Journal of Visualized Experiments

Measuring ascending aortic stiffness in vivo in mice using ultrasound

--Manuscript Draft--

Manuscript Number:	JoVE52200R2
Full Title:	Measuring ascending aortic stiffness in vivo in mice using ultrasound
Article Type:	Methods Article - JoVE Produced Video
Keywords:	Aortic stiffness; ultrasound; in vivo; aortic compliance; elastic modulus; mouse model; cardiovascular disease
Manuscript Classifications:	1.7: Cardiovascular System; 1.7.231: Blood Vessels; 93.39.19: elasticity
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Abstract:	We present a protocol for measuring in vivo aortic stiffness in mice using high-resolution ultrasound imaging. Aortic diameter is measured by ultrasound and aortic blood pressure is measured invasively with a solid-state pressure catheter. Blood pressure is raised then lowered incrementally by intravenous infusion of vasoactive drugs phenylephrine and sodium nitroprusside. Aortic diameter is measured for each pressure step to characterize the pressure-diameter relationship of the ascending aorta. Stiffness indices derived from the pressure-diameter relationship can be calculated from the data collected. Calculation of arterial compliance is described in this protocol. This technique can be used to investigate mechanisms underlying increased aortic stiffness associated with cardiovascular disease and aging. The technique produces a physiologically relevant measure of stiffness compared to ex vivo approaches because physiological influences on aortic stiffness are incorporated in the measurement. The primary limitation of this technique is the measurement error introduced from the movement of the aorta during the cardiac cycle. This motion can be compensated by

	adjusting the location of the probe with the aortic movement as well as making multiple measurements of the aortic pressure-diameter relationship and expanding the experimental group size.
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Dear Editor:

We would like to submit our manuscript titled "Measuring ascending aortic stiffness *in vivo* in mice using ultrasound" for publication in the *Journal of Visualized Experiments*. Our manuscript was solicited by associate editor Jane Hannon.

Successful performance of this protocol depends on recognizing specific anatomical features of the mouse. Because these features are best shown visually, this protocol is well-suited for *JoVE*'s multimedia format. Moreover, the procedures described can be used in a range of applications besides the one described in our protocol. We believe visual documentation of these procedures would greatly benefit other investigators wanting to use these procedures for their studies.

We request that filming takes place prior to July 31, 2014. The author who performs these procedures, Maggie Kuo, will be finishing her PhD work and graduating by that date.

We would like to suggest the following as reviewers for this manuscript:

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Thank you for your consideration. We look forward to hearing from you.

Sincerely,

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TITLE:
Measuring ascending aortic stiffness *in vivo* in mice using ultrasound

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KEYWORDS:
Aortic stiffness; ultrasound; *in vivo*; aortic compliance; elastic modulus; mouse model; cardiovascular disease

SHORT ABSTRACT:
We describe a technique for measuring aortic stiffness from its pressure-diameter relationship *in vivo* in mice. Aortic diameter is recorded by ultrasound and aortic pressure is measured invasively with a solid-state pressure catheter. Blood pressure is changed incrementally and the resulting diameter is measured.

LONG ABSTRACT:
We present a protocol for measuring *in vivo* aortic stiffness in mice using high-resolution ultrasound imaging. Aortic diameter is measured by ultrasound and aortic blood pressure is measured invasively with a solid-state pressure catheter. Blood pressure is raised then lowered incrementally by intravenous infusion of vasoactive drugs phenylephrine and sodium nitroprusside. Aortic diameter is measured for each pressure step to characterize the pressure-diameter relationship of the ascending aorta. Stiffness indices derived from the pressure-diameter relationship can be calculated from the data collected. Calculation of arterial compliance is described in this protocol.

This technique can be used to investigate mechanisms underlying increased aortic stiffness associated with cardiovascular disease and aging. The technique produces a physiologically relevant measure of stiffness compared to *ex vivo* approaches because physiological influences on aortic stiffness are incorporated in the measurement. The primary limitation of this technique is the measurement error introduced from the movement of the aorta during the

cardiac cycle. This motion can be compensated by adjusting the location of the probe with the aortic movement as well as making multiple measurements of the aortic pressure-diameter relationship and expanding the experimental group size.

INTRODUCTION:

Increased aortic stiffness is a hallmark of cardiovascular disease. Aging[1], smoking[2], diabetes[3], hyperlipidemia[4], and other risk factors of cardiovascular disease have been shown to increase aortic stiffness. Epidemiological studies have further demonstrated aortic stiffness as a powerful independent predictor of the onset of coronary heart disease and stroke, as well as the occurrence of cardiovascular events and mortality[5-8]. Because of the clinical and public health significance of increased aortic stiffness, current research is focused on understanding the mechanisms underlying the development and progression of vascular stiffness. Great interest therefore exists in developing accurate measures of vascular stiffness in experimental models of cardiovascular disease.

A material's stiffness can be characterized by its stress-strain relationship and quantified as elastic modulus. A linear elastic material deforms reversibly and its stress increases proportionally to strain. The aorta and the large arteries are nonlinear elastic bodies: when stretched, the stiffness of the artery does not remain constant but increases with the degree of distension. This nonlinearity in the mechanical properties of large arteries is due to the different stiffness properties of the load-bearing elements, namely elastin and collagen, which constitute the vessel wall. Elastin is highly extensible with an elastic modulus of 0.6 MPa. In comparison, collagen is very stiff with an elastic modulus of 1 GPa[9]. The initial stiffness exhibited by the aorta at lower strain values is attributed to elastin while the high stiffness exhibited at high strain values is due to collagen. Load is transferred from elastin to collagen as the vessel distends and this region of load transferring is where the vascular system operates. Therefore, at physiologic pressures, arterial stiffness depends on the contribution of both elastin and collagen[10].

The distribution and orientation of elastin and collagen vary by layer within the arterial wall. In the media, the elastin, collagen, and smooth muscle cells are bundled into tight helices that are layered concentrically. This arrangement allows the artery to resist high loads in the circumferential direction. The adventitia is predominantly collagen with little elastin and the collagen fibers are organized in a net-like fashion. These collagen fibers are wavy in an unstressed state and straighten out as load increases. Stiffness increases as the collagen fibers straighten out, thereby preventing the artery from overstretching and rupturing. Because of the structural organization and varying orientation of the collagen fibers, arteries are anisotropic: the stiffness exhibited depends on if the vessel is stretched longitudinally or circumferentially[11]. *In vivo* stiffness is therefore a composite of the aorta's longitudinal and circumferential stiffness.

Arterial stiffness is generally quantified *in vivo* as compliance or pulse wave velocity (PWV). Arterial compliance is defined as $C = \Delta D / \Delta P$ where ΔD is change in diameter and ΔP is the corresponding change in pressure. Lower values of compliance indicate stiffer vessels.

Compliance is calculated from the pressure-dimension relationship of the artery and is therefore a direct measure of stiffness. As stiffness is disseminated non-uniformly in the vasculature[12], compliance should be measured at the same/similar location in each subject to make meaningful comparisons between experimental groups.

The difference between compliance and elastic modulus is that elastic modulus is normalized to the material's dimensions. Compliance therefore reflects structural stiffness, whereas elastic modulus reflects material stiffness. With aging, arterial wall thickness increases and elastin/collagen ratio decreases, so both structural stiffness and material stiffness are greater.

Compared to compliance, PWV is an indirect measure of arterial stiffness. PWV is the speed at which a pressure pulse travels along a length of artery and is influenced by the properties of the vessel wall. The Moens-Korteweg equation is used to model the relationship between PWV and elastic modulus: $PWV^2 = E\ h\ /\ (2\ \rho\ r)$ where E is incremental elastic modulus, h is wall thickness, ρ is blood viscosity, and r is vessel radius. A higher PWV value therefore suggests a stiffer vessel.

Compliance and elastic modulus can be measured experimentally *ex vivo* on an excised segment of vessel. To determine compliance, the vessel segment is mounted on a pressure myograph[13,14]. Pressure within the vessel is increased step-wise and the resulting change in diameter is tracked using video microscopy. Compliance is determined from the pressure-diameter data. Incremental elastic modulus can be measured by tensile testing. In these experiments, the vessel is pulled apart step-wise and force-displacement data is collected until the vessel ring breaks. Stress and strain values can be calculated and plotted to determine incremental elastic modulus. These *ex vivo* approaches can be used to evaluate changes in the passive properties that influence stiffness.

