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Delayed intramyocardial delivery of stem cells after ischemia reperfusion injury in a murine model

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TITLE

Delayed Intramyocardial Delivery of Stem Cells After Ischemia Reperfusion Injury in a Murine Model

AUTHORS AND AFFILIATIONS

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KEYWORDS

cardiovascular, ischemia reperfusion, left anterior descending artery, infarction, stem cells, microsurgery, mice

SUMMARY

Stems cells are continuously investigated as potential treatments for individuals with myocardial damage, however, their decreased viability and retention within injured tissue can impact their long-term efficacy. In this manuscript we describe an alternative method for stem cell delivery in a murine model of ischemia reperfusion injury.

ABSTRACT

There is significant interest in the use of stem cells (SCs) for the recovery of cardiac function in individuals with myocardial injuries. Most commonly, cardiac stem cell therapy is studied by delivering SCs concurrently with the induction of myocardial injury. However, this approach presents two significant limitations: the early hostile pro-inflammatory ischemic environment may affect the survival of transplanted SCs, and it does not represent the subacute infarction scenario where SCs will likely be used. Here we describe a two-part series of surgical procedures for the induction of ischemia-reperfusion injury and delivery of mesenchymal stem cells (MSCs). This method of stem cell administration may allow for the longer viability and retention around damaged tissue by circumventing the initial immune response. A model of ischemia reperfusion injury was induced in mice accompanied by the delivery of mesenchymal stem cells (3.0×10^5), stably expressing the reporter gene firefly luciferase under the constitutively expressed CMV promoter, intramyocardially 7 days later. The animals were imaged via ultrasound and

bioluminescent imaging for confirmation of injury and injection of cells, respectively. Importantly, there was no added complication rate when performing this two-procedure approach for SC delivery. This method of stem cell administration, collectively with the utilization of state-of-the-art reporter genes, may allow for the in vivo study of viability and retention of transplanted SCs in a situation of chronic ischemia commonly seen clinically, while also circumventing the initial pro-inflammatory response. In summary, we established a protocol for the delayed delivery of stem cells into the myocardium, which can be used as a potential new approach in promoting regeneration of the damaged tissue.

INTRODUCTION

Cardiovascular disease remains the most common cause of morbidity and mortality worldwide. Cardiac ischemic events have been found to be detrimental to the overall function of the myocardium and surrounding cells[1]. Only ~0.45-1.0% of cardiomyocytes will regenerate every year after myocardial damage occurs[2]. Despite the growing demand and inherent focus on developing treatments, therapies aiding in the regeneration of injured tissue have been difficult to establish and still require further optimization[3-5]. Stem cell therapies have been introduced as an alternative path to rejuvenate damaged tissue after an ischemic event; however, advancement of these therapies has been challenged by the limited survival and retention of the cells to an injured area[6].

The microenvironment of the heart after an ischemic event can be characterized as hypoxic, pro-oxidant, and pro-inflammatory, presenting hostile conditions for therapeutic stem cells to adapt to for survival[7,8]. As an immune response is triggered following injury, naïve lymphocytes, macrophages, neutrophils and mast cells attempt to repair the damage by removing dying cells and modulating the process for tissue remodeling[9-11]. Within the first 3 days post-ischemia, inflammation is at its peak with the release of pro-inflammatory cytokines with high numbers of neutrophils and monocytes in the area[10,12]. After 7 days much of the inflammation has subsided and the transition to reparative cells begins, continuing until the remodeling cascade is complete, approximately 14 days in mice[13]. Our surgical method is a potential alternative approach to the introduction of biologics into the myocardium to bypass the peak innate immune response after ischemia reperfusion injury. At the same time, it will allow for the study of any treatments in a condition of subacute/chronic ischemia where there may be different variables to consider compared to acute myocardial infarction.

PROTOCOL

The experiments were performed on female C57BL/6 mice, age 10-12 weeks and 20-25 g body weight. All animal procedures complied with the standards stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD, USA) and were approved by the Mayo Clinic College of Medicine Institutional Animal Care and Use Committee (IACUC).

1. Preparation and intubation

1.1. Autoclave all surgical instruments before surgery. If multiple surgeries are to be performed in one session, clean the instruments after each animal and re-sterilize using a hot bead sterilizer.

1.2. Anesthetize the mice with 3.5-4% isoflurane at 1 L/min O_2 in an induction chamber.

1.3. Administer Buprenorphine SR 1 mg/kg (analgesic) subcutaneously, weigh the animal, and input the weight into the ventilator.

1.4. Shave the left side of the chest from the sternum to the level of the shoulder and apply depilatory cream to remove excess fur.

1.7. For the ischemia reperfusion procedure maintain the positive end-expiratory pressure (PEEP) on the ventilator at 2 cmH_2O. For the delayed injection of cells procedure change the PEEP to 3 cmH_2O to prevent lung collapse.

1.8. Intubate the animal using a 20 G endotracheal tube, transfer to a controlled heating pad to maintain a body temperature of 35-37 °C.

1.9. Place the mouse on a ventilator in lateral recumbency with cranial end on the left and caudal end on the right.

1.10. Maintain anesthesia at 2-2.5% isoflurane at 1 L/min O_2 for the remainder of the procedure.

1.11. Scrub the surgical area alternating between povidone-iodine and alcohol swabs three times and apply ophthalmic ointment to both eyes.

2. Ischemia reperfusion injury

2.1. Using a #10 blade scalpel make a vertical incision 2.5 mm to right of the leftmost nipple in the field of view.

2.2. Using scissors cut through the superficial muscle layers until the intercostal muscles and ribs are visible.

2.3. While lifting the ribs and surrounding tissue, cut through the intercostal space between the 4th and 5th ribs, then insert the eyelid retractor into the open space.

2.5. Retract the pericardium using curved forceps, moving the lung upwards and out of view.

2.6. Visualize LAD artery and, using a 9-0 nylon suture, pass through the myocardium beneath the artery 2.5 mm distal to the left auricle and tie a loose square knot.

