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Echocardiographic Assessment of the Right Heart in Mice

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Abstract

Transgenic and toxic models of pulmonary arterial hypertension (PAH) are widely used to study the pathophysiology of PAH and to investigate potential therapies. Given the expense and time involved in creating animal models of disease, it is critical that researchers have tools to accurately assess phenotypic expression of disease. Right ventricular dysfunction is the major manifestation of pulmonary hypertension. Echocardiography is the mainstay of the noninvasive assessment of right ventricular function in rodent models and has the advantage of clear translation to humans in whom the same tool is used. Published echocardiography protocols in murine models of PAH are lacking.

In this article, we describe a protocol for assessing RV and pulmonary vascular function in a mouse model of PAH with a dominant negative BMPRII mutation; however, this protocol is applicable to any diseases affecting the pulmonary vasculature or right heart. We provide a detailed description of animal preparation, image acquisition and hemodynamic calculation of stroke volume, cardiac output and an estimate of pulmonary artery pressure.

Keywords

Medicine; Issue 81; Anatomy; Physiology; Biomedical Engineering; Cardiology; Cardiac Imaging Techniques; Echocardiography; Echocardiography; Doppler; Cardiovascular Physiological Processes; Cardiovascular System; Cardiovascular Diseases; Echocardiography; right ventricle; right ventricular function; pulmonary hypertension; Pulmonary Arterial Hypertension; transgenic models; hemodynamics; animal model

Introduction

Elevated pulmonary pressure and right ventricular (RV) dysfunction are the hallmarks of pulmonary vascular disease in animal models and human patients with pulmonary arterial hypertension (PAH). Transgenic and toxic (*e.g.* monocrotaline or hypoxia) models of PAH are widely used to study the pathophysiology of PAH and to investigate potential therapies.

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Video Link

The video component of this article can be found at http://www.jove.com/video/50912/

Disclosures

The authors have nothing to disclose.

Given the expense and time involved in creating animal models of disease, it is critical that researchers have tools to accurately assess phenotypic expression of disease.

Echocardiography is the mainstay of the noninvasive assessment of ventricular function in rodent models[1,2]. Echocardiography has the advantage of clear translation to humans in whom the same tool is used. In addition, some genetic models exhibit incomplete penetrance[3]; the ability to noninvasively identify affected animals saves valuable time and resources. Noninvasive assessment of disease severity without sacrificing an animal also allows researchers to serially study the effects of investigative therapies. This is especially important given the rapidity with which translational therapies can progress to human trials[4,5].

In humans, echocardiographic assessment of RV size and pulmonary hypertension is particularly challenging due to the retrosternal position and irregular shape of the RV[6]. Rodent models have the added challenges of small size and extremely rapid heart rates (300–700 beat/min). Recent advances including higher frame rates and smaller transducers have improved image quality and even allowed conscious imaging in some experimental protocols, though most rodent imaging is done under anesthesia[7,8]. Excellent experimental protocols of echocardiography in rat models of PAH have been described and validated against both MRI and invasive hemodynamics[1,9]. However, published echocardiography protocols in murine models of PAH are lacking.

In this article, we describe a protocol for assessing RV and pulmonary vascular function in a mouse model of PAH with a dominant negative BMPRII mutation and a model of isolated RV afterload after pulmonary artery banding; however, this protocol is applicable to any diseases affecting the pulmonary vasculature or right heart. We will describe animal preparation and detailed assessment of RV size and function as well as main pulmonary artery (PA) size. We also demonstrate the techniques and calculations needed to estimate stroke volume and cardiac output. Technical limitations preclude accurate Doppler estimates of pulmonary pressure, but we have applied a well-validated human surrogate, pulmonary artery acceleration time, to estimate PA pressure.

Protocol

1. Equipment Preparation

1. Examine the ultrasound transducer for defects. Depending on the equipment used, this step may be unnecessary.
    1. If an air bubble is observed, remove the screw located on the right side of the transducer head, and add sterile water through the hole with a 26 G needle. Air bubbles inside the transducer head are common. They will impede the acquisition of quality images.
    2. Check the membrane covering the probe for leaks or holes. Replace if necessary.
2. Open the software and initialize the probe.

1. Choose the cardiac package from the drop down menu, along with the appropriate transducer. Click “initialize”. Use a 20–60 MHz probe for mice under 35 g and a 15–45 MHz probe for mice greater than 35 g.
2. Select the operator, animal & date on the demographics screen, and select “start”.

