Journal of Visualized Experiments

A Modified Simple Method for Induction of Myocardial Infarction in Mice

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE63042R2
Full Title:	A Modified Simple Method for Induction of Myocardial Infarction in Mice
Corresponding Author:	Chenglin Jiang Bengbu Medical College bengbu, anhui CHINA
Corresponding Author's Institution:	Bengbu Medical College
Corresponding Author E-Mail:	alisajiang@126.com
Order of Authors:	Chenglin Jiang
	Jianqing Chen
	Yichao Zhao
	Dasheng Gao
	Hongju Wang
	Jun Pu
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.	Shanghai, China
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please confirm that you have read and agree to the terms and conditions of the video release that applies below:	I agree to the Video Release
Please provide any comments to the journal here.	

TITLE:

A Modified Simple Method for Induction of Myocardial Infarction in Mice

AUTHORS AND AFFILIATIONS:

Chenglin Jiang[1,2#], Jianqing Chen[1,2#], Yichao Zhao[2#], Dasheng Gao[1], Hongju Wang[1*], Jun Pu[1,2*]

[1]Department of Cardiology, The First Affiliated Hospital of Bengbu Medical College, Bengbu 233004, Anhui, China

[2]Department of Cardiology, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, China

Email addresses of the authors:

Chenglin Jiang (alisajiang@126.com)
Jianqing Chen (chjq5879@hotmail.com)
Yichao Zhao (frankysuperior@hotmail.com)
Dasheng Gao (gaodasheng612@163.com)
Hongju Wang (hongjuwang@outlook.com)
Jun Pu (pujun310@hotmail.com)

*Email addresses of the corresponding authors:

Hongju Wang (hongjuwang@outlook.com)
Jun Pu (pujun310@hotmail.com)

[#]These authors contributed equally to this work

KEYWORDS:

Heart, mouse, myocardial infarction, cardiac remodeling

SUMMARY:

Under adequate anesthesia with a simplified anesthesia device, the mouse heart was externalized through the intercostal space, and myocardial infarction was successfully induced by ligating the left anterior descending artery (LAD) using materials readily available in most laboratories.

ABSTRACT:

Myocardial infarction (MI) represents one of the leading causes of death. MI models are widely used for investigating the pathomechanisms of post-MI remodeling and evaluation of novel therapeutics. Different methods (e.g., isoproterenol treatment, cryoinjury, coronary artery ligation, etc.) have been used to induce MI. Compared with isoproterenol treatment and cryoinjury, coronary artery ligation may better reflect the ischemic response and chronic remodeling after MI. However, traditional methods for coronary ligation in mice are technically

challenging and require commercially available apparatus. The current study describes a simple and efficient process for induction of MI in mice with readily available materials. The mouse chest skin was cut open under stable anesthesia with a simplified anesthesia device made of centrifuge tubes. The heart was immediately externalized through the intercostal space after blunt separation of the pectoralis major and pectoralis minor. The left anterior descending branch (LAD) was ligated with a 6-0 suture 3 mm from its origin. Following LAD ligation, staining with 2,3,5-Triphenyl tetrazolium chloride (TTC) indicated successful induction of MI and temporal changes of post-MI scar size. Meanwhile, survival analysis results showed overt mortality within 7 days after MI, mainly due to cardiac rupture. Moreover, post-MI echocardiographic assessment demonstrated successful induction of contractile dysfunction and ventricular remodeling. Once mastered, an MI model can be established in mice within 2-3 min with readily available materials.

INTRODUCTION:

Myocardial infarction (MI) represents one of the significant causes of death and disability worldwide[1-5]. Despite timely reperfusion, there is currently a lack of effective therapies to treat post-MI cardiac remodeling. Correspondingly, considerable efforts have been made to mechanistic exploration and therapy exploitation for MI[6-8]. Of note, the establishment of MI models is a prerequisite to meet these ends.

Several methods (e.g., isoproterenol treatment, cryoinjury, coronary artery ligation, etc.) have been proposed to induce MI models in small animals. Isoproterenol treatment is a simple method for MI induction, but it cannot induce infarction of the targeted area[9]. Cryoinjury leads to myocardial necrosis *via* the generation of ice crystals and disruption of the cell membrane rather than direct ischemia[10]. By contrast, coronary artery ligation permits precise control of occlusion site and extent of infarct area and faithfully recapitulates remodeling response following infarction[11,12]. Coronary artery ligation is typically performed following intubation, mechanical ventilation, and thoracotomy, which is technically challenging[13,14]. Several modified protocols for coronary artery ligation (eg., ventilation free) were reported and potentiated the induction of MI, but commercial apparatus is required[15-17]. These issues pose a significant financial and technical barrier for groups wishing to engage in research using MI models. This report presents a unique approach for induction of MI in mice. The current method is easy, timesaving, and uses surgical tools and equipment found readily in most laboratories.

PROTOCOL:

The experiments involving animal work are performed with all necessary approvals from the Laboratory Animal Welfare Ethics Committee of Renji Hospital, Shanghai Jiao Tong University, School of Medicine (R52021-0506). Female and male C57BL/6J mice aged between 8-10 weeks were used in the study.

1. Preparation of the simplified anesthesia equipment

1.1. Take a 15 mL centrifuge tube, and make a cut perpendicular to the long axis of the tube about 3 cm from the opening (**Figure 1A-a**).

NOTE: Ensure that the cut is greater than half of the circular circumference of the tube lumen so that the valve can be successfully inserted.

1.2. Drill holes (diameter, 2 mm) at the centrifuge tube wall between the cut and the tube opening (**Figure 1A-a**).

1.3. Cut a suitably sized piece of the valve from a plastic sheet and insert the valve into the cut on the tube wall (**Figure 1A-a**).

NOTE: The valve can be used to control the release rate of isoflurane by changing the depth of the insertion.

1.4. Fill the bottom of the tube with a cotton ball; add 0.5 mL of isoflurane (as obtained, see **Table of Materials**) into the cotton ball and close the valve.

1.5. Test the anesthesia efficacy by masking the mice with tubes prepared as described above. Monitor the breathing rate and anesthesia depth by toe pinch response.

NOTE: A breathing rate less than 10 times/10 s suggests excessive anesthesia, and the insertion depth of the valve should be adjusted. In the current study, the tube with 20 holes was used for subsequent study. For all the procedures involving anesthesia, a gas filter filled with activated charcoal sheets must be used (**Figure 1A-j**), and surgery should be performed within a hood.

