Journal of Visualized Experiments

Intramyocardial Transplantation of MSC-Loading Injectable Hydrogels after Myocardial Infarction in a Murine Model

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61752R1
Full Title:	Intramyocardial Transplantation of MSC-Loading Injectable Hydrogels after Myocardial Infarction in a Murine Model
Section/Category:	JoVE Medicine
Keywords:	myocardial infarction; Injectable hydrogels; Intramyocardial injection; Gelatin; Stem cell therapy; Mesenchymal stem cells
Corresponding Author:	Chan Joon Kim Catholic University of Korea School of Medicine Uijeongbu, KOREA, REPUBLIC OF
Corresponding Author's Institution:	Catholic University of Korea School of Medicine
Corresponding Author E-Mail:	godandsci@catholic.ac.kr
Order of Authors:	Chan Woo Kim
	Chan Joon Kim
	Eun-Hye Park
	Eunmi Lee
	Eunhwa Seong
	Kiyuk Chang
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Seoul, Korea

TITLE:

Intramyocardial Transplantation of MSC-Loading Injectable Hydrogels after Myocardial Infarction in a Murine Model

AUTHORS AND AFFILIATIONS:

Chan Woo Kim[1,2], Chan Joon Kim[1,3,*], Eun-Hye Park[1,2], Eunmi Lee[1,2], Eunhwa Seong[1,2], Kiyuk Chang[1,2]

[1]Cardiovascular Research Institute for Intractable Disease, College of Medicine, The Catholic University of Korea, Seoul, Republic of Korea
[2]Division of Cardiology, Department of Internal Medicine, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea
[3]Division of Cardiology, Department of Internal Medicine, Uijeongbu St. Mary's Hospital, College of Medicine, The Catholic University of Korea

Email addresses of co-authors:
Chan Woo Kim (cw.kim@catholic.ac.kr)
Eun-Hye Park (park1119@catholic.ac.kr)
Eunmi Lee (dmsaltks@naver.com)
Eunhwa Seong (seongeh84@gmail.com)
Kiyuk Chang (kiyuk@catholic.ac.kr)

Corresponding author:
Chan Joon Kim (godandsci@catholic.ac.kr)

KEYWORDS:

Myocardial infarction, Injectable hydrogels, Intramyocardial injection, Gelatin, Stem cell therapy, Mesenchymal stem cells

SUMMARY:

Stem cell-based therapy has emerged as an efficient strategy to repair injured cardiac tissues after myocardial infarction. We provide an optimal in vivo application for stem cell transplantation using gelatin hydrogels that are able to be enzymatically cross-linked.

ABSTRACT:

One of the major issues facing current cardiac stem cell therapies for preventing postinfarct heart failure is the low retention and survival rates of transplanted cells within the injured myocardium, limiting their therapeutic efficacy. Recently, the use of scaffolding biomaterials has gained attention for improving and maximizing stem cell therapy. The objective of this protocol is to introduce a simple and straightforward technique to transplant bone marrow-derived mesenchymal stem cells (MSCs) using injectable hydroxyphenyl propionic acid (GH) hydrogels; the hydrogels are favorable as a cell delivery platform for cardiac tissue engineering applications due to their ability to be cross-linked in situ and high biocompatibility. We present a simple method to fabricate MSC-loading GH hydrogels (MSC/hydrogels) and evaluate their survival and

proliferation in three-dimensional (3D) in vitro culture. In addition, we demonstrate a technique for intramyocardial transplantation of MSC/hydrogels in mice, describing a surgical procedure to induce myocardial infarction (MI) via left anterior descending (LAD) coronary artery ligation and subsequent MSC/hydrogels transplantation.

INTRODUCTION:

Cardiac stem cell therapy has emerged as a potential approach for myocardial repair and regeneration[1,2]. Despite the recent positive results in animal models and clinical trials, the application of stem cell-based therapy for myocardial repair is limited due to low retention and poor survival of injected cells at the infarcted heart tissues[3,4]. As a result, the use of cell-based tissue engineering, including injectable biomaterials[5], cardiac patches[6], and cell sheets[7], has been intensively studied to improve cell retention and integration within the host myocardium.

Among the various potential approaches to bioengineered cardiac tissue repair, injectable hydrogels combined with appropriate cell types, such as mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs), are an attractive option to effectively deliver cells into myocardial regions[8,9]. Gelatin, a well-known natural polymer, can be used as an injectable matrix due to its great biocompatibility, considerable biodegradability, and reduced immunogenicity when compared with a wide range of biomaterials used in biomedical applications. Although gelatin-based injectable platforms have great potential, their applicability in vivo remains limited based on their low mechanical stiffness and easy degradability in the physiological environment.

To overcome these limitations, a novel and simple design of gelatin-based hydrogels consisting of hydroxyphenyl propionic acid has been proposed for in vivo applications. Gelatin-hydroxyphenyl propionic acid (GH) conjugates can be cross-linked in situ in the presence of an enzyme, horseradish peroxidase (HRP), and subsequently encapsulate various drugs, biomolecules, or cells within the hydrogel, suggesting great potential in tissue engineering applications[10-14]. In addition, we have recently investigated the therapeutic effects of GH hydrogels containing encapsulated MSCs and demonstrated their use in successful cardiac repair and regeneration after MI in a murine model[15]. In this protocol, we describe a simple technique for the encapsulation and in vitro three-dimensional (3D) proliferation of MSCs within GH hydrogels. We also introduce a surgical procedure designed to generate a murine MI model via coronary artery ligation and intramyocardial transplantation of MSC-loading GH hydrogels into the infarcted heart.

PROTOCOL:

All animal research procedures were provided in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals and the Guidelines and Policies for Rodent Experiments provided by the Institutional Animal Care and Use Committee (IACUC) in the School of Medicine of The Catholic University of Korea.

1. Preparation of MSCs and injectable gelatin hydrogels

1.1. Culture MSCs in a 100 mm culture dish at 37 °C and 5% CO_2. When MSCs growth reaches 80% confluence, wash the dish twice with DPBS and add 1 mL of trypsin-substitute at 37 °C for 3 min.

NOTE: MSCs were isolated from murine bone marrow following conventional procedures[16], cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic solution, and used between passage 7–9 for this study.

1.2. Add 9 mL of culture medium and centrifuge at 500 x *g* for 3 min. Next, discard the resulting supernatant, resuspend the cells in 1 mL of PBS, and maintain the cell suspension on ice.

1.3. Dilute 10 µL of cell suspension with 10 µL of Trypan blue and obtain the cell concentration using an automated cell counter.

1.4. Resuspend and transfer MSCs to a 1 mL tube at a density of 1 x 10^7 cells/mL.

1.5. Prepare a 6.25 wt% of GH conjugate solution in PBS and separate into 2 vials. Next, mix the GH solutions with either 6 µg/mL of HRP (GH solution A) or 0.07 wt% of H_2O_2 (GH solution B).

NOTE: Prepare gelatin-hydroxyphenyl propionic acid (GH) conjugates according to published protocols[12,15].

1.5.1. Keep a 9:1 volumetric ratio of GH conjugate solution to HRP (GH solution A) and GH conjugate solution to H_2O_2 (GH solution B), respectively.

1.6. Prior to mixing the MSCs with GH solution A, briefly centrifuge the cell suspension at 1,000 x *g* and carefully aspirate the resulting supernatant. Subsequently, mix the pellet containing MSCs with GH solution A.

2. In situ MSC-loading and three-dimensional in vitro culture

2.1. Load GH solution A (containing MSCs) and GH solution B into either side of a dual syringe. Plate 300 µL of the combined GH solutions with MSCs at a final density of 5 x 10^6 cells/mL onto an eight-well chamber slide.