2. Mouse Preparation Including Anesthesia, Hair Removal, and Positioning

1. **Anesthesia**: place the mouse in an induction chamber and anesthetize with a portable, table top anesthesia machine containing an isoflurane vaporizer and a waste gas container.
    1. Set the vaporizer to 3% with an oxygen flow rate of 3 L/min. This relatively high anesthesia rate is used to achieve rapid anesthetic effect and therefore minimize stress response that may influence cardiac function. The short duration of the protocol minimizes any potential risk to the animal. It is very important to always keep the anesthesia rate the same. This protocol has been optimized for exclusive use of isoflurane as the anesthetic, therefore optimal conditions for other agents may vary from this protocol. A lower depth of anesthesia may be chosen, depending on experimental needs, but once an anesthetic protocol is established, it must not be changed. Anesthesia affects heart rate and other hemodynamic measurements. Therefore, if the depth of anesthesia is changed during an experiment, the data may not be useful for analyses. If several mice are to be imaged in one day, anesthetize them separately.
    2. Monitor room temperature to ensure it is the same between experimental groups. Room temperature can affect vasoreactivity, even when the mouse is on a heated table, so it should be monitored and kept the same between experimental groups that will be compared. Although this protocol does not measure animal temperature directly, constant ambient and table temperature ensures that there is little variation in temperature between experimental groups.
2. **Hair removal**: Remove hair from the chest with a depilatory cream after the mouse is anesthetized. Begin application with a cotton-tipped applicator, at the clavicles, and continue to just below the diaphragm.
    1. Place the mouse back in the anesthesia chamber for 1 min to allow the depilatory to work. To determine if anesthesia is effective, firmly press your thumbnail against one of the mouse’s paws. If there is no withdraw, anesthesia is adequate. If the limb withdraws, place the mouse back in the anesthesia chamber for another minute.
    2. Apply a small amount of lubricating ointment to the mouse’s eyes to avoid damage to the cornea.

3. Remove hair from the chest with a 2 in x 2 in gauze pad. The chemical used in the depilatory cream is caustic, and will harm skin if it is left on for too long, so care should be taken to remove all product from the skin.
4. Apply a skin moisturizer following hair removal.

3. **Positioning:** Place the mouse in a ventrodorsal position on a heated table set to 37 °C. Correct positioning is imperative to the acquisition of quality images. Use a table that can capture body temperature, respiration, and heart rate. Use of an integrated rail system allows for precise positioning and subsequently, image optimization.
    1. Gently tape down all four paws and apply a dime size amount of transduction gel to the chest.

3. Acquisition of Images: Imaging in Parasternal Long Axis View

1. Lock the ultrasound transducer into place inside the mount on the rail system, and rotate it 10° counterclockwise, so that the metal probe of the transducer is positioned directly over the heart. More specifically, the probe should be on the left side of the chest, in the 2nd or 3rd intercostal space, and lateral to the sternum.
    1. Manipulate the x- and y-axes located on the rail system, until the correct view is obtained.
    2. Select “B Mode”. This is found in the upper right portion of the system console, in order to project a 2D live image.
    3. View the following anatomic structures on the monitor:
        1. The entire heart from apex to aorta - The apex will be visualized on the far left of the screen, and the aorta on the far right.
        2. The lumen of the left ventricle (LV)
        3. Posterior wall of the left ventricle (LPW)
        4. Interventricular septum (IVS)
        5. The lumen of the right ventricle (RV)
        6. Anterior and posterior mitral valve leaflets (AML & PML)
        7. Ascending aorta (AO)
        8. Left atrium (LA)
2. Obtain one diametric measurement of the aorta in this view by pressing the scan/freeze button to “freeze” the image. Then use the mouse to pull back through the video loop at the bottom of the image until the left ventricle is in systole, and the aorta is at its greatest diameter.
    1. Click the measurement tool in the upper left corner of the screen and select the icon that looks like a diagonal line. Left click your mouse and draw a

straight line from the anterior to the posterior wall of the aorta, perpendicular to its long axis. Save by pressing the “Frame Store” button.

