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Invasive Hemodynamic Assessment for the Right Ventricular System and Hypoxia-induced Pulmonary Arterial Hypertension in Mice

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TITLE:

Invasive Hemodynamic Assessment for the Right Ventricular System and Hypoxia-Induced Pulmonary Arterial Hypertension in Mice

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KEYWORDS:

Pulmonary hypertension, hemodynamics, right ventricular pressure, pulmonary artery pressure, mouse, catheterization, hypoxia

SUMMARY:

Here, we present a protocol to perform an invasive hemodynamic assessment of the right ventricle and pulmonary artery in mice using an open-chest surgery approach.

ABSTRACT:

Pulmonary arterial hypertension (PAH) is a chronic and severe cardiopulmonary disorder. Mice are a popular animal model used to mimic this disease. However, the evaluation of right ventricular pressure (RVP) and pulmonary artery pressure (PAP) remains technically challenging in mice. RVP and PAP are more difficult to measure than left ventricular pressure because of the anatomical differences between the left and right heart systems. In this paper, we describe a stable right heart hemodynamic measurement method and its validation using healthy and PAH mice. This method is based on open-chest surgery and mechanical ventilation support. It is a complicated procedure compared to closed chest procedures. While a well-trained surgeon is

required for this surgery, the advantage of this procedure is that it can generate both RVP and PAP parameters at the same time, so it is a preferable procedure for the evaluation of PAH models.

INTRODUCTION:

Pulmonary arterial hypertension (PAH) is a chronic and severe cardiopulmonary disorder with elevation in pulmonary artery pressure (PAP) and right ventricular pressure (RVP) that is caused by cellular proliferation and fibrosis of small pulmonary arteries[1]. Pulmonary artery catheters, also called Swan-Ganz catheters[2], are commonly used in the clinical monitoring of RVP and PAP. Furthermore, a wireless PAP monitoring system has been used clinically[3,4,5]. To mimic the disease for study in mice, a hypoxic environment is used to simulate human clinical manifestations of PAH[6]. In the evaluation of PAP in animals, large animals are relatively easy to monitor through pulmonary artery catheters using the same technique as for human subjects, but small animals such as rats and mice are difficult to assess because of their small body size. Hemodynamic measurement of the right ventricular system in mice is possible with an ultrasmall size 1 Fr catheter[7]. A method for measuring RVP and PAP in mice has been reported in the literature[8,9], but the methodology lacks a detailed description. RVP and PAP are more challenging to measure than left ventricular pressure because of the anatomical differences between the left and right heart systems.

To get both PAP and RVP parameters in the same mouse, we describe an open-chest surgery–based approach for right heart hemodynamic measurements, its validation with healthy and PAH mice, and how to avoid generating artificial data during the complicated open-chest surgery. Although this technique is best performed by a well-trained surgeon, it has the advantage of being able to assess PAP and RVP in the same mouse.

PROTOCOL:

The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee at Fuwai Hospital, Chinese Academy of Medical Science, Peking Union Medical College (NO.0000287). The experimental animals were housed and fed according to the guidelines of animal welfare in China.

NOTE: Eight- to 12-week-old male C57BL mice were housed in an environment with a 12 h dark/ 12 h light cycle. The PAH mice were housed for 4 weeks under an oxygen concentration of 10%, maintained by an oxygen-controlled hypoxia chamber to induce pulmonary hypertension, and control mice were housed in room air (21% oxygen) under identical conditions. RVP and PAP measurements were performed at the end of the 4 weeks of hypoxia challenge.

1. Preoperative preparation

1.1. Soak the pressure transducer catheter (size: 1 Fr) in 0.9% saline at room temperature for at least 30 min before the hemodynamic experiment.

1.2. Prepare 10 mL of 1.0% digestive enzyme solution for catheter cleaning.

1.3. Connect the pressure transducer catheter to a pressure-volume system.

1.4. Calibrate the pressure transducer prior to obtaining pressure measurements for each mouse.

1.4.1. Turn the calibration knob to 0 mmHg and 25 mmHg to send a verification pressure signal to the data acquisition software and configure the calibration setting in the software.

1.4.2. Turn the knob to **Transducer** and adjust the **Balance** knob to zero baseline.

1.5. Set up a standard stereomicroscope and a temperature-controlled small animal surgical table for body temperature maintenance during the surgery.

1.6. Set up a light illumination system for microsurgery to provide enough light over the surgical area.

2. Open-Chest surgery and hemodynamic measurement

2.1. Anesthetize mice with 250 mg/kg of 2,2,2-Tribromoethanol via intraperitoneal (i.p.) injection. If needed, repeat supplemental doses at 1/3 to 1/2 of the original dose during the procedure.

2.2. Remove chest and neck fur using a shaver and hair removal lotion (**Figure 1A**, **2A**).

2.3. Secure each mouse in the supine position on a temperature-controlled small animal surgical table to help maintain body temperature (37 °C) during surgery.

2.4. Once anesthesia is in effect, confirm adequate anesthesia induction using a toe pinch.

2.5. Make a midline incision on the neck skin (**Figure 1A**).

2.6. Dissect the skeletal muscle using curved forceps and expose the trachea (**Figure 1B, 1C**).

2.7. Perform intubation through the mouth using a modified 22 G intravenous sheath catheter. Confirm that the tubing is in the trachea using forceps (**Figure 1D**).

2.8. Connect the tubing to a small animal ventilator. Calculate and set respiration rate and tidal volume based on body weight according to the ventilator user manual[10]. For example, set respiration rate to 133/min and tidal volume to 180 µL for a 30 g mouse based on the described calculation.

2.9. Secure the tubing for ventilation using tape.

2.10. Make a midline incision on the chest skin and carefully dissect the chest muscles using a cautery tool (**Figure 2B, 2C**).

