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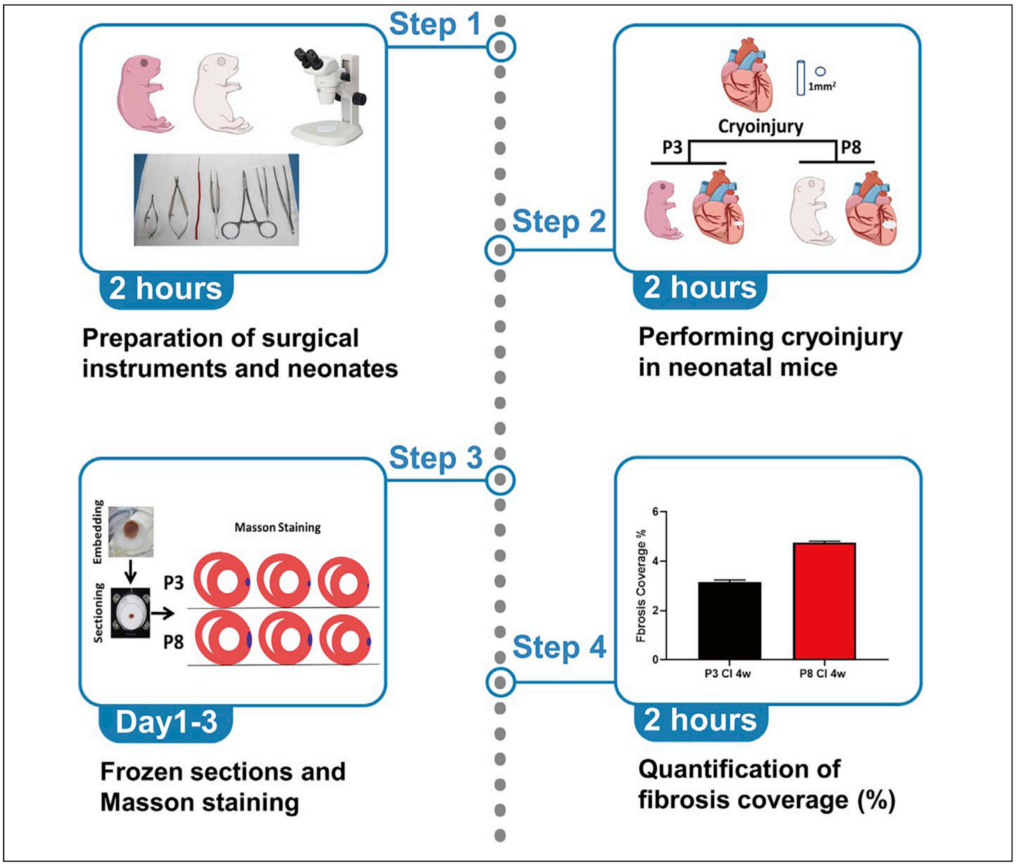
Protocol for cryoinjury model in neonatal mice for heart regeneration and repair research



The variability of animal experimental groups and high maternal cannibalization are two major limitations in cardiac injury models. A cryoinjury model could be an ideal model in heart regeneration and repair research as it can provide reproducible results and the injury size can be scaled. Here, we describe a simple and successful cryoinjury model (rate of mouse survival >90% and rate of maternal cannibalization <5%) for evaluating heart injury in regenerating and non-regenerating mice.

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Highlights

Low-maternal cannibalization and reproducible cryoinjury model in neonatal mice

Tissue processing and sectioning for Masson's trichrome staining

Apply the ratio of scar area and heart area in quantification of heart cryoinjury

Zhao et al., STAR Protocols 2, 100623
September 17, 2021
https://doi.org/10.1016/j.xpro.2021.100623

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Protocol for cryoinjury model in neonatal mice for heart regeneration and repair research

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https://doi.org/10.1016/j.xpro.2021.100623

SUMMARY

The variability of animal experimental groups and high maternal cannibalization are two major limitations in cardiac injury models. A cryoinjury model could be an ideal model in heart regeneration and repair research as it can provide reproducible results and the injury size can be scaled. Here, we describe a simple and successful cryoinjury model (rate of mouse survival >90% and rate of maternal cannibalization <5%) for evaluating heart injury in regenerating and non-regenerating mice.
For complete details on the use and execution of this protocol, please refer to Zhao et al. (2021).

