Journal of Visualized Experiments

Induction of myocardial infarction and myocardial ischemia-reperfusion injury in mice

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE63257R2
Full Title:	Induction of myocardial infarction and myocardial ischemia-reperfusion injury in mice
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Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Medicine
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Wuhan, Hubei, China
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TITLE:

Induction of Myocardial Infarction and Myocardial Ischemia-Reperfusion Injury in Mice

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SUMMARY:

Here we describe a simple and reproducible method that can induce myocardial infarction or myocardial ischemia-reperfusion injury in mice by precision ligation of the left anterior descending coronary artery through micromanipulation.

ABSTRACT:

Acute myocardial infarction is a common cardiovascular disease with high mortality. Myocardial reperfusion injury can counteract the beneficial effects of heart reflow and induce secondary myocardial injury. A simple and reproducible model of myocardial infarction and myocardial ischemia-reperfusion injury is a good tool for researchers. Here, a customizable method to create a myocardial infarction (MI) model and MIRI by precision ligation of the left anterior descending coronary artery (LAD) through micromanipulation is described. Accurate and reproducible ligature positioning of the LAD helps obtain consistent results for heart injury. ST-segment changes can help to identify model accuracy. The serum level of cardiac troponin T (cTnT) is used to assess the myocardial injury, cardiac ultrasound is employed to evaluate the myocardial systolic function, and Evans-Blue/triphenyl tetrazolium chloride staining is used to measure infarct size. In general, this protocol reduces procedure duration, ensures

controllable infarct size, and improves mouse survival.

INTRODUCTION:

Acute myocardial infarction (AMI) is a common cardiovascular disease worldwide and carries high mortality[1]. Advances in technologies make early and efficacious revascularization available for AMI patients. After these treatments in some patients, myocardial ischemia-reperfusion injury (MIRI) can occur[2]. Thus, it is of great significance to understand the mechanisms of actions and how to ameliorate MI/MIRI. Mice are widely used as models because of their low cost, rapid breeding time, and ease for making genetic alterations[3]. Scholars have developed different methods to model MIRI and MI in animal[4–9]. This strategy promotes research, but the different criteria and methods employed complicate the interpretation of results among research teams.

In mice, MI has been induced by isoproterenol[10], cryoinjury[11–12,] or cauterization[13]. MI can be induced readily by isoproterenol, but the pathophysiological process is different from that in clinical MI. Cryoinjury-induced MI has poor consistency, elicits excessive myocardial damage around the left anterior descending coronary artery (LAD), and can easily induce arrhythmia. Cauterization-induced MI is quite different from the natural process of myocardial infarction, and the inflammatory reaction in the burning area is more intense; in addition, the operation is difficult. Moreover, there are some labs[14] developing MI model in minipigs using balloon blocking or embolization or thrombosis method through interventional technique. All these methods can cause coronary artery occlusion directly, but needing coronary angiography devices and, above all, the too-thin mouse coronary arteries makes these operations not practical. For MIRI, the differences among different models were quite modest, such as using respirators /micromanipulation or not[5–6].

Here, a simple and reliable method that can induce MI and the MIRI model, adapted from previously published methods[4–9,15], is described. This method can simulate pathophysiological processes by direct blockade of the LAD through ligation. Moreover, by relieving the ligation, this model can also simulate reperfusion injury. In this protocol, a dissecting microscope is used for LAD visualization. Then, the researcher can identify the LAD readily. Subsequently, accurate ligation of the LAD leads to reproducible and predictable blood occlusion and ventricular ischemia. Moreover, electrocardiography (ECG) changes can be used to confirm ischemia and reperfusion in addition to the color changes of the LAD observed under a microscope. This strategy leads to a shorter procedure duration, lower surgical complications risk, and fewer experiment mice needed. The methods for the troponin-T test, cardiac ultrasound, and triphenyl tetrazolium chloride (TTC) staining are also described. Overall, this protocol is useful for studies of MI/MIR mechanism, as well as for drug discovery.

PROTOCOL:

Animal studies have been approved by the Animal Care and Utilization Committee of Huazhong University of Science and Technology (Wuhan, China).

NOTE: Male C57BL/6J mice (8–10 weeks) are used as models. Mice have free access to food and water and are bred in specific pathogen-free conditions. The room is maintained under controlled temperature (22 °C ± 2 °C) and humidity (45%–65%). Mice are exposed to a 12-h light/dark environment at the Animal Care Facility of Tongji Medical School (Wuhan, China) according to the guidelines set by this institution. Microsurgical instruments and surgical supplies must be previously sterilized. Surgical gloves and masks are required throughout the procedure. The experimental workflow is shown in **Figure 1A**.

1. Preoperative preparation

1.1 Use a rectangular operating table (OT) with a prewarmed heating pad (37 °C) throughout the surgical procedure (**Figure 1B**). Disinfect the board with ultraviolet light and 70% alcohol before procedure initiation.

1.2 Weigh all mice accurately to calculate the dose of anesthetic drugs needed. Then, anesthetize the mice with ketamine (80 mg/kg) and xylazine (10 mg/kg) via intraperitoneal injection. Ensure appropriate depth of anesthesia by the absence of a withdrawal reflex to toe pinching and blink reflexes.

1.3 Place the mouse supine on the OT with gauze under the head to avoid overheating of the eyes. Apply ophthalmic ointment to the eyes to prevent them from drying out.

1.4 Shave the fur on the left precordial chest with an electric razor. Use a fur removal cream on the pre-shaved thorax and massage evenly with a sterile cotton swab for ~1 min. Wipe the excess loose fur with gauze.

