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ORIGINAL RESEARCH - NOVEL TRANSLATIONAL METHODS

The Technique of Permanent Pericardial Catheter in Mice

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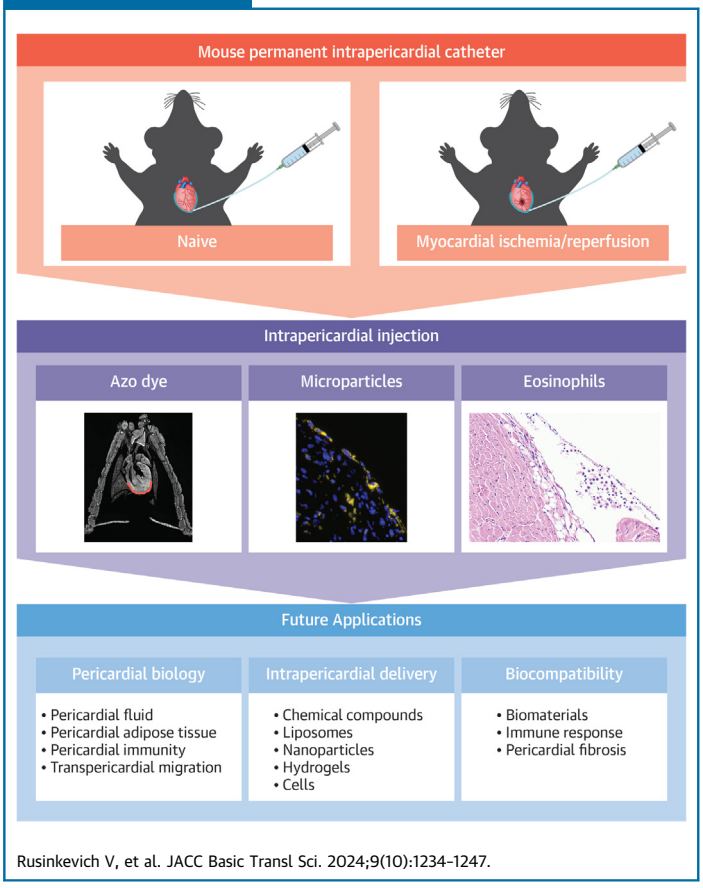
VISUAL ABSTRACT



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HIGHLIGHTS

- **A microsurgical method for implanting an intrapericardial catheter was developed specifically for mice.**
- **The successful application of a permanent pericardial catheter allowed for the delivery of lipophilic particles and immune cells into the pericardial cavity of mice.**
- **The insertion of a permanent pericardial catheter was combined with a closed chest model of prolonged myocardial ischemia/reperfusion in mice.**

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The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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SUMMARY

Intrapericardial delivery offers a route for heart therapies. Mouse heart size and membrane thickness pose catheterization challenges, hampering pericardium-targeted treatments. The objectives were to develop a mouse surgical technique for pericardial catheter insertion and to assess its suitability for intrapericardial delivery, including use with a myocardial ischemia/reperfusion model. We used successful catheter implantation in BALB/cJ and ΔdblGATA1 mice, showcasing intrapericardial delivery with fluorescent beads and eosinophils. We demonstrated a combination of pericardial catheterization with myocardial ischemia/reperfusion model. A reliable catheterization technique, enabling intrapericardial delivery of therapeutic agents in mice provides a valuable tool for studying the pericardial space and mediastinum in basic and translational research.

ABBREVIATIONS AND ACRONYMS

ECG = electrocardiogram
HBSS = Hank's Balanced Salt Solution
IL = interleukin
IP = intraperitoneal
I/R = ischemia/reperfusion
IV = intravenous
LAD = left anterior descending coronary artery
LV = left ventricular
MI = myocardial infarction
SC = subcutaneous

Up till now the delivery of heart therapeutics has mainly relied on systemic administration and to a lesser extent on intracoronary or intramyocardial administration. At the same time, experimental intrapericardial therapeutics are associated with higher heart deposition/retention, higher residence time, lower clearance rates, higher potency/efficacy, and lower, if any, systemic side effects.[1] Therefore, intrapericardial delivery of bioactive compounds, drugs, genes, and stem cells is a promising avenue to bypass the limitations of more traditional routes such as intravenous, intracoronary, and intramyocardial. Despite the fact that different techniques of catheterization of pericardial space are available for humans[2] and larger animals,[3-5] however, the size of the mouse heart and thickness of the mouse pericardial membrane put some serious restrictions on the use of such catheters in mice. At the same time, the number of proof-of-the-concept studies targeting pericardial space in mice[6-8] is growing. So, the absence of a well-reproducible and reliable surgical technique for pericardial catheter placement in mice is a serious obstacle in the way of the development of therapies targeting pericardial space. We developed a surgical approach for permanent pericardial catheter insertion in mice, and showed several examples of the delivery into pericardial space using the developed technique alone and in combination with a closed-chest mouse model of myocardial ischemia/reperfusion (I/R).

METHODS

ANIMALS. Wild-type BALB/cJ (JAX 651) and ΔdblGATA1 (JAX 5653) mice were obtained from the Jackson Laboratory. Interleukin (IL)-5Tg (founder line NJ.1638; Lee et al, 1997) mice were provided on the BALB/c background by J. and N. Lee (Mayo Clinic, Scottsdale, Arizona). Mice were housed in specific pathogen-free animal facilities at the Johns Hopkins University. Experiments were conducted on 9- to 12-week-old mice in compliance with the Animal Welfare Act and the principles set forth in the Guide for the Care and Use of Laboratory Animals. All methods and protocols were approved by the Animal Care and Use Committee of Johns Hopkins University. For terminal experiments, mice were euthanized by cervical dislocation after achievement of deep anesthesia status with a single intraperitoneal dose of Avertin (Tribromoethanol 2.5% wt/vol; dose of 0.02 mL/g of body weight).

THORACOTOMY. Ten minutes before the initiation of surgery, mouse was preanesthetized with Ketamine (80-100 mg/kg; intraperitoneal [IP]). Then the animal was fixed to a heated surgical board, intubated with a 22-G catheter, and connected to a respirator Mini Vent 845. The mouse was ventilated throughout the procedure with a stroke volume of 200 μL at a rate of 130-150 strokes/min and further anesthetized with Isoflurane (1.0-2.0 vol %). The respirator was connected to oxygen all the time during the operation and until the animal establish spontaneous breathing. Body temperature was monitored using a rectal probe and kept constant (~37°C). The hair over the appropriate skin area on the chest of the mice was removed using depilation cream Nair. For analgesia and proper myorelaxation slow-release formula Buprenorphine (SR-LAB, 0.5-1.0 mg/kg; subcutaneous [SC]) and Succinylcholine (0.05-0.1 mg/kg; IP) were administered, respectively. Then the shaved part of the thorax was disinfected with 10% Betadine, and lateral thoracotomy was performed as follows. The skin over the fourth rib was incised. The rectus abdominis and pectoralis muscles were kept intact and put aside. The ventral portion of the intercostal muscle between the third and fourth ribs was bluntly penetrated with

the forceps. A custom-made wound retractor was applied to spread the wound and expose the heart. After that, implantation of a permanent pericardial catheter alone or in combination with a coronary artery occluder was performed. Details of these procedures are described in the Results section. After either procedure, the thorax was closed with 1 stitch of a 6-0 monofilament polypropylene suture. The thoracic muscles released, moistened with 0.9% saline, and the skin over the thorax closed with an 9-0 polypropylene suture. Animals were weaned from the ventilator and extubated once independent breathing was visually verified. All mice were placed in a clean cage on a warming pad to assist recovery, until ambulatory motion was regained.