2. Create a video loop by pressing “Cine Store”

4. Acquisition of Images: Imaging in Parasternal Short Axis View

1. Reposition the transducer to the 3 and 9 o’clock positions (transverse). Angle slightly caudally by manipulating the transducer mount to achieve the best view of the aorta and LV lumen. The metal probe will be positioned horizontally and directly over the sternum.
    1. Manipulate the x- and y-axes on the rail system until the correct view is obtained. The LV lumen will be seen, along with the anterolateral and posteromedial papillary muscles, which are visible on the right of the monitor. This is the standard reference point for the short axis, indicating the mid portion of the left ventricle, where dimension measurements are made. Deviating slightly from the reference point with the x- and y-axes to bring different anatomical structures in view will be necessary, but positioning is explained by referencing the above view.
2. Obtain the following measurements in the short axis view:
    1. **B mode**
        1. Two more diametric measurements of the aorta.
        2. Three measurements of the pulmonary outflow tract.
    2. **Pulsed wave Doppler mode (PW)**
        1. Three velocity time integral measurements (VTI) of the aorta
        2. Three VTI measurements of the pulmonary artery measured just proximal to the pulmonary valve.
        3. Measure pulmonary artery acceleration time by tracing the VTI curve from the beginning of blood flow to peak velocity.
    3. **M mode**
        1. Three measurements of the left ventricular internal diameter in diastole (LVIDd)
        2. Three measurements of the left ventricular internal diameter in systole (LVIDs)
        3. Three measurements of the right ventricular internal diameter (RVID). The RV lumen will only be visible in this view if it is dilated.
        4. Measure heart rate three times using m-mode by tracing the distance between two diastolic peaks of the anterior wall of the LV during three different cardiac cycles.

3. **B mode measurements:**

    1. Manipulate the Y axis cranially from the papillary muscle view, until the semilunar valve of the aorta comes into focus.
    2. Obtain measurements of the aorta just above the valve at the greatest diameter.
    3. Click the measurement tool in the upper left corner of the screen, and select the icon that looks like a diagonal line.
    4. Left click your mouse and draw a straight line from the anterior to the posterior wall of the aorta.
    5. Manipulate the x- and y-axes until the main pulmonary artery bifurcates. This structure will be seen anteriorly, and to the right of the aorta on the monitor.
    6. Manipulate the y-axis cranially until the annulus of the main pulmonary artery comes into view. It will not be as clearly defined as the aorta.
    7. “Freeze” the image and obtain the measurement in systole.
    8. Collect three measurements in total.

4. Pulsed wave (PW) Doppler measurements: PW Mode is primarily used for hemodynamic assessment of blood flow through arteries and veins. In this protocol, it will be used to retrieve three velocity time integral measurements of the aorta and pulmonary artery.

    1. Bring the aorta back into view as described in step 4.3.1, and bullet point 1.
        1. Select “PW Mode”. This is located on the upper right corner of the system console and will produce a Doppler reading of blood flow through the aorta.
        2. Place the sample volume just above the level of the aortic valve. The x- and y-axes may need to be adjusted slightly to obtain sufficient Doppler envelopes. The envelopes should have white borders, and a hollow inside indicating laminar blood flow.
        3. Once a sufficient view is obtained, “freeze” the image and trace the border of the Doppler envelope. This will calculate the VTI.
        4. Rotate the “Angle” knob located on the system console clockwise until the segmented yellow line seen in the image on the upper right-hand side of the monitor is at 0°. This yellow line represents the direction of blood flow through the vessel. Since the transducer itself is angled in such a way to produce a cross-sectional or transverse view of the heart, the line must be adjusted to 0°, to align with the vertical flow of blood through the ascending aorta.
    2. Place the sample volume proximal to the level of the pulmonary valve in the center of the right ventricular outflow tract and repeat VTI measurements as

above. The flow of blood should appear inverted, or opposite the flow of blood in relation to the aorta on the monitor.

