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TITLE:

Generation and Characterization of Right Ventricular Myocardial Infarction Induced by Permanent Ligation of the Right Coronary Artery in Mice

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SUMMARY:

There are several differences between the right and left ventricles. However, the pathophysiology of right ventricular infarction (RVI) has not been clarified. In the present protocol, a reproducible method for RVI mouse model generation is introduced, which may provide a means to explain the mechanism of RVI.

ABSTRACT:

Right ventricular infarction (RVI) is a common presentation in clinical practice. Severe RVI can lead to fatal hemodynamic dysfunction and arrhythmia. In contrast to the extensively used mouse myocardial infarction (MI) model generated by left coronary artery ligation, the RVI mouse model is rarely employed due to the difficulty associated with model generation. Research on the mechanisms and treatment of RVI-induced RV remodeling and dysfunction requires animal models to mimic the pathophysiology of RVI in patients. This study introduces a feasible procedure for RVI model generation in C57BL/6J mice. Further, this model was characterized based on the following: infarct size evaluation at 24 h after MI, assessment of cardiac remodeling

and function with echocardiography, RV hemodynamics assessment, and histology of the infarct zone at 4 weeks after RVI. In addition, a coronary vasculature cast was performed to observe the coronary arterial arrangement in RV. This mouse model of RVI would facilitate the research mechanisms of right heart failure and seek new therapeutic targets of RV remodeling.

INTRODUCTION:

The right ventricle (RV), long thought to be a simple tube connected to the pulmonary artery, has been wrongfully neglected for many years[1]. However, there has been an increasing interest in RV function recently since it plays an essential role in hemodynamic disorders[2,3] and may serve as an independent risk predictor of cardiovascular disease[4-7]. RV diseases include RV infarction (RVI), pulmonary artery hypertension, and valvular disease[8]. In contrast to the immense interest in pulmonary artery hypertension, RVI has remained neglected[7,9].

RVI, usually accompanied by inferior-posterior myocardial infarction[10,11], is caused by right coronary artery (RCA) occlusion. According to clinical investigations, severe RVI likely induces hemodynamic disturbances and arrhythmias, such as hypotension, bradycardia, and atrioventricular block, associated with higher hospital morbidity and mortality[12-14]. RV function could recover spontaneously to a certain extent even in the absence of reperfusion[15,16]. Several morphological and functional differences exist between the left ventricle (LV) and RV[17]. RV is believed to be more resistant to ischemia than LV[8], partially due to the more extensive collateral circulation formation after RVI. Clarifying the differences between LV infarction (LVI) and RVI and identifying the underlying mechanisms would provide new therapeutic targets for cardiac regeneration and ischemic heart failure. However, owing to the difficulty of RVI mouse model generation, basic research on RVI is mainly limited.

A large animal model of RVI has been generated by ligating RCA in swine[18]**,** which is easier to operate because of the visible RCA. Compared with the large animal model, the mouse model has the following advantages: more accessibility in gene manipulation, lower economic cost, and shorter experimental period[19,20]. Although a mouse RVI model focusing on the influence of RVI on LV function was reported previously, the detailed steps of the procedure, the difficulties and key points of operation, and the model characteristics such as hemodynamic changes were not fully introduced[9,21].

This article provides detailed surgical procedures for generating a mouse model of RVI. Moreover, this model was characterized by echocardiographic measurement, invasive hemodynamic evaluation, and histological analysis. Furthermore, a coronary vasculature cast was performed to observe the coronary arterial arrangement in RV. The technique introduced in this paper would facilitate the junior learners to quickly grasp the generation of the mouse RVI model with acceptable operation mortality and reliable evaluation approaches. The mouse model of RVI would help research the mechanisms of right heart failure and seek new therapeutic targets of RV remodeling.

PROTOCOL:

All procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised in 1996). Male C57BL/6J mice (8-10 weeks old; bodyweight, 25–30 g) were obtained from the Animal Center of Southern Medical University. After arrival, the mice were housed under a 12-h/12-h dark/light cycle, with ad libitum food and water.

1. Preparation for surgery

1.1. Sterilize surgical instruments by autoclaving and soaking them in 75% ethanol before the surgery. Adjust the heating pad to 37 °C.

1.2. Anesthetize the mice by an intraperitoneal injection of 50 mg/kg of pentobarbital (see **Table of Materials**) to relieve surgical pain. Place the mice in separate boxes for anesthesia induction. Ensure the depth of anesthesia by the disappearance of the toe-retreat reflex.

NOTE: It is also recommended to use 1.5% isoflurane for inhalation anesthesia because it is better for analgesia.

1.3. Place the mice supine on the pad by fixing their incisors with a suture and immobilizing their limbs with adhesive tape. Ensure the depth of anesthesia again by checking the reflex.

1.4. Remove hair from the neck to xiphoid with a depilatory cream. Disinfect the surgical area with 75% alcohol.

1.5. Perform intubation following the steps below.

1.5.1. Adjust the breathing frequency of the animal with a mini ventilator (see **Table of Materials**) to 150/min and the tidal volume to 300 µL.

NOTE: It is unnecessary to use positive end-expiratory pressure mode.

1.5.2. Pull out the tongue slightly with tweezers, lift the mandible with a tongue depressor to expose the glottis, and insert a laboratory tracheal with a 22 G cannula into the glottis.

1.5.3. Switch on the mini ventilator and connect the tracheal cannula to the ventilator. The phenomenon of thoracic undulation becoming equal to the ventilator frequency indicates successful intubation. Fix the cannula with tape to prevent slipping during the operation.

2. Permanent ligation of the right coronary artery

2.1. Connect the electrocardiography (ECG) electrodes (see **Table of Materials**) to the mouse limbs correctly and record the ECG.

NOTE: One of the II, III, or AVF lead is selected as a monitoring lead; Lead III is more appropriate.

2.2. Open the chest.

2.2.1. Make a 1 cm long incision in the skin parallel to the third right rib with ophthalmic scissors. Determine the third rib gap again and ensure adequate intercostal space according to the sternum angle.

NOTE: The direction of the skin incision is made from the sternum angle to the right anterior axillary line.

2.2.2. Separate and cut the pectoralis major and pectoralis minor muscles with scissors and micro forceps above the third intercostal space. After that, bluntly separate the intercostal muscle with elbow forceps to expose the surgical field.

NOTE: Only a small part of pectoral muscles needs to be cut, and then a blunt separation is recommended to expose the heart.

2.2.3. Tear the pericardium apart. Lift the right atrium with cotton and ligate the RCA with an 8-0 nylon thread with a ligation range of 3–5 mm. After ligating the RCA, the monitoring ECG (lead III) shows ST-segment elevation.

NOTE: Because the mouse RCA is invisible, its anatomic location must be carefully confirmed. The myocardium of the RV is much thinner than that of the LV. Therefore, it is difficult to grasp the depth of the inserted needle. It is easy to induce sinus bradycardia and atrioventricular block if the depth of the inserted needle is too deep and the ligation range is too large.

2.3. Suture muscles and skin with 5-0 nylon thread and disinfect the skin again with 75% alcohol.

NOTE: Analgesics such as buprenorphine (0.1 mg/kg body weight, subcutaneously injection) are recommended to reduce the animals' pain after the surgery.