2. Operative preparation and anesthesia

2.1. Prepare and sterilize all the required instruments on the day of surgery, including a pair of forceps, a micro-mosquito hemostat, a pair of surgical scissors, two pairs of needle holders, 4-0 silk surgical suture, 6-0 silk surgical suture, a gas filter, and a light source (see **Table of Materials**) (**Figure 1A**).

2.2. Put on a surgical mask and sterile gloves.

2.3. Apply the depilatory cream to the mouse chest and wait for 1 min. Gently wipe off the depilatory cream and hair with wet gauze.

2.4. Hold the mouse with the dominant hand after depilation, open the valve in the anesthesia tube and mask the mouse with the tube. Confirm adequate anesthesia by the lack of toe pinch response.

NOTE: Masking the mouse with the anesthesia tube for about 15 s will be adequate to induce anesthesia.

2.5. Apply sterile eye cream to both eyes to prevent corneal dryness.

2.6. Secure the mice on a surgery platform in the supine position. Apply povidone-iodine swabs (see **Table of Materials**) to the chest three times and cover the disinfected chest with a sterile drape.

3. Induction of myocardial infarction

3.1. Change the contaminated gloves to ensure sterility.

3.2. Make a 0.5 cm skin cut along the line connecting the xiphoid and armpit.

3.3. Bluntly separate the pectoral major and pectoral minor muscles using forceps and a micro-mosquito hemostat to expose the fourth intercostal space.

3.4. Open the fourth intercostal space using a micro-mosquito hemostat.

3.5. Externalize the heart by pushing the heart toward the fourth intercostal space with the index finger of the left hand.

3.6. Secure the heart with the left hand, and ligate the left anterior descending branch with a 6-0 suture 3 mm from its origin.

3.7. Place the heart back into the thoracic cavity quickly.

NOTE: It is safe to externalize the heart for less than 30 s.

3.8. Evacuate the air out of the thoracic cavity by a gentle press of the chest cavity manually.

3.9. Close the skin with a 4-0 silk suture.

3.10. Place the mice on a pad (37 °C) immediately after the operation.

3.11. Return the operated mice to cages when fully recovered.

NOTE: The mice will be fully recovered within 3-5 min after surgery.

3.12. Inject buprenorphine (3 mg/kg) subcutaneously twice a day to reduce post-operative pain for the first 48 h after operation.

4. Harvesting the tissues

4.1. Sacrifice the mice at different time points after MI establishment by cervical dislocation.

4.2. Secure the sacrificed mice on the surgery platform in the supine position.

4.3. Make a ventral incision (~3-4 cm) in the upper abdomen. Cut off the ribs from both sides of the thorax cavity, and remove the diaphragm.

4.4. Perfuse the heart with 10 mL cold phosphate-buffered saline (1x PBS, 4 °C) through intraventricular injection.

4.5. Collect the heart by cutting off the aortic root and immediately store the heart at -80 °C.

NOTE: According to the authors' experience, it is feasible to perform TTC staining within two weeks of storage.

4.6. Stain the heart with 2,3,5-Triphenyte-trazoliumchloride (TTC).

4.6.1. Slice the frozen heart into 1 mm thick sections on ice using razor blades.

4.6.2. Incubate the prepared heart slices in 1% TTC solution (dissolved in 1x PBS) at 37 °C for 10-15 min.

NOTE: After 15 min incubation, discard the TTC solution and immerse the stained heart slices into 1x PBS.

4.7. Photograph the slices using a digital camera.

REPRESENTATIVE RESULTS:

The experimental protocol and some of the critical steps are shown in **Figure 1**. The simplified anesthesia equipment induced anesthesia. As shown in **Figure 2A**, the anesthesia was stable, as reflected by the regular breathing rates (varied from 90-107 breaths/min in the tested mice). Following coronary artery ligation, TTC staining analysis indicated successful induction of myocardial infarction and temporal changes of post-MI scar size (**Figure 2B**). Meanwhile, survival analysis results showed overt mortality within 7 days after MI in male and female C57BL/6J mice (**Figure 2C,D**). Ventricular rupture (56% in male mice; 40% in female mice) was a common reason

for post-MI death. Moreover, post-MI echocardiographic assessment demonstrated successful induction of contractile dysfunction and ventricular remodeling (**Figure 2E,F**).

FIGURE LEGENDS:

Figure 1: Materials and critical steps in the modified methods for MI induction. (**A**) Surgical instruments and materials needed for this protocol. (**a**) The simplified anesthesia device was made from a 15 mL centrifuge tube. (**b**) 4-0 silk suture. (**c**) 6-0 silk suture. (**d**) Forceps. (**e**) Scissors. (**f-g**) Needle holders. (**h**) Micro-mosquito hemostat. (**i**) Light source. (**j**) Gas filter. (**B**) Representative images showing key steps for inducing MI in mice. (**a**) The mouse is held with the dominant hand after depilation, and anesthesia was induced by masking the mouse with the anesthesia tube. (**b**) The mouse was secured, and povidone-iodine was applied to the surgical site. (**c**) The surgical site is draped. (**d**) A 0.5 cm cut at the surgical site. (**e**) Exposed ribs. The arrow indicates the ribs. (**f**) Dissected the pectoral major and pectoral minor muscles to expose the fourth intercostal space. (**g**) Externalized heart. (**h-i**) Ligated LAD with a 6-0 silk suture. The arrow indicates LAD. (**j**) The heart is placed back into the chest cavity. (**k**) Air was evacuated from the thoracic cavity. (**l**) The skin closed with 4-0 silk sutures.

Figure 2: Histological and functional changes after coronary artery ligation. (**A**) Breathing rates in mice anesthetized by the simplified anesthesia equipment (n=10). **(B)** TTC staining results of heart slices (4 slices from each heart) were collected at different time points post-MI. The white area indicated an infarcted area, and the red area revealed viable myocardium. **(C)** The Kaplan-Meier curve shows the post-MI mortality rate in male mice (n=20 per group). **(D)** The Kaplan-Meier curve shows the post-MI mortality rate in female mice (n=20 per group). (**E**) Representative images of echocardiographic analysis at different time points after MI (sham, 3 days, 7 days, 21 days, and 28 days post-MI). (**F**) The quantitative analysis of the left ventricular ejection fraction (LVEF), left ventricular fractional shortening (LVFS), left ventricular end-systolic diameter (LVsD), and left ventricular end-diastolic dimension (LVdD) values among the indicated groups (n=5 per group). **p<0.01 or ***p<0.001 *vs*. sham; ##p<0.01 or ###p<0.001 *vs*. 3 days post MI. One-way analysis of variance with posthoc Tukey HSD (Honestly Significant Difference) test was performed for statistical analysis.