In vivo, in addition to wall content, vascular stiffness is influenced dynamically by smooth muscle tone and blood pressure[13,15,16]. PWV is the most widely used method for measuring *in vivo* aortic stiffness in experimental models. PWV can be determined non-invasively using Doppler ultrasound or applanation tonometry[17]. Pressure pulse is measured at two separate locations and the time required for the pulse to traverse the distance is the pulse wave velocity. Because PWV is measured over a length of aorta, it is an averaged value of stiffness. Large arteries are nonlinear elastic, so stiffness and therefore PWV will vary with arterial pressure. A higher PWV value could therefore arise from increased stiffness or elevated pressure. PWV values therefore must be normalized to blood pressure to derive conclusions about the vessel's stiffness. Measurement methods that incorporate the influence of blood pressure with the passive properties of the vascular wall and the effects of vasoactive mediators that alter tone would yield a physiologically relevant index of arterial stiffness. This approach is implemented by measuring PWV invasively using a catheter with two pressure sensors separated at a fixed distance[13]. This dual-pressure catheter is inserted into the aorta and vasoactive drugs, such as phenylephrine or sodium nitroprusside, are infused intravenously through a venous catheter to raise and lower arterial pressure.

This protocol describes a method to determine aortic stiffness *in vivo* from its pressure-

dimension relationship in a mouse model. This approach offers several advantages over the invasive PWV measurement. Stiffness indices, such as compliance, can be calculated from the pressure-dimension data collected by this procedure. Moreover, this technique allows for measurement of local aortic stiffness because stiffness is measured from a single location. This approach is particularly useful in measuring ascending aortic stiffness as the short length of this region makes a PWV measurement difficult to obtain. Research interest exists specifically in the ascending aorta because its mechanical properties influence the perfusion of the coronary circulation and the cardiac response to vascular dysfunction.

To measure the pressure-diameter relationship of the aorta *in vivo*, the ascending aorta is visualized and its diameter is measured by ultrasound imaging. Aortic blood pressure is measured invasively with a pressure catheter. Blood pressure is changed incrementally by intravenous infusion of vasoactive drugs. Phenylephrine constricts blood vessels and is used to raise aortic pressure. Sodium nitroprusside dilates blood vessels and is used to lower aortic pressure. Systolic and diastolic aortic diameters and corresponding aortic pressures are measured for each pressure increment. Compliance can be calculated from the pressure-diameter data collected.

PROTOCOL:

This protocol has been approved by the Institutional Animal Care and Use Committee at Johns Hopkins University.

1. Preparation of solutions, materials, and animal

1.1) Prepare a 300 µg/ml solution of phenylephrine (PE) and 300 µg/ml solution of sodium nitroprusside (SNP) in 0.9% saline. Prepare a separate heparin-saline solution by mixing 1 ml of 1000 U/ml heparin into 10 ml of 0.9% saline.

Note: Drugs should be at room temperature before use.

1.2) Make the catheter for intravenous drug infusion from two 30G x ½" hypodermic needles and PE 10 polyethylene tubing. To make the catheter, insert one needle into one end of the tubing. Remove the needle portion of the other hypodermic needle and insert the blunt end into the other end of the tubing. Attach the catheter to a 1 ml syringe and fill the catheter with the heparin-saline solution.

1.3) Place mouse in the anesthesia induction chamber containing 2-2.5% isoflurane in 100% oxygen. Leave the mouse in the induction chamber until it is unresponsive to external stimuli. Remove the mouse from the induction chamber and place it on the heated electrocardiogram (ECG) pad. Maintain the animal at 2% isoflurane.

1.4) If necessary, apply vet ointment or saline solution to the animal's eyes to prevent dryness during the procedure.

2. Insertion of catheter into tail vein

2.1) Since the tail veins are located laterally on both sides of the tail, place the animal on its side for better access. Secure the mouse onto the ECG pad with tape. Make sure the animal is kept warm to promote vasodilation of the tail veins.

2.2) Using a piece of silastic tubing as a tourniquet, tie the tourniquet around the base of the tail. Tie the tourniquet tight enough to collapse the veins but not enough to cut off the arterial circulation. After 2-3 minutes, the vein should bulge out and become more visible.

2.3) Gently pull the tail taut. Bend the tail at an angle with one hand and hold the needle parallel to the tail with the other. Pierce the needle where the tail is bent through the skin into the vein. Blood will push back into the catheter if the needle is inserted into the vein.

2.4) Place one drop of tissue glue where the needle is inserted to secure the catheter. Remove the tourniquet and confirm patency by injecting saline with little resistance.

3. Insertion of blood pressure catheter through femoral artery

3.1) Place the pressure catheter into a 30 ml syringe filled with distilled water and connect the catheter to the pressure control unit. Soak the catheter in water, plugged in, for 30-45 minutes during the set-up and surgery procedures.

3.2) Place the animal supine and tape its paws onto the ECG pad. Apply depilatory cream on the chest and area over the femoral artery.

3.2.1) Wait 3-5 minutes and remove cream and hair. Thoroughly remove hair from the chest to prevent artifacts during the ultrasound. Wipe both the chest and hind limb regions with a moistened pad to remove excess depilatory cream.

3.3) Using fine scissors, make an incision in the skin above the location of the femoral artery. Dissect through the subcutaneous fat tissue to reveal the femoral artery. The femoral artery is partially covered by the abdomen. Use hemostats to move the abdomen away.

3.4) Using fine forceps, separate the nerve away from the artery-vein bundle. Gently pierce through the sheath around the artery-vein bundle to separate the artery from the vein. Pass one suture around the artery at the proximal end and place two sutures at the distal end.

3.5) Securely knot the most distal suture to stop distal blood flow. Use hemostats to pull the proximal suture to temporarily stop blood flow into the femoral artery. Use microscissors to make a small incision into the femoral artery. Make the incision near the distal knot.

3.6) Calibrate the data acquisition software to the catheter using the calibration settings on the

pressure control unit. Switch the pressure control unit back to reading the transducer and balance the pressure catheter so that the catheter outputs 0 mm Hg in the water-filled syringe.

3.7) Insert the catheter into the femoral artery. Open the incision with fine forceps with one hand and insert the catheter head into the artery with the other hand.

3.7.1) Knot the middle suture around the catheter wire to secure the catheter into the artery. Relax the proximal suture and advance the catheter forward into the abdominal aorta. Knot the proximal suture to further secure the catheter and to prevent bleeding.

3.8) Carefully move the ECG pad with mouse, pressure catheter and saline syringe to the ultrasound imaging stage. Connect the blood pressure catheter to the pressure control unit. Place the saline syringe in the syringe pump. Allow the animal and the catheter to equilibrate for 20 minutes.

4. Measuring aortic diameter over a range of blood pressures

4.1) Reduce isoflurane to 1.5%. Visualize the ascending aorta longitudinally on B-mode using a long axis view. Mount the transducer onto the rail system so that the same view is maintained for the duration of the experiment.

4.2) On the ultrasound mainframe, place the M-mode cursor over the section of aorta to be tracked. Track the aortic diameter change over the cardiac cycle using M-mode.

4.3) Change the saline in the syringe to the PE solution and place the syringe into the syringe pump.

4.3.1) Record M-mode at baseline aortic pressure. Begin infusion at 360 µg/kg/min and infuse for 1 minute for aortic pressure to reach a plateau. For a 25 g mouse, this dose equates to 30 µl/min.

4.3.2) Record the M-mode, then stop the infusion, and wait 2 minutes for blood pressure to return to baseline.

4.4) Lower infusion rate to 240 µg/kg/min. For a 25 g mouse, this dose equates to 20 µl/min. Start infusion, infuse for 1 minute for blood pressure to plateau, and record M-mode. Stop the infusion, and wait 2 minutes for blood pressure to return to baseline.

4.5) Repeat step 4.4 for 120 µg/kg/min PE (10 µl/min for a 25 g mouse).

4.6) Replace PE with saline and infuse the saline at the rate used for the 360 µg/kg/min infusion (30 µl/min for a 25 g mouse). Infuse for 2-3 minutes, until further infusion does not produce an increase in aortic pressure and pressure is returning to baseline. Wait 5 minutes for the blood pressure to stabilize at baseline.

