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A cryoinjury model in neonatal mice for cardiac translational and regeneration research

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Abstract

The introduction of injury models for neonatal mouse hearts has accelerated research on the mechanisms of cardiac regeneration in mammals. However, some existing models such as apical resection and ligation of the left anterior descending artery produce variable results, which may be due to technical difficulties associated with these methods. Here, we present an alternative model for studying cardiac regeneration in neonatal mice in which cryoinjury is used to induce heart injury. This model yields a reproducible injury size, does not induce known mechanisms of cardiac regeneration, and leads to a sustained reduction of cardiac function. This protocol uses reusable cryoprobes that can be assembled in 5 minutes, with the entire procedure taking 15 minutes per pup. The subsequent heart collection and fixation takes 2 days to complete. Cryoinjury results in a myocardial scar, and the size of injury can be scaled by use of different cryoprobes (0.5 and 1.5 mm). Cryoinjury models are medically relevant to diseases in human infants with heart disease. In summary, the myocardial cryoinjury model in neonatal mice described here is a useful tool for cardiac translational and regeneration research.

Keywords

Cryoinjury; neonatal surgery; regeneration; heart disease

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Author contributions

BDP, BG, BH, and BK designed research. BDP, BG, and BH performed and analyzed experiments. BH and JP provided data for Fig. 4A,C, and Fig. 5. BDP, BG, and BK wrote and all authors edited the manuscript.

Competing financial interests

The authors declare that they have no competing financial interests.

INTRODUCTION

Adult mammalian hearts show little evidence for cellular regeneration, however research on growing mouse, rat, pig and human heart has revealed active mechanisms of cellular proliferation^{1–6}. Since the introduction of mouse models of cardiac injury^{7–11}, it has become possible to investigate to what degree growing mammals are capable of cardiac regeneration. Myocardial injury models in neonatal mice also provide a useful tool for research on the mechanisms of heart failure in human infants and could be helpful to identify and evaluate new therapies¹².

Development of the protocol

Research on the mechanisms of cardiac regeneration in mammals has greatly benefitted from the use of injury models for neonatal mouse hearts. However, different groups have seen apparently contradictory results despite using a similar method. For example, using a model of amputation of the neonatal mouse heart, some researchers have observed full regeneration⁸, others have reported some regeneration¹³ while Anderson *et al* report that no regeneration occurs¹⁴ after amputation of a large piece of myocardium. Similarly, using the model of ligation of the left anterior descending coronary artery (LAD) to create a myocardial infarction in neonatal mice, we and other researchers have found scarless repair below the ligation site^{9,10}, while others have found scar formation and aneurysms¹⁵. In adult mice, multiple reports have shown that cardiac cryoinjury induces scar formation^{11,16,17}, while one report indicated scarless repair¹⁸. We and others have shown that cryoinjury in neonatal mouse hearts resulted in scar formation^{11,19,20}, while another report indicated scarless repair²¹. Cardiac cryoinjury performed on adult MRL/MpJ^{+/+} (Jackson Labs) mice has been reported to heal within 60 days post injury with restored myocardial function and structure²². However, the technique was a trans-diaphragmatic right ventricular cryoinjury, in which the cardiac injury cannot be visually confirmed²². This report has been convincingly refuted by another study that shows that C57BL/6 and MRL mice both exhibit scar formation following cryoinjury that was induced through the open chest and visually confirmed¹⁷. Furthermore, myocardial cryoinjury in adult zebrafish resulted in collagen deposition, which was completely resorbed over the course of 60–90 days^{23–26}. The extent of collagen deposition was greater and the duration until complete resorption longer than after apical amputation in adult zebrafish²⁷, suggesting that the regenerative response may vary between different injury types. The extent to which neonatal mouse hearts can regenerate has been difficult to ascertain with the available methods and several recent publications^{28,29} suggest that the conflicting results are due to technical challenges associated with these two models.

We present a protocol for a different model for studying cardiac regeneration in neonatal mice, by inducing heart muscle cell death by cryoinjury. We recognize the technical complexity of performing cryoinjury procedure and how technical differences may lead to varying results. Therefore, we hope reproducibility will be improved by access to this detailed description of how to learn and reproducibly perform this method. To resolve and account for technical differences, we have summarized relevant cardiac cryoinjury papers in

mice (Table 1). Variables that may potentially determine the tissue response after cardiac cryoinjury are summarized (Table 2).