BEFORE YOU BEGIN

Animal procedures

All animals were approved by the Ethics Review Committee of Guangdong Medical University in accordance with the principles of animal welfare from the Institutional Animal Care.

Preparation of surgical instruments, equipment, and reagents

Timing: 1 day

Prepare the sterile equipment and materials prior to performing this protocol.

CRITICAL: Aseptic processing of all surgical instruments and solutions

1. Prepare surgical instruments, equipment, and reagents

Instrument or equipment	Model	Quality
Corneal scissors A	11.5 cm	1
Corneal scissors B	8.5 cm	1
Toothed corneal forceps	10 cm	1
Forceps	12.5 cm/14 cm	2
Sutures	5-0/7-0/10-0	3
Zoom-stereo microscope	SZ650	1

(*Continued on next page*)

Continued		
Instrument or equipment	Model	Quality
Warming light	100 watt	1
Freezing microtome	LEICA, CM1950	1
REAGENT		
1% acetic acid solution	Sangon Biotech, China	1
30% sucrose	Sangon Biotech, China	1
1× PBS buffer solution (pH 7.4)	Sangon Biotech, China	1

Note: To prepare 1% acetic acid working solution, 100% acetic acid solution is diluted in 1× Phosphate Buffered Saline (PBS) buffer solution.

Note: To prepare 30% sucrose solution, 30 g solid sucrose is dissolved in 100 mL 1× PBS buffer solution.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
Masson's Trichrome Stain Kit	Meilun Biotech, China	MAO123-1
Chemicals, peptides, and recombinant proteins		
75% Medical alcohol	Guilin Lifeng Medical Supplies Co., Ltd., China	N/A
Iodine tincture	Guilin Lifeng Medical Supplies Co., Ltd., China	N/A
4% PFA	Solarbio Biotech, China	P1110
30% Sucrose	Sangon Biotech, China	A100335
20× PBS solution (pH 7.4)	Sangon Biotech, China	B548117
OCT	SAKURA, Japan	4583
Bouin's solution	Meilun Biotech, China	MB9889
Acetic acid solution	Sangon Biotech, China	A501931
Experimental models: Organisms/strains		
Mouse: ICR-CD-1(6- to 8-week-old males and females)	Guangdong Medical Laboratory Animal Center	N/A
Software and algorithms		
ImageJ	NIH	N/A
Other		
Freezing microtome	Leica	CM1950
Zoom stereo microscope	Chongqing Optec Instrument Co., Ltd	SZ650
Corneal scissors A(11.5 cm)/B(8.5 cm)	Shanghai Medical Instruments Co., Ltd. China	N/A
Toothed micro-forceps	Shanghai Medical Instruments Co., Ltd. China	N/A
Sutures (7-0/10-0)	Ethicon	W8702/W1770
BD insulin syringe	BD	328421
12-Well plate	Corning	353043
MX35Ultra	Thermo	3053835
Adhesion microscope slides	Citotest, China	188105
Microscope cover glass	Citotest, China	10212450C
Neutral balsam	Shanghai Yiyang, China	N/A
Warming light	100watt	N/A

Note: The working solution of reagents is diluted in 1× PBS buffer solution.

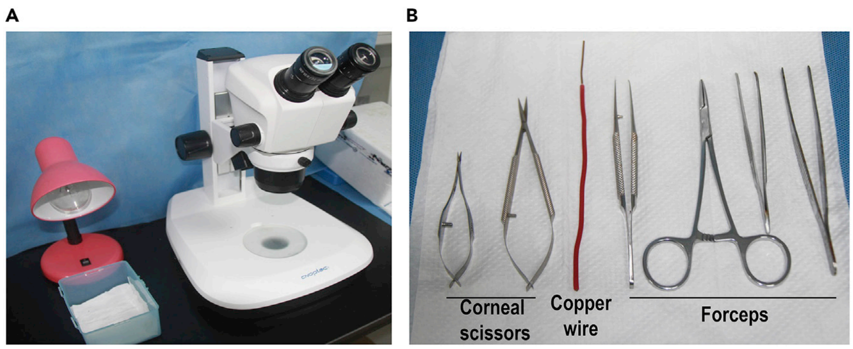

Figure 1. Preparation of surgical instruments and equipment
(A) Zoom stereo microscope and warming light.
(B) Corneal scissors, copper wire, and forceps.