5. **M mode measurements:** M-mode imaging provides high temporal resolution of tissue motion along a single ultrasound beam, and is used to quantify cavity dimensions, as well to study valvular, myocardial, and vessel wall movement.
    1. Resume “B Mode” and reposition the transducer to obtain the “reference view” (the cross sectional view of the left ventricle at the level of the papillary muscles).
    2. Press “M-mode”. This will produce a continuous video feed in which the motion of the following anatomical structures will be visible as a “ribbon”. If dilated, the RV lumen will appear at the top of the feed as a very thin black ribbon. The interventricular septum (IVS) will be visible as an opaque ribbon directly below the RV lumen. The LV lumen will be seen directly below the IVS. It is the large black space that occupies the majority of the feed. Below the LV lumen is the LV posterior wall (LVPW) which will be seen as an opaque ribbon.
    3. Freeze the image and pull back through the video loop if needed to a point where respiration is not occurring. When the mouse respires, the image acquisition is disrupted by movement of the diaphragm and chest wall, thereby producing a distorted “smeared” artifact in the feed that occurs with regular frequency.
    4. Obtain the following measurements using the diagonal line icon:
        1. Three measurements of LV end diastolic dimension, which appears as the largest distance between the IVS and LVPW.
        2. Three measurements of LV end systolic dimension, which appears as the shortest distance between the IVS and LVPW.
        3. Three measurements of heart rate, which is done by clicking the heart icon and measuring from systolic peak to systolic peak of the LVPW.
        4. If the RV lumen is dilated, obtain three measurements using the diagonal line icon.
    5. Record a video loop of the short parasternal axis view in “B mode” by pressing the “Cine Store” button.
    6. Go to “File”, select “Browse Study” to recap your measurements, click “End Session”, and then “Commit Session Data.”
    7. Recover the mouse as outlined by the IACUC protocol, and clean up.
    8. Export data as a CSV file to a thumb drive for subsequent analysis.
6. Calculate the following parameters of cardiac function (Table 1):
    1. Left ventricular outflow tract area

2. Left ventricle stroke volume
3. Left ventricle cardiac output
4. Fractional shortening
5. Pulmonary artery area
6. Pulmonary artery acceleration time
7. Right ventricle stroke volume
8. Cardiac index

Representative Results

The principal goals of this protocol are to quantify RV size and function, and to understand the degree to which the pulmonary vasculature is diseased. Appropriate preparation of both the mouse and echocardiography equipment is essential to obtaining accurate and reproducible results. Mice should have their chest depilated and limbs secured to the imaging platform with tape. Anesthesia, in this case isoflurane, is administered via nose cone. The transducer should be checked for defects, particularly air bubbles, which can degrade image quality. Obtaining good quality 4-chamber views of the heart is quite difficult in mice so this protocol focuses on RV assessment using the parasternal short and long axes. Relevant anatomy in these views is shown in Figures 1A and 1B.

RV size is best assessed in the parasternal long axis view and is measured as the distance from the free wall to the interventricular septum using M-mode (Figure 2). This measurement is only possible when the RV is dilated as the normal RV is very small. In mice, it is not possible to accurately measure the usual metrics of RV function in humans such as fractional area change and the tricuspid annular plane systolic excursion. These measurements require high quality views of the RV free wall which are very difficult to obtain in mice. However, using PW Doppler to measure the velocity time integral (VTI) at the level of the right ventricular outflow tract (RVOT) and the diameter of the pulmonary artery, it is possible to estimate RV stroke volume (Figure 3). Stroke volume and cardiac output are calculated from the formulae in Table 1. Heart rate is obtained from m-mode imaging.

Main PA diameter reflects PAH severity in humans[10] and can be measured in mice in the parasternal short-axis view (Figure 3). It is important to have clear view of both sides of the main PA because this value is squared in the equation used to calculate cardiac output. If PA size cannot be accurately measured, left ventricular outflow tract diameter and LVOT VTI can be inserted into the equations above as RV and LV output are equal in the absence of shunting.

The RV VTI can be further interrogated to estimate PA pressure by measuring the time to peak velocity (pulmonary artery acceleration time [PAT], Figure 4). In humans PAT is used to dichotomize PA pressure as high or low[11], and may be used to estimate PA pressure when a tricuspid regurgitant jet is not present.[12]

Discussion

Mouse models of disease, either transgenic or toxin-related, require phenotypic validation that the model actually recapitulates the human disease it is intended to emulate. This validation can often be accomplished by the presence or absence of a particular feature, for example development of a tumor. However, models that result in hemodynamic abnormalities such as aortic constriction models of left ventricular hypertrophy or our transgenic model of PAH are more difficult to validate. These models require either terminal measurement of hemodynamics or tools to noninvasively measure hemodynamics and abnormalities in cardiac function. Echocardiography is critical to such models because it allows real-time quantification of hemodynamics and cardiac function without requiring sacrifice of diseased animals[14]. In addition, individual animals can be imaged serially to follow the natural history of a disease or response to therapy. We estimate that proficiency in echocardiography of the right heart according to this protocol can be gained after performing approximately 20 examinations.