2.7. Cut 1 cm of polyethylene tubing and place it within the loose knot.

2.8. Secure the suture around the tubing, confirm ischemia, then release after 35 min.

NOTE: Confirm ischemia by pallor and ventricular arrhythmia.

2.9. After releasing the ligation and removing the tubing, wait for 5 min to confirm reperfusion of the myocardium.

2.10. Place a 24 G IV catheter tube into the thoracic cavity one intercostal space to right of the opening.

2.11. Close the intercostal incision with a 6-0 absorbable suture in a simple interrupted pattern.

2.12. Close the muscle layer with a 6-0 absorbable suture in a continuous suture pattern.

2.13. After closing the superficial muscle layer, remove the chest tube while withdrawing the air from thoracic cavity using a 1 mL tuberculin syringe.

2.14. Close the skin incision with a 6-0 absorbable suture in a continuous horizontal mattress pattern

NOTE: Nylon sutures and a discontinuous suture pattern may also be used for the skin layer.

2.15. Administer 1.5 mL of warm saline subcutaneously and apply triple-antibiotic ointment to the incision site to prevent infection.

2.16. Turn off isoflurane and allow the animal to breathe through the ventilator on 100% O_2 until it can breathe continuously without aid.

2.17. Transfer the mouse to a bedding-free cage or a cage with covered bedding (paper towel or drape) on a warm pad with a temperature of 35-37 °C until fully recovered.

3. Mouse mesenchymal stem cell delivery

NOTE: The strain of mice used for the procedure are an inbred line and are deemed genetically identical. The mesenchymal stem cells were obtained from animals of the same strain and, by protocol design, immunosuppression was not induced[1].

3.1. Complete the preparation and intubation steps as done previously for the first procedure.

3.2. Remove the suture from the skin layer using scissors and forceps.

3.3. With a #10 scalpel, make an incision in the same location as the previous surgery.

3.4. Continue to use the scalpel to cut through scar tissue until muscle layer suture is visible

3.5. Using the scissors and forceps remove the suture and cut the muscle layer open.

3.6. Visualize and remove the sutures holding the ribs together and continue cutting through the intercostal muscle from the previous incision.

NOTE: The lungs may have adhered to the chest wall, if this occurs, use blunt or curved forceps to carefully separate and release them.

3.7. Place the eyelid retractor into the intercostal space and locate the area of the previous ligation.

3.8. Load the mesenchymal stem cells (3.0×10^5), suspended in 20 µL PBS, into a 30 G insulin syringe, bend the needle slightly as needed for the proper angle to inject.

NOTE: Mesenchymal stem cells (MSCs) were isolated from the adipose tissue of 4-6-week-old C56BL/6 mice. Early passage cells (p3) were transduced with a vector expressing the firefly luciferase gene under the CMV promoter to allow in vivo cell viability monitoring. Adipose-derived mouse MSC were characterized by flow cytometry and the cells were positive for CD44, CD29, CD90 and CD105 but negative for the hematopoietic marker CD45[14]. Prior to the injection, MSCs were cultured for at least one passage to avoid the loss of cells from the thawing process.

3.9. Moving in the direction from the apex towards the base of the heart insert the syringe into the peri-infarct region until the needle opening is completely inside the myocardium.

3.10. Once inside slowly inject the cells into the myocardium, wait 3 s, then remove the needle.

3.11. Observe the heart closely for 3 min to be sure of no abnormal reactions to the cells such as ventricular fibrillation.

3.12. Place a 24 G IV catheter tube into the thoracic cavity one intercostal space to right of the opening.

3.13. Close the intercostal, muscle, and skin layers and remove the chest tube in the same method as the first procedure.

3.17. Administer 1.5 mL of warm saline subcutaneously and apply triple-antibiotic ointment to the incision site to prevent infection.

3.18. Turn off isoflurane and allow animal to breathe through the ventilator on 100% O_2 until it is able to breathe continuously without aid.

3.19. Transfer the mouse to a bedding-free cage or a cage with covered bedding (paper towel or drape) on a warm pad with a temperature of 35-37 °C until fully recovered.

4. Post-operative care following both procedures

4.1. Observe the animal continuously until spontaneous breathing, sternal recumbency and normal movement is established.

4.2. Continue observation every 15-30 min for at least 3 h on the day of the surgery.

4.3. Check the mice for wound dehiscence or abnormal pain once daily for 5 days, then 2-3 times weekly.

4.4. If the animal shows signs of pain (i.e. arched back, minimal movement, grimacing, or scruffy fur) after 72 h post-op, provide an additional dose of the Buprenorphine SR analgesic.

REPRESENTATIVE RESULTS

Ischemia reperfusion injury was induced in mice on day 0, followed by a post-operative echocardiogram and electrocardiogram on the day preceding stem cell implantation. Ultrasound and electrocardiogram analysis confirmed infarction and decreased ventricular contractile function **(Figure 1A-D)**. Further examination of the data showed the ejection fraction and fractional shortening were decreased in mice that received ischemic injury, while the end-diastolic and systolic volumes increased **(Table 1)**. Compared to a normal mouse heart (**Figure 2A**), Masson Trichrome staining of myocardial tissue 7 days post-injury **(Figure 2B)** showed increased collagen deposition and thinning of the left ventricular wall. The second procedure was performed 7 days after injury; mice were given an intramyocardial injection of mesenchymal stem cells (3.0×10^5 in 20 µL PBS) stably expressing the reporter gene firefly luciferase under the constitutively expressed CMV promoter. In vivo bioluminescent imaging (BLI) of these mice was completed the day after stem cell implantation for confirmation of a successful injection. The successful delivery of MSCs is exemplified by the BLI signal, compared to mice that had induced ischemia reperfusion injury but did not receive MSCs **(Figures 3A,B)**. This dual interventional procedure had an attrition rate of 22%, similar to that observed in animals that received MSCs in the acute scenario.