STEP-BY-STEP METHOD DETAILS

Establishment of a cryoinjury model in neonatal mice

⏲ Timing: 2 h (within 10–15 min per neonatal mouse)

1. Surgical setup: make sure that the surgical instruments are autoclaved before surgery, re-used surgical instruments and equipment are disinfected with 75% medical ethanol (Figures 1A and 1B)
2. Hypothermic anesthesia for neonatal mice: take P3 neonatal mice (postnatal day 3) from the mouse cage and place them in crushed ice for 2–3 min (Figure 2A).

⚠ CRITICAL: Do not perform hypothermic anesthesia for more than 5 min.

3. Prepare for cryoinjury: disinfect neonatal mice with 75% medical alcohol and iodine tincture for two times and place them on a pre-cooled table; meanwhile, focus on the chest of neonatal mice by using a Zoom stereo microscope (Figure 2B).
4. Fix mouse limbs with forceps and disinfect the surgical area of neonatal mouse with iodine tincture for two times (Figure 2C).
5. Make an incision (<1 cm) on the skin to expose the musculature underneath and then cut the chest muscles (incision <0.5 cm) once to expose the sternum under a Zoom stereo microscope (Figures 2D and 2E).

Note: Do not cut the blood vessel of muscles during surgery.

6. Make an intercostal incision (<0.5 cm) between the 3rd and 4th intercostal spaces with corneal scissors and expose the left ventricle (LV) of the heart (Figures 2F and 2G).

Note: The intercostal incision should be less than 0.5 cm.

7. Gently place a pre-cooled 1 mm^2 blunt copper wire on the LV for 3–5 s and the area of white frostbite appears in the coronary vasculature of the LV (Figures 2H and 2I).

Note: The 1 mm^2 blunt copper wire should be placed in liquid nitrogen for at least 5 min.

Note: The pre-cooled copper wire should be gently placed on the coronary vasculature of the LV.