2.11. Cut the sternum using scissors across the middle and expose the thoracic cavity (**Figure 2D**).

2.12. Prevent any bleeding using the cautery tool during the open-chest surgery procedure.

2.13. Expose the right ventricle with retractors (**Figure 2E**).

2.14. Insert the saline-soaked pressure transducer catheter through a small tunnel created with a 25 G needle into the right ventricle to measure RVP (**Figure 2F** and **Figure 3A, 3C**).

2.15. Hold the catheter cable and cross the pulmonary valve in a coaxial manner with the pulmonary artery. Observe the pressure waveform and obtain a stable PAP signal (**Figure 3B, 3D**).

2.16. Record hemodynamic data using the data acquisition system and software.

2.17. After the final measurements, euthanize mice humanely through i.p. injection of an excess dose of 2,2,2-Tribromoethanol solution.

2.18. Carefully remove catheter from the right heart system and place into a 1 mL syringe containing 1% digestive enzyme solution.

2.19. Use distilled water to continuously flush the catheter carefully and store it in the original box.

3. Data analysis for hemodynamics

NOTE: The hemodynamic data were recorded and analyzed using analysis software[11] (**Table of Materials**).

3.1. For each mouse, select at least 10 continuous and stable heartbeat cycles without noise to obtain the average data of RVP or PAP data for each parameter.

3.2. Use Student's *t*-test to compare the normal air control and hypoxia groups. NOTE: $p < 0.05$ was considered statistically significant. Data are presented as the mean ± SD.

REPRESENTATIVE RESULTS:

The pressure transducer catheter was inserted into the right ventricle (**Figure 3A**) through a tunnel expanded by a 25 G needle, and a typical RVP waveform (**Figure 3C**) was obtained. The catheter was continually adjusted and slowly advanced and kept in the same axis as the pulmonary artery while passing through the pulmonary valve (**Figure 3B**). When the pressure sensor was successfully inserted into the pulmonary artery, a typical PAP waveform with a

characteristic dicrotic notch appeared (**Figure 3D**). To avoid the generation of artificial data, we observed whether the waveform had noise (**Figure 4**) or whether the zero level of the catheter had drifted (**Figure 5**). If this occurred, corrections were made, and these segments with noise were excluded from data analysis.

PAH is characterized by a sustained elevation in PAP and RVP, caused by increased resistance in small pulmonary arteries. PAH is defined by a mean PAP of ≥25 mmHg at rest, measured during right heart catheterization in the clinic[12]. We measured the RVP and PAP in the mice with the induced chronic hypoxia (kept at 10% oxygen for 4 weeks) or a control group (kept in normal air). The results are shown in **Figure 6**. Compared to those in the normal air control group, systolic PAP (**Figure 6A**), diastolic PAP (**Figure 6B**), mean PAP (**Figure 6C**), and right ventricular systolic pressure (**Figure 6D**) were all significantly increased in the chronic hypoxia group. Investigators have also reported that compared with hypoxia alone, a combination of a VEGFR inhibitor with chronic hypoxia for 3 weeks to induce severe PAH in mice can result in significantly increased RVP[13,18].

FIGURE LEGENDS:

Figure 1: Intubation for mechanical ventilation support in mice. (**A**) The neck fur is removed using hair removal lotion to obtain a clean area for surgery. A midline incision is made on the skin of the neck. (**B**) The skeletal muscle covering the trachea is exposed. (**C**) The skeletal muscles are bluntly dissected to expose the trachea. The yellow arrow indicates the trachea. (**D**) The tubing (modified using a 22 G intravenous catheter) is inserted into the airway, with placement confirmed using forceps. The yellow arrow indicates the tubing inside the trachea.

Figure 2: Open-chest surgery for hemodynamic measurement at the right ventricular system. (**A**) The chest fur is removed using hair removal lotion to obtain a clean area for surgery. (**B**) A midline incision is made to expose chest skeletal muscles and the sternum. (**C**) A cautery tool is used to minimize bleeding during chest opening (the arrow indicates the cautery tip). (**D**) The sternum is cut along the midline (the yellow dash line). (**E**) Two retractors are used to expose the heart (the upper arrow indicates the right atrial wall, and the lower arrow indicates the right ventricular free wall). (**F**) A pressure transducer catheter (the lower arrow) is inserted into the right ventricular chamber using a puncture tool (25 G size needle, the upper arrow) to produce a small tunnel on the right ventricular free wall.

Figure 3: Representative RVP and PAP curves. The pressure transducer catheter is inserted into the right ventricular chamber (**A**) to obtain the RVP waveform (**C**). The pressure transducer catheter goes through the pulmonary valve and then stays in the pulmonary artery (**B**) to generate the PAP waveform. The arrows indicate the characteristic dicrotic notch on the PAP waveform (**D**), which is a sign of a pulmonary valve closure. RA = right atrium, RV = right ventricle, PA = pulmonary artery, LV = left ventricle.

Figure 4: RVP waveform noise caused by touching of the pressure sensor surface to the ventricular wall. The arrow point shows a sharp increase in pressure on the RVP curve (the upper

channel), which simultaneously produces an artificial change in dP/dt (the lower channel). dP/dt is calculated from RVP. The dashed lines indicate dP/dt noise. If the noise is constantly present, adjustment of the catheter sensor position in the ventricle can prevent noise.

Figure 5: Zero drift of pressure transducer during RVP measurement. The left window shows artificially slightly elevated end-diastolic RVP. The right expanded window shows increased end-diastolic RVP (arrows indicate end-diastolic RVP).

Figure 6: Hypoxia-induced pulmonary artery hypertension in C57BL mice. (**A**) Systolic PAP (sPAP). (**B**) Diastolic PAP (dPAP). (**C**) Mean PAP (mPAP). (**D**) Right ventricular systolic pressure (RVSP). (**E**) and (**F**), Representative PAP waveforms for control and PAH mice respectively *p < 0.05; Student's *t*-test; control group n = 10; hypoxia group n = 3. Data are presented as the mean ± SD. PAP = pulmonary artery pressure, RVP = right ventricular pressure.