4.7) Replace saline with SNP.

4.7.1) Record M-mode at baseline aortic pressure. Begin infusion at 240 µg/kg/min (20 µl/min for 25 g mouse)and infuse for 1 minute. When aortic pressure reaches a plateau, record the M-mode. Stop the infusion and wait 2 minutes for blood pressure to return to baseline.

4.8) Lower infusion rate to 120 µg/kg/min (10 µl/min for 25 g mouse). Start infusion, infuse for 1 minute for blood pressure to plateau, and record M-mode. Stop infusion and wait 2 minutes for blood pressure to return to baseline.

4.9) Repeat step 4.8 for 60 µg/kg/min SNP (5 µl/min for 25 g mouse).

5. Terminating the experiment

5.1) To euthanize the animal, increase isoflurane to 4%. When breathing has slowed, usually in 1-2 minutes, cut through the sternum with scissors to open the thoracic cavity and expose the heart.

5.2) Grasp the heart with medium forceps and excise it from the body by cutting at the ascending aorta with scissors.

REPRESENTATIVE RESULTS:

A longitudinal image of the left ventricle and ascending aorta is captured on B-mode, as shown in **Figure 1**. Alternatively, a longitudinal image of only the aorta can be obtained, as in **Figure 2**. The movement of the aortic wall during the cardiac cycle appears as two white lines on the M-mode, as shown in **Figure 3**. The aortic lumen is the area in between the lines. Aortic pressure is modulated by infusion of vasoactive drugs. PE raises the aortic pressure, as shown in **Figure 4A**, and SNP lowers pressure, as shown in **Figure 4B**. M-mode is recorded when blood pressure plateaus, 1 minute after the start of infusion. Aortic pressure is changed incrementally through changing the dose of the drug administered, as shown in **Figure 5**. Dose of drug is controlled through the rate of infusion. All drug doses are in µg/kg/min. Maximum and minimum diameters are measured from the M-mode, shown in **Figure 3**. These diameters correspond to the systolic and diastolic aortic pressures recorded by the pressure catheter.

Systolic and diastolic diameter and pressure values of three cardiac cycles are measured at baseline and for each PE and SNP dose. The standard deviation between three diameter measurements at one drug dose ranges from 0.01 mm to 0.04 mm. Aortic diameter can be plotted against its corresponding aortic pressure to illustrate the pressure-diameter relationship, as shown in **Figure 6A**.

These pressure-diameter values are used to calculate aortic compliance. Arterial compliance is calculated by

$$C = (D_{sys} - D_{dia}) / (P_{sys} - P_{dia}) \qquad (1)$$

where D_{sys} and D_{dia} are systolic and diastolic diameters and P_{sys} and P_{dia} are systolic and diastolic pressures. Compliance and mean aortic pressure (MAP) are calculated at baseline and for each PE and SNP dose. Compliance is plotted against MAP to demonstrate the pressure-dependency of stiffness. Because of the nonlinear elastic behavior of the aorta, compliance decreases with increasing MAP, as seen in **Figure 6B**.

Figure 1: Longitudinal view of ascending aorta on B-mode. Diameter measurements are taken from a longitudinal image of the ascending aorta leaving the left ventricle. LV: left ventricle; PA: pulmonary artery; AA: ascending aorta. Visualization of the pulmonary artery depends on the probe placement. Aortic diameter is measured distal to the aortic valve. Frequency of the probe used to capture this image is 40 MHz.

Figure 2: Alternate view of ascending aorta on B-mode. The ascending aorta is featured more prominently and the left ventricle and heart walls are less distinct. AA: ascending aorta; LV: left ventricle. Frequency of probe used to record this image is 40 MHz.

Figure 3: Aorta visualized on M-mode. Aortic diameter is measured from the M-mode image. The movement of the aortic wall appears as two wavy lines. The space in between the two lines is the aortic lumen. Systolic and diastolic aortic diameters of three cardiac cycles are measured from the M-mode. In this image, aortic pressure recorded by the pressure catheter, ECG signal, and respiratory cycle are displayed in red, green, and yellow on the M-mode. Probe frequency used to record this image is 40 MHz and the acquisition sweep speed is 1200 Hz.

Figure 4: Modulating aortic pressure with vasoactive drugs. Aortic pressure is increased with infusion of vasoconstrictor phenylephrine (PE) and decreased with infusion of vasodilator sodium nitroprusside (SNP). Aortic pressure plateaus 1 minute after the start of the drug infusion. M-mode of the aortic diameter is recorded at the plateau. (A) shows the rise in aortic pressure with 360 µg/kg/min PE infusion. (B) shows the decrease in aortic pressure with 240 µg/kg/min SNP infusion. The time the infusion is begun and the time the M-mode is recorded are labeled on the traces.

Figure 5: Changing aortic pressure incrementally. Aortic pressure is changed incrementally by the dose of drug infused. Drug dose is modulated by the infusion rate. All doses are in µg/kg/min.

Figure 6: Diameter vs. pressure and compliance vs. mean aortic pressure plots. Aortic diameter can be plotted against its corresponding aortic pressure to show the pressure-diameter relationship (A). Compliance can be calculated for each pressure increment and plotted against the mean aortic pressure (MAP) to show the pressure dependency of aortic stiffness (B).

DISCUSSION:

Taking diameter measurements at several pressure increments over a wide range of pressure values is necessary for accurate characterization of the pressure-diameter relationship. The

upper and lower pressure limits that can be pharmacologically induced may vary by the experimental group but the ideal range is around 25 mm Hg to 125 mm Hg diastolic and 50 mm Hg to 200 mm Hg systolic. Doses of 360 µg/kg/min PE and 240 µg/kg/min SNP generally elicit the limits of the pressure range. However, doses of PE can be increased to 480 µg/kg/min and SNP to 360 µg/kg/min to verify that the limits have been reached. Working concentrations of PE and SNP can be decreased to achieve finer pressure increments. As the diameter will change with aortic pressure, inducing the same pressure values between animals and experimental groups is not important.

Venous and arterial cannulation can be performed at other locations with the same outcomes. Tail vein cannulation can be challenging because of the small size of the tail vein. Moreover, the tail vein is not readily visible in dark-colored mice. The femoral vein can be cannulated as an alternative. This route may be easier since the femoral vein is more accessible. For pressure catheter insertion, besides the femoral artery, the catheter can be inserted through the carotid. The femoral artery is preferable over the carotid artery, however, because the chest region remains intact for the ultrasound imaging. Femoral artery cannulation can be more difficult because the femoral artery is smaller. Using a 1.2F catheter and introducing the catheter in the proximal femoral artery beneath the abdominal cavity will facilitate the cannulation process. Placing a few drops of a vasodilating agent like lidocaine onto the femoral artery or using a catheter introducer can also help enlarge the vessel to facilitate catheter insertion. The pressure catheter should be handled and used according to the manufacturer's instructions.

Location of the catheter within the aorta does not need to be consistent between animals as the pressure drop within the aorta is insignificant. However, placing the catheter in the abdominal aorta may be better to minimize interference with the ultrasound imaging of the thoracic aorta. Some ultrasound mainframes can record pressure real-time with the M-mode trace, thereby giving a pressure measurement for every diameter measured on the M-mode. Unfortunately, because the location where the diameter is measured is not the same location as where pressure is recorded, a lag exists between the pressure recorded at the catheter and the actual pressure in the ascending aorta. As a result, only maximum and minimum diameter measurements can be used for the data analysis.

The primary limitation of this method is the uncertainty in measurement introduced by the aorta shifting in and out of the ultrasound plane during the cardiac cycle. Motion-introduced error is common to all imaging-based studies, including MRI and CT. Compensation strategies include using anatomical features to shift the frame of reference with the movement[18] and are implemented during data processing. As motion compensation software is not readily available, the investigator has to be vigilant about adjusting the location of the probe to track the shift in location of the aorta as blood pressure rises and decreases. Diameter measurements should also be taken through the center of the aorta. However, determining whether the M-mode recording location is passing through the center can be difficult to judge on the ultrasound image, especially with the aorta shifting positions. The uncertainty introduced by these limitations manifest in the degree of scatter in the data, as evident in **Figure 6**. Obtaining an image of the cross-section instead of longitudinal axis of the ascending aorta could be a

solution. However, obtaining this view can sometimes be more challenging and the resulting M-mode trace can be less clear. The cross-sectional circumference from the B-mode image could be measured instead of the diameter from the M-mode image. However, determining when maximum and minimum circumference has been achieved will be limited by the B-mode frame rate and may be more difficult to judge than on the M-mode.