1.5 Use povidone-iodine, followed by 70% alcohol to clean the area. Cover the thorax with gauze.

1.6 Use a 4-0 suture under the upper incisors and secure it to the anchor point (close to the edge of the OT over the nose) to keep the mouth slightly open and facilitate cannulation.

1.7 Pull the tail to keep the body straight, and secure the tail to the OT using tape. Secure the four limbs with rubber bands and tighten them on the other anchor points. Importantly, do not over-stretch the front limbs; otherwise, respiratory compromise may occur.

1.8 Use curved forceps and forceps to open the jaw and lift the tongue. Use an illuminator to clearly visualize the throat and glottis.

1.9 Insert a 22-G cannula gently with a blunted and truncated needle into the trachea through the mouth ~1-cm down the throat. Use one hand to hold the tongue, move it slightly upwards with blunt forceps, and, simultaneously, use the other hand to gently insert the tube into the trachea. Be careful not to insert the tube into the esophagus.

1.10 Remove the needle gently. Check the intubation by placing the tube into the water for bubbles to form before connecting to the ventilator.

1.11 Connect the endotracheal tube to a ventilator set to 120/min and tidal volume adjusted to 250 µL.

NOTE: The ventilator setting is adjusted by body weight (in general, a higher body weight requires a higher tidal volume).

1.12 Verify intubation by checking bilateral symmetrical chest expansion. Then, the connection is fixed to the OT with tape to avoid the tube falling off.

1.13 Place ECG electrodes on the paws and connect them to the ECG recorder. Monitor cardiac electrophysiology throughout the procedure.

2. Thoracotomy

2.1 Remove the gauze on the thorax. Disinfect again with 70% alcohol for the incision areas using three scrub cycles. Then, cover the mouse with a sterile surgical drape with a hole over the surgical field to reduce contamination of the surgical site.

2.2. Make an oblique skin incision (0.8–1.0 cm) along the left midclavicular line with sterile scissors.

2.2 Undertake blunt dissection of subcutaneous tissues to expose the ribs underneath. Be careful not to injure vessels, ribs, and lungs. Stop the bleeding by using cotton applicators.

2.3 Identify and make an incision of about 6–8 mm in the third intercostal space. Then, carry out blunt dissection of tissues in the intercoastal space to open the chest cavity. Be careful not to injury the internal thoracic artery.

2.4 Use forceps to span the intercostal space. Insert pre-sterilized homemade retractors (**Figure 1C**) into the rib cage and pull back to spread the incision to ~6 mm in width. Attach the retractors to the OT with rubber bands.

2.5 Remove the surrounding tissues carefully to expose the heart fully. Pull off the pericardium gently with curved forceps without injuring the heart. Now a clear view of the heart is available.

3. LAD ligation

NOTE: The LAD appears as a thin red line running perpendicular from near the apex and down through the left ventricle. The LAD is bright-red color, so be careful not to mistake it for a vein. Usually, the ligation site is ~1–2 mm below the left auricle. This ligation position will produce

about 40%–50% of the ischemia in the left ventricle. A higher position will create a more extensive infarct zone. A more distal site will create a smaller infarct zone.

3.1 Use a dissecting microscope and direct a focused and appropriate light for LAD visualization. Press the site below the chosen ligation position gently to enlarge the LAD temporarily (≤5 s per time). Recheck the LAD in this way.

3.2 Use a tapered needle (3/8, 2.5 x 5) to pass an 8-0 silk ligature underneath the LAD under a dissecting microscope. Be careful with the needle depth: not too deep to enter the left ventricle and not too shallow to avoid damaging the LAD.

3.3 Tie the ligature with a loose double-knot. The loop diameter is about 2–3 mm.

3.4 Place a 2–3 mm PE-10 tubing into a loop parallel to the artery.

3.5 Tighten the ligature loop gently until it is around the artery and tubing. Then, secure the loop with a slipknot. Take care not to damage the myocardial wall with excessive tightening pressure.

NOTE: Ligation is not carried out for the sham-operation group.

3.6 Confirm cessation of blood flow in the LAD: observe a paler color in the anterior wall of the LV after ligation. In addition, significant ST-elevation within a few heartbeats also indicates occlusion[16]. If permanent ligation is required (e.g., MI), remove the PE-10 tubing and tie the LAD directly with a knot. Resume the remaining procedure as mentioned in step 4.3 below.

3.7 Remove the retractors from the incision. Then, close the wound temporarily with a bulldog clamp. Ischemia duration is according to the experimental design. Ensure that the mouse continues to be connected to the ventilator.

4. Reperfusion

4.1 When the period of ischemia ends, remove the bulldog clamp and insert the retractors again to open the incision and expose the heart (especially the ligation site).

4.2 Untie the slipknot and remove the PE-10 tubing. Confirm the restoration of blood flow in this step by observing the color change back to pink-red within 20 s. Simultaneously, watch the ECG carefully: a potential dissolution of ST-elevation also suggests reperfusion.

4.3 Leave the 8-0 ligature in *situ* for subsequent Evans-Blue and TTC staining. In other cases, remove the suture at this step.

4.4 Remove the retractors and close the incision by suturing the third and fourth ribs with a 4-0 silk suture. Be careful not to injure the lung. Push out the air that might be trapped in the

chest cavity by pressing the chest gently while tying the suture knots.

4.5 Close the muscle layers with continuous sutures (4-0 silk). For skin closing, continuous sutures and interrupted sutures are acceptable.