2.2. After in situ hydrogel formation and subsequent MSC encapsulation via enzymatic cross-linking, add 700 µL of DMEM containing 10% FBS and 1% antibiotic–antimycotic solution.

2.3. Incubate the slide at 37 °C and 5% CO_2 and replace the culture medium every 2–3 days.

3. Confirmation of in vitro proliferation and survival of MSCs within GH hydrogels

3.1. To determine the viability of 3D cultured MSCs within GH hydrogels, use a live/dead cell staining assay after the predetermined incubation time.

3.2. Following incubation of the encapsulated MSCs in GH hydrogels for 3, 5, 7 or 14 days, aspirate the medium and wash the well twice with PBS.

3.3. Prepare a staining solution containing 5 µL of calcein AM and 20 µL of ethidium homodimer-1 (EthD-1) in 10 mL of DPBS.

3.4. Add 200 µL of the staining solution to the well and incubate for 30 min in the dark at room temperature.

3.5. Aspirate the staining solution and wash the well twice with PBS.

3.6. Carefully separate the chamber from the slide and place a full coverslip over the GH hydrogels. Use a confocal microscopy to visualize the degree of proliferation and morphological changes of the encapsulated MSCs.

NOTE: Fluorescent images were acquired under 200x magnification and imaged at the excitation/emission wavelengths of 470/540 nm for calcein and 516/607 nm for EthD-1.

4. Induction of myocardial infarction in mice

4.1. Anesthetize 7-week-old male C57BL/6 mice (20–22 g) with intraperitoneal injection of Zoletil and Rompun in saline (2 mL/kg).

4.2. Prior to surgery, depilate the mouse chest using hair removal cream and sterilize the skin with iodine.

4.3. Place the mouse on an operating table and intubate by inserting a catheter into the trachea to provide supplemental oxygen via mechanical ventilation.

4.4. Gently cut through the skin using surgical scissors and then penetrate the intercostal muscles by micro scissors. Separate the 2nd and 3rd left ribs using a 5-0 silk suture to maintain an open chest cavity.

4.5. Carefully ligate the left anterior descending (LAD) coronary artery using a needle holder with an 8-0 polypropylene suture and cut the suture using electrocautery.

4.6. Observe an immediate color change in the anterior left ventricular wall.

5. Intramyocardial transplantation of MSC-loading GH hydrogels

5.1. After inducing the myocardial infarction by LAD ligation, inject 10 µL of MSC-loading GH solutions into two different points at the infarct border zone (total: 2 x 10^5 MSCs/20 µL) using a dual-syringe equipped with a 26G needle.

5.1.1. Following the same procedure described in Step 1, prepare and transfer MSC-loading GH solutions into a dual syringe.

NOTE: To assess the engraftment of MSC-loading GH hydrogels within the infarcted area, MSCs and GH conjugates were pre-labeled with PHK26 and fluorescein isothiocyanate (FITC), respectively.

5.2. Restore the opened chest cavity and close the muscles and skin using 5-0 sutures.

NOTE: Prior to chest closure, remove the air using a catheter syringe.

5.3. Remove the tracheal tube and place the mouse in a cage under an infrared lamp during recovery.

6. Echocardiography

6.1. Four weeks following transplantation, initially anesthetize the mouse with 5% isoflurane and then adjust the isoflurane concentration to 1%.

6.2. Depilate the chest using hair removal cream and place the mouse on a heating pad. Apply ultrasound transducer gel onto the chest.

6.3. Acquire two-dimensional parasternal short axis views and record M-mode tracings at the level of the papillary muscle.

NOTE: Place a linear array transducer (7–15 MHz) in the left parasternal line and view the anatomical structures.

6.4. Measure corresponding lines for LVAW, LVID, and LVPW to obtain cardiac wall thickness, chamber dimension, and fractional shortening.

NOTE: Compare cardiac function including the ejection fraction (EF), fractional shortening (FS), and end-systolic volume (ESV) at the level of the papillary muscle to ensure proper assessment at the same anatomic location.

7. Histological evaluation

7.1. At the predetermined time after transplantation of MSC-loading GH hydrogels into the infarcted heart, euthanize the mouse in a CO_2 chamber and collect the heart for histological analysis[15].

7.2. For hematoxylin and eosin (H&E) and Masson's trichrome (MT) staining, fix the dissected heart tissues in 4% paraformaldehyde (PFA) and embed in paraffin. Next, cut paraffin-embedded

heart blocks into 4 µm serial sections using a microtome and stain the sections with MT stain according to standard protocols[17].

7.3. Acquire images on a slide scanner at 20x magnification and calculate the infarct size of the treatment groups.
Infarct size (%) = total infarct circumference / total LV circumference x 100

7.4. Calculate both circumferences by midline length measurement. For LV midline circumferences, measure the centerline lengths between the endocardial and epicardial surfaces. For midline infarct circumferences, measure the lengths of infarct including more than 50 % of the whole thickness of myocardium[18].

NOTE: All image analyses were performed using ImageJ software.

7.5. Measure the wall thickness of the scar at the papillary muscle levels.

7.6. Calculate the fraction of collagen area.
Collagen area (%) = total area of interstitial fibrosis/myocyte area x 100

REPRESENTATIVE RESULTS:

To effectively deliver MSCs to the infarcted myocardium, MSC-loading in situ cross-linkable hydrogels described in **Figure 1** were used in this protocol. Prior to in vivo transplantation, the proliferation and survival of MSCs in GH hydrogels were confirmed by a 3D in vitro live/dead cell staining assay (live: green; dead: red). As shown in **Figure 2**, representative images exhibited sufficient MSCs proliferation, showing branched networks within GH hydrogels. In addition, an extensive multicellular 3D structure of MSCs was clearly observed at day 14, indicating that GH hydrogels could provide a proper microenvironment for the encapsulated cells.

After the induction of MI via LAD ligation, MSC-loading GH hydrogels were intramyocardially transplanted into peri-infarct areas (**Figure 3A**). As shown in **Figure 3B**, the MSCs and gel were appropriately sustained within the infarcted region. MSCs, stained with PHK26 (red), were well integrated into GH hydrogels, stained with FITC (green), presenting successful engraftment and retention in the infarcted hearts for in vivo application.

To verify the therapeutic effects of MSC-loading GH hydrogels in a murine MI model, the changes in cardiac function and structure were evaluated by echocardiography and histological analysis at day 28 post-transplantation and compared among the different treatment groups. The representative echocardiography showed improved cardiac functions, including FS, EF, and ESV, in the MSC/gel treated group compared with the other groups (**Figure 4**). In addition, histological analysis exhibited less fibrosis, thicker infarcted walls, and a smaller infarct size in the MSC/gel treated group than in the other groups, indicating that this protocol contributed beneficial effects by significantly attenuating LV remodeling (**Figure 5**).

[Place **Figure 1** here]

[Place **Figure 2** here]
[Place **Figure 3** here]
[Place **Figure 4** here]
[Place **Figure 5** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Scheme of the process for improving stem cell retention and engraftment using injectable hydrogels. In situ cross-linkable GH hydrogels containing bone marrow-derived MSCs were prepared and transplanted by intramyocardial injection into the infarcted heart.

Figure 2: In vitro 3D MSC proliferation within GH hydrogels. Representative images of live (green)/dead (red) MSCs obtained via a confocal microscopy following live/dead cell staining after 3, 5, 7, and 14 days of incubation (200x magnification; scale = 100 μm). The images and video were partly adapted with permission from Kim et al.[15].