Making multiple measurements of the pressure-diameter plot and increasing experimental group size can improve accuracy of the data. The pressure-diameter data can be collected from several locations along the chest. This protocol would first be carried out with the probe placed on one location on the chest. The aorta would then be visualized with the probe placed on another location and the protocol repeated.

Vasoactive agents used to modulate blood pressure could potentially affect aortic smooth muscle tone, which in turn would affect stiffness. However, manipulation of aortic pressure by venous return has been shown to produce similar changes in invasively measured PWV as pharmacologic manipulation in rats. These findings demonstrate that infusion of vasoactive drugs act primarily on the peripheral resistance arteries and do not significantly affect aortic smooth muscle tone[19].

This protocol can be performed in rats with a few minor modifications. The chest is shaved prior to applying depilatory cream. A commercially available 27G x ½" catheter is used for drug infusion. The drug doses used to modulate aortic pressure are 40, 80, and 120 µg/kg/min of PE and 40, 80, and 120 µg/kg/min of SNP.

Besides the ascending aorta, regional differences in aortic stiffness can be determined with this protocol. Regional stiffness measured by this approach would be more precise than by PWV as measurements are taken from one location as oppose to two locations for PWV. However, regions along the aorta that can be measured with this technique are limited to those that can be visualized by ultrasound.

Elastic modulus can also be calculated from the data collected by this method if a wall thickness measurement can be obtained. Accurate *in vivo* measurement of the mouse aorta is limited by the resolution limits of current ultrasound technology. Future improvement of ultrasound technology could make *in vivo* wall thickness measurement more feasible. As an alternative, thickness measurements can be performed *ex vivo*. Pressure myography would provide the most accurate measurements because thickness can be measured at each pressure increment.

ACKNOWLEDGEMENT:
This work was supported by a National Heart, Lung, and Blood Institute grant 1RO1-HL-105296-01 (to D.E. Berkowitz) and an Australian Research Council Grant DP110101134 (to A. Avolio).

DISCLOSURES:
The authors have nothing to disclose.

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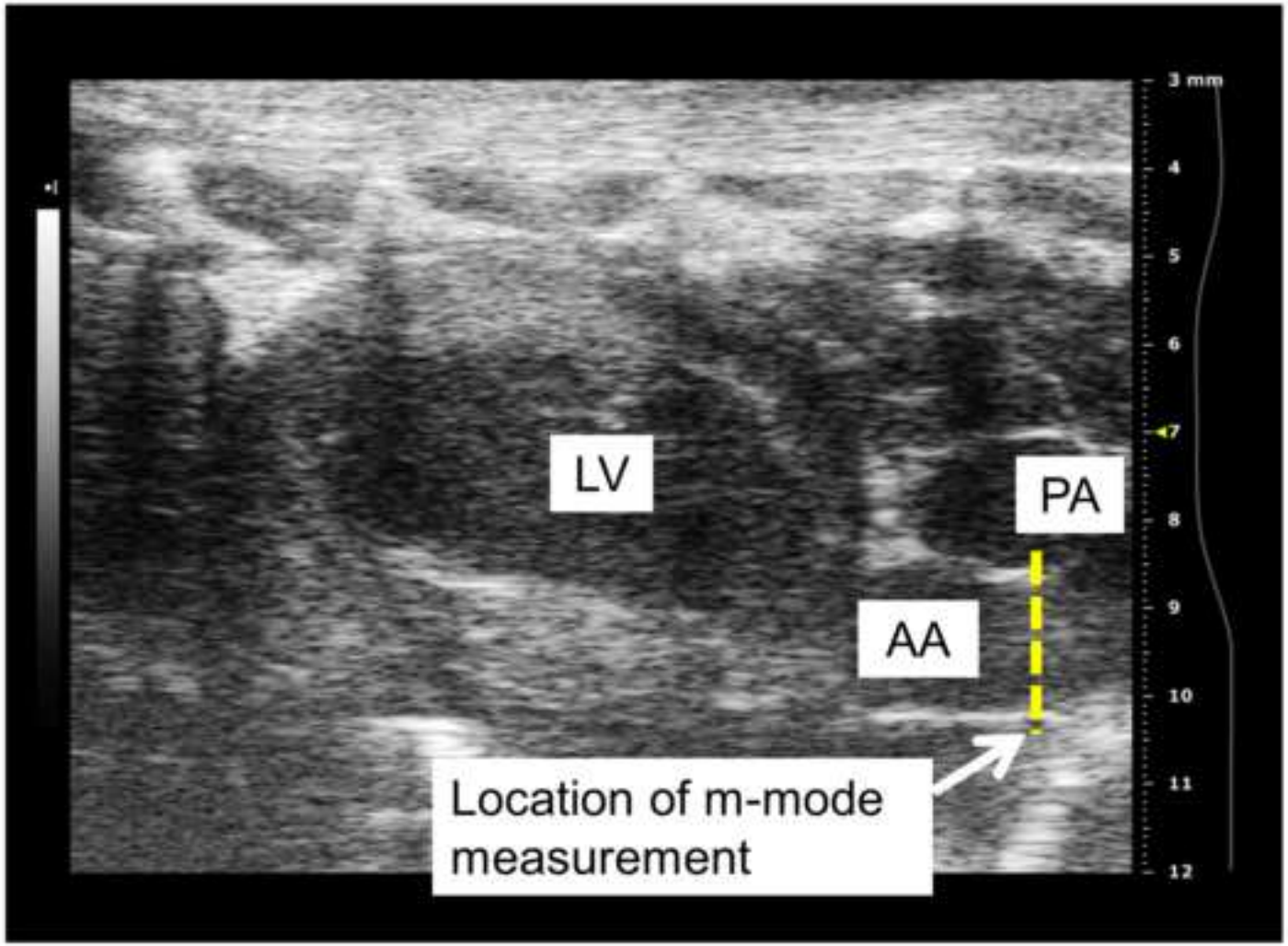

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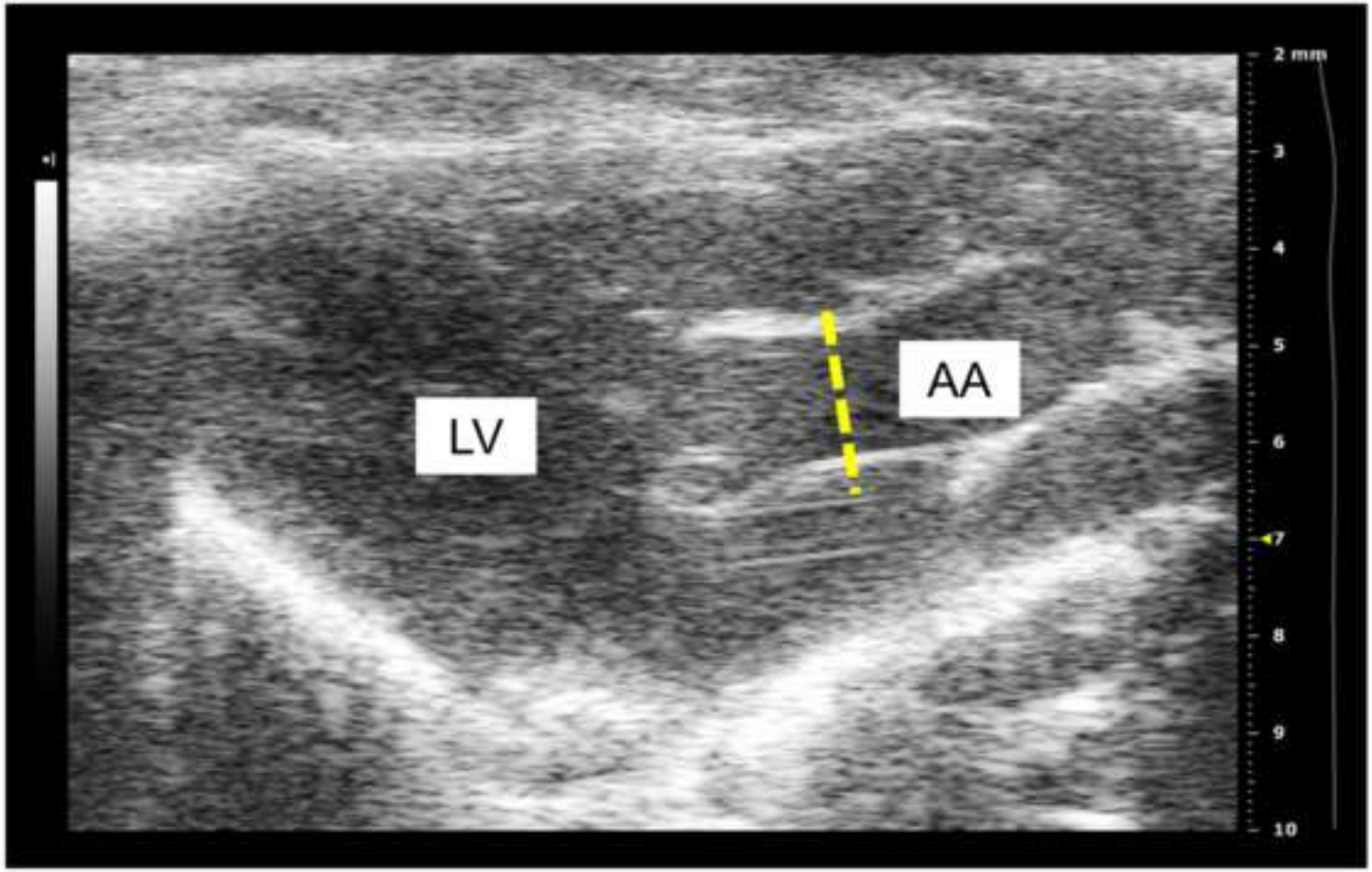

