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A Murine Model of Hind Limb Ischemia to Study Angiogenesis and Arteriogenesis

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Abstract

Therapeutic angiogenesis offers promise as a novel treatment that is complementary to surgical or endovascular procedures for peripheral arterial diseases (PAD). Appropriate development and use of hind limb ischemia models is necessary for successful studies of therapeutic angiogenesis and/or arteriogenesis. In this chapter, we describe two commonly used murine unilateral hind limb ischemia models, the femoral artery transection model and the femoral/saphenous artery excision model.

Keywords

Hind limb ischemia; Animal model; Angiogenesis; Arteriogenesis; Ischemia; Peripheral arterial disease

1 Introduction

Peripheral arterial disease (PAD) affects approximately eight million people in the USA, although it has poor awareness and recognition in the general population, and thus the prevalence of PAD is likely to be underestimated [1]. While surgical revascularization remains the most effective treatment for limb ischemia, many patients with advanced disease are not suitable for surgical or endovascular management. Therapeutic angiogenesis and stem cell therapies are newer treatments that may be appropriate for these difficult patients [2, 3]. However, defining optimal parameters for these gene and cell therapies is expensive to perform as clinical trials in human patients, and therefore may be more suitable in animal models [4, 5]. The murine hind limb ischemia model is a very useful model to use for testing some of these parameters and therapies [5, 6].

In this chapter, we describe the methodology for the two most commonly used murine models of unilateral hind limb ischemia, e.g., the femoral artery transection model and the femoral/saphenous artery excision model. The femoral artery transection model is a model that produces only a mild-to-moderate amount of ischemia that induces mainly arteriogenesis in the thigh, with minimal calf angiogenesis. The femoral/saphenous artery excision model produces more severe ischemia that induces both thigh arteriogenesis as well as calf angiogenesis. The materials and procedures for establishing and evaluating these models are described, including several notes that come out of our own experience with these models.

2 Materials

2.1 Surgical Tools (See Note 1)

1. Dissecting microscope.
2. Pointed forceps.
3. Surgical scissors.
4. Retractor (*see* Note 2).
5. Fine pointed forceps.
6. Spring scissors.
7. Needle holder.
8. Cautery tool.
9. Sterile cotton swabs.
10. 7-0 and 6-0 nonabsorbable sutures.
11. Heating pad.

2.2 Blood Flow Measurement Equipment

1. PeriFlux Laser Doppler Perfusion Measurement Unit with deep penetration probe.
2. Laser speckle imaging system (moorFLPI-2 or higher resolution moorLDI2-HIR).
3. Power lab.

3 Methods

3.1 Femoral Artery Transection Model (See Note 3)

1. Anesthetize the mouse by placing it into an anesthesia induction chamber containing 2% isoflurane at a flow rate of 2 L/min.
2. Remove the mouse from the induction chamber when it is unresponsive to external stimuli. Confirm proper anesthetization by pinching its toe. Apply some artificial tears ointment on the eyes to prevent dryness (*see* Note 4).
3. Place the mouse in the preoperating area and connect it to a continuous flow of isoflurane. Remove the hair from the lower abdomen to the foot using an electric shaver. Then apply hair removal cream to thoroughly remove the fur.

¹Surgical tools need to be sterilized prior to surgery with a hot-bead sterilizer. It is required that the surgical tools be resterilized between each operation when multiple mice are operated on the same day.

²We use our own retractors since most commercial available retractors are too big for surgery on a mouse limb.

³This is a mild hind limb ischemia model that mainly induces arteriogenesis in the thigh without calf angiogenesis.

⁴Flush the anesthetic from the induction chamber prior to opening the lid to decrease the operator's exposure to isoflurane.