3. Echocardiographic assessment of the RV function after surgery

NOTE: For echocardiography, use an MS400D probe with a center frequency of 30 MHz, connected to a high-resolution ultrasound imaging system (see **Table of Materials**).

3.1. Anesthetize the mouse with 3% isoflurane *via* inhalation.

3.2. Place the mouse in the supine position on an ultrasonic platform for animal fixation and ultrasonic operation. Tape its claws to the electrode to obtain an ECG recording through a system attached to the ultrasonic machine.

3.3. Monitor heart rate through ECG and maintain it between 450-550 beats/min by adjusting the anesthetic concentration between 1.5% and 3%.

3.4. Remove the hair from the mouse's chest with a depilatory cream and apply ultrasound gel to the skin of the chest.

3.5. Set the platform to the horizontal position. Orient the transducer parallel to the left leg and obtain the left ventricular long-axis image. Rotate the probe 90° clockwise to obtain the LV short-axis view. Press the **Cine store** button to save the images.

NOTE: The upper-left of the platform is tilted at the lowest point. The LV short-axis rotation angle of the transducer is maintained while the transducer is oriented toward the right shoulder of the mouse.

3.6. Move down the transducer vertically, maintaining its position over the upper abdomen and below the mouse's diaphragm under **B-mode**. Adjust the platform position slightly by rotating its x- and y-axes until the RV, right atrium (RA), left atrium (LA), and LV are clearly seen on the screen. Save apical four-chamber images by pressing the **Cine store** or **Frame store** button.

NOTE: B-mode is used to show the two-dimension (2D) view of the heart.

3.7. Press **M-mode**; after the 2x indicator line appears, locate the indicator line at the tricuspid valve orifice to obtain the movement of the tricuspid annular plane. Press the **Cine store** or **Frame store** button to save data and images.

NOTE: M-mode means motion mode, which reveals the motion of the heart or vessel in a curve form.

3.8. Press **Measure** button to enter measurement mode. Click on **Area measurement** button to zone into RV and LV. Calculate the area of RV and LV to obtain the area ratio of RV to LV.

3.8.1. Click on **Timeline** button and make two baselines to define the movement range of the tricuspid annular plane during the systolic and diastolic periods. Click on **Distance** button and measure the distance between two baselines to obtain tricuspid annular plane systolic excursion (TAPSE).

3.9. Tilt the left side of the platform at the lowest point. Keep the probe at a 30° angle to the horizontal axis along the right anterior axillary line. Rotate the x- and y-axes of the platform to display the RV.

3.9.1. Press **M-mode** button and locate the indicator line at the septum's hyperechoic point to obtain the M-mode image of the RV interface. Press **Cine store** button to save the picture.

3.10. Open the M-mode image of the RV interface, press **Measure** button to enter measurement mode. Measure the RV inner distance at the end of diastole (RVIDd), RV ejection fraction (RVEF), and RV fraction shortening (RVFS) using the in-built measurement tool of the echocardiographic system.

3.11. Stop administering isoflurane and place the mouse on the heating pad for 3-5 min until it regains consciousness. After that, return the mouse to its cage with 12 h light/dark cycle.

4. Invasive measurements of RV hemodynamic

NOTE: RV hemodynamic is assessed through right heart catheterization 4 weeks after RVI. A 1.0 F catheter together with a monitoring system is applied.

4.1. Anesthetize the mouse with an intraperitoneal injection of 50 mg/kg of sodium pentobarbital (see **Table of Materials**).

4.2. After confirming the disappearance of the pedal withdrawal reflex, keep the mouse in the supine position and immobilize it with adhesive tape.

4.3. Shave the chest hair from the sternal angle to the xiphoid. Disinfect the operating area with 75% alcohol.

4.4. Perform tracheal intubation and set the parameter of the animal ventilator as described in steps 1.5.2-1.5.3.

4.5. Make a 1 cm bilateral incision on the skin above the xiphoid process and transect the diaphragm and rib with ophthalmic scissors to expose the heart.

4.6. Puncture the right ventricular free wall with a 32 G needle. Remove the needle and press the wound with cotton to stanch bleeding.

4.7. Insert the tip of the catheter into the right ventricle through the puncture site and push the catheter forward slowly. Adjust the position of the tip to obtain a typical RV pressure waveform

shown on a monitor and recording system.

NOTE: Right jugular vein is also an appropriate route for hemodynamic measurement.

4.8. After 10 min of stabilization, record the data of RV systolic blood pressure (RVSBP), RV end-diastolic pressure (RVEDP), and RV dP/dt. Click on **Select** button to select cardiac cycles for calculation and then click on **Analyze** button to calculate the mean values of the selected cycles.

4.9. Remove the catheter after completion of recording and then place it inside Normal saline solution.

4.10. Euthanize the mouse with an intraperitoneal injection of overdose pentobarbital sodium (150 mg/kg) and then sacrifice it by cervical dislocation.

4.11. Collect the heart and tibia for histological analysis.

5. Coronary vascular cast using a vascular casting agent

5.1. Heparinize the mouse with an intraperitoneal injection of 200 IU/mL of heparin sodium at 2000 IU/kg (see **Table of Materials**).

5.2. Anesthetize the mouse with an intraperitoneal injection of 50 mg/kg of sodium pentobarbital.

5.3. Place the animal supine on the pad and intubate for artificial ventilation following steps 1.5.2-1.5.3.

5.4. Open the chest with surgical scissors as described in step 4.5 and expose the heart.

5.5. Make a 3 mm notch with ophthalmic scissors on the right atria and perfuse the heart with 5 mL of normal saline through the cardiac apex with an injector.

5.6. Block the blood from the aorta with an aortic clamp and perfuse 0.1 mL of nitroglycerin (1 mg/mL) through the cardiac apex with an injector to dilate the coronary artery.

5.7. Prepare the cast reagent by mixing the ingredients in the kit according to the manufacturer's instructions (see **Table of Materials**).

NOTE: It is recommended to prepare the cast reagent and perfusion with normal saline and nitroglycerin for simultaneous preparation and to prevent microvessel closure.

5.8. Perfuse the heart with 1 mL of cast reagent through the cardiac apex and wait for 2–3 h.

5.9. Erode the heart with 50% sodium hydroxide for 2–3 days and remove the muscle tissue or connective tissue by rinsing with normal saline.

5.10. Take pictures under a camera.

CAUTION: The cast reagent is harmful to the eyes, skin, and respiratory tract. Sodium hydroxide is corrosive. Wearing protective gloves, goggles, and a lab coat is recommended. The cast reagent must be prepared in a fume hood.

REPRESENTATIVE RESULTS:

In this study, mice were randomly assigned to the RVI (n = 11) or sham operation (n = 11) group. The coronary cast in 2 normal mouse hearts is shown in **Figure 1A**. In response to RCA ligation, ST-segment elevation was seen in lead III of the ECG (**Figure 1B**). Moreover, 2,3,5-triphenyl tetrazolium chloride (TTC) staining showed that the infarct area accounts for 45% of the RV free wall at 24 h postoperatively (**Figure 1C,D**). The above data indicated the successful generation of the RVI mouse model.