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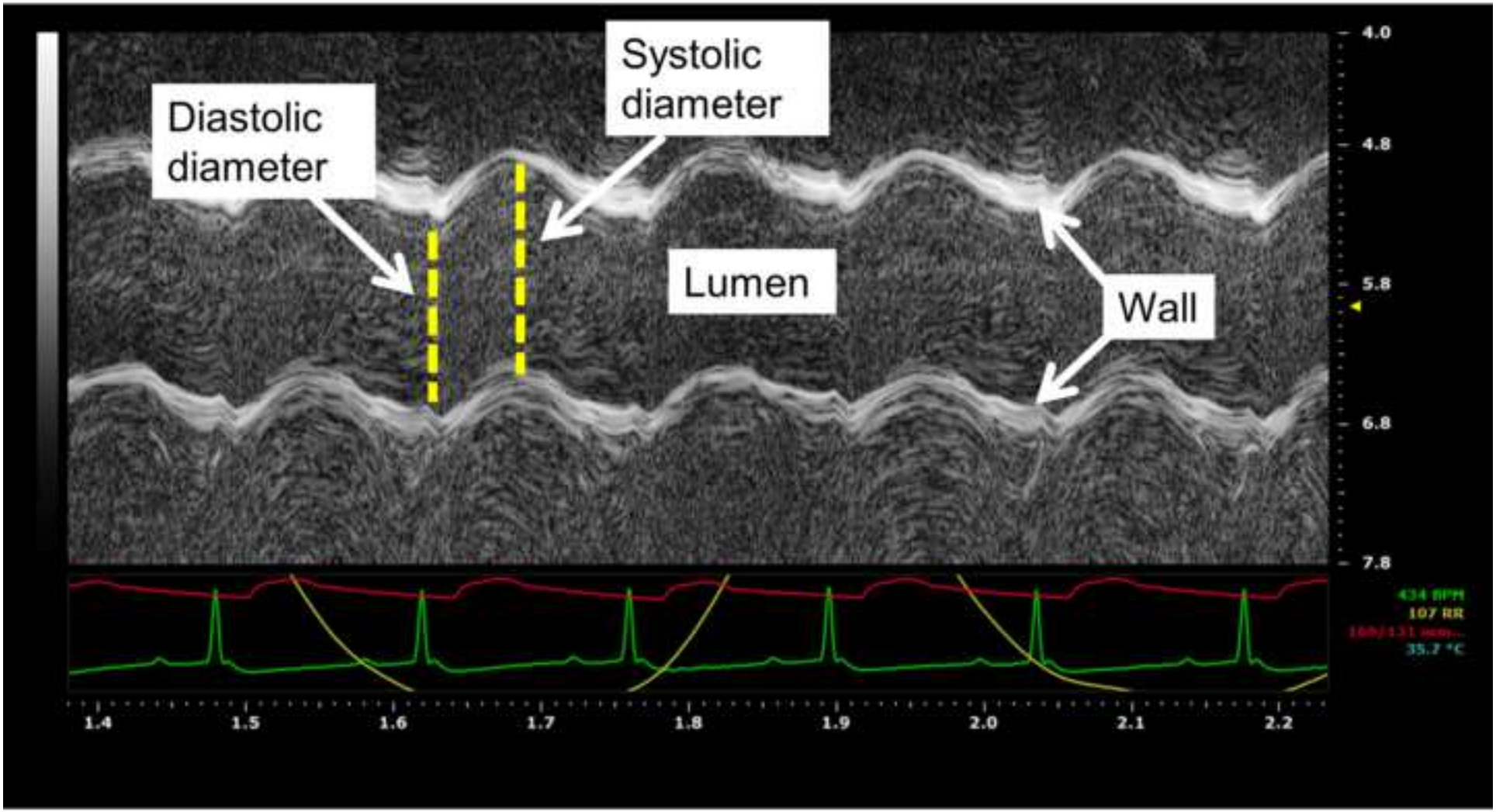

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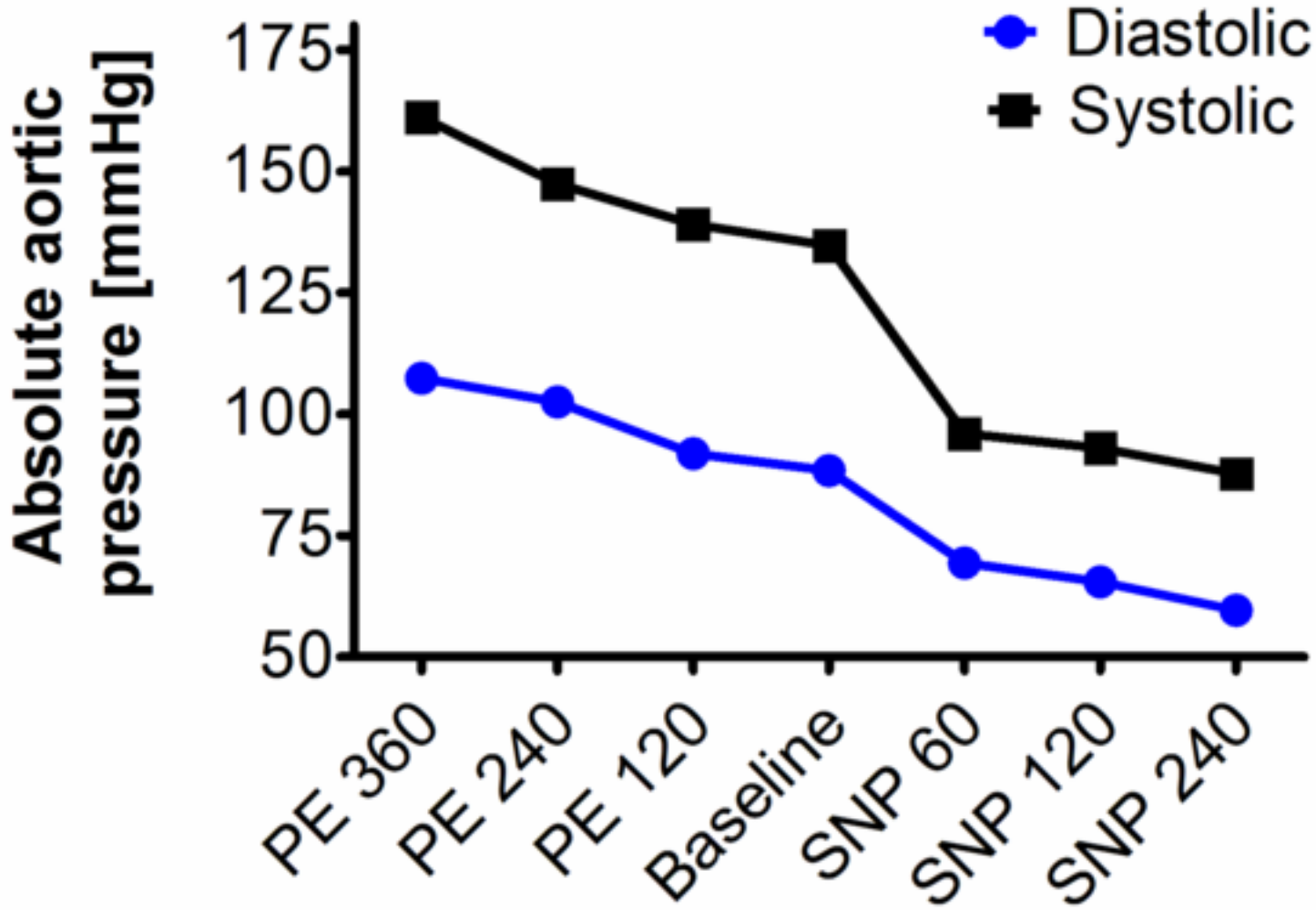

