Video Article

Methods for Acute and Subacute Murine Hindlimb Ischemia

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URL: https://www.jove.com/video/54166
DOI: doi:10.3791/54166

Keywords: Medicine, Issue 112, Ischemia, peripheral artery disease, angiogenesis, skeletal muscle regeneration, atherosclerosis, vascular surgery, mouse hindlimb, ameroid constrictor

Date Published: 6/21/2016

Citation: Padgett, M.E., McCord, T.J., McClung, J.M., Kontos, C.D. Methods for Acute and Subacute Murine Hindlimb Ischemia. *J. Vis. Exp.* (112), e54166, doi:10.3791/54166 (2016).

Abstract

Peripheral artery disease (PAD) is a leading cause of cardiovascular morbidity and mortality in developed countries, and animal models that reliably reproduce the human disease are necessary to develop new therapies for this disease. The mouse hindlimb ischemia model has been widely used for this purpose, but the standard practice of inducing acute limb ischemia by ligation of the femoral artery can result in substantial tissue necrosis, compromising investigators' ability to study the vascular and skeletal muscle tissue responses to ischemia. An alternative approach to femoral artery ligation is the induction of gradual femoral artery occlusion through the use of ameroid constrictors. When placed around the femoral artery in the same or different locations as the sites of femoral artery ligation, these devices occlude the artery over 1 - 3 days, resulting in more gradual, subacute ischemia. This results in less substantial skeletal muscle tissue necrosis, which may more closely mimic the responses seen in human PAD. Because genetic background influences outcomes in both the acute and subacute ischemia models, consideration of the mouse strain being studied is important in choosing the best model. This paper describes the proper procedure and anatomical placement of ligatures or ameroid constrictors on the mouse femoral artery to induce subacute or acute hindlimb ischemia in the mouse.

Video Link

The video component of this article can be found at https://www.jove.com/video/54166/

Introduction

Peripheral artery disease (PAD) is a leading cause of cardiovascular morbidity and mortality in developed countries [1]. PAD results from atherosclerotic obstruction of the peripheral arteries that leads to limb ischemia with resultant exertional or rest pain and occasionally non-healing ulcers and gangrene that necessitate limb amputation. Therapies targeting PAD are directed primarily towards endovascular [2] or surgical revascularization [3], as essentially no effective medical therapies exist [4].

Unfortunately, revascularization is often of limited benefit, as bypass grafts have high failure rates (up to 50% within 5 years) [5] that are worse in some populations (*e.g.*, smokers, women, non-saphenous vein grafts) [6,7]. Endovascular approaches, such as angioplasty and stenting, are also compromised by high restenosis rates (in excess of 50% within 1 year), particulary in femoropopliteal disease [8], although the use of drug-eluting balloons and stents has improved outcomes somewhat [9-11]. In order to develop new treatments for PAD it is essential to develop animal models that reliably reproduce the human disease.

To date, the most common model of PAD is the hindlimb ischemia model (HLI), which is most frequently performed in mice [12,13]. In its most common manifestation, the model entails surgical ligation of the proximal and distal femoral artery and its intervening side-branches followed by excision of the vessel, resulting in occlusion of blood flow and induction of acute limb ischemia. HLI has been used primarily to study the angiogenic and arteriogenic responses in peripheral limb muscle tissue and the effects of various therapies (*e.g.*, drugs, gene delivery, stem cells) on these responses. More recently, our group has used this model to examine the role of skeletal muscle cells in the response to limb ischemia and the effects of genetic differences on outcomes [14].

The HLI model has facilitated our current understanding that the vascular and muscle responses to ischemia are dependent on genetics (*i.e.*, inbred strain) [15], age [16], and the presence or absence of other diseases or conditions relevant to atherosclerosis, including diabetes mellitus [17] and hypercholesterolemia [18]. However, an important weakness of the traditional HLI model is that it is a model of acute limb ischemia [12,13], whereas human PAD causes chronic ischemia as a result of the gradual development of occlusive atherosclerotic lesions in the peripheral arteries.