[Place **Figure 1** here]

[Place **Table 1** here]

[Place **Figure 2** here]

[Place **Figure 3** here]

FIGURE AND TABLE LEGENDS

Figure 1: Imaging of mice heart function. Ultrasound analysis of mouse at baseline (**A**) shows uniform contraction of left ventricle myocardium compared to a mouse after ischemia reperfusion injury (**B**), which shows decreased ventricular movement. When compared to the baseline electrocardiogram of a normal mouse (**C**), there are significant shifts in the ST segment of a mouse with ischemia reperfusion injury (**D**), indicating a decrease in ventricular function.

Table 1: Echocardiography analysis. Variables are expressed as Mean ± Standard Error of the Mean. EF: Ejection Fraction, FS: Fractional Shortening, EDV: End-Diastolic Volume, ESV: End-Systolic Volume, SV: Stroke Volume.

Figure 2: Histological Staining of Heart Tissue. Masson's Trichrome staining of the myocardium in normal mouse (**A**) shows no injury to the cardiac tissue, whereas the mouse with ischemia reperfusion injury (**B**) shows increased collagen deposition and thinning in the myocardium of left ventricle, supporting the determination of a successful infarction.

Figure 3: In vivo bioluminescent imaging. A mouse with ischemia reperfusion injury that did not receive intramyocardial injection of stem cells showed no bioluminescent signal (**A)**. A mouse with ischemia reperfusion injury which received a delayed injection of mesenchymal stem cells (CMV-FLUC) showed a significant amount of signal (**B**).

DISCUSSION

Over 85 million people worldwide are affected by cardiovascular disease[3]. The high prevalence of these ischemic events warrants further development and expansion of alternative therapies for promoting the regeneration of damaged tissue. Traditional methods utilize the ischemia reperfusion procedure in an acute setting with subsequent administration of therapeutics[1]. Inflammatory reactions are at its peak between 3-4 days postdating a cardiac ischemic event, with infiltration of neutrophils, macrophages, and increased cytokine signaling[10,12]. After this period of degradation of dead cells, the primary immune response begins to subside and transition towards remodeling phases[13]. Furthermore, it is important that treatments are investigated within the same scenario as presented in the clinical setting. In this manuscript, we are showing representative results obtained from ischemic mice to demonstrate the feasibility and the safety of the double surgical procedure, with delayed injection of MSCs. We believe that this approach can be used not only for myocardial ischemia animal models, but also for animal models of any disease where inflammation may play a critical role, altering the success of therapeutic strategies that involve biologics, such as cell or drug therapies.

Therefore, in this manuscript we describe a surgical method for delivering stem cells into a subacute infarction, 7-10 days after inducing ischemia reperfusion injury in mice. This technique will be useful in studying stem cell viability and biology in connection to different stages of the immune response and in the subacute/chronic phase of the ischemic disease process. Murine models are ideal subjects for this method of study in terms of reproducibility and convenience, however, they may bear some disadvantages. The size of the animal warrants a certain degree of surgical skill although, with practice, these procedures can be completed successfully.

To perform the procedures presented in this manuscript, it is important to note some key steps and observations essential to the successful completion of these surgeries. A critical step of the first procedure is the ligation of the left anterior descending coronary artery (LAD) and placement of polyethylene tubing to achieve temporary ischemia of the myocardium. Use of sterile tapered tip cotton swabs to place pressure on the cardiac tissue distal to the atrium allows for enhanced delineation of the LAD. Once the tubing is in place and the suture tightly secured, observation of arrhythmia and pallor of the tissue is essential to determining successful induction of ischemia. The period of ischemia and the subsequent reperfusion, once the suture is released, is important for consistency of injury across multiple animals. Additionally, during the second described procedure, the injection of mesenchymal stem cells must be performed with horizontal movements distal to proximal direction. Due to resulting fibrosis from the first procedure, significant but steady pressure is required to insert the needle followed by a slow consistent injection of the cells to prevent shock. Finally, providing continuous heat and supplemental subcutaneous fluids before waking mice from anesthesia will prevent heat loss and aid in the replacement of any blood lost during the procedures, as well as the animal's overall recovery.

In this manuscript, we provide a protocol for completing multiple procedures as a method of administering stem cells as a therapeutic treatment in a murine model of chronic ischemia reperfusion injury. Utilization of these surgical procedures offers a new approach for the delivery of stem cells into the hostile ischemic environment after injury to enhance their viability over time. Use of this approach for the study of stem cell therapy will significantly complement other studies focusing on the use of SCs in the acute setting. In conclusion, the described protocol is successful in inducing ischemic injury and the ensuing delayed implantation of stem cells for use as a model in preclinical studies.

ACKNOWLEDGMENTS

None.

DISCLOSURES

The authors have nothing to disclose.