DISCUSSION:

Tracheal intubation is the first important step for open-chest surgeries. The classic method of tracheal intubation for small animals, such as rats or mice, involves making a T-shaped incision on the trachea and directly inserting Y-type tracheal tubing into the trachea. In practice, we find that this method is not easy during operation. The Y-type tracheal tubing is too large for small animals and forms an angle with the trachea. Thus, it is difficult to fix the tubing in place. Additionally, once the intubation tubing accidentally comes out from the airway during open-chest surgery, it usually results in animal death because of loss of mechanical ventilation support. Therefore, we modified the method of endotracheal intubation[14] by making an incision on the skin, separating the muscle layer to expose the trachea (**Figure 1C**), and directly inserting the tracheal tubing into the airway through the animal's mouth. The placement of tubing in the trachea can be conveniently confirmed by clamping the trachea using forceps (**Figure 1D**). After removing the guide needle and only using the sheath catheter, a 22 G intravenous catheter is used as the intubation tubing. The tubing can be easily secured after intubation. This is a safe way to manage intubation during surgery and can significantly improve the success rate of small animal open-chest surgery. However, this method requires training and practice.

The closed-chest approach for right heart hemodynamic measurement has been described in detail[15,16]. One limitation of the closed-chest method is that it can be used to only evaluate RVP, because the catheter cannot access the pulmonary artery in mice. We use a midline chest incision where the right ventricular free wall is located, just below the sternum (**Figure 2D**). After right ventricular catheterization to obtain RVP, it is easy to insert the catheter in a coaxial manner with the pulmonary artery to get PAP (**Figure 2E**). When the sternum is cut during the open-chest surgery, an electrocoagulation tool is used to avoid sternal cutting section bleeding to prevent artificial blood pressure decrease caused by blood loss (**Figure 2C**). It is optional for this open-chest surgery to use a P-V loop catheter to get both RVP and volume information[16]. However, it is best to not use it to obtain PAP because of its bigger size. Although this method is best performed by a well-trained surgeon, it is preferable to the closed-chest approach because it allows for the maintenance of tracheal intubation and prevention of bleeding during open-chest surgery to avoid animal death.

Additionally, the right ventricular free wall is punctured with a 25 G or smaller needle to reduce resistance during the insertion of the catheter into the ventricle. During catheterization, the pressure sensor surface must not deviate from the bevel of the needle to prevent accidental damage to the catheter sensor by the sharp metal surface. It is preferable to not use a large needle to puncture the ventricular free wall, as it usually causes further bleeding, and the insufficient blood volume in circulation also causes artificial pressure data.

Because of the small volume of the ventricle and the irregular size of the right ventricular chamber in mice, the pressure sensor of the catheter easily touches the right ventricular free wall during the high heartbeat rate. This generates noise on the ventricular pressure curve (**Figure 4**), directly affecting ventricular pressure analysis. In this case, the angle and depth of the catheter should be adjusted until the noise disappears to obtain a smooth ventricular pressure waveform again.

The small size of the 1 Fr pressure transducer catheter[7] makes it a very precise, accurate pressure transducer. Zero drift is generally not experienced during a standard catheter test in saline solution in vitro unless the catheter is faulty or damaged. However, in the presence of body blood, blood components adhering to the pressure sensor surface may cause the catheter to undergo zero drift during an in vivo experiment (**Figure 5**). To address this issue, we do the following: temporarily remove the catheter out from the ventricular chamber and place the sensor tip of the catheter into warm 1.0% digestive enzyme solution; incubate it to digest the blood components attached to the sensor surface; and after gently wiping the catheter with saline-soaked gauze, insert the catheter back to the ventricular chamber to obtain a stable, non-zero drift ventricular pressure waveform.

The preparation of a pressure transducer catheter is also essential to obtain stable data. The pressure sensor tip of the catheter must be soaked for at least 30 min in 0.9% saline at room temperature before the in vivo procedure to maintain the stability of the catheter. In this way, the electrical characteristics of the pressure transducer catheter can be optimally stabilized.

Finally, the hypoxia period is viable from 3 to 4 weeks for the hypoxia-induced hypertension model in mice[6,13,17,18]. Our data showed that 4 weeks of hypoxia can induce a stable pulmonary hypertension model in C57BL mice, and the PAP and RVP levels are comparable with the literature. Further study is needed to address how long the PAH model can be maintained if the mice are put back in normoxic conditions for different hypoxia protocols.

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DISCLOSURES:

The authors have nothing to disclose.

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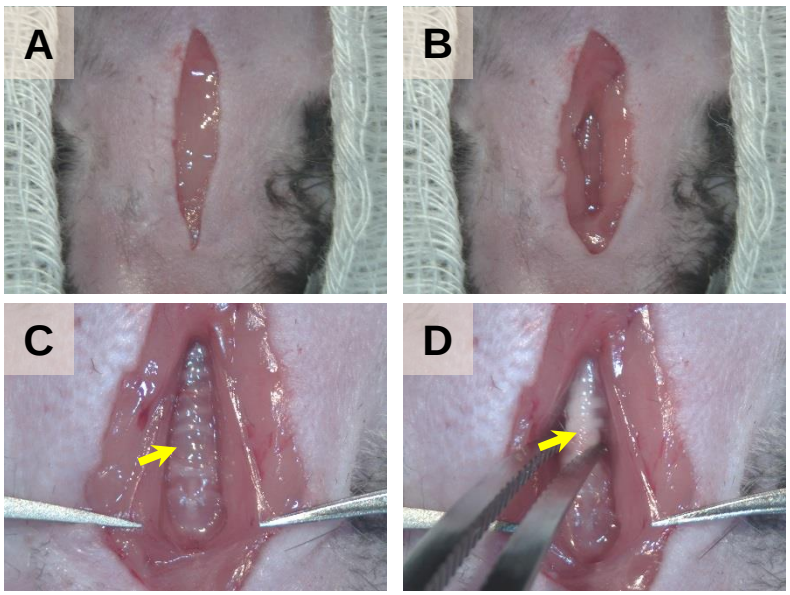
Figure 1



