Chapter 20

Cryoinjury Models of the Adult and Neonatal Mouse Heart for Studies of Scarring and Regeneration

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Abstract

A major limitation in studies of the injured heart is animal-to-animal variability in wound size resulting from commonly used techniques such as left anterior descending coronary artery ligation. This variability can make standard errors sufficiently large that mean separation between treatment and control groups can be difficult without replicating numbers (n) of animals in groups by excessive amounts. Here, we describe the materials and protocol necessary for delivering a standardized non-transmural cryoinjury to the left ventricle of an adult mouse heart that may in part obviate the issue of injury variance between animals. As reported previously, this cryoinjury model generates a necrotic wound to the ventricle of consistent size and shape that resolves into a scar of uniform size, shape, and organization. The cryo-model also provides an extended injury border zone that exhibits classic markers of remodeling found in surviving cardiac tissue at the edge of a myocardial infarction, including connexin43 (Cx43) lateralization. In a further extension of the method, we describe how we have adapted the model to deliver a cryoinjury to the apex of the heart of neonatal mice—a modification that may be useful for studies of myocardial regeneration in mammals.

Key words Cryoinjury, Mouse, Scarring, Regeneration, Myocardial infarction, Cardiac wound healing

1 Introduction

Animal models of cardiac injury facilitate the study of wound healing processes and the evaluation of therapies that may improve or hasten this response. One of the most widely used models of cardiac injury is the left anterior descending artery (LAD) ligation model [1–4]. LAD ligation mimics myocardial infarction (MI) seen in ischemic heart disease by artificially occluding a major coronary artery, causing ischemia in a portion of the myocardium and recapitulating aspects of the characteristic pathologic progression found subsequent to MI, beginning with necrosis, and ending with the formation of a mature scar.

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LAD ligation is a reliable and useful method of cardiac wound healing, but it has limitations [1]. First, there is consideration of the extent to which ligating a coronary artery in a healthy animal actually models ischemic heart disease in humans. Perhaps of greater importance is that coronary artery distribution and arterial supply are not consistent between individuals within the same species. Such normal anatomic variations can result in variability of the injury size observed within a group of animals. This variability becomes particularly important in studies of the wound healing response, where measurement of injury size is a key parameter for discerning differences between experimental groups. The efficiency of using LAD ligation is also limited by higher postoperative mortality, as compared to other myocardial injury models [5].

In earlier work, we reported that a peptide based on the carboxyl terminus of the gap junction (GJ) protein Cx43 (αCT1) inhibited remodeling of GJs in cultured cells [6], as well as beneficially affecting the progression of healing of skin wounds [7, 8]. In follow-up studies, we sought to determine whether αCT1 had similar effects on GJ remodeling and recovery from injury to the mouse heart [9]. In initial experiments, it was found that coronary arterial ligation was problematic in our hands owing to difficulties in achieving a repeatable injury to the left ventricle (LV). To circumvent this, a cryoinjury model that provided a wound on the LV of the mouse heart of uniform size and geometry was developed. The method was based on one described by Van den Bos and coworkers who used a liquid nitrogen-cooled cryoprobe [5]. We modified their protocol to include probe prechilling and a non-transmural injury of LV, as opposed to the more severe transmural injury generated by this group (Fig. 1). We did so as non-transmural injuries provide extended IBZs—a tissue of particular interest in our experiments because of its putative causal role in reentrant arrhythmia mechanisms.

In this chapter, we describe the materials and protocol that were used to deliver a standardized non-transmural cryoinjury to the LV of an adult mouse. In addition to providing an injury and extended IBZ of uniform size and shape, this approach has further advantages over LAD of being technically straightforward, providing high postoperative survival and generating healed scars of relatively consistent volume and organization. As we have also reported, mouse hearts receiving cryoinjury demonstrated loss of mechanical LV function as assessed by echocardiography, slowed

Fig. 1 (continued) scar immunolabeled for Mlc2a—normally expressed in the embryonic ventricle (*gray*). Dapi nuclear signal (*dark gray*). *Inset*. Cx43 immunolabeled sister section at IBZ. Note lateralized Cx43 in IBZ. *Inset*. *Dashed lines* represent border of injury/scar. Scale (**c**) = 500 μm, (**d**) = 25 μm, (**c**, **d**) inset = 10 μm