Figure 3. In vivo transplantation of MSC/Hydrogels. (A) A schematic diagram showing intramyocardial transplantation after the induction of MI. (B) Representative images of transplanted MSCs and GH hydrogels labeled with PKH26 (red) and FITC (green), respectively. Mice were sacrificed after 1, 3, 5, or 7 days of transplantation and their hearts were then excised to assess the degree of MSC and GH hydrogel engraftment. The excised hearts were cryo-fixed, prepared into serial-sections, and imaged via a confocal microcopy (200x magnification; scale = 100 μm). The images were partly adapted with permission from Kim et al.[15].

Figure 4. Improvements in cardiac function following MSC/Hydrogels transplantation. (**A**) Representative video of echocardiography. (**B**) Representative short axis M-mode image with measurements, including left ventricular anterior wall thickness in diastole (LVAWd) and systole (LVAWs), internal diameter in diastole (LVIDd) and systole (LVIDs), and posterior wall thickness in diastole (LVPWd) and systole (LVPWs). (**C–E**) Functional improvements in the ejection fraction (EF), fractional shortening (FS), and end-systolic volume (ESV) after 28 days of transplantation of all treatment groups. Data were represented as mean ± standard deviation (*p < 0.05, **p < 0.001, ***p < 0.0001; n = 9–12 per group). The videos and results were partly adapted with permission from Kim et al.[15].

Figure 5. Improvements in cardiac structure following MSC/Hydrogels transplantation. (**A**) Representative images of histological evaluation. (**B–D**) Structural improvements were observed in infarct size, along with less infarcted wall thinning and fibrosis. Scale = 1 mm. Data are represented as the mean ± standard deviation (*p < 0.05, **p < 0.001, ***p < 0.0001; n = 4–7 per group). The images and results were partly adapted with permission from Kim et al.[15].

DISCUSSION:

Injectable GH hydrogels have great potential for in vivo applications because of their ability to homogenously incorporate diverse therapeutic agents in situ. Furthermore, their physical and biochemical properties can be easily manipulated based on disease-dependent requirements. In this respect, injectable hydrogels have been proposed to address the major limitations in current

cardiac stem cell therapy hampered by poor survival and cell retention (i.e., < 10% within 24 h post-transplantation) in the injured heart[19,20]. To overcome this poor outcome, the protocol described herein provides a simple and feasible method to improve cell retention and survival using GH hydrogels that can be cross-linked in situ after myocardial transplantation, which have demonstrated favorable effects on cardiac structure and function in a murine MI model.

The primary advantage feature of this technique is its broad in vivo applicability with any type of cell and biomolecule, which can be loaded by simply mixing with the pregel GH solution prior to injection. Furthermore, to obtain a comprehensive understanding of donor-to-host interactions, a straightforward labeling approach of the GH conjugates and/or encapsulated biomolecules can be adapted to track changes in their in vivo stability, host integration, and resorption kinetics. To our knowledge, the use of injectable gelatin-based hydrogels combined with therapeutic stem cells was the first to validate the restorative potential of cardiac tissue in vitro and in vivo[15].

At the current stage in this research, GH hydrogels that are loaded with MSCs, injected, and cross-linked in situ were used as a proof of concept to assess their applicability in a murine MI model. Although this method seemingly improved MSC engraftment and retention in the transplanted heart tissues, the detailed conditions during injection should be considered for optimizing therapeutic efficacy, such as the location of injection site (i.e., peri-infarct zone or infarct zone), volume and number of injections, and stiffness of the hydrogel (i.e., hard to inject or easy to leak).

In conclusion, we have demonstrated a protocol for a representative murine MI model by LAD ligation and a practical method for intramyocardial transplantation of stem cells using in situ cross-linked hydrogels to improve the retention and engraftment of transplanted MSCs. These techniques provide an effective method for intramyocardial transplantation of MSC-loading injectable hydrogels and highlight their great potential for application in large animals and clinical translation.

ACKNOWLEDGMENTS:
This research is supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by Ministry of Education (NRF-2018R1D1A1A02049346)

DISCLOSURES:
The authors have no conflicts of interest to declare with this work.

REFERENCES:
1 Jhund, P. S., McMurray, J. J. Heart failure after acute myocardial infarction: a lost battle in the war on heart failure? *Circulation.* **118** (20), 2019-2021 (2008).
2 Cahill, T. J., Kharbanda, R. K. Heart failure after myocardial infarction in the era of primary percutaneous coronary intervention: Mechanisms, incidence and identification of patients at risk. *World Journal of Cardiology.* **9** (5), 407-415 (2017).
3 Cambria, E. et al. Translational cardiac stem cell therapy: advancing from first-generation to next-generation cell types. *npj Regenerative Medicine.* **2** 17 (2017).
4 Lemcke, H., Voronina, N., Steinhoff, G., David, R. Recent Progress in Stem Cell

Modification for Cardiac Regeneration. *Stem Cells International.* **2018** 1909346 (2018).
5 Alagarsamy, K. N., Yan, W., Srivastava, A., Desiderio, V., Dhingra, S. Application of injectable hydrogels for cardiac stem cell therapy and tissue engineering. *Reviews in Cardiovascular Medicine.* **20** (4), 221-230 (2019).
6 Gaetani, R. et al. Epicardial application of cardiac progenitor cells in a 3D-printed gelatin/hyaluronic acid patch preserves cardiac function after myocardial infarction. *Biomaterials.* **61** 339-348 (2015).
7 Gao, L. et al. Myocardial Tissue Engineering With Cells Derived From Human-Induced Pluripotent Stem Cells and a Native-Like, High-Resolution, 3-Dimensionally Printed Scaffold. *Circualtion Research.* **120** (8), 1318-1325 (2017).
8 Hasan, A. et al. Injectable Hydrogels for Cardiac Tissue Repair after Myocardial Infarction. *Advanced Science.* **2** (11), 1500122 (2015).
9 Wu, R., Hu, X., Wang, J. Concise Review: Optimized Strategies for Stem Cell-Based Therapy in Myocardial Repair: Clinical Translatability and Potential Limitation. *Stem Cells.* **36** (4), 482-500 (2018).
10 Lee, Y. et al. In situ forming gelatin-based tissue adhesives and their phenolic content-driven properties. *Journal of Materials Chemistry B.* **1** (18), 2407-2414 (2013).
11 Lee, Y., Bae, J. W., Lee, J. W., Suh, W., Park, K. D. Enzyme-catalyzed in situ forming gelatin hydrogels as bioactive wound dressings: effects of fibroblast delivery on wound healing efficacy. *Journal of Materials Chemistry B.* **2** (44), 7712-7718 (2014).
12 Lee, S. H. et al. In situ Crosslinkable Gelatin Hydrogels for Vasculogenic Induction and Delivery of Mesenchymal Stem Cells. *Advanced Functional Materials.* **24** (43), 6771-6781 (2014).
13 Jung, B. K. et al. A hydrogel matrix prolongs persistence and promotes specific localization of an oncolytic adenovirus in a tumor by restricting nonspecific shedding and an antiviral immune response. *Biomaterials.* **147** 26-38 (2017).
14 Kim, G. et al. Tonsil-derived mesenchymal stem cell-embedded in situ crosslinkable gelatin hydrogel therapy recovers postmenopausal osteoporosis through bone regeneration. *PLoS One.* **13** (7), e0200111 (2018).
15 Kim, C. W. et al. MSC-Encapsulating in situ Cross-Linkable Gelatin Hydrogels To Promote Myocardial Repair. *ACS Applied Bio Materials.* **3** (3), 1646-1655 (2020).
16 Meirelles Lda, S., Nardi, N. B. Murine marrow-derived mesenchymal stem cell: isolation, in vitro expansion, and characterization. *Br J Haematol.* **123** (4), 702-711 (2003).
17 Ojha, N. et al. Characterization of the structural and functional changes in the myocardium following focal ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol.* **294** (6), H2435-2443 (2008).
18 Takagawa, J. et al. Myocardial infarct size measurement in the mouse chronic infarction model: comparison of area- and length-based approaches. *Journal of Applied Physiology.* **102** (6), 2104-2111 (2007).
19 Terrovitis, J. et al. Noninvasive Quantification and Optimization of Acute Cell Retention by In vivo Positron Emission Tomography After Intramyocardial Cardiac-Derived Stem Cell Delivery. *Journal of the American College of Cardiology.* **54** (17), 1619-1626 (2009).
20 Dib, N., Khawaja, H., Varner, S., McCarthy, M., Campbell, A. Cell Therapy for Cardiovascular Disease: A Comparison of Methods of Delivery. *Journal of Cardiovascular Translational Research.* **4** (2), 177-181 (2011).

