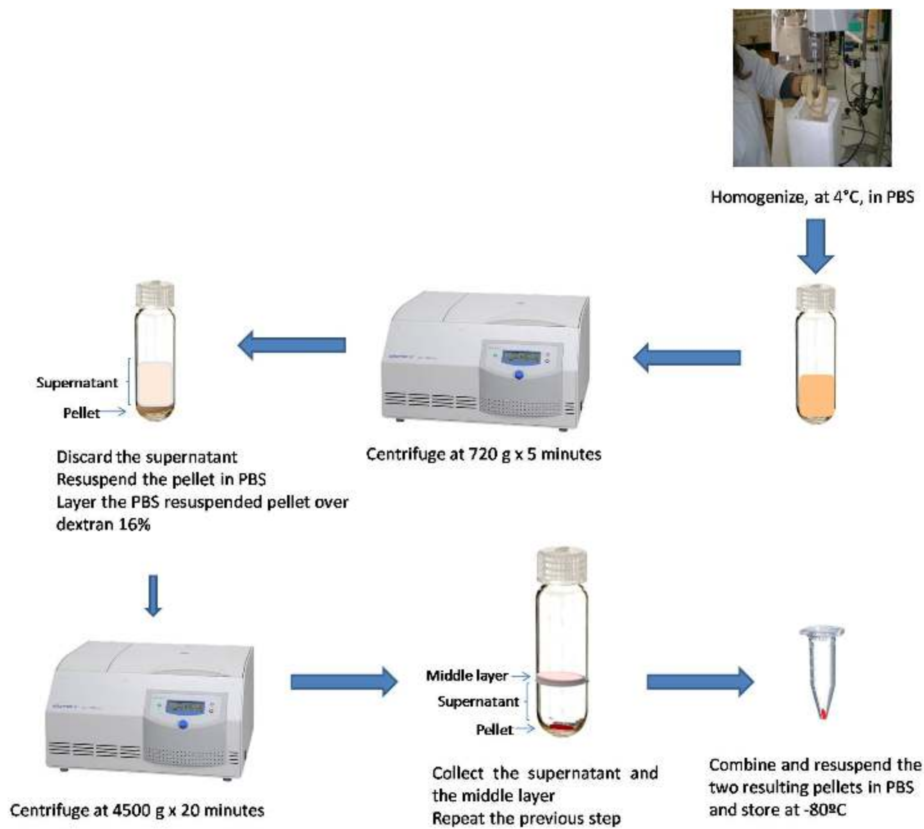

Figure 3. Schematic illustration of mice brain vessels isolation

8. Centrifuge at 720 *x g* for 5 min, at 4 °C (Figure 3).
9. Discard the supernatant by decantation (Figure 3).
10. Resuspend the pellet in 10 ml PBS using a soft hair brush, 3 mm dia (Figure 3).
11. Repeat the steps 8-10 two more times.
12. Layer the PBS resuspended pellet over 20 ml 16% dextran (Video 2; see Recipes).
    Note: This step must be gently done to avoid mixing the two layers (Figure 3).

Video 2. Brain vessels isolation

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13. Centrifuge at 4,500 *x g* for 20 min, at 4 °C (Figure 2).
14. Collect the supernatant and the middle layer, and keep the pellet on ice (Figure 3).
15. Repeat step 14.
16. Discard the supernatant and resuspend the two resulting pellets in 100 µl PBS.
    Note: Resuspend the first pellet in 100 µl PBS and use the suspension to resuspend the second pellet. The vessels can be visualized at optic microscope (Figure 4) using the DiffQuick staining as described by Mota and Ramalho-Santos (2006).
17. Store the vessels at -80 °C until use (used until 2 years upon isolation; Figure 3).

Figure 4. Representative images of isolated mice brain vessels after DiffQuik staining. Unstained (A) and stained (B, C) brain vessels observed under the optic microscope. 100x amplification; scale bars = 50 µM.

Notes

1. In order to minimize the day-to-day variability that can bias the results, in studies using animal models of disease, researchers must always isolate vessels from control and diseased animal models.
2. Minimum number of animals required for the isolation of brain vessels: 1 rat brain or 3 mice brains. It is possible to use only 1 mice brain. However, the pellet size will be very small, which will difficult its visualization with the naked eye.

Recipes

1. Phosphate buffer (0.01 M)
   8.5 g/L NaCl
   1.42 g/L Na_2HPO_4
   pH = 7.4
2. Dextran (16%)
   160 g/L Dextran from *Leuconostoc mesenteroides*

Notes:

a. Distilled water type 1 must be used in the preparation of the solutions.

b. *For short periods of time solutions must be conserved at 4 °C (e.g., one week); for longer periods solutions must be conserved at -20 °C (except Dextran solution).*
c. *Dextran solution must not be frozen; it is stable for one week at 4 °C.*

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