Video Article

Deep Vein Thrombosis Induced by Stasis in Mice Monitored by High Frequency Ultrasonography

Ryan N. Rys[1], Mark D. Blostein[1,2], Catherine A. Lemarié[1,2]

[1]Lady Davis Institute for Medical Research, Jewish General Hospital, McGill University

[2]Department of Medicine, Jewish General Hospital, McGill University

Correspondence to: Catherine A. Lemarié at catherine.lemarie@mcgill.ca

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Abstract

Venous thrombosis is a common condition affecting 1 - 2% of the population, with an annual incidence of 1 in 500. Venous thrombosis can lead to death through pulmonary embolism or results in the post-thrombotic syndrome, characterized by chronic leg pain, swelling, and ulceration, or in chronic pulmonary hypertension resulting in significant chronic respiratory compromise. This is the most common cardiovascular disease after myocardial infarction and ischemic stroke and is a clinical challenge for all medical disciplines, as it can complicate the course of other disorders such as cancer, systemic disease, surgery, and major trauma.

Experimental models are necessary to study these mechanisms. The stasis model induces consistent thrombus size and a quantifiable amount of thrombus. However, it is necessary to systematically ligate side branches of the inferior vena cava to avoid variability in thrombus sizes and any erroneous data interpretation. We have developed a non-invasive technique to measure thrombus size using ultrasonography. Using this technique, we can assess thrombus development and resolution over time in the same animal. This approach limits the number of mice required for quantification of venous thrombosis consistent with the principle of replacement, reduction, and refinement of animals in research. We have demonstrated that thrombus weight and histological analysis of thrombus size correlate with measurement obtained with ultrasonography. Therefore, the current study describes how to induce deep vein thrombosis in mice using the inferior vena cava stasis model and how to monitor it using high frequency ultrasound.

Video Link

The video component of this article can be found at https://www.jove.com/video/57392/

Introduction

Venous thromboembolism (VTE), which is comprised of deep venous thrombosis (DVT) and pulmonary embolism, is the third leading cause of cardiovascular death after myocardial infarction and stroke. It is a common condition affecting 1 - 2% of the population, with an annual incidence of 1 in 500[1]. VTE can lead to: 1) death through pulmonary embolism; 2) post-thrombotic syndrome, characterized by chronic leg pain, swelling, and ulceration; or 3) chronic pulmonary hypertension resulting in significant chronic respiratory compromise. VTE is a multi-factorial disease and may result from stasis of blood flow, damage to vessel walls, and/or hypercoagulable states due to a disruption of the balance between the coagulation and the fibrinolytic systems, as it has been described over a hundred years ago by Virchow and is known as the Virchow's Triad.

Because in most cases it is impossible to obtain human DVT samples, researchers have developed experimental animal models of DVT. Several animals including rat[2], mouse[3], rabbit[4], pig[5], dog[6], and non-human primate[7] have been used. Mice can be genetically modified and are the most frequently used animal to study DVT. However, as in all animals, spontaneous DVT is not observed in mice. Thus, physical or chemical alterations of the vein wall are used to create thrombosis in mice. We have previously used the ferric chloride model to induce thrombosis in the inferior vena cava (IVC) of mice[8,9,10]. This model has the advantage of reliably producing occlusive thrombi within minutes and can be used to investigate the role of anti-coagulant and anti-platelet drugs during acute DVT. However, it is a terminal procedure. Thus, to study acute and chronic DVT, the stasis model is more suitable. In this model, thrombus formation is induced by the complete interruption of blood flow in the IVC, one of the factors in Virchow's triad for DVT development. This model can be used to study DVT formation and resolution, which is an advantage compared to the $FeCl_3$ model[11].

We have developed a non-invasive method to follow thrombus formation and resolution over time using a micro-imaging high-frequency ultrasound system[12]. We have previously demonstrated that measurement of venous thrombosis by ultrasound correlates favorably with thrombi obtained pathologically. We have confirmed in two subsequent studies that measurements obtained with ultrasound correlate with thrombus weight and thrombus area quantified by histochemistry[9,10]. More importantly, we have showed that high frequency ultrasound can be used to monitor the formation of deep venous thrombosis in mice[12]. It may also be used to quantify thrombus resolution in a non-invasive way.

