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TITLE:

A New Model of Heart Failure Post-Myocardial Infarction in the Rat

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KEYWORDS:
heart failure model, myocardial infarction, LAD ligation

SUMMARY:

We successfully developed a reliable and reproducible model of heart failure post-myocardial infarction in the rat without ventilation or exteriorization of the heart. This simplifies the procedure and benefits the further studies about the potential mechanisms behind the heart failure.

ABSTRACT:

Ligation of the left anterior descending (LAD) coronary artery has been widely used to establish the rat model of heart failure (HF) post myocardial infarction (MI). However, the disadvantages of this model include high mortality rate after ligation and larger variations both in the infarct size and the degree of impaired cardiac function. In addition, a ventilator or exteriorization of the heart is indispensable for the previous models, which complicates the procedure during the ligation. In this study, we developed a reliable and reproducible model without the ventilator or exteriorization of the heart by ligating the LAD coronary artery. Four weeks after the procedure,

we found that the serum concentrations of CK-MB, NT-proBNP, and Renin, which were used to assist diagnoses of MI and HF, were significantly higher in the MI group compared to the sham group. In contrast, the value of left ventricle ejection fraction (LVEF) in the MI group was obviously less than in the sham group. Furthermore, the infarct size and cardiac fibrosis area were individually confirmed and quantitatively analyzed by TTC staining and Masson's trichrome staining. Smaller variations were found in either infarct size or fibrosis area in the MI group, which helped to develop a reliable and reproducible model of HF post-MI. This new model of HF post-MI in the rat is vital for studying the potential mechanisms of MI and HF. This new method can be used to develop the new drug for treatment of MI and HF in rats by using pharmacological strategies.

INTRODUCTION:

Heart failure (HF) is considered a global public health issue that affects over 26 million patients and the prevalence is still increasing[1]. HF is defined as when the heart does not pump blood as well as it should. There are many risk factors for HF, including ischemic heart disease, diabetes, hypertension, LV hypertrophy, obesity, smoking, etc.[2-4]. Myocardial infarction (MI) is one of the most common causes of HF[5]. For almost 50 years, HF following MI has been the major driver of late morbidity, mortality, and healthcare cost[6]. For better understanding of the pathophysiological mechanism of HF and to prevent and treat HF more effectively, large numbers of animal HF models post-MI have been developed for preclinical study. Rats are typically used due to easy maintenance, lower costs, ability for controls, and high homology with humans[7-9]. Left coronary artery (LCA) ligation, especially the left anterior descending (LAD) branch ligation, is widely used to develop the model of HF post-MI in the rat[8].

However, surgeries that induce MI in rats are often technically challenging to perform due to high variation, complicated operation, and high mortality[10, 11]. Even though quite a few procedures have been reported, the vast majority of surgeries required either ventilation after tracheal intubation or exteriorization of the heart. Those methods increased the cost of surgery, made the operation more difficult, and reduced the safety of the animal. In addition, the extent of HF is dependent on the site of the ligation of LCA. The anatomic diversity of the LCA in rats leads to inconsistency in infarction sizes and functional parameters in the rat model. Different ligation sites of LCA cause special infarct size, which determines the degree of HF and the survival rate.

In this study, we aimed to create a reliable and reproducible HF post-MI model. Our findings allowed for surgeries of rats without a ventilator or exteriorization of the heart, which resulted in a high survival rate. Also, the relatively fixed site of the ligation of LAD led to less variations of HF 4 weeks post-surgery. Furthermore, we have evaluated the effects of the HF model by using histopathological staining, biochemical detection, and cardiac function measurement, which helped to study the potential mechanisms behind the disease and develop new drugs to treat HF.

PROTOCOL:

The protocol was approved by the Animal Ethics Research Committee of Shandong University of Traditional Chinese Medicine.

NOTE: Adult male Sprague-Dawley rats, 10-weeks-old weighing 180-220 g, were randomly divided into 2 groups, sham (n = 25) and MI (n = 35). Animals were kept on a 12/12 h light/dark cycle and received water and food ad libitum.

1. Anesthesia and continuous supply of oxygen

1.1. Place rats into an induction chamber and anesthetize with 3% isoflurane.

1.1.1. To confirm whether the anesthetization was ready, observe the toe pinch reflex. The anesthetization is sufficient when the toe pinch reflex disappears.

1.2. Individually move the rats to a small temperature-controlled surgery board. Tape the rat's paws to the board.

1.3. Attach a mask to the rat face that provides a constant supply of 3% isoflurane and 1% oxygen.

2. Induction of MI without ventilation or exteriorization of heart

2.1. Use depilatory creams to remove the thorax hairs for sterilization.

2.2. Use a sterile drape with a 1 cm x 3 cm hole at the surgical field of the rat.

2.3. Confirm sufficient depth of anesthesia with a toe pinch before operating.

2.4. Open the chest with a transverse 2.5 cm incision between the third and fourth intercostal space on the left edge of the sternum without cutting the ribs.

2.5. Separate the pectoralis major and pectoralis minor muscles by using two vascular forceps without cutting the tissue or small blood vessels. Position the Weitlaner self-retaining retractor into the thorax to separate the third and fourth ribs to gain enough exposure of the heart while keeping rib integrity.

2.6. Delicately dissect the pericardium with two forceps.

2.7. Use a 6/0 nylon suture with a curved needle to permanently ligate the LAD 2.0-2.5 mm below the midpoint of the conus arteriosus and left atrial appendage connection. LAD can be easily recognized and found in some rats.

2.7.1. Confirm appropriate ligation of the LAD after the anterior wall of the left ventricle turned pale.

2.7.2. In the sham group, pass only the suture under the LAD, but do not ligate.

2.8. Remove the retractor and squeeze the air in the thorax out before suturing the muscle and skin layer by layer.

2.9. After surgery, place the rats on a 37 °C heating pad for recovery. Monitor all animals, and provide ketoprofen (5 mg/kg SC) for analgesia every 24 hours up to 72 hours.

NOTE: For technical reasons, rats with smaller body weights (less than 250 g) are preferred for this operation. The time of keeping chest open, from step 2.4-2.8, was limited to 5 minutes.

3. Assessment of cardiac function

NOTE: To confirm whether HF developed successfully, the cardiac function was measured 4 weeks post-procedure using a TE7 ultrasound system equipped with a 13 MHz electronic transducer. During the echocardiography test, the rats were under the same anesthesia protocol used for the initial procedure.

3.1. After shaving the anterior chest hair, position the rats on the board used for the previous operation.

3.2. Place the probe on the anterior chest wall and obtain images from the left parasternal long-axis (PLAX) views of the left ventricle (LV) (at the level of papillary muscles).

3.3. Measure the left ventricular internal dimensions at end-diastole (LVIDd) and end-systole (LVIDs) by M-mode from three consecutive cardiac cycles.

