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TITLE:

Pre-Clinical Model of Cardiac Donation after Circulatory Death

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KEYWORDS:

Cardiac transplantation, donation after circulatory death, ischemic conditioning, ischemia-reperfusion injury, ex vivo perfusion, Langendorff, functional evaluation.

SUMMARY:

This protocol shows a simple and flexible approach for the evaluation of new conditioning agents or strategies to increase the feasibility of cardiac donation after circulatory death.

ABSTRACT:

Cardiac transplantation demand is on the rise; nevertheless, organ availability is limited due to a paucity of suitable donors. Organ donation after circulatory death (DCD) is a solution to address this limited availability, but due to a period of prolonged warm ischemia and the risk of tissue injury, its routine use in cardiac transplantation is seldom seen. In this manuscript we provide a detailed protocol closely mimicking current clinical practices in the context of DCD with continuous monitoring of heart function, allowing for the evaluation of novel cardioprotective

strategies and interventions to decrease ischemia-reperfusion injury.

In this model, the DCD protocol is initiated in anesthetized Lewis rats by stopping ventilation to induce circulatory death. When systolic blood pressure drops below 30 mmHg, the warm ischemic time is initiated. After a pre-set warm ischemic period, hearts are flushed with a normothermic cardioplegic solution, procured, and mounted onto a Langendorff ex vivo heart perfusion system. Following 10 min of initial reperfusion and stabilization, cardiac reconditioning is continuously evaluated for 60 min using intraventricular pressure monitoring. A heart injury is assessed by measuring cardiac troponin T and the infarct size is quantified by histological staining. The warm ischemic time can be modulated and tailored to develop the desired amount of structural and functional damage. This simple protocol allows for the evaluation of different cardioprotective conditioning strategies introduced at the moment of cardioplegia, initial reperfusion and/or during ex vivo perfusion. Findings obtained from this protocol can be reproduced in large models, facilitating clinical translation.

INTRODUCTION:

Solid organ transplantation in general and cardiac transplantation, in particular, are on the rise worldwide[1,2]. The standard method of organ procurement is donation after brain death (DBD). Given the strict inclusion criteria of DBD, less than 40% of the offered hearts are accepted[3], thereby limiting the offer in face of increasing demand and extending the organ waiting list. To address this issue, the use of organs donated after circulatory death (DCD) is considered a potential solution[4].

In DCD donors, however, an agonal phase following withdrawal of care and a period of unprotected warm ischemia before resuscitation are inevitable[5]. The potential organ injury after circulatory death can lead to organ dysfunction, explaining the reluctance to routinely adopt DCD heart transplantations. It is reported that only 4 centers use DCD hearts clinically, with stringent criteria that includes very short warm ischemia times and young donors without chronic pathologies[6,7]. For ethical and legal reasons, limited or no cardioprotective interventions can be applied in donors prior to circulatory death[5,8,9]. Thus, any mitigation to alleviate the ischemia-reperfusion (IR) injury is limited to cardioprotective therapies initiated during early reperfusion with cardioplegic solutions, and do not allow for proper functional assessment. Ex vivo heart perfusion (EVHP) and reconditioning of the DCD heart using dedicated platforms has been proposed as an alternative solution and studied by various scholars[10-13]. EVHP offers a unique opportunity to deliver post-conditioning agents to DCD hearts to improve functional recovery. However, for efficient clinical translation, many technical and practical issues remain to be addressed, and this is further compounded by a lack of consensus on a range of perfusion and functional criteria to determine transplantability[6,8].

Herein we report the development of a reproducible pre-clinical small animal DCD protocol combined with an ex vivo heart perfusion system that can be used to investigate organ post-conditioning initiated at the time of procurement, during initial reperfusion, and/or throughout EVHP.

PROTOCOL:

All animal care and experimental protocols conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the institutional animal care and use committee of the Centre Hospitalier de l'Université de Montréal Research Center.