5. Postoperative care

5.1 Observe the mouse carefully for signs of recovery from anesthesia, for example, movement of the tail or whiskers. After that, the mouse usually resumes a normal breathing pattern with a respiration rate of around 150 bpm. Extubate the mouse by removing the tube slowly.

5.2 Monitor the mouse for an additional 3–5 min to ensure respiratory distress is absent.

5.3 Administer 100 µL of buprenorphine (0.1 mg/mL, s.c.) after the mouse begins to breathe. For the next 24 h, provide an additional dose every 4–6 h. Provide ibuprofen as additional pain relief in drinking water as a 0.2 mg/mL solution for 2 days before and ≤7 days after surgery.

5.4 Keep the mice warm and reduce mortality risk by using thermal insulation blankets as mice are prone to hypothermia after the anesthesia.

6. Validation after the procedure

6.1 Troponin-T test

6.1.1 Collect blood samples from the retroorbital plexuses and isolate the serums by centrifugation (3,000 × *g*, 10 min, room temperature).

6.1.2 Dilute 20 µL of serum to 100 µL with saline solution for the troponin-T test. Store the remainder of the samples at -80 °C.

6.1.3 Detect the Troponin T (cTnT using a commercial kit following the manufacturer's instructions.

6.2 Cardiac ultrasound

NOTE: Cardiac ultrasound is used to evaluate cardiac function and wall-motion abnormalities at different stages before and after surgery according to the experimental design[17–18]. Different parameters such as ventricular wall thickness, ventricular volume, ventricular-cavity diameter, ejection fraction, and short-axis shortening fraction are measured.

6.2.1 Anesthetize the mice with ketamine (80 mg/kg) and xylazine (10 mg/kg) via intraperitoneal injection.

6.2.2 Shave the chest with an electric razor. Use fur removal cream and massage evenly. Wipe the excess loose fur with gauze.

6.2.3 Place the mouse on the OT and secure the four limbs with adhesive tape.

6.2.4 Place the ultrasound probe (30 MHz) on the anterior region of the heart at ~ 30° to the sternum. The probe in this view is aligned with the long axis of the heart. Set the ultrasound in **B-Mode**; the left ventricle, left atrium, mitral valve, and ascending aorta can be identified clearly. Use video capture to obtain data for subsequent analysis.

6.2.5 By rotating the transducer 90°clockwise, obtain a parasternal short-axis view at the level of the papillary muscles to clearly detect the left and right ventricles. Then use **B-Mode** and **M-Mode** to assess cardiac function and morphometry.

6.2.6 Calculate the left ventricular end-diastolic diameter (Dd), end-systolic diameter (Ds), and interventricular septal thickness by specifying the corresponding location in the ultrasound images.

NOTE: The machine would manually calculate left ventricular end-diastolic volume (LVEDV) and end-systolic volume (LVESV). Also, the machine would calculate the values for fractional shortening (FS) and ejection fraction (EF) using formulas FS = (Dd-Ds)/Dd × 100% and EF= (LVEDV-LVESV)/LVEDV × 100%. Choose five consecutive cardiac cycles and obtain their mean values.

6.3 Measurement of myocardial infarct size

NOTE: Evans-Blue/TTC staining is used to measure the infarct size because it can evaluate tissue viability[19]. It is recommended to stain within 72 h of reperfusion because the scar will shrink. This step is performed after euthanizing the animal with 200 mg/kg pentobarbital sodium via intraperitoneal injection.

6.3.1 Expose the heart again following the previous procedures from steps 2.2–2.5. Then, re-ligate the LAD at the initial site validated by the suture mentioned in step 4.3 at the end of the desired reperfusion duration.

6.3.2 Cannulate the aorta and then perfuse the heart with 0.3 mL of 1% Evans Blue solution. The myocardium of the non-ischemic region is stained blue. After perfusion, remove the heart rapidly by cutting the aorta with scissors.

6.3.3 Then, wash the heart in KCl solution (30 mM) to stop the heart from beating. Store at -20 °C for ≥4 h after removing the surrounding fatty tissue.

6.3.4 Cut the heart in the transverse direction into five slices of thickness 1 mm using a sharp scalpel. Weigh the slices and then incubate them with 2% TTC for 40 min at 37 °C.

NOTE: After the incubation, the infarct areas are demarcated as white, whereas viable tissues in non-infarct areas remain red.

6.3.5 Fix the slices with 4% formaldehyde overnight.

NOTE: This action will enhance the contrast between the infarct area and the non-infarct area. It will also shrink the slices.

6.3.6 Photograph the slices with a digital camera. Then, calculate the area at risk (AAR), infarct area, and non-ischemic zone using graphics software.

NOTE: After Evans-Blue/TTC double-staining, the blue area is the "normal" area. The remaining areas (including white and red) are the "ischemia risk" areas: the white area is the myocardial infarction area (IA), and the red area is the ischemic (but not infarcted) area. Taking the inconsistency of sizes of heart slices into account, the results are adjusted for weight.

Assign:
A1–A5 for Area of infarct zone /Area of the heart slice;
B1–B5 for Area of non-infarct zone/ Area of the heart slice;
W1–W5 for Weight of the heart slice.