Applications of the cryoinjury protocol

The neonatal mouse cardiac cryoinjury model has multiple applications. First, the model enables comparative research with other neonatal mouse models⁷ and between species, with zebrafish²⁴, which do show myocardial regeneration after cryoinjury^{23,25,26}. The different mechanisms activated in zebrafish and mice may hold the key for understanding how the ability to regenerate myocardium is regulated. Second, it provides an animal model for testing interventions that stimulate mechanisms of cardiac protection and regeneration, meaning the cryoinjury model enables testing of molecular interventions to stimulate regeneration. Third, cardiac cryoinjury could be used to determine whether the specific mouse strain can influence cardiac regeneration. Fourth, the cryoinjury model decreases cardiomyocyte cell cycle activity and induces scar formation^{19,20}. Decreased cardiomyocyte cell cycling¹⁹ and increased scar formation are also present in patients with pediatric heart disease^{30–32}. Thus, the ability to model these myocardial changes should enable a new line of translational heart failure research toward pediatric-specific disease mechanisms and therapies¹².

Advantages and adaptations of this cryoinjury protocol

Myocardial injury in neonatal mice can also be induced with apical resection and ligation of the LAD^{8–10}, however cryoinjury differs in multiple aspects. Compared with the amputation model, cryoinjury does not involve cutting the myocardium. As a result, blood loss is negligible which increases survivability and the injury is more reproducible. In addition, this enables an adaptation of our original protocol¹⁹, which consists of scaling the injury size, without alteration of the principal outcome, *i.e.* scar formation. Scaling the size of the cryoinjury may be advantageous for experimentation in strains that differ in the size of pups.

The method of LAD ligation requires visualization of an extremely small vessel. This leads to some operators using microsurgical loupes or dissecting microscopes. Even with these magnifying tools, threading thin surgical suture material around the LAD and tying a secure knot is extremely challenging because of the small size of the neonatal heart. The cryoinjury model does not require identification and manipulation of the LAD, thereby reducing the technical challenge for researchers.

The cryoinjury model enables comparisons with the other neonatal mouse heart injury models that exhibit features of regeneration, *i.e.* amputation and LAD-ligation. Because the comparison between regenerating and non-regenerating neonatal models is direct it should be possible to relate the molecular differences to myocardial regeneration³³.

Although very useful for basic regeneration research, the amputation model is criticized as being non-physiologic due to there being no type of myocardial disease in human patients that involves a traumatic amputation of a piece of myocardium. Likewise, while ischemic injury is common in adults with coronary artery disease, in infants and children, it is restricted to rare disorders, including Anomalous Left Coronary Artery from the Pulmonary Artery (ALCAPA, a congenital malformation of the origin of the left coronary artery) and

Kawasaki disease, an acquired disease of the coronary artery. Although the precise mechanism of inducing tissue damage with cryoinjury does not exist in human heart disease, the induced cellular changes, such as myocardial cell death, inflammation, scar formation, and decrease in cycling, are clinically relevant. For example, scar formation^{30–32} and decreased cardiomyocyte cycling¹⁹ is reported in patients with heart disease such as Tetralogy of Fallot/Pulmonary Stenosis (ToF/PS).

Experimental design

Due to the multiple technical challenges associated with surgical manipulations of neonatal mouse hearts, we recommend using a structured and didactic approach to learn this surgery. The suggested sequence of steps and metric for evaluating progress have been provided (Table 3). In our lab, we have predominantly used the ICR strain for cryoinjury experiments due to the fact that the dams have better nursing behavior (leading to decreased cannibalism), pups are bigger in size (easier to perform surgery), and the litter size is consistently larger. These advantages may lead to better survival rates. We have also utilized this cryoinjury protocol in mice up to 7 days old (P7). While we believe that the surgery itself may be easier performed in slightly larger pups, the duration of hypothermia-anesthesia may have to be optimized. Due to the inherent variability between animals, we recommend an experimental design that allows for at least 6 mice per data point. A corresponding number of sham operated pups (chest opened and closed) should be included in every experiment. It is advisable to have a team of two experienced researchers working together during the steps of inducing cryoinjury. One researcher can perform the surgery while the other researcher can anesthetize the pups and revive them after surgery. This neonatal cryoinjury protocol can be learned by following a recommended sequence of steps during a dedicated period for training. Potential variables that may determine the tissue response after cardiac cryoinjury experiments is provided (Table 2). Researcher 1 developed and optimized this protocol. To validate the proposed didactic steps (Table 3) of this protocol, we assessed the learning curve of researchers 2 and 3 who had no prior experience with mouse surgery (Supplementary figure. 1, left and right panels).

Below we discuss parts of the protocol which have been explained in further detail so the individual steps can be adapted according to experimental conditions.

Assembly of cryoprobes

The size and type of metal used to fabricate the tip cryoprobe is an important parameter. Several companies manufacture cryoprobes fabricated from various metals and with continuous liquid nitrogen feeds. Alternatively, custom-made probes can be made using metal filaments that can be purchased from a local hardware store. Probes of varying diameters are easily fabricated by grinding stock filaments to the desired diameter. It is important to note that metals with low thermal conductivities will remain colder for a longer period of time, and are capable of generating a more severe injury. We used a Vanadium probe due to its low thermal conductivity ($\kappa = 30.7 \text{ Wm}^{-1}\text{K}^{-1}$) compared to aluminum ($237 \text{ Wm}^{-1}\text{K}^{-1}$) and copper ($\kappa = 401 \text{ Wm}^{-1}\text{K}^{-1}$) filaments.