DISCUSSION:

The present report demonstrated a new protocol for MI induction in mice with readily available materials, which was modified from a method reported by Gao[16]. Murine MI models are indispensable for mechanistic exploration and drug screen for post-MI dysfunction and remodeling[12]. Among the existing techniques for MI induction, coronary artery ligation represents the most commonly practiced one. Coronary artery ligation faithfully recapitulates the ischemia nature of myocardial infarction and leads to a scar healing and remodeling response similar to the clinical scenario[18,19]. However, the conventional protocol for coronary artery ligation involves intubation, ventilation, and a wide opening of the chest, which is technically challenging and time-

consuming. Over the past years, different protocols for coronary artery ligation have been reported and potentiated the establishment of MI to some extent[15-17]. The current study presented a simple and efficient protocol using surgical tools and equipment readily found in most laboratories.

Critical steps and troubleshooting

For optimal performance in practicing this method, several key steps are worth noting. To externalize the heart, the chest cavity should not be squeezed fiercely, which would negatively affect the coronary blood flow and obscure the coronary artery, leading to the invisibility of the coronary artery and failure of LAD ligation. Moreover, this may result in severe lung injury. For most cases, a gentle push against the right side of the chest wall will successfully externalize the heart through the opened intercostal space. Occasionally, a feeling of resistance during heart externalization may indicate a mismatch of the heart apex and the intercostal opening. This may be addressed by slight movements of the micro-mosquito hemostat along the midaxillary line. Another critical point is the adequate evacuation of the residual air in the thorax cavity before suturing the skin. Failing to do so will increase post-operative mortality due to pneumothorax.

Advantages and limitations

Conventional methods for coronary ligation require intubation, mechanical ventilation, ribs being cut, and are not easy for coronary artery identification due to high heart rate. These issues dramatically prolong the operation time and increase operation-related mortality. Compared to conventional methods, the modified protocol presents the following advantages: (1) it is free from ventilation, which is one of the causes of operation-related death in conventional practice; (2) it is timesaving (i.e., it takes approximately 3 min from anesthesia, LAD ligation to successful skin suturing); (3) the surgical tools and materials required are readily available in most laboratories. However, a significant limitation of this unique method is the limited time allowed for LAD ligation after heart externalization due to the lack of mechanical ventilation support. Thus, high mortality caused by pneumothorax may be expected for beginners. Based on the authors' experience, heart externalization for less than 30 s is well tolerated by all the tested mice. This time window is adequate for an experienced technician to finish MI induction with low perioperative mortality (<5%).

DISCLOSURES:

The authors have nothing to disclose.

ACKNOWLEDGEMENTS:

This work was supported by grants from the National Natural Science Foundation of China (81930007, 81625002, 81800307, 81470389, 81500221, 81770238), the Shanghai Outstanding Academic Leaders Program (18XD1402400), the Science and Technology Commission of Shanghai Municipality (201409005200), Shanghai Pujiang Talent Program (2020PJD030), and China Postdoctoral Science Foundation (2020M671161, BX20190216).

REFERENCES:

1. Fu, Y. et al. A simple and efficient method for in vivo cardiac-specific gene manipulation by intramyocardial injection in mice. *Journal of Visualized Experiments.* **134**, e57074 (2018).
2. Pell, S., Fayerweather, W. E. Trends in the incidence of myocardial infarction and in associated mortality and morbidity in a large employed population, 1957-1983. *The New England Journal of Medicine.* **312** (16), 1005-1011 (1985).
3. Ramunddal, T., Gizurarson, S., Lorentzon, M., Omerovic, E. Antiarrhythmic effects of growth hormone--in vivo evidence from small-animal models of acute myocardial infarction and invasive electrophysiology. *Journal of Electrocardiology.* **41** (2), 144-151 (2008).
4. Tabrizchi, R. β-blocker therapy after acute myocardial infarction. *Expert Review of Cardiovascular Therapy.* **11** (3), 293-296 (2013).
5. Virani, S. S. et al. Heart disease and stroke statistics-2020 update: A report from the American Heart Association. *Circulation.* **141** (9), e139-e596 (2020).
6. Cahill, T. J., Choudhury, R. P., Riley, P. R. Heart regeneration and repair after myocardial infarction: Translational opportunities for novel therapeutics. *Nature Reviews Drug Discovery.* **16** (10), 699-717 (2017).
7. Froese, N. et al. Anti-androgenic therapy with finasteride improves cardiac function, attenuates remodeling and reverts pathologic gene-expression after myocardial infarction in mice. *Journal of Molecular and Cellular Cardiology.* **122**, 114-124 (2018).
8. Wang, W. et al. Defective branched chain amino acid catabolism contributes to cardiac dysfunction and remodeling following myocardial infarction. *American Journal of Physiology-Heart and Circulatory Physiology.* **311** (5), H1160-H1169 (2016).
9. Acikel, M. et al. Protective effects of dantrolene against myocardial injury induced by isoproterenol in rats: Biochemical and histological findings. *International Journal of Cardiology.* **98** (3), 389-394 (2005).
10. van den Bos, E. J., Mees, B. M. E., de Waard, M. C., de Crom, R., Duncker, D. J. A novel model of cryoinjury-induced myocardial infarction in the mouse: A comparison with coronary artery ligation. *American Journal of Physiology-Heart and Circulatory Physiology.* **289** (3), H1291-H1300 (2005).
11. Guo, Y. et al. Demonstration of an early and a late phase of ischemic preconditioning in mice. *American Journal of Physiology-Heart and Circulatory Physiology.* **275** (4), H1375-H1387 (1998).
12. Kumar, M. et al. Animal models of myocardial infarction: Mainstay in clinical translation. *Regulatory Toxicology and Pharmacology.* **76**, 221-230 (2016).
13. Das, S., MacDonald, K., Chang, H. Y., Mitzner, W. A simple method of mouse lung intubation. *Journal of Visualized Experiments.* **73**, e50318 (2013).
14. Johns, T. N., Olson, B. J. Experimental myocardial infarction. I. A method of coronary occlusion in small animals. *Annals of Surgery.* **140** (5), 675-682 (1954).
15. Ahn, D. et al. Induction of myocardial infarcts of a predictable size and location by branch pattern probability-assisted coronary ligation in C57BL/6 mice. *American Journal of Physiology. Heart and Circulatory Physiology.* **286** (3), H1201-1207 (2004).