Then:
Total weight of infarcted myocardium: $W1 \times A1 + W2 \times A2 + W3 \times A3 + W4 \times A4 + W5 \times A5$;
Total weight of non-infarcted myocardium: $W1 \times B1 + W2 \times B2 + W3 \times B3 + W4 \times B4 + W5 \times B5$;
Total weight of AAR
$= (W1 + W2 + W3 + W4 + W5) - (W1 \times A1 + W2 \times A2 + W3 \times A3 + W4 \times A4 + W5 \times A5)$

Finally:
The area of myocardial ischemia is calculated as the percentage of AAR in the left ventricle:

$$\frac{(W1 + W2 + W3 + W4 + W5) - (W1 \times A1 + W2 \times A2 + W3 \times A3 + W4 \times A4 + W5 \times A5))}{(W1 + W2 + W3 + W4 + W5)}$$

The area of myocardial infarction is calculated as the percentage of IA in the AAR:

$$\frac{(W1 \times A1 + W2 \times A2 + W3 \times A3 + W4 \times A4 + W5 \times A5)}{((W1 + W2 + W3 + W4 + W5) - (W1 \times A1 + W2 \times A2 + W3 \times A3 + W4 \times A4 + W5 \times A5))}$$

REPRESENTATIVE RESULTS:

The experimental workflow is shown in **Figure 1A**. The researcher can schedule the time nodes according to the experimental design upon study initiation. The duration of LAD ligation is according to the research purpose. For MI, the research can ignore the reperfusion step. Cardiac ultrasound is available at different stages of the study because it is non-invasive, while Evans-Blue/TTC staining can be performed only when the mouse is sacrificed. For research

that focuses on fibrosis and ventricular remodeling, the observation time is much longer.

The typical images for part of the experiment process are shown in **Figure 2A**, from endotracheal intubation, skin incision, thoracotomy, LAD identify, LAD ligation to reperfusion. To verify myocardial ischemia and reperfusion, the representative ECG images with significant ST-elevation after ligation and dissolution of ST-elevation when the slipknot is untied are shown in **Figure 2B**.

After obtaining blood samples from all mice, the troponin-T test can be undertaken to validate infarction. **Figure 3A** shows a significant increase of cTnT in MIRI and MI groups when compared with the sham groups. **Figure 3B** demonstrates the double-staining of Evans-Blue and TTC for five consecutive transverse sections of the heart between the sham group and MIRI group. The blue area suggests the normal area, the white area suggests the myocardial infarction area, and the red area suggests the ischemic but not infarcted area. **Figure 3C** represents the long-axis images of cardiac ultrasound between the sham group and MI group. Software applications can be used to calculate different functional parameters, such as a higher value of ejection fraction for the sham group in Figure 3C compared with that in the MI group.

FIGURE AND TABLE LEGENDS:

Figure 1: Surgical setup. (**A**) Overview of the experimental timeline. (**B**) Operating table with a prewarmed heating pad and connection for ECG electrodes. (**C**) Homemade retractors.

Figure 2: Experimental process and ECG changes. (**A**)Images of endotracheal intubation, skin incision, thoracotomy, LAD identification, LAD ligation, and reperfusion are shown in 1, 2, 3, 4, 5, and 6, respectively. (**B**) Typical ECG images of MI and MIRI after ligation and reperfusion.

Figure 3: Validation after the procedure. (**A**) Expression of cardiac troponin among sham, MIRI 24 h, and MI 3 d groups. (**B**) Evans -Blue/ TTC double-staining for sham and MIRI 24 h groups. (**C**) Cardiac ultrasound for sham and MI groups. LVID; d, end-diastolic left ventricular internal dimension; LVID; s, systolic left ventricular internal dimension.

DISCUSSION:

In recent years, the creation of models for MI and MIRI in clinical and scientific research has developed rapidly[20,21]. However, there are still some questions, such as the mechanisms of actions and how to ameliorate MI/MIRI, that must be resolved. Here, a modified protocol for establishing a murine model of MI and MIRI is described. Several key points must be considered carefully.

The first key point is endotracheal intubation. Some procedures[6,9] involve incision of the cervical skin, tissue separation, followed by exposure of the sternohyoideus muscle to view the trachea. In that way, the researcher can visualize tube insertion into the trachea. This is a good step to reduce the risk of respiratory distress. In the current method, the researcher can clearly visualize the glottis's closing and opening with breathing under an illuminator and then

insert the tube into the trachea easily. Hence, a cervical incision is not made to reduce skin trauma and potential infections, which is important in research on inflammatory signaling. Visual laryngoscopes are used widely in clinical tracheal intubation: maybe they can be used in mice too. Mares et al.[22] reported the continuous mask inhalation anesthesia without endotracheal intubation, which was performed by 2% isoflurane inhalation after 5% isoflurane induction with oxygen administered through a non-invasive mask placed over the nose and mouth of the animal. It can avoid tissue damages and improve the safety and efficiency of anesthesia. However, a special inhalation anesthesia machine is needed. Moreover, volatile anesthetics can cause physical harm to the operator.