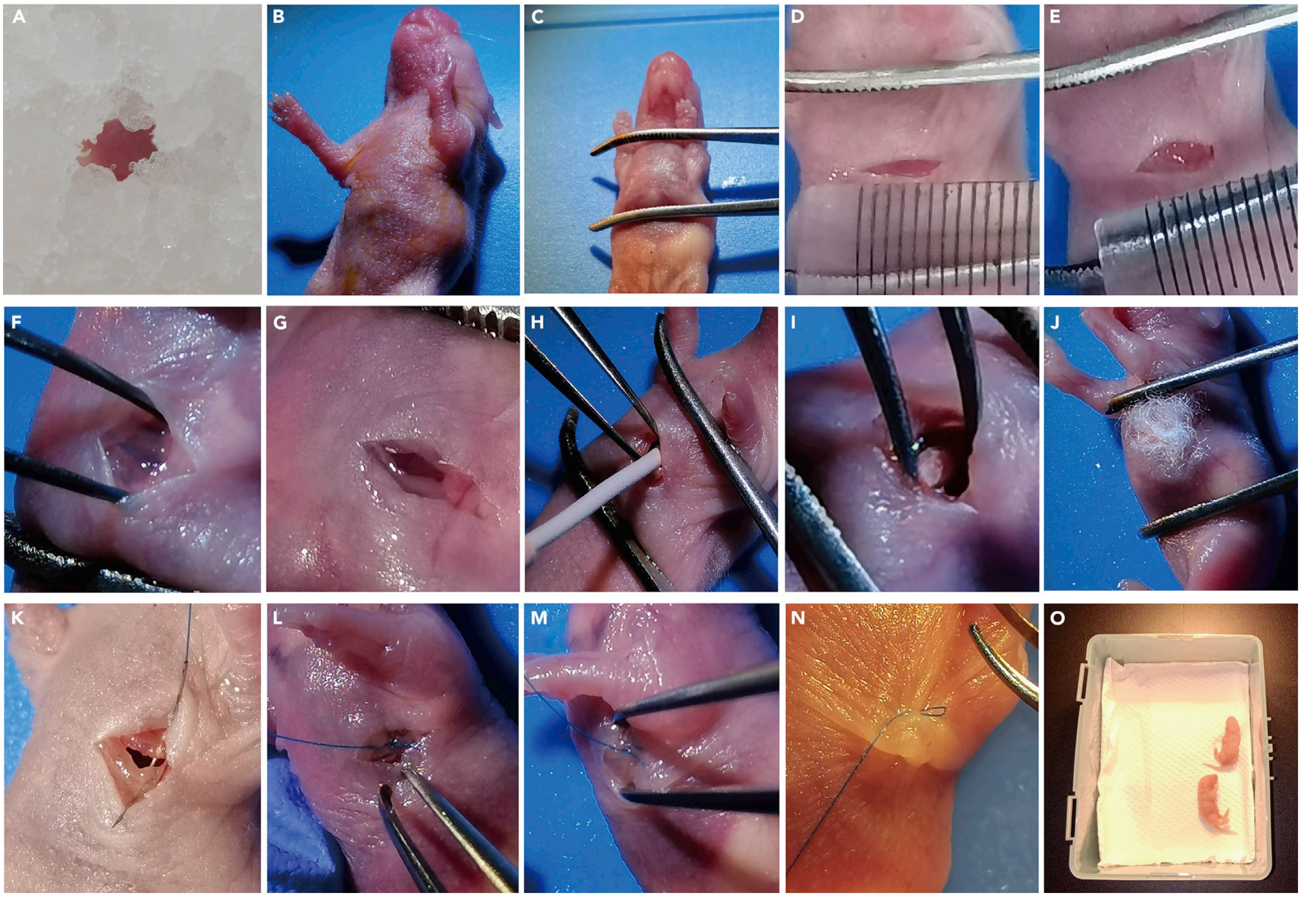

Figure 2. Establishment of cryoinjury in P3 neonatal mice
(A) Hypothermic anesthesia.
(B and C) Disinfection and fixation of neonatal mouse.
(D–F) Make an incision on the skin and chest muscles (The intervals between lines: 0.08 cm).
(G) Exposing the LV of the heart.
(H) Placement of a frozen copper wire on the coronary vasculature of the LV.
(I) White area of the injured heart.
(J) Removal of excess blood and bubbles.
(K and L) Suture the 3rd and 4th ribs together.
(M and N) Close the pectoral muscle groups and skin with sutures.
(O) Keep all the pups warm after surgery under a warming light.

⚠ CRITICAL: Make sure that the pre-cooled copper wire is perpendicularly attached to the surface of the neonatal heart.

8. After cryoinjury, remove excess blood and bubbles in the chest with a medical cotton swab (Figure 2J).
9. Suture the 3rd and 4th ribs together and the pectoral muscle with 7-0 sutures (9.3 mm, 3/8c taper ends) and close the skin with 10-0 sutures (6.5 mm, 3/8c taper ends) (Figures 2K–2N).

Note: Suture the chest and muscle with 7-0 sutures in P3 and P8 neonatal mice and close the skin with 10-0 (7-0) sutures in P3 neonatal mice (5-0 sutures [13 mm, 3/8c, cutting needles] in P8 neonatal mice).

Note: Excessive blood loss should be avoided during surgery.

