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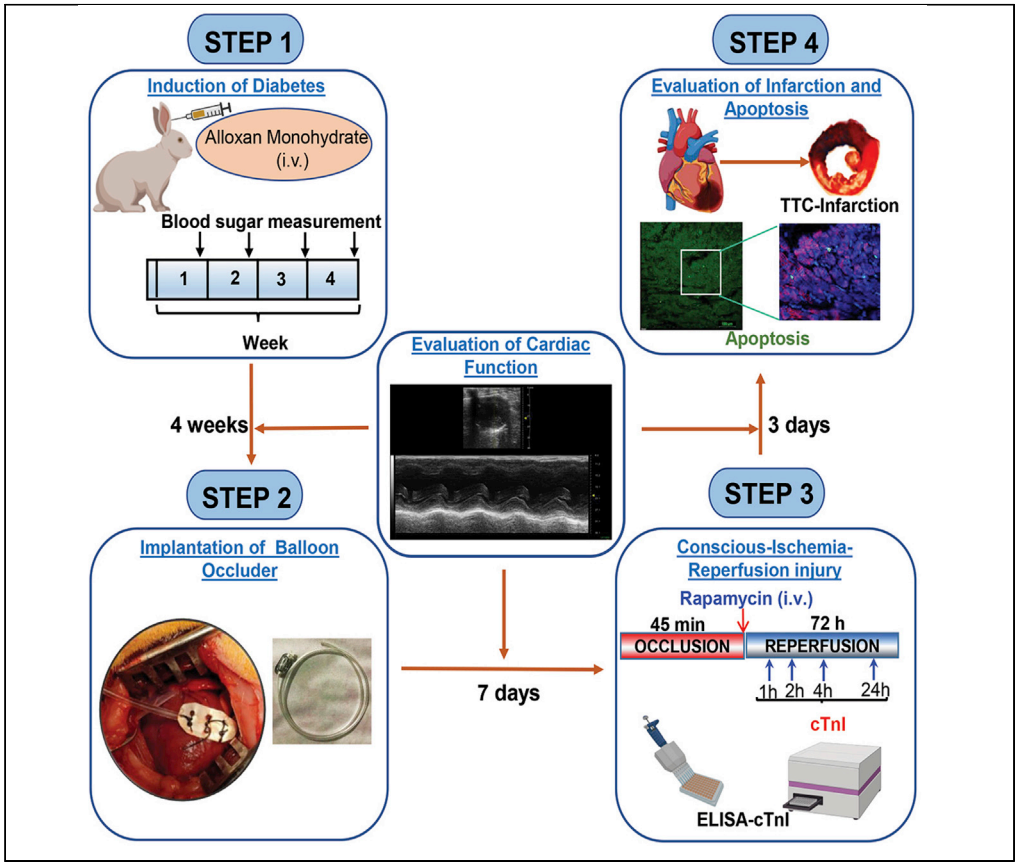
Preclinical model of type 1 diabetes and myocardial ischemia/reperfusion injury in conscious rabbits—demonstration of cardioprotection with rapamycin



We developed a preclinical model of myocardial ischemia/reperfusion (I/R) injury in conscious diabetic rabbits to identify an early pharmacological intervention for patients with diabetes and acute myocardial infarction (AMI). Here, we describe a reproducible protocol for induction of diabetes with subsequent manifestation of myocardial I/R injury in conscious rabbits to mimic the real-life scenario observed in clinical settings. Further, we demonstrate the efficacy of rapamycin at the onset of reperfusion to limit the adverse effect of AMI.

Arun Samidurai, Ramzi Ockaili, Chad Cain, ..., Donatas Kraskauskas, Rakesh C. Kukreja, Anindita Das

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Highlights

Detailed protocol for the generation of type 1 diabetic rabbit model

Method for ischemia/reperfusion injury in conscious diabetic rabbit

Measurement of cardiac function, myocardial infarction, cardiac troponin, and apoptosis

Reperfusion therapy with rapamycin

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Preclinical model of type 1 diabetes and myocardial ischemia/reperfusion injury in conscious rabbits—demonstration of cardioprotection with rapamycin

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SUMMARY

We developed a preclinical model of myocardial ischemia/reperfusion (I/R) injury in conscious diabetic rabbits to identify an early pharmacological intervention for patients with diabetes and acute myocardial infarction (AMI). Here, we describe a reproducible protocol for induction of diabetes with subsequent manifestation of myocardial I/R injury in conscious rabbits to mimic the real-life scenario observed in clinical settings. Further, we demonstrate the efficacy of rapamycin at the onset of reperfusion to limit the adverse effect of AMI.

For complete details on the use and execution of this protocol, please refer to Samidurai et al. (2020).

BEFORE YOU BEGIN

Acute myocardial infarction (AMI) is the result of occlusion of coronary artery, which obstructs the blood flow to the myocardium, a clinical scenario widely known as ischemia. Although, timely and adequately re-establishing blood supply after myocardial ischemia, known as reperfusion of the coronary artery, is mandatory to salvage or prevent the demise of ischemic myocardium, reperfusion itself causes additional ischemic damage by excessive formation of reactive oxygen species (ROS) as a result of mitochondrial dysfunction, intracellular calcium overload and proteolysis as well as other metabolic changes (Heusch, 2013; Yellon and Hausenloy, 2007). Diabetes mellitus (DM) is the major risk factor for developing cardiovascular diseases, including AMI, which is associated with poor prognosis and eventually higher mortality (Grundy et al., 1999; Hinkel et al., 2017).

A chronic activation of mammalian target of rapamycin (mTOR) signaling contributes to the pathogenesis of diabetes and leads to worsening diabetic-associated cardiac complications, including the myocardial ischemia/reperfusion (I/R) injury (Das et al., 2015; Stamateris et al., 2016). We previously demonstrated that mTOR is persistently activated in the hearts of diabetic mice (Das et al., 2014) and chronic pre-treatment with the mTOR inhibitor, Rapamycin (RAPA), improved cardiac function and reduced myocardial infarct size following I/R injury (Das et al., 2014; Samidurai et al., 2019; Samidurai et al., 2017). We also showed that treatment with Rapamycin at the onset of reperfusion reduced myocardial infarct size following ischemia in diabetic mice (Das et al., 2015) and diabetic rabbits (Samidurai et al., 2020).

Here, we describe extensive protocols to provide stepwise procedure for the induction of type 1 diabetes (T1D) in rabbit and method for induction acute myocardial I/R injury in the conscious

diabetic rabbit. To mimic the clinical scenario of myocardial I/R injury, the conscious rabbit myocardial I/R model was used to avoid the confounding effect of anesthesia during myocardial ischemia. The implantation of balloon to conduct I/R injury under sedation could also minimize the postsurgical complications of acute opening and closing of the chest for performing coronary artery occlusions. We also provide technical details for assessment of myocardial infarct size, apoptosis and cardiac function. In addition, we provide an outline for the administration of Rapamycin at the onset of reperfusion and demonstrate its effect in attenuating I/R injury. The conscious diabetic rabbit myocardial I/R protocol can serve as a powerful preclinical platform for identification and testing the efficacy of novel infarct-sparing therapies.

Acclimation of rabbit

Timing: 1 week

All experimental protocols were conducted in male New Zealand white rabbits approximately 3–4 months of age and weighing about 2.8–3.0 kg (Robinson Services Incorporated (RSI, NC, USA). After arrival to our facility, the animals were allowed to acclimate to the new housing environment for at least a week before the start of experiment. Standard food and water were freely accessible for the rabbits. The care and use of the animals were conducted in accordance with the Guidelines on Humane Use and Care of Laboratory Animals for biomedical research (National Institutes of Health), which was approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

All personnel performing surgical procedures should wear proper Personal Protective Equipment (PPE) while in the surgical suit.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse Troponin T antibody (1:200 dilution)	Sigma-Aldrich	Cat# T6277-100UL
Anti-mouse IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 594 Conjugate) (1:500 dilution)	Cell Signaling Technology	Cat# 8890S
Chemicals, peptides, and recombinant proteins		
Alloxan monohydrate	Sigma-Aldrich	Cat# 2244-11-3
Ketamine	Covetrus North America	Cat# 071069
Atropine	Covetrus North America	Cat# 002452
Xylazine	Covetrus North America	Cat# 061035
Gentamycin	Covetrus North America	Cat# 006913
Diazepam	Covetrus North America	Cat# 072709
Ketoprofen	Covetrus North America	Cat# 40028371
Buprenorphine- SR-LAB	Zoopharm	IZ-73000-192703
Triphenyltetrazolium chloride (TTC)	Sigma-Aldrich	Cat# T8877
Phthalo blue dye	Quantum LLC	Cat# QWP-7387
Novalin-R -Insulin	Novo Nordisk	NDC 0169-1833-11
Krebs-Henseleit buffer	Prepared	n/a
Potassium chloride	Sigma-Aldrich	Cat# P3911
Formalin	Thermo Fisher Scientific	Cat# SF100-4
Dextrose 50%	Covetrus North America	Cat# 068511