1. Preliminary preparations

1.1. Turn on the water bath to heat the cardioplegia delivery system (**Figure 1A**) and the Langendorff ex vivo perfusion system (**Figure 1B**). Set the water temperature to 38.5 °C for a solution temperature of 37 °C. Setup photographs can be seen in **Supplementary Figure 1A,B**.

1.2. Prepare 1 L of cardioplegic solution. Add 1 mL of 2% lidocaine hydrochloride and 10 mL of 2 mM KCl (final concentration 20 mM) to 1 L of Plasma-Lyte A (140 mM Na, 5 mM K, 1.5 mM Mg, 98 mM Cl, 27 mM acetate, 23 mM gluconate). Correct pH to 7.4 using 6 N HCl.

CAUTION: This model is highly sensitive to pH. A wrong pH correction (outside the 7.3-7.4 physiological range) or pH unstable solutions may compromise the experiment or provide unreliable data.

1.3. Prepare 4 L of Krebs solution (113 mM NaCl, 4.5 mM KCl, 1.6 mM NaH_2PO_4, 1.25 mM $CaCl_2$, 1 mM $MgCl_2{\cdot}6H_2O$, 5.5 mM D-Glucose, 25 mM $NaHCO_3$). Substrate masses per 1 L of solution should be as follows: 6.1 g of NaCl, 0.3355 g of KCl, 0.2035 g of $MgCl_2{\cdot}6H_2O$, 0.192 g of NaH_2PO_4, 0.1387 g of $CaCl_2$, 0.99 g of D-Glucose, 2.1 g of $NaHCO_3$, final volume of 1 L in ultrapure deionized water. **Add the $NaHCO_3$ last to avoid precipitation**. Filter the solution using a 0.22 µm filter and store overnight. Correct the pH to 7.4 when the solution is at 37 °C and bubble with 5% CO_2/95% O_2.

1.4. Fill the Langendorff circuit with Krebs solution and start the system pump. **Make sure that no bubbles are left inside the tubing**. Adjust the peristaltic pump speed to 80 rpm (equivalent to 1 L/minute). Using the two way stop cock, adjust the flow to maintain a slow drip through the aortic cannula until the heart is attached (**Figure 1B**). Keep a sample of Krebs solution (15 mL) in a 50 mL conic tube on ice for heart transportation.

1.5. Fill the cardioplegia delivery system with the cardioplegic solution. Once the bubbles are removed, switch the circuit to saline using a 3 way stop cock (**Figure 1A**). Adjust the drip rate. Saline must be slowly dripping from the tip of the catheter to assure that no cardioplegic solution is injected before the animal's death.

2. Animal preparation

2.1. Using an inhalation chamber, induce anesthesia with 3% isoflurane. Once the animal is unresponsive, perform an intraperitoneal injection of ketamine (75 mg/kg) and xylazine (5 mg/kg) to maintain anesthesia for the rest of the procedure. Confirm proper anesthesia by a

negative toe pinch test (no movement) and the absence of corneal reflex[14].

2.2. Intubate the animal using a 14G, 2-inch I.V. catheter. Start ventilation at 50 breaths per minute, with airway pressure limited to 20 cmH_2O.

2.3. Place the animal on a heating pad set to "medium" and cover with an absorbent pad to maintain body temperature. Insert a rectal temperature probe and attach a transdermal pulse oximeter sensor to one of the feet. Maintain rectal temperature at 37 °C throughout the procedure.

2.4. Vascular access

2.4.1. Make a 3 to 4 cm midline skin incision in the neck using scissors. Using blunt tip curved scissors, blunt dissect the subcutaneous tissue and expose the right sternohyoid muscle. Using non traumatic forceps, move the muscle laterally until the right carotid artery (pulsating), jugular vein (non-pulsating) and the vagus nerve (white) are visually identified (**Supplementary Figure 2A**). Carefully separate the vagus nerve from the carotid artery using blunt tip curved scissors.

2.4.2. Inject heparin (2,000 IU/kg) via the right jugular vein. Apply pressure to the injection site after needle retraction to avoid blood leakage.

