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A cryoinjury model to study myocardial infarction in the mouse

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Corresponding Author:	Sonja Schrepfer University of California San Francisco San Francisco, CA UNITED STATES
Corresponding Author's Institution:	University of California San Francisco
Corresponding Author E-Mail:	Sonja.Schrepfer@ucsf.edu
Order of Authors:	Sonja Schrepfer
	Dong Wang
	Grigol Tediashvili
	Xiaomeng Hu
	Alessia Gravina
	Sivan Marcus
	Hao Zhang
	Jeffrey E Olgin
	Tobias Deuse
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TITLE:

A Cryoinjury Model to Study Myocardial Infarction in the Mouse

AUTHORS AND AFFILIATION:

Wang, Dong[1,2,*], Grigol Tediashvili[1,2,*], Xiaomeng Hu[1,2], Alessia Gravina[2], Sivan G. Marcus[1,2], Hao Zhang[4], Jeffrey E Olgin[4], Tobias Deuse[1,2,5], Sonja Schrepfer[1,2,3,5]

[1]Transplant and Stem Cell Immunobiology Lab, University Heart Center, Hamburg, Germany.
[2]Department of Surgery, Transplant and Stem Cell Immunobiology Lab, University of California San Francisco (UCSF), San Francisco, USA.
[3]Cardiovascular Research Center (CVRC) and DZHK German Center for Cardiovascular Research, partner site Hamburg/Kiel/Luebeck, Hamburg, Germany
[4]Division of Cardiology, Cardiovascular Research Institute, University of California, San Francisco, 505 Parnassus Avenue, M1182, San Francisco, CA, USA
[5]Cardiovascular Surgery, University Heart Center, Hamburg, Germany.

*Shared first authorship.

CORRESPONDING AUTHOR:

Sonja Schrepfer (Sonja.Schrepfer@ucsf.edu)

Email Addresses of Co-Authors:

Wang, Dong (Dong.wang@ucsf.edu)
Grigol Tediashvili (g.tediashvili@uke.de)
Xiaomeng Hu (xiaomeng.hu@ucsf.edu)
Alessia Gravina (alessia.gravina@ucsf.edu)
Sivan G. Marcus (sivan.marcus@ucsf.edu)
Hao Zhang (Hao.Zhang@ucsf.edu)
Jeffrey E Olgin (Jeffrey.Olgin@ucsf.edu)
Tobias Deuse (Tobias.Deuse@ucsf.edu)

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Heart failure, cardiac injury, myocardial infarct, mouse model, cryoinjury, heart surgery

SUMMARY:

This article demonstrates a model to study cardiac remodeling after myocardial cryoinjury in mice.

ABSTRACT:

The use of animal models is essential for developing new therapeutic strategies for acute coronary syndrome and its complications. In this article, we demonstrate a murine cryoinjury infarct model that generates precise infarct sizes with high reproducibility and replicability. In brief, after intubation and sternotomy of the animal, the heart is lifted from the thorax. The probe

of a handheld liquid nitrogen delivery system is applied onto the myocardial wall to induce cryoinjury. Impaired ventricular function and electrical conduction can be monitored with echocardiography or optical mapping. Transmural myocardial remodeling of the infarcted area is characterized by collagen deposition and loss of cardiomyocytes. Compared to other models (e.g., LAD-ligation), this model utilizes a handheld liquid nitrogen delivery system to generate more uniform infarct sizes.

INTRODUCTION:

Acute coronary syndrome (ACS) is the leading causes of death in the Western world[1,2]. Acute occlusion of the coronary arteries leads to activation of ischemic cascade and necrosis of the affected cardiac tissue[3]. Damaged myocardium is gradually replaced by non-contractile scar tissue, which manifestz clinically as a heart failure[4,5]. Despite recent advances in the treatment of ACS, the prevalence of ACS and ACS-related heart failure is rising, and therapeutic options are limited[6,7]. Therefore, developing animal models to study ACS and its complications are of immense interest.

To date, the most widely used animal model to study ACS and ACS-induced myocardial remodeling is the ligation of the left descending coronary artery (LAD). Ligation of the LAD leads to acute ischemia of the myocardium, similar to human myocardial tissue during ACS. However, inconsistent infarct sizes remain the Achilles' heel of LAD ligation. Surgical variation and anatomical variability of the LAD lead to inconsistent infarct sizes and hinder the reproducibility and replicability of this procedure[8-10]. In addition, LAD ligation has a high intra- and postsurgical mortality. Despite recent endeavors to improve reproducibility and reduce mortality[11,12], large numbers of animals are still needed to properly evaluate anti-remodeling therapies.