3.4. Calculate the LV ejection fraction (LVEF) and the LV fractional shortening (LVFS) by the software of the machine.

4. Further analysis of the HF model 4 weeks after LAD ligation

4.1. Sacrafice the rats 4 weeks after LAD ligation by administering an overdose of isoflurane. Measure the serum concentrations of CK-MB, N-terminal pro-brain natriuretic peptide (NT-proBNP), Renin, Angiotensin (AngII), Aldosterone (ALD), TNF-α, VEGF, IL-6, and HIF-1α by using the ELISA kits according to manufacturer's protocol.

4.2. To harvest the hearts for histopathological analysis, perfuse the rat hearts with 4 °C physiologic saline before being removed.

4.3. Set the hearts in 10% formalin for 24 hours, embed in paraffin, and slice at 5.0 μm thick sections transversely along the left ventricular axis.

4.4. Mount all sections of the heart on glass slides and individually stain with Hematoxylin and Eosin (HE) and Masson's trichrome.

4.5. For the purpose of infarct size measurement, stain hearts with 2,3,5-Triphenyl tetrazolium chloride (TTC).

4.5.1. Slice the hearts transversally at 3 mm thick from apex to base in a semi-frozen state and incubated in 2% TTC solution for 20 minutes at 37 °C.

4.5.2. When the color was established, fix slices in 4% paraformaldehyde solution for 15 minutes and take pictures.

4.5.3. Mark the infarct size and calculate by using ImageJ. Express the infarct size as a percentage of the infarct area versus the total LV area.

4.6. To quantitatively analyze the collagen content in the middle of the infarcted area of LV, stain the rat heart tissue sections with Masson's trichrome.

4.7. After staining, scan the images and analyze. Evaluate the degree of fibrosis by the collagen volume fraction (CVF) which was expressed as a percentage of the fibrotic area versus the total LV area.

REPRESENTATIVE RESULTS:

The procedure was performed with a low mortality rate. The key instruments used for this experiment are the Weitlaner Self-Retaining Retractor (13.5 cm) and isoflurane vaporizer shown in **Figure 1**. The MI model was developed without ventilation or exteriorization of the heart as described in the protocol. During the whole procedure, all ribs were kept in integrity and the entire procedure took about 10 minutes. The schematic diagram of the surgical ligation site is shown in **Figure 2**. In this study, 2 rats died from ventricular fibrillation during the ligation procedure in the MI group, and 1 rat died due to bleeding after the heart was accidently pierced by the curved needle in the sham group. The mortality rate was about 5% throughout the experiment.

The cardiac function of rats was reduced significantly in the MI group, and HF was successfully developed. Echocardiographic measurements were obtained in rats 4 weeks after the procedure to evaluate the effects of the HF models (**Figure 3**). Based on the 2016 ESC guidelines for the diagnosis and treatment of acute and chronic heart failure[12], the rats with LVEF less than 50% are considered as successful HF models. The main parameters related to heart failure were summarized in **Table 1**. When comparing the MI group to the sham group, LVEF in the MI group reduced significantly (32.7% ± 8.0 VS 75.3% ± 4.9, $P<0.001$). These significant decreases of FS and increases of LVIDd and LVIDs in the MI group were good signs of HF. Additionally, the changes in the ventricular structure were seen from the ultrasound images (**Figure 3**). The chamber of LV got larger, and the wall of LV got thinner and stiffer in the MI group compared to the sham.

Assessment of biomarkers of MI and HF post-MI by ELISA

As shown in **Figure 4** and **Figure 5**, the serum concentrations of the cardiac marker CK-MB used to assist the diagnosis of MI rose significantly by more than 3 times in the MI group. Meanwhile, some parameters related to heart failure such as renin, AngII, and ALD serum concentrations were higher compared to the sham group 4 weeks post-procedure. The concentrations of NT-proBNP in the MI group was 13 times higher than in the sham group. Also, the concentrations of pro-inflammatory cytokines in the MI group, including TNF-α and IL-6 were increased by 400% and 300% compared to in the sham group. Meanwhile, as the representative angiogenesis-related factors like VEGF, and HIF-1α were also significantly higher by 2 and 5 times in the MI group compared to in the sham group.

Morphological alterations and histopathology analyses

In the MI group, the morphological analysis of the hearts revealed a thin and pale LV wall as well as fibrous scar formation (**Figure 6**). Additionally, MI was also verified using TTC staining and the infarct size was tested (**Figure 6**). The infarct size was 40.7±4.4% 4 weeks after the procedure in the MI group, which showed the reliability and stability of the new method of HF post-MI. For HE staining, microscopic evaluation displayed a neat arrangement of myocardial fibers without inflammatory change in the sham group. However, the myocardial fibers became a loose and irregular arrangement with inflammatory cellular infiltrates in the MI group (**Figure 7a**). In addition, Masson's trichrome staining revealed the areas of cardiac fibrosis were increased in the MI group (**Figure 7a**), and the collagen volume fraction (CVF) was 39.2±6.9% in the MI group. The Masson's trichrome staining results were consistent with the TTC staining, which further confirmed the successful development of MI and HF models (**Figure 7b, 7c**).

Figure 1. Key instruments were used to establish the MI model. (**a**) The Weitlaner Self-Retaining Retractor (13.5 cm) (Third from left); (**b**) The oxygen supply equipment; (**c**) The isoflurane vaporizer.

Figure 2. Experimental schematic. (**a**) Exposure of the heart with Weitlaner Self-Retaining Retractor; (**b**) The ligation location is indicated. Asterisk illustrates the ligation position. LCA, left coronary artery; LAD, left anterior descending.

Figure 3. Echocardiographic measurements. (**a**) The representative images of left ventricle structures from the sham and MI group tested by M-Mode during 3 cardiac cycles after 4 weeks of the procedure; (**b**) LVEF of rats after 4 weeks of the procedure from the sham (n= 24) and MI (n=33) group. MI, myocardial infarction. LVEF, left ventricle ejection fraction. ***P < 0.001 compared with the sham group.

Figure 4. Concentrations of CK-MB, NT-proBNP, ALD, Renin and AngII were increased 4 weeks after LAD ligation. Data were expressed as mean **±** SD (n = 8 animals in each group). MI, myocardial infarction; LAD, left anterior descending. ***P < 0.001 compared with the sham group. ****P < 0.0001 compared with the sham group.

Figure 5. Concentrations of TNF-α, IL-6, VEGF, and HIF-α were **increased 4 weeks after LAD ligation.** (a, b) Concentrations of TNF-α and IL-6 associated with inflammation response were increased 4 weeks after LAD ligation; (c, d) Concentrations of VEGF and HIF-α associated with angiogenesis were increased 4 weeks after LAD ligation. Data are expressed as mean ± SD (n = 8 animals in each group). LAD, left anterior descending; MI, myocardial infarction. **P < 0.01 compared with the sham group. ***P < 0.001 compared with the sham group.