In an attempt to circumvent this weakness, Tang and colleagues initially developed a rat model of gradual femoral arterial occlusion using ameroid constrictors [19], and the same group subsequently developed a similar mouse model [20]. Ameroid constrictors were described initially in the 1950s in a canine model of chronic myocardial ischemia [21,22]. These devices have an outer metal sleeve encasing an inner layer of a hygroscopic material, usually casein, and when placed around an artery they induce gradual vessel occlusion as they absorb moisture from the surrounding tissues. In their modification of the model, Yang *et al.* placed constrictors on both the proximal and distal femoral artery at sites

analogous to the surgical ligation sites, and they ligated the side branches of the femoral artery, as in the traditional model. Compared to acute HLI, ameroid constrictor-induced ischemia resulted in lower expression of inflammatory and shear stress-dependent genes, lower blood flow recovery 4 - 5 weeks post-operatively, and less muscle necrosis [20]. Based on these observations, it was felt that gradual arterial occlusion might provide a model of PAD more relevant to the human disease.

Notably, in the original report, effects of ameroid constrictor-induced ischemia were examined only in C57BL/6 mice [19], which are relatively resistant to ischemia-induced muscle necrosis [15]. We recently modified the gradual ischemia model further and explored its effects in the more ischemia-susceptible BALB/c mouse strain [23]. In the first manifestation of the model, we placed constrictors on both the proximal and distal femoral artery but left all side-branches intact. In a second, milder modification, we placed a single constrictor only on the proximal femoral artery and again left all side-braches of the artery intact. In both modifications of this model, we found that BALB/c mice, but not C57BL/6 mice, displayed significant muscle necrosis despite having similar blood flow and vascular density. Similar to our previous study [14], these findings demonstrated that limb muscle injury is not solely influenced by blood flow, but is in part dependent on genetic background. Moreover, we found that limb blood flow fell to its nadir within 3 days, thus the model appears to be more one of 'subacute' rather than gradual limb ischemia.

Based on these prior studies, it appears clear that a single method for inducing hindlimb ischemia may not be suitable in all cases. Because a variety of conditions (*e.g.*, genetic differences and presence or absence of co-morbid conditions) influence both the vascular and skeletal muscle-specific responses, investigators may find it necessary to modify the chronicity and/or the severity of hindlimb ischemia to best suit their purposes. Furthermore, prior descriptions of the model typically lacked suitable anatomical landmarks to facilitate reliable inter-investigator reproducibility of the technique. In this paper, methods for inducing either acute or subacute hindlimb ischemia in the mouse are described, and precise anatomical landmarks are provided.

Protocol

All animal experiments were performed according to protocol approved by the Duke Institutional Animal Care and Use Committee. Male mice were used in this study, although either sex can be used as indicated for the scientific purpose of the study.

1. Hair Removal

1. Prior to induction of anesthesia, set up a pre-surgical preparation area consisting of a covered heating pad set at 37 °C and a nosecone port connected to continuous flow of isoflurane.
2. Place the mouse in the anesthesia induction chamber. Set the O_2 flow meter to 1 L/min and isoflurane to 1 - 3%.
   NOTE: Anesthesia is typically induced in a 25 g mouse with 2% isoflurane.
3. Check the mouse's stimulus response by gently rocking the chamber and observing a lack of a righting reflex.
4. Flush the chamber with O_2 to clear the isoflurane prior to opening. Quickly move the mouse to the heating pad and connect it to isoflurane via the nosecone.
5. Adjust the isoflurane to 1.5%. Check the stimulus response by pedal reflex (toe pinch).
6. Apply ophthalmic lubricant liberally to both eyes to avoid drying during surgery.
7. Shave the hair from both hind limbs using a small electric trimmer. Hold the skin taut while shaving to avoid lacerating the skin.
8. Apply pre-warmed hair removal cream and let sit for 1 min. Gently wipe away using a moistened gauze pad.
9. For surgical procedure at a later time, turn off the isoflurane and move the mouse to an empty paper towel-lined recovery cage to ensure the mouse does not aspirate the cage bedding. Monitor the animal until it is able to maintain sternal recumbency. Otherwise, move the mouse to the surgical table.
   NOTE: The hair removal process can irritate the skin and affect perfusion measurements. It is recommended to wait 1 - 2 days after removing the animal's hair before performing a pre-surgical perfusion scan or performing surgery.