Anesthesia by hypothermia, steps 5–6

We commonly use the thumb of a disposable nitrile glove as protective barrier to prevent direct contact between the pup skin and the ice-water slurry. P1 pups are remarkably resilient to hypothermia and can remain in a hypothermic state for at least 15 min before mortality rates begin to rise. Pups older than P4 typically experience much higher mortality rates. The key parameter to maximizing survivability is to minimize the duration of hypothermia. Once the pup is anesthetized, the surgeon should be able to perform the complete thoracotomy (including skin closure) in approximately 5–8 min. Complications commonly occur during exteriorizing the heart, application of the cryoprobe, and suturing the chest wall and skin. These complications prolong the duration of hypothermia and can lead to increased mortality.

Exteriorizing the heart, step 9

Exteriorizing the heart is a critical step that allows visualization of the left ventricular surface and reproducible application of the cryoprobe. Common complications associated with exteriorizing the heart are dissection of the wrong intercostal space, lung injury, and heart laceration. To effectively exteriorize the heart, one must first determine the optimal entry site. Typically, the ideal place to enter the thoracic cavity is between the 4th or 5th intercostal space, however care must be taken to avoid the lung. Using microdissection scissors make a series of small cuts until the thoracic cavity is exposed. Then using a pair of blunt tipped tweezers, open the cavity and expose the heart. Once the heart is exposed, the pericardial sac can be removed using a pair of blunt tipped tweezers. Alternatively, one can gently rub the surface of the heart with a sterile cotton swap to rupture the sac and free the heart. Once the pericardial sac is removed, the heart can be exteriorized by applying gentle pressure to the abdominal cavity.

Application and reproducibility of cryoinjury, step 10

Once the heart is exteriorized the left ventricular surface can be identified and the cryoprobe applied to its surface. Common complications associated with this step include an inability to identify the left ventricle, excessive application pressure of the cryoprobe, and adhesion of the heart muscle to the cryoprobe. The left ventricle can be easily identified, once the heart is exteriorized, by looking at the color of the ventricle. The right ventricle, which contains deoxyhemoglobin, will appear dark red, whereas the left ventricle, which contains oxyhemoglobin, will appear bright red.

MATERIALS

Reagents

- Mouse strain - ICR (Taconic)
! CAUTION All animal experiments must be performed in accordance with all relevant institutional and governmental ethics guidelines and regulations. This protocol was approved by the Institutional Animal Care and Use Committee of Boston Children's Hospital and University of Pittsburgh.
- Sodium heparin (5,000 USP units per mL)

- Sodium phosphate dibasic (Na_2HPO_4 , Sigma-Aldrich, cat. No. 255793)
- Sodium phosphate dibasic (NaH_2PO_4 , Sigma-Aldrich, cat. No. S3139)
- Formaldehyde (Sigma-Aldrich, cat. No. F8775)
- Liquid nitrogen
- **! CAUTION** Extremely cold. Do not touch. Wear appropriate personal protection equipment.
- Phosphate buffered saline (PBS, 1X)
- Cardioplegia solution: 2.5 M KCl (74.6 g +1,000 mL water)= 50× stock
PBS²⁻/50 mM KCl (4°C): 500 mL PBS²⁻ + 10 mL 2.5 M KCl. Pre-cool on ice or 4°C.
- Bupivacaine (0.25%)

Equipment

- Micro-dissecting spring scissors (angular, ROBOZ, cat. no. RS-5658)
- Micro-dissecting scissors (curved, ROBOZ, cat. no. RS-5913)
- Angled forceps (Fine Science Tools, cat. no. 11251-35).
- Micro-needle holder (ROBOZ, cat. no. RS 6437)
- Graefe forceps (straight, ROBOZ, cat. no. RS-5112)
- Dewar vessel for liquid nitrogen
- Halsey micro needle holder (Fine Science Tools, cat. no. FST-12500-12)
- Sterile gloves
- 8-0 Nylon sutures (Ethicon, cat. no. 1716G)
- Insulin syringe, 3/10cc (Becton Dickinson, cat. no. 309301)
- Dissecting microscope or surgical loops (10x, Design for Vision)
- Gaymar T pumps with heated water blanket, model TP-500
- Q-tips
- Polydrapes
- Glass Petri dish filled with ice as operating table
- Sterile gauze
- Web glue (Webster Veterinary, cat. no. 07-8566128)
- Styrofoam platform
- Surgical tape
- Heating lamp

- Bucket filled with ice-water bath
- Betadine swabs
- Bead sterilizer
- Light source
- Vanadium probe (made from screwdriver WIHA, Sp 353 SW 1.5 mm screwdriver)
- Aluminium probe (Hardware store, 1.0 mm filament ground down to 0.5 mm using a Dremmal tool equipped with a grinder bit)

Equipment Setup

Assembly of cryoprobes ● Timing 5 min—Assemble a disposable 5 mL pipet, 200 μ L pipette tip, 5 cm piece of Tygon tubing, and a vanadium or aluminum metal filament (Fig. 1A). Pass the metal filament through a 200 μ L pipette tip and fix it tightly to the tip of the 5 mL disposable pipet (Fig. 1B). Serially cut the pipette tip until a tight fit is achieved to minimize probe movement. Secure the metal filament to the disposable pipet by placing the 2" piece of Tygon tubing where the pipette tip intersects with the disposable pipet (Fig. 1C).