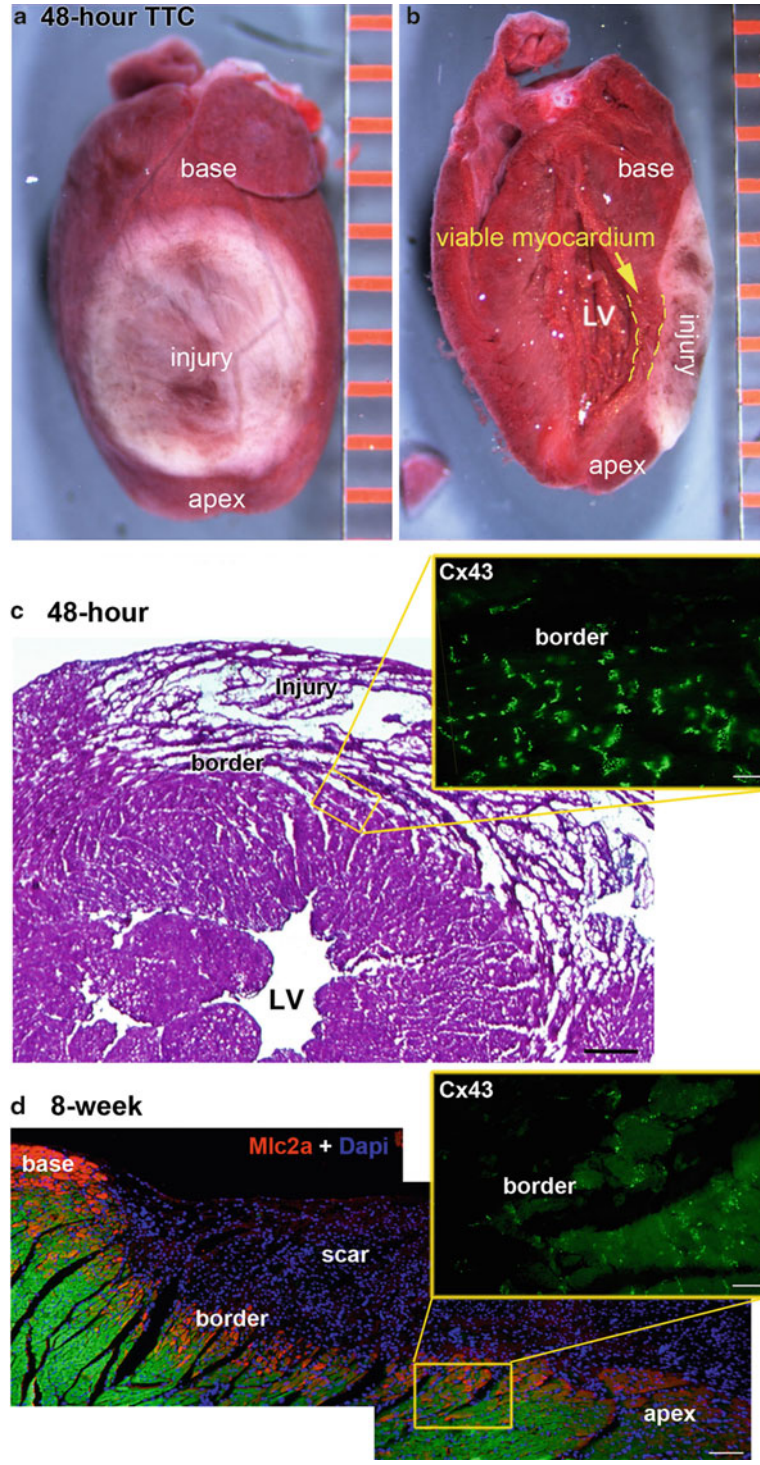

Fig. 1 Cryoinjury of the LV of an adult mouse heart. (**a**, **b**) Whole mount and cross section of a TTC-stained heart 48-h after 5-s exposure to cryoprobe. (**c**) Montage of H&E section through LV 48 h after standard 5-s exposure. *Inset*. Cx43 immunolabeled (*gray*) sister section at IBZ. (**d**) Montage of section from 8-week LV

action potential conduction velocity, and increased propensity to develop ventricular arrhythmias consistent with pathological changes seen following myocardial infarction [9, 10]. Moreover, the cryo-IBZ exhibits classic markers of remodeling seen in surviving myocardium at the edge of the MI, including Cx43 lateralization, interspersion of scar tissue with myocardial tissue, and reexpression of markers normally only found in the embryonic ventricle [9, 11]. In a further extension of the method, we describe how we have adapted cryoinjury to deliver an injury to the apex of the neonatal heart useful for studies of myocardial regeneration.