2. Pre-Surgical Preparation

1. Use the following tools during this procedure; small straight surgical scissors, 2 fine-tipped angled forceps, small Graefe forceps, needle driver forceps, 3 retractors, small spring scissors, and fine-tipped cotton swabs.
2. Sterilize all tools using an autoclave prior to the initiation of surgery. Use a hot-bead sterilizer before and between each surgical procedure, for up to 5 animals. Sterilize additional surgical tool packets for surgeries of groups larger than 5.
3. Prepare a sterile surgical field consisting of a covered heating pad and an isoflurane port. Perform all work under a 10 stereo dissection microscope.
4. Anesthetize and prepare the mouse as described in steps 1.1 to 1.5.
5. Check that the mouse is fully sedated and place into a supine position on the surgical table. Secure both legs using surgical tape.
6. If using a temperature-controlled heating pad, attach the temperature probe and secure it to the base of the surgical platform using surgical tape to ensure that it will not be accidentally pulled out during the procedure.
7. Clean the incision site using 3 alternating povidone-iodine and alcohol wipes. Cover the animal with a sterile surgical drape and cut a hole to expose the incision site.

3. Induction of Limb Ischemia

1. Use a scalpel to make an initial incision along the center of the medial thigh, running from the knee towards the abdomen, and lengthen the incision to approximately 1 cm with fine scissors (**Figure 1A**).

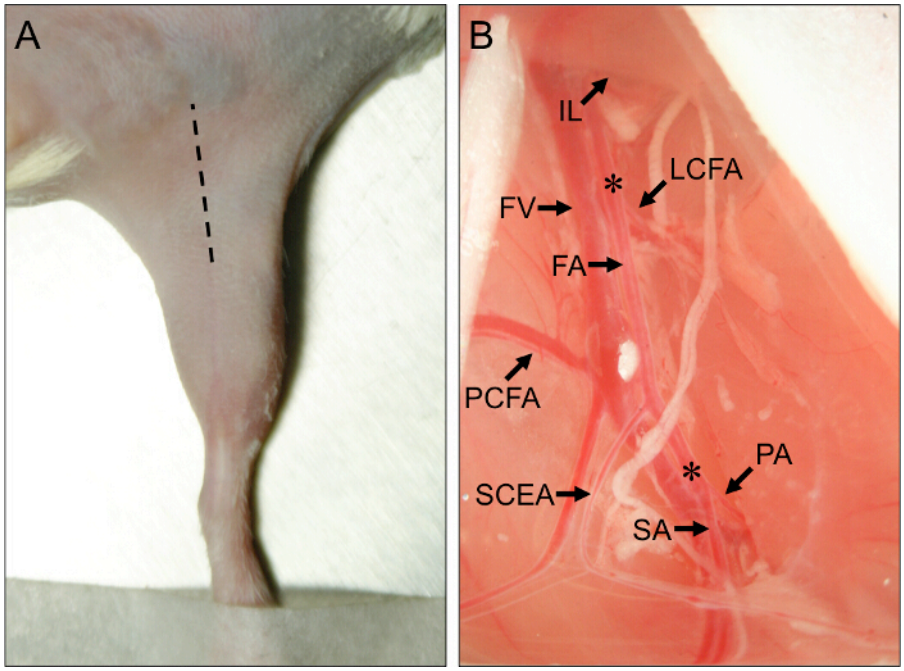

Figure 1. Surgical Site and Vascular Anatomical Landmarks for Mouse Hindlimb Ischemia Surgery. (A) External view of the hindlimb of a mouse in the supine position. The hatched line indicates the incision site to properly perform the hindlimb ischemia procedures. **(B)** View of the proximal mouse hindlimb vasculature. The proximal end of the femoral artery (FA) arises from beneath the inguinal ligament (IL). The distal end of the FA is located at its bifurcation into the popliteal artery (PA) and saphenous artery (SA). The major collateral arteries off of the FA are the lateral circumflex femoral artery (LCFA), the proximal caudal femoral artery (PCFA), and the superficial caudal epigastric artery (SCEA). The femoral vein (FV) runs adjacent to the FA, and venous branches can be seen parallel to the major arterial branches. Asterisks (*) denote the proximal and distal sites for placement of ameroid constrictors or ligatures, depending on whether subacute or acute ischemia will be induced. Please click here to view a larger version of this figure.