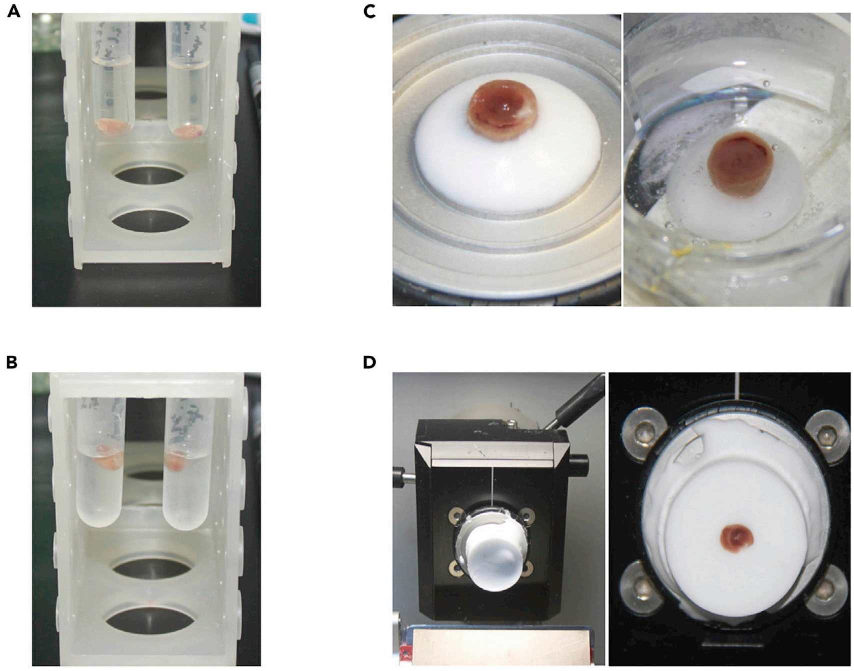

Figure 3. Tissue processing and sectioning for Masson's trichrome staining
(A) Fixation with 4% PFA.
(B) Dehydration of tissue with 30% sucrose.
(C) Embedding tissue with OCT.
(D) Serial frozen sectioning.

⚠ CRITICAL: The optimal time for the entire surgical procedure is about 10–15 min.

10. After surgery, place the injured neonatal mice on warming light for 10–20 min and then transfer them to the mouse cage containing feeding mice after finishing all the pups' surgery. (Figure 2O)

Note: Keep the mouse cage clean and do not change the mouse cage before or after operation.

Note: Take all the pups (the total amount of neonates is less than 10) from feeding mice and then transfer them to another mouse cage without feeding mice before surgery.

Pause point: A pause can be taken for 5 min before performing the surgery of another neonatal mouse

Tissue processing and sectioning for Masson's trichrome staining

Timing: 2–3 days

Heart tissue is complicated and needs to be embedded in a correct section orientation. This step describes the processing and serial sectioning of heart tissue for Masson's trichrome staining.

⚠ CRITICAL: Make sure that the surface of the sectioned tissue is in cross-section orientation.

11. Take heart tissue at 4 weeks after injury and place it into 4% PFA solution for 24 h at 4°C (Figure 3A).

Note: Inject pre-cooled 1× PBS into the right ventricle of the heart (next to the right ventricular apex) to wash the heart before taking heart tissue.

12. After fixing the heart tissue, wash the tissue with pre-cooled 30% sucrose once and place it into pre-cooled 30% sucrose solution for 24–48 h at 4°C (Figure 3B).

Note: The heart tissue should be located at the bottom of the tube, if the dehydration is sufficient.

13. Perfuse heart tissue with OCT compound and then fix the heart tissue with OCT on a freezing microtome (Figure 3C)
14. After finishing step 13, place the fixed heart tissue into pre-cooled 12-well plate containing 4 mL OCT. Embed the tissue with OCT at −80°C until adequate fixation (Figure 3C).

Note: The 12-well plate containing OCT should be pre-cooled at −20°C.

15. Fix the perfused heart tissue with OCT on the sample plate (Figure 3D).

Note: Sample component temperature: −20°C, working platform: −15°C

16. Perform serial sectioning to perfused heart tissue with OCT (Figure 3D).

Note: Make 6 or 10 μm frozen sections prior to staining.

Pause point: The slides of frozen sections can be stored at −20°C for 1–3 months

Masson's trichrome staining for injured heart

Timing: 1 day

CRITICAL: Make sure that all solutions are freshly prepared.

17. Perform Masson's trichrome staining to P3 and P8 heart tissue (Table 1; Figure 4A).

Note: All reagents are obtained from Masson's trichrome staining kit unless otherwise noted.

Note: Weigert's Iron Hematoxylin working solution is mixed Weigert's Iron Hematoxylin A and Weigert's Iron Hematoxylin B at a 1:1 ratio before performing Masson's trichrome staining.

18. Mount with neutral resin and glass coverslips.
19. Calculate the fibrosis coverage (%) of the injured heart (Figures 4A and 4B).