2 Materials

2.1 Cryoinjury of the Left Ventricle of an Adult Mouse Heart

Anesthesia

1. Anesthetic: Isoflurane, USP.
2. 2 % Lidocaine solution, USP.
3. Oxygen cylinder and regulator.
4. Compact anesthesia vaporizer system, Harvard Apparatus.
5. MiniVent Type 845 mouse ventilator, Harvard Apparatus.

Surgical Preparation and Procedure

1. Cork surgery board.
2. Umbilical tape.
3. Surgical stereomicroscope (Wild M3Z) and adjustable (snake) light source.
4. Sterile gauze, tape, and cotton-tipped applicators.
5. Nair® hair removal product.
6. Betadine® solution (10 % providone-iodine).
7. 70 % (v/v) EtOH in H_2O.
8. Surgical gloves.
9. Liquid nitrogen.
10. 16-G angiocatheter (needle bevel removed).
11. Brymill Cryo-Gun apparatus with 3 mm circular, flat copper cryoprobe.

Surgical Tools (Sterilized)

1. Small surgical scissors.
2. Small surgical retractor.
3. 2× small surgical forceps.
4. Needle holder.

Suture

1. 4–0 Silk suture.
2. Gluture® skin glue.

Postoperative Care

1. Heating pad.

2.2 Cryoinjury of the Ventricular Apex of a Neonatal Mouse Heart

Anesthesia

1. Ice water bath.
2. Latex barrier.

Surgical Preparation and Procedure

1. Surgical stereomicroscope (Wild M3Z) and adjustable (snake) light source.
2. Sterile gauze, tape, and cotton-tipped applicators.
3. Betadine® solution (10 % providone-iodine).
4. 70 % (v/v) EtOH in H_2O.
5. Surgical gloves.
6. Liquid nitrogen.
7. Brymill Cryo-Gun apparatus with 1 mm circular, flat copper cryoprobe.

Surgical Tools (Sterilized)

1. Small surgical scissors.
2. 2× Small surgical forceps.
3. Needle holder.

Suture

1. 6–0 Prolene suture.
2. Gluture® skin glue.

Postoperative Care

1. Heating pad.

3 Methods

3.1 Cryoinjury of the Left Ventricle of an Adult Mouse Heart

Intubation and Anesthesia

1. Begin airflow to the anesthesia induction chamber. Use a mixture of 100 % oxygen and 2 % isoflurane and a flow rate of 0.8–1.0 L/min.

2. Place animal in the induction chamber, and wait until all voluntary motion ceases, approximately 5 min.
3. Remove the mouse from the induction chamber and place in a supine position on the table with the nose of the mouse slightly overhanging the edge of the table. Run the umbilical tape behind the upper middle two incisors and secure the umbilical tape to the edge of the table using tape.
4. Adjust the arms of the adjustable "snake" light so they overlie the neck of the mouse. This will illuminate the upper airway and facilitate visualization of the subtle opening and closing of the vocal folds as the mouse breathes.
5. Use a cotton swab to gently brush the tongue out of the mouse's mouth. Using the opposite end of the same cotton swab, using gentle pressure, press the tongue against the floor of the mouth and hold the mouth open.
6. Place a small amount of 2 % lidocaine on another cotton swab, and apply to the vocal folds as a local anesthetic.
7. With the blunted catheter introducer still in place, guide the catheter past the vocal folds and the epiglottis, and into the trachea.
8. Remove the catheter introducer from the catheter and attach the catheter to the tubing running from the ventilator. Switch the valves of the ventilation system to redirect airflow from the induction chamber to the ventilator. Turn on the ventilator, using a stroke volume of 260 μL and a stroke rate of 350 breaths/min. If the intubation catheter has been properly placed in the trachea, the chest wall will rise and fall in synchrony with the ventilator; if the intubation catheter has been erroneously placed in the esophagus, there will be no rhythmic rising and falling of the chest wall.

Surgical Procedure

1. Position the mouse supine on the cork board on the operating table, taking care to not remove the intubation catheter. Tape the mouse's fore- and hindlimbs to the operating table to prevent any muscular movements during the course of the operation.
2. Apply a hair removal product, such as Nair®, on the left side of the anterior chest wall. Wipe away after about a minute to remove the hair from the surgical area.
3. Apply Betadine followed by 70 % ethanol to the now-hairless area of skin to sterilize the surgical area.
4. Make a transverse skin incision using scissors. Palpation of the point of maximum impact of the apex against the chest wall provides a useful landmark for the location of the skin incision.