▲ **CRITICAL** The type of metal and diameter of the wire used to fabricate the cryoprobe is an important parameter that affects injury size.

▲ **CRITICAL** It is important that the metal filament be between the 200 μ L pipet and the 5 mL pipet so that it is held in place by a tight fit.

? TROUBLESHOOTING

Ordering of timed-pregnant dams ● Timing 6–14 days prior to surgery—Order E17 pregnant ICR dams and house them in individual cages prior to surgery. Ordering of time-pregnant dams provides the mothers with sufficient time to acclimate to their new environment and significantly reduces maternal cannibalism after birth. Neonatal regeneration is thought to be age-dependent and is highest within the first few days of life. It is important to closely monitor when the pups are born to ensure reproducibility of the injury model. Dams should be checked daily for births. Pups born after 5 pm are considered to be day of life 0 (P0) on the following day and subjected to cryoinjury on day of life 1 (P1).

PROCEDURE

Surgery ● Timing 15 min per mouse pup

- 1 ∟ Prepare Bupivacane working solution (0.1% in sterile saline).

! **CAUTION** Follow your institutional guidelines regarding administration of analgesics pre- and post-operatively. While it is known that neonatal mice lack centralized pain reflexes, your institution may require administration of analgesics.

- 2 ∟ Pre-cool the cryoprobe by immersing it in liquid nitrogen for at least 10 min prior to the first surgery.

▲ **CRITICAL STEP** Remove the probe only to induce injury. It is important to maintain adequate liquid nitrogen levels in the Dewar.

- 3 ∟ Remove half of the pups from the nursing mother and place them on a heated water blanket set to 37°C. Cover the pups with bedding from the mother's nest.

▲ **CRITICAL STEP** Removing all the pups from the mother increases her stress level and may lead to increased cannibalism. Leave half of the litter with the mother at all times.

- 4 ∟ If analgesics are required, administer now. Some institutions may require administration of 20 µL of 0.1% Bupivacaine (2 mg/kg body weight) sub-cutaneously to the thoracic region.

- 5 ∟ Place the neonatal pup into a protective sleeve and anesthetize by placing the pup into an ice/water bath for approximately 3–5 minutes.

▲ **CRITICAL STEP** The sleeve provides a physical barrier between the ice-water and the pup skin which is required to prevent frostbite.

▲ **CRITICAL STEP** Check the pup frequently while on ice until there is no paw reflex. Prolonged exposure to hypothermia can lead to increased mortality. Hypothermia-anesthesia is challenging in pups older than P4. It is important to note that the anesthesia has to remain consistent in depth and duration to prevent the pups from waking up during the course of surgery. The exact duration of hypothermia needed to induce anesthesia may vary between strains and hence the researchers should titrate the depth of anesthesia (Table 3). However, once optimal durations of hypothermia have been established, this duration should not be changed. ! **CAUTION** Hypothermia-anesthesia must be performed in accordance with all relevant institutional and governmental ethics guidelines and regulations. This protocol was approved by the Institutional Animal Care and Use Committee of Boston Children's Hospital and University of Pittsburgh.

? TROUBLESHOOTING

- 6 ∟ Remove the pup from the ice/water bath and dry using sterile gauze pad. Place the pup in the surgical area (Fig. 1D) in the supine position and tape the arms, legs, and tail to immobilize the pup.

▲ **CRITICAL STEP** To maintain a hypothermic state during surgery, place the pup on top of a cold surface such as an ice pack or a chilled Petri dish.

- 7 ∟ Sterilize the surgical area by gently wiping it with a Betadine swab (Fig. 2A)
- 8 ∟ Make a transverse skin incision across the chest using a pair of micro-scissors and separate the skin from the underlying muscle using blunt dissection (Fig. 2B–D).

▲ **CRITICAL STEP** Carefully dissect the skin from the underlying muscle using a blunt tipped tool such as a Halsey microneedle holder. Skin tears complicate wound closure and may increase maternal cannibalism.

▲ CRITICAL STEP Care should be taken to minimize the length of the incision site. Larger incisions greatly enhance the visibility of the rib cage, which will help with selecting the point of entry. However, larger incisions may require additional time to suture, lead to increased sternal scar formation that may complicate ultrasound sound-based functional studies, and may increase maternal cannibalism.