Alternative models of ACS have been proposed and studied over the recent years, including radio-frequency[13], thermal[14] or cryogenic injuries[15-18]. Current cryoinjury methods apply a metal rod pre-cooled in liquid nitrogen to damage the subject´s cardiac tissue[15,16]. However, this procedure needs to be repeated several times to generate a sufficient infarct size. Due to the high conductivity and low heat capacity of the rod compared to the tissue, the probe warms quickly, and the tissue is cooled (and thus infarcted) heterogeneously. To overcome these limitations, we describe herein a cryoinfarction model utilizing a hand-held liquid nitrogen delivery system. This model is reproducible, easy to perform and can be established fast and reliably. A reproducible transmural infarct lesion independent of coronary anatomy is generated, which eventually leads to cardiac failure. This method is especially suitable to study the remodeling process for the evaluation of novel therapeutic pharmacological and tissue engineering-based strategies.

PROTOCOL:

Animals received humane care in compliance with the Guide for the Principles of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources, and published by the National Institutes of Health. All animal protocols were approved by the responsible local authority (the University of California San Francisco (UCSF) Institutional Animal Care and Use Committee).

1. Animal care

1.1. Obtain mice at the age of 14 weeks weighing approximately 27 g (e.g., from the Institute of Laboratory Animals).

NOTE: BALB/c mice are used for this article.

1.2. Keep mice under conventional conditions in ventilated cabinets, feeding them standard mice chow and autoclaved water ad libitum.

2. Mouse preparation

2.1. Use an induction chamber to anaesthetize mouse with isoflurane (3.5%).

2.2. Remove the hair over the chest and neck using a hair trimmer.

2.3. Place mouse in supine position on a heated pad and maintain anesthesia with a facemask covering mouth and nose of the mouse.

2.4. Check for sufficient depth of anesthesia by pinching the hind feet and tail to verify an absence of reflexes.

2.5. Inject subcutaneous buprenorphine (0.03 mg/kg) for analgesia.

2.6. Spread the hind and fore limbs and fix their position using tape.

2.7. With povidone iodine, disinfect the shaved area, followed by scrubbing with 80% ethanol. Repeat this step twice.

2.8. Use a small scissor to make a midline skin incision from the lower third of the sternum to the chin.

2.9. Use curved forceps and carefully separate the muscles around the neck to expose the trachea.

2.10. Use a micro-scissor to perform a tracheotomy between the second and third cartilage rings.

2.11. Set the ventilator to a ventilation frequency of 110/min with a tidal volume of 0.5 mL.

2.12. Remove the facemask and insert a plastic cannula (20 G), connected to the ventilator, into the trachea. Ventilate the animal.

NOTE: Ensure that the ventilation cannula is not inserted too deep by confirming bilateral lung ventilation.

2.13. Use cautery to detach the right pectoralis muscle from its sternal origin between the third and seventh ribs.

2.14. Use side angled spring scissors to cut the fourth to sixth ribs as close as possible to the sternum.

2.15. Cauterize the mammary artery, if bleeding is visible.

2.16. Decrease isoflurane to 2.5%.

2.17. Dissect underlying connective tissue to obtain a clear view into the chest cavity.

2.18. Use blunt forceps to open the pericardium and expose the heart.

2.19. Use a mini Goldstein retractor to spread the ribs and keep the chest cavity open.

2.20. Lift the heart from the thoracic cavity with a blunt rod.

2.21. Decrease the tension of the retractor to reduce chest opening and to keep the heart from falling back.

2.22. Precool the cryoprobe (3 mm diameter) for 10 s.

2.23. Apply the cryoprobe on the anterior left ventricle wall and freeze for 10 s to generate a left ventricular cryo-injury infarct.

NOTE: The cryoprobe can be applied to different heart walls depending upon the scientific question and need.

2.24. Irrigate the cryoprobe with room temperature saline to detach the probe from the left ventricular wall.

2.25. Use the retractor to enlarge the chest opening.

2.26. Gently return the heart to the thoracic cavity with a blunt rod.

2.27. Remove the retractor and connect the sternotomy with a single knot using 6-0 suture.

2.28. Close the chest cavity using 6-0 running suture. Use a 10 mL syringe to evacuate any remaining air from the chest before tying the knot.