Figure 1

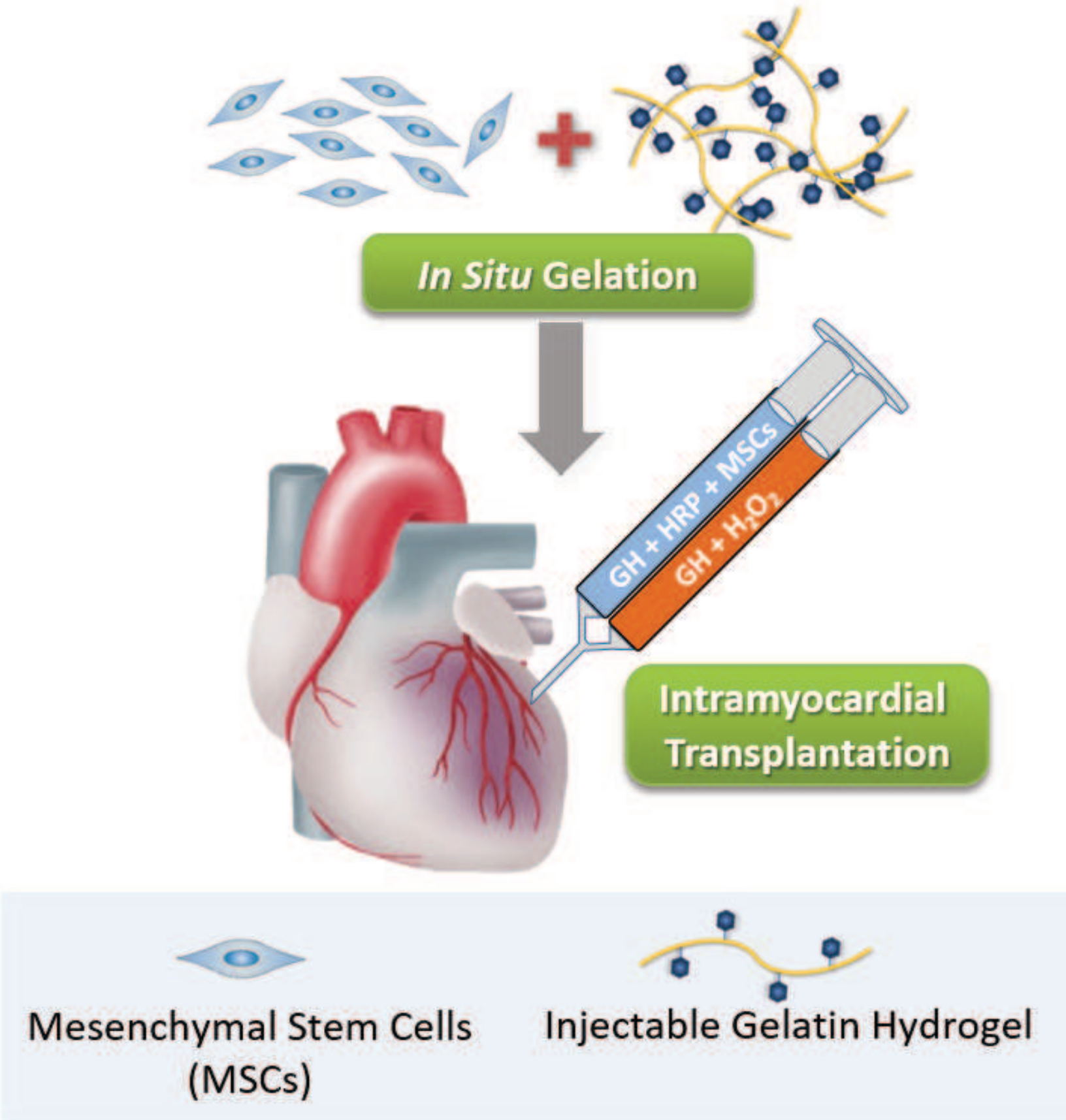
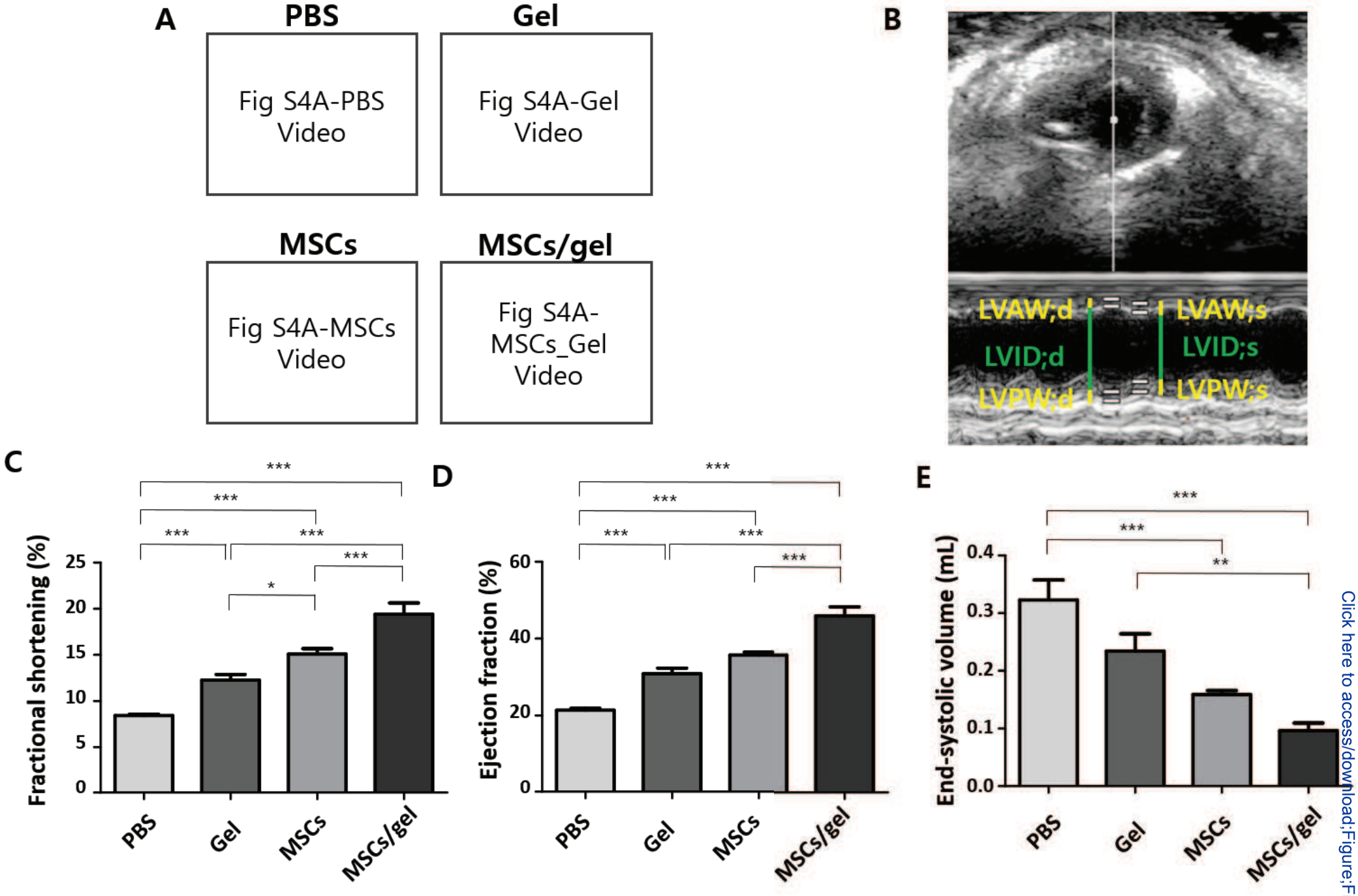