- 9 ∟Perform lateral thoracotomy (micro-dissecting spring scissors) by making a small incision at the 4th or 5th intercostal space (Fig. 2E, F). Separate the intercostal muscles using Graefe forceps (Fig. 2G) and carefully remove the pericardial sac. Exteriorize the heart by gently pressing on the abdomen (Fig. 2H).

▲ CRITICAL STEP Selecting the correct entry site is critical to the success of the procedure. Select the intercostal space that provides the best access to the heart while minimizing lung injury. Visualization of the entry site with surgical loops or a dissecting microscope may greatly enhance accuracy and reproducibility of the surgeon.

▲ CRITICAL STEP Removing the pericardial sac is critical for exteriorization, but care must be taken not to lacerate the heart.

? TROUBLESHOOTING

- 10 ∟Identify the left ventricle and carefully apply the precooled cryoprobe once to the left ventricular surface for 2 sec (Fig. 2I, J).

▲ CRITICAL STEP The metal type, application pressure and duration of exposure will determine the extent and reproducibility of injury (Table 3). Excessive pressure or prolonged exposure will increase pup mortality.

? TROUBLESHOOTING

- 11 ∟Use 8-0 nonabsorbable Prolene sutures (micro-dissecting spring scissors and angled forceps) to close the chest wall (Fig. 2K, L).

▲ CRITICAL STEP Care should be taken to avoid excessive tightening of the knot, which can break the ribs and increase pup mortality. Care should be taken not to nick the lungs with the suture needle. Lung injury becomes evident if pups are gasping after rewarming.

- 12 ∟To close the skin, webglue or 8-0 nonabsorbable Prolene sutures may be used (Fig. 2N). After closure, wash the surgical area with wet gauze (or Q-tips) to remove any residual blood (Fig. 2O).

▲ CRITICAL STEP When using acrylate-based webglue, it is important to release the tape restraining the hind paws before skin closure. Ensure that there is an overlap of skin to prevent wound reopening due to normal animal movement. Acrylate-based web glues may be associated with increased maternal cannibalism. If this occurs, close the skin with 8-0 nonabsorbable Prolene sutures, being careful to avoid excessive tension and unnecessary tearing which may also lead to increased maternal cannibalism.

- 13 ∟Rapidly warm the pup by placing it in your hands and warming it under a heat lamp for several minutes.

▲ **CRITICAL STEP** Exposure to excessive heat can increase neonatal mortality.

▲ **CRITICAL STEP** Sterilize the surgical instruments in dry bead sterilizer for the next surgery.

- 14 ∠Return the pup to the heating blanket with the other pups and cover with bedding from the mother's cage.

▲ **CRITICAL STEP** Use a cotton swab soaked with sterile saline to remove all traces of blood from the wound area immediately after surgery. Any remnants of blood around the injury may lead to increased maternal cannibalism.

- 15 ∠Repeat Steps 4–16 for all the pups on the heating blanket.
- 16 ∠Once all the pups have fully recovered from the surgery, swap them with the uninjured pups in the mothers cage.

▲ **CRITICAL STEP** Placing pups after surgery with a mother that is nursing pups without prior surgery will result in maternal cannibalism. The remaining healthy pups must be removed first before the injured pups can be returned to their mother.

- 17 ∠Repeat Steps 8–18 for the remaining pups in the litter. Once the pups are fully recovered from surgery, return them to their injured littermates in their mother's den. Leave pups with their mother until the chosen time point for heart collection and fixation, at which point proceed to the next step.

▲ **CRITICAL STEP** A typical litter of ICR mice yields 12–16 pups, which we break up into two groups with 6–8 pups each. Higher 24-hr survivability is achieved when >10 pups are placed with a single mother following surgery. Adjusting litter size is suggested.

▲ **CRITICAL STEP** Follow your institutional guidelines regarding the use and administration of analgesics following recovery from surgery.

Heart collection and fixation ● Timing 2d, Variable after injury

- 18 ∠Prepare a working solution of cardioplegia (PBS²⁻ with 50 mM KCl) in phosphate buffered saline and place on ice.
- 19 ∠For neonatal hearts (P1–P7) place 1 mL of 3.7% formaldehyde into a microcentrifuge tube. For older animals place 5 mL of 3.7% formaldehyde into a 15 mL conical tube.
- 20 ∠To perform the resection, gather dissection scope or surgical loops, Graefe forceps, one curved forceps, one curved micro-dissecting scissors, and a micro-dissecting spring scissors.
- 21 ∠Administer 50 µL of heparin (5000 USP units per mL) to the mouse *via* intraperitoneal injection 10 min prior to resecting the heart.
- 22 ∠Euthanize the mouse using a technique approved by your institution. Carbon dioxide should be avoided due to its negative effects on the heart.