CLOSED-CHEST MYOCARDIAL I/R MODEL. A closed-chest model of prolonged myocardial I/R was used as described previously.[9] In brief, mice underwent surgical implantation of an occluding device around the left anterior descending (LAD) coronary artery. This time placement of suture on LAD was combined with insertion of permanent pericardial catheter during the thoracotomy described in the previous section. The mice assigned to SHAM surgery received the same surgery with the same instrumentation as the mice assigned to I/R injury, except that the ends of the suture encircling the LAD were not pulled. SHAM-treated mice had the same duration of anesthesia, ventilation, and surgical manipulations as mice subjected to I/R injury.

ELECTROCARDIOGRAM. For the electrocardiogram (ECG) recording mice were preanesthetized with 3 vol % isoflurane in an induction chamber, secured to a heated board, and further anesthetized with 1-1.5 vol % isoflurane through the nosecone. ECG signals were captured using a PowerLab16/35 unit (AD Instruments), with data acquisition facilitated through Lab Chart Pro 8 (AD Instruments). The mouse ECG lead I, II, and III were recorded with a sampling rate of 4 kHz.

ECHOCARDIOGRAPHY. Echocardiography was conducted on conscious mice using an MS-400 imaging transducer paired with the Vevo 2100 Imaging System (Visual Sonics). The imaging process commenced by positioning the transducer probe along the left sternal border to capture the parasternal short-axis view. Various measurements were obtained, including left ventricular (LV) end-systolic and end-diastolic internal diameters (LVID;s, LVID;d), posterior wall thickness (LVPW), and LV fractional shortening (FS).

ISOLATION OF EOSINOPHILS FOR ADOPTIVE TRANSFER. Eosinophils were isolated from the blood of IL-5Tg mice as described previously. In brief, blood from donor IL-5Tg mice was collected in phosphate-buffered saline with 100 U/mL heparin and overlaid on Histopaque-1119 (Sigma Aldrich) to remove red blood cells. Anti-CD90.2 and anti-CD45R(B220) microbeads (Miltenyi Biotec) were used to deplete lymphocytes and enrich eosinophils. Eosinophil purity (80%-90%) was determined using flow cytometry.

FLUORESCENT MICROSCOPY. For fluorescent microscopy whole hearts with residual pieces of pericardial membrane containing patches of adipose tissue were rinsed in phosphate-buffered saline and fixed in optimal cutting temperature compound (Sakura Finetek), frozen at −80°C, and sectioned transversely (4-μm thick) at 500-μm intervals beginning from the apex. Then the slides were fixed in 4% paraformaldehyde, washed, and incubated with 4′,6-diamidino-2-phenylindole (1:2,000). Fluorescent imaging was performed with an Axio Imager A2 microscope (Zeiss).

WHOLE MOUNT MOUSE CHEST HISTOLOGY. Mice were injected with 100 U of heparin IP 30 min before the procedure and were anesthetized with Avertin IP. Then the abdominal cavity was exposed and inferior vena cava was cannulated and the mouse was perfused with cold Hank's Balanced Salt Solution (HBSS). After that, the whole rib cage with attached diaphragm was separated from the rest of the body and placed in SafeFix II (Fisher Scientific) for fixation. After proper fixation, the rib cage was decalcified. Parafin-embedded whole rib cage was used for slide preparation and subsequent hematoxylin and eosin staining (Histoserv).

MOUSE HEARTS HISTOLOGIC ASSESSMENT. Mice were injected with heparin, anesthetized with Avertin, and euthanized by cervical dislocation. Immediately after that chest cavity was exposed and heart ventricles were cannulated and perfused with cold HBSS. After excision mouse hearts were fixed in SafeFix (Fisher Scientific), embedded in paraffin, cut into 5-μm-thick sections, and stained with hematoxylin and eosin stain (Histoserv).

PREPARATION OF SINGLE-CELL SUSPENSIONS AND FLOW CYTOMETRIC ANALYSIS. Mice were injected with 100 U of heparin IP 30 min before the procedure, anesthetized with Avertin IP, and euthanized by cervical dislocation. Chest cavities were exposed and mice were intracardially perfused with 20 mL of ice-cold HBSS. Heart ventricles and pericardial membranes along with pericardial adipose patches were carefully dissected under the microscope, minced with fine scissors, and enzymatically digested with a cocktail of type II collagenase and DNAse I (Worthington

Laboratories) in HBSS for 40 min at 37°C. To generate single-cell suspensions, heart ventricles were mechanically dissociated in gentleMACS C Tubes using GentleMACS dissociator (Miltenyi Biotec) following the manufacturer's protocols. Pericardial adipose tissues were mechanically dissociated by passing through the 1,000-μL pipet tip several times. Cells from the heart ventricles and pericardial adipose tissues were then passed through a 40-μm nylon mesh (BD Falcon), and reconstituted with a staining buffer. Single-cell suspensions were stained with LIVE/DEAD Fixable Aqua (Thermo Fisher Scientific). FcγRII/III was blocked with anti-mouse CD16/CD32 (eBioscience), and markers of interest were stained with flourochrome-conjugated antibodies (BD, BioLegend, and eBioscience). Eosinophils were gated as CD45+/CD11b+/Ly6G-/SiglecF+ cells. Samples were acquired on BD LSR II or Fortessa (BD Biosciences) and data were analyzed using FlowJo V9 (Tree Star).

STATISTICAL ANALYSIS. Continuous data presented using the mean ± SD. Comparisons between 2 groups were performed using 1-way analysis of variance or Kruskal-Wallis test followed by Dunn multiple comparisons test dependent on normality of data. All statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad Software Inc.), and a P value <0.05 was considered statistically significant.

RESULTS

IMPLANTATION OF THE PERMANENT PERICARDIAL CATHETER. All surgical procedures were executed by a proficient animal surgeon, with thoracotomy being a routine and frequently practiced procedure. The inclusion of an additional step, namely, pericardial catheter implantation, was deemed relatively uncomplicated and necessitated only minimal supplementary skill sets.