2.29. Adapt the skin at the caudal edge and suture it to the point of the tracheal opening with running suture (5-0).

2.30. Set isoflurane to 1.5% and wait until the animal gains spontaneous breathing.

2.31. Remove the tracheal catheter and reapply the facemask onto the animal mouth and nose to maintain anesthesia.

2.32. Close the tracheal incision with one 8-0 suture.

2.33. Reposition the ventral neck muscles back to their position to cover the trachea.

2.34. Complete the skin suture.

2.35. Add metamizole to the drinking water (50 mg metamizole per 100 mL) for pain analgesia for 3 days and monitor the animal daily.

NOTE: The observation period for this model is 8 weeks.

REPRESENTATIVE RESULTS:

The cryoinjury infarct model is suitable to study ACS and its complications. Low mortality rates and efficient postsurgical recovery is seen in this model. Cryoinjury induced myocardial damage leads to reduced cardiac function, electrical uncoupling, and transmural remodeling.

Echocardiography can be used to monitor cardiac function noninvasively in vivo. In cryo-injured hearts, echocardiography demonstrates significantly reduced ejection fraction and fractional area change (**Figure 1a–c**). Functional impairment continues from day 7 post-surgery until the observational endpoint of 56 days.

Detailed cardiac function can be assessed invasively through pressure volume loop (PV-loop) analysis. A 1.2 Fr conductance catheter is introduced into the left ventricle, and the left ventricular pressure is plotted against the left ventricular volume. Hemodynamic parameters such as stroke volume, stroke work, cardiac output, and preload-adjusted maximal power can be calculated. As shown in **Figure 1d–h**, cryoinfarction leads to impaired left ventricle (LV0 function, which is reflected as a decrease in stroke volume, stroke work, cardiac output and preload-adjusted maximal power.

To study cardiac electrophysiology, optical mapping can be performed ex vivo. Hearts are removed, perfused with Langendorff perfusion technique, and stained with a fluorescent voltage sensitive dye. Cryoinjured hearts demonstrate blockage of electrical conduction at the border of injury, indicating local electrical uncoupling (**Figure 1i**).

Histological staining with Masson's trichrome demonstrates transmural fibrotic tissue formation at the site of injury (**Figure 2a**). Infarct size can be calculated by measuring infarct scar area or midline scar length[19] (**Figure 2b**). Immunofluorescence staining against alpha- sarcomeric actinin

(cardiomyocyte marker) and collagen-I confirm fibrotic remodeling and loss of cardiomyocytes at the site of injury (**Figure 2c**).

FIGURE LEGENDS:

Figure 1: Functional and electrophysiological analysis of cryoinjured heart. Representative two-dimensional echocardiography images taken pre-operatively (D0) and at post-operative day 7 (D7), 28 (D28), and 56 (D56). (**a**) The top panel shows the parasternal long-axis view at end-diastole and the bottom panel at end-systole. (**b**, **c**) Ejection Fraction (EF) and Fractional Area Change (FAC) decrease after cryo-infarction and remained diminished over time Cardiac function was assessed invasively by pressure volume curve analysis. (**d–g**) Day 56 post injury stroke volume (SV), stroke work (SW), cardiac output (CO), and preload-adjusted maximal power (PAMP) were significantly lower than in pre-operative native animals. (**h**) Representative PV-loops from native and 56 days post-surgery animals showed characteristic right shift and decline in amplitude of the pressure signal following thoracic vena cava (TVC) occlusion. (**i**) Isochrone map of cardiac optical mapping from native and cryoinjured hearts 14 days post-surgery. Top and bottom panels show hearts paced from the apex and base, respectively. Infarct area is marked by dashed white line. Intergroup differences were assessed by one-way analysis of variance (ANOVA) with Bonferroni's post-Hoc test or Student's *t*-test. N = 3 animals. * indicates $p < 0.05$. The error bars represent the standard deviation (SD). ESPVR = end-systolic pressure volume relationship; EDPVR = end-diastolic pressure volume relationship.

Figure 2: Histologic assessment of native and cryo-injured hearts. (**a**) Masson's trichrome staining shows collagen deposition (green) in the infarcted area. The infarcted percentage of the left ventricle was measured as (**b**) area and (**c**) midline infarct length. (**d**) Immunofluorescence staining demonstrates increased collagen-I deposition with concomitant loss of cardiomyocytes in infarcted area. LV = left ventricle; RV = right ventricle; endo = endocardial; epi = epicardial. N = 3 animals. Error bars show SD.

DISCUSSION:

This article describes a mouse cryoinjury model to investigate ACS and related pharmacological and therapeutic options.