▲ **CRITICAL STEP** Mouse pups (P0–P7) may be euthanized by decapitation, whereas mice older than P7 can be euthanized by isoflurane and cervical dislocation. It is the researcher's responsibility to follow institutional guidelines regarding accepted forms of euthanasia.

- 23 ∠Place the mouse in the supine position and tape the arms, legs, and tail to immobilize the mouse. Using the micro-dissection spring scissors, make a transverse incision across the entire abdomen and extend vertically until the diaphragm is exposed (Fig. 3A–D).
- 24 ∠Place the Graefe forceps in the non-dominant hand and lift the chest wall by the sternum to expose the diaphragm (Fig. 3E). Place curved forceps in the dominant hand and carefully dissect the diaphragm from the anterior chest wall to expose the thoracic cavity (Fig. 3F).
- 25 ∠Place the curved forceps in your non-dominant hand and position it under the base of the heart, pinch down on the main vessels, and carefully lift the heart (Fig. 3G). Using your dominant hand, place the curved micro-dissecting scissors under the tweezers and cut the vessels to free the heart (Fig. 3H). Place the heart in the ice-cold cardioplegia solution (Fig. 3I).

▲ **CRITICAL STEP** Make sure the heart is not adhered to the rib cage before removing it from the thoracic cavity. If adhesions are present, carefully dissect the heart free under a dissection microscope using fine tipped tweezers and microdissection scissors. When in doubt, leave adhesions attached to the heart. They can be resected after fixation.

- 26 ∠Place the curved forceps in your dominant hand and gently squeeze excess blood from the heart.
- 27 ∠Place the heart into the appropriate conical tube containing 3.7% formaldehyde, put it on a rocker and incubate for 2–3 hours or at 4°C overnight.

▲ **CRITICAL STEP** For pups P1–P3, incubate for 2–3 hours, for adult hearts incubate overnight at 4°C.

- 28 ∠Remove the formaldehyde from the tube containing the hearts by vacuum suction and replace with PBS. Place the tube on a rocker at room temperature for 5 minutes. Remove the PBS by vacuum suction. Perform this washing step two more times.
- 29 ∠The hearts are now ready to be either (1) dehydrated for paraffin embedding or (2) soaked in a 30% sucrose solution for OCT embedding according to standard procedures¹⁹.

● **Timing**—Equipment setup, assembly of cryoprobes, 5 min per probe; ordering of timed-pregnant dams, 6–7 days prior to surgery

Steps 1–17, surgery, 15 min per mouse pup

Steps 18–29, heart collection and fixation, 1–30 d after injury, takes 2 d

? TROUBLESHOOTING: See Table 4 for Troubleshooting advice.

ANTICIPATED RESULTS

We have previously published results obtained with the large (1.5 mm) cryoprobe (Fig. 4A, first and third panel)¹⁹, demonstrating that cryoinjury induces myocardial cell death in neonatal mice. Cryoinjury leads to the formation of a hematoma at the application site with both a 0.5 mm and 1.5 mm probe (Fig. 4A, middle and right panels). Cryoinjury with the 0.5 mm probe leads to a much smaller hematoma size (Fig. 4A, middle panel) while the 1.5 mm probe damages approximately 15–20% of the left ventricle (Fig. 4A, **right panel**)¹⁹.

Quantification of hematoma size after application of the 1.5 mm cryoprobe showed damage to $17.5 \pm 8.6\%$ ($n=5$, mean $\pm$ SD) of the surface of the heart¹⁹. We use whole organ staining with triphenyltetrazolium chloride (TTC) to demarcate the injury zone on the heart surface (Fig. 4B). Sections after TTC staining show that the degree of penetration of injury is higher with the large cryoprobe compared to the 0.5 mm probe (Fig. 4C **middle and right panels**). Using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL, Fig. 5) we estimated the myocardial volume damaged by the 1.5 mm probe to be approximately $0.58 \pm 0.02 \text{ mm}^3$ ($n=5$), which corresponds to approximately 18% of the myocardium¹⁹.

Echocardiography revealed that myocardial dysfunction was evident within 2 dpi (ejection fraction after 1.5 mm probe 55.1% ($n=13$) vs 84.4% after sham injury, $n=19$)¹⁹. At 30 dpi the ejection fraction was $45.7 \pm 3\%$ for cryoinjured animals (large probe) vs $57.9 \pm 3.5\%$ for shams ($n=13$, $P < 0.05$, ANOVA)¹⁹. Staining with acid fuchsin orange-G revealed fibrin deposition at 1 dpi and significant fibrosis at 7 dpi, which matured into transmural scar after the 1.5 mm probe (i.e. the entire myocardial wall thickness is composed of scar) at 30 dpi (approximately 3% of the total myocardium)^{19,20}. Cryoinjury with the 0.5 mm probe resulted in a non-transmural scar at 30 dpi (Suppl. Fig. S2), approximately 2% of the myocardium). Note transmural scars were present only after application of the 1.5 mm probe but not after the 0.5 mm probe (Fig. 6). We have previously published results obtained with the large (1.5 mm) cryoprobe (Fig. 6, lower right 2 panels for the 7dpi and 30 dpi time point)¹⁹.