Recordings of the 4-chamber apex view (**Figure 2A**) and 2-chamber view at LV short axis and the corresponding M-mode echocardiography (**Figure 2B**) measurements were performed at 4 weeks after the surgery to evaluate the RV remodeling and function. Compared with that in the sham group, the RV internal dimension at the end of diastole (RVIDd) increased in the RVI group (**Figure 2C**), and it was more than 2 times in the sham group (**Figure 2A**). RV ejection fraction (RVEF), RV fraction shortening (RVFS), and tricuspid annular plane systolic excursion (TAPSE) were significantly smaller in the RVI group than in the sham group (**Figure 2D-F**). The RV/LV area ratio increased by approximately 50% relative to the sham group (**Figure 2G**).

The mice were subjected to RV hemodynamic measurement 4 weeks after surgery. In the RVI group, RVSBP, dp/dt max, dp/dt min, and RV contractility were significantly smaller. At the same time, RVEDP and τ index were considerably more significant than those in the sham group (**Figure 3A-E**).

Four weeks after the surgery, the mice were sacrificed. An RV aneurysm was visible in the infarcted area (**Figure 4A**). The heart weight to body weight (HW/BW) ratio and heart weight to tibia length (HW/TL) ratio in the RVI group were slightly larger (without statistical significance) than those in the sham group (**Figure 4B,C**). Masson staining[22] indicated significant fibrosis in the RV-free wall, and seldomly fibrosis occurred in the septum in the RVI group (**Figure 4D,E**). In contrast, a few surviving cardiomyocytes were in the infarct area (**Figure 4F**).

FIGURE LEGENDS:

Figure 1: Electrocardiography (ECG) changes and infarct size after ligation of the right coronary artery (RCA). (**A**) Representative images of mouse coronary vascular cast. Scale bar = 4 mm. (**B**) Lead III ECG change in response to RCA ligation. (**C**) Representative pictures of 2,3,5-triphenyl tetrazolium chloride (TTC) staining (white indicates infarct area, red indicates viable tissue). Scale bar = 4 mm. (**D**) Quantitation of myocardial infarct size of RVI mice. Data are presented as mean ± SEM, **P* < 0.01 *vs*. sham group, n = 6 per group (two independent sample *t*-test).

Figure 2: Echocardiography assessment of right ventricular (RV) remodeling and function in mice subjected to RCA ligation. (**A**) Representative B-mode images in four-chamber view 4 weeks after RCA ligation; scale bar = 2 mm. (**B**) Typical pictures of B-Mode at right ventricle interface (upper) and the corresponding M-Mode (lower) showing both LV and RV at 4 weeks after RCA ligation; scale bar = 2 mm. (**C**) RV internal dimension at the end of diastole (RVIDd). (**D**) RV fraction shortening (RVFS). (**E**) RV ejection fraction (RVEF). (**F**) Tricuspid annular plane systolic excursion (TAPSE). (**G**) RV/LV area ratio. Data are presented as mean ± SEM. **P* < 0.01 *vs*. sham group, n = 6 per group (two independent sample *t*-test). LV, left ventricle; RVI, right ventricular infarction.

Figure 3: Right ventricular (RV) hemodynamics at 4 weeks after right coronary artery ligation. (**A**) Representative pressure curves were obtained with a pressure catheter. (**B**) Right ventricular systolic blood pressure (RVSBP) and right ventricular end-diastolic pressure (RVEDP). (**C**) The maximum and minimum rising rate of RV pressure (dp/dt max, dp/dt min). (**D**) RV contractility. (**E**) The exponential time constant of RV relaxation (τ). **P* < 0.01 *vs*. sham group, n = 6 per group (two independent sample *t*-test). Data are presented as mean ± SEM. RVI, right ventricular infarction; RVP, right ventricular pressure.

Figure 4: Histological results at 4 weeks after RVI. (**A**) Pictures of whole representative heart from sham and RVI group (red circle indicates infarct wall; scale bar = 3 mm). (**B**) Heart weight to body weight ratio (HW/BW), *P* = 0.0536 between RVI and sham group. (**C**) HW to tibia length ratio (HW/TL), *P* = 0.1682 between RVI and sham group. (**D**) Represent pictures of hematoxylin-eosin staining and Masson staining of heart sections (scale bar = 3 mm). (**E**) Quantitative results of myocardial fibrosis. (**F**) Representative Masson staining pictures showing survival cardiomyocytes in the infarct area (the right picture (scale bar = 100 μm) is an enlargement of the tissue in the left box (scale bar = 1 mm). **P* < 0.01 *vs*. sham group, n = 6 per group (two independent sample *t*-test). Data are presented as mean ± SEM. RVI, right ventricular infarction.

DISCUSSION:

Sicard and colleagues from France first reported a mouse model of RVI in 2019, which described the surgical process and focused on the interaction between LV and RV after RVI[9]. However, to date, no study has reported using this model for further studies. A more detailed procedure would be helpful for researchers to use the mouse model of RVI for investigation. In contrast to the report by Sicard et al.[9], we provided step-by-step information for model generation and strategy for quality control and further evaluated the anatomical distribution of RCA, RV hemodynamics,

and the survival of cardiomyocytes in the infarct area. A recent report demonstrated that cardiomyocytes in the infarct area play an essential role in myocardial regeneration[23]. The RV function in patients with RVI would recover spontaneously within 3–12 months, even without reperfusion[16,24]. These findings suggest that the mouse RVI model would help search for potential therapeutic targets for right heart failure or cardiac regeneration. Therefore, it is necessary to popularize the model.

Due to the invisibility of RCA and the variation of RCA distribution, it would be difficult for the junior operators to generate RVI models with stable infarct sizes. To overcome this limitation, controlling the ligation level and range and ensuring sufficient elevation of ST-segment in II or III lead of ECG is recommended. The most critical step for successfully generating a mouse RVI model is to locate the anatomical structure of RCA. As shown in **Figure 1A**, mouse RCA may contain a primary or several parallel arteries; thus, the infarct size depends on how many arteries are blocked. Therefore, intraoperatively, the position of the RCA can be confirmed according to the anatomical characteristics of neighboring the right atrium and the visible vein. RVI mice usually exhibit myocardial infarction in the free wall of RV. Still, the septum can also be seldomly involved if the septal artery originates from the RCA, as shown in **Figure 4D**. The septum can be irrigated in mice by its own septal coronary artery branch[25] or a branch of RCA or LCA[26,27]. After ligating the RCA, the classical ECG change of ST-segment elevation in EEG leads II or III leads is the gold standard to judge the success of RVI.

Since RV dilation induced by RCA ligation would increase intrapericardial pressure and then restrain cardiac filling, which would result in aggravation of hemodynamic disorder[9,10], the pericardium should be torn apart during the operation. In contrast to the high incidence of cardiac rupture in mice with LCA ligation, no cardiac rupture was observed in the RVI mice. However, surgical mortality due to bleeding and atrioventricular block could be as high as 50% for beginners, which can be avoided by decreasing the piercing depth of needle stitch and myocardial range of suture ligation, lowering the ligation position, and gentle manipulation. Experienced technicians in our laboratory can complete the generation of an RVI mouse model in about 30 min with an 80%-90% success rate calculated by the survival proportion of mice with significant infarct size. Operation success was judged by instant elevation of ST-segment in Lead II or III of ECG after RCA ligation, TTC negative staining of myocardium in the 1st 24 h after surgery, and RV dilation measured by echocardiography at 3 days or 1 week after surgery. ST elevation in ECG leads of inferior wall and echocardiographic dilation of RV at 3 days after surgery may be used as the inclusion criteria for studies using mouse RVI model.