The ability to estimate cardiac output on echocardiography is also critical to the calculation of pulmonary vascular resistance (PVR) at the time of sacrifice. Measurement of cardiac output using conductance catheters is often unreliable in our model because of the small size of the RV. At the time of sacrifice, we measure invasive PA systolic pressure using a conductance catheter and combine this with cardiac output from echocardiography to determine PVR (pulmonary wedge pressure is assumed to be low and is ignored). This allows us to further quantify the degree of pulmonary vascular disease in our model.

Theoretical and Practical Limitations of Echocardiography

It is important to recognize that the application of ultrasound physics to humans and live animals has limitations. Accurate measurement of blood velocity using Doppler is dependent on the angle of flow relative to the angle of insonation (angle at which transducer is aimed). For every degree those two angles are unaligned, the measurement of blood velocity will be decreased by the cos (θ)[15]. Clinically, if the two angles are off by more than 20° the measurement is felt to be unreliable. This has potentially important implications for this protocol in the measurement of the LVOT and RVOT VTI. If the PW angle cannot be well-aligned with the direction of blood flow in the LVOT and RVOT, the measured SV and cardiac output will be falsely low.

Another potential measurement error is in the calculation of PA and aortic area which are then used to calculate SV and cardiac output. Because the area of a circle is πr^2, any inaccuracy in the measurement of the diameter of the aorta or pulmonary artery is squared and the error compounded. In humans, the RVOT and LVOT diameters are used to calculate SV instead of the diameter of the aorta and pulmonary arteries; however, in mice it is very difficult to accurately identify the LVOT and RVOT so we substitute the aorta and pulmonary artery areas. Provided the same technique is used in one animal to the next, this minor difference should not impact study results.

Right ventricular dysfunction is the major manifestation of pulmonary hypertension in humans. A number of practical limitations pertain to the noninvasive assessment of the right

heart. In humans and mice, the right ventricle is situated adjacent to the chest wall. This close proximity to the transducer makes imaging the anterior RV free wall very difficult. The RV is an irregular crescent shape which precludes volumetric assumptions like those used to determine LV size and function. RV size in mice can usually only be determined as normal or enlarged due to the difficultly in seeing the RV free wall. However, this categorization is still helpful to validate the presence or absence of pulmonary vascular disease.

Echocardiography may be performed on mice with or without anesthesia. We prefer to use anesthesia to maximize the quality and accuracy of our measurements but recognize that anesthesia will lower the heart rate. When performed without anesthesia, image quality may suffer and the process is a source of stress for the animals which will elevate the heart rate and blood pressure. We perform all echocardiograms with an identical degree of anesthesia to allow comparison of results between and within mice.

Quantification of RV and pulmonary vascular function in mice relies on Doppler estimation of beat to beat flow across the pulmonary valve, the RVOT VTI. This can be used as a dichotomous variable (high/low), but when measured carefully can be used as a serial measurement in a mouse or compared between groups with different interventions. Advanced equipment is commercially available to use color Doppler assessment for the presence and velocity of a tricuspid regurgitation (TR) jet, which is used in humans to quantify the severity of pulmonary hypertension. In humans without a measureable TR jet, the PAT is used as a surrogate to determine whether pulmonary hypertension is present or absent[11]. PAT will shorten as PH worsens because RV ejection will stop sooner against increased pressure. This method has also been validated in rat models of PAH as an accurate estimate of the severity of pulmonary hypertension[1]. Finally, the shape of the RVOT VTI envelope can shed light on coupling between the RV and the pulmonary vasculature in humans[16]. Notching of the envelope is consistent with elevated pulmonary vascular resistance with later notching indicating higher resistance. However, we have not observed these patterns in mice in our model of PAH, even in mice subsequently confirmed to have severe PH by invasive measurement.

Aside from echocardiography, cardiac magnetic resonance imaging (CMR) is the only noninvasive alternative for the assessment of RV function. In rats, CMR provides accurate measurement of RV thickness, mass and volumes (and thus ejection fraction and cardiac output)[17]. In addition, CMR-measured PAT and flow-time curves (analogous to VTI) correlate strongly with echocardiography and invasively measured hemodynamics. Despite some obvious advantages, CMR is more expensive and time-intensive than echocardiography and for those reasons is rarely used in our experiments. To our knowledge no study has validated the echocardiographic measurements described here with invasive measurements or CMR. However, we routinely use the measurements presented in this protocol to assess disease penetrance and severity[18–20].