Here, we will describe the protocol allowing thrombus formation using the stasis-induced thrombosis mouse model and how thrombus formation can be monitored non-invasively over time using high frequency ultrasound.

Protocol

All procedures were approved by the institutional Animal Care Committee of McGill University Montréal, QC, Canada. All the equipment required is listed in Table I.

1. Murine (C57BL/6J) IVC Stasis Protocol

1. **Surgery preparation**
   NOTE: This is a survival surgery and therefore, proper aseptic technique needs to be followed at all times. This includes making sure all materials are at hand, cleaning and sterilizing instruments before and between use, and designating a sterile area on the working surface for all sterile material.
   1. Sterilize all surgical instruments and materials by autoclave. A glass bead sterilizer is sufficient to sterilize instrument tips between animals, if more than one procedure is being done at one time.
   2. Anesthetize 8 - 10 week old C57Bl/6 male mice with a mixture of 100% oxygen and 2.5% isoflurane.
      NOTE: Adjust the flow of oxygen and isoflurane as necessary.
   3. Confirm anesthetization by pinching the rear paw of the animal between the toes with forceps. No response from the pinch indicates the animal is anesthetized.
   4. Administer slow release analgesic (buprenorphine) via subcutaneous injection. Give mice a dose of 1 mg of analgesic per 1 kg of body weight (1 mg/kg).
   5. Place eye ointment on both eyes of the animal to ensure no corneal desiccation for the duration of the procedure.
   6. Administer 0.2 - 0.5 mL of isotonic fluids per 10 g of body weight subcutaneously.
   7. Fix the animal in place to the surgery table using surgical tape. Place the animal in a supine position to expose the abdomen. Perform surgery on a heating pad to prevent hypothermia.
   8. Remove hair from the abdomen of the mouse by application of a hair removal cream for 1 - 2 min. Wipe the abdominal area clean with gauze and distilled water, if necessary.
   9. Alternatively, remove the hair from the abdomen by shaving with a small electric razor.
   10. Sterilize the abdomen with Baxedin (Chlorhexidine Gluconate BP Solution) prior to making any incisions. Apply ethanol lightly with gauze to avoid excessive heat loss caused by the evaporation of the alcohol.

2. **Exposure of the Inferior Vena Cava (IVC)**
   1. Using surgical scissors, perform a laparotomy to open the abdominal cavity.
      1. Lift skin with forceps at the lower abdomen and make a vertical incision parallel to either side of the linea alba. Make the first incision to the left or right of the linea alba, depending on the handedness of the operator. The incision is made away from the midline to assist in ultrasound imaging later on.
      2. Make a second, horizontal incision at the top of the abdomen.
         NOTE: Upon penetration into the abdomen, the musculature should be “tented” and then penetrated with surgical scissors to prevent any damage to the abdominal organs.
      3. Repeat the vertical and horizontal incisions on the abdominal muscle layer of the animal. Be sure to tent the musculature during penetration into the abdomen so as not to damage the organs beneath.
         Caution: Lift skin and muscle away from intestines and other internal organs when making incisions so as not to puncture them.
   2. Fold the skin and muscle away from the incision to expose the abdominal cavity.
   3. Apply gauze, moistened with isotonic solution, to the sides of the wound opening and externalize the intestines. Apply pressure to both sides of the abdomen to assist the externalization of the intestines. Use gentle movements with a cotton tip applicator to move the intestines. Soak gauze in isotonic solution and place it over the externalized intestines.
      1. Gauze should be pre-moistened prior to placement on tissue.
   4. Move aside any peritoneal fat, and expose the IVC between the renal and iliac veins.
   5. Make an initial identification of obvious side branches. A side branch will appear as smaller, but significant vein that branches away from the IVC, between the renal and iliac veins. Vasculature can vary greatly between mice. Mice may have side branches present on one or both sides of the IVC.