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Figure 1

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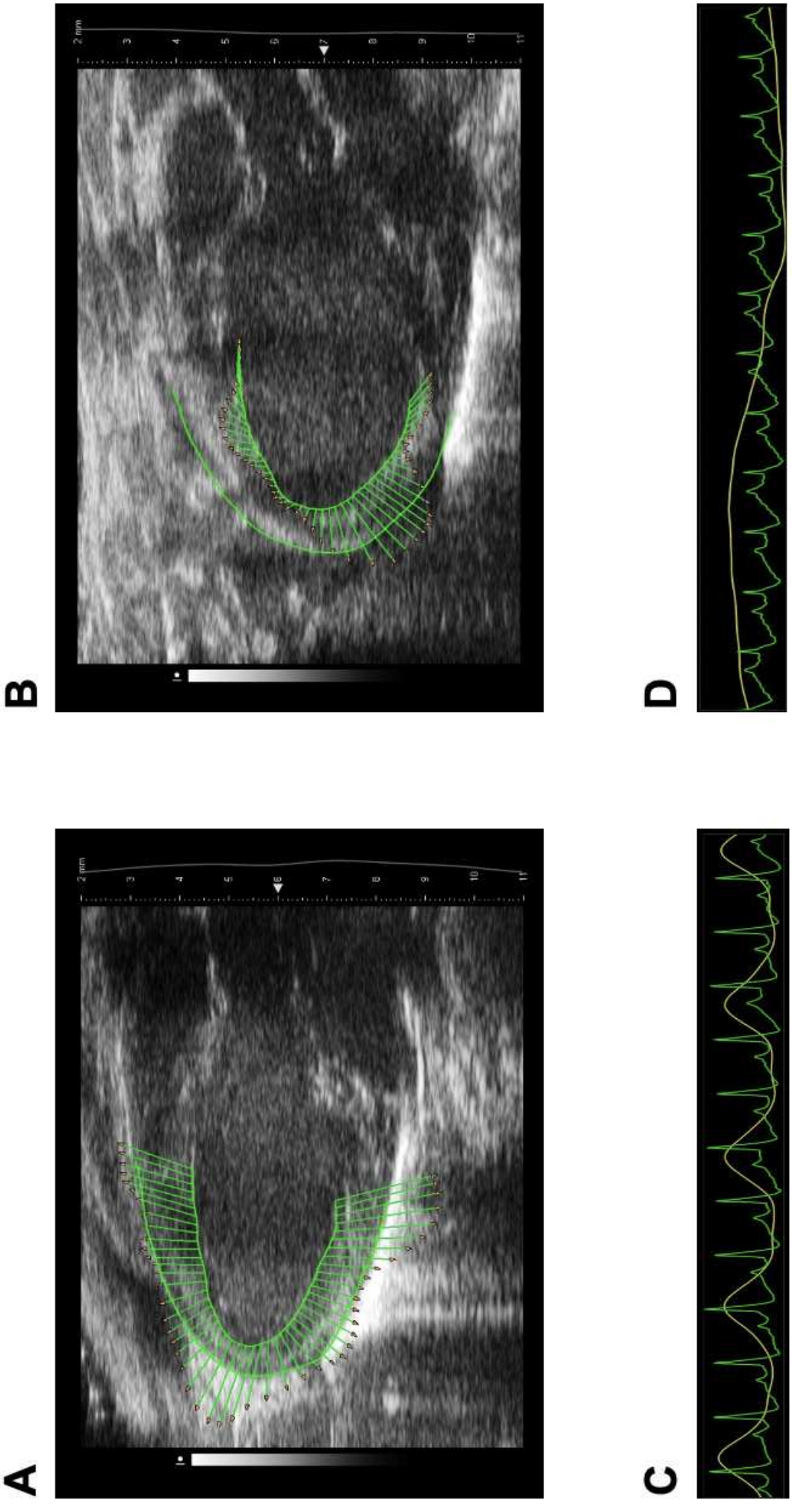

Figure 2

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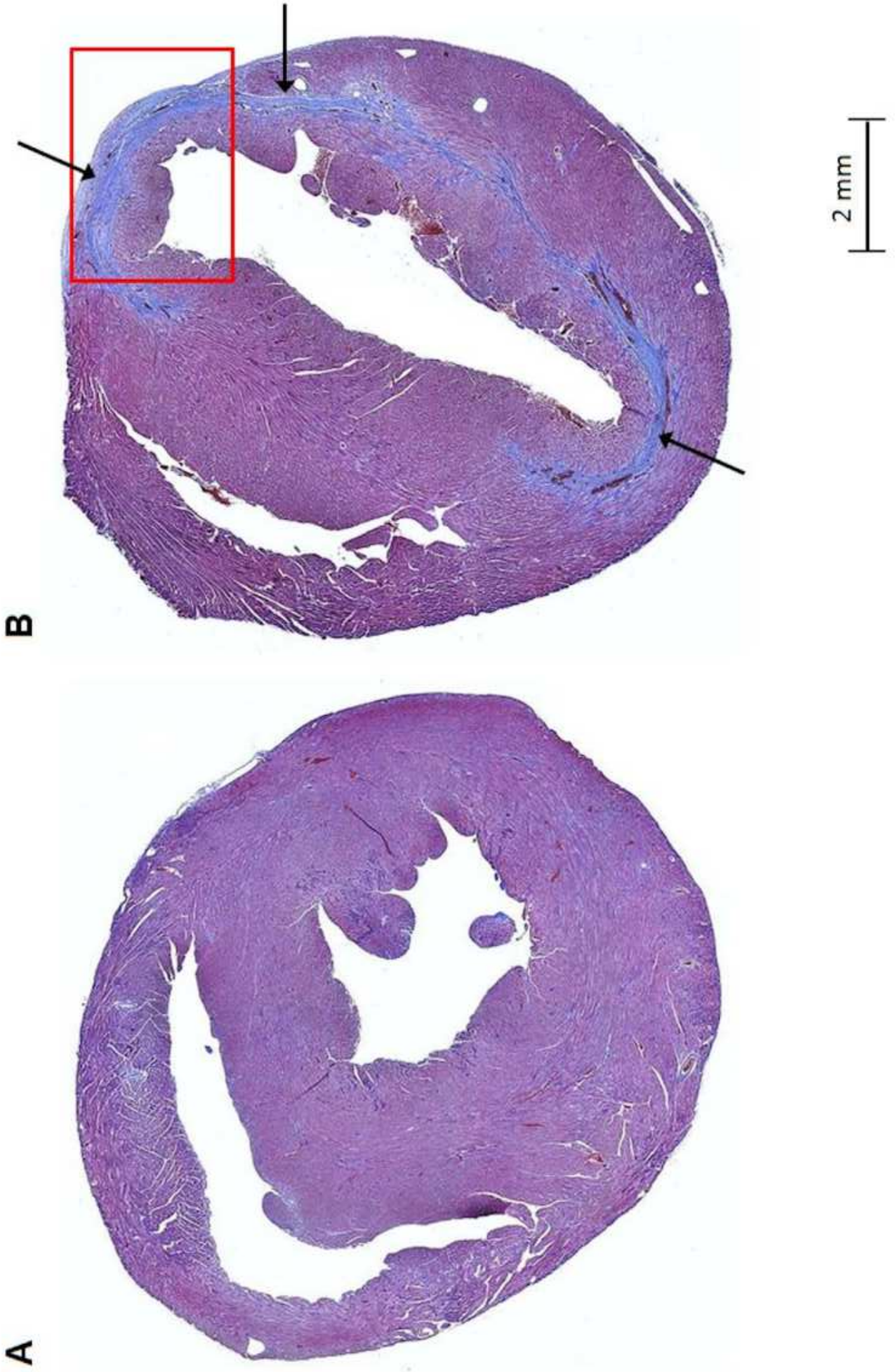