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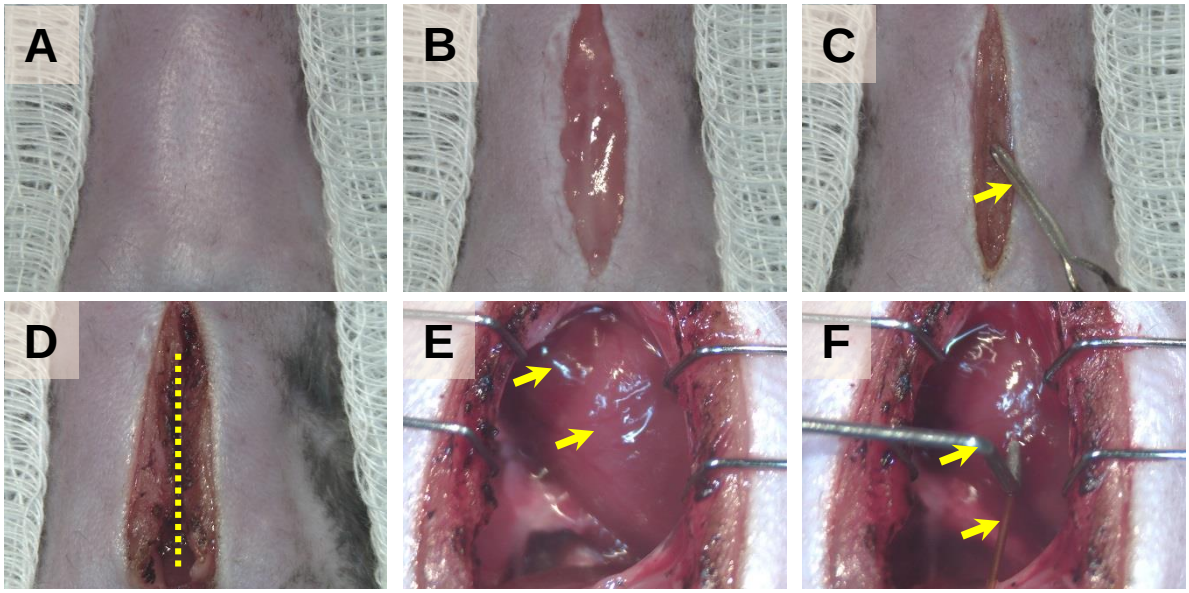

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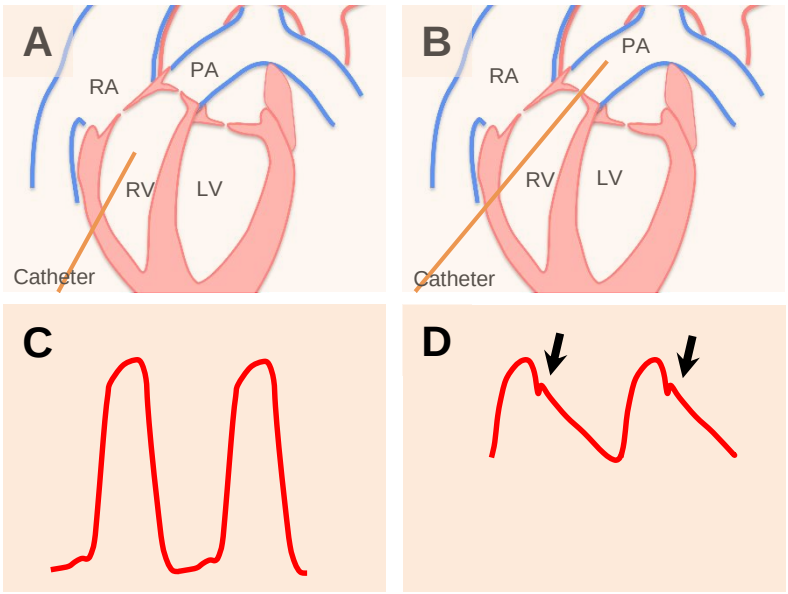

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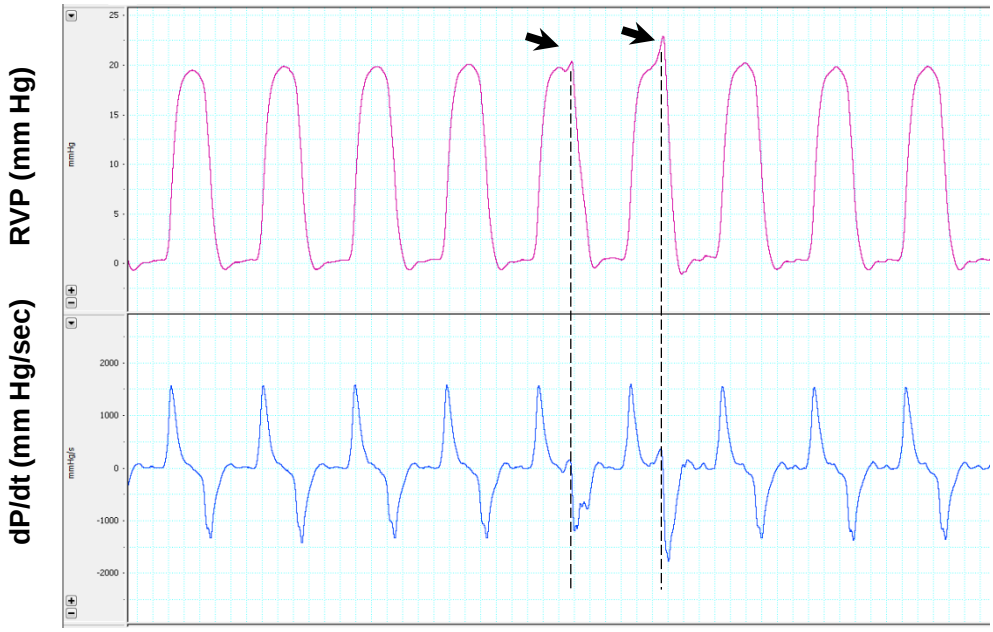