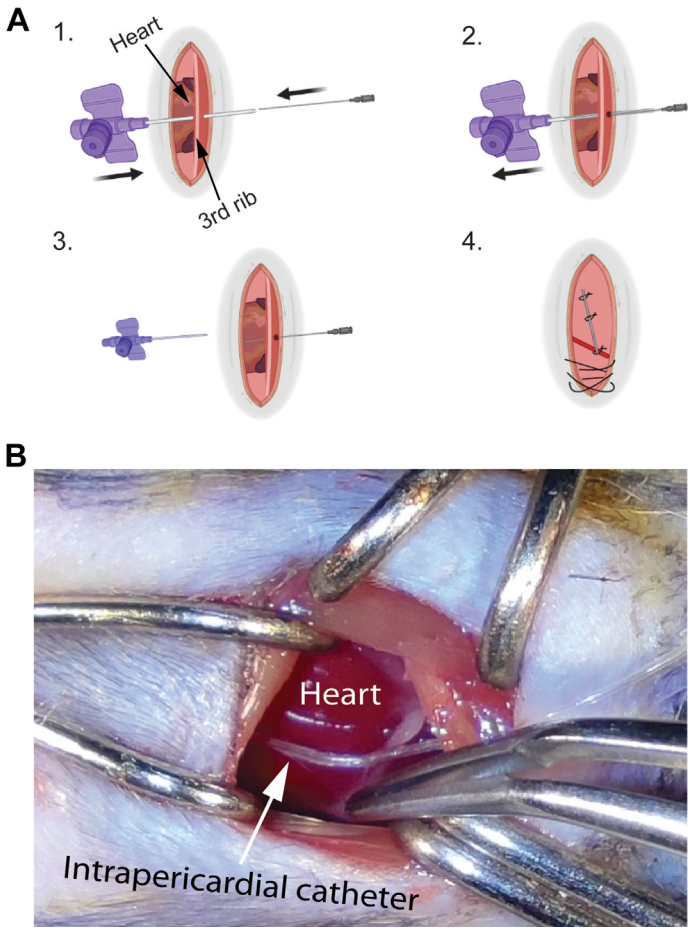
After a thoracotomy guided PU-254 microcatheter (Doccol Corp) was inserted intrapericardially, it was exteriorized through the intercostal muscles and fixed under the skin. A detailed explanation of the technique of catheter insertion is illustrated in **Figure 1A** and Video 1.

1) For catheter insertion the third intercostal was penetrated with a 20-G intravenous (IV) catheter. After that, the metal guide was withdrawn from the 20-G catheter, and the microcatheter with its guide wire was inserted into the lumen of the 20-G catheter through the tip as depicted.
2) Then the 20-G catheter was gently pulled backward and finally removed to leave the pericardial microcatheter penetrating the third intercostal. The 20-G catheter served as an external tunnel guide to facilitate the microcatheter insertion through the intercostal space.
3) In the next step the pericardial membrane was gently pulled up with microforceps, and the microcatheter tip with its protruding guidewire were inserted into the pericardial space (**Figure 1B**). After that the guidewire should be pulled backward until its tip reaches the external surface of the thorax. So, the intrathoracic portion of the microcatheter stays without internal wire, but the subdermal portion of the catheter still has it. Then, the thoracic length of the microcatheter was adjusted to settle the tip of the catheter closer to the apex of the heart.
4) After the catheter insertion, the thorax was closed with 1 stitch of a 6-0 monofilament polypropylene suture. The thoracic muscles were released, moistened with 0.9% saline, and closed with continuous 9-0 polypropylene suture. Then the subdermal portion of the microcatheter containing guidewire was fixed with several 9-0 polypropylene sutures on the external surface of the thoracic muscles. Then the skin over the catheter was closed with a continuous 9-0 polypropylene suture.

To prove the reproducibility and the reliability of the technique of pericardial catheter insertion we implanted permanent pericardial catheters in 5 mice. All mice survived the procedure, and no complications were observed during their recovery period.

INTRAPERICARDIAL INJECTION OF FLUORESCENT BEADS. Mice were allowed to recover for 7 days; after that fluorescent microparticles were injected into the previously implanted permanent pericardial catheter. On the day of injection, a mouse with a preimplanted catheter received a dose of slow-release formula Buprenorphine SR-LAB, (0.5-1.0 mg/kg; SC), was secured to a heated board, and further anesthetized with 1-1.5 vol% Isoflurane through the nosecone. Then the skin over the pericardial catheter was disinfected with 10% Betadine and incised (1-mm to 2-mm cut). The external tip of the implanted microcatheter was identified and isolated as shown in **Figure 2A** and Video 2. Next the catheter's internal guidewire was pulled out using fine microforceps and placed on a sterile gauze. After that, the tip of the microcatheter was connected to the adapter - TF-508 microcatheter (Doccol Corp) set together with 1 mL syringe via a 1-way stopcock. Then ~$1*10^6$ fluorescent microparticles Fluoresbrite (Polysciences Inc.) were slowly injected into the pericardial cavity. After the

FIGURE 1 Technique of Implantation of the Permanent Pericardial Catheter in Mice



(A) Step-by-step instructions for mouse intrapericardial catheter insertion. A guided microcatheter was implanted intrapericardially and exteriorized using 20-G intravenous (IV) catheter as an external guide. The chest was closed in layers and the extrapericardial part of the microcatheter was left under the skin. (B) Image of the implanted and exteriorized permanent pericardial catheter. A thin pericardial layer was elevated by the tips of the microforceps (arrow).

injection, the guidewire was inserted back into the catheter lumen serving as a plug. The tip of the catheter was hidden under the skin and the skin was closed with 1 9-0 polypropylene suture. It usually took 10 to 15 minutes to accomplish the intrapericardial injection in 1 mouse.

Because of the large size of the standard Luer lock and significant tissue damage associated with its subcutaneous implantation in mice it was decided to not keep it, and, therefore, the intrapericardial injections were achieved by using connector catheter TF-508, whose internal diameter equals the external diameter of the implanted intrapericardial catheter. Despite that, such a connector-based technique of injection has a significant disadvantage - leak from the site of catheters connection, which makes difficult precise dosing of injected agents. However, we solved this issue by application of a microscopic amount of BioMed Clear Resin (Formlabs) as a sealing glue for the junction (**Figure 2B**). Resin application and ultraviolet exposure are fast and guarantee the secure connection between 2 catheters for precise injection. After injection, the catheters' junction and short adjacent parts of the catheters were trimmed out to save the tips of the catheters for repeated injections. However, if precise dosing is not necessary, intrapericardial injection may be achieved without sealing catheters' junction with glue.