During the 4-week follow-up period, quite a few surviving cardiomyocytes were observed in the infarct area of RVI mice, which may be a reasonable basis for regenerative research. RV remodeling and dysfunction recovery at 4 weeks were not noted after RVI in this model, suggesting that this model is also feasible for basic research on right heart remodeling and failure.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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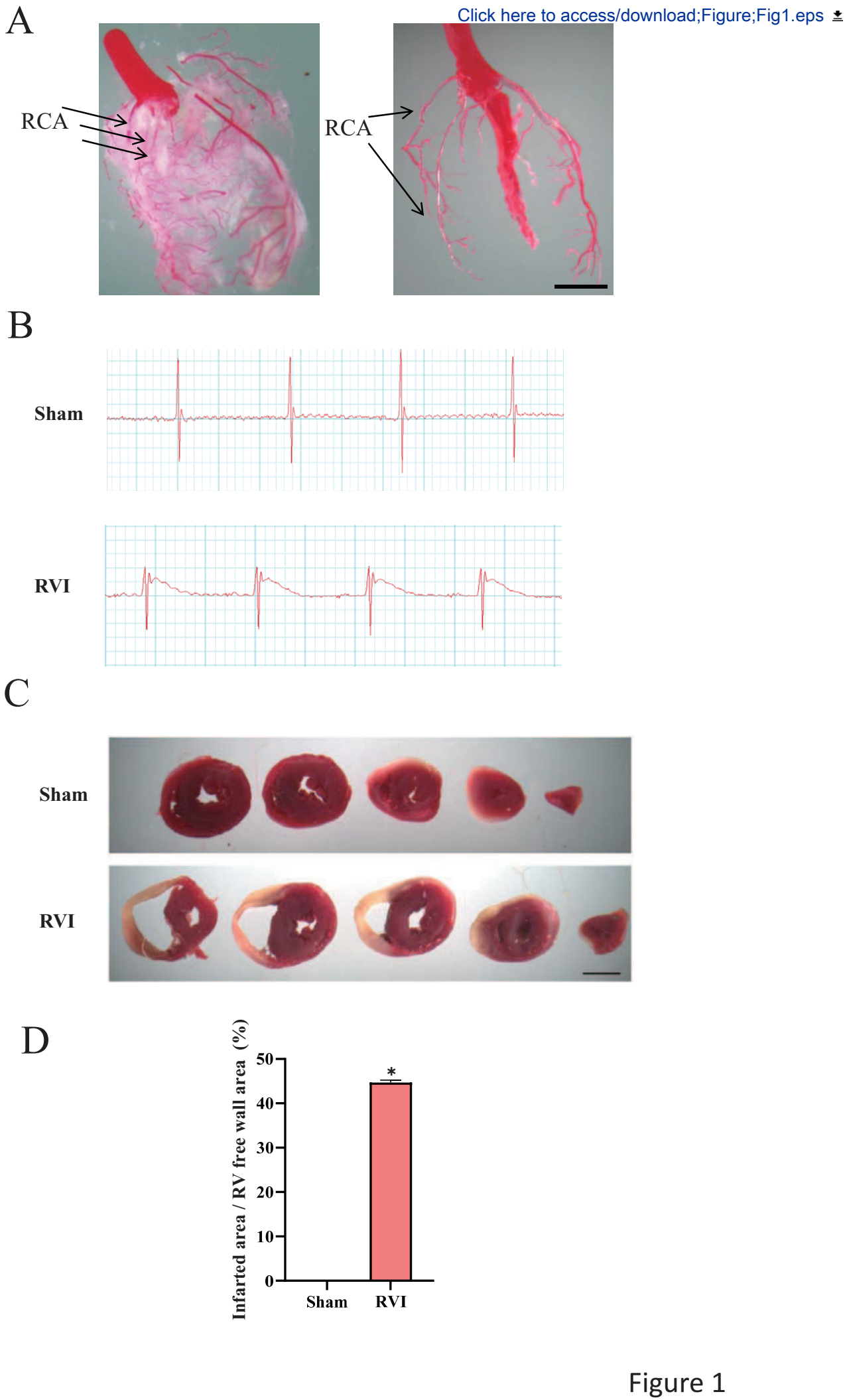