Figure 2

Figure 3



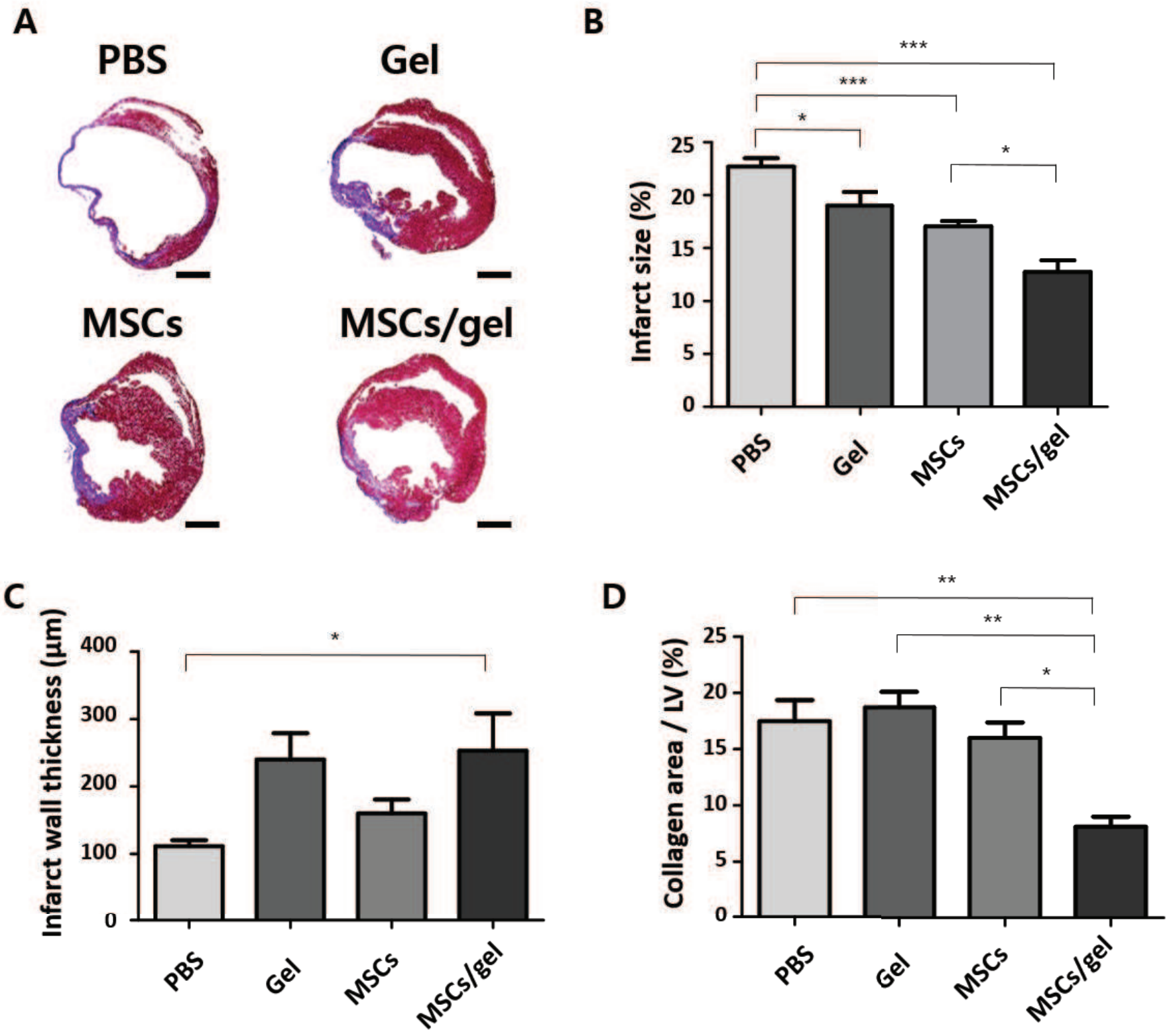


Figure 2 (day14) Video

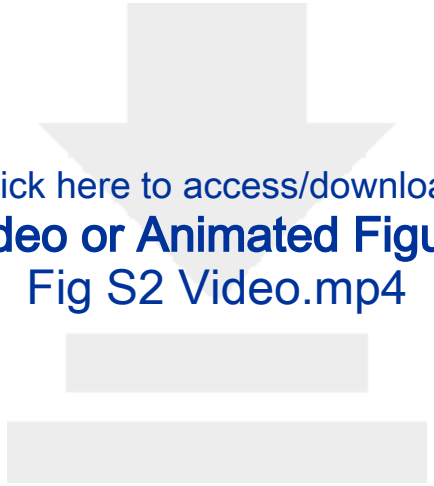


Figure 4A (PBS) Video

Click here to access/download

Video or Animated Figure

Fig S4A-PBS.mp4

Figure 4A (Gel) Video

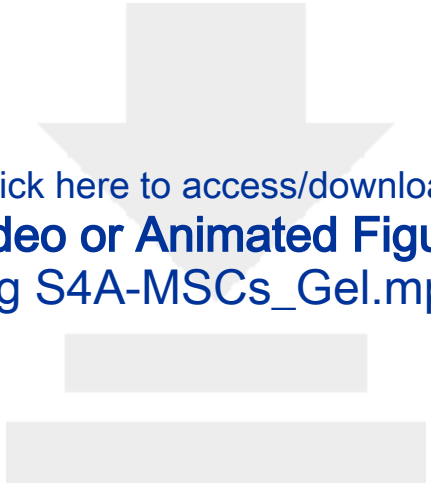
Figure 4A (MSCs) Video

Click here to access/download

Video or Animated Figure

Fig S4A-MSCs.mp4

Figure 4A (MSCs/gel) Video



Click here to access/download

Video or Animated Figure

Fig S4A-MSCs_Gel.mp4

Name of Material/ Equipment	Company	Catalog Number
4 % paraformaldehyde (PFA)	Intron	IBS-BP031-2
5-0 silk suture	AILEE	SK534
8-0 polypropylene suture	ETHICON	M8732H
8-well chamber slide	Nunc LAB-TEK	154534
Angiocath Plus (22GA) catheter	BD Angiocath Plus	REF382423
Antibiotic-antimyocotic	Gibco	15240-062
Centrifuge	GYROGEN	1582MGR
Confocal microscope	Zeiss	LSM 510
Cover slipe	MARIENFELD	101242
Deluxe High Temperature Cautery kit	Bovie	QTY1
DMEM	Gibco	11995-065
DPBS	Gibco	14040-133
Dual-syringe		
EOSIN	SIGMA-ALDRICH	HT110116
Ethanol	EMSURE	K49350783 739
FBS	Gibco	16000-044
Fechtner conjunctiva forceps titanium	WORLD PRECISISON INSTRUMENTS	WP1820
Fluorescein isothiocyanate isomer I (FITC)	SIGMA-ALDRICH	F7250
Forcep	HEBU	HB0458
Hair removal cream	Ildong Pharmaceutical	
Heating pad	Stoelting	50300 50301
Hematoxylin	SIGMA-ALDRICH	HHS80
Horseradish peroxide (HRP; 250-330 U/mg)	SIGMA-ALDRICH	P8375
Hydrogen peroxide (H2O2; 30 wt % in H2O)	SIGMA-ALDRICH	216763
Iodine	Green Pharmaceutical	
LIVE/DEAD cell staining kit	Thermo Fisher	R37601
Mechanical ventilator	Harvard Apparatus	
Micro centrifuge	HANIL	Micro 12
Micro needle holder	KASCO	37-1452

Micro scissor	HEBU	HB7381
Microscope	OLYMPUS	SZ61
MT staining kit	SIGMA-ALDRICH	HT1079-1SET
		HT15-1KT
Paraffin	LK LABKOREA	H06-660-107
PBS buffer	Gibco	10010-023
PHK26 staining kit	SIGMA-ALDRICH	MINI26
Slide scanner	Leica	SCN400
Surgical scissor	HEBU	HB7454
Surgical tape	3M micopore	1530-1
Tissue cassette	Scilab Korea	Cas3003
Transducer gel	SUNGHEUNG	SH102
Trout-Barraquer needle holder curved	KASCO	50-3710c
Ultrasound system	Philips	Affiniti 50
Xylene	JUNSEI	25175-0430

Comments/Description

Homeothermic Blanket System
Replacement Heating Pad for 50300 (10 X 12.5cm)

Weigert’s iron hematoxylin solution
Trichrome Stain (Masson) Kit

RESPONSE TO REVIEWERS' COMMENTS

We appreciate the reviewers' time and efforts toward the review of our manuscript. The comments provided by the editor and reviewers' were carefully studied and reflected in the revised manuscript. All comments were taken into consideration in the revision, as described in detail below.

Response to Editor

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Response to Comment) We have carefully checked grammatical mistakes and sentence construction in English by utilizing an academic editing service (https://www.aje.com/), which is reflected in the revised manuscript in blue.

• **Protocol details:** 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 add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps**. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Comment 1) 3.1: What is the source of MSCs? How are they cultured and prepared for injection? Mention all media usage and culture conditions. How are the cells counted?
Response to Comment 1) We described the steps for MSC preparation and *in vitro* culture in "Step 1 and 2"; however, it seems to not cover all of the details necessary to generate the script for MSC-loading and *in vitro* 3D culture. As noted by the editor, we have explained and clarified additional details for this comment in the revised manuscript as follows.

Q) What is the source of MSCs?
A) Murine bone marrow-derived MSCs were used in this work and we have described it in a note following Step 1.1.

Q) How are they cultured and prepared for injection?
A) The details are described in Step 1.

Q) Mention all media usage and culture conditions.
A) The details are described in Step 1.

Q) How are the cells counted?
A) We counted the cells using an automated cell counter and have explained this in Step 1.3.

Comment 2) 4.1: Mention animal age.
Response to Comment 2) We have added information regarding animal age and weight to