3. **Ligation of side branches**
   NOTE: If side branches are present, the following procedure is followed for each one. If not present, proceed to step 4: Initial suture placement under the IVC. The ligation site of the IVC will be immediately distal to the left renal vein, regardless of the presence of side branches.
   1. Perform blunt dissection on each side of the side branch. Blunt dissection is the process of repeatedly opening blunt forceps with gentle pressure, so as to break through fascia without damaging surrounding vessels.
      Caution: Avoid puncturing or damaging the vein.
   2. Lift the branch and pass a section of 6-0 silk suture underneath the vein. Always take care when lifting or moving vessels. It is best to grab the fat around the vessels, otherwise there is a risk of damaging the vessel.
   3. Using suture forceps, make a surgical ligation around the side branch using standard surgical knot tying technique. Full ligation is done to ensure 100% occlusion of the vein. Use at least three tying throws to ensure the ligation is secure.
   4. Repeat step 1.3 for any remaining side branches.

4. **Dissection of the abdominal aorta from the IVC**

1. Perform blunt dissection around the IVC immediately distal from the left renal vein. The abdominal aorta is attached to the IVC via fascia, therefore perform initial blunt dissection around both the IVC and abdominal aorta.
   Caution: Avoid puncturing or damaging the vein. Do not rupture or puncture either vessel. This is the most critical part of the procedure, with the highest risk for vessel damage.
2. Continue to apply gentle pressure via blunt dissection until a clear window is made between the aorta and IVC.

5. **Suture placement and IVC ligation**
   1. Pass a section of 6-0 silk suture underneath the IVC and abdominal aorta.
   2. Locate the suture through the window created between the IVC and aorta. Use forceps to pull the suture and thread it between the abdominal aorta and IVC. Thread the suture through carefully, trying not to pull/puncture the IVC and abdominal aorta.
   3. Make a surgical ligation around the IVC with suture forceps, ensuring a total occlusion of the vein. Again, make the ligation immediately distal from the left renal vein.
   4. Make three suture throws to secure the ligation.
      NOTE: It is also possible to pass the suture underneath the IVC and aorta prior to separating the two as an alternative option. Alternatively, 7-0 Prolene sutures can be used for ligation of the side branches and the IVC.

6. **Wound closure and post-operative care**
   1. Verify that the abdominal aorta has been left uninterrupted and that all side branches have been ligated. The IVC will appear dilated distal from the ligation site and no blood flow will be visible.
   2. Place all peritoneal fat and the intestines back in the abdominal cavity.
   3. Close the wound using suture forceps and 6-0 silk suture. Use a running suture (simple continuous) and ensure that the suture will not be too tight. A tight suture will rupture when the animal wakes up and moves around its cage.
      1. Alternatively, use PDS, Ethilon, Vicryl, Dexon, or stainless steel clips for wound closure.
   4. Suture the layer of abdominal muscle first using appropriate suture tying technique.
   5. Repeat suture technique for the skin and verify wound closure is sufficient.
      NOTE: If using the same suture needle and thread for multiple animals, sterilize both in 70% ethanol prior to each use.
   6. Remove the animal from anesthetic gas and place them in a 34 °C incubator for at least 30 minutes.
   7. Again, administer 0.2 - 0.5 mL of isotonic fluids per 10 g of body weight subcutaneously. Fluids can be given in following days to maintain pre-operative body weight, if necessary.
   8. Monitor the animal until recovery to ensure successful surgery and assess the overall health of the animal.
      Caution: Expect a slight hunched posture for an invasive procedure such as this, but the animal will behave normally as early as 30 minutes post-operation.
   9. Place the animal in its own cage and do not place it with other animals until fully recovered.
   10. Re-administer analgesic daily for up to 48 h postop.
   11. Place food on the floor of the cage for the animal while in recovery. Other special care is generally not needed.
   12. Examine the wound site for redness, swelling, or discharge and any other signs of infection.
   13. If necessary, remove sutures after 7 - 10 days.
   14. Perform euthanasia immediately if surgery is unsuccessful (*e.g.,* significant vessel damage and/or blood loss) or animal does not improve with post-operative care. Euthanasia is generally performed at 48 h postop. For longer experiments, use 7-0 Prolene to ligate side branches and the IVC and 5-0 Vicryl sutures to close the abdominal cavity.
      1. Perform euthanasia by anesthetization of the animal in an induction as previously described, except at 5% isoflurane. This is followed by asphyxiation with 100% CO_2 while anesthetized.
      2. After confirmation of euthanasia by asphyxiation (loss of heartbeat and no breathing), perform cervical dislocation to ensure complete euthanasia.