16. Gao, E., Koch, W. J. A novel and efficient model of coronary artery ligation in the mouse. *Methods in Molecular Biology.* **1037**, 299-311 (2013).
17. Most, P. et al. Cardiac S100A1 protein levels determine contractile performance and propensity toward heart failure after myocardial infarction. *Circulation.* **114** (12), 1258-1268 (2006).
18. Christia, P. et al. Systematic characterization of myocardial inflammation, repair, and remodeling in a mouse model of reperfused myocardial infarction. *Journal of Histochemistry & Cytochemistry*. **61** (8), 555-570 (2013).
19. Frantz, S., Bauersachs, J., Ertl, G. Post-infarct remodelling: Contribution of wound healing and inflammation. *Cardiovascular Research*. **81** (3), 474-481 (2008).

Figure 1

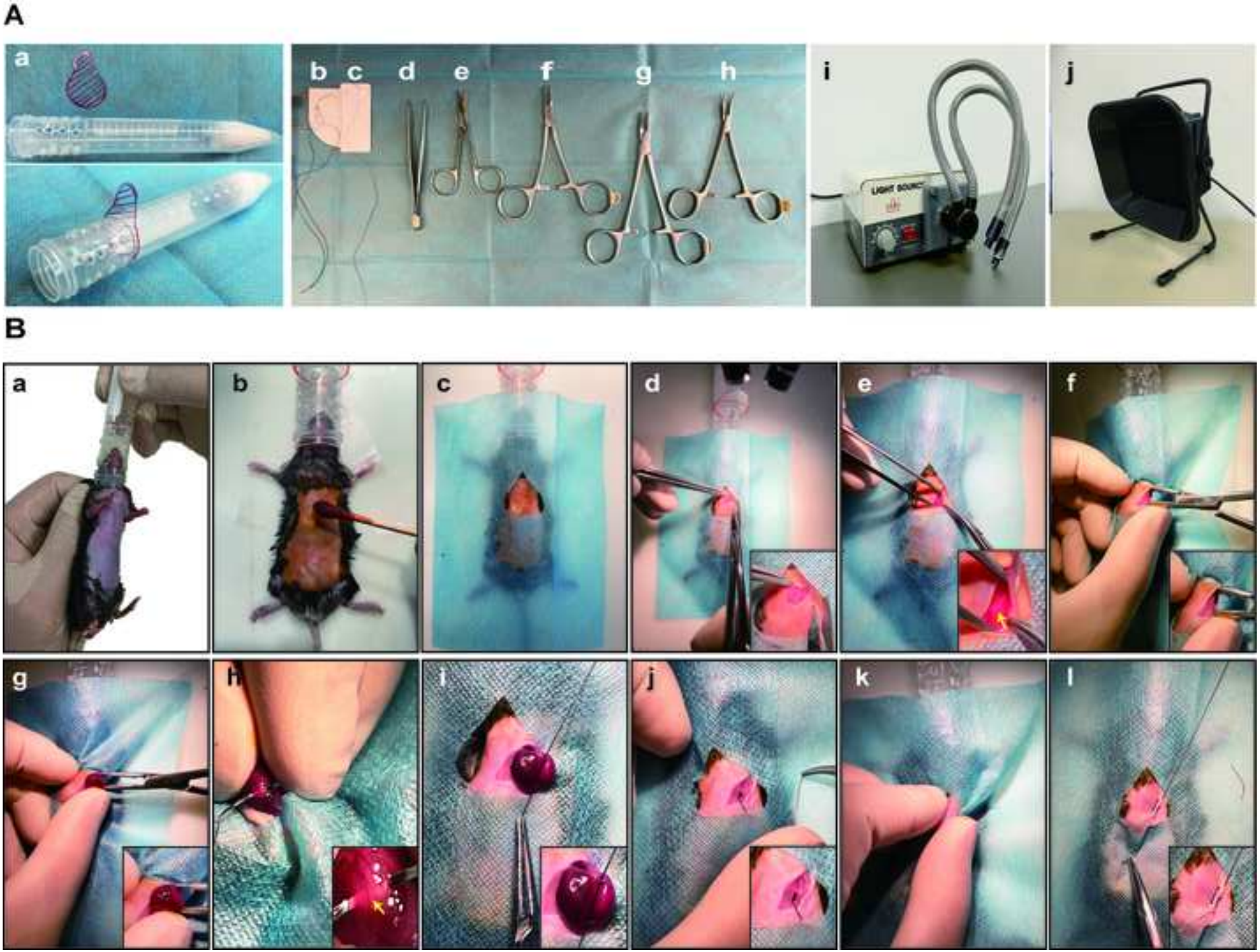
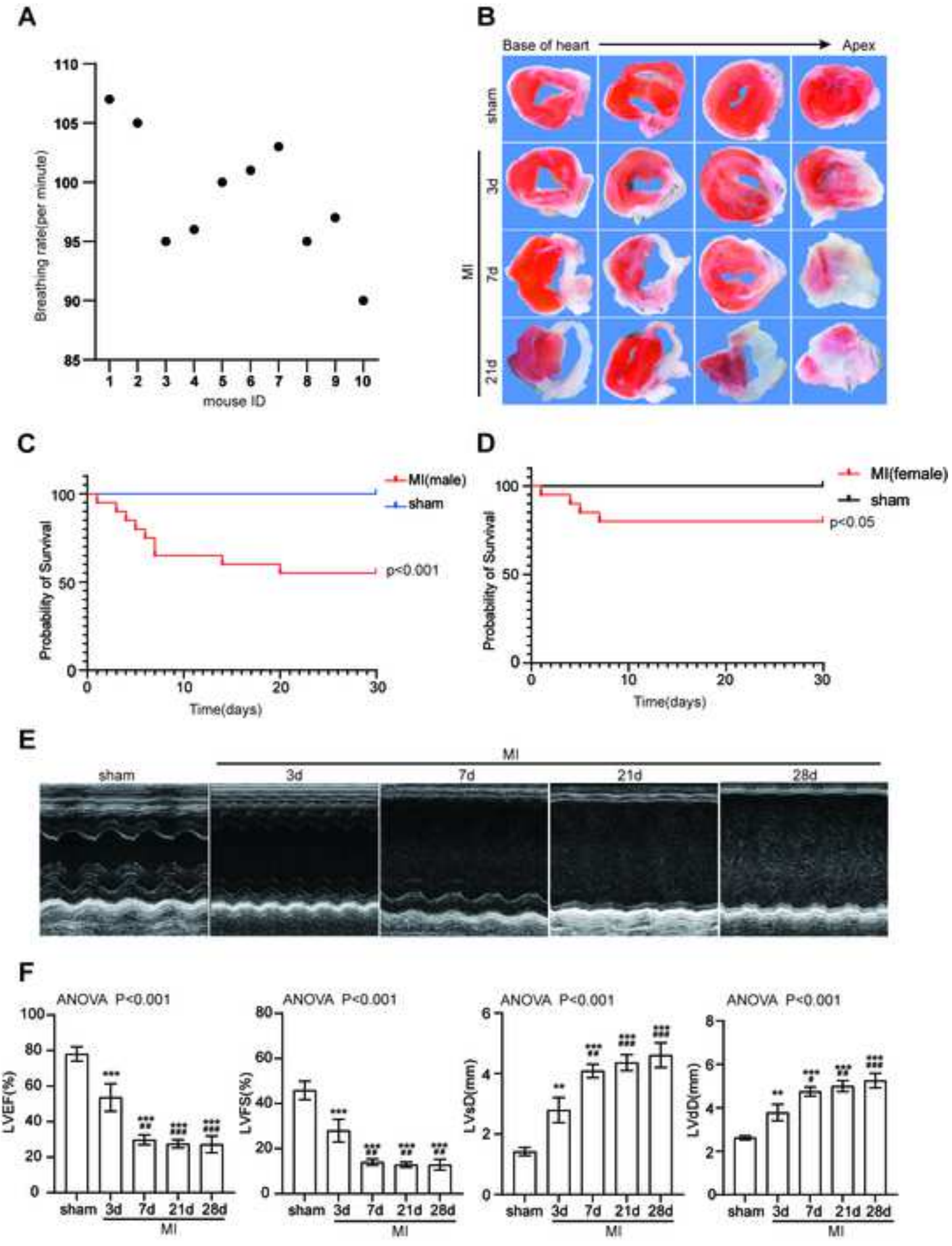