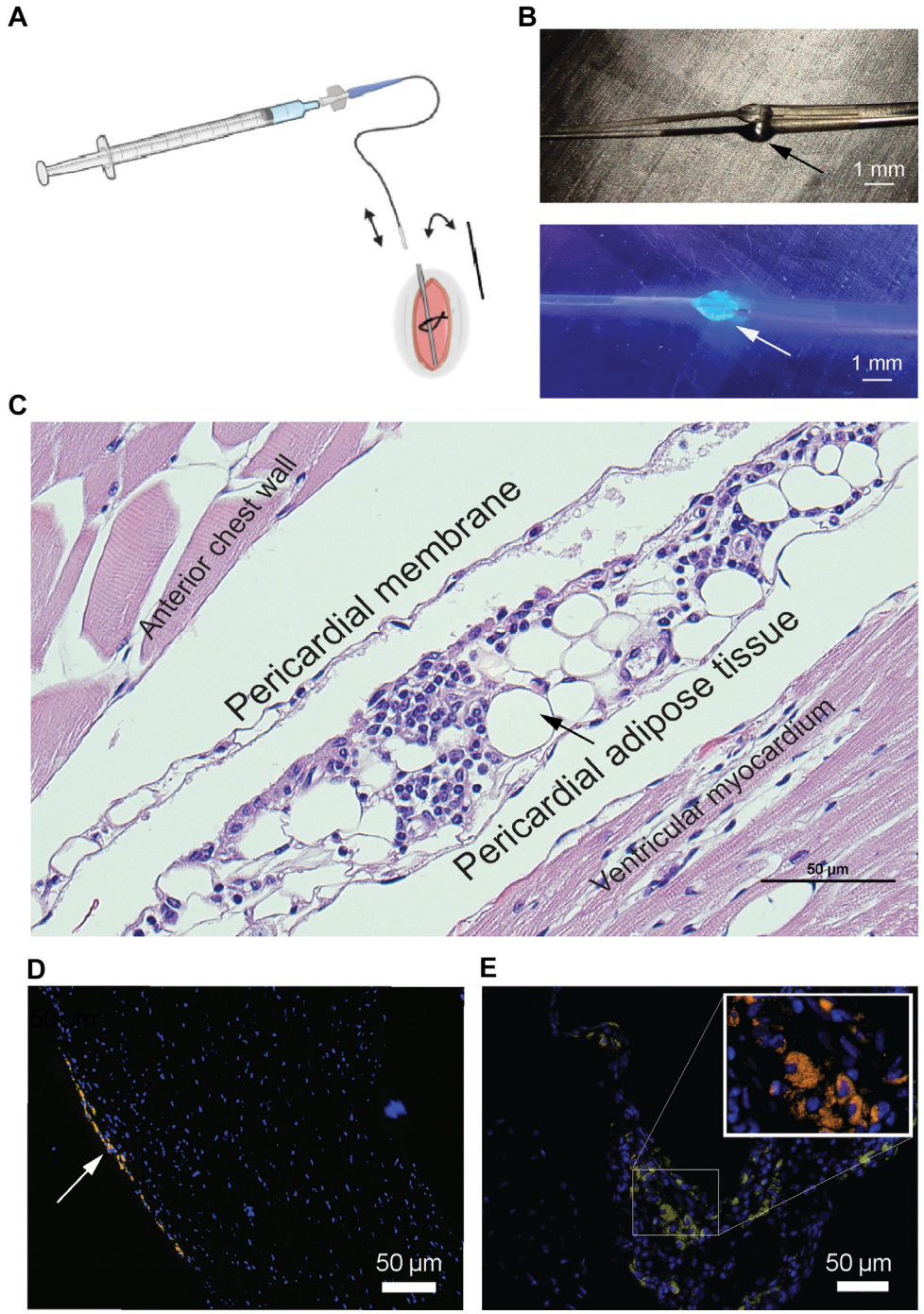
To verify the presence of microparticles inside the pericardial cavity mice were sacrificed 4 days after intrapericardial injections and hearts along with residual pieces of pericardial membranes were subjected to fluorescent microscopy. **Figure 2C** shows the anatomy of the pericardial cavity of a naive mouse. In contrast to humans, the mouse heart almost lacks adipose tissue on its epicardial surface. However, large patches of adipose tissue with incorporated immune cells are present on the pericardial membrane. Intrapericardially injected fluorescent microparticles were detected both on the epicardial surface of the heart (**Figure 2D**) and in cells of pericardial adipose tissue (**Figure 2E**) 4 days after injection in all 5 mice.

MODELING MYOCARDIAL I/R WITH THE PREIMPLANTED PERMANENT PERICARDIAL CATHETER. After confirmation of reproducibility of the techniques of permanent pericardial catheter insertion and intrapericardial delivery we decided to test whether implantation of a pericardial catheter may be combined with the closed chest model of prolonged myocardial I/R. This time operation of insertion of permanent pericardial catheter was associated with the placement of the LAD occluder during the thoracotomy.

After exposure of the fourth left intercostal space, 8-0 polypropylene suture attached to a U-shaped tapered needle was passed under the proximal LAD artery ~2-3 mm from the tip of the left auricle. Then the needle was cut from the suture. For the next step, a 20-G IV catheter was used as an external guide for both LAD sutures and pericardial microcatheter as shown in **Figure 3A** and Video 3.

It is important to note that for pericardial catheter insertion without placement of LAD occluding device it is more convenient to use the method where the microcatheter is inserted through the 20-G IV catheter tip as shown in **Figure 1A**, however, when the catheter insertion is combined with LAD occluding device

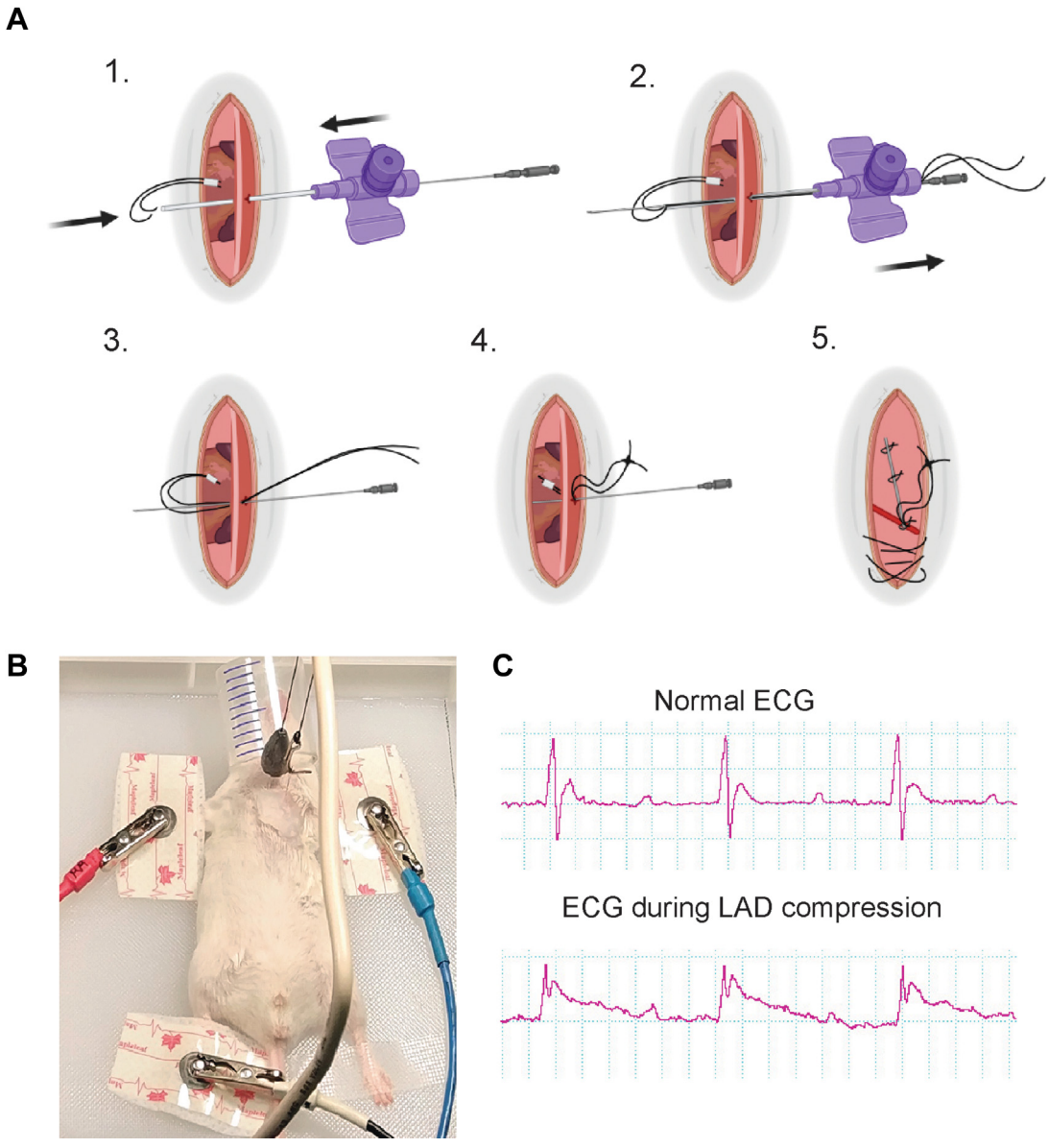
FIGURE 2 **Intrapericardial Injection of Fluorescent Beads**