Figure 6. Morphological analysis of the hearts. (**a**) Gross observation and histology of rat hearts from sham and MI group 4 weeks after procedure. The MI heart showed a thinner and bigger left ventricle wall compared to the sham; For the TTC staining hearts of the sham and MI group, viable tissue was stained red and infarct area was pale and unstained. (**b**) MI infarct size was expressed as the percentage of the infarct area relative to the whole LV. Data are expressed as mean ± SD (n = 10 animals in each group).MI, myocardial infarction; TTC, triphenyl tetrazolium chloride; LV, left ventricle. Scale bar= 5mm. ****P < 0.0001 compared with the sham group.

Figure 7. HE and Masson's trichrome staining of the rat heart tissue 4 weeks after the procedure. (**a**) The MI heart LV wall became thinner than the sham group (HE×10, Scale bar = 2 mm). Microscopic evaluation displayed a neat arrangement of myocardial fibers without inflammatory change in the sham group and displayed a loose and irregular arrangement with inflammatory cellular infiltrates in the MI group (HE×200, Scale bar = 100 μm); (**b**) Masson's trichrome staining of heart tissue shows the myocardial fibrosis as blue in the MI group (Scale bar = 2 mm). (**c**) Collagen volume fraction for Masson's trichrome staining in left ventricular tissue slices from the sham and MI groups. Data are expressed as mean ± SD (n = 6 animals in MI group). HE, hematoxylin and eosin; MI, myocardial infarction; LV, left ventricle. ****P < 0.0001 compared with the sham group.

Table 1: Echocardiographic data of rats in the sham and MI group 4 weeks after LAD ligation. Data were expressed as mean ± SD. MI, myocardial infarction; LVIDd, left ventricular internal dimensions at end-diastole at end-diastole; LVIDs, left ventricular internal dimensions at end-systole; FS%, percent fractional shortening. *P < 0.05, compared with the sham group. ***P < 0.001 compared with the sham group.

DISCUSSION:

Although there have been many existing models of MI or HF in rodents[13-15], this study has developed a novel and efficient LAD ligation procedure for inducing HF post-MI in rats. However, in this new rat HF model, the needs for intubation and ventilation or exteriorization of the heart were eliminated, which significantly increased the survival rate of the rats. To develop this new rat HF model, the induction of MI is a crucial step. In comparison to the conventional protocols which usually involved intubation and ventilation or exteriorization of the heart during ligation[9, 16, 17], we developed an improved approach with neither ventilation nor exteriorization of the heart for the first time allowing for a higher survival rate. It is worth noting that the improved procedure leads to less tissue damage. Therefore, rats have a much faster post-surgery recovery and higher survival rate. The following two points allowed us to achieve these results: First, the continuous inhalation of oxygen during the whole procedure allowed for sufficient oxygenation

and ligation without ventilation. Second, the Weitlaner self-retaining retractor, a key surgical instrument, was used to gain enough exposure to the heart while keeping rib integrity, which helped to avoid the exteriorization of the heart.

It is well known that successful development of HF post-MI in rat largely depends on the infarct size, which is related to the ligation site of the LCA. As early as 1979, Marc A. Pfeefer and his colleagues reported that LCA occlusion in rats could readily provide left ventricular free wall infarctions of varying sizes[18]. To reduce the variation of infarct sizes and to develop a more stable HF model post-MI, LAD ligation has been commonly used in rats to induce left ventricular infarction for research purposes[19]. The major advantage of LAD occlusion is to allow for accurate ligation of this artery to induce a stable MI that can cause HF while keeping a much higher survival rate. In our procedure, we chose the ligation position 2.0-2.5 mm below the midpoint of the connection between the conus arteriosus and left atrial appendage, which proved to be successful and stable with less variations in LVEF among all the rats in the MI group. In addition, we individually determined the infarct size by TTC staining and the degree of fibrosis by Masson's staining, both of which have been widely used to evaluate this kind of model[20-22]. Meanwhile, the less variations of this model were also illustrated through morphological alterations and histopathology analyses.

We also studied some parameters related to MI and HF after 4 weeks of the LAD ligation to help confirm the development of HF after MI. Echocardiography has the ability to accurately and noninvasively measure ventricular function and assess causes of structural heart disease[23]. The LVEFs of the mice in the MI group were all under 50% which could be considered as HF[12]. In line with these results, the concentration of NT-proBNP was increased accordingly which was also an important indicator of impairment of heart function. Ample evidence exists for inflammation and apoptosis in the ischemic heart[24, 25]. Inflammation and heart failure are strongly interconnected and mutually reinforce each other[26]. In our study, we evaluated the significant increase in inflammation factors, IL-6 and TNF-a. The renin-angiotensin-aldosterone system (RAAS) activated by renal hypoperfusion and sympathetic activation is a central feature in the pathophysiology of heart failure[27]. We evaluated the parameters involved in the RAAS and we found that the serum level of renin was significantly higher in the MI group compared to the sham group, which further verified the development of HF.

There are two main points of emphasis during the procedure of LAD ligation. First, during the whole procedure and until they recover from anesthesia, provide a continuous supply of oxygen to the animals while also keeping them warm. Secondly, the position of ligation on the LAD has a key role in the infarct size and the degree of HF. The site 2.0-2.5 mm below the midpoint of the connection between the conus arteriosus and left atrial appendage is appropriate for a stable and successful HF model with less mortality rate. However, the limitation of the new HF model in rats is that the surgical procedure for developing MI without ventilation is technically demanding and challenging. The key step for achieving this model is to gain enough exposure of the heart to fully visualize the heart inside the thorax and limit the time the chest is open to 5 minutes, which requires more practice before the formal experiment.

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DISCLOSURES:

The authors have nothing to disclose.

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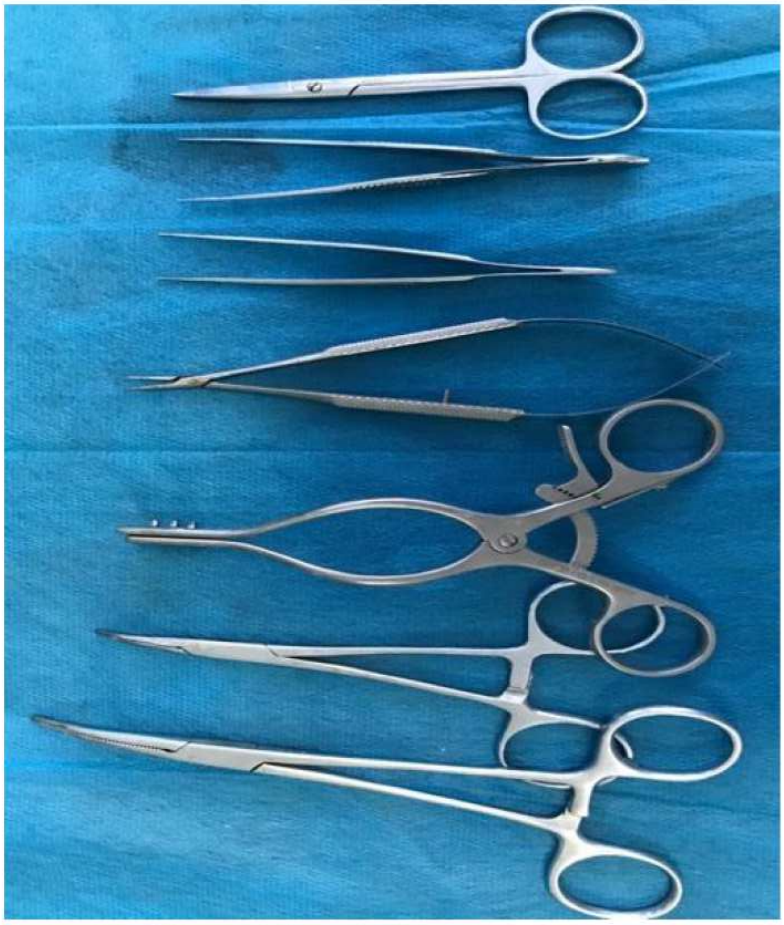
Figure 1