Figure 2



Nilanjana Saha, Ph.D.
Review Editor
JoVE
E-mail: nilanjana.saha@jove.com

Dear Prof. Saha,

Thank you for your comments on our manuscript entitled “A modified simple method for induction of myocardial infarction in mice” (Original manuscript number: JoVE63042).

We have answered and addressed all the queries in the commented text. We hope that this manuscript is now acceptable for publication in **Journal of visualized experiments**.

With best regards,
Sincerely yours,

Jun Pu, MD, PhD, FACC, FAHA, FESC
Department of Cardiology, Renji Hospital
School of Medicine, Shanghai Jiao Tong University
160 Pu Jian Road, Shanghai 200127, China.
Tel: 86-21-68383064;
Fax: 86-21-68383477;
E-mail: pujun310@hotmail.com

Response to Editorial comments:

Q1). Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
Response: Thanks for the comment. We have defined all abbreviations at first use, and asked for professional copy-editing services to correct spelling or grammar issues.

Q2). Please reduce the word count of the "short abstract" or SUMMARY to be 10-50 words.
Response: As suggested, the word count of the "short abstract" was reduced to 38 words (underlined, lines 31-34).

Q3). Please revise the following lines to avoid overlap with previously published work: 121-122, 124, 130-134, 181-183, 213.
Response: Thanks for the suggestions. We have revised the indicated lines.

Q4). Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Response: As suggested, the use of any personal pronouns was avoided in the revised text.

Q5). 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: Thanks for the comment. All text in the protocol section has been described in the imperative tense. We have also included all safety procedures.

Q6). Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
Response: Thanks for the suggestions. We provided enough detail in each step to supplement the upcoming video.

Q7). Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.

Response: Thanks for the comment. We have formatted the manuscript as required, and highlighted (in yellow) protocol text for inclusion in the protocol section of the video.

Q8). As we are a methods journal, please add any limitations of the technique to the Discussion.

Response: As suggested, the limitations of the technique have been added in the discussion section to read: "However, a major limitation of this novel method is the limited time allowed for LAD ligation after heart externalization, due to the lack of mechanical ventilation support. Thus, high mortality caused by pneumothorax may be expected for beginners. Based on the authors' experience, heart externalization for less than 30 seconds is well tolerated by all the tested mice, and this time window is adequate for an experienced technician to finish MI induction with low perioperative mortality (<5%)" (underlined, lines 219-224).

Q9). Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.

Response: Thanks for the comment. The references have been revised as suggested.

Response to Reviewer #1:

Q1). please state advantages and disadvantages of this method compared to other method. Moreover, manuscript should be checked by a native speaker for grammatical errors and typos.

Response: Thanks for the comments. We have asked for professional copy-editing services to correct grammatical errors and typos. Moreover, advantages and disadvantages of this method compared to others have been added in the revised Discussion section to read: "Compared to conventional methods, the modified protocol presents the following advantages: (1) it is free from ventilation, which is one of the causes of operation-related death in conventional practice; (2) it is timesaving (i.e., it takes approximately 3min from anesthesia, LAD ligation to successful skin suturing); (3) the surgical tools and materials required are readily available in most laboratories. However, a major limitation of this novel method is the limited time allowed for LAD ligation after heart externalization, due to the lack of mechanical ventilation support. Thus, high mortality caused by pneumothorax may be expected for beginners. Based on the authors' experience, heart externalization for less than 30 seconds is well

tolerated by all the tested mice, and this time window is adequate for an experienced technician to finish MI induction with low perioperative mortality (<5%)" (underlined, lines 215-224).

Response to Reviewer #2:
Jiang et al. report a modified method for induction of myocardial infarction in mice. The main advantage proposed by the authors is that the procedure can be performed with readily available materials.

Q1). How is the initial anesthesia in the conscious mice induced? Since the simplified anesthesia equipment is the only difference of this method compared to the procedure reported from Gao et al. (Circ. Res. 2010) the authors should provide more details (also pictures) in this regard.