5. Using a pair of small forceps, bluntly dissect between the layers of skeletal muscle overlying the chest wall. In most cases, the muscle layers can be divided and individually retracted without the need for transection.
6. Use blunt dissection to open the chest wall by transecting the intercostal muscles between the fourth and fifth ribs. Retract the ribs. Blunt dissection during this step will prevent transection of the internal mammary artery, which will run along the medial edge of the resulting thoracotomy. Following this step, the surgeon should be able to visualize the heart through the pericardial sac.
7. Gently open the pericardial sac using blunt dissection. Move the thymus off of the surface of the heart if it is overlying the area of the ventricle to be injured.
8. After the proposed injury site is totally visualized and free from overlying obstructions, prechill the cryoprobe for 10 s using the Brymill Cryo-Gun apparatus—prefilled with liquid nitrogen (**Note 1**).
9. Cease chilling the cryoprobe and apply the cryoprobe to the surface of the heart for 5 s (**Notes 2** and **3**).
10. Gently remove cryoprobe, making sure not to tug on the heart or disrupt the epicardium in the process. Excess moisture on the heart could cause the cryoprobe to stick to the surface, potentially creating a traumatic injury.
11. Apply gentle pressure to the chest wall to remove excess air in the chest cavity. Close the chest wall incision using 4–0 silk suture with two or three sutures tied in an interrupted fashion. Be sure to include the superior and inferior ribs within this closure, as the intercostal muscle and parietal pleura will tear under the force of the sutures. Close any transected skeletal muscle layers using 4–0 silk suture in a running continuous fashion. Close the skin incision using Gluture skin glue with standard techniques.

Postoperative Care

1. Remove tape restraints from the mouse's limbs and turn the isoflurane to 0 % on the gas mixer, leaving the oxygen flow and ventilator running.
2. Allow the mouse to pull itself off of the intubation tube as it ascends from anesthesia, rather than removing it immediately following incision closure.
3. Place the mouse on a heating pad until it regains sufficient movement to be placed into its cage.

3.2 Cryoinjury of the Ventricular Apex of a Neonatal Mouse Heart

Rather than forming scar tissue as observed in the adult mouse heart, the ventricular apex of newborn mouse hearts has been reported to possess a transient regenerative potential similar to that seen in newts

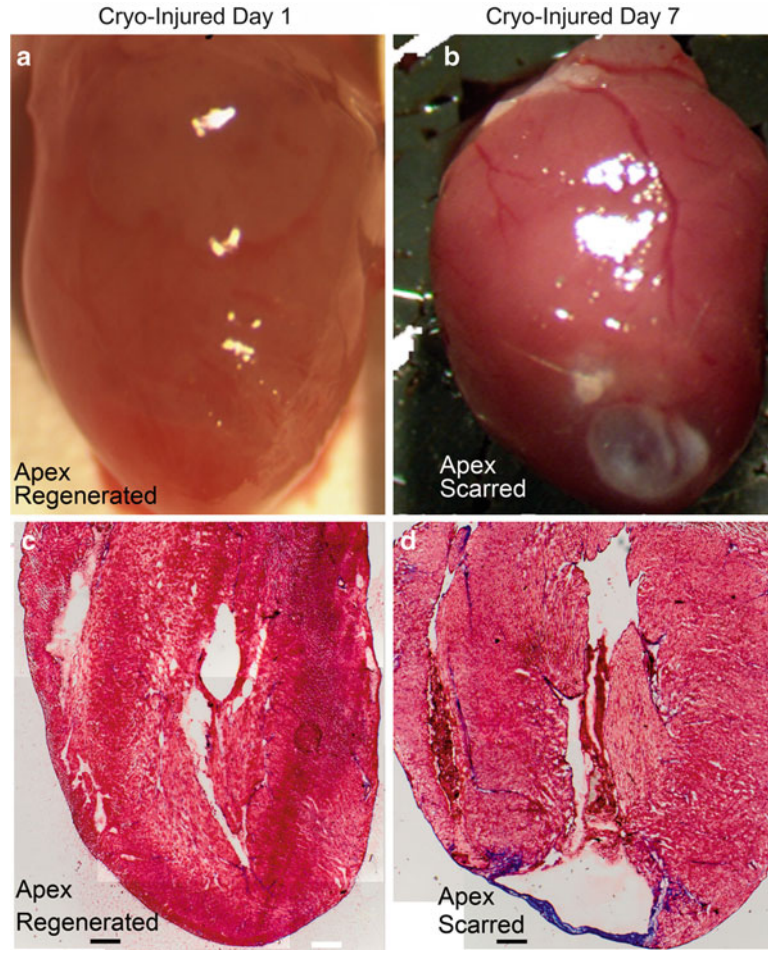

Fig. 2 Cryoinjury of the ventricular apex of neonatal mouse heart. (**a**, **b**) 21-day-old postnatal mouse hearts cryoinjured at either 1 (**a**) or 7 (**b**) postnatal days. Note only the 21-day heart injured 7 days after birth has a visible scar. (**c**, **d**) H&E sections through 21-day-old postnatal hearts cryoinjured at either 1 (**c**) or 7 (**d**) postnatal days. Note the heart cryoinjured at 1 day has no visible scar and fully regenerated myocardium at its ventricular apex, whereas the heart injured at 7 days displays transmural scar tissue (*black*). Scale 100 μm