Cardiomyocyte proliferation was previously reported to contribute to neonatal heart regeneration following apical resection and myocardial infarction^{8,9,34,35}. Staining with an anti-phospho-histone H3 antibody revealed that cryoinjury inhibited the endogenous proliferation of cardiomyocytes within the injury and border zones at 1, 4, and 7 dpi¹⁹, suggesting that neonatal mouse hearts do not regenerate after cryoinjury to the same degree as reported after amputation and LAD ligation injury. The decreased local cardiomyocyte cell cycle activity is consistent with the unchanged global cardiomyocyte cell cycle activity after cryoinjury reported by Lien and colleagues²⁰. In conclusion, this protocol describes a useful model for the cardiac translational and regeneration researchers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Main steps of construction of cryoprobes and experimental setup

(A–C) Materials and assembly of cryoprobes. Materials needed for construction of cryoprobes (A): 1. Disposable 5 mL pipet, 2. 200 µL pipette tip, 3. 5 cm piece of tygon tubing, and 4. Vanadium or aluminum metal filament. Metal filament passed through the 200 uL pipette tip and attached to the disposable 5 mL pipette (B). Tygon tubing is used to secure the metal filament to the 5 mL pipette (C). (D) Photograph of the surgical setup used during the procedure (represented by arrows): 1. light source, 2. surgical stand, 3. sterile polydrape placed over the surgical area, and 4. ice cooled surgical bed.

Figure 2. Detailed description of cardiac cryoinjury in a neonatal mouse

(A) Sterilization of the surgical site with Betadine swabs. (B) Transverse skin incision across the chest. (C) Separation of skin from underlying muscle using blunt dissection. (D–E) Lateral thoracotomy at the 4th intercostal space. (F–G) Separation of the intercostal muscles using blunt tipped forceps. (H) Exteriorization of the heart (inset illustrates the clear demarcation of right and left ventricles). (I) Application of the liquid nitrogen cooled cryoprobe to the surface of the left ventricle (inset, cryoprobe is placed on the left ventricular surface). (J) Visualization of the hematoma following cryoinjury. (K–M) The chest wall is closed with 8-0 nonabsorbable sutures. (N–O) The skin is closed with webglue. This protocol was approved by the Institutional Animal Care and Use Committee of Boston Children’s Hospital and University of Pittsburgh.

Figure 3. Resection of heart following cryoinjury at day of life 1 (P1)

(A) Schematic representation of the main cuts required to expose the heart. 1. Abdominal transverse incision, 2. left, and 3. right lateral incisions extending from abdominal cavity to thoracic cavity. (B) Pups are injected with heparin (50 μ L of heparin, 5000 USP units per mL) prior to euthanization to prevent clot formation in the heart. (C) Position of the first abdominal incision underneath the chest bone. (D–E) Position of second and third incisions (D), which extend from the abdomen to the diaphragm (E). (F) Removal of the diaphragm to expose the heart. (G) Clamping of the major vessels prior to excision. (H) Excision of the heart using tapered microscissors. (I) Resected heart in cardioplegia solution. This protocol was approved by the Institutional Animal Care and Use Committee of Boston Children's Hospital and University of Pittsburgh.

Figure 4. Cryoinjury in neonatal mice induces tissue damage

Mice underwent sham surgery or cryoinjury on day of life 1 (P1) with either a 0.5 mm or 1.5 mm probe and their hearts were resected the next day (P2). (A) Hematoma is seen at the site of injury, (B) Vital staining with 1% TTC (w/v; in phosphate buffer, pH 7.4) at 37°C for 20 min, and then fixed in 10% phosphate-buffered formaldehyde overnight shows injury zone indicated with white arrow in whole hearts. (C) Vital staining with TTC shows the degree of penetration of injury varies with probe size in sliced heart sections indicated by white dotted lines. Scale bars, 1 mm (A,B,C). Note the scalability of the injury size with different probes. Abbreviations: LV, Left ventricle, RV, Right ventricle, LA, Left atrium, RA, Right atrium. Figure 4A (first and third panel) was previously published¹⁹.

Figure 5. Cryoinjury in neonatal mice induces apoptosis in myocardial cells

Cryoinjury was performed on day of life (DOL) 1 with either a 0.5 mm or a 1.5 mm probe and hearts were resected DOL 2. The scalability of injury is shown with myocardial cell death as visualized by TUNEL staining (green, ApopTag Red in Situ apoptosis detection kit, EMD Millipore Corporation, CA) and DNA staining with Hoechst (blue). Scale bars, 1 mm.