Response: Thanks for the comment. We have added description for the initial anesthesia to read: "Hold the mouse with the dominant hand after depilation, open the valve in the anesthesia tube and mask the mouse with the tube" (underlined, lines 106-107). Moreover, we added a corresponding picture for initial anesthesia in the revised Figure 1B-a.

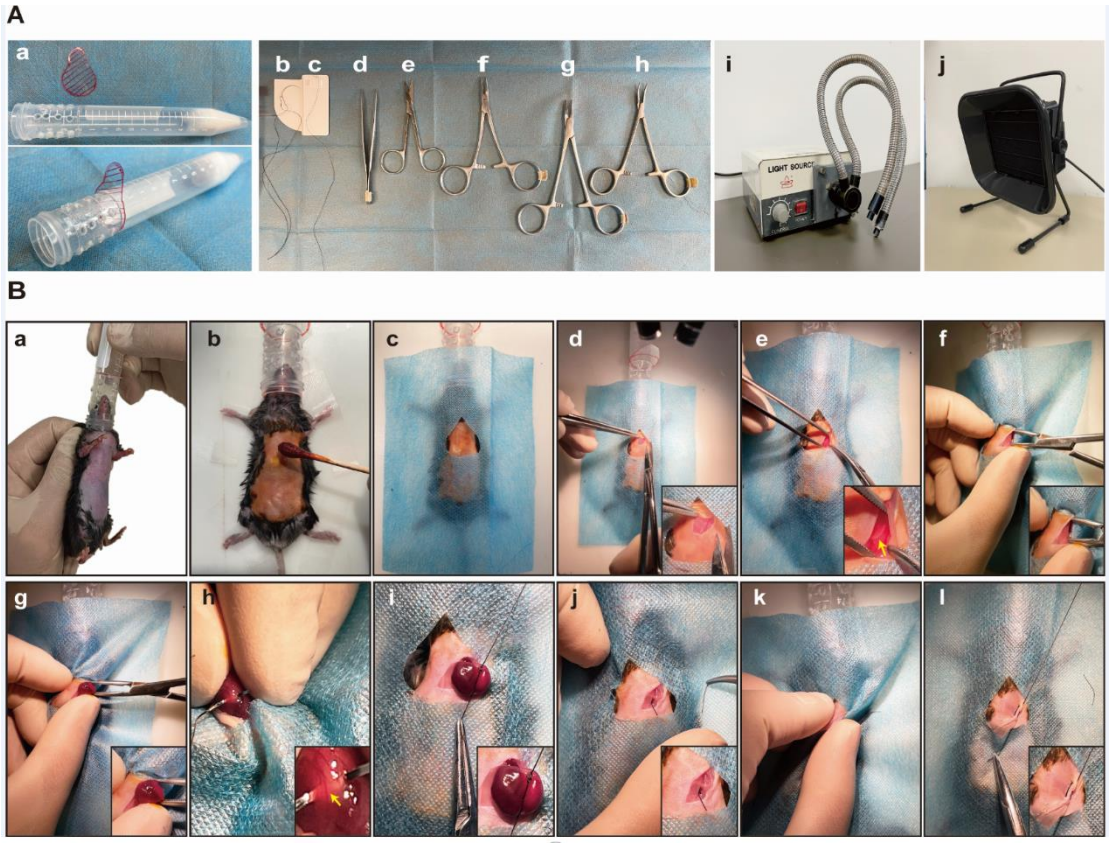


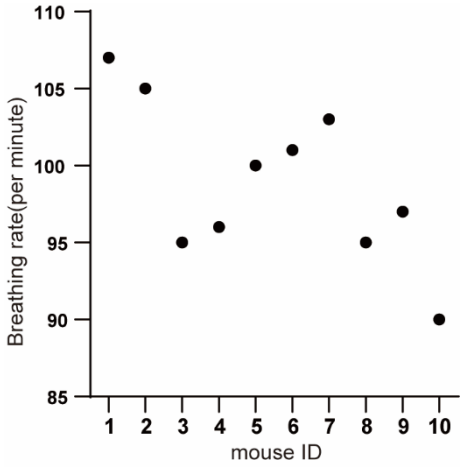
Figure 1. Required materials and key steps in the modified methods for MI induction.

Q2). The authors should also provide more information on the breathing rates with the used anesthesia equipment. How was the variability of the breathing rate within

the operated mice? How well can the anesthesia be controlled with the use of one prepared tube (e.g. with 20 holes)? How long will the anesthesia last if the tube is filled with 0.5 ml isoflurane?

Response:

(1) During operation, the mice were covered by drape and breathing rate is hard to be counted. Instead, we recorded the breathing rate in another batch of mice (n=10) with the prepared tube. As shown in the figure below, the breathing rate varied from 90 to 107 per minute.



(2) Masking the mice with the tube for about 15s is adequate for initial anesthesia, then observe the breathing rate. A breathing rate less than 10 times/10s suggests excessive anesthesia, and insertion depth of the valve should be adjusted (lines 94-95). With the use of the prepared tube (with 20 holes), all the tested mice exhibited adequate and stable anesthesia.

(3) If the tube is filled with 0.5 ml isoflurane, the anesthesia would last for 47.3±4.2 min (evidenced by lack of toe pinch response).

Q3). Can the procedure be performed without microscope or magnifying glasses? Since the authors stress the simplified equipment, the manuscript should include all necessary tools. It is hard to imagine that the LAD can be seen/ligated without microscope.

Response: As suggested, we included all necessary tools in the revised manuscript, including a pair of forceps, a micro-mosquito hemostat, a pair of surgical scissors, two pairs of needle holders, 4-0 silk surgical suture, 6-0 silk surgical suture, a gas filter, and a light source (Figure 1A). The LAD can be seen and ligated without a microscope or magnifying glasses, and the illumination of the light source is sufficient for LAD identification (LAD indicated by arrow in Figure 1B-h). After heart externalization, securing the heart with the left hand would lead to temporary congestion of coronary artery, and this may potentiate the visualization of LAD.