Figure 3

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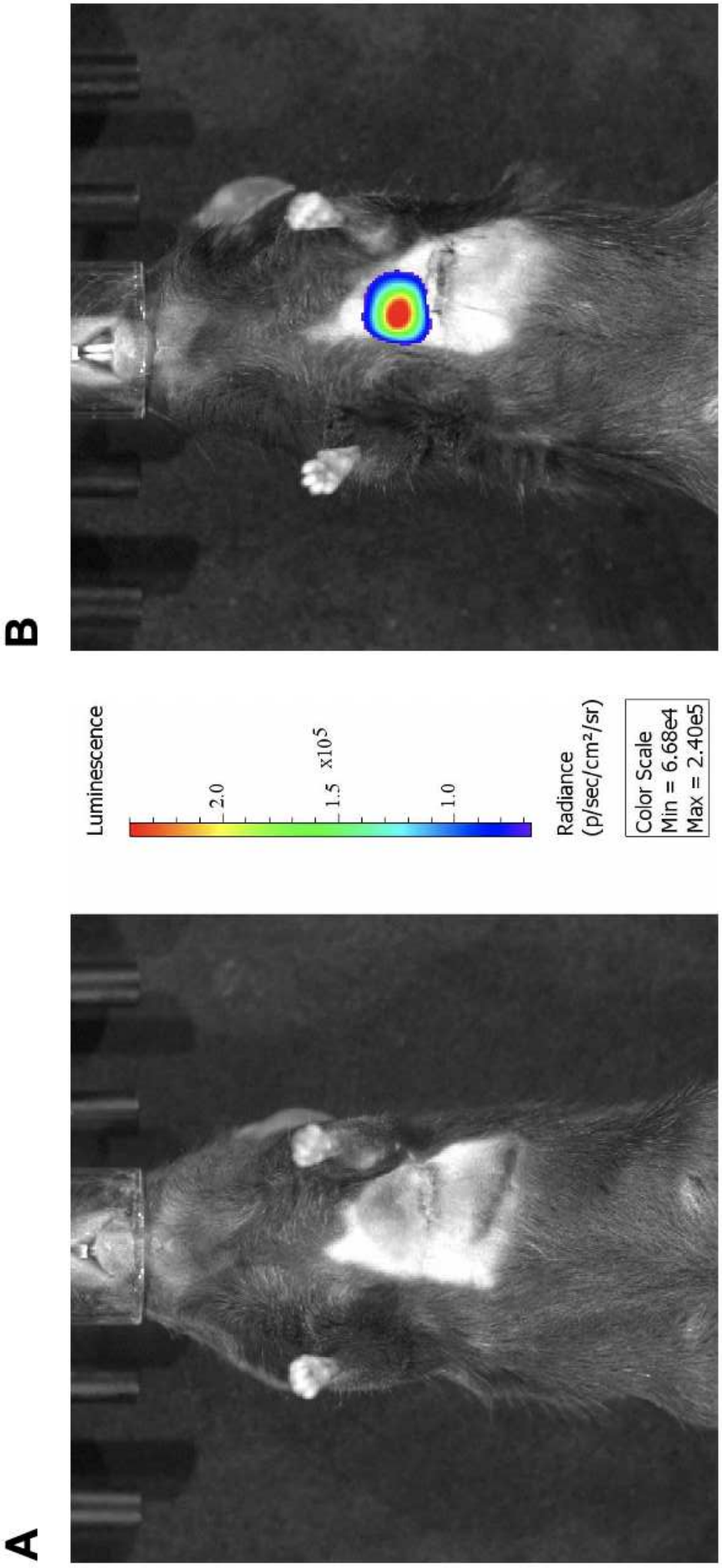

Table 1

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	EF%	FS%	EDV (µl)	ESV (µl)	SV (µl)
Baseline	74.19±1.2	44.67±2	23.8±3.6	6.14±0.98	17.68±2.7
Post-IR	43.9±3.8	30.65±3.8	33.88±4.4	18.11±1.4	15.74±3.2