The most crucial step is the application of the cryoprobe on the cardiac tissue. Contact duration must be tightly controlled in order to obtain the optimal infarct size and to guarantee reproducible results. Prolonged cooling of the myocardium will lead to oversized infarcts or ventricular perforation. In contrast, shortened cooling time generates limited epicardial lesions and does not eliminate all resident cells. Hence, this can be confounding when studying regenerative cell transplantation.

Compared to other cryoinfarction methods[20], the open chest approach described in this article has the advantage that the infarct can be induced freely on different positions of the heart. Moreover, this approach facilitates therapeutic cell injection or patch applications, as the infarct border is visible, and the site of cell transplantation can be chosen accordingly.

A drawback of this model is the etiology of myocardial injury. Cryoinjury results in cell death due to the generation of ice crystals disrupting the cell membrane rather than a direct ischemia. In addition, the direction of injury is usually from epicardium inwards, whereas ischemic infarcts tend to propagate outwards from the endocardial to the epicardial layer. Therefore, this model is limited to study the pathophysiological mechanisms of myocardial ischemia or to imitate the ischemia-reperfusion setting.

In conclusion, the model described here is inexpensive, easy to perform, can be established fast and reliably. Cardiomyocyte necrosis and subsequent scar formation develop over time, resulting in progressive impaired pump function and electrical conductance. Well-controllable infarct size, shape and location make this model ideal to evaluate experimental interventions aiming to restore cardiac function or cardiac regeneration. Successfully tested treatment options should be further confirmed in large animal studies.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure

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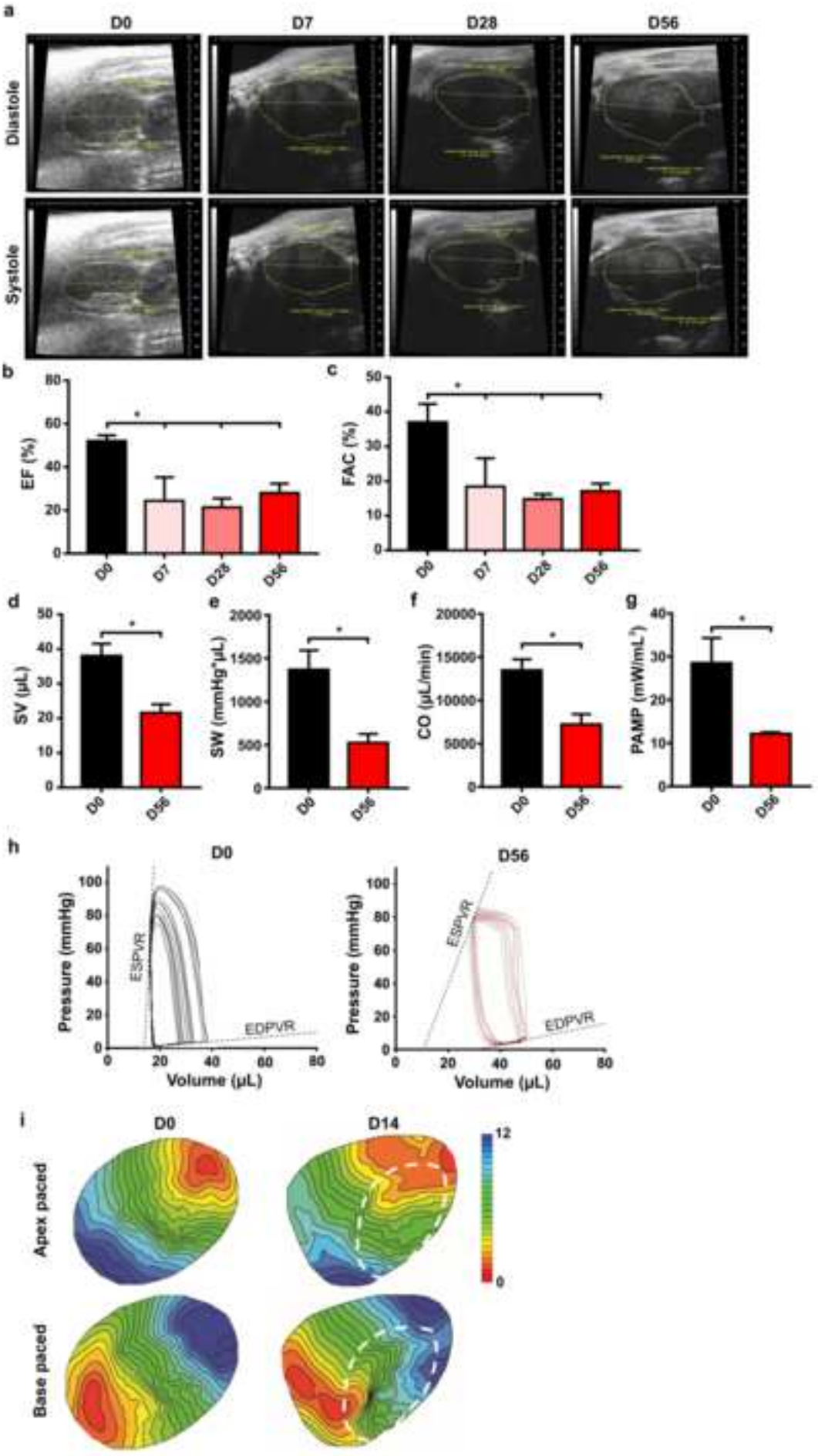