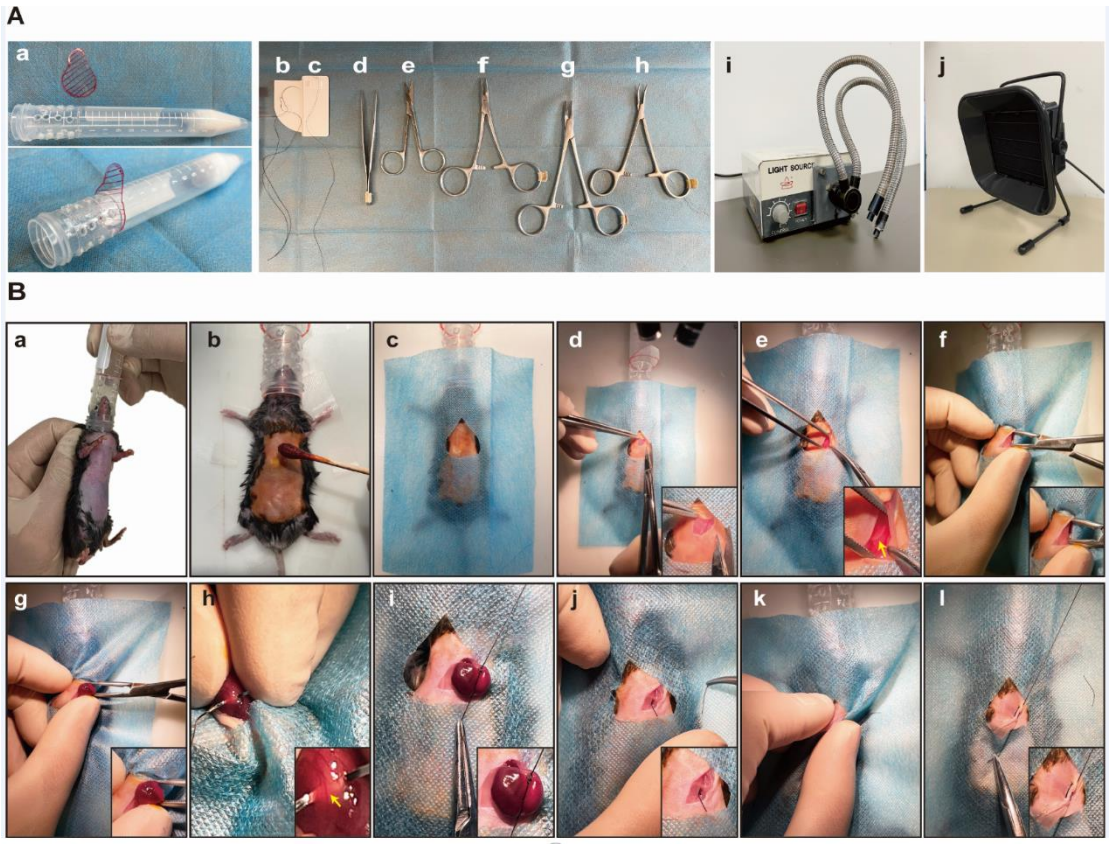


Figure 1. Required materials and key steps in the modified methods for MI induction.

Q4). Line 104 Please specifiy which kind of gas filter is necessary.

Response: As suggested, we revised the description for the gas filter to read: "For all the procedures involving anesthesia, gas filter filled with activated charcoal sheets must be used", and the picture for the gas filter was shown in Figure 1A-j (underlined, lines 96-97).

Q5). Figure 2D The authors should revise the included statistics (e.g. indicate significant differences between each groups rather than one p value and specify which multiple comparison test was used). Also please report sample size or display single data points for each animal.

Response: As suggested, one-way analysis of variance with post-hoc Tukey HSD (Honestly Significant Difference) test was performed for statistical analysis and sample size was reported in the figure legend.

Q6). Figure 2C/D The functional differences in fig 2D are not reflected in the representative m-mode pictures in fig. 2C (e.g. day 3 seems almost like baseline).

Response: As suggested, more representative M-mode pictures were presented.