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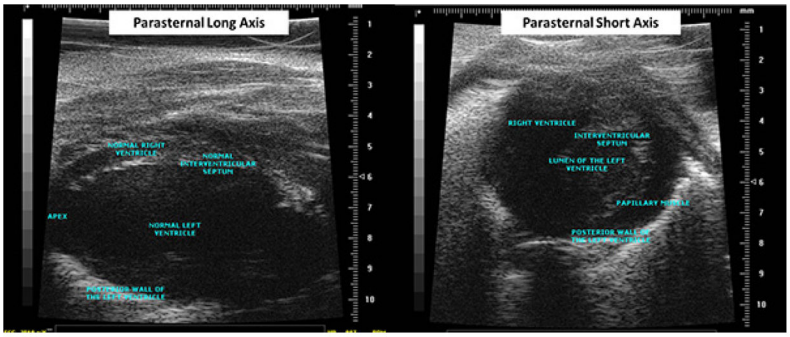

Figure 1. Echocardiographic Views of Murine Anatomy

Panel **A** shows normal anatomy in the parasternal long-axis view. Panel **B** shows anatomy in the parasternal short axis view. The right ventricle is enlarged in panel **B**.

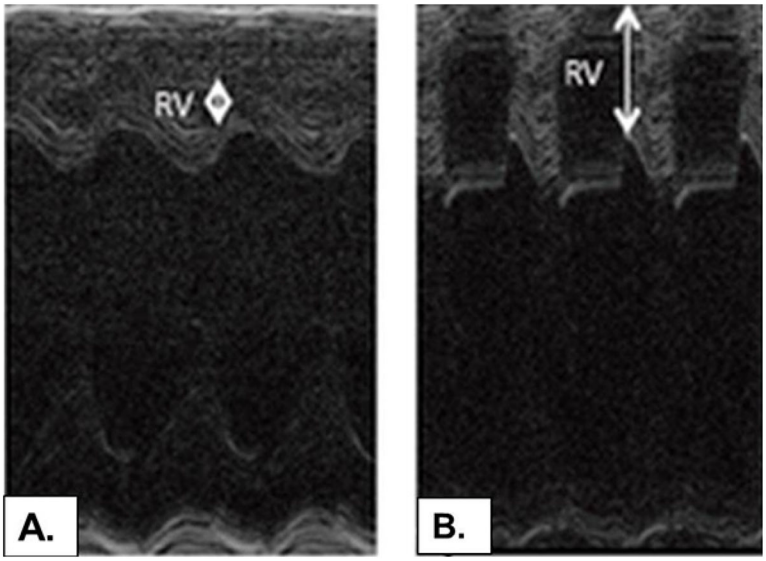

Figure 2. Measurement of Dilated Right Ventricle

This figure demonstrates (**A**) normal RV size in a control mouse (**B**) severe RV enlargement in a mouse that underwent pulmonary artery banding model.

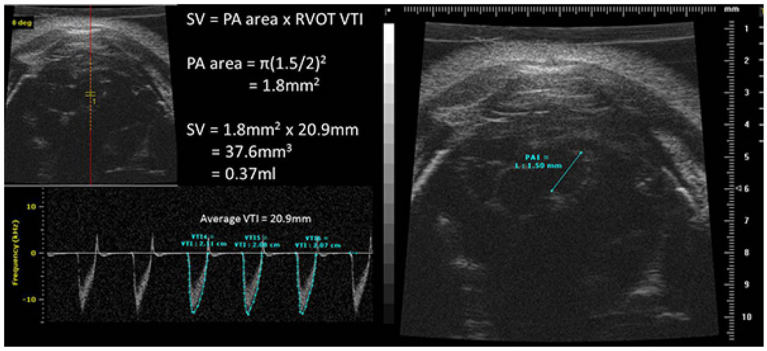

Figure 3. Measurement and Calculation of Right Ventricular Stroke Volume

This figure shows the measurements for both right ventricular VTI and pulmonary artery diameter. The method for calculating stroke volume with these data is also demonstrated.