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cidex Ortho-Phthalaldehyde (OPA)	Thermo Fisher Scientific	Cat# NC0948940
Isoflurane	Covetrus North America	Cat# 029405
Rapamycin	LC Laboratories	Cat# R-5000
Heparin Sodium -1000 USP/mL	Sagent Pharmaceuticals	Cat# 055737
Betadine -Cotton Swap	Thermo Fisher Scientific	Cat# 19-061617
Normal Saline	VWR	Cat# 76285-236
Phosphate-Buffered Saline (PBS) pH 7.4	Thermo Fisher Scientific	Cat# 10010023
Aquasonic CLEAR -Ultrasound Gel	Parker Laboratories INSOURCE INC	Cat# 1005242
DAPI anti-fade mount solution (Vectashield Mounting Medium for Fluorescence with DAPI)	Vector Laboratories	Cat# H-1200
Critical commercial assays		
Ultra-sensitive rabbit cardiac troponin-I ELISA	Life Diagnostics, Inc.	Cat# CTNI-10-US
Terminal Deoxynucleotidyl Transferase dUTP Nick end Labeling (TUNEL) kit	ApoAlertTM DNA Fragmentation assay Kit, BD Bioscience	Cat# 630108
Experimental models: organisms/strains		
New Zealand white rabbit-male; Age- 3–4 months weight- 2.8–3.0 kg	Robinson Services Incorporated	NZW
Software and algorithms		
ImageJ bundled with 64-bit Java 1.8.0_172	National Institutes of Health	https://imagej.nih.gov/ij/
Sonic Vevo-2100-Echocardiography analysis	FUJIFILM VisualSonics	https://www.visualsonics.com/product/imaging-systems/vevo-2100
GraphPad Prism 8	GraphPad Software LLC	https://www.graphpad.com/
NIS-Elements Viewer- Microscope image Analysis	Nikon Instruments Inc	https://www.microscope.healthcare.nikon.com/products/software/nis-elements/viewer
SoftMax® Pro Software Version 5	Molecular Devices	https://www.moleculardevices.com/products/microplate-readers/acquisition-and-analysis-software/softmax-pro-software#gref
Other		
Metzenbaum Scissors-7" Curved	Roboz Surgical Instrument Co	Cat# RS-6956
Micro Dissecting Scissors- 4.5" Curved	Roboz Surgical Instrument Co	Cat# RS-5983
Moloney Forceps-5" Long Slight Curve Serrated	Roboz Surgical Instrument Co	Cat# RS-8254
Mayo Scissors- 5.75" TC, Curved	AmerisourceBergen Corp.	Cat# 045240
KELLY Hemostatic forceps- 6-1/4"	MPM Medical Supply	Cat# 115-7116
KELLY Forceps- 5.5" Curved, tips	Roboz Surgical Instrument Co	Cat# RS-7131
SEMKEN Tissue Forceps- 5" Long Serrated	Roboz Surgical Instrument Co	Cat# RS-5240
Thumb Dressing Forceps- 4.5" Serrated Tip	Roboz Surgical Instrument Co	Cat# RS-8100
Weitlaner Retractor - 3×4 5.5"	Roboz Surgical Instrument Co	Cat# RS-8612
Micro Dissecting Scissors- 4.5" Curved Blunt/Blunt	Miltex Adair	Cat# RS-5983
Tissue Holding Tenaculum Forceps- 6.75"	Medical Device Depot, Inc	Cat# 16-51-MLTX
Metzenbaum Needle Holder - 7" Straight	Roboz Surgical Instrument Co	Cat# RS-7900
Betadine solution -Swap tick - 4	Fisher Scientific	Cat# 19-061617
Sterile latex surgical gloves	Covetrus North America	Cat# 7068956
2×2 Sterile non-woven gauze -2	Thermo Fisher Scientific	Cat# 22028556
4×4 Sterile non-woven gauze-2	Thermo Fisher Scientific	Cat# 22028558

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sterile surgical drape cover-1	Steri-Drape	Cat# 1092
Sterile cotton tipped applicators – 2 packs	Puritan	Cat# 25–806 10WC
0 Perma-Hand™ Silk Suture	Ethicon, INC.	Cat# 678G
3-0(30")-75cm Nylon-Black monofilament -non-absorbable surgical suture-1	Ethicon, INC.	Cat# 669H
4-0(18")-75cm-Silk- Black braided micro point surgical needle suture-1	Ethicon, INC.	Cat# 789G
Sterile Scalp Vein Set 21gX3/4" -1	EXELINT International, Co	Cat# 26704
24GX3/4"- 27G needle - I.V Catheter	Terumo Surflo	Cat# SR-OX2419CA
BD needle- 26GX5/8	Becton, Dickinson and Company	Cat# BD 305115
BD-vacutainer- Gel- blood collection tubes -5	Becton, Dickinson and Company	Cat# 367960
Filter Unit 0.22 µm -1	SARSTEDT	83.1826.001
Sterile scalpel blades-1	Integra Miltex	Cat# 4-122
Tissue Slicer Blades-1	Thomas Scientific	Cat# 6727C18
Absorbent towels	Thermo Fisher Scientific	50-118-4365
Disposable pipette tips	GeneMate	P-1237-20 P-1237-200 P-1237-1250
Polypropylene microcentrifuge tubes (1.5 mL)	USA Scientific	1615-5500
18G tubing adaptor	Becton, Dickinson and Company	408208
Tygon S-54-HL Microbore Tubing	TygonÂ®	AAQ04127
3-way Stopcock; Swivel Male Luer Lock	Smiths Medical ASD, Inc.	MX5311L
Small canine mask, SurgiVet	Smiths Medical	32393B4
Neck Collar XS	Covetrus	023679

⚠ CRITICAL: Diazepam is an amnestic drug, used to reduce tension and anxiety and to induce amnesia during ischemia in our conscious rabbit model of myocardial infarction. It is toxic if swallowed or in contact with skin. Protective gloves, clothing and eye/protection are recommended while handling this chemical and thorough hand wash with plenty of soap and water is needed after handling.

⚠ CRITICAL: Alloxan monohydrate was used to induce T1D in rabbit. It is harmful if swallowed, in contact with skin or if inhaled. It should be used in a well-ventilated area. Protective gloves, facemask, clothing and safety eyeglass are recommended while handling this chemical. Thorough hand wash with soap and plenty of water is needed after handling.

⚠ CRITICAL: Triphenyltetrazolium chloride is used to identify the area of myocardial infarction. It is a flammable and causes skin and eye irritation. It should be kept away from open flames, hot surfaces and sources of ignition. Protective gloves, eyeglasses and face protection are recommended to handle this chemical. Thorough hand wash with plenty of soap and water is needed after handling. All contaminated clothing should be immediately taken off. After any contact with skin or eye, skin or eye should be immediately rinsed with plenty of water and then the person should consult with a physician.

⚠ CRITICAL: Formalin is used to fix the cardiac tissue. It is harmful if inhaled, causes genetic defects, cancer and allergic skin reaction. It should be used under well-ventilated area. Protective gloves, clothing, eyeglasses and facemask are recommended to handle this chemical. After any contact with skin or eye, the person should rinse skin or eye with plenty of water. If inhaled, the person should get fresh air for breathing and consult with a physician.

MATERIALS AND EQUIPMENT

Equipment	Source	Identifier
Glucose monitor meter: Contour blood glucose monitoring system	Bayer HealthCare	Cat# 7151G
Rodent Ventilator	Harvard Apparatus	Model 55-3438
Hemodynamic machine: Blood Pressure Analyzer (BPA)	Digi-Med	BPA-400
Veterinary Anesthesia System	VETLAND	EX3000
Confocal microscope	NIKON	Nikon D-Eclipse C1
VersaMax™ Tunable Microplate Reader and SoftMax® Pro Software Version 5	Molecular Devices	89429-538
Sorvall Legend XTR centrifuge	Thermo Fisher Scientific	75004521
Incubator – for 37°C	Thermo Fisher Scientific	HERAcell 150i
Multi-channel pipette	VWR	*89079-954*
Ergonomic High Performance Single-Channel Variable Volume Pipettors	VWR	89079-962 89079-970 89079-974
Analog Vortex Mixer	VWR	97043-562
Recovery Cage, Therm-O-Matic	Suburban Surgical Co. Wheeling, IL	12013-00-FNLBEI
Echocardiography imaging system	VisualSonics Inc., Toronto, Canada	Vevo2100TM

⚠ CRITICAL: All surgical tools (key resources table and Figure 1), used for *in vivo* implantation of balloon occluder, were sterilized using Cidex OPA, activated dialdehyde solution for at least 30 min before the start of surgical procedure. Cidex OPA contains phthalaldehyde and direct contact with skin and eye causes irritation. After any contact with skin or eye, the person should rinse skin or eye with plenty of water. Careful handling of this chemical with protective gloves is recommended.

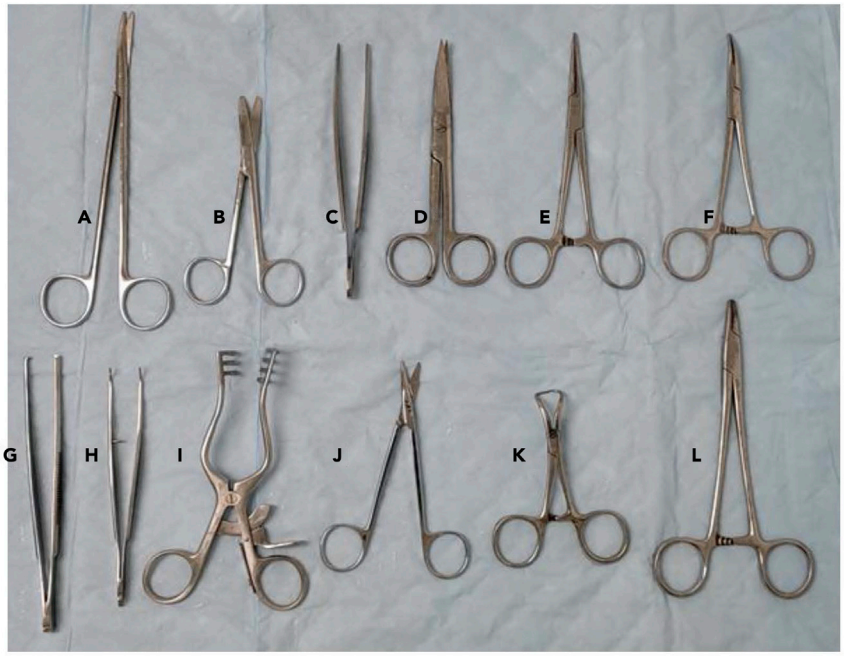

Figure 1. Major surgical tools

(A–L) (A) Metzenbaum Scissors-RS-6956, (B) Micro Dissecting Scissors-ROBOZ RS-5983, (C) Moloney Forceps-ROBOZ RS-8254, (D) Mayo Scissors, (E) KELLY Hemostatic forceps, (F) Kelly Forceps-ROBOZ RS-7131, (G) SEMKEN Tissue Forceps, RS-5240, (H) Thumb Dressing Forceps, RS-8100, (I) Weitlaner Retractor - ROBOZ RS-8612, (J) Micro Dissecting Scissors, RS-5983, (K) Miltex Adair Tissue Holding Tenaculum Forceps, (L) Metzenbaum Needle Holder - RS-7900.