(c)



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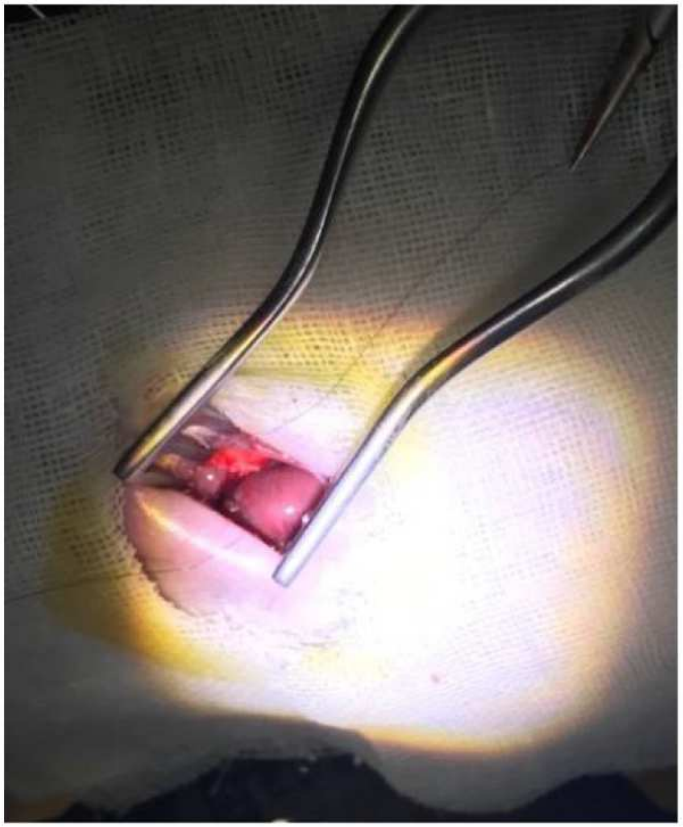

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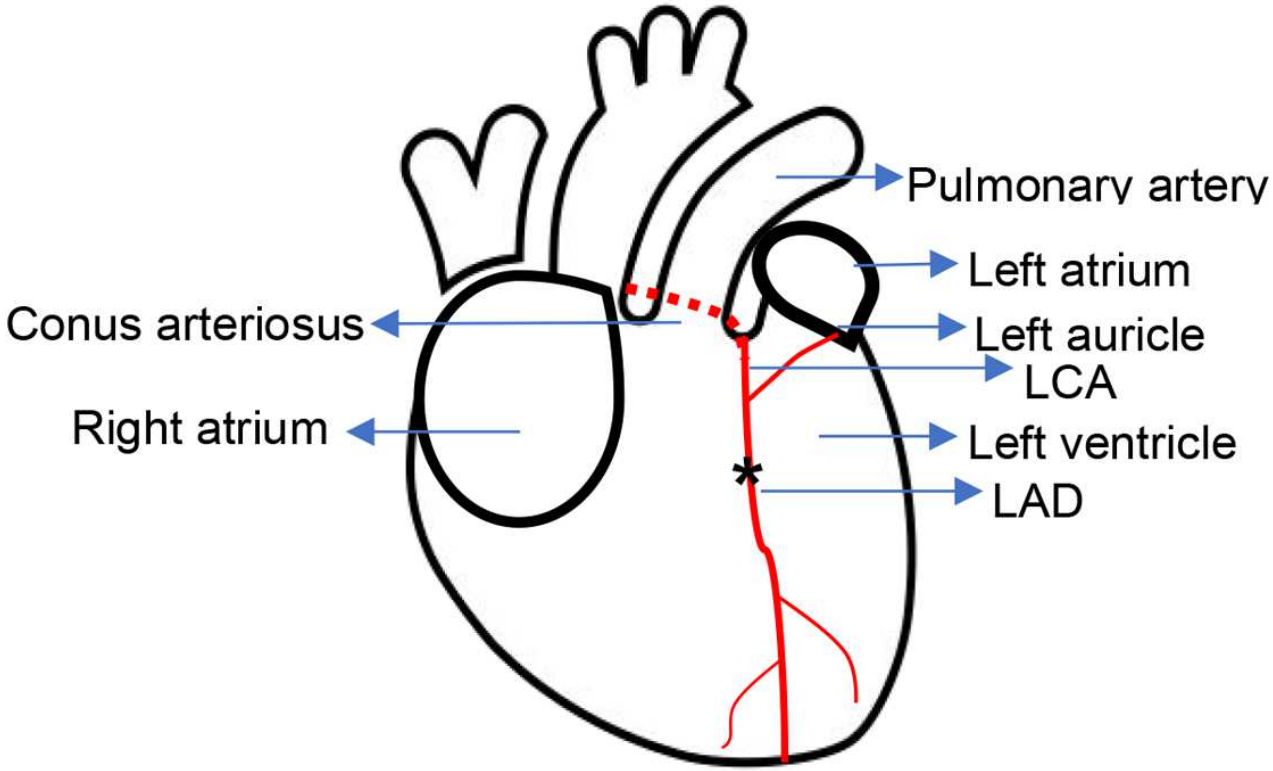