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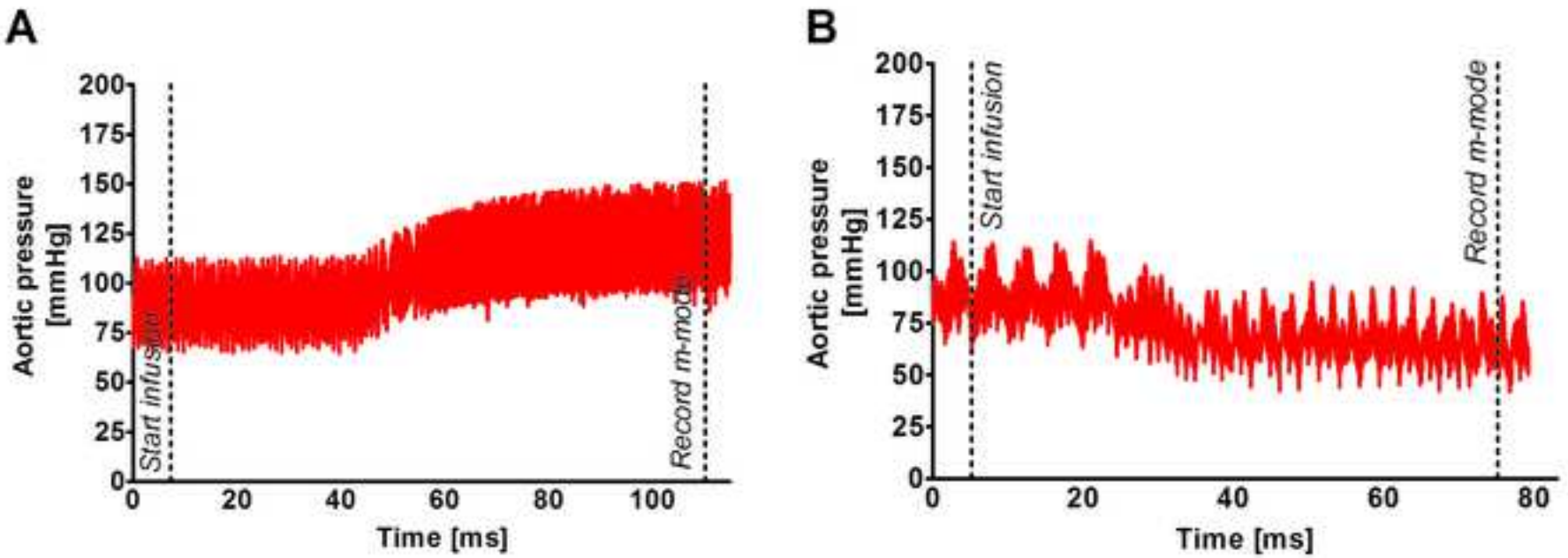

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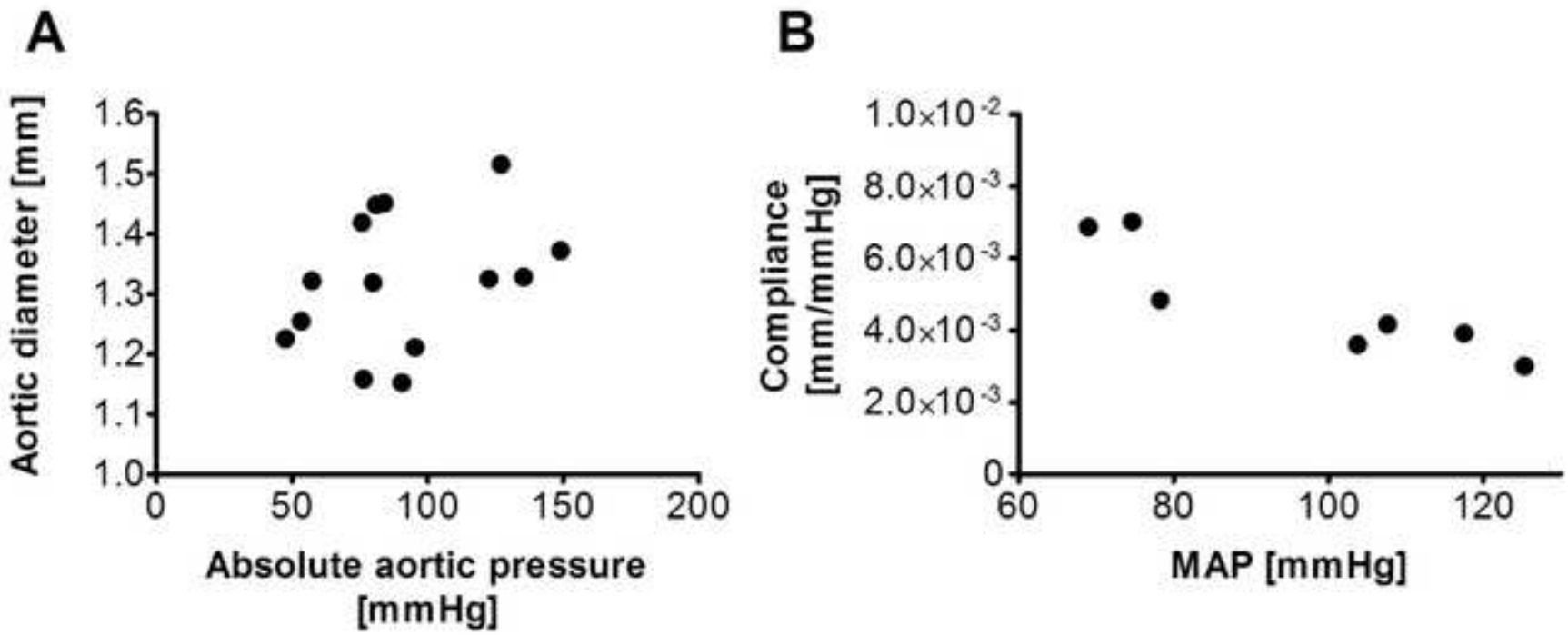

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Name of Material/ Equipment	Company	Catalog Number
Equipment		
High-resolution ultrasound machine	Visual Sonics	Vevo2100
13-24 MHz transducer	Visual Sonics	MS250
22-55 MHz transducer	Visual Sonics	MS550D
Imaging Station	Visual Sonics	Imagine Station 1
1.2F Pressure catheter	Transonic	FTH-1211B-0018
SP200 pressure control unit	Transonic	FFS-095-DP01
Standard Infusion Only Harvard Pump 11 Plus syringe pump	Harvard Apparatus	702208
Isoflurane vaporizer	VetEquip	911103
Induction chamber	VetEquip	941443
100% O2	Airgas	OX USP200
Single Stage Brass 0-50 psi General Purpose Cylinder Regulator CGA540	Airgas	Y11215B540
Stereo Boom Stand Microscope	National Optical	420-BMSQ
Fiber optic illuminator & light pipe	Cole Palmer	EW-41500-50
Supplies		
30G x 1/2" BD PrecisionGlide Needle	BD	305106
Polyethylene Tubing PE10	Becton Dickinson	427401
27Gx1/2" Surfloe winged infusion set	Terumo	SV*27EL
Signa Gel Electrode Gel	Parker	15-25
Aquasonic Clear Ultrasound Gel	Parker	03-08
1mL Sub-Q Syringes, 26G x 5/8"	BD	309597
Nair	Nair	
Histoacryl	TissueSeal	TS1050071FP
Braided Silk Suture 6-0	Teleflex	104-S
Dumostar P55 fine forceps	Roboz	RS-4984