2. Using forceps, open the incision and expose the membrane covering the inguinal fat tissue (IFT).
3. Using closed-forceps, pierce through the membrane into the separation between the IFT and the abdomen. Gently release pressure on the forceps to separate the IFT from the abdominal muscles, exposing the neurovascular bundle underneath. Observe the proximal and superficial caudal branches as important anatomical landmarks (**Figure 1B**).
4. Insert a retractor and pull the abdominal tissue proximally to expose the proximal ameroid constrictor or ligation site, just proximal to the lateral circumflex femoral artery (**Figure 1B**). The lateral circumflex artery lies about 5 mm proximal to the proximal and superficial caudal arteries.
    1. Insert two more retractors into the distal part of the incision, one medial and one lateral, to pull the IFT distally away from the surgical site to widen the surgical field.
5. Use two fine forceps to remove the outermost membrane covering the neurovascular bundle. Gently insert one half of the fine forceps tip between the vein and artery, sliding the forceps tip under the membrane that binds them together. Close the forceps and gently tear away the membrane.
6. Insert the tip of a closed forceps between the vein and artery and create a gap between them by releasing pressure on the forceps. Repeat this technique to create a gap between the artery and nerve.
7. For subacute limb ischemia, place an ameroid constrictor on the proximal femoral artery (**Figure 2**).
    1. To install the proximal ameroid constrictor, slide the tip of a forceps under the femoral artery to isolate it from the neurovascular bundle. Use a second set of angled- forceps to grip the edge of the constrictor and guide it under the femoral artery.
    2. Lay the femoral artery into the slot in the constrictor. Repeat for the distal constrictor, positioning it immediately proximal to the bifurcation of the femoral artery into the popliteal artery and saphenous artery (**Figure 2**).

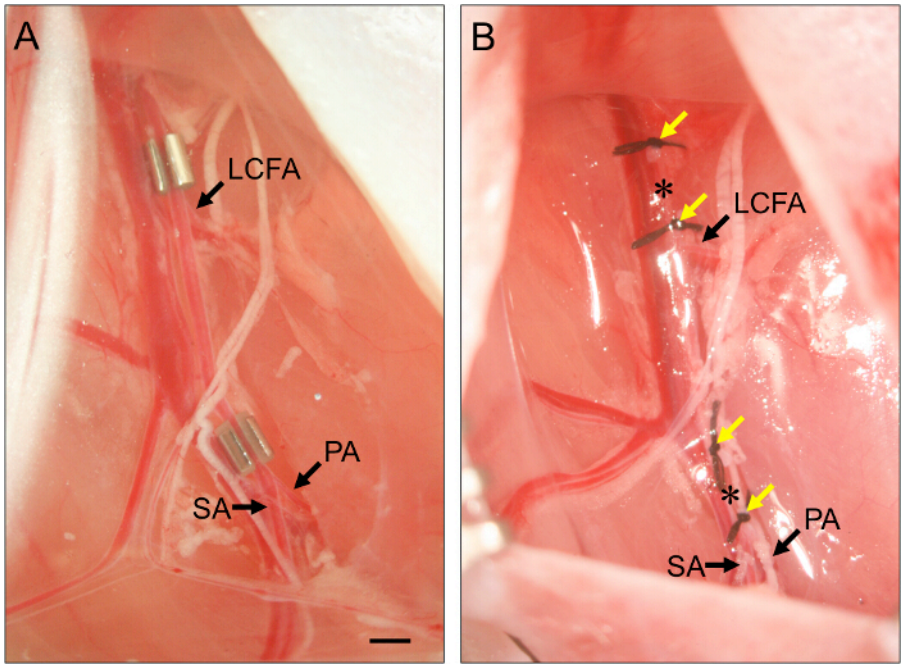

Figure 2. Placement of Ameroid Constrictors and Ligatures. (A) Example of two ameroid constrictors placed on the femoral artery to induce subacute hindlimb ischemia. The proximal constrictor is placed just proximal to the lateral circumflex femoral artery (LCFA). The distal constrictor is placed just proximal to the bifurcation of the popliteal (PA) and saphenous arteries (SA). Constrictors are installed with the slot facing up to ensure the artery is properly set within the constrictor. **(B)** Example of ligatures of the femoral artery to induce acute hindlimb ischemia. Ligatures (yellow arrows) are placed such that they flank the position of the constrictors in panel (B), and the femoral artery is transected between each set of two ligatures (asterisks). Bar, 1 mm. Please click here to view a larger version of this figure.