The second and most important key point is the identification and ligation of the LAD**.** Every mistake in the LAD identification and ligation will lead to inconsistent results: either too large infarct size resulting in death or too small infarct size resulting in failure. Various methods can be applied to identify the LAD and verify its ligation. Here, a dissection microscope is used to locate the LAD. The LAD usually appears as a thin red line running perpendicular from near the apex and down through the left ventricle. By pressing the site below the chosen ligation position gently to enlarge the LAD temporarily (≤5 s per time), the LAD can be checked again. After ligation, LAD occlusion is verified by a paler color in the anterior wall of the left ventricle and significant ST-elevation within a few heartbeats. Then, the ligation is untied, and reperfusion is validated by a color change back to pink-red within 20 s and potential dissolution of ST-elevation upon ECG. Finally, the troponin-T test, TTC staining, and cardiac ultrasound are employed to evaluate the myocardial injury. These multiple insurances and mutual verifications make the experimental results highly reliable. Moreover, micromanipulation elicits higher accuracy and fewer complications (e.g., bleeding). Another important issue is the assumption that the blood vessels of mice are normal, but in fact, some coronary arteries vary greatly, and even collateral circulation can present[23–24]. Hence, the infarct sizes are sometimes not consistent even though the ligations are deemed to be at the same level. The advantages of the microscope are exhibited here. Ligation cannot be done based only on experience or anatomical landmarks: the LAD and its direction must be verified clearly before ligation otherwise, the results will be unreliable. In some experiments[6,8], mice are in the right lateral decubitus position for the convenience of observing the anterior wall of the left ventricle and coronary arteries after heart exposure.

This model has two main limitations. First, LAD ligation cannot simulate occlusion of the right coronary artery. In fact, due to anatomical differences among animals[25], the LAD usually extends to the apex of the heart in mice and rats, and the left circumflex branches are not developed, so the models in mice and rats are established by LAD ligation. For large and medium-sized animals such as rabbits and pigs, the LAD is relatively short, whereas the left circumflex artery covers a large area of the heart, so ligation of the left circumflex artery is selected to establish the model. Sicard et al.[26] reported a novel method to investigate right-ventricular dysfunction and biventricular interaction by ligating the right coronary artery in mice, which could remedy this limitation. The second limitation is an inconsistent infarct size due to variability in coronary-artery anatomy[27] and the experience of the surgeon. As discussed above, the microscope is very important for increasing consistency by verifying the

LAD and its direction before ligation, and for an experienced researcher, adjusting the ligation position after a complete assessment of vascular anatomy can be achieved.

Some other issues merit mention. For example, thoracotomy and needle-piercing will inevitably cause slight damage to muscles and the myocardium, which may have effects upon inflammation. In addition, analgesic agents were reported to have effects on MI[28]. Hence, these factors must be taken into consideration when analyzing inflammation or its effects on MI. For troubleshooting, there are several factors that would lead to mice death. For example, complications related to myocardial infarct, anesthetic accident, and bleeding. Moreover, the inconsistent results mainly come from inappropriate ligation positions: too high a ligation position would induce too large infarct size even mice death; meanwhile, the false identification of LAD would result in model failure. Some details need to be improved in this method. For example, it would be better if a rectal probe could be inserted to monitor the temperature during the procedure. Last but not least, the experimenter should keep in mind the differences between animal studies and clinical realities, especially that the 30 min ischemia time is indeed quite short for clinical. We encourage the researcher to arrange the steps according to their experiment design, including the ischemia time. Only in this way can this protocol be useful for studies of the mechanism and treatment of MI/MIRI and drug discovery.

In short, a simple and reproductive murine model for MIRI and MI is provided. This model can be used for the study of MI/MIRI mechanisms and therapeutic research.

ACKNOWLEDGMENTS:

This work was supported by the National Natural Science Foundation of China (82070317, 81700390 to Jibin Lin, 8210021880 to Bingjie Lv and 82000428 to Boyuan Wang) and the National Key R&D Program of China (2017YFA0208000 to Shaolin He).

DISCLOSURES:

The authors declare no conflict of interest.

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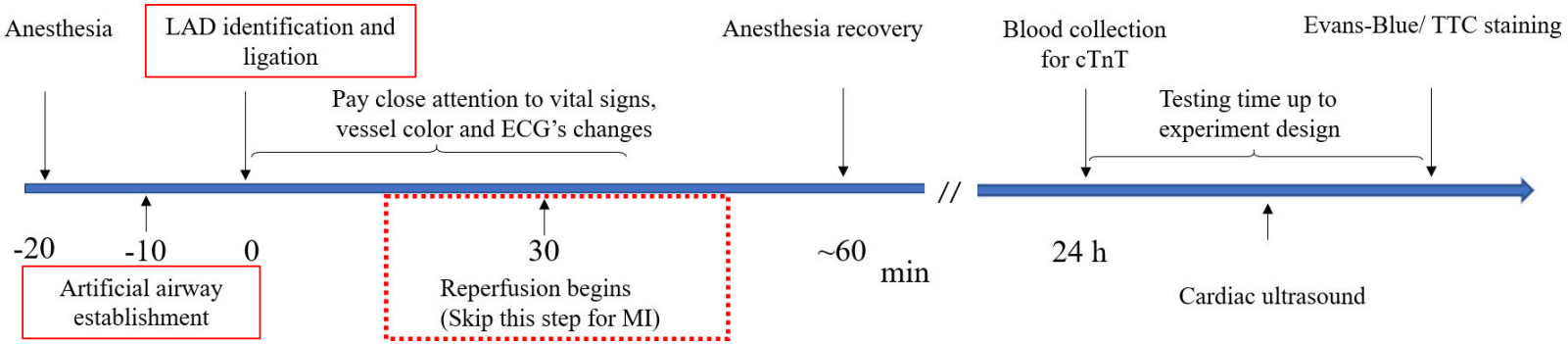
Figure 1