Table of Materials

Click here to access/download;Table of Materials;Materials.xlsx

Name of Reagent/ Equipment	Company	Catalog Number
0.9% NaCl Irrigation, USP	Baxter	0338-0048-04
11x12" Press n' Seal surgical drape, autoclavable	SAI Infusion Technologies	PSS-SD
24G 3/4" IV catheter tube	Jelco	4053
28G x 1/2" 1mL allergy syringe	BD	305500
30G x 1/2" 3/10cc insulin syringe	Ulticare	08222.0933.56
6-0 S-29, 12" Vicryl suture	Ethicon	J556G
9-0 BV100-4, 5" Ethilon suture	Ethicon	2829G
Absorbent underpad	Thermo Fischer Scientific	14-206-64
Alcohol prep pads, 2 ply, medium	Coviden	6818
Anti-fog face mask	Halyard	49235
Bonn Strabismus scissors, curved, blunt	Fine Science Tools	14085-09
Buprenorphine HCL SR LAB 1mg/ml, 5 ml	ZooPharm Pharmacy	
Castroviejo needle holders, curved	Fine Science Tools	12061-01
Curity sterile gauze sponges	Coviden	397310
Delicate suture tying forceps, 45 angle bent	Fine Science Tools	11063-07
Electric Razor	Wahl	
Isoflurane 100 ml	Cardinal Health	PI23238
Lab coat		
Monoject 1 mL hypodermic syringe	Coviden	8881501400
Moria iris forceps, curved, serrated (x2)	Fine Science Tools	11370-31
Moria speculum retractor	Fine Science Tools	17370-53
Mouse endotracheal intubation kit	Kent Scientific	
Nair depilatory cream	Johnson & Johnson	
Optixcare eye lube plus	Aventix	
Physiosuite ventilator	Kent Scientific	
PolyE Polyethylene tubing	Harvard Apparatus	72-0191
Povidone-iodine swabs	PDI	S41125
Scalpel, 10-blade	Bard-Parker	371610
Sterile 3" cotton tipped applicators	Cardinal Health	C15055-003
Sterile 6" tapered cotton tip applicators	Puritan	25-826-5WC
Sterile gloves	Cardinal Health	N8830
Sterilization pouches	Medline	MPP100525GS
Surgery cap		
Surgical Microscope	Leica	M125
Suture tying forceps, straight (x2)	Fine Science Tools	10825-10
Transpore surgical tape	3M	1527-1
Triple antibiotic ointment	G&W Laboratories	11-2683ILNC2
Vannas-Tübingen Spring Scissors, curved	Fine Science Tools	15004-08
Vetflo vaporizer	Kent Scientific	

Comments/Description
Injection of analgesic Injection of stem cells Intercostal, superficial muscle and skin layer incision closure Ligation of the LAD artery For underneath the animal
Buprenorphine narcotic analgesic formulated in a polymer that slows absorption extending duration of action (72 h
Fur removal Anesthetic
Fur removal Sterile ocular lubricant
Temporary compression of LAD artery
Topical application to prevent infection

ours duration of activity).

Rebuttal Letter

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May 20, 2020

Dear Bajaj,

Please find enclosed a revised version of the manuscript entitled “Delayed intramyocardial delivery of stem cells after ischemia reperfusion injury in a murine model” by Olthoff et al. We appreciate the expert reviewers’ comments about our manuscript, which gave us the opportunity to improve its quality.

We are hopeful that this work will be considered for publication in the *Journal of Visualized Experiments* and look forward to a positive response.

Sincerely,

Michaela Olthoff

Editorial 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. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

The manuscript has been reviewed for grammatical errors.

2. 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

The format of the manuscript has been examined to ensure it follows the guidelines provided.

3. Please revise the Introduction to include all of the following with citations:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application

The introduction has been examined to ensure all of the above listed standards have been met.

4. JoVE cannot publish manuscripts containing commercial language. 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 and Reagents.
For example: Ethilon, vicryl, etc.

All commercial language has been removed from the manuscript

5.

We did not receive any comment in place of #5

6. 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."

All text has been reviewed to ensure all text is in the imperative tense.

7. The Protocol should contain only action items that direct the reader to do something.

The protocol has been reviewed to verify action items are present throughout.

8. Please ensure you answer the "how" question, i.e., how is the step performed?

All steps within the protocol have been reviewed to make certain they clearly provide this information to the reader.

9. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

Steps within the protocol have been double checked to confirm that 3 or less actions are performed in each.

10. 1: Please include the strain, age, sex of the animal used in the study.

This information can be found in the first sentence of the protocol section in line 80

"The experiments were performed on female C57BL/6 mice, age 10-12 weeks and 20-25g body weight."

11. 3: How are mice immunocompromised before the stem cell delivery?

The strain of mice used for the procedure are an inbred line and are deemed genetically identical. The mesenchymal stem cells were obtained from animals of the same strain and, by protocol design, immunosuppression was not induced.

12. 3.8: Which stem cells are injected? Number of cell injected per animal? Volume of injection?

These details have been added to the protocol section for step 3.8 in line 188

This information can also be found in the results section in line 231: *"The second procedure was performed 7 days after injury; mice were given an intramyocardial injection of mesenchymal stem cells (3.0 x 10^5 in 20µL PBS) stably expressing the reporter gene firefly luciferase under the constitutively expressed CMV promoter."*

13. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

The section of the protocol we are requesting be translated to the video are highlighted in yellow.

14. Please ensure the results are described in the context of the presented technique, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

The representative results section has been examined to ensure the listed stipulations have been met.

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None of the figures we provided in this manuscript have been used in another publication.

16. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

The discussion section has been reviewed to ensure the above listed guidelines have been met.

17. Please do not abbreviate the journal titles in the references section.

References have been changed to reflect the full-length journal titles

18. Figure 2: Please include a scale bar.

A scale bar has been added to Figure 2 as requested

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
Olthoff et al. developed an alternative stem cell (SC) transplantation protocol by inducing the ischemia-reperfusion (I/R) injury, followed by cell delivery 7 days later. The viability and retention of transplanted cells are still a serious obstacle for therapeutic cell transplantation. The authors reported that transplantation of SCs 7 days post I/R injury may circumvent the

initial hostile immune response and improve viability and retention of transplanted cells in the infarct area. Moreover, these procedures are more clinically relevant compared to the traditional method of delivery right after the induction of ischemic injury. Using a reporter gene such as firefly luciferase to trace the transplanted cells provided an alternative way to evaluate the survival and retention of the transplanted cells in vivo. Finally, the authors claimed that the new approach will enhance the SC viability over time after transplantation and will significantly complement other SC transplantation studies used with an acute myocardial infarction model. This approach is a well-established technique that many stem cell research groups have been applying on both small and large animal models. In this article, the authors provided the details for the whole procedure, but as a surgical protocol, there remain several limitations in this manuscript.

Major Concerns:
1) As this protocol is for stem cell transplantation study, and the recipient animals are not immunodeficient, immunosuppressants should be used before and after cell transplantation to protect the cells. The authors did not use those medications, please explain the reason.