Microscissors	WPI	501839	
Fine scissors	FST	14060-11	
Medium forceps	Ted Pella		5665
Hemostatic forceps	Roboz	RS-7131	
Non-sterile cotton gauze sponge	Fisherbrand	22-362-178	
Cotton tipped applicators	Oritan	803-WC	
Label tape	Fisherbrand	15-901-20	
Drugs			
Sodium chloride	Sigma Aldrich	S7653	
R-Phenylephrine hydrochloride	Sigma Aldrich	P6126	
Sodium nitroprusside dihydrate	Sigma Aldrich		71778
Software			
Prism	GraphPad		
Excel	Microsoft		

	Comments/Description
Used for imaging rats	
Used for imaging mice	
For tail vein cannulation in mice	
For tail vein cannulation in mice	
For tail vein cannulation in rats	
Use for ECG recording	
Use for ultrasound	
Depilatory cream	
Tissue glue	

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Author(s): Maggie M. Kuo, Dan E. Berkowitz, Lakshmi Santhanam et. al

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Department: Biomedical Engineering

Institution: Johns Hopkins University

Article Title: Measuring ascending aortic stiffness in vivo in mice using ultrasound

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Rebuttal Comments
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Dear Dr. Nguyen:

Please find attached the revised version of our manuscript titled “Measuring ascending aortic stiffness *in vivo* in mice using ultrasound.” We would like to thank the reviewers for their time and feedback which we found to be thoughtful and insightful. We have revised our manuscript according to their comments and added content in the introduction and discussion sections as suggested to improve clarity. We believe the manuscript to be greatly improved as a result.

Specifically, we included background information on the mechanical properties of the aorta. We also increased the working concentration of the drugs to reduce total volume infused and changed the infusion rates and figures to reflect the new working concentrations. We focused the data analysis section on compliance and removed the elastic modulus calculations due to concerns about the inaccuracy of the wall thickness measurements. We included additional figures (Figures 2 and 6) to improve the accessibility of this method. The figures and numbering have changed as a result. Finally, we expanded our discussion of the limitations of this method. Our response and the corresponding change to the manuscript are below each reviewer’s comment in italics. We look forward to publishing this manuscript with the *Journal of Visualized Experiments*.

Sincerely,
Maggie Kuo
Dan Berkowitz
Lakshmi Santhanam

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
In this article, an experimental protocol to measure the pressure diameter relation of the ascending aorta of mice based on ultrasound imaging and invasive pressure measurements is described. Vascular biomechanical properties are an important factor in the pathophysiology of the cardiovascular system. These material properties of the arterial wall are not easily identified in vivo because not all neccessary factors can be measured. Therefore different indices have been developed which describe the structural stiffness of arteries based on clinically available measurements. The authors dexcribe a measurement protocol in which blood pressure is raised and lowered by two drugs to measure the diameter change over a wide range of blood pressure.
The pressure diameter relation is used to calculate several indices of (local) arterial rigidity.

Major Concerns:
The presented approach adequately describes the measurement of the diameter change over a wide range of blood pressure. The experimental protocol for conducting the measurements on the animals is well described. Important mechanical background information is not clearly presented or ommitted. The presented evaluation of the measured data in part is inadequate.

1) The limitations of the proposed method are not fully discussed, however: the accuracy of the ultrasound based diameter measurements are not given (resolution of the ultrasound images). This is especially important for the determination of wall thickness. The authors identify the artifacts

introduced by rigid body movement of the ascending aorta as another source of uncertainty. This close to the heart this is especially pronounced and the measurements should be done distal to the aortic arch instead.

We agree entirely that the main limitation of this method is the measurement uncertainty introduced by the movement of the ascending aorta, particularly given its proximity to the heart.

Stiffness measurement could be performed on the abdominal aorta because it can readily visualized by ultrasound. The thoracic aorta would be more difficult due to the rib cage. However, the ascending aorta is preferable for a number of reasons. First, it is easily identifiable by its proximity to the heart, which affords methodological benefits including ease of experimentation and measurement of stiffness in the same location between different animals. Second, ascending aorta stiffness has direct implications on cardiac function and coronary circulation, making these measurements clinically relevant. We have interest in investigating coronary function and developed this method in that context. Finally, ascending aorta stiffness is measured for many of the aortic stiffness studies in patient populations. Therefore experiments in animal models using this method could be directly compared to epidemiological findings.

We have now included an example of a pressure-diameter plot and corresponding compliance-mean aortic pressure plot in the Results section (Figure 6) to better illustrate the data spread. We have also included the standard deviation between diameter measurements (line 346). We have expanded our discussion of the limitations and approaches to addressing them (lines 430-452).

2) The basics of vascular mechanics are not adequately presented, but are needed to understand the posibilities and limitations of this approach: elastic properties of the aortic wall (nonlinear elasticity, anisotropy); the different indices used to describe material (elastic modulus) and structural (compliance) stiffness.

We have substantially revised the introduction to include background on vascular mechanics. We have included descriptions of the nonlinear elastic and anisotropic properties of the aortic wall (lines 104-128), as well as a comparison between elastic modulus and compliance (lines 138-141).

3) The analysis of the measured data is confusing: (arterial) Compliance usually is calculated from the diameter difference (Dsys-Ddia) and the pulse pressure (Psys-Pdia). This compliance might be plotted versus the mean arterial pressure (MAP) to give a compliance / pressure plot which should show a nonlinear dependency of compliance on blood pressure (because of nonlinear elastic behaviour of the aortic wall). Therefore, using a linear fit on these data is not adequate.

We have revised our data analysis and reevaluated our data in terms of this definition of arterial compliance (dD / dP) to minimize confusion (lines 350-357). We present plots of diameter as it changes with pressure and compliance as it changes with mean aortic pressure in the Results section (Figure 6). The compliance plot confirms the pressure-dependency of stiffness.

We derived the equation of $C = d(Diameter)^2 / dP$ from the definition of compliance C = (change in volume) / (change in pressure). Previously, we had analyzed our data by this definition of compliance and used a linear fit because this approach is done to calculate ex vivo stiffness by pressure myography. We saw our ultrasound method as an in vivo version of the pressure myograph method.

4) The calculation of an elastic modulus is based on the (inaccurate) measurement of wall thickness and on simplifying assumptions concerning the boundary conditions of the aorta. It does not add new information over the calculation of compliance and should thus be avoided.

We appreciate this concern, which was also echoed by Reviewer 3. For the data we had included, we excised the ascending aorta and measured the thickness by microscopy. We used one thickness value in our calculations based on the assumption that wall thickness does not change appreciably with pressure. We included elastic modulus because we thought that one of the novel aspects of this method compared to the invasive PWV approach is the ability to measure stiffness indices used to characterize mechanical properties of materials. Elastic modulus calculation was intended to illustrate how the pressure-diameter data collected can be used to calculate other stiffness indices besides compliance.

We have removed the elastic modulus calculations from the Results section because of this wall thickness measurement concerns. In the Discussion section, we included suggestions on how to obtain wall thickness measurements to calculate elastic modulus should an investigator be interested or better imaging technologies or techniques come along that make accurate in vivo thickness measurements feasible (lines 472-477).

Minor Concerns:
li 130: "However, because large arteries are viscoelastic, PWV varies with arterial pressure." This variation is due to the non-linear elastic properties of the arterial wall and not the viscoelastic properties.

We thank the reviewer for this important and insightful comment. We have incorporated this. (line 165).

li 140-144: The indices presented in this approach (compliance) are indicative of the structural stiffness (not material). A major difference to PWV measurements is that PWV is averaged over the length of the aorta whereas the pressure / diameter measurement allows for locally varying measurements.