Figure 1

Figure 2

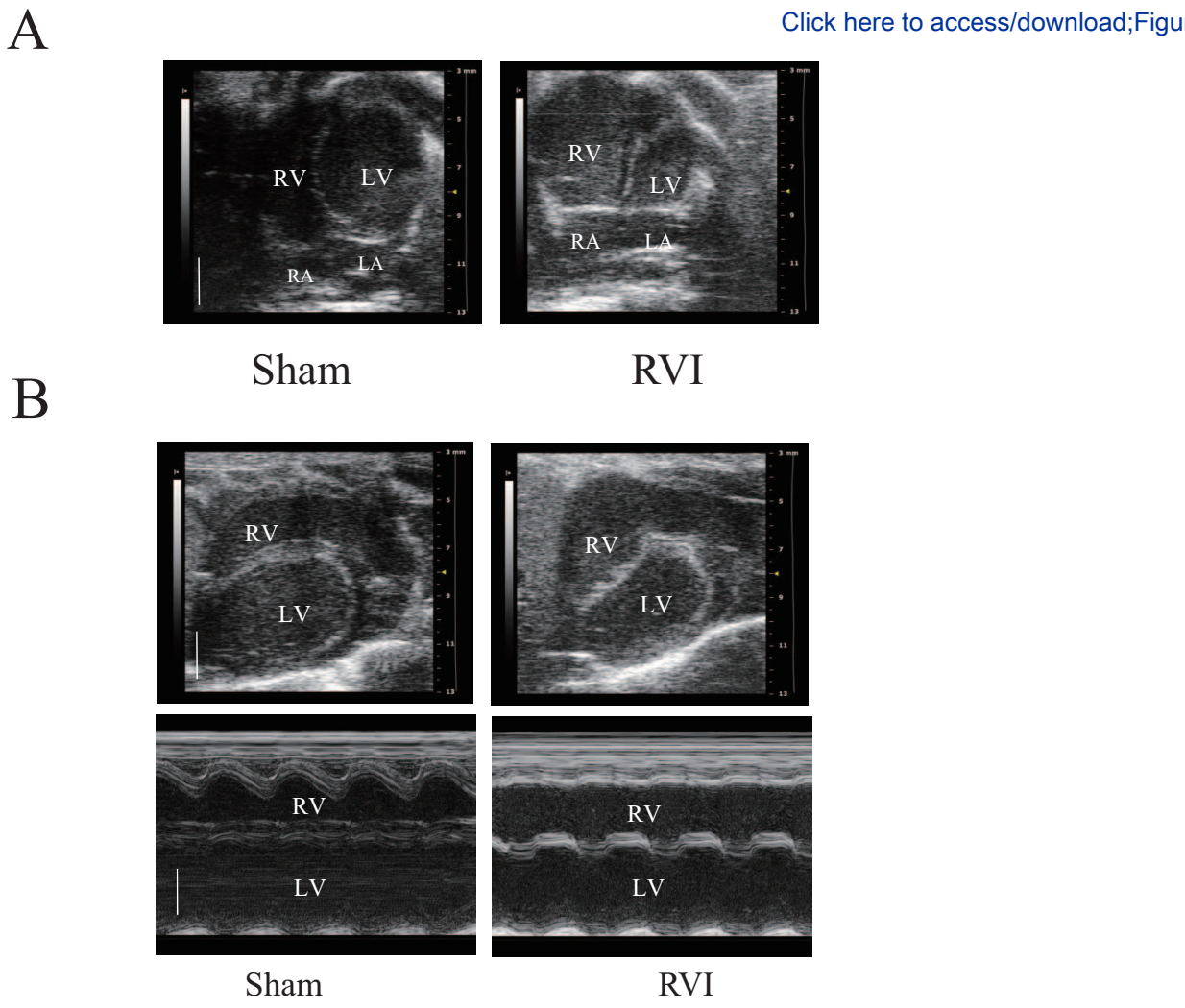
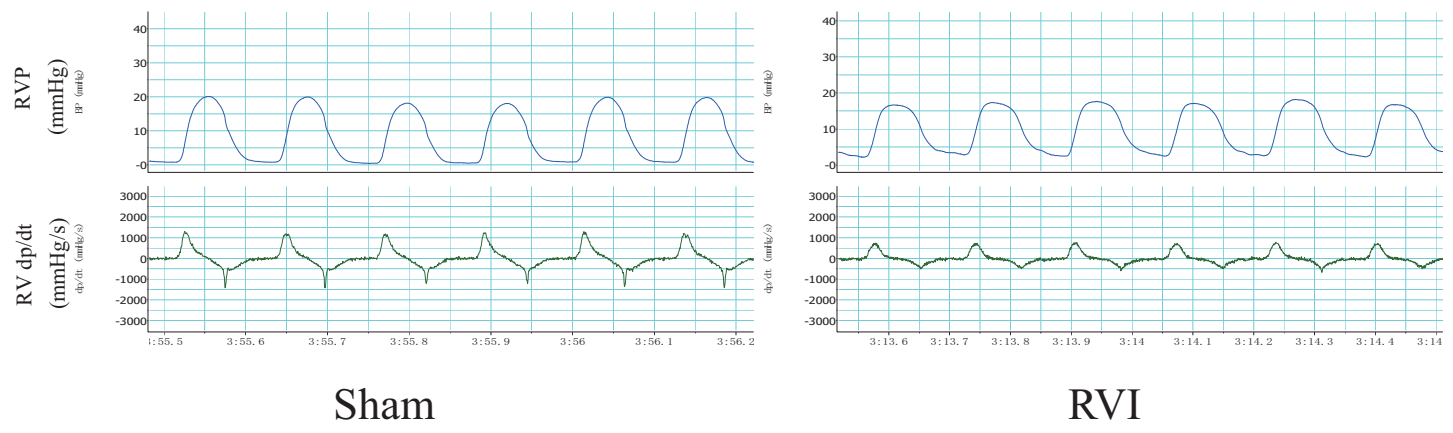
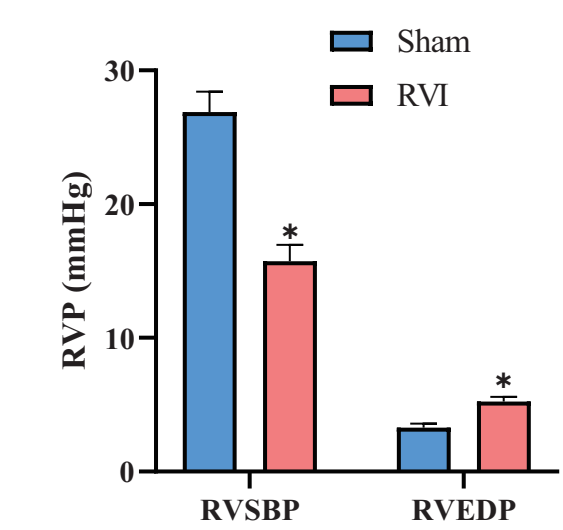