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Figure 3

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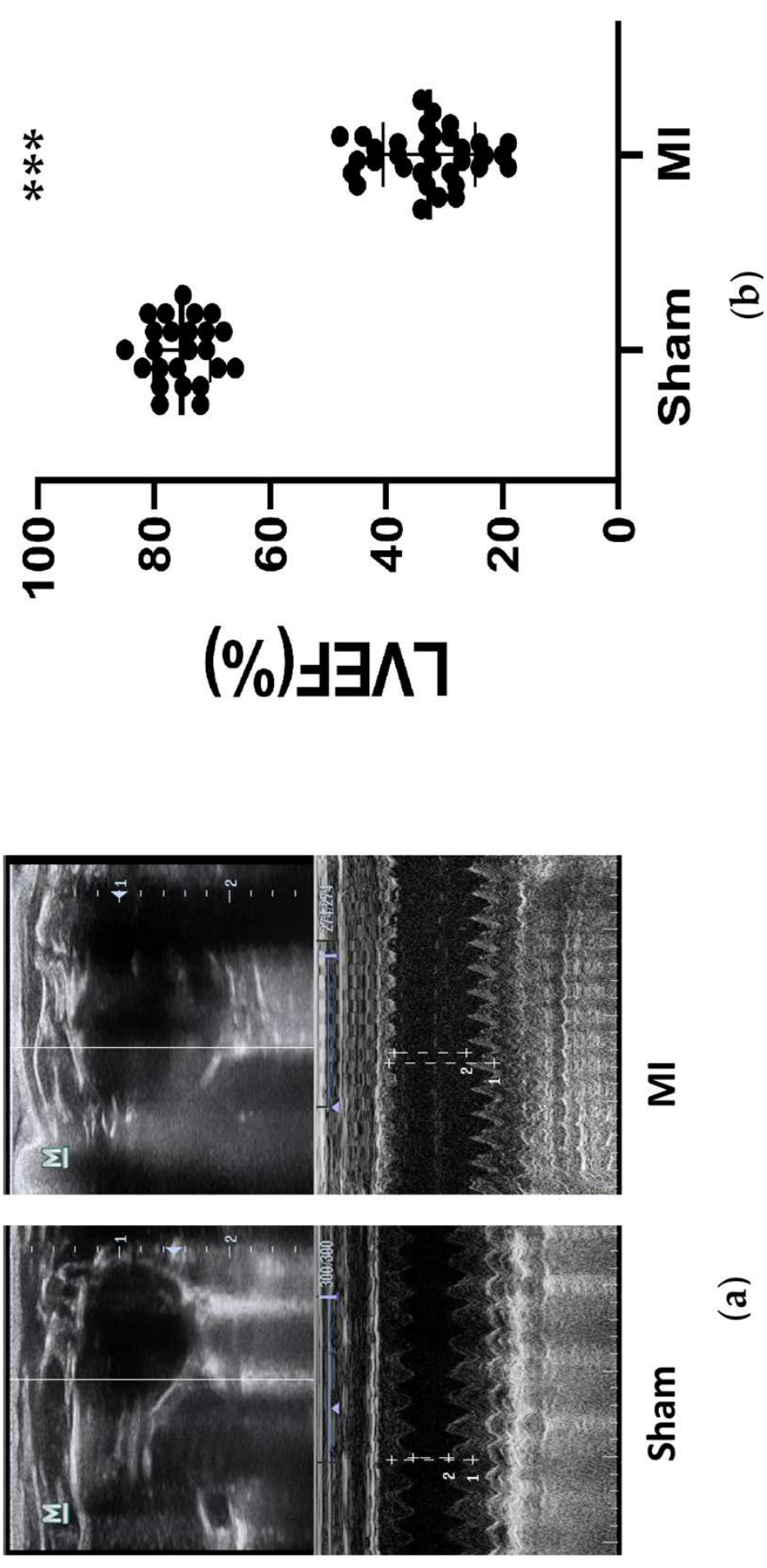

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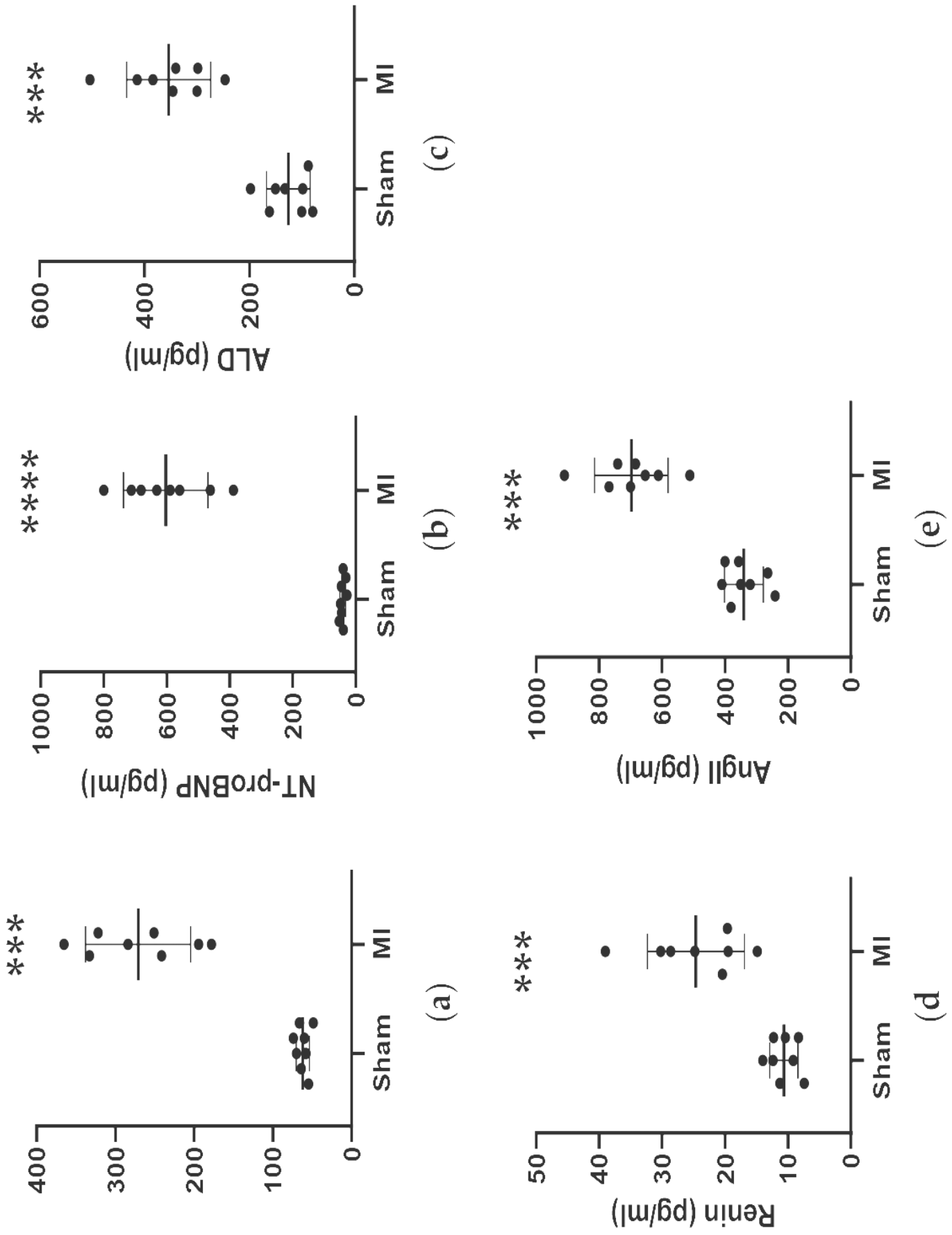