Table 1. Krebs-Henseleit buffer

Reagent	Source	Catalog number	MW/FW	Final concentration
Sodium Chloride	Sigma-Aldrich	S9888	58.44	118 mM
Sodium Bicarbonate	Sigma-Aldrich	S6014	84.01	24 mM
Potassium Chloride	Sigma-Aldrich	P3911	74.55	4.7 mM
Potassium Phosphate Monobasic	Sigma-Aldrich	P-0662	136.09	1.20 mM
Magnesium Sulfate	Sigma-Aldrich	M-9397	120.37	2.5 mM
Dextrose / Glucose	Sigma-Aldrich	G-5767	180.16	11.1 mM
EDTA	Sigma-Aldrich	E1644	18.02	10 mM
Calcium Chloride Dihydrate	Sigma-Aldrich	C3881	147.01	2.5 mM

Preparation of Krebs-Henseleit buffer

Timing: 1 h

The following amount of chemicals are need for the preparation of 1L Krebs-Henseleit buffer.

- The following ingredients without calcium chloride (in Table 1) were added sequentially in 800 mL of double distilled (DD) water in a beaker and mixing with a magnetic stir bar. Wait for each component to dissolve before adding the next chemical.

- After dissolving all ingredients, volume was adjusted to1L by adding DD water.
- Solution was aerated with 5% CO_2 and 95% O_2 for 15 min.
- Calcium Chloride Dihydrate was added and completely dissolved.
- The solution was filtered through 0.22 μm or 0.45 μm filter unit (PVDF) to avoid obstruction of perfusion by any insoluble chemicals in Langendorff system.

Note: Buffer should be freshly prepared prior to use or buffer can be stored at 4°C–8°C for 1 week.

Preparation of balloon occluder

Timing: 2 h

Inflatable balloon occluder was prepared according to the procedure standardized in our laboratory (Figure 2) (Jones et al., 2015; Samidurai et al., 2020). Video demonstrates the preparation of balloon occluder (Methods video S1). The design involves following steps:

1. Tygon S-54-HL Microbore Tubing (TygonÂ® Cat#AAQ04127 .040 I.D. x .070 I.D. x .015" Wall (S-54-HL) was used to prepare the balloon occluder. The tube was cut to approximately 15 cm in length and a knot was tied at one end of the tubing.
2. The other end of the tubing was connected to a 10 cm^3 syringe using a three-way stopcock attached to 18G tubing adaptor (Becton Dickinson, 408208) with Swivel Male Luer Lock (Smiths Medical ASD, Inc. MX5311L).
3. Air pressure was forcibly introduced in to the tube using the syringe.

⚠ CRITICAL: Additional care should be taken when inflating the tube, since too much pressure can burst the tube.

4. The knot side of the tube was placed in boiling water in a beaker to aid in expanding the tube and form a balloon.

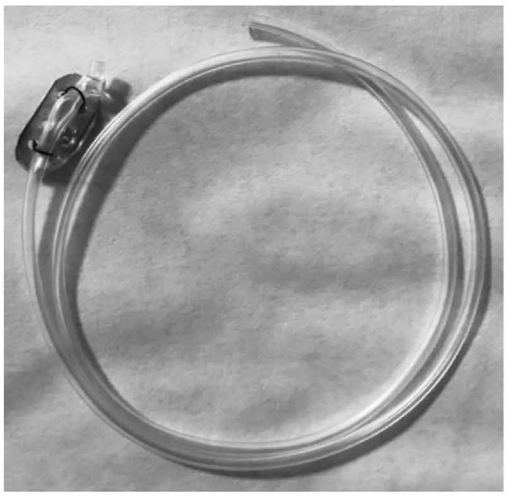

Figure 2. Inflatable balloon occluder

5. The balloon was immediately cooled on ice. In the meantime, a blister aluminum foil (aluminum package of suture, ETHICON Inc.) was cut to an oval shape and two pairs of holes (adjacent to each other) each on top, middle and bottom of the aluminum foil was made with an 18 G needle.
6. The aluminum foil was mounted on a holder and 3-0 silk suture (ETHICON, INC. 669H) was used to tightly secure the balloon occluder in place.
7. The balloon occluder was sealed in a gas sterilization pouch (Kimberly-Clark self-seal Plus-sterilization pouch) and sterilized using a gas sterilization instrument.

STEP-BY-STEP METHOD DETAILS

Induction of type I diabetes mellitus in rabbit

Timing: 4 weeks

A careful adherence to the following procedure results in the classical symptoms of reliable type 1 diabetes mellitus (T1D) in rabbit with consistent elevated blood sugar levels of 220 mg/dL for up to 8 weeks (Samidurai et al., 2020).

1. Body weight and blood glucose level were monitored before the induction of diabetes.
2. Rabbits were fasted 12 h before the procedure and supplemented with drinking water.
3. The rabbits were lightly sedated by intramuscular (i.m. in Biceps femoris muscle) administration of Ketamine (35 mg/kg), Xylazine (5 mg/kg) and Atropine (5 mg/kg) (as a cocktail).

   Note: It is advisable to start the procedure early in the morning around 8–9 AM, so that the animal health can be monitored throughout the day.

4. Alloxan monohydrate (10 mL; 50 mg/mL) was freshly prepared by dissolving in sterile saline right before its administration and kept on ice all the time.
5. This solution was aspired in 10 cc syringe and loaded on microprocessor multiple syringe infusion/withdrawal pump unit (Harvard apparatus, Model series '22' Infuse/withdraw, Male Luer Lock, Cat # MA1 55–2226) (Figure 3C). The syringe was connected to standard 6-inch bore extension tube with pinch Clamp. Rabbit was infused with appropriate volume of alloxan monohydrate, according to the body weight of the animal, to achieve final concentration 125 mg/kg.
6. The ear was cleaned with sterile alcohol pads and the ear vein was cannulated with a 24G X $^{3}/_{4}$ inch Safelet IV catheter and connected to standard bore extension set with pinch clamp, Male Luer

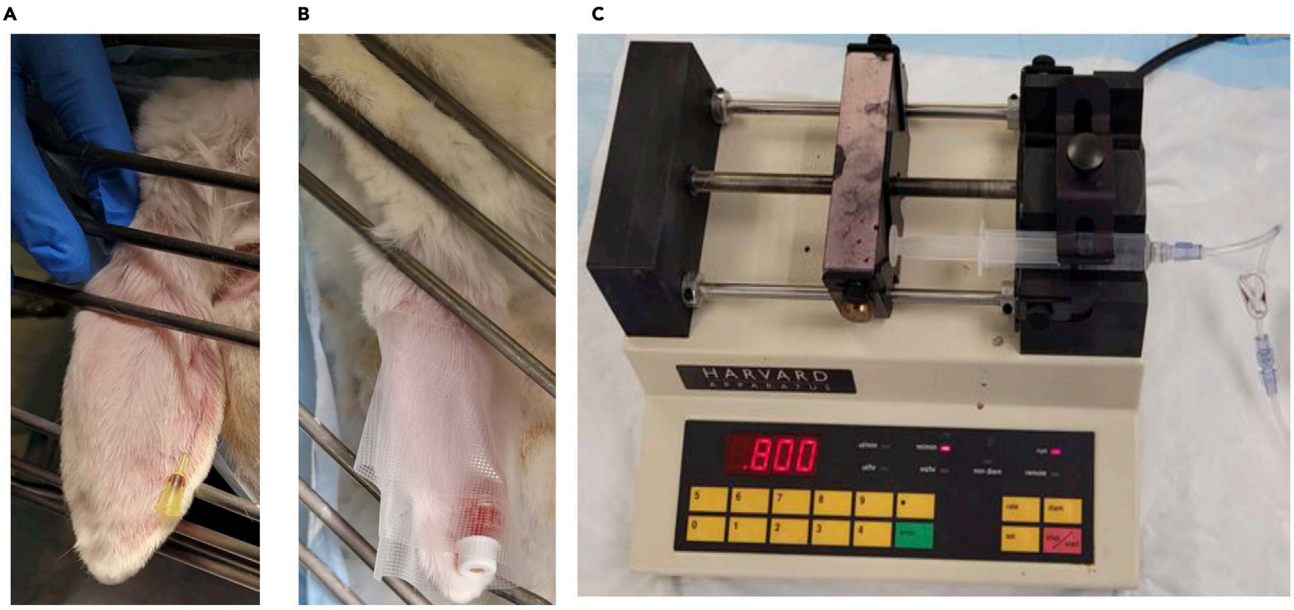

Figure 3. Induction of type I diabetes in rabbit

(A) Cannulation of the marginal ear vein with a catheter.

(B) Securing the cannulation by taping to the earlobe using surgical tape.