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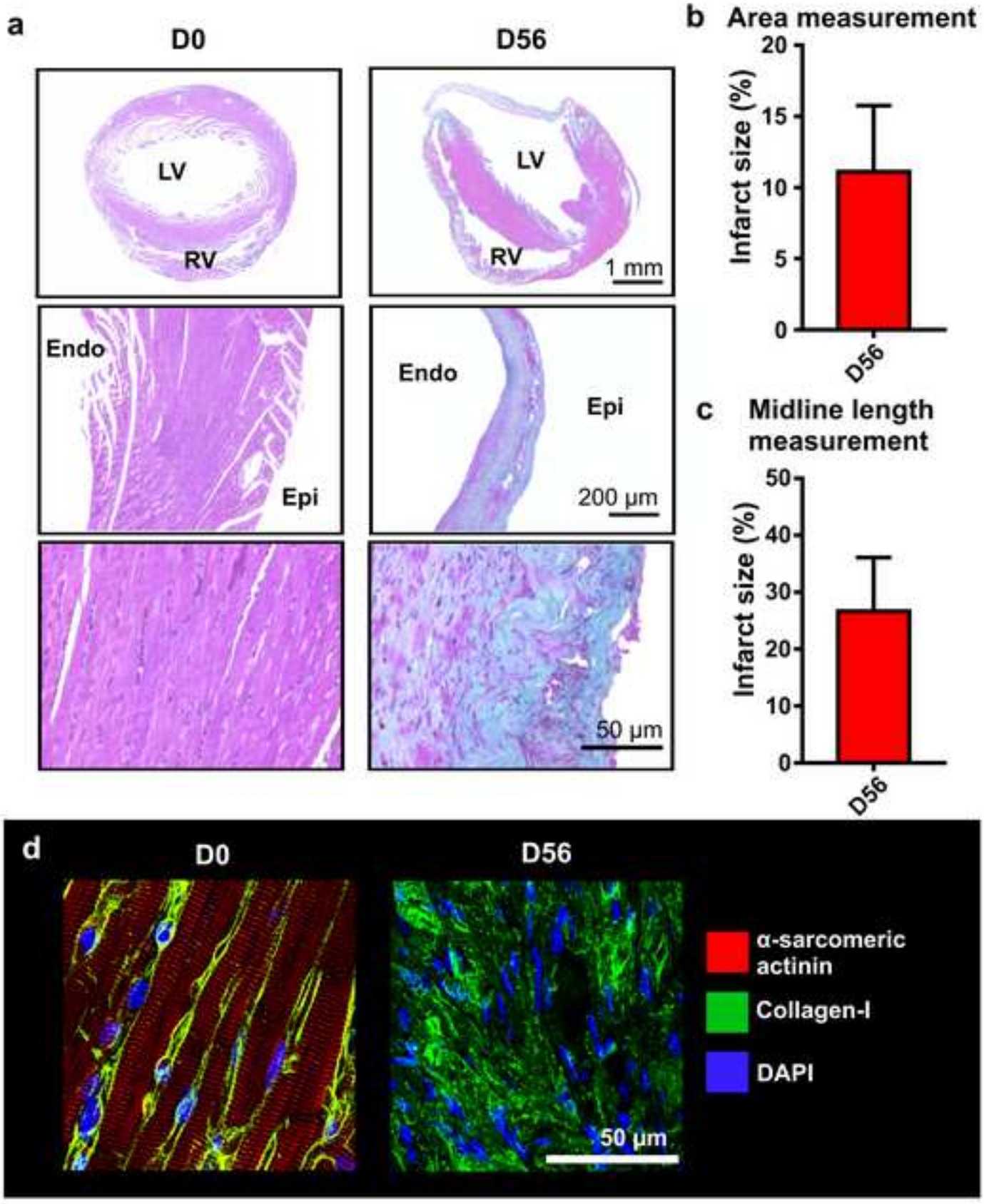