Figure 5

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Figure 6

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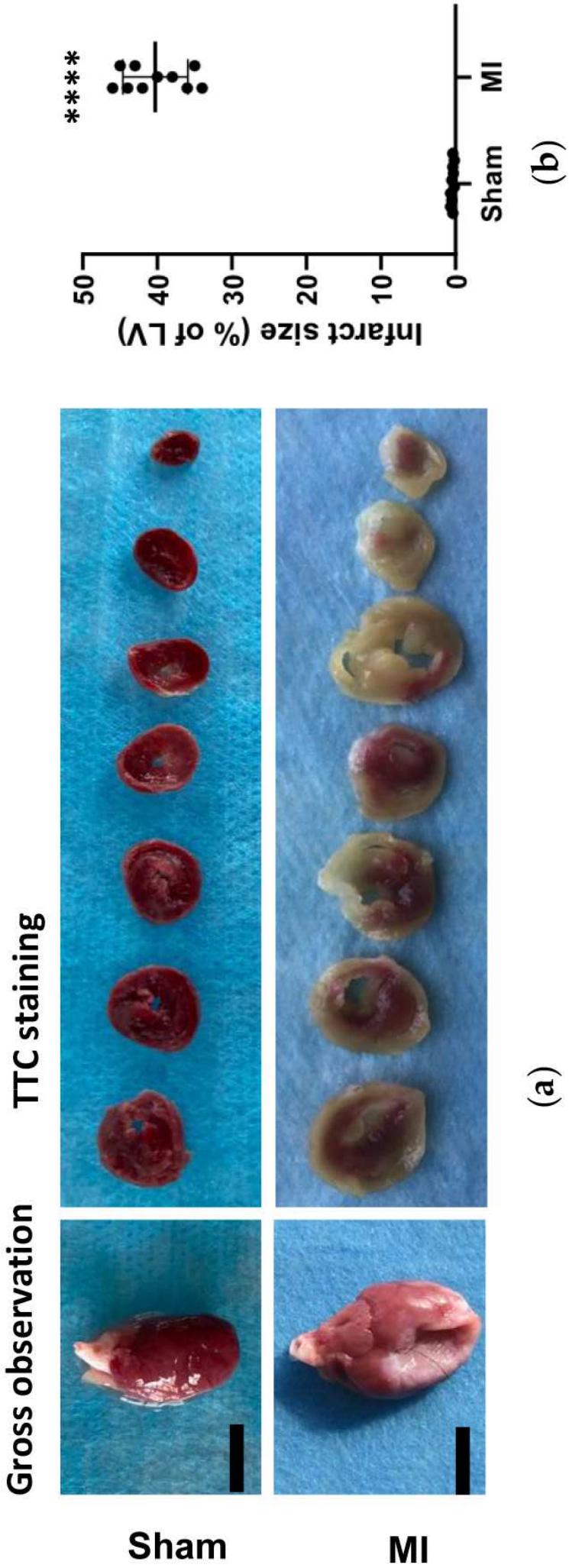

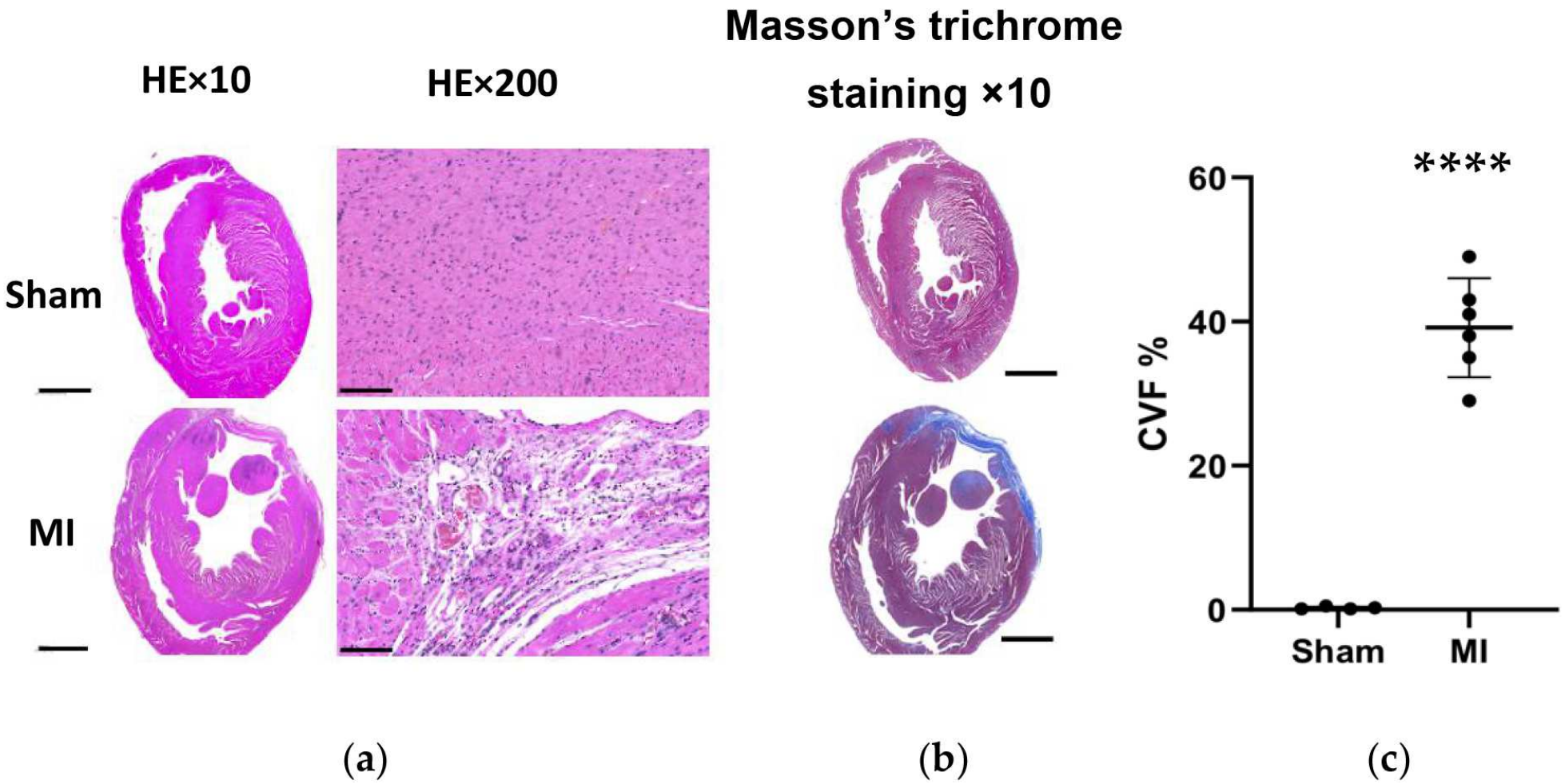

Table 1

Parameters	Sham group(n=24)	MI group(n=33)
LVIDd (mm)	8.3±1.3	10.1±2.9*
LVIDs (mm)	4.1±0.9	7.7±1.5***
FS (%)	42.5±7.8	22.2±4.4***

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2% TTC solution	Solarbio	T8170-1	
ELISA kits	Shanghai BlueGene Biotech CO., LTD		
Forceps	shinva		
H&E Staining Kit	abcam	ab245880	
Isoflurane	RWD		100 mL
Kotoprofen	Zoetis		KETOFEN
nylon suture 6-0	AD surgical	#S-N618R13	with needle
Scalpel blades	shinva	s2646	
Scalpel Handles	shinva		
Trichrome Stain (Masson) Kit	Sigma-Aldrich	HT15-1KT	
Ultrasound	Mindray	TE7	
Veterinary Vaporizer	Matrix	vip-3000	
Weitlaner Self-Retaining Retractor	shinva	ZV077RN	

We appreciate the Editorial comments and reviewer's insightful suggestions. We believe that the revised manuscript has been substantially improved by addressing these comments.