(C) Infusion of alloxan monohydride with adjusted flow rate through the pump (Harvard Apparatus, Model '22' Syringe Pump MA1 55–2222 -constant pressure dispensing system).

Lock. The catheter was safely secured by taping to the earlobe using surgical tape (Figures 3A and 3B).

7. The infusion flow rate of alloxan monohydrate was adjusted through the pump to deliver it slowly for a duration of 10 min. Volume was adjusted with body weight of the rabbit. Example: For a rabbit weighing 3.2 kg, 8 mL of alloxan (125 mg/kg X 3.2 kg, i.e., total 400 mg of aloxan; stock concentration; 50 mg/mL) will be administered, average flow rate 0.8 mL/min through the pump (Figure 3C).
8. The animal was transferred to recovery cage and blood glucose level was monitored using Contour glucose meter (Bayer, NJ, USA) at 1, 2, 3 and 4 h post-alloxan injection to prevent hypoglycemic shock.

   Note: We use 26G X 3/8 needle to pierce the small vein in the earlobe for blood sugar measurement using a glucose monitor meter.

9. When the blood sugar level drops below 70 mg/dL, animals were supplemented with 5% dextrose (10 mL, i.m.).

   Note: In our experiments we observed the blood sugar level starts decreasing to 70 mg/dL around 3.5–4 h after alloxan administration.

10. After 4 h of alloxan treatment, the animals were transported back to the vivarium with normal food and 20% glucose in drinking water for next 3 days.
11. The health of the animals and blood sugar were checked further after 10–12 h of alloxan administration, if again blood sugar level drops below 70 mg/dL, 5% dextrose was administered (i.m.) at 10–12 h of post alloxan infusion.
12. The animals were maintained for 4 weeks and during this time blood sugar was measured twice (morning/evening) every day. When blood glucose level exceeded 450 mg/dL, rabbits were

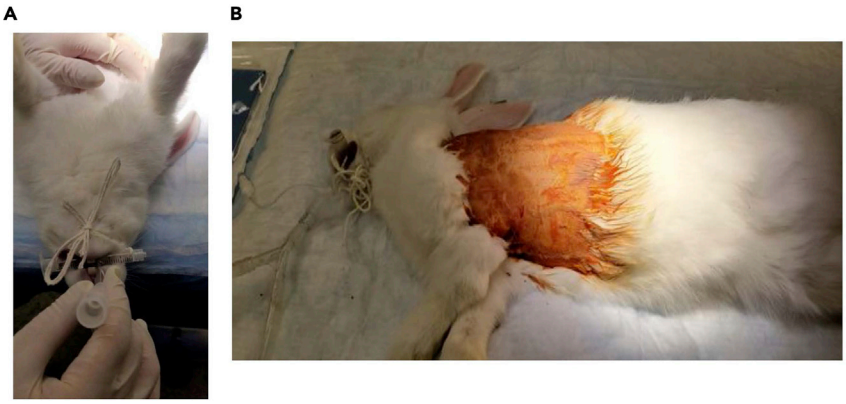

Figure 4. Pre-surgical preparation of rabbit for implantation of balloon occlude

(A) Rabbits were intubated with an endotracheal tube to mechanically ventilated with oxygen enriched air.

(B) Betadine was applied to the surgical area.

injected (i.m.) insulin (Novolin-R, Novo Nordisk Pharmaceuticals Inc. Princeton, NJ). For example, rabbits with blood glucose level (BGL) 400–500 mg/dL received 1–2 U/kg of insulin; BGL 500–600 mg/dl received 3 U/kg, and BGL > 600 mg/dl received 4 U/kg.

Implantation of balloon occluder

Timing: 1 h

The balloon occluder was implanted on top of the left anterior descending artery (LAD) of the diabetic rabbit before conducting myocardial I/R injury.

13. The rabbits were anesthetized by intramuscular (i.m.) administration of ketamine (35 mg/kg), Xylazine (5 mg/kg) and Atropine (5 mg/kg).
14. The appropriate surgical area from the sternum to left shoulder blade of rabbits were shaved using handheld vacuum assisted hair removal system (Clipper Vac -MDC-Romani Inc.).
15. Rabbits were intubated with an endotracheal tube, and mechanically ventilated with oxygen enriched air (Figure 4A). The ventilation rate was maintained between 28–30 breaths per minute.
16. All rabbits were given Buprenorphine SR LAB (0.1 mg/kg, i.m.) prior to surgery.
17. Gentamicin was also administered before surgery and on the first and second postoperative days (0.7 mg/kg; i.m.; each day).
18. Body temperature was monitored continuously during the procedure with a rectal probe attached to a thermocouple (TRACEABLE Digital Thermometer, VWR). A heating blanket (K&H Lectro-Kennel^{TM} heated Pad & Cover) was used to maintain body temperature between 36°C–39°C.
19. Betadine was applied with sterile gauze to the surgical area and allowed to dry (Figure 4B). A sterile, adhesive drape (3M) was used as a barrier on the surgical site.
20. Under sterile conditions, a left thoracotomy in the fourth intercostal space was performed (Figures 5A–5D) to expose the heart (Figure 5E). Then the pericardium was opened and LAD was identified (Figures 5F–5I). A 4-0 taper-needled silk suture was passed beneath a major branch of the left coronary artery, perpendicular to the vessel (Figures 5J and 5K).
21. To estimate a risk area of nearly 25%, the suture was slightly lifted against a cotton-tip applicator to briefly test the coronary occlusion (Figure 5J).
22. A balloon occluder (using Tygon tubing) attached with the aluminum sheet [0.2–0.3 mm thick] was placed on top of the coronary artery and secured with the 4-0 silk suture on the anterior left ventricular (LV) wall (Figures 5L–5N)

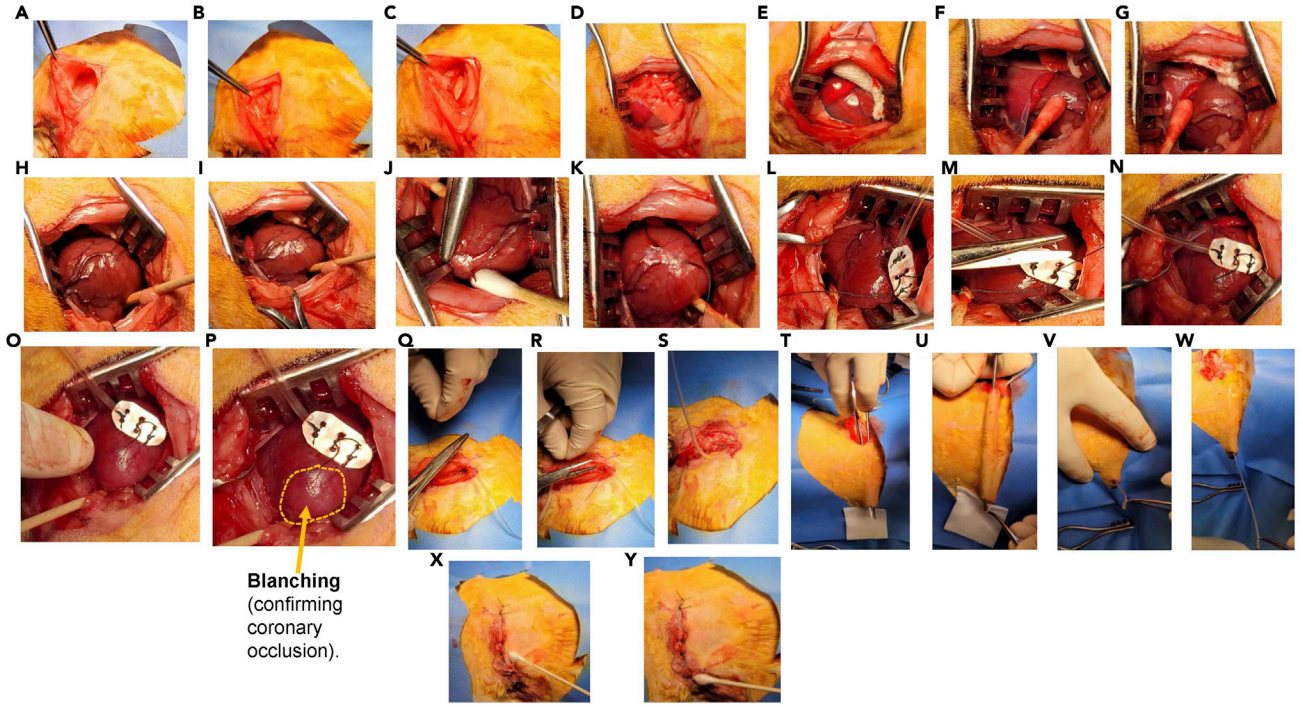

Figure 5. Sequential images of implantation of balloon occluder

(A–C) Surgical incision in the chest.
(D) Thoracotomy.
(E) Exposure of heart after thoracotomy.
(F–I) Opening of the pericardial sac to access the LAD (left anterior descending artery).
(J and K) Placing suture around LAD.
(L–N) Implantation of Balloon Occluder.
(O and P) brief inflation of the balloon occluder to confirm the blanching of the distal myocardium.
(Q and R) Suturing of inner muscle.
(S) Completion of muscle suture.
(T–W) Tunneling balloon occluder under the skin layer between shoulder blades.
(X) Suturing outer skin layer.
(Y) Application of antiseptic in the incision area.

⚠ CRITICAL: (a) Careful placement of the balloon is critical for a later successful conscious ischemia induction, i.e. the suture knot should not be too tight (which would cause blood flow restriction after balloon deflation) or too loose (which would fail to induce complete coronary occlusion during balloon inflation). (b) Proper function of the occluder should be confirmed by the appearance of cyanosis (bluish or grayish color) of the distal myocardium upon brief inflation of the balloon (10 s) and hyperemia after deflation (Figures 5O and 5P).