(A) Principle of intrapericardial injections. The external part of the catheter was connected to the adapter catheter set together with a 1-mL syringe via a 1-way stopcock. Microcatheter internal guide wire was pulled out before injection and inserted back right after injection. (B) Visible light (upper) and ultraviolet (UV) light (lower) images of PU-254 and TF-508 catheters' junction sealed with BioMed Clear Resin. Black and white arrows point at the junction. (C) Microphotograph of pericardial adipose tissue patch situated between the left ventricle and the anterior chest wall. Black arrow points at adipocytes. (D) Fluorescent image of the intrapericardially injected Fluoresbrite microparticles distributed on the edge of the left ventricle (white arrow). (E) Fluorescent image of the intrapericardially injected Fluoresbrite microparticles found in cells of pericardial adipose tissue.

JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 9, NO. 10, 2024
OCTOBER 2024:1234–1247

FIGURE 3 **Technique of Implantation of the Permanent Pericardial Catheter in Combination With Modeling of Myocardial I/R**



(A) Step-by-step instructions for the combinatorial procedure of intrapericardial catheter insertion and left anterior descending (LAD) artery coronary occluder implantation. During thoracotomy 8-0 suture was placed under LAD, and both ends of the suture were threaded through the LAD occluder and then through the tip of a 20-G IV catheter. At the same time, the microcatheter was pulled toward the tip of the 20-G catheter from the port side of the IV catheter. After that, the guided microcatheter was implanted intrapericardially and exteriorized using 20-G IV catheter as an external guide. The chest was closed in layers and the extrapericardial part of the microcatheter and external portion of the LAD suture were left under the skin. (B) Image of the mouse with implanted permanent pericardial catheter during the induction of the prolonged myocardial ischemia/reperfusion (I/R). The lead weight was hung on the bearing, allowing permanent compression of the LAD for 90 minutes. (C) Elevation of ST-segment was visible on the electrocardiogram (ECG) indicating successful occlusion of the coronary artery. Abbreviations as in Figure 1.

implantation, the port of the 20-G IV catheter should be placed on the same side with pericardial microcatheter:

1) Immediately after placement of the LAD occluder the 20-G IV catheter was used to penetrate the third intercostal from the cranial side. After that, the metal guide was withdrawn from the 20-G catheter, and the microcatheter with its guide wire was inserted into the lumen of the 20-G catheter through its port.
2) Then both ends of the 8-0 suture placed on LAD were threaded through the tip of the 20-G IV catheter until they come out from the port side, and at the same time the microcatheter was gently pulled toward the tip of the 20-G catheter, until the port of the microcatheter reaches the port of 20-G IV catheter;
3) Next the port of the microcatheter was cut off, and the 20-G catheter was pulled backward releasing 2 LAD sutures along with the microcatheter penetrating the rib cage. After that, the thoracic length of the microcatheter was adjusted to settle the tip of the catheter closer to the apex of the heart. Then the microcatheter tip was inserted into the pericardial space and adjusted. The microcatheter guidewire should be pulled backward until its tip reaches the external surface of the thorax;
4) After the insertion of the catheter intrapericardially the rib cage was immediately closed with 1 stitch of a 6-0 monofilament polypropylene suture. The thoracic muscles were released, moistened with 0.9% saline, and closed with continuous 9-0 polypropylene suture. The LAD sutures were knotted together; and
5) Finally, the subdermal portion of the microcatheter containing guidewire was fixed on the external surface of the thoracic muscles, and the skin over the catheter was closed with a continuous 9-0 polypropylene suture.

Animals were weaned from the ventilator and extubated once independent breathing was visually verified. All operated animals were allowed to recover for at least 14 days before induction of infarction.

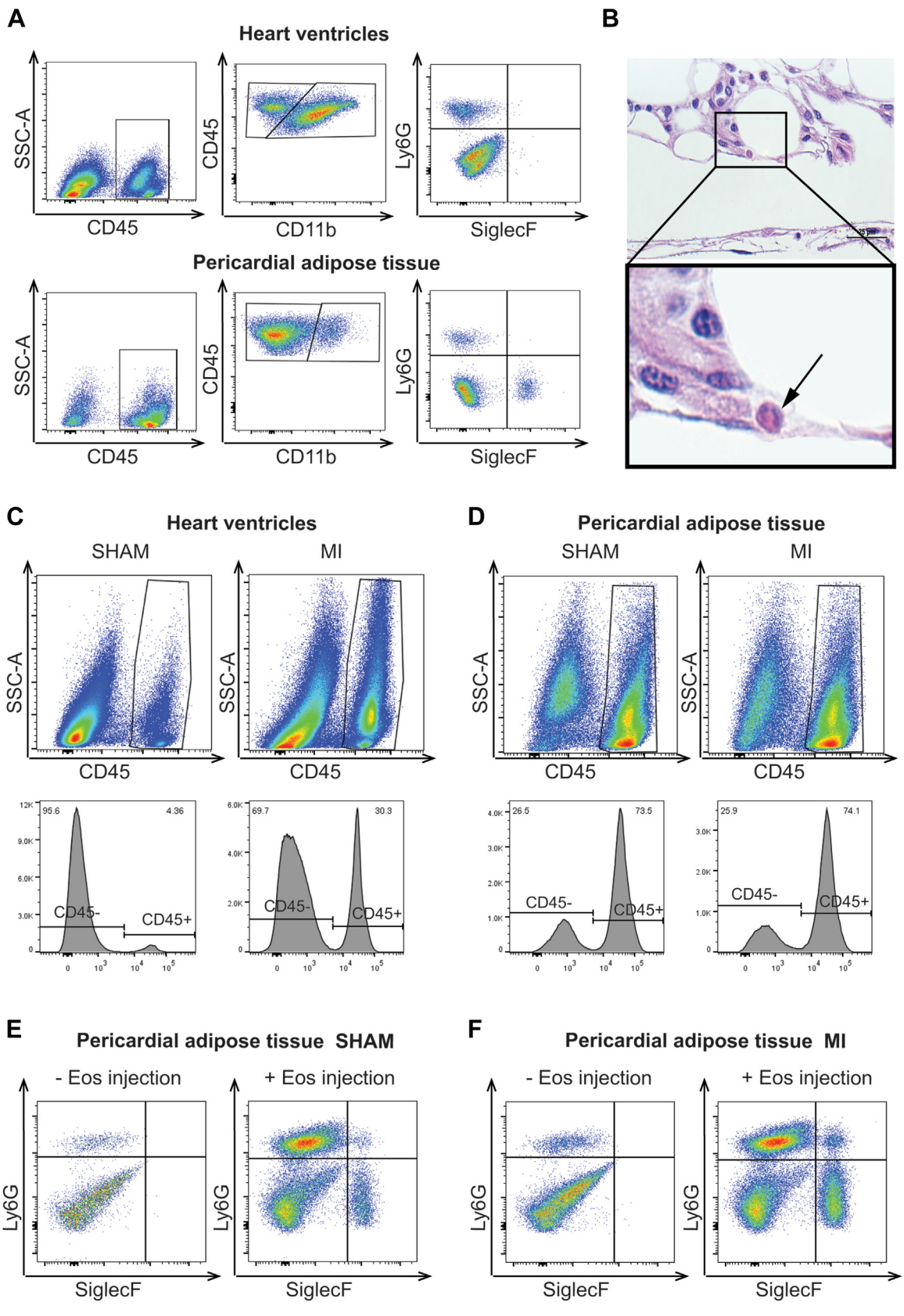
On the day of induction of myocardial I/R mice with the preimplanted occluding device and permanent pericardial catheter received a dose of slow-release formula Buprenorphine SR-LAB, (0.5-1.0 mg/kg; SC), were secured to a heated ECG board, and were further anesthetized with 1-1.5 vol% Isoflurane through the nosecone. The mice were breathing spontaneously throughout the experiment (**Figure 3B**). The skin over the old scar was opened, and both ends of the LAD artery suture were taped to a 5 g lead weight. The lead weight was hung on the bearing, allowing permanent compression of the LAD. ST-segment elevation was visible on the ECG (**Figure 3C**), indicating successful occlusion of the coronary artery; ischemia was continued for 90 min. To induce reperfusion, the LAD sutures were cut close to the chest wall, releasing the tension. Then the skin incision on the chest was closed with a single 9-0 polypropylene suture.