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10 ml Syringe	Thermo Scientific	03-377-23	
5-0 prolene suture	Ethicon	EH7229H	
6-0 prolene suture	Ethicon	8706H	
8-0 Ethilon suture	Ethicon	2808G	
Absorption Spears	Fine Science Tools	18105-01	
BALB/c	The Jackson Laboratory	Stock number 000651	
Bepanthen Eye and Nose ointment	Bayer	1578675	Eye ointment
Betadine Solution	Betadine Purdue Pharma	NDC:67618-152	
Blunt Forceps	Fine Science Tools	18025-10	
Buprenex	Reckitt Benckiser	NDC Codes: 12496-0757-1, 12496-0757-5	Buprenorphine
Cryoprobe 3mm	Brymill Cryogenic Systems	Cry-AC-3 B-800	
Ethanol 70%	Th. Geyer	2270	
Forceps curved	S&T	00284	
Forceps fine	Fine Science Tools	11251-20	
Forceps standard	Fine Science Tools	11023-10	
Gross Anatomy Probe	Fine Science Tools	10088-15	
Hair clipper	WAHL	8786-451A ARCO SE	
High temperature cautery kit	Bovie	18010-00	
ISOFLURANE	Henry Schein Animal Health	029405	
IV Catheter 20G	B. Braun	603028	
Mini-Goldstein Retractor	Fine Science Tools	17002-02	
NaCl 0.9%	B.Braun	PZN 06063042 Art. Nr.: 3570160	saline
Needle holder	Fine Science Tools	12075-14	
Needle Holder, Curved	Harvard Apparatus	72-0146	
Novaminsulfon	Ratiopharm	PZN 03530402	Metamizole
Operating Board	Braintree Scientific	39OP	
Replaceable Fine Tip	Bovie	H101	
Scissors	Fine Science Tools	14028-10	
Small Animal Ventilator	Kent Scientific	RV-01	
Spring Scissors - Angled to Side	Fine Science Tools	15006-09	
Surgical microscope	Leica	M651	
Transpore Surgical Tape	3M	1527-1	
Vannas Spring Scissors	Fine Science Tools	15400-12	
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CORRESPONDING AUTHOR

Name:	Sonja Schrepfer
Department:	Surgery
Institution:	UCSF
Title:	Professor
Signature:	Schrepfer
Date:	march 6th, 2019

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University of California
San Francisco

UCSF

Department of Surgery

TSI Lab
Transplant and Stem Cell Immunobiology Lab

Sonja Schrepfer, MD, PhD
Associate Professor

Department of Surgery
Medical Sciences S 1207
513 Parnassus Avenue
San Francisco,
CA 94143-2205
USA

Sonja.Schrepfer@ucsf.edu
www.tsi-lab.de

April 18, 2019

Dear Dr. DSouza,

Thank you very much for editing and reviewing our manuscript. Please find enclosed our revised protocol entitled “A cryoinjury model to study myocardial infarction in the mouse”.

All questions and comments of the editor have been addressed in the following with a line-by-line response.

We hereby certify that the material submitted for publication, nor parts of it, have been previously published or are currently under consideration for publication by any other journal. We declare no other competing interest.

We hope that you will view our revised manuscript favorably, and look forward to hearing from you at your earliest convenience.

Please do not hesitate to contact us with any questions or concerns you might have!

Yours sincerely,
Sonja Schrepfer

Response to Editorial:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Answer: The manuscript has been thoroughly proofread and spelling and grammar issues corrected.

2. Keywords: Please provide at least 6 keywords or phrases. More keywords has been added.

Answer: More keywords has been added: Heart failure; cardiac injury; myocardial infarct; mouse model; cryoinjury; heart surgery

3. Please revise the Protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Answer: The protocol has been revised and personal pronouns removed from the protocol.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by “(Table of Materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: FST, Cry-AC-3 B-800, Brymill Cryogenic Systems, prolene, ethilon, etc.

Answer: Commercial sounding language has been removed from the protocol.

5. Figure 1: Please abbreviate liters to L (L, mL, µL) to avoid confusion. Please define error bars and asterisk symbols in the figure legend.

Answer: Abbreviation and definition of symbols has added to the protocol.

6. Figure 2: Please include a space between the number and the units of the scale bar.

Answer: A space has been added in the figure.

7. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

Answer: Table of materials has been sorted alphabetically.

8. References: Please do not abbreviate journal titles.

Answer: Reference style has been changed into the style provided on the JoVE webpage.

Response to Reviever #1:

Major Concerns:

1. The cryoinjury method is very hard to control the variation of infarct size on the heart wall. If the author could develop a device or some experiment tricks to control the infarction size would be more helpful.

Answer: We thank the reviewer for this comment. To generate infarcts of the same size, cooling duration can be controlled with a timer. A timer is set to 10 sec., started, and the nitrogen probe is applied immediately onto the heart. Probe should be removed immediately, after the timer rings. By utilizing a timer during the injury step, identical cooling duration and injury extent can be achieved.

2. How long a mouse surgery will be taken? 15-20min. How many mice surgery could be done in a workday?

Answer: The surgery will take 15-20min. 12-15 animal surgeries can be routinely done on a workday.

3. What is the mortality rate of the mice during the surgery?

Answer: The mortality rate is ~10%.

4. How long it will take for the mice get conscious?

Answer: It will take 3-5min for the animal to gain consciousness.

Minor Concerns:

Please show the sham group data rather than d0 data in both figure 1 and 2.

Answer: We thank the reviewer for this comment. We have performed sham operations in the past and did not observe changes in heart function or histological remodeling. Hearts of sham-operated animals behave the same as native animal hearts. Due to the long observation time of 56d and the upcoming revision deadline as well as additional animal costs, we cannot add a sham group.

Response to Reviever #2:

Major Concerns:

1. The major weakness is that no data are provided to support the claim that this protocol results in higher reproducibility of infarct size compared to LAD ligation and previous cryoinjury methods. Although the authors measured infarct size by two different techniques, it is not indicated how precisely this was achieved (e.g. area in how many cross-sections apex/mid/base etc.). The technique should be described in more detail, preferably as part of the protocol. In Figure 2b,c the standard error or deviation (not even indicated in the Figure legend what exactly the bars depict) appears rather high and it is

not known how many animals were used. In addition, overall infarct size as measured by area appears rather low (10%).

Answer: We thank the author for this comment. The comparable high standard deviation is due to the small number included in the measurement (n=3). Infarct sizes were measured using the midline infarct length method and infarct area method according to Takagawa et al. (2007). Images of 20 Masson's trichrome stained sections per heart (interval of 150 µm between sections) were evaluated.
For the midline infarct length method: The LV myocardial midline was drawn at the center between the epicardial and endocardial surfaces and the length of the midline was measured as midline circumference. Midline infarct length was taken as the midline of the length of infarct that included greater than 50% of the whole thickness of the myocardial wall. Infarct size derived from midline length measurement was calculated by dividing the sum of midline infarct lengths from all sections by the sum of midline circumferences from all sections and multiplying by 100.
For infarct area measurenment: Infarct scar area and the total area of LV myocardium were measured in the digital images and calculated automatically by the computer. Infarct size, expressed as a percentage, was calculated by dividing the sum of infarct areas from all sections by the sum of LV areas from all sections (including those without infarct scar) and multiplied by 100.
Comparably low infarct area values are typical for infarct models. Cardiac remodeling after infarction leads to thinning of the infarct region with concomitant compensational hypertrophy of the viable region. Thinning of infarct area and hypertrophy of the viable region leads to possible underestimation of the severity of the infarct. In contrast, length based approaches measures the circumferential extent of the infarct scar and is not influenced by the thinning of the wall.

Takagawa, J. et al. Myocardial infarct size measurement in the mouse chronic infarction model: comparison of area- and length-based approaches. J Appl Physiol (1985). 102 (6), 2104-2111, (2007).

2. Another weakness is that the protocol does not include a sham control. Just comparing morphology and function to pre-surgery assessments might not be sufficient. In addition, surgery per se can induce a pro-inflammatory response and affect cardiac function.

Answer: We thank the reviewer for this comment. We have performed sham operations in the past and did not observe changes in heart function or histological remodeling.

3. Is there a reason why Balb/c mice are used? Given that many (i.e. most) genetically modified mice are on a C57BL/6 background, why not use C57BL/6 mice?

Answer: Both BALB/c and C57BL/6 mice can be used for this procedure. We have successful experiences with both animal strains.