8. For acute limb ischemia, ligate and transect the proximal femoral artery.
    1. To transect the femoral artery, thread 7 - O suture under the artery just proximal to the position of the proximal constrictor (see Step 3.7) and ligate. Tie a second ligature about 1 mm distal to the first.
    2. Use spring scissors to transect the artery between the two ligatures. For the distal arterial transection, repeat these steps, placing two ligatures about 1 mm apart, just proximal to the bifurcation of the femoral artery into the popliteal artery and saphenous artery but ensuring that they are distal to the superficial caudal epigastric artery (see **Figure 1**)
9. Close the incision using interrupted 5 - O vicryl sutures.

4. Perfusion Imaging

1. Move the mouse to a 37 °C heating pad set beneath the laser doppler perfusion imager (LDPI) and connect via a nosecone to the isoflurane source. If no temperature monitor is available, allow 5 min for the mouse to warm up to 37 °C.
2. Turn on the imager and launch the image capture software.
3. Click the 'New Single Image' icon to open the 'Scanner Setup' window. Set the 'Scan Size' to 'Large' and the 'Scan Speed' to '4 ms/pixel'. Set the scan area by changing the x and y values under the 'Scan Area (units)' pane.
4. Click the 'Video and Distance' tab to view the video feed, and arrange the mouse to fit into the scan area indicated by a red outline. Click 'Auto Distance' to calibrate the distance from the laser to the subject. Click 'Next' to open the 'Subject Details' window.
5. Enter the subject information and any relevant comments. Click the 'Next' button to move on to the scanning window.
6. Click the 'Start Measurement' button to open the 'Confirm or Override Scan Distance' dialog. Click 'OK' to confirm scan distance. Observe the scanning process begin and run for 4 - 8 min depending on the size of the scan area.
7. After the scanning is complete, observe the 'Save As' window. Name the file and save it.
8. Shut off the isoflurane and move the mouse to an empty recovery cage and monitor until the animal is able to maintain sternal recumbency. Never place a mouse recovering from anesthesia into a cage with other mice.
9. Open the image analysis software. Click the 'Open' icon and browse to and open the image file for analysis. On the file window, observe the flux, photo, and color images of the mouse.
10. To mark the region of interest (ROI) on the flux image, click the 'Show ROIs' icon. Next click the 'Add Polygon' button and drag the cursor around the non-surgical limb to draw the ROI. Right-click to close the shape. Select 'Add Polygon' again and draw a matching ROI around the surgical limb.
11. Click the 'Statistics' icon to open the 'Image ROIs Statistic Results (PU)' window. Observe the percent difference in flux in the 'Flux %' column.
    NOTE: The first ROI drawn will serve as the reference.
    NOTE: Prior to each subsequent perfusion scan follow the steps outlined in Section 1 to anesthetize the mouse and in steps 4.1 to 4.11 to image the animal.

Representative Results

Proper identification of the mouse hindlimb vasculature is critical to ensuring reproducibility of the techniques for inducing both subacute and acute hindlimb ischemia, as described here. In addition to the variation inherent in animal studies, other factors can introduce variability in laser Doppler perfusion imaging (LDPI), including the type of anesthesia, position of the animal (supine *vs.* prone), and body temperature (see **Figure 3**). In addition, the subacute hindlimb ischemia model is dependent on the quality of the ameroid constrictors, which can vary widely within a given batch (see **Figure 4**). Each of these issues can have substantial effects on the quantitation of flow measured by LDPI and are discussed in more detail below.

Following placement of ameroid constrictors in the subacute hindlimb ischemia model or ligation and transection of the femoral artery in the acute hindlimb ischemia model, LDPI images should be obtained immediately post-operatively while the animal is still anesthetized to demonstrate effects of the surgery and establish a baseline post-operative level of perfusion (**Figure 5**). Quantitation of perfusion is performed by drawing a region of interest (ROI) around the ischemic hindlimb and a comparable ROI around the non-ischemic hindlimb. Perfusion is most commonly expressed as a ratio of the perfusion in the ischemic limb to that in the non-ischemic limb, and changes in this ratio are measured over time. We have found that imaging mice in the prone position results in less variability due to animal movement and changes in positioning of the feet from one time point to the next. Moreover, perfusion in the upper thigh tends to be more variable when mice are in the supine position. A complete loss of hindlimb perfusion should be observed after induction of acute limb ischemia, whereas only a mild decrease in perfusion is typically observed after placement of ameroid constrictors in the subacute model (**Figure 5**). In some cases, we have observed rapid constrictor occlusion with resultant dramatic loss of perfusion immediately post-operatively.