and zebrafish [12]. The earlier study in neonatal mouse used partial surgical resection as an injury model. Below we describe an approach to reproducing a similar regenerative outcome by adapting the cryoinjury approach we describe for adult hearts for 1 day neonates (Fig. 2). Cryoinjury has the advantage of reducing perioperative mortality by preventing excess bleeding from surgical breach of the ventricular wall and leaving a scaffold of extracellular matrix in place for repair processes to proceed upon. This approach may also enable

improved resolution of steps in the wound healing process including inflammation, granulation tissue deposition and scar differentiation, remodeling, and resorption compared to what is observed following a mechanical injury of the ventricle.

Animal Preparation and Anesthesia

1. Remove litter of mice from mother's cage and place in a new, clean cage.
2. Induce anesthesia by inducing hypothermia. Using a latex barrier to protect the mouse's skin, submerge the mouse in an ice water bath until an anesthetic state is reached (**Note 4**). This may occur at any point between 4 and 20 min, so close observation is required.
3. Place the mouse in a supine position on the surgical table and clean the skin using Betadine solution followed by 70 % ethanol (**Note 5**).

Surgical Procedure

1. Make a horizontal incision in the dermis using a small scissors from the midline to the left axilla, about 1 cm in length.
2. Gently separate the ribs by inserting the closed forceps through the intercostal muscles and slowly opening the forceps such that the ribs separate.
3. Apply gentle pressure to the dorsum of the mouse, such that the heart extrudes through the opening in the rib cage with the apex leading.
4. Prechill the cryoprobe for 10 s and apply the probe to the surface of the heart's apex for 5 s (**Note 6**). Remove pressure from the dorsum of the mouse and allow the heart to reenter the chest cavity.
5. Close the chest cavity using one or two discontinuous sutures of 6–0 prolene. Close the skin incision using Gluture skin glue.

Postoperative Care

1. Clean any blood or topical antiseptic from the skin of the mouse.
2. Allow the mouse to recover from hypothermic anesthetic state by placing on a heating pad. After the mouse resumes voluntary movement, place the mouse into a new, clean cage until operations on the entire litter are complete.
3. Return the postoperative mice to the mother's cage as a complete group (**Note 7**).

4 Notes

1. Gentle rotation of the exposed heart such that the cryoprobe is in contact with the left ventricular surface of the heart may be required.
2. We used a 5-s exposure to the cryoprobe to generate a non-transmural injury in adult mice. We noted that the blanched frozen area of the heart took just over 19 s to recover a pink reperfused appearance if cryoinjury had been successful. Shorter or longer periods of recovery generally indicated an unsuccessful injury that was either too little or too extensively injured. Such hearts were discarded.
3. The size of the injury and the extent of its transmurality can also be controlled by adjusting the length of time the cryoprobe is in contact with the surface of the heart.
4. A latex surgical glove serves as an effective and convenient barrier between the mouse's skin and the ice water bath during hypothermia induction of neonates.
5. Time to anesthetic induction of neonates can vary widely from animal to animal. It is, therefore, extremely important to closely monitor each animal independently as overcooling can result in mortality. After induction of anesthesia, the surgeon oftentimes has less than 5 min before the mouse begins to awaken, so the procedure must be performed expediently.
6. It is important for the surface of the heart to be dry at the time of application of the cryoprobe. If the surgeon encounters the issue of the cryoprobe adhering to the surface of the heart during the wounding process, it is likely that there is excess moisture on the surface of the heart.
7. Postoperative maternal cannibalization is a major problem with neonates, but several measures can be taken to limit morbidity and mortality. Precondition to the scents acquired during the procedure by having the surgeon handle the mother daily prior to the birth of the pups and place a small gauze containing a few drops of Betadine solution in the cage. Remove and replace the entire litter before and after the operation, respectively. Careful cleaning of all traces of blood from the animals' skin can also reduce this form of mortality.

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