A



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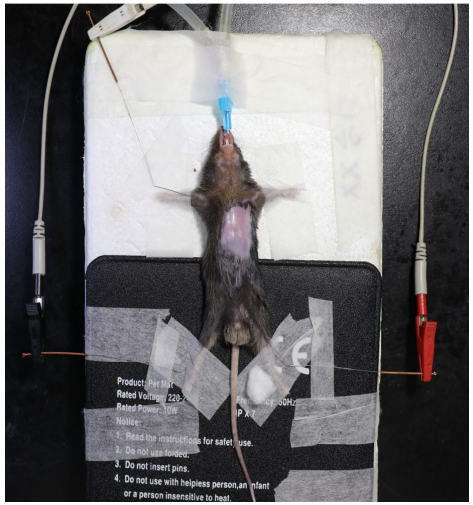


C

Figure 2

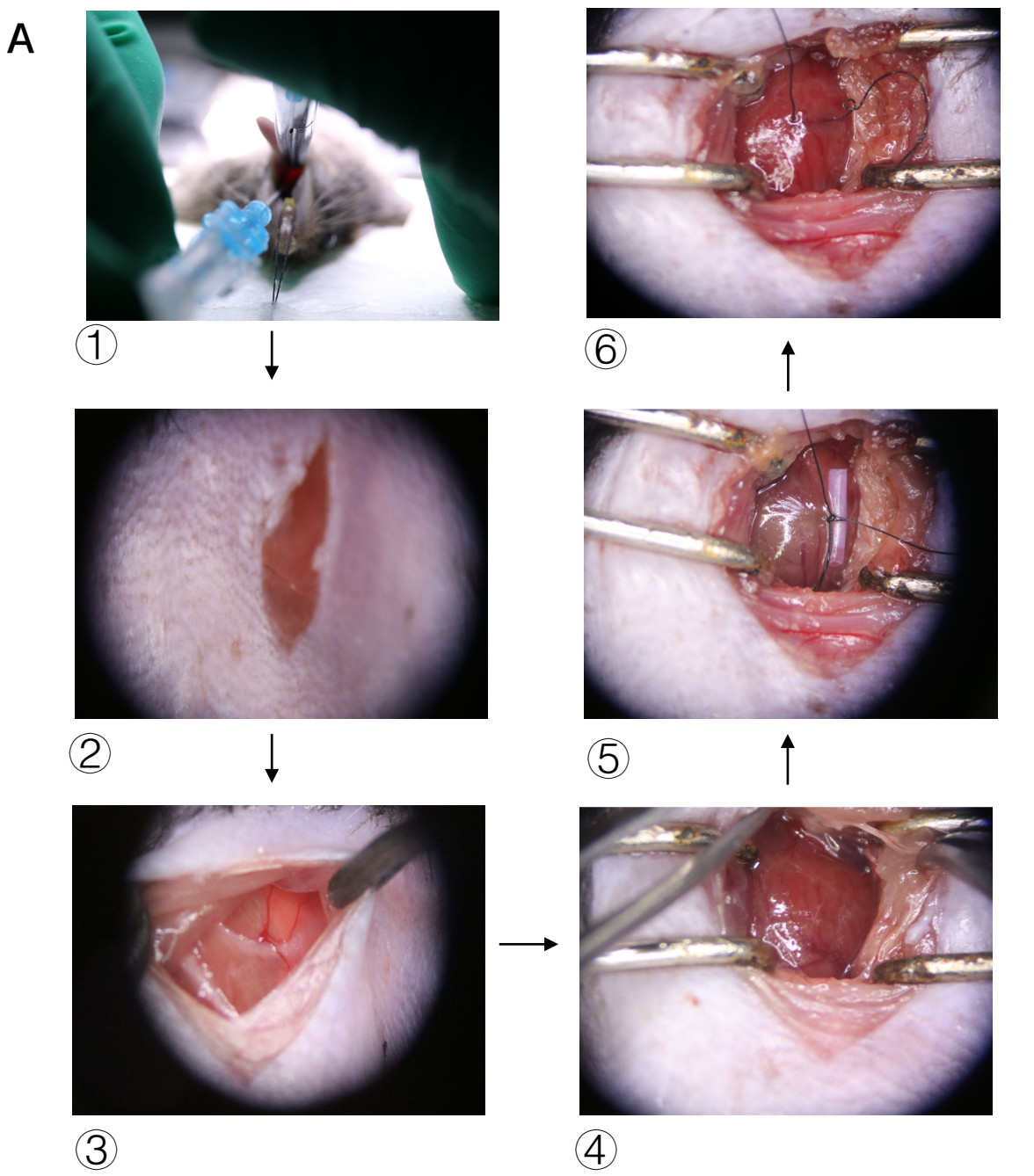
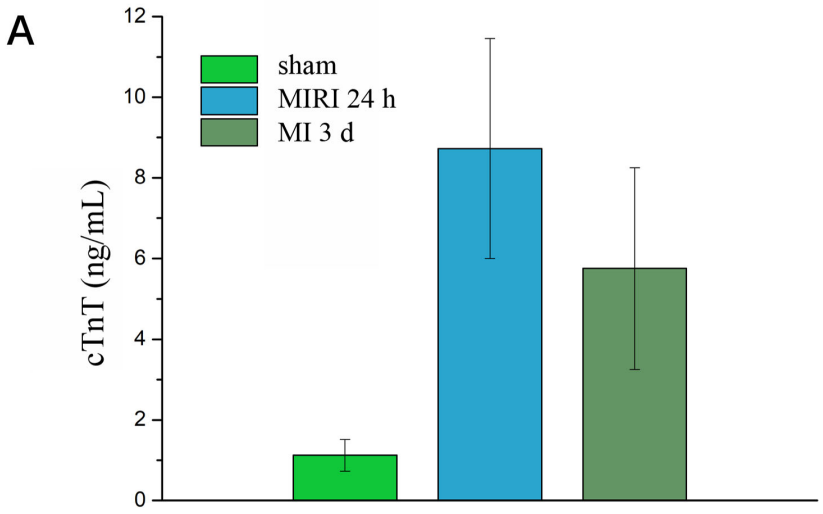
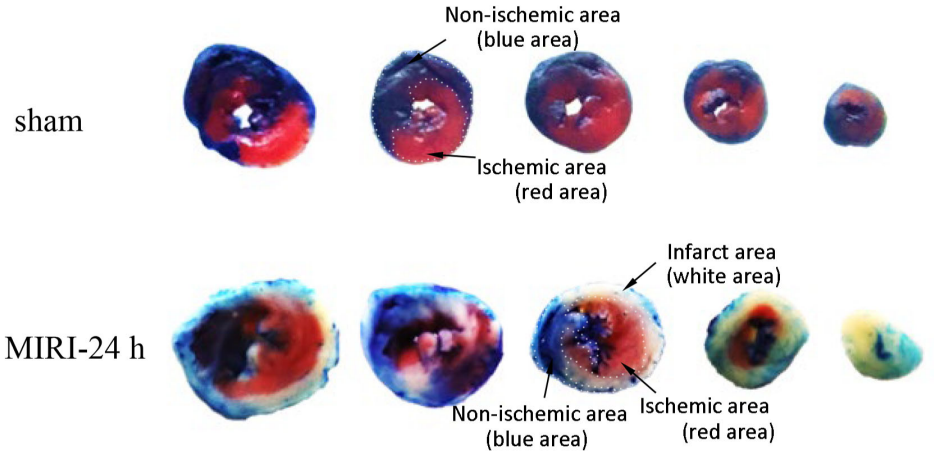
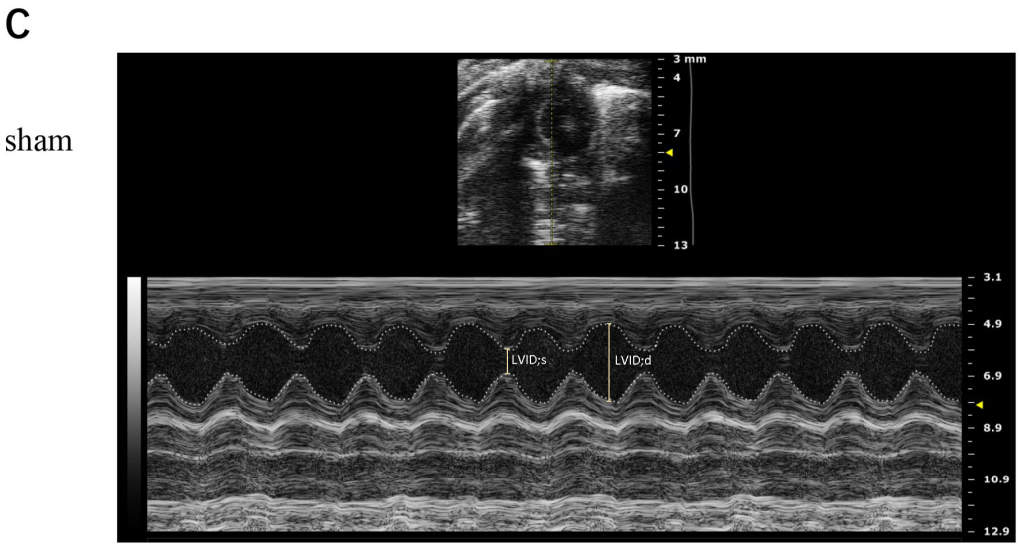