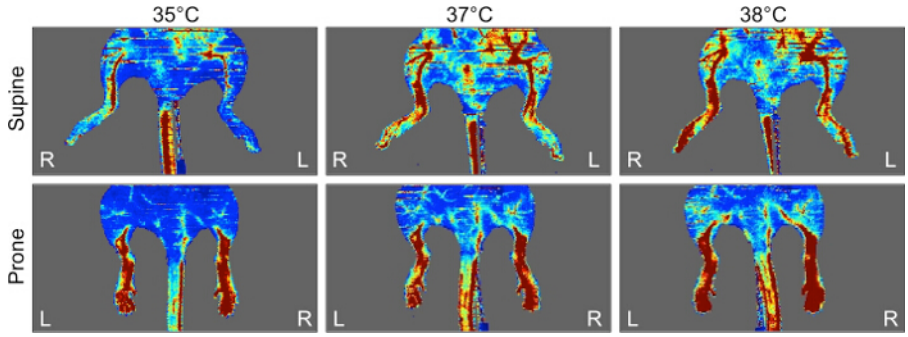

Figure 3. Variation in Perfusion Imaging Based on Hindlimb Position and Body Temperature. Laser Doppler perfusion images of a single BALB/c mouse, anesthetized with 1.5% isoflurane and scanned in both the supine and prone position at 35 °C, 37 °C, and 38 °C. With isoflurane anesthesia, the animal's hindlimbs tend to move with respiration, resulting in an artifact (horizontal lines) that are observed with greater frequency on supine compared to prone images. In the prone position, it is much easier to reproduce the precise angle of the feet, making quantification of perfusion over time more precise. Perfusion is substantially reduced as the body temperature drops below 37 °C, whereas the images are saturated at higher temperatures. Please click here to view a larger version of this figure.

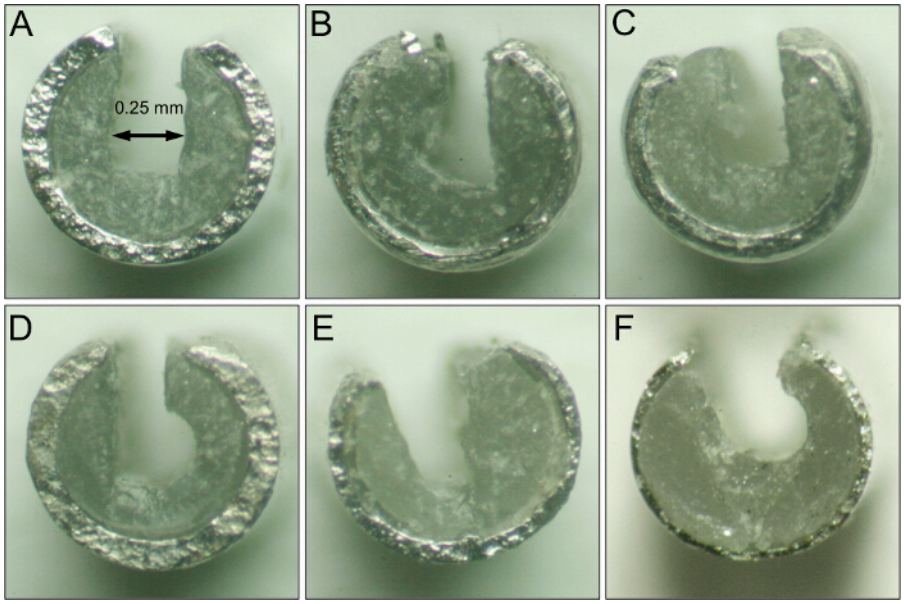

Figure 4. Variability of Commercially Available Ameroid Constrictors. (A) An example of an ideal 0.25 mm inner diameter (double-headed arrow) constrictor with uniform slot shape and casein thickness. **(B - D)** Examples of slot shape variation. Slots may sometimes display a 'd' shape **(B and C)** or a 'b' shape **(D)**. In these cases, the casein is fairly evenly distributed and the slots are deep enough hold the artery. **(E)** An example of uneven casein thickness. This constrictor would be discarded. **(F)** An example of a slot that is too shallow to hold the artery in place during constriction. This constrictor would also be discarded. Please click here to view a larger version of this figure.