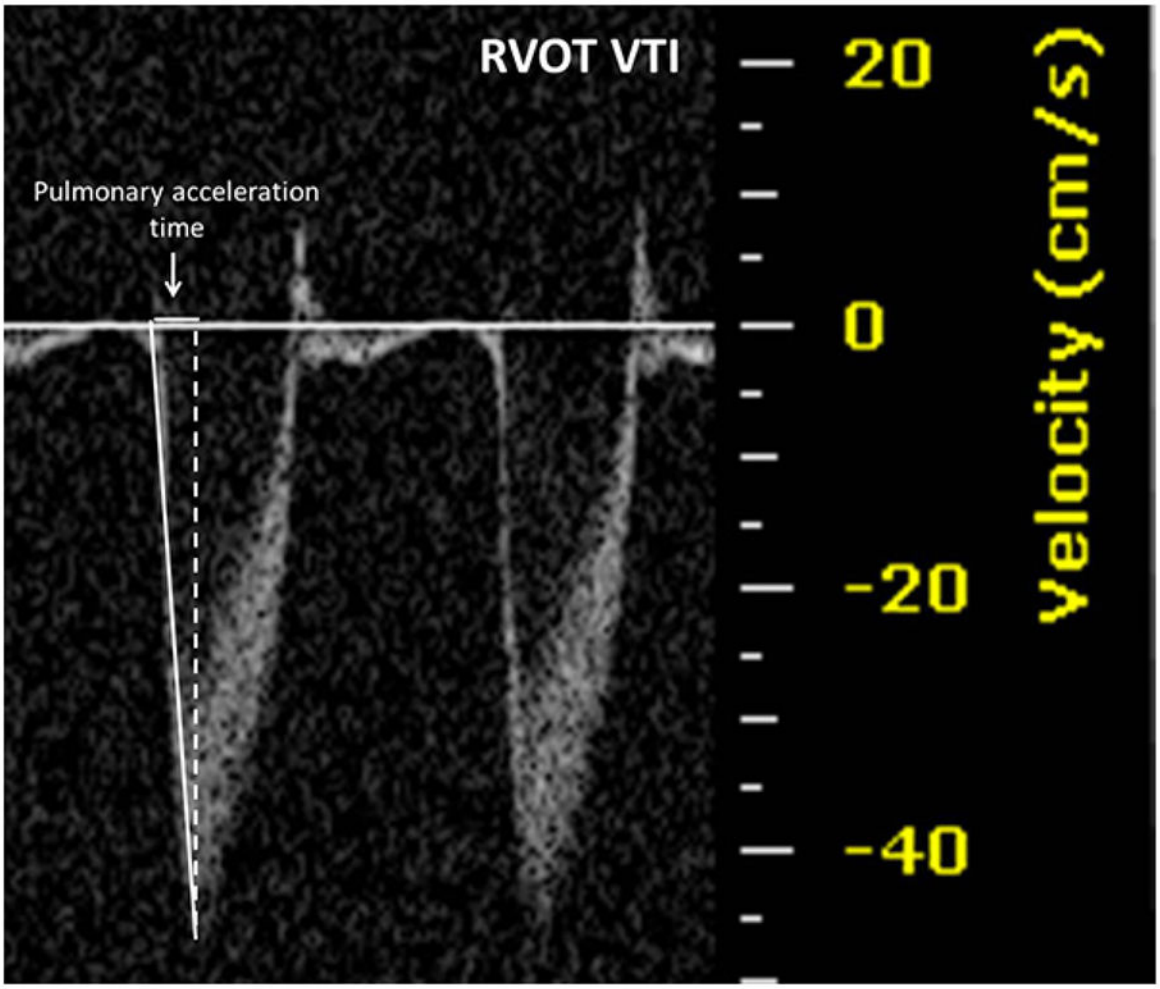

Figure 4. Measurement of PAT

Pulmonary acceleration time is measured as the time to peak velocity in the RVOT VTI.

Table 1

Useful Calculations in Echocardiography.

Measurement	**Formula**
Pulmonary artery/aortic area	$\pi(\text{diameter}/2)^2$
Right Ventricular Stroke volume	PA area x VTI
Cardiac Output	heart rate x stroke volume
Cardiac Index	cardiac output / body surface area
Fractional Shortening	(LV end-diastolic dimension − LV end-systolic dimension) / LV end-diastolic dimension

Body surface area = $10.5\ (\text{grams})^{2/3}$ [13]