23. Lungs were inflated using an Ambu bag (Spur II Pediatric Resuscitation bag, Ambu®).
24. The surgical incision in the chest was closed according to the standard procedures. Briefly, pericardium was closed using 4-0 silk suture, rib was closed using 0 Perma-Hand™ silk suture, muscle and skin were closed with 3-0 monofilament suture (Figures 5Q–5Y). Only a few millimeters of the incision was left accessible for catheter retrieval.
25. The occluder tubing was then tunneled under the skin, and exteriorized through a small incision between the scapulae (Figures 5T–5W).
26. Prior to closing the chest, a sterile chest tube was placed to remove fluid and air to avoid a pneumothorax (Figures 5X and 5Y).
27. Triple-antibiotic (Neosporin) ointment was applied after the surgery (Figure 5Y).

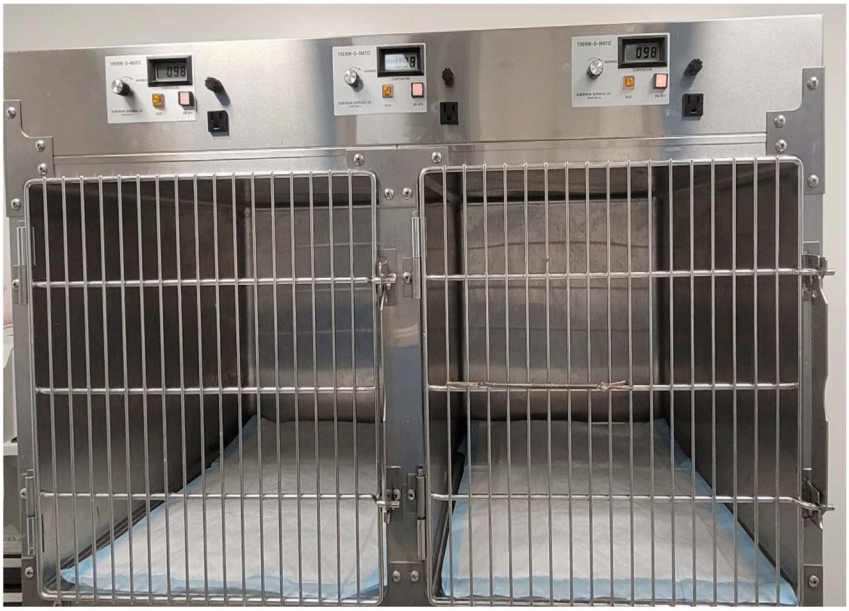

Figure 6. Recovery Unit with automated temperature control

28. Rabbit was extubated after waking from anesthesia by checking their eyeball movement to light and diaphragm movement for abdominal breathing. Then rabbit was transferred to the recovery unit (Figure 6) and continuously monitored until they awake. The average time taken by animals to awake is 1 h.
29. After post-operative recovery (when animal was fully awoken, i.e., when they could stand on their legs and start moving without imbalance), the rabbit was transported back to the vivarium with normal food and drinking water.
30. Necessary measurements were taken to ensure that the animals could not get access or pull the catheter at the exit site. These include minimizing the incision size between the shoulder blades, periodic close monitoring, at least 2 times a day and the use of protective neck collar (in order to make it least accessible).

Conscious I/R injury

Timing: 3 days

Myocardial I/R injury was conducted under sedation to avoid the confounding effects of anesthetics as well as the postsurgical complications of acute opening and closing of the chest for performing coronary artery occlusions.

31. Seven days after successful implantation of balloon occluder, rabbits were prepared for performing I/R protocol.
32. Throughout the coronary occlusion-reperfusion sequence, rabbits were kept in a cage (in a restrainer to control movement) in a quiet and dimly lit room (Figure 7).
33. To alleviate discomfort, rabbits received ketoprofen (3.0 mg/kg; i.m.) and diazepam (4 mg/kg; i.m.) separately 2 h before inflating the balloon for inducing ischemia.
34. The ear was cleaned with sterile ethanol pads. A venous line was established by placing a 24-gauge angiocatheter on a marginal ear vein to draw blood samples and administer drugs. Under local anesthesia (Lidocaine Hydrochloride Jelly USP, 2%), the ear dorsal artery was cannulated with a 24-gauge angiocatheter to monitor arterial pressure (Figure 7B).

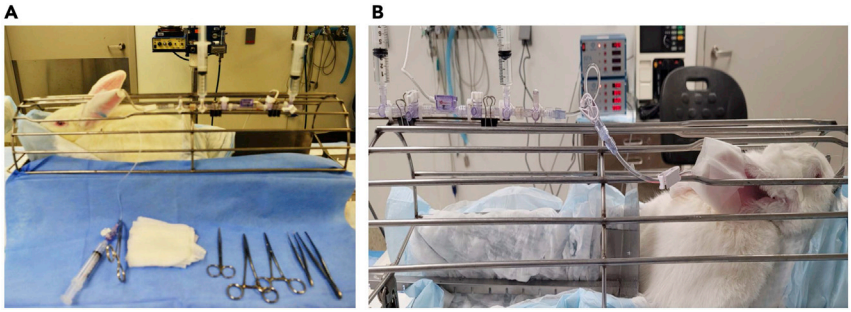

Figure 7. Myocardial ischemia in conscious diabetic rabbit

(A) Picture of conscious diabetic rabbit during myocardial ischemia by inflating the hydraulic balloon occluder.

(B) A transducer sensor probe was secured to arterial line of the ear connected to Blood Pressure Analyzer during conscious ischemia/reperfusion

35. Under sedation, rabbits were subjected to a 45-min coronary artery occlusion (to induce 45 min of ischemia) by inflating the pre-implanted hydraulic balloon occluder. After 45 min of ischemia, the hydraulic balloon was deflated to allow reperfusion for 3 days.

Note: Vital cardiac parameters including mean arterial pressure, systolic pressure, diastolic pressure and heart rate were monitored throughout the conscious I/R procedure via a transducer sensor probe secured to arterial line of the ear connected to Blood Pressure Analyzer (BPA, Digi-Med, KY USA).

36. Blood sample (0.5 mL) was collected in heparinized tube (BD-vacutainer- Gel- blood collection tubes - Becton, Dickinson and Company) at base line and at 1, 2, 4 and 24 h of reperfusion. Blood in the tube was centrifuged to separate plasma using Thermo Scientific Sorvall Legend XTR centrifuge at 2000g for 10 min under refrigerated condition (4°C). The plasma was stored at -20°C to measure cardiac troponin (cTnI).
37. Rabbit was transferred to the recovery unit and continuously monitored until they awoke (Figure 6). The rabbit was closely monitored to ensure adequate breathing and to prevent hypothermia and dehydration. If animal will encounter severe breathing problem, it will be again intubated and mechanically ventilated with oxygen enriched air. Visual signs of dehydration are not easy to detect during the course of surgery under anesthesia. After completion of ischemia, the rabbit was monitored for dehydration by checking symptoms such as the sticky saliva, crusty eyes or dark color urine. To avoid dehydration and compensate the blood loss/withdrawal, saline (5 mL/h) was injected intravenously during surgery and post-op recovery period. Animal was provided with unrestricted supply of drinking water in recovery chamber as well as vivarium.
38. When animal was fully awoken, i.e., when they could stand on their legs and start moving without imbalance, the rabbit was transported back to the vivarium with normal food and drinking water.

Reperfusion therapy with Rapamycin

39. In the sedated rabbit, undergoing 45 min of ischemia (through inflation of balloon occluder) and 5 min before the onset of reperfusion (deflation of the balloon occluder), Rapamycin (0.25 mg/kg, in 1 mL of saline) was slowly infused for 10 min intravenously through marginal ear vein.

Assessment of cardiac function by echocardiography

Timing: 2 h

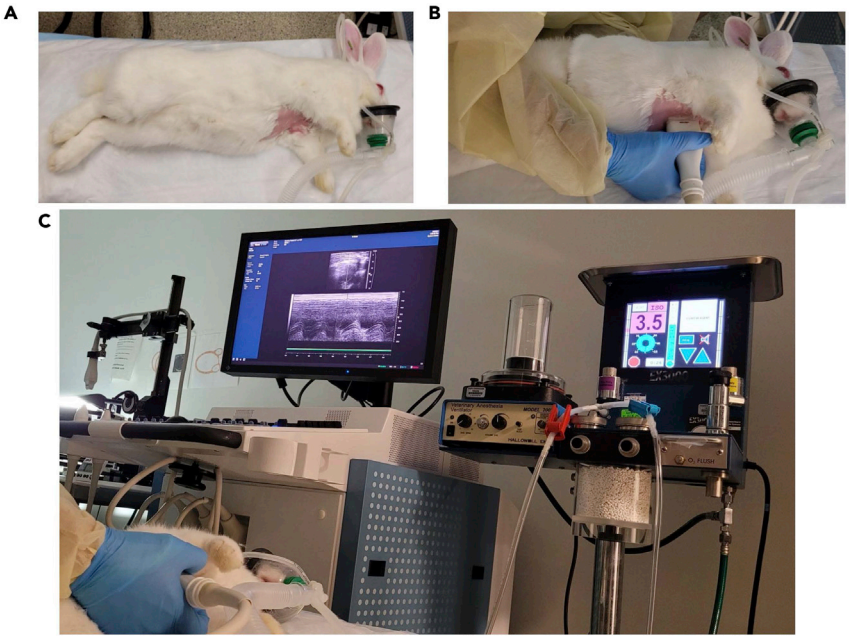

Figure 8. Cardiac function was assessed with echocardiography

(A) Chest hair was removed in rabbit while anesthetized with isoflurane.