Note: Quantification of fibrosis coverage (%):100 × scar area/total area

EXPECTED OUTCOMES

Using this cryoinjury model protocol, we can successfully establish cryoinjury models (mouse survival rate of >90%) in regenerating and non-regenerating mice. In addition, the rate of maternal cannibalization is less than 5% after surgery because of optimized suture techniques and additional treatment. The cryoinjury model can be an ideal injury model to evaluate heart regeneration and repair in neonatal mice because the injury size can be scaled and the results are reproducible (Gonzalez-Rosa and Mercader, 2012; Polizzotti et al., 2016; van den Bos et al., 2005). In addition, 1 mm^2 blunt copper wire has been well documented in heart regeneration and repair research (Strungs et al.,

Table 1. The procedures of performing Masson's trichrome staining

Step	Reagents	Temperature	Time
Dying and washing slides	Tap water	Room temperature (RT; 22°C–25°C)	20 min
Bouin's solution staining	Bouin's solution	RT	24 h
	Tap water		1 min
The nuclear staining	Weigert's Iron Hematoxylin Working solution	RT	10 min
	Tap water		1 min
	Masson blue solution		3–5 min
	Tap water		1 min
Muscle fiber staining	Biebrich scarlet-acid fuchsin solution	RT	30 min
	1% Acetic acid solution washing		1 min
	Phosphotungstic/Phosphomolybdic acid solution		2 min
	1% Acetic acid solution washing		1 min
Collagen staining	Aniline blue solution	RT	2 min
	1% Acetic acid solution washing		1 min
Dehydration and clearing	95% Ethanol	RT	1 min
	100% Ethanol		1 min
	100% Xylene		1 min
	100% Xylene		1 min

2013; Yu et al., 2016). We used 1 mm^2 blunt copper wire to perform cryoinjury as the model of copper wire can be useful to evaluate the potential role of celecoxib treatment in cardiac repair (Zhao et al., 2021). Other types of copper wires may produce small or severe scar formation, resulting in different results in our protocol.

During tissue processing, we used frozen method to embed heart tissue as the frozen section is more convenient for embedding heart tissue in cross-section orientation. Moreover, the frozen section may be more sensitive to antigen-antibody reaction because of complicated tissue processing in paraffin sections, which would be beneficial for saving samples and materials for future studies (Krenacs et al., 2010). To calculate the injury degree of cryoinjury models, serial histological sectioning can be applied for Masson's trichrome staining, which can be used to accurately evaluate the injured degree of heart in different cross-section (Aurora et al., 2014).

LIMITATIONS

Using this protocol, the cryoinjury model should be practiced before evaluating the regenerative capacity in neonatal mice. We failed to establish the injured model at first because of blood loss and long operation time, resulting in the low survival rate of neonatal mice after injury. In addition, Masson's trichrome staining was the only method used to evaluate fibrosis coverage in our protocol. Other methods should be considered.

TROUBLESHOOTING

Problem 1

Different injury areas in different heart zones (step 7).

Potential solution

The main reason of different injury areas is that hypothermic anesthesia of neonatal mouse could be insufficient. Hypothermic anesthesia of neonatal mice should be checked by using with animal's pain stimulus (Zhao et al., 2019).

Problem 2

Keeping identical injury size with 1 mm^2 blunt copper wire in cryoinjury processing (step 7).

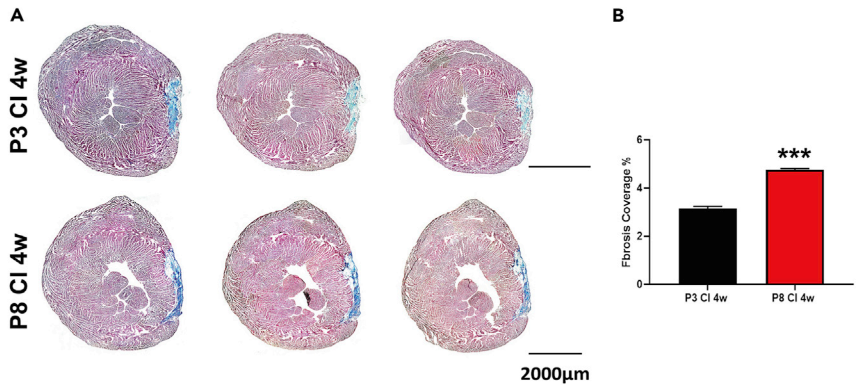

Figure 4. Masson's trichrome staining for injured heart

(A) Masson's trichrome staining for P3 and P8 injured heart tissue (Scale bar: 2000 μm).