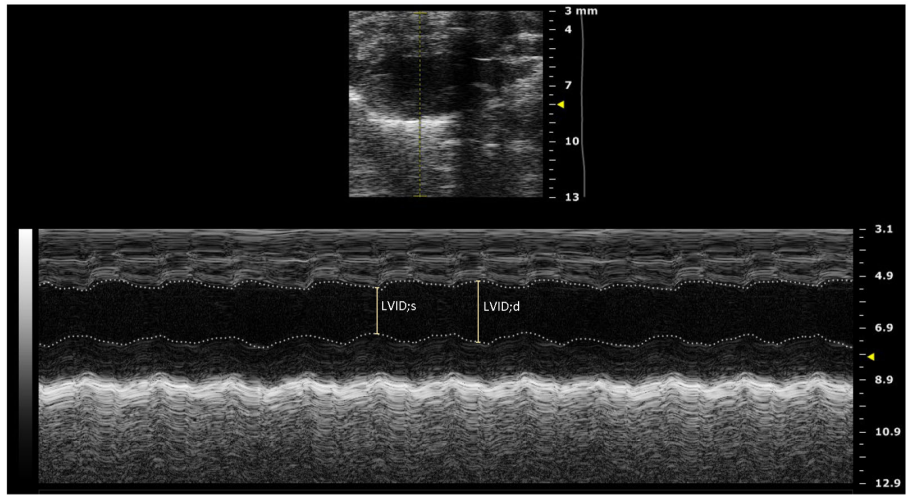
Figure 3

Click here to access/download;Figure;Revised Figure3.pdf









Response to Reviewer/Editor Comments

We thank the reviewers and editor for the excellent suggestions for our manuscript "JoVE63257". Below we provide a point-by-point response to each question raised.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please note that the manuscript has been formatted to fit the journal standard. Some comments to be addressed are included within the manuscript. Please review and revise accordingly.