(B) Cardiac function was measured by placing the MS201 probe on the parasternal view.

(C) Cardiac left ventricle (LV) function was measured by two-dimensional ultrasound B-Mode (Short Axis) as well as M-Mode.

Cardiac function was evaluated by echocardiography in the diabetic rabbits following myocardial I/R injury.

40. The rabbits were sedated with inhalation of isoflurane (2.5%) and echocardiographic measurements were performed using a Vevo2100TM (VisualSonics Inc., Toronto, Canada) at different time points, including before (baseline) and after alloxan treatment, after balloon implantation, and 3 days post I/R as per the protocol.
41. Two operators, blinded to rabbit cohort allocation, performed repeated rounds of echocardiography to minimize inter- and intra-observer variations.
42. Rabbit was lightly sedated in anesthesia chambers supplied with mixture of oxygen and isoflurane 4.0% (VETLAND-EX3000-Veterinary Anesthesia System).
43. Rabbit was placed on the table in left lateral recumbent position (shown in Figure 8) and supplied with isoflurane (2.5%–3.5%) through small canine mask (SurgiVet, Smiths Medical, 32393B4) to keep them sedated during the echocardiography acquisition process.
44. Chest hairs were removed using handheld vacuum assisted hair removal system (Clipper Vac). The remaining hairs were removed by applying hair removal cream/gel (Veet -Gel Cream hair remover) to the chest region and using 4×4 non-sterile non-woven gauze (Figure 8A).
45. Prewarmed ultrasound gel (Aquasonic CLEAR -Ultrasound Gel, Parker Laboratories) was applied to the chest, primarily in the area overlying the heart.

Note: Additional precautions are taken to avoid air bubbles in the gel, which can interfere with ultrasonic imaging. Generally, a 60cc syringe, filled with ultrasound gel, was centrifuged for 10 min at 2000g to remove air bubbles.

46. Cardiac left ventricle (LV) function was measured by two-dimensional ultrasound B-Mode (Short Axis) as well as M-Mode by placing the MS201 probe on the Parasternal view to access the LV

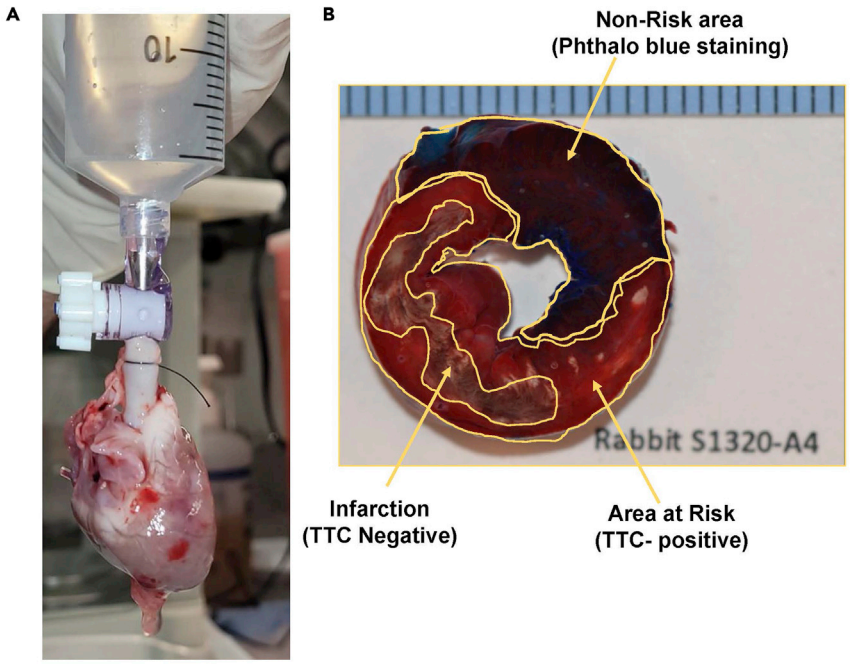

Figure 9. Infarct size measurement

(A) Perfusion of heart with Krebs-Henseleit buffer to wash out most of the blood and infusion of Phthalo blue dye for demarcation of risk and non-risk area.

(B) Image of heart section to measure infarct size.

anterior (AW) and posterior (PW) walls, the intraventricular septal wall (SW), and lateral wall (LW) (Figures 8B and 8C). The diameter of the LV lumen was measured as LV internal diameter (LVID).

47. The LV ejection fraction (LVEF), LV end-diastolic diameter (LVEDD), end-systolic diameter (LVESD) and stroke volumes (SV) were calculated by tracing the end- and epicardial boarder during contraction (Torrado et al., 2018). The obtained images were analyzed using Vevo LAB 3.2.0 software.

Infarct size measurement

Timing: 6 h

After myocardial I/R injury, histological measurement of the infarcted area in tissue sections of the LV is a standard approach to determine the extent of cardiac damage.

48. After completion of I/R protocol and echocardiography, the rabbits were anesthetized by administration (i.m.) of ketamine (35 mg/kg), Xylazine (5 mg/kg) and Atropine (5 mg/kg) (as a cocktail).
49. The ear was cleaned with sterile ethanol pads and heparin (500 U/kg) was infused through the ear vein.
50. Thereafter, the rabbits were euthanized by administration of saturated potassium chloride (10 mEq/10 mL) via catheter connected to ear auricular artery.
51. After excision, the heart was mounted through the aortic root on a 60 c.c. syringe and perfused with Krebs-Henseleit buffer (~30 mL, 37°C) to remove all of the blood (Figure 9A). The balloon occluder was removed. Using a 4-O suture, a double knot is tied around the coronary artery at the same location where the balloon occluder was placed. Thereafter, a 5% solution of phthalo blue dye in normal saline (~10 mL over 3 min) was infused through the aortic root.

52. The heart was cut into 5–6 transverse slices (~2-mm-thick/slice) using tissue slicer blades, which were stained with 1% triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4) for 15 min at 37°C.
53. The heart slices were then fixed in 10% formalin for 4 h.
54. The atrial and RV (right ventricle) tissues were removed. The LV slices were numbered from apex to base as 1 through 5/6 and photographed on both sides of the slices with a ruler (Figure 9B).
55. Using image J software (Bethesda, NIH, USA), the area of total LV, red region (viable tissue within the ischemic zone, TTC positive region), blue tissue (non-ischemic myocardium, phthalo blue positive region) and white region (necrotic area, TTC negative region) were measured (Figure 9B). The region at risk was expressed in a percentage of the LV and the infarct was calculated as percentage of the risk area (Samidurai et al., 2020).

Cardiac troponin I measurement

Timing: 5 h

Cardiac troponin I is the established biomarker of cardiac injury, which is released into the circulation in response to cardiomyocyte damage following I/R injury.

56. Cardiac troponin I (cTnI) was measured in the plasma samples using Ultra-Sensitive Rabbit Cardiac Troponin-I ELISA kit (Life Diagnostics Inc, USA, Cat. No. CTNI-10-US) (Jones et al., 2015; Samidurai et al., 2020; Torrado et al., 2018). Each assay was performed in duplicate in a blinded fashion.
57. Briefly, the plasma samples were diluted 10× with cTnI diluent before measuring cTnI.
58. Standards of cTnI (1.25, 0.625, 0.312, 0.156, 0.078, 0.039 and 0.019 ng/mL) were prepared from the cTnI stock by serial diluting according to the instructions provided in the kit.
59. The standards and diluted plasma samples (200 μL) were dispensed into the wells of ELISA plate (standards and samples were run in duplicate).
60. The plate was incubated for 2 h at 25°C on a plate shaker at 150 rpm.
61. After the completion of incubation, the solutions were discarded from the wells of the plate and the wells were washed five times with 1× wash solution (400 μL/well/each time).
62. After removing all residual droplets, 100 μL of diluent was added to each well. Then 100 μL of horseradish peroxidase (HRP)-conjugate was added to each well.
63. The plate was incubated at 25°C for one hour on a plate shaker at 150 rpm.
64. After the completion of incubation, the solutions were discarded and the wells were washed five times with 1× wash solution (400 μL/well/each time).
65. All residual droplets were removed by tapping the plate on clean paper towels.
66. TMB (3,3′, 5,5″-tetramethylbenzidine; 100 μL) was dispensed into each well.
67. The plate was incubated at 25°C for 20 min on a plate shaker at 150 rpm.
68. After 20-min, the reaction was stopped by adding 100 μL of Stop solution to each well. The blue color should be changed to yellow.
69. Then, the absorbance was measured at 450 nm with a plate reader within 5 min.
70. A standard curve was prepared by plotting absorbance values of the standards versus $\log_{10}$ of the concentration.
71. The concentration of the samples were determined using this standard curve, with multiply by 10 (dilution factor).

Evaluation of cardiac apoptosis

Timing: 5 days

Apoptosis, a programed cell death, plays a vital role during the myocardial I/R injury. Post-I/R myocardial apoptosis was assessed by measuring DNA fragmentation using a Terminal Deoxynucleotidyl Transferase dUTP Nick end Labeling (TUNEL) kit.