We have incorporated this distinction between material and structural stiffness (lines 138-141). We have also included the distinction between stiffness measured by PWV and stiffness measured by this method (lines 162-164 and lines 179-180).

li 146-149: PWV measurements usually rely on the "foot to foot" distance (phase shift of the end-diastolic time point of the pressure curve). This is not influenced by wave reflection.

We have reread our original explanation and agree that an occlusion would alter the pressure wave form but not the time at which it arrives. We have removed this section.

li 302 / 306: The adequate definition for arterial compliance for the measured data should use the diameter change over pressure change ($C=\Delta D/\Delta P$).

We have reinterpreted our data according to this definition (lines 131 and 352).

li 332 / Fig. 1: Please mention the ultrasound frequency used in the measurements. Add a scale bar to the image.

Ultrasound probe frequency has been added (line 367 and 375). The entire recorded image from the ultrasound mainframe has been included to improve clarity. A scale bar is on these images (Figures 1, 2, and 3).

li 336 / Fig. 2: Frequency of image acquisition (frames / s) should be mentioned.

Acquisition sweep speed information has been included (line 375).

Fig. 4: Add a title to the y-axis.

We have improved our plots and labeled the axes more explicitly (Figure 4 and 5).

li 354 / Fig. 6: Replace figure (cf. 3 above).

We have removed the elastic modulus data as suggested.

li412-416: This approach relies on using vasoconstrictors or vasodilators to (indirectly) modulate blood pressure. Changing the tone of vascular smooth muscle cells (VSMC) will significantly change the stiffness, thus limiting the use of this technique to elastic arteries. Even in elastic arteries modification of VSMC will change wall stiffness to some extent.

This is a possible limitation to this protocol. However, a study by our collaborator, A. Avolio, showed that PWV measured by changing blood pressure pharmacologically or by venous return was not different. Their findings demonstrate that the vasoactive effects of the drugs are in the resistant arteries. We have included this in our discussion (line 454-459).

Reviewer #2:
Manuscript Summary:
This study describes a methodology for assessing aortic stiffness in mice in vivo using ultrasound. Clearly, if validated, such a technique could be useful in studying aortic pulse wave velocity in mouse models in vivo. However, it still involves anaesthetising the animals and invasive insertion of a catheter into the aorta.

1. How does the aortic pulse wave velocity, measured using this technique, equate to aortic pulse wave velocity measured using the transit time methodology?

This method measured stiffness from the aorta's pressure-dimension relationship and does not measure aortic pulse wave velocity (PWV).

2. A recent study (Leloup et al, Hypertension 2014;64:ePub) has used applanation tonometry to assess pulse velocity in mice. The authors should compare and contrast their technique with that of the Leloup group.

The data collected by the tonometry technique described by Leloup et al. is the same as the data collected by non-invasively measuring PWV. The main limitation of measuring PWV non-invasively, whether by Doppler ultrasound or applanantion tonometry, is that the measurement yields a single point value of stiffness. Because PWV is influenced by blood pressure, single point PWV measurements must be normalized to mean arterial pressure to make meaningful comparisons between experimental groups.

We have now referenced this study in this manuscript and expanded our explanation on the limitations of single point measurements of PWV in the introduction (lines 160-168).

Reviewer #3:
Manuscript Summary:
This study describes a protocol by which to measure aortic stiffness in vivo that is an alternative to pulse wave velocity, which is plagued by dependence on blood pressure and heart rate. The authors should be commended for a nice study and should consider the following comments:

Major Concerns:
1. One of the major confounds of measuring vascular stiffness other than by pulse wave velocity is that many of the equations, including elastic modulus, require some input of wall thickness, which is difficult to accurately obtain by echo. The resolution of the Vevo2100 is around 25 um (1 pixel is about 25 um square), and a typical mouse aorta has a wall thickness of around 50-75 um, so measuring wall thickness in these mice at only 2-3 fold higher than the resolution seems like it would introduce a good bit of variability into the data. The authors should check the accuracy of their Vevo wall thickness measurements by collecting aortas after ultrasound measurements to determine how the aortic morphometrics compare with those measured in vivo (may require vessel myograph to pressurize vessel to similar extent as in vivo).

This is an important concern and we agree that wall thickness is difficult to measure accurately using ultrasound. Please also see our response to Reviewer 1, #4. We removed the elastic modulus calculation and focused on compliance calculation instead (lines 350 – 357 and Figure 6).

2. It may be useful to also compare elastic modulus with another clinically-used index, beta stiffness, which can be calculated from diameter and pressure measurements. Moreover, how to the elastic modulus measurements compare with those obtained by pulse wave velocity?

We have removed the elastic modulus calculation and focused the analysis on the calculation of arterial compliance.
Stiffness measured by this technique is different than PWV in that PWV is stiffness averaged over a length of aorta while this technique is local stiffness. We have made this distinction in the introduction (line 164 and lines 179-182)

3. When in vivo infusions are performed, it is best to keep the total infusion volume at/below 10% of the estimated total blood volume (at/below about 200 uL in the normal mouse) to avoid activating baroreceptor reflex systems controlling blood pressure. The authors infused about 660 uL total into each mouse, which is well above that required to elicit a central response. Admittedly, the doses of drugs infuse apparently were sufficient to overcome any central responses as evidenced by the blood pressure data. Nonetheless, there should be some effort to increase the stock concentration of Phe and SNP so that less volume can be infused into each mouse. This may also affect the BP stabilization time for each dose infused. Finally, it's best to represent the Phe and SNP doses per kg body weight (e.g. ug/kg/min).

This is an important methodological consideration, and we thank the reviewer for bringing this to our attention. We have changed the stock concentrations and infusion rates to decrease the amount of total

volume infused (lines 290 – 318). Total volume infused with this new protocol is ~100 µL. Dosing units have also been changed to µg/kg/min.

4. Protocol questions/concerns:
-1.2: Do you heparinize the saline for venous cannulation?

Heparin saline is used to prevent potential clotting in the cannula during the catheter insertion procedure, but PE and SNP are prepared in saline alone. We have included this in the protocol (line 203-204 and line 210-211).

-3.4: What size suture is used to secure the catheter?

We use braided silk 6-0 suture. We find that silk is easiest to knot securely. The materials and vendors we use are in the materials list.

-3.6: In this reviewer's experience, inserting a 1.2F pressure-tip catheter into the mouse femoral artery almost always requires the topical administration of a vasodilator such as lidocaine to make the artery sufficiently large enough to accommodate the catheter. Did the authors find this necessary? If not, the authors may consider mentioning the potential use of vasodilators to aid in catherterization. I also find that bending the bevel of a 30-Ga needle (using fine needle drivers) about 60 degrees makes a nice introducer for cannulation. Also, it is unclear how far the catheter is advanced up the aorta? Given that the mechanical measurements are made at the ascending aorta, it would be best to measure pressure closest to that point, but the pressure drop down the aorta is likely negligible. In any case, some clarification of catheter position should be provided.

We have incorporated your techniques as suggestions to facilitate femoral cannulations for other investigators (lines 416-417).

We find that lidocaine is not necessary to insert the catheter. Our experience may be because the insertion point is done quite proximally along the femoral artery. Also, the mice are anesthetized at 2% isoflurane, which has vasorelaxing properties that may also aid in the cannulation procedure. We also find that opening the membrane sheath surrounding the femoral artery-vein bundle dramatically increases the femoral artery diameter.

In general, we advance it into the abdominal aorta to leave the thoracic region clear for imaging. However, as you mention, since pressure difference along the aorta is not significant, we do not find it necessary to be exacting in the catheter placement location. We have included this clarification into the protocol (line 270) and discussion (line 420-428).

-Some commentary about catheter setup and calibration would be useful.

We have included catheter setup and calibration in the protocol (lines 240-242 and 263-265).

Minor Concerns:
1. Are the blood pressure values reported as mean arterial pressure? Please clarify in Figure 2, and provide y-axis scale bar on Figure 4.

We have labeled the pressure measurement more precisely as "mean aortic pressure' or "absolute aortic pressure" (Figure 5 and Figure 6). Y-scale axis bar has been included in the blood pressure traces (Figure 4.)

2. Methods 1.4: Saline solution is not an ointment for eyes. Do you use either ointment or saline?

We do not find it necessary to add saline solution or vet ointment. Their use was included in the protocol to follow the format of this journal.