The strain of mice used for the procedure are an inbred line and are deemed genetically identical. The mesenchymal stem cells were obtained from animals of the same strain and, by protocol design, immunosuppression was not induced.

2) Multiple sites (peri-infarct area and infarct area) cell transplantations are a common way for most stem cell research groups to perform now, as enough survival cells are one of the key factors for successful cell transplantation studies. If the authors can include the details on the procedures in this protocol, it will be better guidance for the stem cell researchers who work on cardiac stem cell transplantation study.

The site of stem cell delivery has been further specified in the protocol section as requested and can be found in line 191

"3.9. Moving in the direction from the apex towards the base of the heart insert the syringe into the peri-infarct region until the needle opening is completely inside the myocardium."

3) The authors indicated that the approach will enhance the stem cell viability over time after transplantation. The authors should show the difference in stem cell retention using their approach compared to transplantation immediately after the injury. For example, Masson Trichrome staining and bioluminescent imaging of the hearts one or two months post cell transplantation.

We appreciate the reviewer's comment. For this study we did not analyze long time points (one or two months). The main aim of this manuscript is to provide a visualized protocol for a successful double surgical procedure that can be used to administer biologics into the ischemic myocardium. We agree that when testing drugs or other biologics, it is critical to perform histochemical analysis at different and longer time points.

4) In the discussion section, the first paragraph should be re-written as most of the contents have been stated in the introduction section. The authors discussed the reasons, applications and key steps of the delayed cell transplantation protocol, but did not discuss the outcomes of this approach. Please add that information.

The first paragraph of the discussion section has been re-written as requested and now states:

"In this manuscript, we are showing representative results obtained from ischemic mice to demonstrate the feasibility and the safety of the double surgical procedure, with delayed injection of MSCs. We believe that this approach can be used not only for myocardial ischemia animal models, but also for animal models of any disease where inflammation may play a critical role, altering the success of therapeutic strategies that involve biologics, such as cell or drug therapies."

Minor Concerns:
1) Line 42, please add "stably expressing the reporter gene firefly luciferase under the constitutively expressed CMV promoter" after mesenchymal stem cells to clarify to readers what the bioluminescent imaging was used for.

The requested addition to the abstract has been made and can be found in line 43.

"A model of ischemia reperfusion injury was induced in mice accompanied by the delivery of mesenchymal stem cells (3.0 x 10^5), stably expressing the reporter gene firefly luciferase under the constitutively expressed CMV promoter, intramyocardially 7 days later."

2) Line 99, please explain what PEEP is.
An adjusted description of the protocol step has been added to include the explanation of PEEP, and is now line 100.

"1.7. For the ischemia reperfusion procedure maintain the positive end-expiratory pressure (PEEP) on the ventilator at 2 cmH_2O, for the delayed injection of cells procedure change the PEEP to 3 cmH_2O to prevent lung collapse."

3) Line 121, please specify which intercostal space and how the authors will avoid hurting the lung when cutting through.
The mentioned line/step of the protocol has been further clarified as requested and can be found in line 123.

"2.3. While lifting the ribs and surrounding tissue, cut through the intercostal space between the 4th and 5th ribs, then insert the eyelid retractor into the open space"

4) Line 187, please describe the method by which the authors avoided the cell suspension leakage after injection.

The method of preventing leakage of cells is described in step 3.10 in which the surgeon slowly injects the cells and is followed by a waiting period before removing the needle. This has prevented loss of the suspension by allowing time for initial absorption.

"3.10. Once inside slowly inject the cells into the myocardium, wait 3 seconds, then remove the needle"

5) Line 190, please describe the abnormal reactions.
The abnormal reaction has been further described in step 3.11 of the protocol in line 197.

"3.11. Observe the heart closely for 3 minutes to be sure of no abnormal reactions to the cells such as ventricular fibrillation"

6) Line 222, is an ultrasound a different test compared to an echocardiogram in this manuscript?
They are the same in this manuscript, there was an accidental error and both terms were used in the same sentence. This has been corrected.

"Ischemia reperfusion injury was induced in mice on day 0, followed by a post-operative echocardiogram and electrocardiogram on the day preceding stem cell implantation."

Reviewer #2:
Manuscript Summary:
Manuscript entitled "Delayed intramyocardial delivery of stem cells after ischemia reperfusion injury in a murine model" by Olthoff et al. demonstrates a protocol for the delayed delivery of stem cells into the myocardium, along with a protocol for the induction of ischemia-reperfusion injury with the ligation of the left anterior descending coronary artery in mice. The protocol can be used as a potential new approach in promoting regeneration of damaged tissue. The manuscript is concisely written. There are only minor concern regarding this manuscript as far as the reviewer considers.

Major Concerns:
None.

Minor Concerns:
Line 79. "The experiments were performed on female C57BL/6 mice,"
It is my personal interest, but why only female did you use?

We appreciate the reviewer's comment. The studies presented in this manuscript represent female mice only, however there is no specific reason as to why this would not apply to male mice. In fact, we would encourage the use of both male and female mice in future studies.

Line 245. "the baseline echocardiogram of a normal mouse"
Isn't it electrocardiogram?

Yes, the error has been corrected.

"When compared to the baseline electrocardiogram of a normal mouse"

Line 251. "increased collagen deposition and thinning"
It would be easier for readers if they were indicated with arrows.

We have added arrows to delineate the areas of collagen deposition and a box encompassing the area of the heart where there is thinning of the ventricle wall.

Line 264. "Inflammatory reactions are at its peak between 3-4 days postdating a cardiac ischemic event"
Is it the same for human? Maybe readers are interested in the comparison between human and mouse.

We appreciate the important comment. As suggested, studies have shown that inflammatory reactions are at its peak within the first week after injury, indicating that both the mouse and human follow the same approximate timeline.