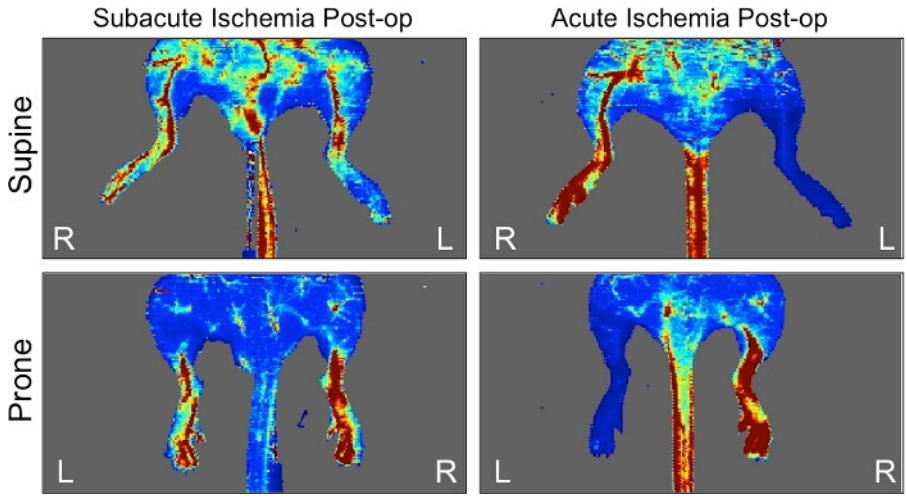

Figure 5. Perfusion Images Immediately Following Induction of Subacute or Acute Hindlimb Ischemia. Representative perfusion images in both the supine and prone position of BALB/c mice that have undergone placement of two ameroid constrictors on the left femoral artery to induce subacute hindlimb ischemia or placement of two ligatures and transection of the left femoral artery to induce acute hindlimb ischemia. Note that perfusion is reduced but still detectable after ameroid constrictor placement, whereas essentially no flow is detected immediately after double ligation/transection of the femoral artery. Please click here to view a larger version of this figure.

Discussion

Perhaps the most challenging step in this procedure is the separation of the femoral artery from the femoral vein. The larger diameter and thinner walls of the femoral vein compared to those of the artery increase its susceptibility to puncture and tearing during surgical manipulation. The likelihood of disrupting the vein can be reduced by keeping the wound moist using a sterile swab moistened with PBS. It is also important to ensure that all forceps are sharpened, aligned, and free of breaks in order to allow precise manipulation of the vessels and surrounding tissues. In the event that bleeding does occur, apply pressure to the area with sterile gauze until the bleeding has stopped. Detailed surgical notes should be maintained for each animal, as what may be perceived as 'minor' bleeding events may represent an unintentionally more severe ischemic injury in the long-term. For data consistency, surgeries involving no venous or collateral arterial bleeding events are crucial.

A limitation of this technique is that the severity and/or onset of ischemia (in the subacute ameroid constrictor model) can vary widely depending on several factors. The proximal constrictor/ligation site will determine how many collateral arteries are affected by the induction of ischemia. Leaving collateral branches intact lessens the severity of the injury, although as noted, in certain contexts, *i.e.*, in animals of certain genetic backgrounds even milder, subacute ischemia can cause substantial tissue necrosis [23]. Furthermore, the addition of a second, distal constrictor

or ligature will decrease perfusion in both the acute and subacute model [23]. Some investigators have ligated or used an electrocautery to ablate collateral branches off the lateral circumflex artery, the proximal caudal femoral artery, and the superficial caudal epigastric artery [24]. This results in more severe ischemia and may induce greater muscle injury, although this also depends on genetic background. It is important to note that electrocautery may be more likely to result in injury to the surrounding tissues and should therefore be used with caution.

In light of these variables, it is critical that the vascular anatomy is accurately identified prior to selecting the site of occlusion. Kochi *et al.* [25] noted a marked lack of consensus among numerous reports regarding the names and locations of blood vessels in the mouse hindlimb, and they have provided a very detailed description of the arterial anatomy that we believe is an essential guide for anyone undertaking this procedure. An earlier publication in this journal demonstrated the technique for inducing acute limb ischemia [13], however in that report the vascular anatomical landmarks were not well defined. An important goal of this report was to provide an improved visual representation of those landmarks in addition to demonstrating a modification of the technique using subacute limb ischemia.