Response to Editor Q1: We have carefully and thoroughly read the manuscript again to correct any potential writing errors. We thank the editor for the comment addressed within the manuscript. We have revised all of them in the new manuscript version.

2. 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.

Response to Editor Q2: Thanks for the reminding. We have rechecked the whole manuscript to write the sentences in the imperative tense wherever possible. The usage of phrases such as “could be,” “should be,” and “would be” were removed. We add some more “NOTE” in the text now.

3. Please ensure that each step contains no more than2-3 actions.

Response to Editor Q3: We have simplified the protocols the make each step contains no more than 2-3 actions.

4. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next and fits the 3-page limit.

Response to Editor Q4: We have highlighted the steps as required.

5. As we are a methods journal, please ensure that the Discussion explicitly covers the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

Response to Editor Q5: The Discussion have already contained all the five details above.

6. Figure 3: Please label 3B and 3C to make them more informative.

Response to Editor Q6: Thanks for the reminding. We have added ischemia area, infarct area and non-ischemia area in the New Figure 3B and added LVID;d , LVID;s in the New Figure 3C.

7. Please ensure that the Table of Materials includes all the supplies (reagents, chemicals, instruments, equipment, software, etc.) used in the study. Please sort the table in alphabetical order.

Response to Editor Q7: We have put all the supplies information in the Table of Materials. We sort the table in alphabetical order now.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors have addressed most questions raised by the reviewers.

Response to Reviewer #1: Thanks for the summary.

Minor comment

1. Although the authors claimed that the paper has been proofread by professional agencies, there are several hard-to-read sentences and grammar mistakes needed to be fixed. e.g., lines 43-44; lines 63-65; lines 70-72; lines 449-450, etc...

Response to Reviewer #1 Q1: We are sorry for the not good enough overall reading experience cause by writing. The image below is the language editing certificate from Charlesworth. We have polished the manuscript carefully again.

EDITORIAL CERTIFICATE

This document certifies that the manuscript below was edited for correct English language usage, grammar, punctuation and spelling by qualified native English speaking editors at Charlesworth Author Services.

Paper Title:

The protocol for myocardial infarction and myocardial ischemia and reperfusion injury in mice

Author:

少林 何

Date certificate issued:

October 13, 2021

2. In Figure 1A, "Blood Collection for cTnT" and "Testing Time Up to experiment design", please correct the caps to lowercase except the first letter.

Response to Reviewer #1 Q2: Thanks for the suggestion. We have corrected them in the new Figure 1A.

Reviewer #2:

Major Concerns:

The revised manuscript did not address my major concern on novelty (procedures or efficacy) of this study. The protocol reported in this manuscript is not different from numerous reports published in JoVE and other journals. The report does not improve efficacy of MI or MIRI models. There is not data to support the authors' claim that the procedures reported in this manuscript would reduce surgical time or improve animal survival. Also, some of the procedures including

fasting and use of anesthetics would not be acceptable by most institutions.

Response to Reviewer #2: Thanks for the bluntly comment. As a well performed experiment model in the past 20 years, we agree that there were numerous reports published in JoVE and other journals. In our last experiment, 50 mice were used. Based on the troponin test result, the success rate for MIRI is 93.3% (14/15) and 94.1% (16/17) for MI. The average surgical time is around 25 minutes per mouse (not including anesthesia recovery). The survival rate is 84% (42/50). An experienced performer should have a success rate higher than 80% in our lab. As far as we know, all these values are better than previous reports. We believe that all these indicators will improve with experience increase. For the issues about fasting and anesthetics, we had accepted the reviewer's comment and revised them in the previous manuscript version.

Reviewer #3:

Manuscript Summary:

This is a very detailed and adequate description of a widely used technique in murine experiments.

Response to Reviewer #3: Thanks for the summary.

Major Concerns:

None

Minor Concerns:

1. The standard of care therapy for ST-segment elevation myocardial infarction (STEMI) is primary percutaneous coronary intervention, performed in a timely fashion, to induce myocardial reperfusion. During the first 30 to 40 minutes of ischaemia, the myocardial changes are reversible and visible only at the electron microscopy level. Infarction can occur secondary to ischaemic injury. However, the process of reperfusion can itself induce cardiomyocyte death, which is reperfusion injury in the strict sense. My point is that the 30 minutes ischemia time is indeed the standard time in animal experiments but quite short compared to clinical reality. Patients are rarely reperfused within 30 minutes. It should be indicated that this 30 minutes period can be amended.

Response to Reviewer #3 Q1: We totally agree with the reviewer about the differences between animal experiments and clinical reality. We encourage the researcher to arrange the steps according to their experiment design, including 30 mins ischemia time. Only in this way can this protocol be useful for studies of the mechanism of action and treatment of MI/MIRI, as well as for

drug discovery. We add this indication in the Discussion:

“Last but not the least, the experimenter should keep in mind about the differences between animal studies and clinical realities. Especially that the 30 minutes ischemia time is indeed quite short for clinical. We encourage the researcher to arrange the steps according to their experiment design, including the ischemia time. Only in this way can this protocol be useful for studies of the mechanism and treatment of MI/MIRI, as well as for drug discovery.”

2. Reference 7 and 8. Prior to this, the model of permanent ligation of the LAD was described in detail in JOVE by Muthuramu et al. PMID: 25489995 PMCID: PMC4354439 DOI: 10.3791/52206

Response to Reviewer #3 Q2: Thanks for the remind. We have added this important paper into the reference.