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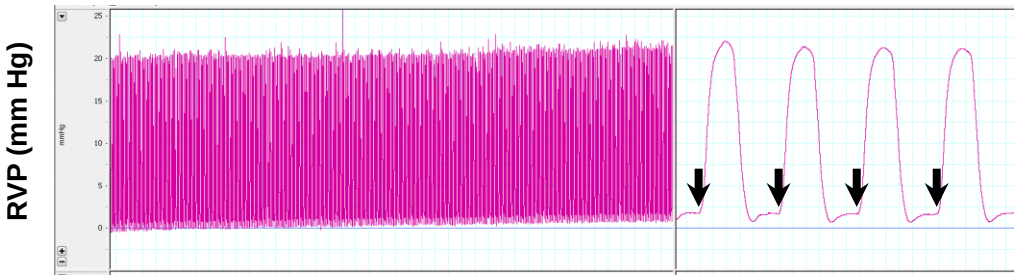
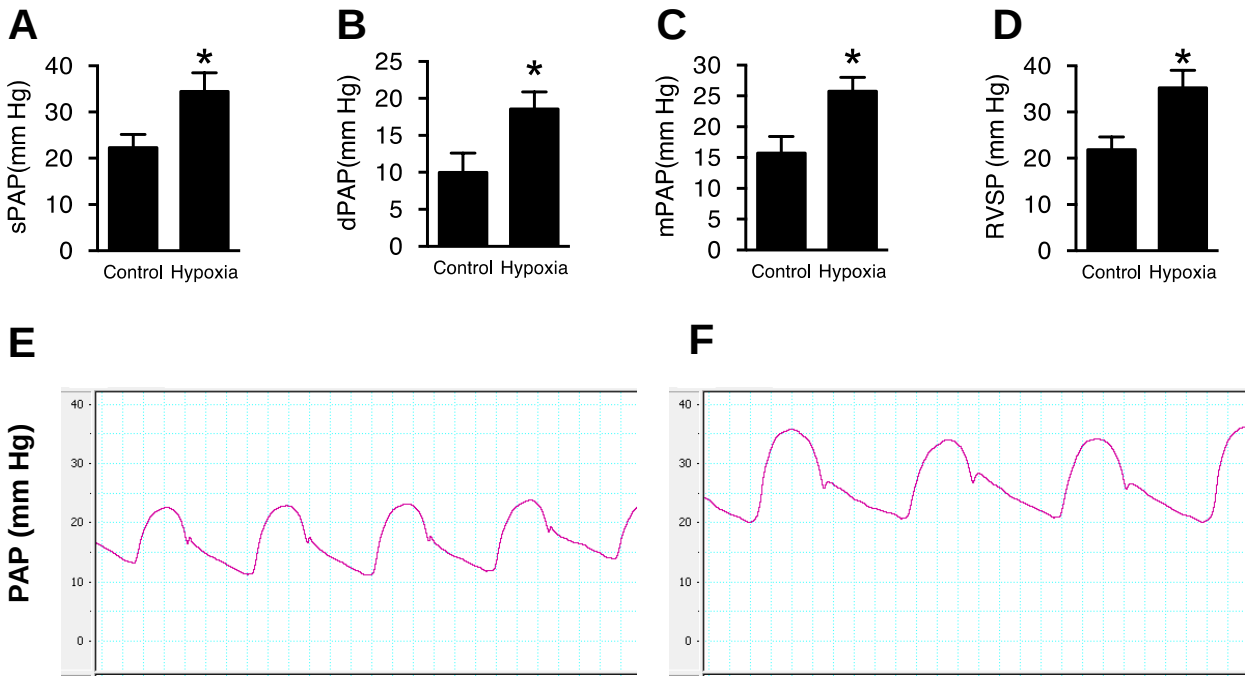


Figure 6



Name of Reagent/ Equipment	Company	Catalog Number
2,2,2-Tribromoethanol	Sigma-Aldrich	T48402-5G
Animal temperature controller	Physitemp Instruments, Inc.	TCAT-2LV
Dissection forceps	Fine Science Tools, Inc.	11274-20
Gemini Cautery System	Gemini	GEM 5917
Intravenous catheter (22G)	BD angiocath	381123
LabChart 7.3	ADInstruments	
Light illumination system	Olympus	
Mikro-Tip catheter	Millar Instruments, Houston, TX	SPR-1000
Millar Pressure-Volume Systems	Millar Instruments, Houston, TX	MVPS-300
O2 Controller and Hypoxia chamber	Biospherix	ProOx 110
PowerLab Data Acquisition System	ADInstruments	PowerLab 16/30
Scissors	Fine Science Tools, Inc.	14084-08
Small animal ventilator	Harvard Apparatus	Mini-Vent 845
Stereomicroscope	Olympus	SZ61
Surgery tape	3M	
Terg-a-zyme enzyme	Sigma-Aldrich	Z273287-1EA

Comments/Description
For anesthesia
For temperature control
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Date:	4-2-2019

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Editorial comments:

Changes to be made by the author(s):

1. The language in the manuscript is not publication grade. Please employ professional copy-editing services.

We have employed professional copy-editing services to improve the language. A native English speaker has edited the manuscript to improve the flow and readability of the text, hopefully, the new version can fit the *JoVE* standard.

2. Please revise lines 57-58, 94-96, and 150-152 to avoid previously published text.

We revised these sentences according to your suggestion.

3. Keywords: Please provide at least 6 keywords or phrases.

We added more keywords as following:

Keywords: Pulmonary hypertension, hemodynamics, right ventricular pressure, pulmonary arterial pressure, mouse, catheterization, hypoxia

4. Please add a Summary section before the Abstract section to clearly describe the protocol and its applications in complete sentences between 10−50 words: "Here, we present a protocol to …"

We added a sentence before the Summary section: “Here, we present a protocol to perform an invasive hemodynamic assessment of the right ventricle and pulmonary artery in mice using the open-chest approach. “

5. Abstract: Please expand to include an overview of the method and a summary of its advantages, limitations, and applications.