Editorial and production comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: We now have thoroughly proofread the manuscript and corrected the grammar mistakes.

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.

Response: We now have followed the instructions and ensured that all text in the protocol section is written in the imperative tense.

3. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

Response: We now have added more details to our protocol steps and ensure the answer the "how" question.

4. Please mention how proper anesthetization is confirmed.

Response: We now have added more details to the proper anesthetization steps. To confirm whether the anesthetization is ready, we observe the toe pinch reflex. It indicates the anesthetization is ready when the toe pinch reflex disappears. See line 76-77 page 3.

5. 2.1: How are the thorax hairs removed?

Response: We now have added more details on removing the thorax hair steps as follow "Depilatory creams were used to remove the thorax hairs" See line 83 page 3.

6. Please submit each figure individually as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps.).

Response: We now have provided each figure individually as an EPS file with high resolution.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Overall, a clear protocol with well defined steps and good evidence of heart failure achieved post surgery. Some claims are made comparing survival rates, tissue damage, recovery times post-surgery and variations in results, which will need either evidencing or some explanation for possible reasons. Otherwise only minor comments to improve the clarity for readers.

Response: We appreciate that the reviewer thought that our manuscript is of importance in the context of heart failure and it is a clear protocol with well-defined steps. We have addressed your insightful comments in detail below, and the manuscript has been revised accordingly. We appreciate the reviewer's insightful and constructive comments. We believe that the revised manuscript has been substantially improved by addressing these comments.

Major Concerns:

In the introduction (line 59) you state that methods using ventilation/intubation or exteriorization reduce safety of the animal. Do you have evidence for this? Ventilation is widely regarded as beneficial for the animal, since it decreases the chances of pneumothorax. You later (line 65) suggest that protocols without ventilation or exteriorization have less variation in results - could you evidence this or suggest a reason?

Response: We regret that we did not adequately explain the safety of procedures without ventilation/intubation or exteriorization of heart of rats in our work, and we now clearly described this by providing the references and comparing them with our work. The reviewer is absolutely right about reduction of pneumothorax with ventilation. It is a general principle that using ventilation/intubation is necessary when performing the ligation of LAD in rats. Previous study has demonstrated that the vast majority of surgeries needed either ventilation after tracheal intubation or exteriorization of the heart in rats (Acute Myocardial Infarction in Rats. Journal of Visualized Experiments. 2011. DOI: 10.3791/2464). Compared with our new protocol, those methods are more complex since the rat needs tracheal intubation or the heart needs to be exposed, both of which increased the cost of surgery, made the operation more difficult, took much more time for the procedures and reduced the safety of the animal. However, in rats operations, the use of ventilation/intubation has not become routine practice. According to

Andrew L. Rivard and his colleagues' report (Rat intubation and ventilation for surgical research. J Invest Surg. 2006 DOI: 10.1080/08941930600778297), "Complications of the intubation and ventilation included mortality from anesthetic over-dose, intubation difficulty, pneumothorax, traumatic extubation, and ventilation disconnection", all of which would reduce safety of the animal. Heart exteriorization may increase the risk of cardiac rupture and arrhythmia. A recent study developed a procedure without exteriorization of the hearts in mice, which reduced the procedure time and significantly increased the survival rate of mice post-surgery (A novel and efficient model of coronary artery ligation and myocardial infarction in the mouse. Circulation research 2010, DOI: 10.1161/CIRCRESAHA.110.223925.) So we basically developed the novel model without exteriorization of the hearts in rats. The survival rate of rats post surgery without ventilation or exteriorization is significantly increased in rats, which is consistent with the similar procedures in mice.

We are sorry for the previous inappropriate description. It's not because the procedure without ventilation or exteriorization resulted in less variation but because the fixed LAD ligation led to less variation. We have modified that description and marked up in red at the change made to the text of revised manuscript. As the site of ligation of LAD is relatively fixed, the infarct size has less variation and so does in LVEF. See line 65-66,page 3.

In the discussion, you claim that the elimination of intubation/ventilation and exteriorization leads to improved survival of the animals, less tissue damage, and faster recovery post surgery. Could you explain why your altered protocol might improve survival vs intubated or exteriorized methods? Is there evidence for reduced tissue damage? Orotracheal intubation should not cause any tissue damage, and the risks for tissue damage from exteriorization are low if done correctly. Could you give examples of recovery times for your protocol vs the traditional methods?

Response: We appreciate the reviewer's insightful and constructive comments. As we mentioned above, our novel protocol can avoid the complications of the intubation/ventilation and heart exteriorization so it can improve survival rate. In our experiment, the mortality is 5% which is much lower than the conventional methods with a very high mortality between 30%-50% (Ref: Involvement of endothelin-1 in acute ischemic arrhythmias in cats and rats. Clin Sci (Lond) 2002;103(Suppl 48):228S– 232S. LAD Ligation in Rats, ST. Vincent Health, AEC SOP.15,Sep.

2004.Enalapril improves arterial elastic properties in rats with myocardial infarction. J Cardiovasc Pharmacol 1999;34(1):102–7.). Furthermore, it is well-known that blind oral endotracheal intubation can cause trauma to the oral cavity and esophagus due to unskilled operation. It is worth noting that the simplified procedures lead to less tissue damage, therefore, rats have a much faster post-surgery recovery and higher survival rate. The following two points allowed us to achieve these results: First, the continuous inhalation of oxygen during the whole procedure allowed for sufficient oxygenation and ligation without ventilation. Second, the Weitlaner self-retaining retractor, a key surgical instrument, was used to gain enough exposure to the heart while keeping rib integrity, which helped to avoid the exteriorization of the heart. The disadvantages to our method is that the surgical procedure for developing MI without ventilation is technically demanding and challenging. The key step for achieving this model successfully is to gain enough exposure of the heart inside the thorax which requires more practice before the formal experiment.

Based on our experience, the recovery time mostly depends on the time of recovery from anesthesia. Our animals got recovery about 5-10 minutes after the procedure. It may take much longer time (20-30 minutes) when using the traditional methods to get recovery.