4. Place the mouse in a supine position over a heating pad with the left hind limb slightly abducted and the knee joint slightly flexed. Extend and secure the limbs with a piece of tape with hind feet facing up. Connect the mouse to a continuous flow of isoflurane.
5. Once the mouse is secure, prepare the skin around surgical area with three alternating betadine and alcohol scrubs.
6. Make a ~1 cm midline incision of the skin from ~7 mm below the inguinal region and ~3 mm above it using forceps and surgical scissors. The remainder of the surgical procedure should be performed under a dissecting microscope to gain a magnified view of the surgical region.
7. Gently push away subcutaneous fat tissue superficial to the neurovascular bundle in the thigh region by using phosphate buffered saline moistened cotton swabs and forceps.
8. Use a retractor to open the wound for easy access to the neurovascular bundle.
9. Gently pierce through the membrane of femoral sheath by blunt dissection using fine forceps to expose the neurovascular bundle. The anatomy of the surgical region should be now clearly exposed as shown in (Fig. 1). Asterisks indicate the locations of ligation for the induction of hind limb ischemia.
10. Push away the nerve from the femoral artery and vein by using a wet cotton swab.
11. Carefully separate the femoral artery from the femoral vein at the ligation sites between the proximal caudal femoral artery and the bifurcation of the deep femoral artery and saphenous artery by blunt dissection using surgical fine forceps. Use caution not to tear the femoral vein (*see Note 5*).
12. Then, pass two 7-0 silk sutures underneath the separated femoral artery segment. Tie off the artery by double knots as shown in Fig. 2a.
13. Transect the femoral artery between the two ties.
14. If a Laser Doppler Perfusion system is used, the blood flow should be assessed at this point, as described below (*see Subheading 3.3*).
15. Remove the retractor and close the incision using 6-0 Nylon sutures.
16. If Laser Doppler imaging system is used, the blood flow should be assessed at this point, as described below (*see Subheading 3.4*).
17. After blood flow measurement, place the mouse in a clean cage with a heating pad and monitor the breath and heart beat continuously until the animal is fully recovered.
18. Return the mouse to animal facility (*see Note 6*).

⁵In the case of accidental disruption of the femoral vein or other vessels occur, a dry sterile cotton tipped applicator should be applied to the site of hemorrhage with moderate pressure until the bleeding stops.

3.2 Femoral Artery and Saphenous Artery Excision Model (See Note 7)

1. Anesthetize the mouse by placing it into an anesthesia induction chamber containing 2% isoflurane at a flow rate of 2 L/min.
2. Remove the mouse from the induction chamber when it is unresponsive to external stimuli. Confirm proper anesthetization by pinching its toe. Apply some artificial tears ointment on the eyes to prevent dryness (*see* Note 4).
3. Place the mouse in the preoperating area and connect it to a continuous flow of isoflurane. Remove the hair from the lower abdomen to the foot using an electric shaver. Then apply hair removal cream to thoroughly remove the fur.
4. Place the mouse in a supine position over a heating pad with the left hind limb slightly abducted and the knee joint slightly flexed. Extend and secure the limbs with a piece of tape with hind feet facing up. Connect the mouse to a continuous flow of isoflurane.
5. Once the mouse is secure, prepare the skin around surgical area with three alternating betadine and alcohol scrubs.
6. Make a ~1 cm midline incision of the skin from ~7 mm below the inguinal region and ~3 mm above it using forceps and surgical scissors. The remainder of the surgical procedure should be performed under a dissecting microscope to gain a magnified view of the surgical region.
7. Gently push away subcutaneous fat tissue superficial to the neurovascular bundle in the thigh region by using phosphate buffered saline moistened cotton swabs and forceps.
8. Use a retractor to open the wound for easy access to the neurovascular bundle.
9. Gently pierce through the membrane of femoral sheath by blunt dissection using fine forceps to expose the neurovascular bundle. The anatomy of the surgical region should be now clearly exposed as shown in (Fig. 1). Asterisks indicate the locations of ligation for the induction of hind limb ischemia.
10. Push away the nerve from the femoral artery and vein by using wet cotton swab.
11. Carefully separate the femoral artery from the femoral vein at the proximal ligation site just below the inguinal ligament. Use caution not to tear the femoral vein (*see* Note 5).
12. Then, pass a 7-0 silk suture underneath the proximal end of the femoral artery just below the inguinal ligament and tie off the artery by double knots as shown in (Fig. 2a).

⁶Consult the Institutional Animal Care and Use Committee (IACUC) at your institution for specific regulations regarding returning post-surgical animals to the animal care facility, as the mouse that undergoes hind limb ischemia surgery may be considered to have a pain level of either D or E, depending on the exact protocol and medications used. Appropriate preoperation and postoperation analgesic agents should be given per local IACUC regulations.

⁷This is a more severe hind limb ischemia model that induces arteriogenesis in the thigh and profound hypoxia induced angiogenesis in the calf muscle groups.