We revised the Abstract section according to your suggestion.

6. Introduction: Please revise to include all of the following:

a) A clear statement of the overall goal of this method

b) The rationale behind the development and/or use of this technique

c) The advantages over alternative techniques with applicable references to previous studies

d) A description of the context of the technique in the wider body of literature

e) Information to help readers to determine whether the method is appropriate for their application

We revised the Introduction section according to your suggestion.

7. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term

followed by “(Table of Materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Beijing Vital River Laboratory Animal Technology Co., Ltd., Millar Mikro-Tip, Terg-a-zyme, PowerLab, ADInstruments, LabChart, etc.

According to your suggestion, Millar Mikro-Tip, Terg-a-zyme, PowerLab, ADInstruments and Beijing Vital River Laboratory Animal Technology Co., Ltd are all deleted in main text and removed to the Table of Materials as needed

The LabChart is a software name, so we only keep this in the main text.

8. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “NOTE.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

We revised the Protocol section according to your suggestion.

9. Lines 80-86, 133-138: Please write the text in the imperative tense. Any text that cannot be written in the imperative tense may be added as a “NOTE”.

These sentences are related to 2 paragraphs, and they are not used to describe the surgery procedures, we checked the other published *JoVE* papers that the authors used a similar style for the description, so we ask for keeping current description.

10. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

We revised and added more details in the Protocol section to ensure viewers can replicate the protocol.

11. Line 89: Please specify the incubation temperature.

We revised the sentence as "Soak the pressure transducer catheter (SPR-1000, size: 1F) in 0.9% saline at room-temperature for at least 30 min before the hemodynamic experiment."

12. Line 91: Please describe how to calibrate the pressure transducer.

The calibration details are added to the Protocol section.

13. Line 93: Please list an approximate volume to prepare.

It is about 10 mL 1.0% digestive enzyme solution, the main text has been updated.

14. Line 96: Please specify the light source used in this protocol.

A standard light source for microsurgery was used, we have updated the information in the Table of Materials.

15. Please specify surgical tools used throughout the protocol.

We described the surgical tools used in the Protocol section and updated the tools information in the Table of Materials.

16. Line 126: Please specify the euthanasia method.

We specify the euthanasia method as “After the final measurements, euthanize mice humanely through i.p. injection of an excess dose of 2,2,2-Tribromoethanol solution.”

17. Figure 6: Please define error bars in the figure legend.

The error bars in the figure legend are defined as standard deviation in the figure legend. The figure and figure legend are all updated.

18. Please remove the titles and figure legends from the uploaded figures. Please include all the figure Legends together at the end of the Representative Results in the manuscript text.

All of titles and figure legends were all moved to the end of the Representative Results section in the main text.

19. Please upload Table of Materials to your Editorial Manager account as an .xlsx file. Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the items in alphabetical order according to the name of material/equipment.

The Table of Materials has been updated to an .xlsx file following the *JoVE* standard.

20. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please do not abbreviate journal titles. See the example below:
Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).
All the references are all formatted to fit the *JoVE* standard.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this paper, the authors established a right heart hemodynamic measurement methodology and validated with healthy and PAH mice. Authors measured only right ventricular pressures, without measuring volumes, and thus the method gives limited information about ventricular function. Even though, this is an interesting, brief, well-structured and useful manuscript. Authors focus also in some troubleshooting, which is very valuable for other researchers to implement the methodology. There are several major points requiring additional workout.

Major Concerns:

-It is not clear on which basis the protocol of 4 weeks of hypoxia was selected. Authors should give a precise justification or provide some reference that justifies their selection.

We added more references and justification in the Discussion section. Based on the published papers, hypoxia period is viable from 3 to 4 weeks to induce pulmonary hypertension in mice. We did pilot chronic hypoxia experiment in C57 wild type mice showed that 4 weeks of hypoxia could induce a stable pulmonary hypertension model.

We have added one more sentence in the Discussion section as following:

" Finally, the hypoxia period is viable from 3 to 4 weeks for the hypoxia-induced hypertension model in mice. Our data showed that 4 weeks of hypoxia can induce a stable pulmonary hypertension model in C57BL mice, the PAP and RVP level are comparable with the literature, further study need to address how long the PAH model can be maintained if put back to normoxia condition for different hypoxia protocols."

References:

1. Ciuclan L., et al. A novel murine model of severe pulmonary arterial hypertension. *American Journal of Respiratory and Critical Care Medicine*. 184 (10), 1171-1182 (2011). 3 weeks of hypoxia
2. Shatat M. A., et al. Endothelial Kruppel-like Factor 4 modulates pulmonary arterial hypertension. American Journal of Respiratory *Cell and Molecular Biology.* 50 (3), 647-653 (2014). 3 weeks of hypoxia
3. Chen M. et al. Berberine attenuates hypoxia-induced pulmonary arterial hypertension via bone morphogenetic protein and transforming growth factor-β signaling. *Journal of Cellular Physiology*. https://doi.org/10.1002/jcp.28370 (2019). 4 weeks of hypoxia

-Did the authors make any anaesthetic maintenance during the hemodynamic procedure? If yes, how? If no, why?

We have added more details about maintenance of anesthesia in the Protocol section:

"Anesthetize mice with 2,2,2-Tribromoethanol, 250 mg/kg, intraperitoneal (i.p.) injection; if needed, repeat supplemental doses at 1/3 to 1/2 of the original dose during the procedure."

-Why did the authors choose to make animal ventilation through an intubation through a "T" shaped incision on the trachea, instead of ventilation through a direct intubation trough the animal mouth? It would be less invasive.