(B) Quantification of fibrosis coverage (%) of P3 and P8 injured heart. Experimental data are presented as the mean ± SEM of biological replicates (n = 3 per group) and independent experiments (n = 2). The statistical significance between groups was calculated using a two-tailed Student's *t*-test. The statistical significance was considered at $p < 0.05$. ***$p < 0.001$.

Potential solution

Make sure that the attached surface is perpendicular when the copper wire is gently attached to the neonatal heart (Figure 5).

Problem 3

The position of injecting cooled 1 x PBS in the right ventricle (step 11) (Figure 6).

Potential solution

We used BD insulin syringe (model: 0.33 mm × 12.7 mm) to avoid changing heart histology as much as possible. Moreover, we controlled the injecting position (next to the right ventricular apex) and degree to prevent changes of the infarct/border zone of the heart.

Problem 4

High cannibalization rates of neonatal cryoinjury model (steps 5, 6, 8, 9, and 10).

Potential solution

Four methods can be used to prevent maternal cannibalization in our protocol.

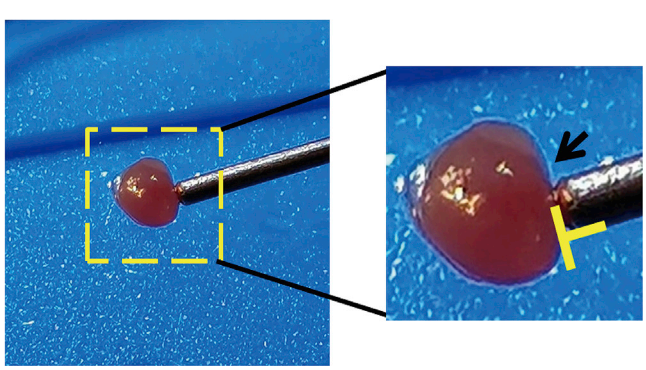

Figure 5. Cryoinjury processing of neonatal heart

Arrow indicates the position of frozen copper wire attached to neonatal heart

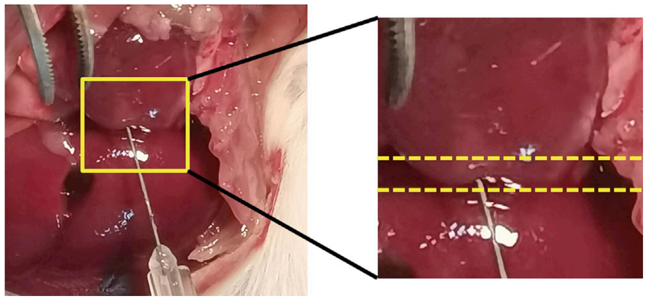

Figure 6. Washing the heart before taking from mice
The location between the two yellow dotted lines is the position of the BD insulin syringe needle.

Avoid cutting the blood vessel around muscle groups. Close the 3rd and 4th ribs together by using 7-0 surgical sutures and then suture all muscle groups in different positions after removing excess blood. Apply mixed solution containing mother's feces and water around the skin wound after disinfecting with 75% medical ethanol (the rate of maternal cannibalization is about zero). Apply Ma-ying-long Musk hemorrhoids ointment around the skin wound after disinfecting with 75% medical ethanol (the rate of maternal cannibalization is about zero).

Problem 5

Bubbles and error cross-section orientation during embedding and sectioning process (steps 13 and 14).

Potential solution

Make sure that the perfused heart tissue is adequately. During the sectioning step, section slides should be observed under a light microscope.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, [Yanli, Zhao., Assistant Research Fellow] (Email: yanlizhao2015@126.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze [datasets/code].

ACKNOWLEDGMENTS

We thank Mr. Dexin Yang for helping to take photos during the process of surgery. This work was supported by Shenzhen Longhua District key laboratory of infection and immunity project (China).

AUTHOR CONTRIBUTIONS

Y.Z. performed experiments; Y.Z. conceptualized the study and analyzed the data; Y.Z. and C.Z. wrote the paper; R.C. and C.Z. supervised the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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