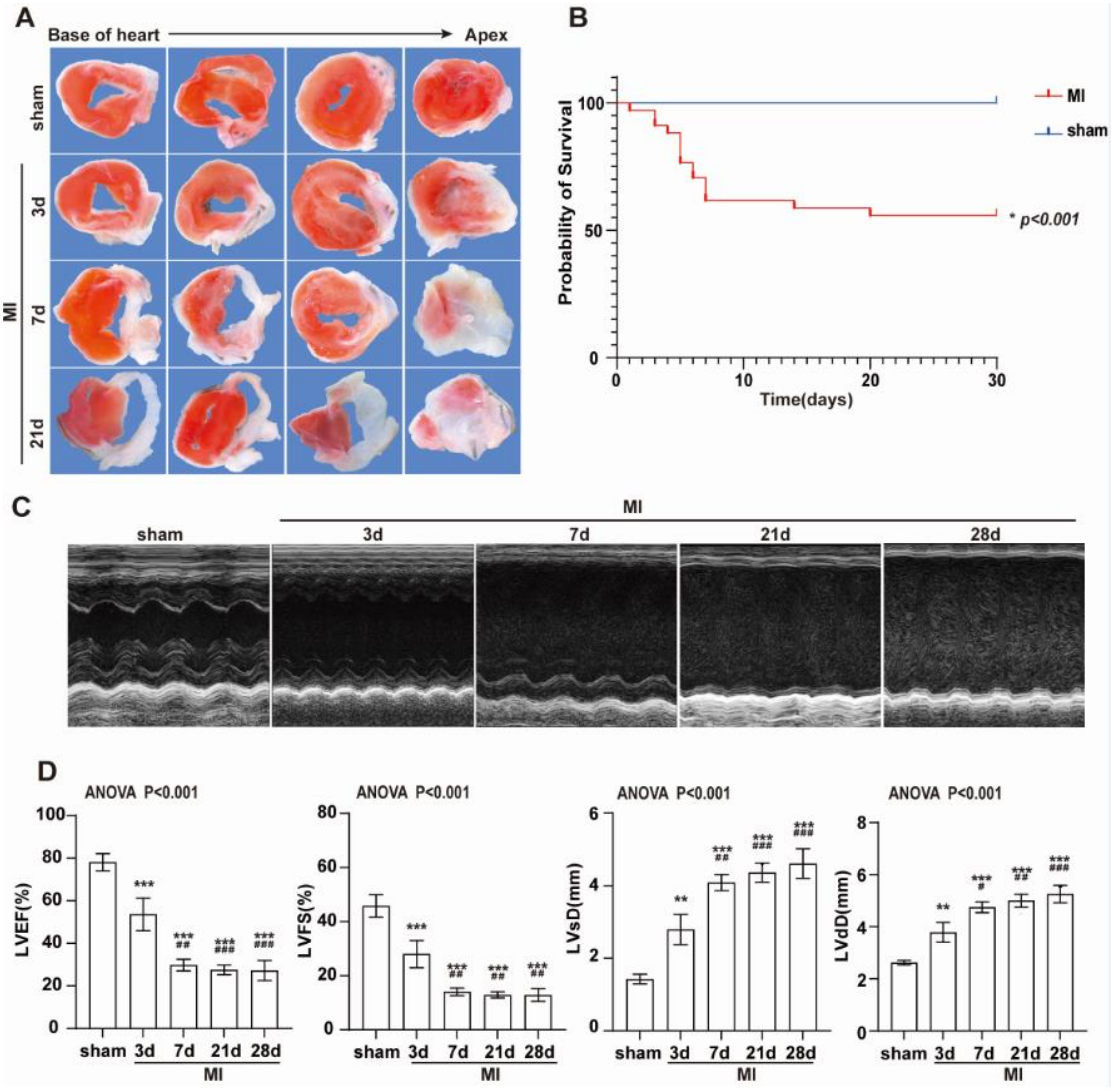


Figure 2. Histological and functional changes after coronary artery ligation

Q7). Figure 2A Please specify if fig2A displays 4 different hearts per each timepoint or 4 slices from the same heart (from base to apex).

Response: Sorry for the confusion. Fig 2A displays 4 slices from the same heart (from base to apex). We have revised Fig 2A.

Q8). Line 168. What were the other reasons for post-MI death?

Response: Cardiac rupture was identified by the presence of blood in the thoracic cavity and signs of ventricular rupture. While some other mice might die due to cardiac arrhythmia or acute heart failure because autopsy showed no blood in the thoracic cavity.

Response to Reviewer #3:
Manuscript Summary:

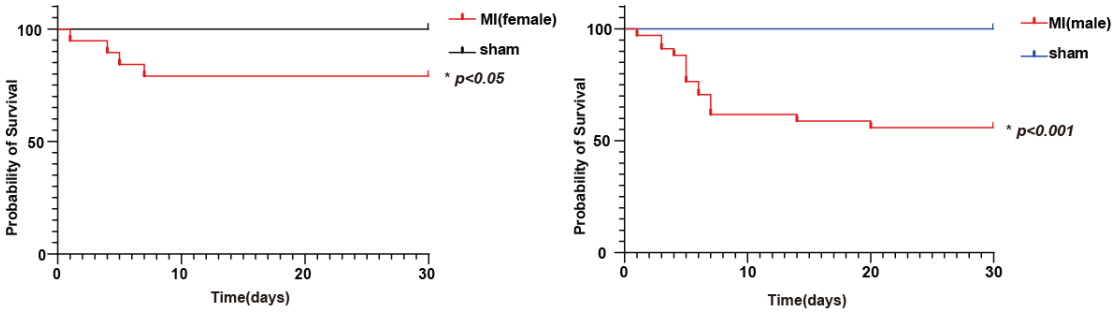
Jiang and co-workers investigated the utility of a ventilation-free, open-chest surgical procedure in mice undergoing permanent coronary artery ligation to induce experimental myocardial infarction. Such a procedure would not require equipment for mechanical ventilation and vaporizer for anesthetics and would thus significantly cut costs, if proven to be equivalent to the standard procedure using endotracheal intubation and mechanical ventilation. In general, the authors demonstrate feasibility of the procedure, but questions concerning procedural complications/mortality remain.

Q1). Figure 2B shows a 28-day post ligation mortality approaching 50% in the MI group, which is high compared to published studies using coronary artery ligation as a method to experimentally induce myocardial infarction in mice. It is not clear whether the high mortality results from sex- and/or strain-related complications (male C57BL/6J mice are prone to myocardial rupture following permanent LAD artery ligation), from procedure-specific complications (i.e., ventilation-free procedure), or from both. To distinguish between the two, experiments should be repeated in female C57BL/6J mice or in a different mouse strain exhibiting lower rates of myocardial rupture. In line 194, it is stated that the number of mice was 20 per group. Does this number include those mice that died during or immediately following the procedure? Since a ventilation-free approach limits the time that is available for the ligation to <1 min, I would expect the procedural mortality and success to be higher and lower, respectively, than in ventilated mice undergoing ligation.

Response:

(1) The mortality rate in our model may differ compared with some studies, but a search in Pubmed indicated that our model presented similar mortality rate with some published studies using coronary artery ligation to induce MI in mice (PMID: 11489778, PMID: 11375279, PMID: 31792327, PMID: 30233357, PMID: 24718482, PMID: 25121738). The initial infarct size may affect post-MI mortality. As shown in the work by Timothy D O'Connell (PMID: 20692266), mortality was significantly increased in mice with proximal ligation of LAD compared with distal ligation. Thus, the difference in mortality between our model and others may be attributable to the difference in the initial infarct size.

(2) As suggested, we further established MI models in female C57BL/6J mice. As shown in the figure below, female mice (left panel) exhibited lower post-MI mortality rate compared with male mice (right panel).



(3) The number of mice (20 per group) includes all the mice that died during or immediately following the procedure. It is natural to think that ventilation-free approach will lead to higher procedural mortality than in ventilated mice undergoing ligation. However, higher procedural mortality is mainly confined to beginners. Once mastered, this novel method is related to high perioperative survival rate (>95%). In fact, all the 20 mice survived during the MI induction procedure in current study. The high perioperative survival by this novel method is possibly attributable to the short operation duration, quick recovery from anesthesia, minimal tissue injury (no intubation, no rib cutting, and blunt dissection of pectoralis major and pectoralis minor muscles). Therefore, high mortality for beginners is a major limitation of current protocol, and this limitation was included in the revised text (underlined, lines 219-224).

Q2). Line 199: ANOVA was used to detect significant differences in echocardiographic outcome. Please indicate which time points differed from each other, using post hoc analyses.

Response: As suggested, one-way analysis of variance with post-hoc Tukey HSD (Honestly Significant Difference) test was performed for statistical analysis, and statistical difference between two groups were indicated in the revised Figure 2 and figure legend.

Q3). Line 120 and 146: supine position instead of prone position.

Response: Thanks for the suggestion. This has been corrected.