INTRAPERICARDIAL INJECTION OF EOSINOPHILS AFTER INDUCTION OF PROLONGED MYOCARDIAL I/R. As was previously shown there is a pool of eosinophils residing in adipose tissue.[10] We isolated single cells from the naive wild-type mouse heart ventricles and from pericardial adipose tissue and subjected them to flow cytometry. It was found that in contrast to ventricular myocardium pericardial adipose tissue hosts a significant number of eosinophils at steady state (**Figure 4A**). Presence of eosinophils in pericardial adipose tissue was also confirmed using histology (**Figure 4B**). Therefore, we decided to use eosinophils for intrapericardial injections because they would potentially home into pericardial adipose tissue. ΔdblGATA1 mice constitutively lack eosinophils, so any presence of eosinophils in pericardial adipose tissue after intrapericardial injection would mean that those cells have an external origin.

Intrapericardial catheters along with LAD occluders were implanted in 10 ΔdblGATA1 mice (experimental group). We compared their postoperative recovery and physiological parameters with 10 ΔdblGATA1 mice receiving only LAD occluders without pericardial catheters. We found that those parameters assessed in the experimental group were indistinguishable from those observed in the group that underwent standard LAD occluder implantation alone (Supplemental Tables 1 to 6). After 2 weeks of recovery, prolonged myocardial I/R was induced in 5 mice from the group where intrapericardial catheters were implanted along with LAD occluders. Twenty-four hours later $1\text{-}2*10^6$ eosinophils in 30 μL of mouse serum were injected into pericardial catheters of 8 mice from this group including 4 mice that previously underwent myocardial I/R.

Next, using flow cytometry, we assessed leukocyte (CD45+ cells) numbers in heart ventricles (**Figure 4C**) and pericardial adipose tissue (**Figure 4D**) 24 hours after injection. Our results revealed a dramatic increase in CD45+ cell numbers in the ventricles of the hearts after induction of myocardial infarction (MI) in comparison with the hearts of animals that were operated on but myocardial I/R was not induced (SHAM). This finding proves that myocardial I/R can

FIGURE 4 **Intrapericardial Injection of Immune Cells After Induction of Prolonged Myocardial I/R**



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be successfully induced in mice with preimplanted permanent pericardial catheter. At the same time, we did not see any differences in CD45+ cell counts in pericardial adipose tissue between the SHAM-operated mice and those with MI.

Then we assessed the presence of eosinophils in the pericardial adipose tissue of mice with induced MI and SHAM. As was stated earlier, ΔdblGATA1 mice constitutively lack eosinophils, and indeed no eosinophils were found in the pericardial adipose tissue of ΔdblGATA1, neither in animals with induced MI nor in SHAM operated mice without pericardial injection (**Figures 3E and 3F**). In contrast, pericardial adipose tissue from mice that have undergone intrapericardial injection of eosinophils showed significant eosinophil infiltration in both MI and SHAM mice. This would suggest that injected eosinophils reached the pericardial space, and moreover homed into pericardial adipose tissue.

MOUSE POSTOPERATIVE RECOVERY AND DISTRIBUTION OF INTRAPERICARDIALLY INJECTED AGENTS. To estimate the potential adverse effects of intrapericardial catheter implantation on the state and function of the mouse heart, we performed intrapericardial catheter implantation alone in 10 mice and in association with the implantation of an LAD occluder in 5 mice (Supplemental Tables 7 to 9). After 4 weeks post-thoracotomy, all mice underwent full recovery. The skin over the exposed portion of the catheter had completely healed in every mouse (**Figure 5A**). Comparing echocardiographic parameters between operated mice and age-matched naive control mice, including LVID;s, LVID;d, LVPW;s, LVPW;d, and FS, we found no differences between the control and operated animals (**Figure 5B**), as the *P* values of multiple comparison tests for all the analyzed parameters were >0.05. Furthermore, the analysis of ECG showed no abnormalities, with normal waveforms observed in all operated animals (**Figure 5C**, upper). Subsequent histologic examination of whole hearts revealed no disparities between control hearts and those with catheter implantation (**Figure 5C**, lower). However, all hearts with LAD occluder implantation exhibited notable pericardial fibrosis at the ligated LAD sites (**Figure 5C**, box C1).

STUDY LIMITATIONS. Previously in this article we have demonstrated an example where injected fluorescent beads and immune cells were validated intrapericardially to prove the feasibility of intrapericardial delivery of various agents. However, we have not questioned whether there is an extra pericardial leak of injected agents and how it is distributed. To explore the leak, we have injected 5% bovine serum albumin mixed with 0.05% Evans Blue intrapericardially in 3 mice, immediately euthanized them, and subjected their whole chests to microscopy. Analyzing a series of longitudinal slides at the different levels of the mouse chest, we observed the most intense fluorescent signal from Evans Blue at the hearts' apexes (**Figure 5D**). Additionally, we observed lower intensity fluorescence scattered along left lung (**Figure 5D**, boxes D1 and D2), indicating potential leakage of the injected solution beyond the pericardium.