Figure 10. Cardiac apoptosis assessment by TUNEL staining was monitored under a fluorescence microscope (Confocal microscope Nikon D-Eclipse C1)

72. The risk area of the LV after 72 h of reperfusion was dissected and fixed in 10% formalin (at least 1 day) after thorough washing with PBS.
73. The 5 μm thick tissue sections (transverse cross section of myocardium) were prepared after paraffin embedding.
74. The sections were stained using a Terminal Deoxynucleotidyl Transferase dUTP Nick end Labeling (TUNEL) kit (ApoAlert™ DNA Fragmentation assay Kit, BD Bioscience, San Jose, CA, Cat no. 630108) according to the instruction provided by the company.
75. The paraffin-embedded tissue sections in slides were deparaffinized using xylene and dehydrated with different concentrations of ethanol (96%, 90% and 80%). Thereafter, the sections were rehydrated in distilled water.
76. The slides with tissue sections were microwaved (high power) for 10 min in 1× Citrate Buffer (pH 6.0, prepared from 10× Citrate Buffer, Sigma-Aldrich Co. LLC. St. Louis, MO, USA; Cat no. C9999-1000mL) and cooled down slowly at 20°C–22°C.
77. Tissue sections were incubated for 1 h with the equilibration buffer (45 μL), nucleotide mix (5 μL) and TdT enzyme (1 μL) provided by TUNEL kit, under dark in humidified 37°C incubator.
78. The slides were washed with PBS three times (5 min per wash) and the tissue sections were incubated with 5% normal goat serum for 1 h at 20°C–22°C.
79. Tissue sections were incubated 16 h at 4°C with troponin antibody (mouse Troponin T antibody, Sigma-Aldrich Co. LLC. St. Louis, MO, USA).
80. Next day, after washing three times with PBS (5 min/each time of washing), the tissue sections were incubated with anti-mouse IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 594 Conjugate, Cell Signaling, MA, USA), and fixed with DAPI anti-fade mount solution (Vectashield Mounting Medium for Fluorescence with DAPI, Vector Laboratories, Burlingame, CA. Cat no. H-1200).
81. The apoptotic cells (with green nuclei) and total cells (with DAPI-blue nuclei) were counted under a fluorescence microscope (Confocal microscope (Nikon D-Eclipse C1, Figure 10), and the data was plotted as the percentage of apoptotic cells to total cells (Figures 15A and 15B). Co-localization of green nuclei with troponin staining in the cells (with red striation) confirmed the cardiomyocyte apoptosis in myocardium.

EXPECTED OUTCOMES

We have established a preclinical cardiac research platform based on the advanced experimental models of I/R in conscious diabetic rabbits, which could offer reliable diagnostic, prognostic and therapeutic information with higher confidence to facilitate translational research in diabetic patients with AMI. Rabbit heart closely resembles to human heart in terms of collateral circulation (Seiler et al., 2013). Another advantage is that the conscious rabbit myocardial I/R model avoided the confounding effects of anesthetics as well as the stress imposed by acute opening and closing of the chest for performing coronary artery occlusions. Considering its low cost, ease of handling and

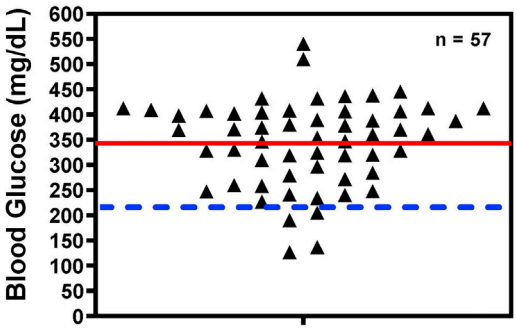

Figure 11. Blood glucose levels after 4 weeks of alloxan treatment in rabbit

Animals with consistent blood glucose level of 220 mg/dL (Blue dashed line) or above were considered diabetic. Red line denoted the average blood glucose level in 57 rabbits.

housing (compared to the large animals such as pigs and dogs), the rabbit has been one of the most commonly used experimental animals for studying complex diseases, such as diabetes and for developing novel therapeutic interventions (Graur et al., 1996). Rabbits are phylogenetically similar to humans than mice and rats. Therefore, the model we developed here can be reliably used in discovering novel therapeutic strategies for cardioprotection in diabetes as well as diabetic cardiomyopathy. Relative to rats and mice, their larger size greatly facilitates surgical procedures (implantation of balloon to conduct I/R under conscious sedation) with minimum postsurgical complications. Following are the successful outcomes of the current protocol:

Development of a reliable diabetic (T1D) rabbit model with alloxan treatment

Out of total 63 rabbits, 53 rabbits had blood glucose level greater than 220 mg/dL, which were considered as diabetic (average glucose level was 339.6 $\pm$ 11.8 mg/dl as indicated by red line in Figure 11). The success rate of induction of diabetes was 84.1% with 9.5% mortality after alloxan treatment (6 out of 63 rabbits died after alloxan treatment).

Reduction of infarct size in diabetic heart by treatment with rapamycin

Histological measurement of myocardial infarct region in the post-MI tissue sections of the LV is a standard approach to assess the efficacy of a potent pharmacotherapy against AMI. Using myocardial I/R injury in conscious diabetic rabbit, this study demonstrates the infarct limiting effect of Rapamycin in diabetic rabbits (Figure12).

Improvement of post-ischemic function by treatment with rapamycin

Infarct-sparing effect of Rapamycin is highly correlated with restoration of cardiac systolic function following I/R injury in diabetic rabbits (Figure 13).

Rapamycin treatment attenuates troponin I levels in the blood

Cardiac-specific TnI concentrations in the plasma is considered as the cornerstones for MI and risk stratification. There was a significant elevation of TnI (until 4 h of reperfusion), which was reduced following Rapamycin treatment in the post-ischemia in diabetic rabbit (Figure 14).

Rapamycin treatment attenuates myocardial apoptosis

Apoptosis, occurs predominantly in the peri-infarcted region, plays an important role in the myocardial loss after AMI. Inhibition of apoptosis with a therapeutic intervention could improve prognosis of patients with AMI. There was significant increase in TUNEL-positive nuclei in cardiomyocytes of the peri-infarct regions in the diabetic hearts following I/R as compared to the non-diabetic hearts (Figure 15). Reperfusion with Rapamycin reduced cardiomyocyte apoptosis in diabetic hearts.

QUANTIFICATION AND STATISTICAL ANALYSIS

- The infarct size was quantified using image J software (Bethesda, NIH, USA). Data was exported to Excel format and calculated.

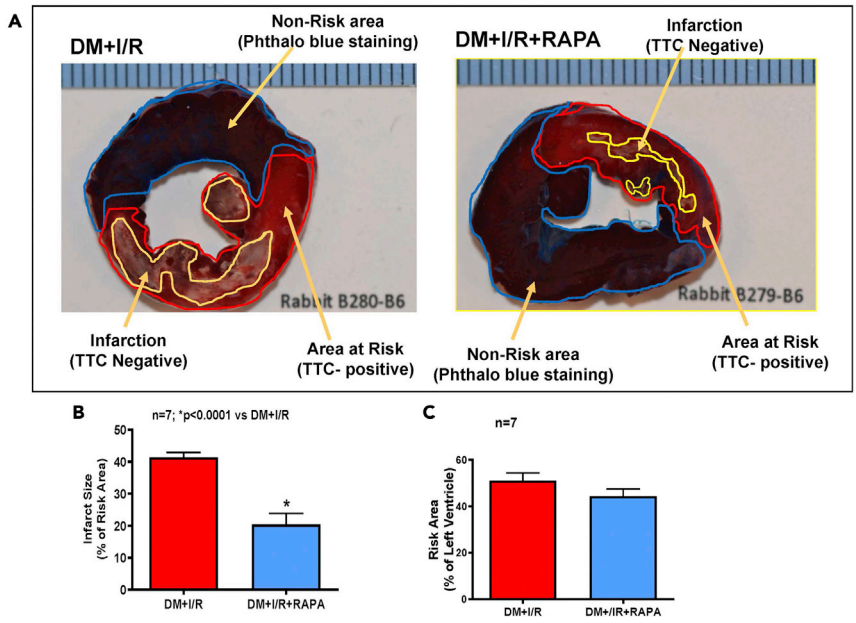

Figure 12. Myocardial infarct size after I/R injury

(A) Representative images of myocardial sections of diabetic rabbits following I/R injury. Each grid in the scale indicates 1 mm.

(B and C) (B) Infarct size and (C) risk area. Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software Inc. La Jolla, CA). The data are presented as mean ± SE for each treatment group. p values <0.05 were considered statistically significant. The difference between two groups was analyzed via Student's t-test.

- The echocardiography images were analyzed using Vevo LAB 3.2.0 software.
- The absorbance of 450 nm for cardiac TnI assay was recorded using Molecular Devices VERSA max microplate reader and SoftMax® Pro Software Version 5. The data was exported to Excel format and calculation of cTnI in each sample was calculated using the standard curve prepared with cTnI (1.25, 0.625, 0.312, 0.156, 0.078, 0.039 and 0.019 ng/mL).
- The pictures of cardiac sectioning after TUNEL staining and counter staining with Troponin and DAPI were captured using a confocal microscope (Nikon D-Eclipse C1). The apoptotic cardiomyocytes (TUNEL positive nuclei: green and Troponin staining: red striation) and total cells (with DAPI-blue nuclei) were counted and the data was plotted in Excel format to calculate the percentage of apoptotic cells to total cells.
- Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software Inc. La Jolla, CA). The data are presented as mean ± SE for each treatment group, along with unadjusted 2-tailed p values <0.05 were considered statistically significant. One-way ANOVA + Bonferroni post-hoc test was used for unpaired data to compare 3 groups, respectively. The difference between two groups was analyzed via Student's t-test.

LIMITATIONS

The long-term effects of diabetes on rabbit heart following myocardial injury was not examined in the current protocol. The E/A ratio (the ratio of peak velocity blood flow from LV relaxation in early diastole (the E wave) to peak velocity flow in late diastole caused by atrial contraction (the A wave)) could not be evaluated, which would have provided early indications of diastolic dysfunction in the T1D rabbits.