13. Carefully separate the femoral artery from the femoral vein all the way down to the site of popliteal artery and saphenous artery bifurcation by blunt dissection using surgical fine forceps. Use caution not to tear the femoral vein.
14. Then, pass a 7-0 suture underneath the femoral artery just proximal to the popliteal artery and saphenous artery bifurcation. Tie off the artery by double knots as shown in (Fig. 2b).
15. Replace the retractor if necessary so the distal region of femoral artery and saphenous artery can be exposed clearly (*see* Note 8).
16. Carefully separate the saphenous artery from the vein for about half way down by blunt dissection using surgical fine forceps. Use caution not to tear the saphenous vein.
17. Then, pass a 7-0 suture underneath the distal site of saphenous and tie off the artery by double knots as shown in (Fig. 2b).
18. Using a cautery tool to cauterize the lateral circumflex femoral artery and proximal caudal femoral artery. Apply the cautery transversely to incise the superficial caudal epigastric artery.
19. Transect and excise the segment of artery between the most distal and proximal knots by using a pair of spring scissors. Use caution not to tear the surrounding vein (Fig. 2c).
20. If a Laser Doppler Perfusion system is used, the blood flow should be assessed at this point, as described below (*see* Subheading 3.3).
21. Remove the retractor and close the incision using 6-0 Nylon sutures.
22. If Laser Doppler imaging system is used, the blood flow should be assessed at this point, as described below (*see* Subheading 3.4).
23. After blood flow measurement, place the mouse in a clean cage with a heating pad and monitor the breath and heart beat continuously until the animal is fully recovered.
24. Return the mouse to animal facility (*see* Note 6).

3.3 Laser Doppler Perfusion Measurement

1. Place the mouse into the anesthesia induction chamber containing 2% isoflurane at a flow rate of 2 L/min.
2. After the mouse is unresponsive to external stimuli, remove it from the induction chamber. Confirm proper anesthetization by pinching its toe. Apply some artificial tears ointment on the eyes to prevent dryness.

⁸The mouse skin on the lower limb is very flexible. It is possible to retract the wound up and down to expose the proximal and distal surgical regions by using a smaller incision. In our experience, this procedure can accelerate the wound closure time, decrease the inflammatory response caused by surgery and diminish the impact of surgical wound on Laser Doppler imaging. A larger incision is shown in the Figures only for illustrative purposes.

3. Place the mouse on preoperating region connected to isoflurane.
4. Apply hair removal cream to thoroughly remove the fur below knee.
5. Place the animal on a 37 °C heated surface for 5 min. Monitor the body temperature to ensure stable.
6. Place the mouse in a supine position over a heating pad with the both hind limbs slightly abducted. Extend and secure the limbs with a piece of tape with paws facing up.
7. Once the mouse is secure, prepare the skin below knee area with three alternating betadine and alcohol scrubs.
8. Make a ~7 mm midline incision of the skin below knee on both limbs.
9. Place the deep penetration probe onto the surface of gastrocnemius muscle group.
10. Record the blood flow trace for 3 min for each limb (*see* Note 9).
11. Close the incision using 6-0 Nylon sutures.
12. Return the mouse to the recovery cage and monitor the animal continuously until fully recovered.

3.4 Laser Doppler Imaging

1. Place the mouse into the anesthesia induction chamber containing 2% isoflurane at a flow rate of 2 L/min.
2. After the mouse is unresponsive to external stimuli, remove it from the induction chamber. Confirm proper anesthetization by pinching its toe. Apply some artificial tears ointment on the eyes to prevent dryness.
3. Place the mouse in the preoperating area and connect it to a continuous flow of isoflurane.
4. Apply hair removal cream to thoroughly remove the fur below knee.
5. Place the animal on a 37 °C heated surface for 5 min. Monitor the body temperature to ensure stable.
6. Place the animal in the supine position on a nonreflective light-absorbing surface, connected to a continuous flow of isoflurane. Extend the hind limbs.
7. Specify the size of the field of view and scanning resolution (*see* Note 10). Using the Laser Doppler imager and the acquisition module to acquire image per the manufacturer's instructions (*see* Note 9).
8. Return the mouse to the recovery cage and monitor the animal continuously until fully recovered.

⁹The body temperature and the light in the measurement area should be kept very stable. Any vibration should be avoided. All these factors may influence the recording.

¹⁰The field of view and scanning resolution should be consistent between animals and all the measurements at different time points.

Both Laser Doppler Perfusion Measurement and Laser Doppler imaging procedures can be repeated to follow the changes in blood flow recovery over time.

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Fig. 1. Anatomy of the mouse hind limb (left). Asterisks indicate the locations of ligation in the models of hind limb ischemia. A indicate artery; v indicate vein

Fig. 2.

(a) The femoral artery transection model. Two ligatures around the femoral artery (not the vein), before transection of the intervening segment. (b) The femoral/saphenous artery excision model. Upper ligature around the femoral artery, middle ligature around the popliteal/saphenous artery bifurcation, and the lower ligature around the saphenous artery. (c) The femoral/saphenous artery excision model, after excision of the femoral and saphenous arteries