DISCUSSION

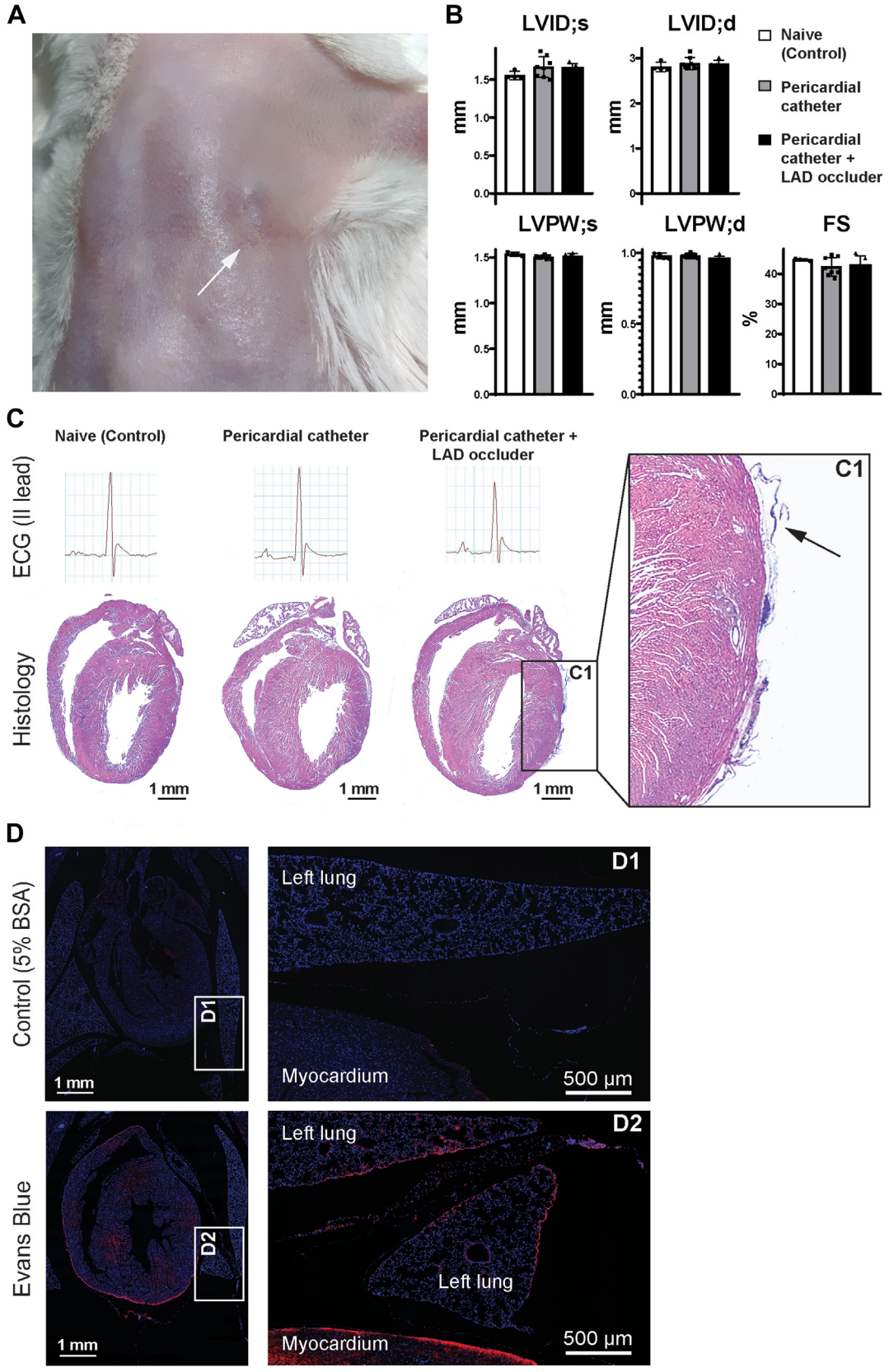
In this paper we have shown the feasibility of pericardial catheterization in mice. Mouse pericardial space was catheterized with a microcatheter during thoracotomy. The external portion of the catheter was left under the skin, and was used for pericardial injections of fluorescent beads and immune cells. In addition, it was shown that this technique may be used along with simultaneous modeling of prolonged myocardial I/R for intrapericardial delivery after MI.

Current interest in intrapericardial delivery of therapeutic agents dictates the need for the development of the corresponding animal models. Various mouse models of cardiac diseases are used in basic and translational studies, but microscopic dimensions of mouse pericardial membrane and pericardial space put serious restrictions on studies where catheterization of pericardial space is

FIGURE 4 Continued

(A) Flow cytometry gating strategy for eosinophils isolated from heart ventricles and pericardial adipose tissue of wild-type (WT) mice. Eosinophils were identified as CD45+, CD11b+, Ly6G-, SiglecF+ population. (B) Microphotograph of the hematoxylin and eosin stained pericardial adipose tissue of WT mice. Arrow points at eosinophil. Scale bar = 50 μm. (C) Flow cytometry plots (upper row) with corresponding histograms (lower row) showing the percent of CD45+ cells in cell suspensions obtained from heart ventricles of the SHAM mice and from the mice after induction of myocardial I/R (myocardial infarction [MI]). (D) Flow cytometry plots (upper row) with corresponding histograms (lower row) showing the percent of CD45+ cells in cell suspensions obtained from pericardial adipose tissue of the ΔdblGATA1 SHAM mice and from the mice after induction of myocardial I/R. (E) Flow cytometry plots showing the percent of eosinophils in cell suspensions obtained from pericardial adipose tissue of ΔdblGATA1 SHAM mice +/- injected with eosinophils. (F) Flow cytometry plots showing the percent of eosinophils in cell suspensions obtained from pericardial adipose tissue of ΔdblGATA1 mice after induction of myocardial ischemia/reperfusion (MI) ± injected with eosinophils. Abbreviations as in **Figure 3**.

FIGURE 5 **Postoperative Recovery and Distribution of Intrapericardially Injected Agents**



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necessary for experimental modeling. At the same time, methods of pericardial delivery, other than the insertion of the permanent pericardial catheter such as echocardiography-assisted intrapericardial injections[11] or intrapericardial application during thoracotomy,[7,12] have been developed for mice as well. However, those approaches have significant limitations. For example, thoracotomy with simultaneous intrapericardial application may cause a significant nonspecific immunologic response that skews other experimental parameters. In turn, echocardiography-assisted intrapericardial injections require an operator with advanced skills and an available mouse echocardiograph. Implantation of a permanent pericardial catheter described here lacks those drawbacks. Moreover, mouse pericardial space has the volume of only a few microliters, and injection of a large volume into it may compromise heart hemodynamics and ultimately lead to cardiac tamponade. In cases where to achieve the therapeutic effect a large volume still should be delivered into the pericardial space, the risk of the tamponade might be ameliorated by use of multiple intrapericardial injections of smaller volumes. This may be achieved by placement of a permanent pericardial catheter but not by the other available for mice methods of pericardial delivery. Moreover, the implanted pericardial catheter can be connected with ALZET Osmotic Pumps, so the intrapericardial cargo might be delivered at a constant rate for days or weeks.

It was shown that a pericardial catheter in mice may be used alone or in combination with the model of myocardial I/R. Because intrapericardial delivery is considered a therapeutic approach in humans for treating the consequences of MI,[6,7] combining procedures further advances the modeling of pericardial delivery in mice.