Step 4.1.

Comment 3) 4.4: mention surgical tools used.
Response to Comment 3) We have clarified the details of tools used in surgical procedures in Step 4.

Comment 4) 5.1: specify needle gauge.
Response to Comment 4) A dual-syringe with a 26G needle was used in this work and we have added this to Step 5.1.

Comment 5) 6.2, 6.3: Mention transducer specifications and imaging settings
Response to Comment 5) We have changed descriptions of transducer and its associated setting to the revised Step 6.3 and 6.4.

Comment 6) 7.1: cite reference for heart collection and handling.
Response to Comment 6) The reference was added to Step 7.1.

Comment 7) 7.2: reference?
Response to Comment 7) To explain heart tissue preparation and its staining, we have merged Steps 7.2-7.3 into a single Step 7.2 and cited an appropriate reference.

Comment 8) 7.3: Using a microtome? Cite references for standard protocols.
Response to Comment 8) We have added a description of the microtome and cited an appropriate reference in the revised Step 7.2.

Comment 9) 7.4: Step describing imaging is missing. Mention magnification and other settings.
Response to Comment 9) We have added a description of imaging and its associated setting to the revised Step 7.3.

Comment 10) 7.5: Which software?
Response to Comment 10) All image analyses were performed using ImageJ software and this was included in a note in the revised Step 7.3.

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

Response to Comment) We have highlighted our selected text in yellow accordingly in the revised manuscript.

• **Figures:**

1) Fig 2,3: Add scale bars for all micrographs.
Response to Comment) We have added scale bars for all images.

2) Remove text " use Figure S…" You may specify this information in the figure placeholder instead.
Response to Comment) We have removed and changed all information regarding the figure placeholder.

• **Figure/Table Legends:** Define all error bars.

Response to Comment) We have added statistics for all data.

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Zeiss LSM 510, Zoletil, Rompun, Angiocath Plus,

1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

Response to Comment) We have changed all commercial sounding language into generic names.

• **Table of Materials:**

1) Please sort in alphabetical order.

Response to Comment) We have changed the table of materials to reflect alphabetical order.

Response to Reviewer #1

Manuscript Summary:
Interesting protocol. Just needs additional detail and clarification to make this more powerful as a learning tool.

Major Concerns:
My biggest concern is that there are an excessive number of minor concerns.

Response to Comment) We appreciate your efforts in reviewing and providing detailed comments for our manuscript. We have addressed the following comments to clarify our claims.

Manor Concerns:

Comment 1. Use of GH acronym in abstract without definition

Response to Comment 1) We are grateful to Reviewer #1 for pointing out our mistake. We have defined the GH acronym in the Abstract.

Comment 2. Line 64: less immunogenicity than what?

Response to Comment 2) To clarify our intention, we rephrased the sentence in the revised manuscript as follows.

" … and reduced immunogenicity when compared with a wide range of other biomaterials used in biomedical applications."

Comment 3. Line 90: Protocol is not clear.

Response to Comment 3) As the reviewer pointed out, the details covered in Step 1 seem to be insufficient. Thus, we have clarified and rephrased more details in the revised Step 1.

Comment 4. Line 100-101: If prior step has 10^7 cell/mL, what is diluting this? I think the confusion starts in step one (line 90). Give the two solutions separate names so the reader has any chance of tracking what is where. Then state specifically which two different samples are loaded into this syringe in step 2.1.

Response to Comment 4) As the reviewer suggested, we have rephrased the protocols in the revised Step 1 and Step 2 to clarify our process.

Comment 5. Line 103: how do you know when crosslinking is complete? Time? Some other approach?