An additional limitation of this approach is that the onset of ischemia in the subacute ischemia model is a function of the quality of the ameroid constrictors that one uses. We have found that commercial constrictors can vary in the depth and shape of their internal slot (**Figure 4**). If the slot is too shallow the artery may be extruded during constriction. Constrictors with cracked casein or malformed slots should be discarded. Additionally, inconsistent distribution of casein within the constrictor can result in accelerated rates of occlusion. The size and age of the mice is another critical factor to consider, as vessel sizes in 'adult' mice may vary considerably between the ages of 8 and 30 weeks of age. This is particularly important when using multiple independent cohorts of mice to generate data sets, as apparently small age ranges (*e.g.*, 10 - 16 weeks) may result in inconsistent rates of occlusion and severity of ischemic limb injury.

Quantification of laser Doppler perfusion imaging can also vary widely depending on the animal's body temperature and position (**Figure 4**), as well as the anesthetic agent used. It is crucial that the mouse maintain a 37 °C body temperature during perfusion imaging. If the temperature is greater than 37 °C the perfusion image will be saturated. If the temperature is below 36 °C the perfusion signal may be too weak. It is also important to keep both legs as symmetrical as possible to obtain an accurate perfusion ratio (perfusion in the ischemic limb compared to that in the non-ischemic limb). Although mice are frequently imaged while lying supine, symmetry may be harder to maintain in this position. Moreover, variation in blood flow in the superior portion of the imaging field, *i.e.*, the proximal thigh, may contribute to variability in quantitation. In addition, anesthesia with isoflurane often results in increased chest movement, which in turn causes leg movement and variability in the signal obtained by LDPI. We have found that placing animals in the prone position gives more support to the limbs, allowing for easier positioning and greatly reduced movement, which results in more reproducible quantification of limb perfusion (**Figure 5**). Furthermore, the prone position facilitates more consistent maintenance of 37°C temperature in the scanned limbs, which also enhances reproducibility of quantitation (**Figure 5**). Compared to isoflurane, mice anesthetized with ketamine display decreased chest movement, making supine imaging less variable. However, ketamine will also increase anesthesia induction and recovery time, resulting in significantly increased absolute perfusion values [16]. Each combination of body position and anesthetic agent can create a distinct perfusion image value, thus it is important to use a consistent technique for each of these methods across all subsequent perfusion measurements.

It is important to recognize several other limitations of this technique. Because the model is often performed in mice without any comorbidities, such as obesity, hyperlipidemia, atherosclerosis, diabetes mellitus, or other factors that predispose to vascular disease, the induction of limb ischemia will never perfectly replicate the pathology of clinical PAD. As noted, the effects of acute limb ischemia have been assessed in aged, diabetic, and hypercholesterolemic mice, and in the future it will be useful to determine the effects of these comorbid conditions on responses to subacute limb ischemia. Moreover, because PAD is a chronic disease, even subacute limb ischemia is an imperfect model of the clinical scenario. Therefore, it will be important to continue to develop models that result in true chronic limb ischemia and to test them in conjunction with models of cardiovascular risk factors. A technical limitation of the laser Doppler imaging modality regardless of the animal model being used is that it measures blood flow velocity and not absolute tissue perfusion, therefore it can be used only to compare relative changes in blood flow within a given animal. Notably, ischemia that causes limb loss will result in a reduced perfusion ratio irrespective of changes in blood flow [23].

In summary, we provide detailed methods for inducing acute and subacute hind limb ischemia in mice for the purpose of analyzing effects on skeletal muscle and vascular remodeling. Special attention is given to identification of critical vascular landmarks to facilitate accurate intra- and inter-operator reproducibility of the model. Further refinement of the technique may eventually lead to the development of a chronic ischemia model that accurately replicates the pathogenesis of clinical PAD.

Disclosures

The authors have no conflicts of interest to disclose.

Acknowledgements

This study was supported by NIH grants R21HL118661, R56HL124444, and R01HL124444 to CDK, and by NIH grants R00HL103797 and R01HL125695 to JMM.

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