We have revised in the first paragraph of the Discussion section to clarify as following:

“Tracheal intubation is the first important step for open chest surgeries. The classic method of tracheal intubation for small animals, such as rats or mice, involves making a "T" shaped incision on the trachea and directly inserting a "Y"-type tracheal tubing into the trachea. In practice, we find that this method is not easy during operation. The “Y”-type tracheal tubing is too large for small animals and forms an angle with the trachea; thus, it is difficult to fix the tubing in place. Additionally, once the intubation tubing accidentally comes out from airway during open chest surgery, it usually results in animal death because of loss of mechanical ventilation support. Therefore, we modified the method of endotracheal intubation by making an incision on the skin, separating the muscle layer to expose the trachea (Figure 1C), and directly inserting the tracheal tubing into the airway through the animal mouth. The placement of tubing in the trachea can be conveniently confirmed by clamping the trachea using forceps (Figure 1D). A 22 G intravenous catheter (after removing the guide needle and only using the sheath catheter) is used as the intubation tubing, and the tubing can be easily fixed after intubation. This is a safe way for management during surgery and can significantly improve the success rate of small animal open chest surgery. However, this method requires a period of training and practice.”

-How did the authors calculate the respiration rate and tidal volume?

Calculate respiration rate and tidal volume based on body weight according the ventilator user manual (on the page 16 and 17: www.harvardapparatus.com/media/harvard/pdf/Inspira_557058_9.pdf).

Page 16:

Safe Range™ Respiratory Profiles

Setting up the ASV ventilator is both easy and safe thanks to the Safe Range™ software. Once the animal's body weight has been entered the software computes the median settings for tidal volume and respiratory rate. These same equations are used to calculate a Safe Range™ around the median values. Inspira uses the entered weight to calculate the correct tidal volume and respiratory rate for the animal. The tidal volume (in liters) is determined by the equation $0.0062 \times M_b^{1.01}$ where M_b is the animal mass in kilograms.

The respiratory rate (in min^{-1}) is determined by the equation $53.5 \times M_b^{-0.26}$. The Safe Range™ is established by computing a range of acceptable values, which are less than or equal to ±10% of the calculated tidal volume and respiratory rate. All the default

Page 17:

Figure 11. Ventilator Graph

Ventilation Parameters Mammals with <70g Body Mass		
Body Mass (g)	Vt (ml)	Rate (BPM)
15	0.1	159
20	0.12	148
30	0.18	133
40	0.24	123
50	0.30	117
60	0.36	111
70	0.42	107

We have added a reference to the Discussion section as following:
www.harvardapparatus.com/media/harvard/pdf/Inspira_557058_9.pdf

-How were the animals euthanized? Which method of euthanasia did the authors used?

We have clarified this in the Protocol section as:
"After the final measurements, euthanize mice humanely through i.p. injection of an excess dose of 2,2,2-Tribromoethanol solution."

-Even for a methodological paper the animal number is too small to show representative results.

We did more control mice (n=10) for the right ventricular hemodynamic measurement to show the reproducibility of this method. However, we are very sorry that we did not add more hypoxia mice data because of the tight revision time and the preparation of animal models takes a long time.

-Authors should provide if there was any mortality during the hypoxia and, most importantly, during the hemodynamic protocol.

The C57BL mice are well tolerated to mild hypoxia (10% oxygen) for 3 to 4 weeks, the surgery mice rarely died during the procedure if maintain the mechanical support well and no severe bleeding during open chest procedure. We added one sentence to the Discussion section:

"Although this method is a safe procedure for a well-trained surgeon, the maintenance of tracheal intubation and prevention of bleeding during open chest is important to avoid animal death."

- The goals of the study are not completely new (even in jove publications: https://www.jove.com/video/53335/hemodynamic-characterization-rodent-models-pulmonary-arterial & https://www.jove.com/video/55065/shunt-surgery-right-heart-catheterization-vascular-morphometry-rat). Having this in mind, authors should better discuss and explain the important differences in their results in comparison with the previous published studies, specially concerning results in the hemodynamic evaluation and hypoxia protocol used.

We have modified the Discussion section to compare the results in RVP and PAP for PAH model.

Minor Concerns:

-Authors should provide the reference for the approval of the animal protocol.

We have added approved protocol information in the Protocol section as following:

"The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee at Fuwai Hospital, Chinese Academy of Medical Science, Peking Union Medical College (NO.0000287)."

-Authors should provide reference to the brand/model of the hypoxia chamber and oxygen sensor.

We have added O_2 controller and hypoxia chamber information to the Table of Materials.

-Authors should provide the reference for the LabChart Software.

We have added the LabChart version information also a reference website of the LabChart to the Protocol section. (LabChart: https://www.adinstruments.com/products/labchart?creative=290739105773&keyword=labchart&matchtype=e&network=g&device=c&gclid=CjwKCAjwxrzoBRBBEiwAbtX1n42I2S06KmccVncUHkmExU8KKOXXREyzx8bvTrxYMSze-ooE0atcbRoCliwQAvD_BwE.)

-Authors should actualize the most recent publications references.

We updated the most recent publications in the Introduction and Discussion section about the hypoxia protocol and hemodynamics.

-The language must be checked throughout the paper by a native speaker.

According to your suggestion, a native English speaker has helped us to edit and polish the manuscript.

Reviewer #2:

Manuscript Summary:

This is a good description of the open-chest right ventricle pressure measurement but the pulmonary artery measurements are barley mention in the discussion.

Major Concerns:

1. Removing the catheter while measuring to clean the electrode. This will cause bleeding through the hole in the RV and they don't mention how long the digestion and rinsing take. The higher the pressure the more bleeding. We always make sure my probe is clean before inserting and when we finish a measurement we wipe it gently with a wet gauze before returning it to the saline beaker. This should remove any protein or debris that it has picked up while reading and pulling it out of the ventricle.

We agreed with your point. If the measurement can be done correctly so it is not necessary to remove the catheter for cleaning, at least it might cause minor bleeding although a cotton-tip can prevent the bleeding.