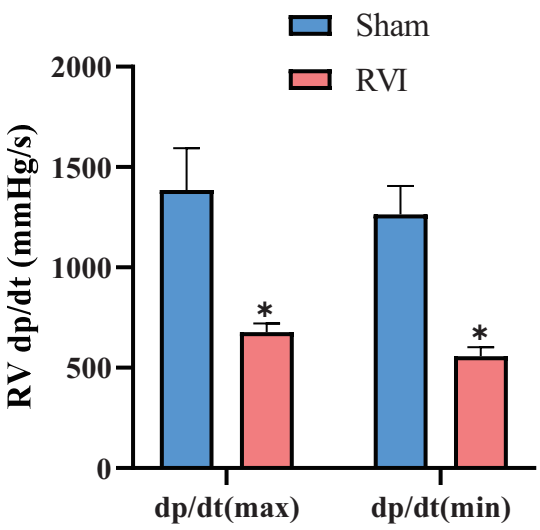
Figure 2

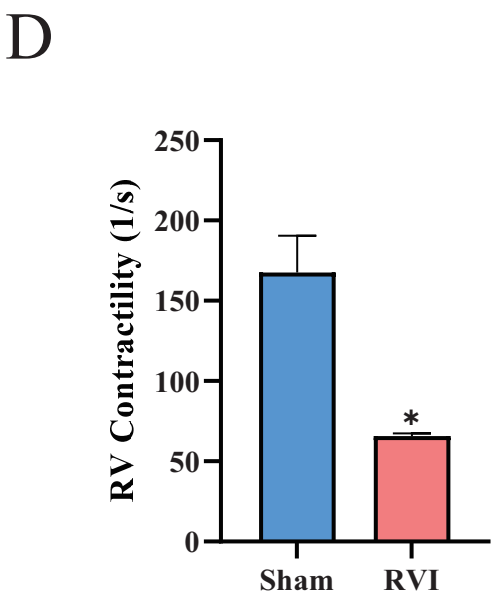
Figure 3

Click here to access/download;Figure;Fig 3.eps









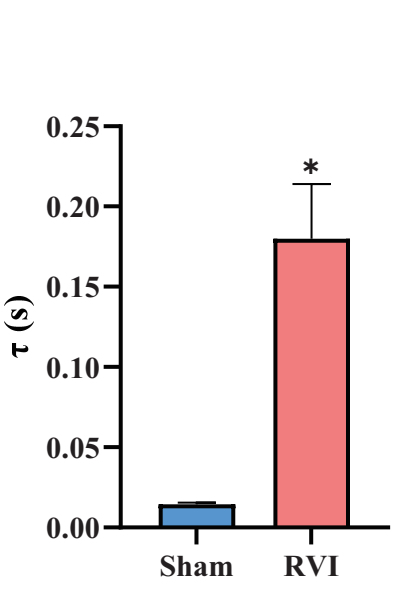


Figure 3

Figure 4

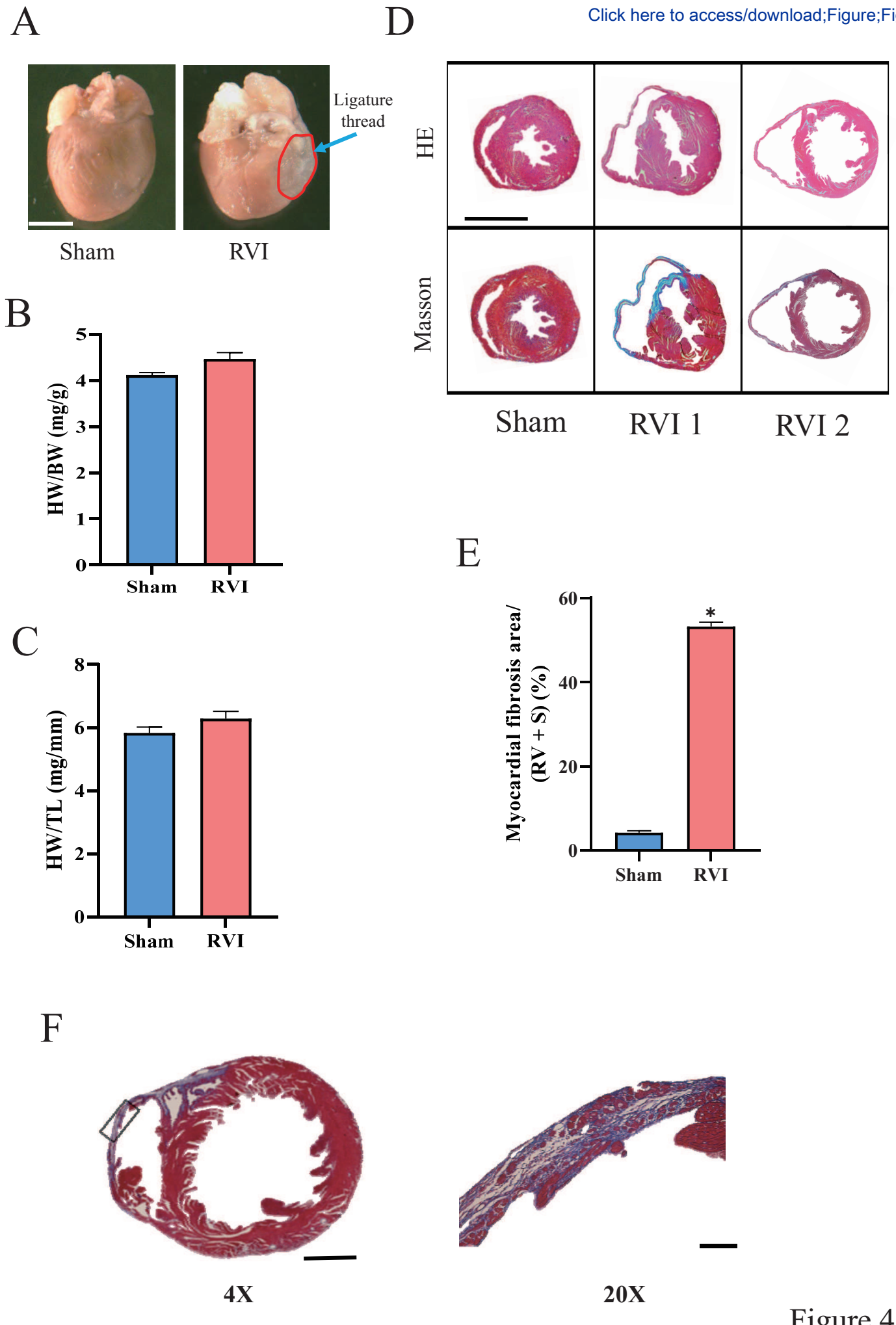


Figure 4

Response to the editorial comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. There are several sentences having grammatical and typographical errors in your manuscript and the meaning conveyed is not very clear to the reader.

Response: Thank you for your kind comment. Although the 1st version has been checked by a professional translating company, grammatical and typographical errors were not completely avoided. This time we thoroughly proofread our manuscript and used a professional copyediting service again to correct the grammatical or typographical errors. The changed parts are indicated by red fonts.

2. Revise 162-164, 176-178, and 200-201 to avoid overlap with published material.

Response: Thank you. We have modified them in the revised version.

3. Revise the text to avoid the use of any personal pronouns.

Response: We have corrected.

4. Remove all commercial language from your manuscript and use generic terms instead.

Response: We have replaced them.

5. Please revise the Introduction to also include the following:

a) The advantages over alternative techniques with applicable references to previous studies b) A description of the context of the technique in the wider body of literature

Response: The advantages of our techniques and related references have been added as follows:

(1) In line 66-72, we added: "Large animal model of RVI has been generated by ligating RCA in swine[18], which should be easier to operate because of the visible RCA. Mouse RVI model would have more advantages than large animal model in gene manipulation, economic cost, and experimental period[19,20]. Although a mouse RVI model focusing on influence of RVI on LV function was reported previously, the detailed steps of the procedure, the difficulties and key points of operation, and the model characteristics such as hemodynamic changes were not fully introduced[9,21]."

(2) In line 78-80, we added: The technique introduced in this paper would facilitate the junior learners to quickly grasp the generation of mouse RVI model with acceptable operation mortality and reliable evaluation approaches.

6. Please revise the Introduction to also include the following: c) Information to help readers to determine whether the method is appropriate for their application.

Response: We have added a description in the end of introduction (line 80-82): **"**The mouse model of RVI would be useful for researching the mechanisms of right heart failure and seeking new therapeutic targets of RV remodeling.**"**

7. Step 1.1: Protocol should contain only action steps. Please move the details of the

equipment and instruments to the Table of Materials.
Response: The details of the equipment and instruments have been moved to the Table of Materials.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.
Respond: Thank you very much. We have modified them according to your suggestion.

9. Step 3.3: please specify how the heart rate is monitored.
Response: The information has been added. Heart rate was monitored by ECG equipped with the echocardiography machine.

10. Step 3.5: what is 'platform'? Please elaborate.
Response: The word "platform" in step 3.5 and following steps indicates an echocardiographic operation platform equipped with the echocardiography machine. That platform serves as a carrier for animal. And if turn on the thermostat, the platform can keep the animal temperature during echocardiographic assessment. Furthermore, the position of the platform can be slightly regulated by rotating the x- and y-axes. In the revised version, we replaced "platform" with "platform for animal fixation ultrasonic operation" at its first appearance.