Pericardial applications of different drugs,[13-15] cells,[16,17] genes,[3] and hydrogels[7,18] have been tested on large animals along with MI modeling. At the same time, pericardial biology has not yet been fully described and still requires further clarification of basic facts. In this regard, mouse models allowing work with the pericardial space either alone or in combination with other models of cardiac diseases might be a good tool for basic and translational studies, especially those related to the pericardial cavity and the mediastinum. Furthermore, in perspective the catheter may not only be used for intrapericardial delivery, but also for sampling from the pericardial cavity. In addition, pericardial catheters are widely used in cardiac surgery in the postoperative period to manage pericardial effusion. Basically, thoracotomy in combination with pericardial catheter implantation is a common procedure for the majority of operations on the heart. So, the described mouse model could contribute to the studies not only related to cardiology but also to cardiac surgery.

Despite all the evident advantages of the model described here it still has a few limitations. One significant limitation is the requirement for the skilled operator being able to perform microsurgical manipulations. However, the level of the complexity of the surgical procedure described here is comparable to the complexity of the widespread mouse model of MI. And most likely anyone who can perform surgical ligation of the coronary artery in mice will be able to insert a pericardial catheter as well. The goal of this work was to show the possibility of use of the pericardial catheter in mice, yet we have not properly assessed the immunologic responses caused by intrathoracic manipulations for catheter insertion. However, we assume that such responses do not exceed the reaction caused by thoracotomy for any other reason.

All the experiments in this study were conducted on adult male mice due to their larger size; therefore, gender distinctions were not investigated. Although we do not anticipate significant gender-related effects on the catheter implantation procedure, potential differences in postoperative recovery, such as thoracotomy-induced inflammatory responses or the development of pericardial fibrosis, cannot be ruled out.

FIGURE 5 Continued

(A) Image displaying the mouse chest with a permanently implanted pericardial catheter 4 weeks post-thoracotomy. The white arrow points at the postoperative scar. (B) Results of echocardiographic assessment of the mice with implanted intrapericardial catheters (n = 4-7), data are shown as the mean ± SD. (C) Representative images of ECG waveforms (upper) and histologic preparations of whole hearts (lower) The black arrow in box C1 points at the fibrotic pericardium. (D) Composite fluorescent images depicting the distribution of Evans Blue in whole-mount mouse chest, accompanied by corresponding higher magnification images. (Blue fluorescence represents DAPI, while red indicates Evans Blue). DAPI = 4′,6-diamidino-2-phenylindole; FS = fractional shortening; LVID;d = left ventricular internal diameter in diastole; LVID;s = left ventricular internal diameter in systole; LVPW;d = left ventricular posterior wall thickness in diastole; LVPW;s = left ventricular posterior wall thickness in systole; other abbreviations as in Figure 3.

JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 9, NO. 10, 2024
OCTOBER 2024:1234–1247

During mouse heart isolation for histologic assessment, we usually observed catheter-induced local fibrosis surrounding the intrathoracic portion of the catheters. It remains unclear whether these fibrotic changes stem from the thoracotomy-related inflammatory response or triggered by the prolonged presence of the catheter within the pericardial space. Fibrosis development is likely associated with pericardial inflammation after thoracotomy, and should be considered one of the limitations of this model.

Another limitation related to post-thoracotomy inflammatory response is associated with development of pericardial adhesions. In our experiments, we usually observed pericardial adhesions in mice that underwent pericardial catheter implantation combined with LAD occluder placement, but not in those that underwent pericardial catheter implantation alone. These adhesions commonly develop at sites where the LAD suture penetrates the myocardium (specifically the anterolateral wall of the left ventricle, approximately 2 mm from the left atrial appendage), whereas the tip of the intrapericardial catheter was positioned near the heart apex. Given this scenario, such pericardial adhesions are unlikely to impede a uniform distribution of agents delivered to the heart apex. Nevertheless, further experiments are warranted to investigate this matter.

Although preserving the integrity of the pericardium is considered crucial during the implantation of intrapericardial catheters, we do not regard it as the sole determinant for the success of intrapericardial injections. We anticipate that puncturing the pericardial membrane during catheter insertion and LAD occluder placement will inevitably result in some degree of pericardial disruption, potentially causing intrapericardially injected substances to leak outside the pericardial space.

We evaluated the extent of extrapericardial leakage from the catheter and used a solution of bovine serum albumin with Evans Blue, which more or less has physical properties similar to pericardial fluid. Notably, in this experiment the most intense Evans Blue fluorescence was detected within the mouse pericardial space around the heart apex, but, in addition, spots of fluorescence in the left lung and mediastinum were detected. This pattern was evident immediately after injection, indicating that some extrapericardial leakage occurs promptly after injection. Therefore, to comprehensively understand the dynamic distribution of any specific agent injected into the pericardial space, further experimentation is warranted.

Obviously, the behavior and distribution of injected agents, both within and outside the pericardium, are significantly influenced by various factors including their molecular weights, composition, hydrophilic/hydrophobic properties, and other physical/chemical characteristics, as well as their ability to move actively (in the case of cell injection). Consequently, the proportion and distribution of extra pericardial leakage hinge on the nature of these elements.

Furthermore, it is essential to consider the structural features of the mouse pericardium. Earlier studies have confirmed the presence of circular fenestrations in the pericardium of rodents, facilitating the passage of certain substances between the pleural and pericardial cavities.[19,20] Consequently, even an intact pericardium may permit leakage of agents capable of traversing pericardial pores. So, this might be considered another limitation and an unknown factor that could influence the use of the model, particularly if cellular therapeutics are being used.

CONCLUSIONS

In summary, the present study displays the possibility of permanent pericardial catheterization in mice for intrapericardial administration of therapeutic agents. The method involves the insertion of a microcatheter into the mouse pericardial cavity during thoracotomy, while leaving the outer part of the catheters beneath the skin. The technique can be used alone or along with the myocardial I/R model for intrapericardial delivery post-MI, thus providing a tool for fundamental and translational research pertaining to the pericardial sac and the mediastinum.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Intrapericardial administration of therapeutic agents is emerging as a promising avenue for treating cardiac disorders. Despite the availability of intrapericardial catheters for human use, none of the proposed interventions have been integrated into clinical practice. This gap underscores the need for suitable tools and animal models, which currently poses significant limitations on both basic research and translation into clinical applications. There is a pressing need to establish novel translational models that enable the simultaneous study of cardiac pathology alongside intrapericardial delivery to advance experimental therapeutics in this domain.

TRANSLATIONAL OUTLOOK: The technique described here suggests bridging the gap between clinical cardiology practice and laboratory experimentation. By demonstrating the feasibility of pericardial catheterization in mice and its integration with myocardial I/R modeling, new avenues for therapeutic development in human cardiac conditions are unveiled. This technique serves as a vital tool for fostering collaboration between basic scientists and clinicians, facilitating the translation of fundamental knowledge into clinical trials and patient care. Identifying challenges such as potential immune reactions and extrapericardial leakage is crucial for refining this technique for future clinical applications.

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APPENDIX For supplemental tables and videos, please see the online version of this paper.