While we trained the beginners such as young residents or fellows to do this open-chest procedure, we noticed that they struggled for the catheterization and the maintenance of the catheter in the ventricle, it took much longer time for the measurement, so it usually causes the catheter zero-drifting. In this case, to avoid to generate artificial data, we have to take the catheter out of ventricle to clean the blood components attached to the sensor. Soak the catheter with warm 1.0%

digestive enzyme solution, gently wipe the catheter with saline-soaked gauze; it is beneficial to remove blood components from the catheter. If soaking in the enzyme in room-temperature (21-24 degree), it usually takes about 15 min for digestion.

We have revised the main text in the Discussion section to clarify this concern.

2. No figure showing an actual pressure tracing when the probe is in the pulmonary artery.

We updated Figure 6 according to your suggestion. The representative pulmonary artery pressure curves for control and PAH mice are shown in Fig 6E and 6F respectively.

3. In the Protocol (Lines 117-120) they say they make a tunnel with the 25 G needle and then put the catheter in through the tunnel but in the discussion (Lines 188-193) the implication is that they leave the needle in and put the catheter through it. If they are leaving the needle in it can cause major problems such as shorting out the probe if the contacts touch the metal of the needle. We make the tunnel, remove the needle and push the probe through the tunnel left by the needle.

We agreed with your point. We prefer the procedure you mentioned above for the safety of the catheter.

When we trained the beginners to do catheterization, it was difficult for them to insert the catheter through the pre-punctured tunnel on the beating heart, so as an option we modified the method to insert the catheter during the needle returning from the ventricular wall. It is helpful for the catheterization, but we should always use a small size needle no more than 25 G to puncture a tunnel, the bigger size needle not only increases the risk of damage catheter but also causes more bleeding.

We did minor revision in the Discussion section.

Minor Concerns:

1. Correcting the zero during a recording is something that we do not do. We zero the catheter at the beginning of the run only.

We agreed that do not correct the zero during recording.

If the catheter zero-drifting does appear during measurement, it means that the data recorded may be artificial, take out the catheter from the ventricle and recheck the zero, then try to do the measurement again.

2. How much blood flow is there once the catheter is introduced into the PA? We know they are using the 1F catheter, but it seems like it would be almost the same diameter as the PA and would impede flow through the valve. It seems that in smaller mice, the catheter would take up the diameter of the PA. We don't do

the closed chest method on mice less that 16 grams and prefer them to be at least 20 grams for the 1.4F probe.

We agreed that it is difficult to hemodynamics in mice less than 16 gram; the mice we used in this experiment are all over 24 gram, so they are big enough to do the measurement.

The catheter size is 1F; it is about 0.33 mm in diameter, considering of the pulmonary artery diameter is about 1.3-1.5 mm in adult mice, the catheter should not significantly affect the pulmonary blood flow during the catheter inserted into the artery. We did not measure the pulmonary blood flow during the pulmonary artery catheterization procedure, so we don't have the data currently to show pulmonary blood flow on this condition.

3. Using 1% Terg-a-zyme is very hard on the electrodes of the catheter. We usually just wipe ours down with a wet (water) gauze after each mouse, before returning the probe to the beaker of saline and again after the experiment before putting the catheter away to prevent salt build up.

We agreed that the Terg-a-zyme might be tricky, we noticed that soak the catheter with warm 1.0% digestive enzyme solution, gently wipe the catheter with saline-soaked gauze, it is beneficial to remove blood components from the catheter, then put the catheter into normal saline until next measurement.

4. Should mention the size of the Millar catheter in the text not just in the materials list.

We added the pressure transducer catheter size in the main text (the Protocol section); the catheter size is 1F in diameter.

5. They didn't discuss it but in Figure 6 they show the systolic pressure in the right ventricle and the systolic pressures in the pulmonary artery and they appear to be the same. They don't mention the variability in pressure between mice which is fairly high with this model and usually requires larger numbers to achieve significance. Are the error bars standard deviation or standard error of the mean?

The RVSP and sPAP are comparable in Figure 6. These data are comparable with references, we did not add more sentences to discuss.

The new Figure 6 is updated, we did more control mice to increase N number (N=10), and the error bars in Figure 6 are all changed to standard deviation.

About the variability such as in systolic pulmonary artery pressure (sPAP) between mice, it is about 17.4 to 25.8 mmHg for room air control mice, and 30.1 to 38.4 for pulmonary hypertension mice. Also, we noticed that while we trained the young residents or fellows to do chronic hypoxia, sometimes failed to maintain a consistent 10% O_2 level in the hypoxia chamber, it might cause significant variation in hypoxia mice, and some mice may not develop to pulmonary hypertension only developed a mild elevation of pulmonary artery pressure. Thus,

for the formal hypoxia experiment, we monitored the hypoxia chamber oxygen level in person at least twice a day, to confirm that the hypoxia process could generate a consistent 10% O_2 level in the hypoxia chamber. In this way, the hypoxia mice can generate a significant increased pulmonary artery pressure and acceptable variation compare to room air control mice.

6. What is the mortality rate with this procedure? The numbers of mice are very small in this experiment. Also they don't mention the variability in pressure between mice which is fairly high with this model and usually requires larger numbers to achieve significance.

About the mortality of this open-chest procedure, it depends on the surgeon. For a well-trained surgeon, animal rarely died during the open-chest surgery if mice could tolerate anesthesia challenge. During the training period, when the young residents or fellows do such open-chest surgery in small mice, sometimes failed for ventilator support such as intubation tubing failure can cause animal die.

We did more control mice (n=10) for the right ventricular hemodynamic measurement to show the reproducibility of this method. However, we are very sorry that we did not add more hypoxia mice data because of the tight revision time and the preparation of animal models takes a long time.

About the variability, we answered the question in question 5.

7. Should mention size of the catheter in the Protocol section.

We added the pressure transducer catheter size in the Protocol section, it is a SPR-1000 catheter, 1F.