Respiratory rate has an impact on cardiac function during echocardiography measurement. In this study, respiratory rate during echocardiography was not accurately measured. During echocardiography,

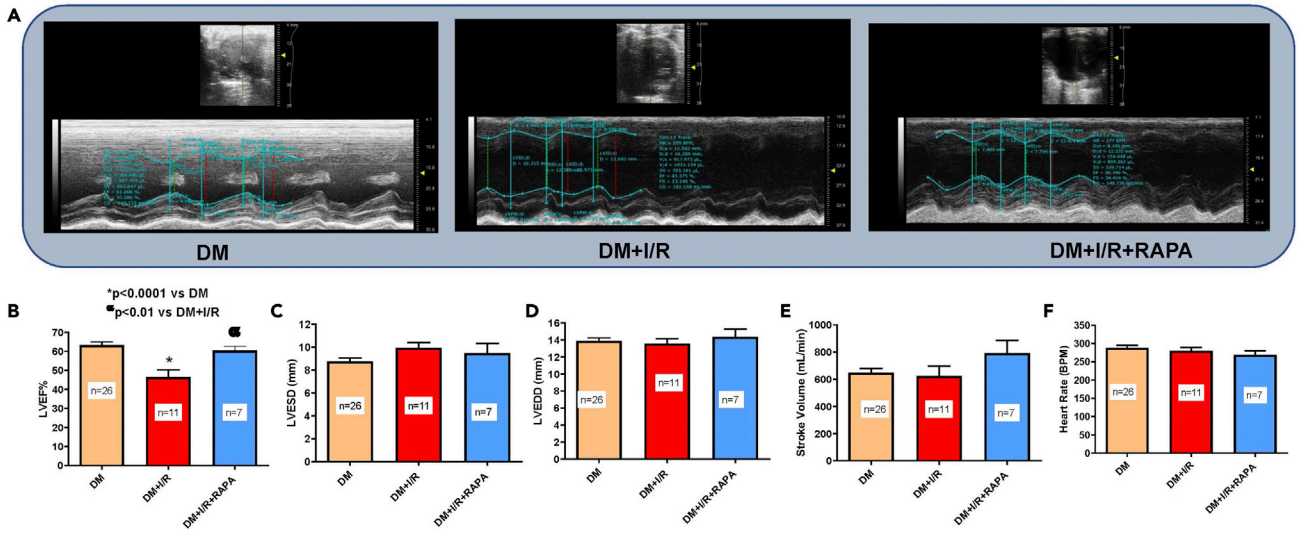

Figure 13. Cardiac function assessed by echocardiography

(A) Representative images of parasternal view (M-mode ultrasound) assessing LVEF (left ventricular Ejection fraction) at baseline (DM) and following I/R in diabetic rabbits (DM+I/R) and diabetic rabbit treated with Rapamycin at the onset of reperfusion (DM+I/R+RAPA). Inner panel, the preview of B-mode image to assess the correct probe positioning.

(B–F) (B) Percentage of LVEF, (C) LV end systolic diameter (LVESD), (D) LV end diastolic diameter (LVEDD), (E) Stroke volume and (F) Heart rate at base line and after I/R injury. Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software Inc. La Jolla, CA). The data are presented as mean ± SE for each treatment group. p values <0.05 were considered statistically significant. The comparison among multiple groups was conducted using one-way analysis of variance with Bonferroni post-hoc test.

rabbits were not anaesthetized (using ketamine, Xylazine and Atropine), which could affect their respiration; they were sedated using isoflurane (adjusted at 2.5–3.5%).

TROUBLESHOOTING

Problem 1

Difficulty to induce T1D in rabbit.

Potential solution

Rabbits whose blood glucose levels remain <220 mg/dL following 7 days of the initial injection of alloxan should receive a second dose of alloxan (100 mg/kg; i.v.) under light sedation as previously described

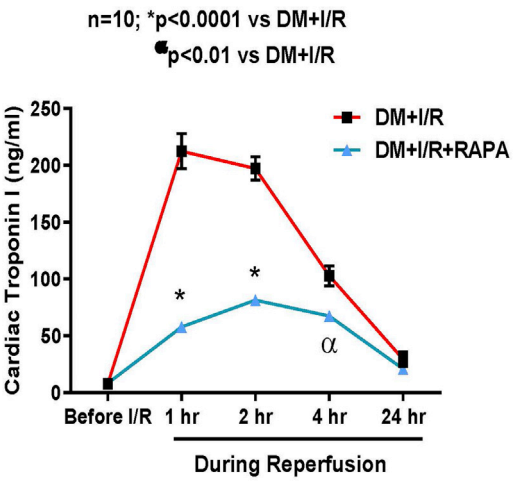

Figure 14. Post-ischemic cardiac troponin I level

Plasma cardiac troponin I following I/R in diabetic rabbits (DM+I/R) and diabetic rabbit treated with Rapamycin at the onset of reperfusion (DM+I/R+RAPA).

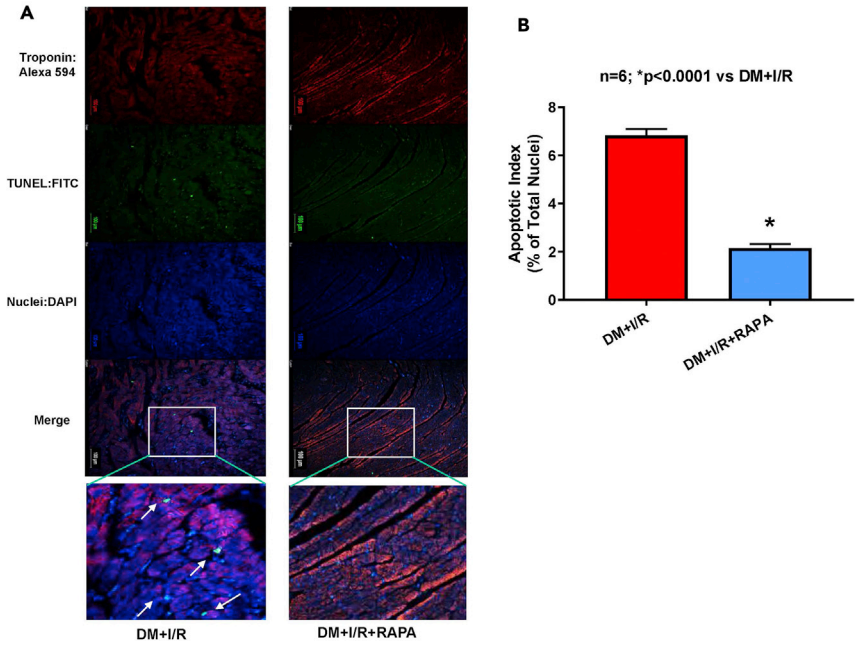

Figure 15. Apoptosis in the LV risk area of rabbit hearts following I/R injury

(A) Representative pictures of TUNEL (Green), Troponin (Red:Alexa 594) and nuclei (DAPI) staining in myocardium of diabetic rabbits (DM+I/R) and diabetic rabbits treated with Rapamycin at the onset of reperfusion (DM+I/R+RAPA). Scale indicates 100 μm.

(B) Percentage of TUNEL positive nuclei to total nuclei. Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software Inc. La Jolla, CA). The data are presented as mean ± SE for each treatment group. p values <0.05 were considered statistically significant. The difference between two groups was analyzed via Student's t-test.

procedure of the induction of T1D in rabbit. The monitoring parameters should be similar to the post-alloxan monitoring procedures listed above until the end of the study. It is essential to freshly prepare the alloxan solution before administration. Precise dose of alloxan solution should be injected into the ear vein. After second dose of alloxan administration, animal should be monitored closely, specifically the body weight was examined at least once every 48 h. If blood glucose level exceeds 450 mg/dL, rabbits should be injected with insulin to control glucose level as previously described.

Problem 2

Adverse effect during I/R in conscious rabbit.

Potential solution

Hemodynamics should be monitored to assess the vital cardiac parameters including mean arterial pressure, systolic pressure, diastolic pressure and heart rate at baseline and throughout the conscious ischemia and reperfusion (1 and 2 h) via a transducer sensor probe secured to arterial line of rabbit ear connected to Blood Pressure Analyzer (BPA, Digi-Med, KY USA). We normally exclude rabbits from the study if blood pressure drops below 30 % and does not return to normal levels during the period of monitoring. We do not administer any vasopressor (e.g., norepinephrine) because they can potentially interfere with the interpretation of our results of ischemia/reperfusion injury and cardioprotection with rapamycin. For example, the administration of norepinephrine activates alpha-adrenergic receptor signaling in the heart which can potentially trigger preconditioning like cardioprotective effect as previously published from our laboratory several years ago (Tejero-Taldo et al., 2002).

Problem 3

Post-surgical complication due to balloon implantation or I/R injury.

Potential solution

The rabbits should be closely monitored post-surgery of balloon implantation to assess their overall behavior, responsiveness, eating/drinking, urination until sacrifice. If any indication of discomfort is noticed, the animal should be immediately sacrificed and excluded from the study. After balloon implantation, cardiac function should be assessed by performing echocardiography. If there is severe cardiac dysfunction, the rabbits should be excluded from the study.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Anindita Das (anindita.das@vcuhealth.org).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate large-scale datasets. All data generated or analyzed during this study are included in this published article (and in supplemental information of iScience. 2020 Nov 26;23(12):101863. https://doi.org/10.1016/j.isci.2020.101863) or are available from the lead author upon request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100772.

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AUTHOR CONTRIBUTIONS

A.D., R.C.K., and A.S. designed experiments. A.S., R.O., C.C., S.K.R., S.M.F., and D.K. performed all surgical experiments and analyzed data. A.D. and A.S. performed biochemical assays and histochemistry and wrote the manuscript. R.C.K. and all other authors edited the document.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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