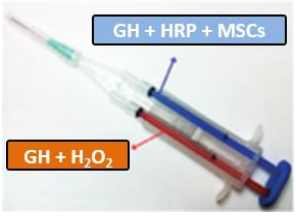
Response to Comment 5) The adjustable stiffness and gelation properties of GH hydrogels used in this work have been verified in our previous work [*Adv. Funct. Mater.* **2014**, *24*, 6771, *ACS Appl. Bio Mater*, **2020**, *3*, 1646]. The scope of this manuscript was focused on intramyocardial transplantation of stem cells with injectable hydrogels and does not extend to detailed gelation properties, such as gelation time and mechanical strength, which can be determined using a vial tilting method and elastic modulus measurement by a rheometer.

Comment 6. Line 126: What magnification and filters should be uses to collect appropriate images for proliferation and morphological studies? No details of proliferation or morphological analysis.

Response to Comment 6) Magnification and filter settings were added to a note in Step 3.6. In regard to fluorescence microscopy, it can simply be speculated that MSCs were well proliferated on the GH hydrogels without any additional description.

Comment 7. Line 150: Again clearly define what is in the dual loaded syringe… concentration of cells and other reagents and total volume in each barrel.

Response to Comment 7) We designed a custom dual syringe that can be effectively used in our lab (see image below). It is equipped with different sized needles and is depicted in Fig. 1. For the loading of the pregel solutions into the syringe, this procedure is conducted in the same manner as explained in the revised Step 1, and we have added an additional Note to address this.



Comment 8. Line 175: No information on how to measure these parameters by Echo. This could be easily achieved by capturing some still images and showing each measurement.
Response to Comment 8) As the reviewer suggested, we have added the information in the revised Step 6.4 and Fig 4. legend.

Comment 9. Line 179-202: Details of histology approach is not clear. It reads as if both H&E and trichrome were done on the same sample. Clarify this and add detail for each.
Response to Comment 9) The authors acknowledge that this wording was confusing. We used MT staining for histology in this manuscript. We have changed this in the revised Step 7.2 and cited the reference for this step.

Comment 10. Line 191: Define what metric you are using to define the infarct zone (color, thickness, other?).
Response to Comment 10) The infarct region is simply defined by scar tissue (colored in dark blue); however, infarcted heart tissue sections usually include both scar and viable tissue in the infarct area. We used midline length measurement to calculate the infarct size based on the details in the original manuscript for the note in Step 7.3. In addition, we cited this reference to provide more details concerning this.

Comment 11. Line 191: Is the histological staining of the collagen in the infarct area specific to collagen and not staining the chemically similar gelatin that was injected?
Response to Comment 11) Since GH hydrogels used in this work is biodegradable, we assume that most of hydrogels are degraded after 28 days in intramyocardial transplantation. This was confirmed in our previous work [*ACS Appl. Bio Mater*, **2020**, *3*, 1646].

Comment 12. No protocol given for sample prep of the fluorescent images in Figure 3.
Response to Comment 12) In this video, we elected to omit this step; therefore, we did not include a detailed procedure.

Comment 13. Injection rate not mentioned. The potential for shear damage to cells is significant with these low diameter syringes.
Response to Comment 13) We agree that injection rate is one of the important factors for cell transplantation. In this work, MSC-loading gels were manually injected into the heart tissue using a dual syringe equipped with a 26G needle. It should be performed carefully to avoid cell damage. At the current stage, the research scope was focused on the proof-of-concept of stem cell-loading injectable hydrogels and the results could provide satisfactory transplantation of cell-loading gels without significant cell damages. For more reliable and accurate applications, the more intensive studies including an accurate injection control technique are currently ongoing.

Comment 14. FITC isn't particularly photostable. In your experience, what is the stability of

fluorophore labeling? Can it be imaged multiple times or does it photobleach?

Response to Comment 14) Since the isothiocyanate group reacts with the amino terminus and primary amines, FITC is widely used to attach a fluorescent label to proteins, antibodies, peptides, etc. In this work, GH hydrogels were simply labeled with FITC, and these hydrogels provided satisfactory outcomes *in vitro* and *ex vivo* without any perturbation to hydrogel properties, such as gelation time and stiffness (data not shown). In addition, the images shown in Fig 3B were obtained from each section, which was harvested from heart tissues at 1, 3, 5, and 7 days under the same conditions following transplantation of fluorescently labeled MSC-loading hydrogels. This is the general method to analyze a change in fluorescence in a time-dependent manner. In this case, we are not concerned about photobleaching because all measurements are processed under the same conditions.

Comment 15. It is not clearly stated which histological approach is used in Figure 5 representative results.

Response to Comment 15) As the reviewer suggested, we have rephrased REPRESENTATIVE RESULTS in the revised manuscript.

<u>Response to Reviewer #2</u>

Comment 1. SUMMARY section, Stem cell-based therapy has emerged as an efficient strategy to repair injured cardiac tissues after myocardial infarction. We provide an optimal in vivo application for cardiac stem cell transplantation using enzymatically cross-linkable gelatin hydrogels. Not cardiac stem cell transplantation, but MSC in this protocol.

Response to Comment 1) We are grateful to Reviewer #2 for pointing out our mistake. We have revised SUMMARY and deleted the highlighted text.

Comment 2. 1.3 Keep a volume ratio of GH:HRP and GH:H2O2 at 9:1. How about the toxicity of H2O2.

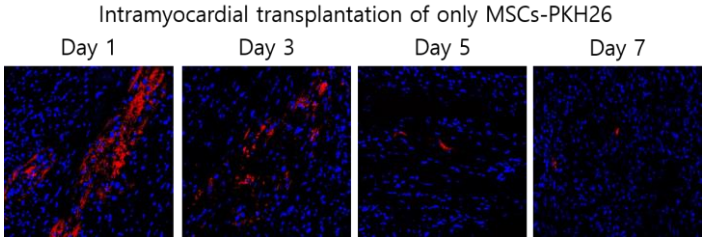
Response to Comment 2) We agree that hydrogen peroxide, a known cytotoxic agent, may induce side effects in its application *in vitro* and *in vivo*. Thus, we have already addressed concerns regarding toxicity in our previous work [*Adv. Funct. Mater.* **2014**, *24*, 6771, *ACS Appl. Bio Mater*, **2020**, *3*, 1646] and no issues arising from toxicity were present in this work.

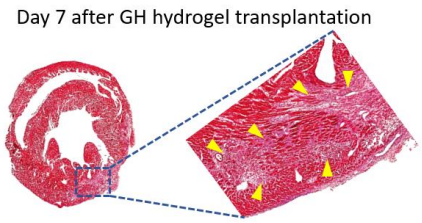
Comment 3. 5.1 To assess the engraftment of MSC-loading GH hydrogels within the infarcted area, MSCs and GH conjugates were pre-labeled with PHK26 and fluorescein isothiocyanate (FITC), respectively. Although authors have published paper, ACS Applied Bio Materials. 3 (3), 1646-1655 (2020), indeed, FITC or PHK26 can leak from gel or cell. Why not use genetic labeling, such as GFP for MSC. According to Fig 3, for hydrogel staining, the green color was not hydrogel, may just dye. HE staining may show the gel in heart.

Response to Comment 3) We agree that fluorescent dyes can leak from the gel or cells. However, it is our opinion that the majority of the signal, as shown in Fig 3, is derived from the MSC/gel because free dye is difficult to retain in beating hearts after 7 days. This is clearly observed when MSCs labeled with PKH26 were applied to hearts in the absence of hydrogel,

and no significant signals was present, as shown in the image below (left). This indicates tha the hydrogel can improve MSC retention within hearts. Furthermore, the PKH26 dye is used for general cell membrane labeling and it has been characterized in a number of model systems for *in vivo* cell tracking [*J. Clin. Invest.* **2012**, *122*, 711].