11. Step 3.7: Please specify what the x- and y-axes correspond to. How to measure RV/LV area ratio? Please provide all button clicks on the software.
Response: X-axes and y-axes correspond to the knob that can adjust the position of the platform horizontally or longitudinally. We made modifications as follows:
(1) 3.7 Move down the transducer vertically, maintaining its position over the upper abdomen and below the diaphragm of the mouse under B-mode. Adjust the position of platform slightly by rotating its x- and y-axes until the RV, right atrium (RA), left atrium (LA), and LV are clearly seen on the screen. Save apical four-chamber images by pressing the button of cine store or frame store.
(2) The protocol to measure RV/LV area ratio has been added in line 199-201: "Press "measure" button to enter measurement mode. Click "area measurement" button to zone into RV and LV. Calculate the area of RV and LV to obtain the area ratio of RV to LV."

12. Steps 3.8, 3.9, and 4.8: Please provide all the button clicks on the software for measuring/recording the specified parameters.

Response: The protocols to measure TAPSE, RVEF, RVFS, RVID and RVSBP,

RVEDP and dp/dt have been added. In line 200-204: Click “timeline” button and make two baselines to define the movement range of tricuspid annular plane during systolic and diastolic periods. Click “distance” button and measure the distance between two baselines to obtain tricuspid annular plane systolic excursion (TAPSE).” In line 212-215: “3.11 Open the M-mode image of RV interface. Press “measure” button to enter measurement mode. Measure the RV inner distance at the end of diastole (RVIDd), RV ejection fraction (RVEF) and RV fraction shortening (RVFS) using the in-built measurement tool of the echocardiographic system.” In line 249-252: “4.8 After 10 min of stabilization, record the data of RV systolic blood pressure (RVSBP), RV end-diastolic pressure (RVEDP), and RV dP/dt. Click “select” button to select cardiac cycles for calculation and then click “analyze” button to calculate the mean values of the selected cycles.

13. Step 3.10: did you administer analgesics to reduce pain after surgery?

Response: Step 3.10 is echocardiographic assessment. Echocardiography is a non-invasive assessment and it is not necessary to give analgesics. We agree with you that analgesics should be added after surgery, thus we added a note following step 2.3: Note: Analgesics such as buprenorphine (0.1 mg/kg body weight, subcutaneously injection) is recommended to reduce pain of the animals after the surgery.

14. Step 4.7: Where is the RV waveform viewed? Specify the instrument in a generic term.

Response: The RV waveform is viewed in a monitor and recording system (Power Lab instrument). We have added the generic term of the instrument in line 245: Adjust the position of the tip to obtain a typical RV pressure waveform shown on a monitor and recording system.

15. Step 5.5, 5.6, 5.8: Please mention the tool used to make the notch. What is the volume of the perfusate and how is it perfused in the heart? Please elaborate on the ‘apical approach’ in step 5.5.

Response: We made modifications as follows:

(1). 5.5 Make a small notch with ophthalmic scissors on the right atria and perfuse the heart with 5 mL normal saline through the cardiac apex with an injector.

(2). 5.6 Block the blood from the aorta with an aortic clamp and perfuse 0.1 mL nitroglycerin (concentration: 1 mg/mL) through the cardiac apex with an injector to dilate the coronary artery.

(3) 5.8 Perfuse the heart with 1 mL cast reagent through the cardiac apex and wait for 2-3 h.

16. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Response: We have highlighted the critical steps.

17. 'ml' should be 'mL' and hours should be abbreviated as 'h'.
Response: Thank you. We have corrected them.

18. Figure 2: Please indicate B-mode and M-mode images in panel B. In panel C, correct the typo in the y-axis title.
Response: The annotation of the images in Figure 2 B has been added in the figure legend of Figure 2. And "RVIDd" in y-axis title of the panel C indicated "right ventricular internal dimension at the end of diastole", which has been elaborated in figure legend.

19. Figure 4, legend: Please indicate the staining used for the images in panel F.
Response: The staining of Figure 4 is Masson staining, which has been elaborated in figure legend of Figure 4.

20. Please submit each figure individually as a vector image file (.psd, ai, .eps, or .pdf) to your Editorial Manager account.
Response: This time we submitted the images in eps format.

21. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.
Response: We have done it.

22. As we are a methods journal, please ensure that the Discussion explicitly covers the following in detail in 3-6 paragraphs with citations: a). Any limitations of the technique; b) The significance with respect to existing methods; c) Any future applications of the technique.
Response: The corresponding content has been added in discussion, which can be listed as follow:
a). In line 389-397, we added the study limitation: "Due to the invisibility of RCA and the variation of RCA distribution, it would be difficult for the junior operators to generate RVI models with stable infarct size. To overcome this limitation, we recommend controlling the ligation level and range, and making sure sufficient elevation of ST segment in II or III lead of ECG. The most critical step for successfully generating mouse RVI model is to locate the anatomical structure of RCA. As shown in Figure 1A, mouse RCA may contain a main or several parallel arteries, thus the infarct size depends on how many arteries are blocked. During operation, the position of the RCA can be confirmed according to the anatomical characteristics of neighboring the right atrium and the visible vein."
b). The significance with respect to existing methods was modified in line 374-381: "Sicard and his colleagues from France first reported a mouse model of RVI in 2019, which described the surgical process and focused on the interaction between LV and RV after RVI[4]. However, to date, no study has reported the use of this model for further studies. A more detailed procedure with the JOVE style would be helpful for researchers to use the mouse model of RVI for investigation. In contrast to the report

by Sicard et al[4], we provided step by step information for model generation and strategy for quality control and further evaluated the anatomical distribution of RCA, RV hemodynamics, and the survival of cardiomyocytes in the infarct area."

c) We have discussed the potential applications of the technique in line 381-387: "A recent report demonstrated that cardiomyocytes in the infarct area play an important role in myocardial regeneration[23]. The RV function in patients with RVI would recover spontaneously within 3-12 months even in the absence of reperfusion[16,24]., These findings suggest that mouse RVI model would be useful for searching potential therapeutic targets for right heart failure or cardiac regeneration. Therefore, it is necessary to popularize the model."

23. Please expand journal names in references

Response: Journal names in references have been expanded.

Response to the reviewer' #1

Manuscript Summary:

In this study Liao R et al, provide detail on a surgical procedure to induce right ventricular infarction by tying permanently the right coronary artery (RCA) in mice. The authors characterize the model using high resolution ultrasound, direct hemodynamic evaluation and histology four weeks after surgery. Here, the RCA ligation in mice induced right ventricular fibrosis associated with chronic right ventricular failure. This will be the second article using and describing the usefulness of tying the RCA to induce right ventricular infarction in mice (Compared to the thousands of articles using left anterior descending artery ligation model in mice to create myocardial infarction). In patients, right ventricular myocardial infarction is recognized in this situation as a major prognostic factor and Liao et al demonstrate that this model is reproducible. Therefore, this work is totally suitable for JoVE publication. The surgical procedure is clearly explained and critical steps are highlighted.

Response: Thank you very much for your encouraging comments. This time we tried to address all of your concerns as possible as we can. The changed parts are indicated by red fonts.

Major Concerns:

In figure 4D, the septum exhibits fibrosis after RCA ligation. However, this result does not correlate with the TTC staining (Figure 1 C) showing that infarct size is localized only in the right ventricle. It has been shown in C57/BL6 mice that the septum is irrigated by its own septal coronary artery branch (10.1002/cmmi.1588). The proximal course of the septal artery may originate from the right CA, from the left CA originates directly from the aortic bulb (10.1111/j.1469-7580.2007.00838.x; 10.1002/cmmi.1588).