Minor Concerns:

In the protocol, point 2, (line 86) the wording here suggests that the chest is opened before the muscles are retracted - consider rewording this to indicate the skin was opened. In point 7 (line 98) - are the ribs themselves sutured closed, or only the muscle and skin?

Response: We appreciate the reviewer's insightful and constructive comments. In our procedure, the chest was opened with a transverse 2.5 cm incision between the third and fourth intercostal space on the left edge of the sternum. The pectoralis major and pectoralis minor muscles were separated by using two vascular forceps without cutting the tissue or small blood vessels. The Weitlaner self-retaining retractor was positioned into the thorax to separate the third and fourth ribs to gain enough exposure of the heart while keeping the heart in the thorax and keeping rib integrity. We separated the ribs without cutting them, so we only need to suture the muscle and skin, which also minimizes the surgery wound.

It may be useful to note somewhere in the protocol that a smaller rat (as used in your protocol) is preferred for a non-intubated method, to help potential readers design experiments.

Response: Thanks for the reviewer's comments, we have revised it in the protocol. See line 103-104 page 4.

In the final part of the discussion (line 230) you mention that surgical time should be minimized. An example of an ideal surgical time would be very helpful here for readers new to the technique. Are there any disadvantages to your method, or adaptations that should be made to the protocol in lieu of the changes? For example, you could note that squeezing the air from the thorax before closure becomes more critical in non intubated animals.

Response: We appreciate the reviewer's insightful comments. We now have added the ideal surgical time to help readers understand it clearly. The ideal surgical time is that the whole procedure should be completed within 10 minutes and keeping the chest open should be less than 5 minutes. (See line 103-104 page 4 and line 232-235,page 9). The disadvantage of our method is that the surgical procedure for developing MI without ventilation is technically demanding and challenging. It requires a lot of practice to complete the procedures as soon as possible, which could potentially minimize the surgery injury. We also included the information on squeezing the air from the thorax before closure. See line 99-100, page 4.

For the figures, consider adding labels to Figure 3a, and Figures 7a and 7b to indicate which group is sham and which MI for better clarity for readers. Please add the n numbers for Figure 6b to the figure legend. For Table 1, mentioning the timepoint for this data (ie 4 weeks post MI) would improve readability. Finally, please check the formatting for Table 1 and the Table of Materials - the figure legend is spilling over into the data cells in Table 1, and the columns need widening in the Table of Materials.

Response: Thanks for the reviewer's comments, we now have added the labels to Figure 3a, and Figure 7a and 7b to indicate the group information and included the n numbers for Figure 6b to the figure legend. We also double-checked the formatting for Table 1 and have widened the Table 1.

Reviewer #2:

Manuscript Summary:

The authors describe a method to induce MI in rats that does not involve ventilation or exteriorization of the heart. According to the authors, this model more reliable and reproducible.

Response: We appreciate that the reviewer thought that our model was more reliable and reproducible and the method we developed in rats have advantages without ventilation and exteriorization. We have addressed your insightful comments in detail below, and the manuscript has been revised accordingly. We appreciate the reviewer's insightful and constructive comments. We believe that the revised manuscript has been substantially improved by addressing these comments.

Major Concerns:

The study has only two groups : a sham group and a MI group. A third group using the traditional MI induction approach is missing. This groups is essential if one wants to ascertain that the new approach is better. In its absence, the claims the authors make can not be confirmed.

Response: The reviewer is absolutely right that providing the third group with traditional MI approach could be more robust, however, the current situation with pandemic doesn't allow us to do that, so we now clearly describe this as the limitation by providing the reference and comparing the previous traditional MI induction model with our new model. In the future study we will consider that three groups are involved to do the study according to the reviewer's comments.

The recent study developed a procedure without exteriorization of the hearts in mice, which reduced the procedure time and significantly increased the survival rate of mice post-surgery (Ref:A novel and efficient model of coronary artery ligation and myocardial infarction in the mouse. Circulation research 2010, DOI: 10.1161) So we basically developed the novel model in rats. The survival rate post surgery without exteriorization is significantly increased in rats, which is consistent with the similar procedures in mice. Previous reports about MI induction using the traditional protocol with the high mortality is between 30%-50% (Involvement of endothelin-1 in acute ischemic arrhythmias in cats and rats. Clin Sci (Lond) 2002;103(Suppl 48):228S– 232S; LAD Ligation in Rats, ST. Vincent Health, AEC SOP.15,Sep. 2004; Enalapril improves arterial elastic properties in rats with myocardial infarction. J Cardiovasc Pharmacol 1999;34(1):102–7). Even in the late studies, the mortality rate was about 6.7%-10% (A novel rat model of chronic heart failure

following myocardial infarction. Methods Find Exp Clin Pharmacol. 2009 Jul-Aug;31(6):367-73. PMID: 19798451. A new technique of coronary artery ligation: experimental myocardial infarction in rats in vivo with reduced mortality. Mol Cell Biochem. 1997 Nov;176(1-2):227-33. PMID: 9406166.). According to our results, the mortality rate was 5% in our study which was much lower than the existing reports and all the survival animals developed MI and heart failure. So, the difference between ours and traditional approach is significant and all these support that our new approach is better than the traditional approach in rat MI model.

Minor Concerns:

There are several other issues to be dealt with :

1) line 94 Explain what is meant with "LAD could be easily visualized in some rats"

Response: Thanks for the reviewer's comment, we now have revised it as "LAD could be easily recognized and found in some rats ". See line 94-95,page 4.

2) lines 132 - 136 Collagen content was measured in the middle of the infarct and then expressed as % of the whole LV area. This is not appropriate. The collagen content will be much lower in the non-infarcted part of the LV.

Response: Thanks for the reviewer's comment and the reviewer is absolutely right about the collagen content in MI group. We now have clarified it by including the quantification. The collagen content in MI group is 39.2±6.9% and it is almost zero in the sham group.

3) line 172 infarct size was huge ~50 %, yet the LV was only mildly dilated : from 8.3 mm to 10.1 mm. Much worse HF would be expected with such MI size.

Response: Thanks for the reviewer's comment. The number of rats in our previous study was relatively low, and we now have turned to a pathologist for analyzing the infarct size and getting 4 more samples quantified to get more robust results. According to the pathologist's result, the infarct size was 40.7±4.4% 4 weeks after the procedure in the MI group. See line 175-176, page 7.

4) lines 228 - 231 explain this better with more details.

Response: Thanks for the reviewer's comment, we now have revised it by including more detailed explanation. "The key step for achieving this model is to gain enough exposure of the heart to

fully visualize the heart inside the thorax and keep chest open within 5 minutes which requires more practice before the formal experiment." See line 233-235, page 9.

5) figure 6 shows the MI size of only six rats or so. This is not appropriate. All MI sizes should be shown.

Response: We agree with the reviewer, we now have revised it by including more quantified results for the experiment.