2. High Frequency Ultrasound Protocol

NOTE: This protocol is adapted from the Lady Davis Institute Rodent Phenotyping Core SOP for high frequency ultrasound imaging. This protocol is carried out 24 h post-operative, but can be done sooner as long as the animal is responding well to the surgery. The protocol can be carried out at any time on healthy mice and is often done so to compare before and after surgery.

1. **Preparation of Animal**
   1. Place the animal in an induction chamber and anesthetize the animal with a mixture of 100% oxygen and 2.5% isoflurane.
   2. Turn on heart rate and temperature monitor. Position heat lamp above table to keep animal warm during procedure.
   3. Remove the animal from the induction chamber and apply eye lubricant to prevent corneal desiccation. Position the animal on the analysis platform and affix the anesthetic tube to the animal's mouth/nose.
   4. Warm ultrasound gel to 37 °C and place the gel on the abdomen of the animal. The ultrasound gel is warmed using a water-heating system that pumps warm water through coils that are wrapped around the bottle of gel.
   5. For heart rate measurements (if needed), put electrode gel on the 4 electrodes of the analysis platform and fix the paws of the animal to the platform/electrodes with surgical tape. Place the animal in a supine position for imaging of the IVC. The ultrasound imaging system includes an analysis platform that is warmed and contains electrodes for monitoring the animal.
   6. For temperature measurements, put electrode gel on thermometer and insert into the rectum of the animal.
   7. Adjust heat lamp and isoflurane as necessary to ensure that animal is comfortable, has a respiration between 30 - 70 bpm, and is kept at 37 °C.
   8. If necessary, shave abdomen of animal or remove hair with hair removal cream. Apply for 1 - 2 minutes and wipe clean with gauze and distilled water.
      NOTE: Too much hair can affect the quality of the images.

9. Place gauze under or to the side of the animal to catch any excess ultrasound gel.

2. **Imaging the IVC and Thrombus**
    1. Turn on the imaging software and begin a new study.
    2. Select an appropriate scanhead for imaging. The scanhead used here is for abdominal organs (Frequency: 35 MHz, Focal length: 12.8 mm).
    3. Record any relevant information about the animal. This may include animal ID, sex, weight, birthdate, strain, *etc*.
    4. Lower the scanhead down until it is touching the ultrasound gel on the animal.
    5. Start the scanhead probe to begin 2-D imaging of the animal. In this mode, images are presented as two dimensional, in varying shades of gray.
    6. Locate the IVC and abdominal aorta by moving the mouse platform throughout the x- and/or y-plane, while also moving the scanhead through the z-plane.
       NOTE: Blood vessels will appear with their walls white and the blood inside nearly black. The abdominal aorta will pulsate intensely and have thicker walls, while the IVC will have thinner walls and compress/expand easily on the image screen when the scanhead is moved up and down. The ligation site will be apparent, as the vein will be dilated and the vein wall will come to a point. Any thrombus formed inside will be solid under ultrasound imaging and will not compress easily like the vein wall does. The vein will compress only above the ligation, where no thrombus is formed.
    7. Upon locating the IVC and the ligation site, center them on the focal point of the ultrasound, in order to achieve the most accurate image.
        1. If the image is too dark to see clearly, adjust the gel and remove any air bubbles with a cotton tip applicator.
        2. If necessary, tilt the scanhead 45° to the left or right and readjust the animal platform to see the vein clearly. This is done if there is interference with the scanhead. In this scenario, the vein needs to be imaged at a side-angle to avoid the suture.
           NOTE: Image interference is most often caused by dark patches of skin on the animal, poor wound suture placement, or bubbles in the ultrasound gel.
    8. Using the software, take photos of any desired structures and make measurements using any measurement tools. Common measurements include cross-sectional area of the thrombus or width of the ligation site.
    9. Change the imaging mode to Pulse-wave Doppler to make blood flow measurements. This mode allows the imaging of vascular blood flow and the measurement of flow velocity, among other variables.
    10. Tilt the analysis platform to lower the posterior end of the animal.
    11. Change the angle/position of the scanhead so that it will contact the gel at an angle of about 30° from the posterior end of the animal.
    12. Reposition the animal platform and scanhead. Relocate the IVC and ligation if necessary.
    13. Make blood flow measurements around the ligation site to confirm stasis and take any images desired. Common measurements of blood flow include average velocity, peak velocity, or median velocity.
    14. Save any measurements or images taken.