Ong SB, Hernández-Reséndiz S, Crespo-Avilan GE, et al. Inflammation following acute myocardial infarction: Multiple players, dynamic roles, and novel therapeutic opportunities. *Pharmacol Ther*. 2018;186:73-87. doi:10.1016/j.pharmthera.2018.01.001

Prabhu SD, Frangogiannis NG. The Biological Basis for Cardiac Repair After Myocardial Infarction: From Inflammation to Fibrosis. *Circ Res*. 2016;119(1):91-112. doi:10.1161/CIRCRESAHA.116.303577

Reviewer #3:
Title: Delayed intramyocardial delivery of stem cells after ischemia reperfusion injury in a murine model
Manuscript ID: JoVE61546
This is a timely report and has significant implication in the field of translational study of myocardial regeneration.

From my perspective, since echocardiography was used to visualize the I/R injury, EF (%), FS (%), LVESD (mm), LVEDD (mm), LVESV (ul), LVEDV (ul) , LVPWS (mm) , LVPWD (mm), IVSS (mm) , IVSD (mm) might be given if available.
To summarize, I am prone to accept this article after minor revision.

Table 1 has been added to reflect the cardiac function of both normal mice and mice with ischemia-reperfusion injury obtained by echocardiography.

Reviewer #4:
Manuscript Summary:
Olthoff et al. describe a protocol for delivering a stem cell load into the myocardium 7 days after the induction of an ischemia reperfusion injury model in the mouse. I would like to see more details about the stem cells themselves, their retention, distribution etc as a baseline for people following this protocol to have some confidence in what to expect.

Major Concerns:
1.To be used appropriately as a methods paper for a stem cell therapy more details should be included on the cells themselves. No details other than the cell number are provided. Things that should be mentioned: cell viability during the procedure and post-op, cell source, cell markers (for cell tracking in the absence of a reporter line), how cells should be prepared, used freshly thawed or after multiple passages. An essential baseline of these details should be provided for a protocol paper.

Mesenchymal stem cells (MSCs) were isolated from the adipose tissue of 4-6-week-old C56BL/6 mice. Early passage cells (p3) were transduced with a vector expressing the firefly luciferase gene under the CMV promoter to allow in vivo cell viability monitoring. We characterized the adipose-derived mouse MSC by flow cytometry and the cells were positive for CD44, CD29, CD90 and CD105 but negative for the hematopoietic marker CD45.

Dominici M., Le Blanc K., Mueller I., Slaper-Cortenbach I., Marini F., Krause D., Deans R., Keating A., Prockop D., Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*. 2006;8:315–317. doi: 10.1080/14653240600855905)

Prior to injection, MSCs were cultured for at least one passage to avoid loss of cells from the thawing process. Mesenchymal stem cells do not express a specific cell marker. Moreover, the use of a reporter gene strategy allows the spatial and temporal in vivo monitoring of cell retention without the need to sacrifice the mice at pre-determined time points.

The intention of this technical manuscript was to provide a detailed step-by-step protocol for the feasibility and performance of a multiple surgery procedure in the same animal to better reflect the clinical settings. Furthermore, characterization of the MSCs is beyond the scope of this manuscript and we also want to emphasize that this methodology could be used for other biologics as well.

2. Post-surgery recovery reporting could be more complete. What was the follow up after the second surgery and for how long?
The post-op observation should be the same as after the first procedure. The title of the post-

op care section has been changed to clarify this (line 215), as well as one of the steps within the section (line 222).

"4. Post-Operative Care Following Both Procedures"
"4.3. Check the mice for wound dehiscence or abnormal pain once daily for 5 days, then 2-3 times weekly"

3. Why is no histology presented showing the distribution of the MSCs in the myocardium after the surgery and over time? I would expect a time course to show the survival and retention of the cells. The primary claim of this paper is that the timing of the procedure may affect the survival and retention of the stem cells. The bioluminescent imaging cannot specify where in the tissue the cells have been deposited, their survival or their impact on the surrounding tissue. Acknowledged, that these details are verging on a traditional research paper, but people following this protocol need to have some idea of what to expect.

Assessment of the fate and biology of cells after delivery to the myocardium traditionally relied on ex vivo assays and molecular techniques (i.e. histology, western blotting), which are both invasive and restricted in their capacity to monitor temporal changes in a living subject. However, developments in molecular imaging techniques, such as reporter gene strategies, have increasingly enabled the non-invasive surveillance of cell fate after transplantation. In this context, bioluminescent imaging is the most commonly used modality in the small animal models of preclinical studies.

Wu JC, Chen IY, Sundaresan G, et al. Molecular imaging of cardiac cell transplantation in living animals using optical bioluminescence and positron emission tomography. *Circulation*.2003;108(11):1302-1305. doi:10.1161/01.CIR.0000091252.20010.6E

Ray P., Gambhir S.S. (2007) Noninvasive Imaging of Molecular Events with Bioluminescent Reporter Genes in Living Subjects. In: Anson D.S. (eds) Reporter Genes. *Methods in Molecular Biology*, vol 411. Humana Press

Wang J, Najjar A, Zhang S, et al. Molecular imaging of mesenchymal stem cell: mechanistic insight into cardiac repair after experimental myocardial infarction. *Circ Cardiovasc Imaging*. 2012;5(1):94-101. doi:10.1161/CIRCIMAGING.111.966424

In recent studies (Psaltis, PJ 2013, Franchi, F 2016, Franchi, F 2020) we have demonstrated that reporter gene imaging can be used to monitor not only cellular viability, but also biological processes (oxidative stress, mitochondrial dysfunction) by using transgenes whose expression is regulated by pathway-specific promoters. Additionally, our lab has previously determined that the amount of signal detected is positively correlated to the number of transplanted cells.

Rodriguez-Porcel, M., Gheysens, O., Chen, I. Y., Wu, J. C. & Gambhir, S. S. Image-guided cardiac cell delivery using high-resolution small-animal ultrasound. *Mol Ther* 12, 1142–1147 (2005).

Minor Concerns:

1. References to the figures in the text are all out of order.

The text has been changed to reflect the figure references in order.