The authors should provide more information on the rate of septum fibrosis observed in this model and further explain why they observed late fibrosis development in the septum.

Response: Thank you for your insightful comment on this issue. We completely agree with your analysis. Surely, the septal infarction or fibrosis is rarely observed in our mouse RVI. In the revised version, we added typical pictures showing fibrosis staining in the free wall of RV without involvement of septum and gave a brief discussion on septal fibrosis after RCA ligation. The modifications are listed as follow:

(1) A picture of coronary vascular cast was added in Figure 1A to show the variation in the origination and distribution of RCA. Text in the discussion and the corresponding figure legend has been modified. In Figure 1 A legend, we modified to "Representative images of mouse coronary vascular cast".

(2) Pictures with Masson staining showing fibrosis only in free wall of RV were added in Figure 4D.

(3) A discussion was added in line 394-401: As shown in Figure 1A, mouse RCA may

contain a main or several parallel arteries, thus the infarct size depends on how many arteries are blocked. During operation, the position of the RCA can be confirmed according to the anatomical characteristics of neighboring the right atrium and the visible vein. RVI mice usually exhibit myocardial infarction in the free wall of RV, but septum can be seldomly involved if the septal artery originates from the RCA as shown in Figure 4D. In mice, the septum can be irrigated by its own septal coronary artery branch[24] or by a branch of RCA or LCA[25,26].

Minor concerns

2. *It is not clear if the author used positive end-expiratory pressure (PEEP) during the protocol*.
Response: We did not use PEEP because it is not necessary. We made a minor modification: 1.5.1 Adjust the breathing frequency of the animal miniventilator to 150/min and the tidal volume to 300 μL (it is unnecessary to use positive end-expiratory pressure mode).

3. *The author must provide the number of animals used in this study and the success rate*.
Response: We performed surgery in 13 mice and 11 of them survived; therefore, the survival rate is 84.6%. Judging by ECG, TTC staining or echocardiography, all of the survived mice had successful RVI. We have made modification in line 411-414: Experienced technicians in our laboratory can complete the generation of an RVI mouse model in about 30 min with an 80-90% success rate which was calculated by the survival proportion of mice with significant infarct size.

4. *The article must undergo new English proofreading*
Response: We have used a new professional copyediting service.

Response to Reviewer #2:

Manuscript Summary:

This is an interesting protocol describing a method of right ventricular infarction model induced by right coronary artery ligation in mice.

The study is well designed and results are straightforward and clearly presented. Overall, the manuscript is well written. However, I would suggest a detailed copyediting by an expert in the field. I will give you only some specific examples: the correct terms are fatal (line 32), tricuspid annular plane systolic excursion (lines 277-278), complete (line 323) and weighing scale (please also check for spelling errors for some words in the table).

Response: Thank you very much for your positive and encouraging comments. The spelling errors have been revised. This time we tried to address all of your concerns as possible as we can. The changed parts are indicated by red fonts.

1. *I would suggest a detailed copyediting by an expert in the field.*

Response: We have used a new copyediting service.

2. *Cutting the pectoral muscles seems too invasive for me*

Response: We agree with you, but we only cut a small part of pectoral muscles. Anyway, it is a surgery. Blunt separation might be better. We added a note after step 2.2.2: Only a small part of pectoral muscles should be cut and then a blunt separation is recommended to expose the heart.

3. *If the authors provide some more advices on how to identify the artery and discuss individual anatomical differences in right coronary artery localization, numbers etc., which all can affect the success of the model.*

Response: According to our result of vasculature casting in several C57BL/6J mice, we determine that the ligation position is under the right atrium and accompanying visible vein. We made modifications as follows: "Due to the invisibility of RCA and the variation of RCA distribution, it would be difficult for the junior operators to generate RVI models with stable infarct size. To overcome this limitation, we recommend controlling the ligation level and range, and making sure sufficient elevation of ST segment in II or III lead of ECG. The most critical step for successfully generating mouse RVI model is to locate the anatomical structure of RCA. As shown in Figure 1A, mouse RCA may contain a main or several parallel arteries, thus the infarct size depends on how many arteries are blocked. During operation, the position of the RCA can be confirmed according to the anatomical characteristics of neighboring the right atrium and the visible vein."

4. What do the authors mean with 80-90% success rate? Survival proportion of mice with significant infarct size? At what time point after the surgery they would recommend to perform assessment of the success of the surgery?

Response: Our success standard of RVI model is that the animals are still survival after a significant infarct size has been induced. Thus, the success rate is the survival

proportion of mice with significant infarct size. Several time points can be used to evaluate the success of the surgery. We made some modification in line 411-417: Experienced technicians in our laboratory can complete the generation of an RVI mouse model in about 30 min with an 80-90% success rate which was calculated by the survival proportion of mice with significant infarct size. Operation success was judged by instant elevation of ST-segment in Lead II or III of ECG after RCA ligation, TTC negative staining of myocardium in the 1st 24 h after surgery, and RV dilation measured by echocardiography at 3 days or 1 week after surgery.

5. Were there cases, where the size of the right ventricular infarction was significantly sm*aller?*

Response: It is sure there were some mice with RVI had smaller infarct size if only a small part of right coronary arteries were blocked, which is not uncommon for the beginners to generate RVI mouse model. On this issue, we added a brief discussion as mentioned in our response to your concern 3: As shown in Figure 1A, mouse RCA may contain a main or several parallel arteries, thus the infarct size depends on how many arteries are blocked. During operation, the position of the RCA can be confirmed according to the anatomical characteristics of neighboring the right atrium and the visible vein.

6. What criteria are used to exclude the animal from the study? Infarct size, right ventricular remodeling and function?

Response: We would like to recommend inclusion criteria, if the animals do not meet the inclusion criteria, they should be excluded from the study. Therefore, we added a brief discussion: ST elevation in ECG leads of inferior wall and echocardiographic dilation of RV at 3 days after surgery may be used as the inclusion criteria for studies using mouse RVI model.

7. Please provide more information on potential complications of the surgery.

Response: In the discussion part, we have given the following information on complications: In contrast to the high incidence of cardiac rupture in mice with LCA ligation, no cardiac rupture was observed in RVI mice. However, surgical mortality due to bleeding and atrioventricular block could be as high as 50% for beginners, which can be avoided by decreasing the piercing depth of suture needle stitch and the myocardial range of ligation, lowering the ligation position, and gentle manipulation.

8. *The authors shall indicate the mouse substrain they used.*

Response: We used C57BL/6J mice. This information has been added in abstract and protocol.

9. *I would recommend isoflurane anesthesia with a proper analgesia as a first choice method of anesthesia, because of the ease of controlling the depth of anesthesia and fast recovery post-surgery.*

Response: We agree with you. Because the cost-effectiveness ratio of inhalation

anesthesia is higher, we didn’t use it for RVI surgery but use it for echocardiographic assessment. We have added a note in the protocol to recommend isoflurane anesthesia:
NOTE: It is also recommended to use 1.5% isoflurane for inhalation anesthesia, because it is better for analgesia.