3. **Clean-up and animal recovery**
    1. Raise the scanhead to initial position and reposition animal platform to its initial position, as well.
    2. Remove excess gel off the animal with gauze. Do this gently so that the wound suture will not be ruptured.
    3. Remove the thermometer from the animal's rectum and take the animal off the platform.
    4. Place the animal in its cage and a 34 °C incubator.
    5. Monitor the animal until recovery. The procedure is very minor and non-invasive. The animal will wake up quickly. Continue monitoring animal in the following days in accordance with section 1.6 of this protocol.
    6. Clean the animal platform with gauze and disinfectant.
    7. Wipe the scanhead clean with gauze and distilled water.
       Caution: Clean the scanhead very gently and **only with distilled water**.
    8. Repeat step 2 for the next animal.
        1. If done with all subjects, verify that all images and recordings have been saved. Then turn off all equipment and software.

Representative Results

Stasis venous thrombosis model

In the stasis model, mice are anesthetized, and an incision is made to expose the inferior vena cava (IVC). The incision is made on the left or right side of the mouse instead of a midline laparotomy in a way that would not interfere with the ultrasound probe. The abdominal muscles and the skin are fold back to expose the IVC (**Figure 1**). First, side branches are ligated with a 6-0 silk suture (**Figure 2**). Then, the IVC is separated from the aorta by blunt dissection and the silk is placed around the IVC (**Figure 3A-C**). The IVC is ligated with a 6-0 silk suture. Dilatation of the IVC below the ligation site is an indication of successful interruption of the blood flow (**Figure 3D**). Finally, the peritoneal cavity and the skin are sutured back in a continuous manner (**Figure 3E**).

Monitoring of thrombus formation using ultrasonography

As we have previously shown, high frequency ultrasound, which is commonly used to assess venous thrombosis in clinical settings, can be used to measure thrombus formation and resolution over time in an experimental murine model. We used a high frequency micro-imaging system[12]. Prior to ligation, the IVC can be identified in the longitudinal view. After ligation of the vein, the success of the procedure can be visualized. The PW-Doppler mode can be used to determine blood flow velocity before (34.8 mm/s) and after (5.6 mm/s) the ligation. Because thrombi are denser than flowing blood, we could appreciate the formed thrombus inside the IVC using ultrasonography, 24 hours after the ligation (**Figure 4A**). Ultrasonography allows for quantification of the velocity of blood flow in the vessels using color Doppler. As shown in **Figure 4B**, we can measure the flow in the vein prior to ligation and appreciate the interruption of the flow after the ligation, when the thrombus is formed. Data from our laboratory show an average thrombus size of 4.85 ± 0.22 mm^2 at 24 hours and 5.05 ± 0.47 mm^2 at 48 hours (mean ± SEM).

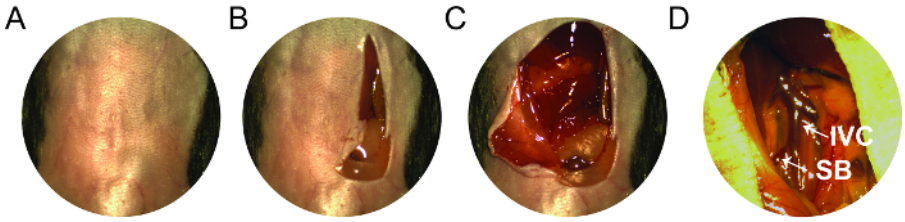

Figure 1. Stasis model: Procedure to expose the inferior vena cava. (A) Mouse hair from the abdomen is removed using hair removal cream. (B) A first incision is made on the left side of the abdomen and a second ventral one (C) from left to right. (D) A wet sterile gaze is used to exteriorize the intestines and expose the inferior vena cava (IVC) and side branch (SB). Please click here to view a larger version of this figure.