Figure 6. Cryoinjury induces scar formation in neonatal mice

Mice underwent sham surgery or cryoinjury on day of life 1 with either a 0.5 mm or a 1.5 mm probe. Time-series of AFOG-stained sections shows fibrin deposition at 1 days post injury (dpi, orange staining, black arrows). Scar (blue) is formed within 7 dpi (black arrow heads) and still present 30 days later. Note transmural scars were present only after 1.5 mm cryoinjury (lower right panel) but not after the 0.5 mm probe. Scale bar 1 mm.

Photomicrographs of the lower right 2 panels (1.5 mm probe) have been published before¹⁹.

Table 1

Summary of reports utilizing myocardial cryoinjury in mice

Age	Strain, provider	Method of cryoinjury	Scar (Yes/No)	Reference
Neonatal	ICR, Jackson Labs	0.5 mm aluminium probe (1s) 1.5 mm Vanadium probe (1 s)	Yes (non-transmural scar) Yes (transmural)	Current manuscript ¹⁹ Polizzotti <i>et al, Sci. Trans. Med.</i> , 2015
	ICR/CD-1 strain, Charles River Laboratories	2 mm metal probe, mild injury (1 s) 2 mm metal probe, severe injury (5 s)	Yes (non-transmural scar) Yes (transmural scar)	²⁰ Darehzereshki <i>et al, Dev. Biol.</i> 2015
	Not provided	Brymill cryo-gun 3 mm probe (5 s)	No	²¹ Strungs <i>et al, Methods in Mol. Biol.</i> , 2013
	C57BL/6	1 mm copper probe (5–10 s)	Initially yes, then replacement over 94 days	¹¹ Jesty <i>et al, PNAS</i> , 2012
Adult	MRL	Trans-diaphragmatic (open abdomen, cryoinjury through diaphragm, 2 mm blunt probe, two times (10 s)	No	²² Leferovich <i>et al, PNAS</i> , 2001
	MRL and C57BL/6	Not trans-diaphragmatic (direct visualization of the heart), 3 mm aluminum probe (10 s)	Yes	¹⁷ Robey <i>et al, Cardiovas Pathol</i> , 2008
	C57BL/6	3 mm probe metal probe, 3 times (10 s)	No	¹⁸ Van Amerongen <i>et al, Am. J. Pathol</i> , 2007
	C57BL/6	1 mm copper probe (5–10 s)	Yes	¹¹ Jesty <i>et al, PNAS</i> , 2012
	C57BL/6	Brymill cryo-gun 2–3 mm cryoprobe (10 s)	Yes	¹⁶ Van den Bos <i>et al, Am. J. Physiol. Heart Circulation</i> , 2005

Table 2

Variables to consider for interpreting results of neonatal mouse cardiac cryoinjury experiments.

Variable	Example
Age	Day of Life 1 vs. day of life 7 vs. adult
Material of cryoprobe type	Vanadium vs. copper vs. aluminium
Size of injury	Size of cryoprobe, duration of probe application, number of times of application
Mouse strain	Under investigation

Table 3

Didactic steps for learning cardiac cryoinjury protocol

Suggested sequence of learning steps	Metric for evaluating progress
1. For researchers with no prior surgical experience, practice suturing on suture pads and/or dead adult animals prior to starting with neonates.	Smooth skin closure
2. Inducing anesthesia on P1 pups by hypothermia.	Paw pinch reflex, survival
3. Rewarming of pups.	Survival
4. Practice opening of chest, popping out the heart, and suture-close chest wall (sham surgery).	Visualization of heart, not breaking of ribs/nicking the lungs, complete chest wall closure
5. Close the skin with webglue.	Smooth skin closure
6. Removal of excessive blood to reduce maternal cannibalism. Return pups to nursing mother.	Survival for at least 24 hours
7. When proficient in steps 1–6, perform cryoinjury.	Survival, cardiac dysfunction, and scar
8. When proficient in steps 1–7, start with actual experiments.	Survival, cardiac dysfunction, and scar

Table 4

Troubleshooting Table

Step	Problem	Possible Reason(s)	Solution(s)
5–6	Mortality due to anaesthesia by hypothermia	Older pups (>P4) typically experience much higher mortality rates than P1 pups.	Minimize the duration of hypothermia; practice complex parts of protocol including exteriorizing the heart, application of the cryoprobe, and suturing the chest wall and skin; use younger mice
9	Lung injury and heart laceration	Dissection of the wrong intercostal space, ineffective exteriorization of the heart	Follow guidelines in Introductions and Figure 2 to ensure entry is at optimal site in the thoracic cavity between the 4th or 5th intercostal space; Ensure sufficient hypothermia conditions to reduce lung injury; Do not dissect the intercostal space aggressively to prevent heart lacerations
10	Myocardial tears	Cryoprobe sticks to the heart	Use vanadium probes instead of aluminium or copper; If probes stick to tissue quickly drip sterile saline over the probe tip to release it