We have already confirmed the integration of transplanted hydrogel by H&E staining, as shown in the image (right), and the result exhibited a similar trend as seen in Fig 3. The purpose of the fluorescent dye used in Fig 3 is not only to observe the improved retention within the infarcted region but also to confirm retention along with colocalization of MSCs and gels, indicating this injectable hydrogel that is cross-linked *in situ* can be effectively used for *in vivo* applications.





Response to Reviewer #3

Comment 1. Abstract: GH hydrogels full name missing. Needs to be addressed at the first instance.

Response to Comment 1) We are grateful to Reviewer #3 for pointing out our mistake. We have rephrased defined the GH acronym in the Abstract.

Comment 2. Figure 1: The chemistry of in situ gel formation is not clear. It is essential to properly represent the chemical interactions on how gelatin-hydroxyphenyl propionic acid (GH) conjugates are formed. Also how it reacts with HRP or H2O2 to form the gel.

Response to Comment 2) The scope of this manuscript is focused on intramyocardial transplantation of stem cell with injectable hydrogels and does not extend to the detailed mechanisms of GH hydrogel formation. A brief description was explained in the Introduction, and relevant references were cited to allow the reader to track the details.

Comment 3. Some background information is needed to justify how the volume (10-20 µL) of gel was decided for injection to the mice heart.

Comment 4. Similarly, how the number of cell concentration to be injected per heart was decided for in vivo studies.

Response to Comments 3 and 4) We have investigated a wide range of conditions to determine the appropriate injection volume and cell number for a murine model. Based on this, we made a selection and used it in this work.

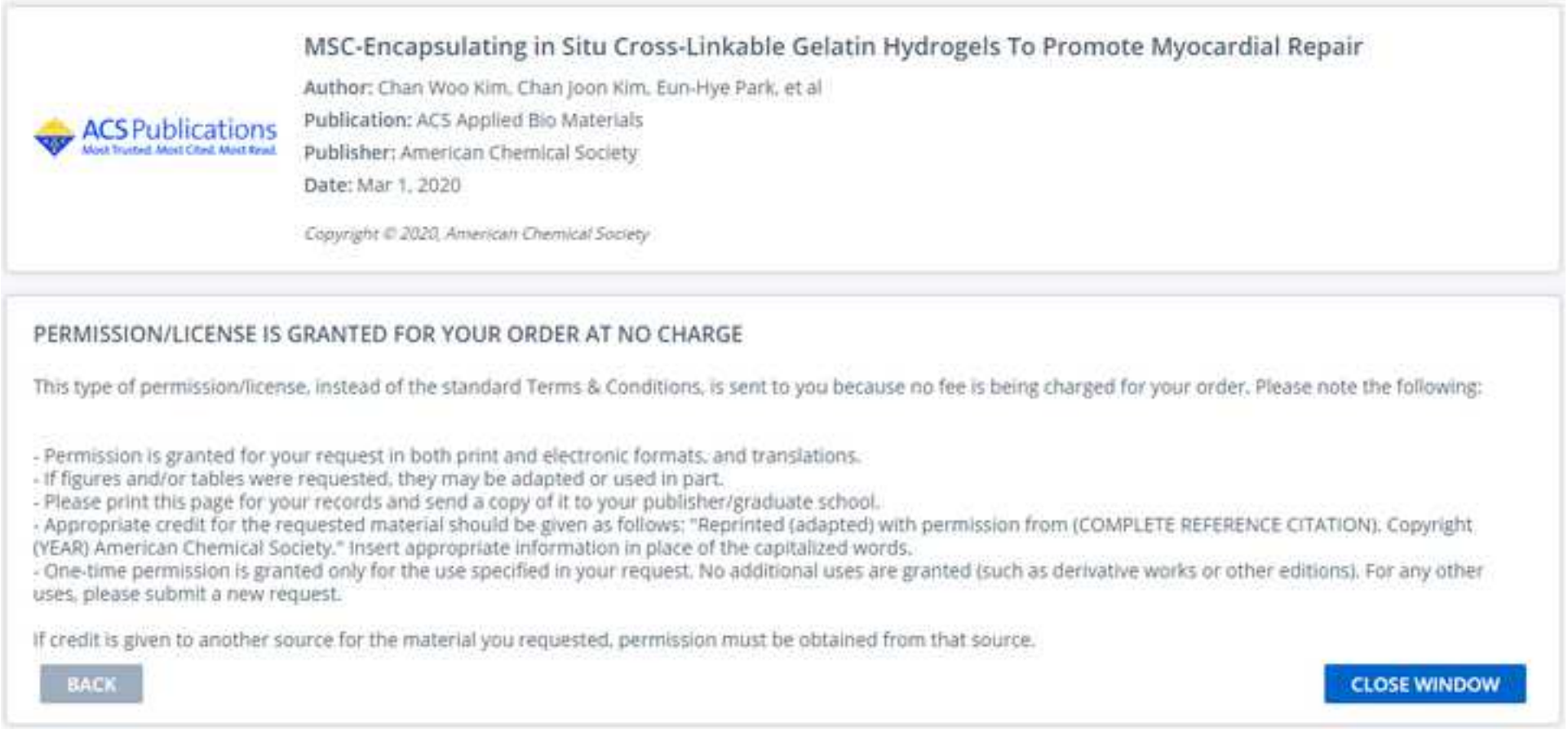
Comment 5. Include specific information of the dual-syringe's needle size.

Response to Comment 5) We have added this detail in the revised Step 5.1.

Comment 6. Figure 4: B, C, D - Y axis labels missing.
Response to Comment 6) The authors should have been more careful. We have included these labels in the revised Fig 4.

Comment 7. The paper is about "Intramyocardial Transplantation" of gel/cells. So it is important to include a video of how the injection experiment in the heart was done. Basically, the video should demonstrate how the gels were injected with dual-syringe to the two different points at the infarct border zone in mice heart.
Response to Comment 7) We agree with the reviewer's comment. We will demonstrate the details of how to prepare MSC-loading hydrogels and their subsequent intramyocardial transplantation in infarcted hearts.

Comment 8. The english language grammar and sentence construction needs to be checked.
Response to Comment 8) As the reviewer suggested, we have carefully checked grammatical mistakes and sentence construction in English by using an academic editing service (https://www.aje.com/), which is reflected in the revised manuscript in blue.



MSC-Encapsulating in Situ Cross-Linkable Gelatin Hydrogels To Promote Myocardial Repair

Author: Chan Woo Kim, Chan Joon Kim, Eun-Hye Park, et al

Publication: ACS Applied Bio Materials

Publisher: American Chemical Society

Date: Mar 1, 2020

Copyright © 2020, American Chemical Society

PERMISSION/LICENSE IS GRANTED FOR YOUR ORDER AT NO CHARGE

This type of permission/license, instead of the standard Terms & Conditions, is sent to you because no fee is being charged for your order. Please note the following:

- Permission is granted for your request in both print and electronic formats, and translations.
- If figures and/or tables were requested, they may be adapted or used in part.
- Please print this page for your records and send a copy of it to your publisher/graduate school.
- Appropriate credit for the requested material should be given as follows: "Reprinted (adapted) with permission from (COMPLETE REFERENCE CITATION). Copyright (YEAR) American Chemical Society." Insert appropriate information in place of the capitalized words.
- One-time permission is granted only for the use specified in your request. No additional uses are granted (such as derivative works or other editions). For any other uses, please submit a new request.

If credit is given to another source for the material you requested, permission must be obtained from that source.

BACK

CLOSE WINDOW