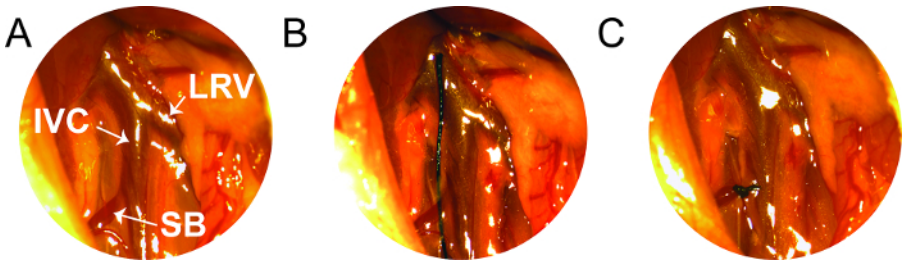

Figure 2. Stasis model: Procedure to ligate the side branch. (A) Exposition of the IVC and SB. (B) Placement of the suture silk underneath the SB. (C) Ligation of the SB. LRV: left renal vein. Please click here to view a larger version of this figure.

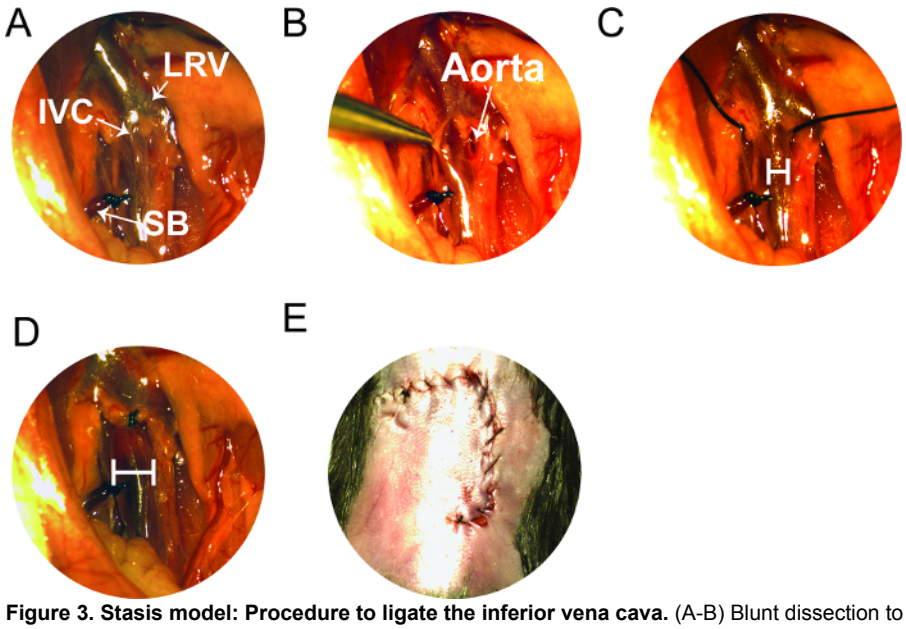

Figure 3. Stasis model: Procedure to ligate the inferior vena cava. (A-B) Blunt dissection to separate the IVC from the aorta. (C) Placement of the suture silk underneath the IVC. (D) Ligation of the IVC. Dilatation of the IVC is observed. (E) The abdominal muscles and skin are closed separately. Please click here to view a larger version of this figure.