4. Please add more procedural information on echocardiography, PV-loop recordings, and optical mapping.

Answer:
For Echocardiography animals were anesthetized with isofluorane (2%), shaved and positioned supine on a warmed echocardiography plate. While under continuous anesthesia, cardiac echography was performed with a Vevo660 system (VisualSonics,

Toronto, Canada) on day 1 prior cryoinjury and on day 7, 28 and 56 post-injury. The ultrasound transducer was immobilized on the shaved area overlying the heart to obtain a parasternal long- and short axis view. Ejection fraction and fractional shortening were calculated using the parasternal long axis view and the Vevo660 Imaging Software (VisualSonics).

For PV-Loop measurement (Open Chest Approach) animals were anesthetized with isoflurane (3.5%) and positioned on the heating pad. A tracheotomy was performed, and the animal mechanically ventilated. The Abdominal wall was opened in the proximity of the sternal manubrium and the diaphragm dissected to expose the heart apex. A 1.2F PV-Loop-Catheter (Transonic, Ithaca, NY, USA) was placed into the LV transapically. After calibration, intraventricular pressure and volume were measured. Data were acquired with an ADV500/ADVantage control unit connected to an amplifier (PowerLab 4/26, AD Instruments, Sydney, Australia) and analyzed with the Pressure Volume Loop Analysis Software (AD Instruments).

Optical Mapping: Mice were injected with heparin (10 U/gram) and anesthetized with Urethane (2 g/kg). The heart was rapidly excised and harvested in cold cardioplegia solution. The aorta was cannulated and retrogradely perfused for retrograde perfusion at a pressure of 80mmHg with modified Krebs-Henseleit solution. The pacing electrodes were punched to the left ventricular free wall below and above MI area. The cannulated heart was then placed in 37 °C Tyrode solution in a temperature-controlled optical recording chamber (maintained at 37 °C). The hearts were perfused with Tyrode solution containing voltage-sensitive dye di-4-ANEPPS (10 µl of 2.5 mM stock). Contractility was blocked using 7 M Blebbistatin.
For optical mapping of isolated mouse hearts, ten thousand simultaneous optical action potentials (APs) were recorded with a 100x100 CMOS camera (Ultima, SciMedia, Costa Mesa, CA, USA) within a 5 mm x 5 mm mapping field for ventricle. The tissue was excited using light from a 1000-W tungsten-halogen light source through an excitation filter of 530 nm and transmitted light collected via the CMOS through an emission long-pass filter of > 630 nm. Fluorescent optical maps were acquired at 1000 Hz during programmed electrical stimulation and were recorded during pacing drives of 150, 120, 90, 80, and 60 ms. Optical activation maps were analyzed using OMproCCD software (courtesy of Bum-rak Choi, Providence, RI).

5. How much oxygen was applied during anesthesia? Or just room air?

Answer: Ventilator was set to a ventilation frequency of 110/min with a tidal volume of 0.5 mL oxygen/isoflurane mix (1.5% – 3.5%).

6. Please add explanation why tracheotomy is used instead of transoral tracheal intubation.

Answer: Both tracheotomy and transoral tracheal intubation can be used for this model. However, we prefer using tracheotomy, because transoral intubation can lead to mucosal injury of the oral pharyngeal area. In addition transoral intubation in mice can be challenging and difficult to perform for a novice surgeon. Tracheotomy has the advantage, that the tube can be placed better accessibly and more securely.

7. In "real" clinical world, the patients go to the cath lab where blood flow to the infarcted area is restored. This can be nicely mimicked by transient ligation of the LAD (ischemia-reperfusion model), but not in a cryoinjury model. This should also be mentioned in the disadvantages of the technique in the discussion section.

Answer: We thank the reviewer for this advice. This limitation was added to the discussion section of the paper.

Minor Concerns:

1. Adaptations to previously described protocols (handheld liquid nitrogen delivery probe) and the overall goal of reaching better reproducibility in comparison to LAD ligation should be mentioned in the abstract.

Answer: We thank the reviewer for this suggestion. This limitation was added to the discussion section of the paper.

2. Please include references in the introduction as appropriate. For example, the claim that ACS and ACS-related complications are the leading cause of death worldwide should be referenced (e.g. WHO stats); a reference where details on the "ischemic cascade" are described etc.

Answer: Additional References were added into the introduction section.

3. Please correct for typos and syntax errors.

Answer: The manuscript has been thoroughly proofread and typos and syntax errors corrected.

4. ACS does not necessarily lead to myocardial infarction. Therefore I suggest to rephrase lines 104-105 in the introduction to ….prevalence of ischemic heart disease and infarct-related heart failure….

Answer: We thank the reviewer for this suggestion. This sentence has been rephrased in the introduction.