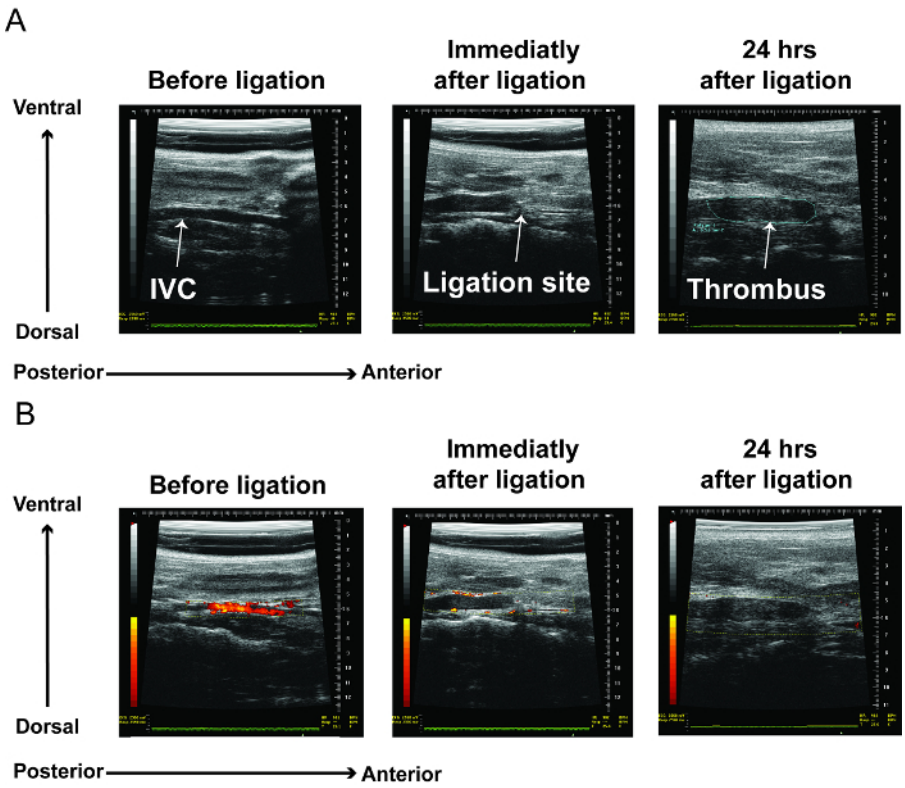

Figure 4. Monitoring of thrombus formation using ultrasound imaging. (A) Representative ultrasound images before, immediately after and 24 hours after ligation of the IVC. (B) Representatives images of blood flow velocity depicted by color Doppler using color processing. The scale of color-coded blood flow ranges from red to yellow to depict high to low flow. In the ligated animals the absence of flow is depicted by the absence of color. Please click here to view a larger version of this figure.

Discussion

There are several critical steps for successful venous thrombus formation using the stasis model. Induction of vein thrombosis is more challenging in old mice due to the accumulation of fat surrounding the inferior vena cava and the aorta. Ideally, mice undergoing this procedure should be 8 - 10-weeks-old. Great care should be taken not to induce endothelial damage in the IVC during the blunt dissection and ligature. In addition, it is crucial to keep the animal in a 34 °C incubator for at least 30 minutes after the surgery and to return it to the company of other animal only after full recovery. When the surgery is done correctly, the animals behave remarkably well during post-operative care. They exhibit no serious side effects such as lameness, paresis, or incontinence. They may exhibit reduced movement and a slightly hunched posture immediately after surgery, but this is not seen often as long as the analgesic is effective and the surgery performed properly.

The stasis model produces a large thrombus with reproducible size measurements from one mouse to another. As in humans, the anatomy of the venous system varies between mice and the issue of IVC branch interruption has recently been addressed in the stenosis and the stasis models of DVT[13,14]. Brandt *et al.* showed that thrombus formation induced by flow restriction was prevented when the side branches were located at <1.5 mm of the IVC ligation site. However, DVT formation induced by flow restriction in mice was not affected by side branch ligation[13,15,16]. It was also reported that the variability of IVC side branches in C57Bl6 mouse strain has an important impact on thrombus formation induced by complete ligation of the IVC[14]. It was found that not ligating side branches results in statistically smaller clot size compared to controls with ligated side branches. Our study also suggest that ligation of the side branches produced consistent thrombus formation and size. However, the most frequent anatomical variation in C57Bl/6 is the presence of 2 back branches (98% of the mice)[14]. It was demonstrated that interrupting back branches has the biggest impact on thrombus size. The present methodology did not address the effect of back branches, which can be interrupted using a low temperature cautery pen[14]. However, we demonstrated that ligation of the IVC and side branches resulted in consistent thrombus formation in C57Bl/6. Finally, as we have previously demonstrated, the high frequency ultrasound system allows the precise measurement of thrombus size and can be used for long-term and translational studies of thrombus formation and resolution[12,13,15].

One major disadvantage of the stasis model is the complete obstruction of blood flow, which reduces the maximal effect of intravenously administered agents on the thrombus and vein wall. This becomes an important issue when one wants to test the effect of a pharmacological agent. If the effect of one specific drug needs to be tested, one would prefer using the stenosis model[13] or the electrolytic model[17]. Both models produce thrombi in the presence of continuous blood flow, which allow testing of new anti-coagulant agents for DVT prophylaxis and treatment.

Disclosures

The authors have nothing to disclose.

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