2.4.3. Using curved forceps, pass two 5-0 silk sutures around the carotid artery. Firmly attach a distal suture to occlude the carotid artery at the superior aspect of the exposed artery. Keep the proximal suture untied. Pulling of the proximal suture will be used for bleeding control in the next step (**Supplementary Figure 2B**). **The distance between sutures should be approximately 2 cm**.

2.4.4. Using a stereomicroscope for better visualization, carefully make a 1 mm incision with microsurgery scissors over the anterior wall of the carotid artery. Insert a 22G, 1-inch closed I.V. catheter towards the aortic arch. The catheter is connected to a 2 way stop cock, allowing for connection to a pressure transducer for constant monitoring, with the possibility of injecting saline or cardioplegia via the cardioplegia delivery system (**Figure 1A**).

3. Initiation of cardiac donation after circulatory death (DCD) protocol

NOTE: A complete protocol timeline can be seen in **Figure 2**.

3.1. Turn off the ventilator and extubate the animal. Using mosquito forceps, clamp the trachea. This moment is considered as the start of the agonal phase. Start counting the functional warm ischemic time (WIT) when the peak systolic blood pressure drops below 30 mmHg, or if asystole or ventricular fibrillation appears, whatever comes first (**Figure 3**).

NOTE: Damage extent should be proportional to WIT. Experiments are needed to optimize WIT time according to animal strain, sex and weight chosen. In control animals, immediately after carotid vascular access is secured, cardioplegia is injected and the heart is procured as described

in the next step (**Figure 2**). The start of perfusion with cardioplegia is considered as the end of WIT.

3.2. At the end of WIT, perform a medial sternotomy. Keep the thorax open by using an Alm retractor. Using scissors, open the inferior vena cava and both atria to avoid myocardial distension or cardioplegia recirculation (Supplementary figure 3). **Clamp the aorta above the diaphragm.** Through the previously catheterized carotid artery, infuse the cardioplegic solution at a constant pressure of 60 mmHg for 5 min using the cardioplegia delivery system. **Infusion pressure can be modified by altering the height of the water column.**

3.3. At the end of cardioplegic infusion, dissect the ascending proximal aorta from the pulmonary artery using curved forceps (**Supplementary Figure 4A**). Cut the aorta distal to the left subclavian artery. **Ensure an aortic length of at least 0.5 cm for cannulation for the Langendorff apparatus.**

3.4. Holding the heart from the aorta, complete the cardiectomy by separating the heart from the pulmonary veins and other thoracic structures (**Supplementary Figure 4B**). Rapidly, submerge the heart in to ice-cold Krebs solution for rapid transportation to the ex vivo system. Keep the dissection and transport times as possible (5 min).

4. Ex vivo heart perfusion system (EVHP) and cardiac functional assessment

4.1. Open the aortic lumen using forceps. Deair the aorta by filling the lumen with the dripping Krebs solution to avoid forcing bubbles in to the coronary vessels. Lower the cannula into the aorta, **taking care not to pass the aortic root or damage the aortic valve leaflets.** Fix the setup with a small clamp.

4.2. Using the 2 way stopcock, increase the flow to search for possible leaks in the aorta. If none are detected, tightly fix the aorta to the cannula using a 2-0 silk suture. Fully open the flow to the cannula. **Maintain aortic pressure at a physiological pressure of 60-70 mmHg (adjusted by changing the height of the system).** At this moment the initial reperfusion and stabilization time is initiated. Aortic pressure can be modified according to the investigator's experimental plan.

4.3. Rotate the heart so the base of the heart (atria) is facing the pressure sensor. Widen the left ventricular atrial opening by dissecting the pulmonary veins. Insert the latex balloon connected to a pressure sensor. Make sure that the balloon is fully positioned inside the ventricle by visual inspection. Slowly fill the balloon with saline until end diastolic pressure (EDP) is set to 15 mmHg. **Adjust as needed to keep EDP constant (pre-determined physiological EDP).** The EDP can be adjusted according to the experimental objectives of each investigator.

4.4. Insert the pacing electrode in the anterior face of the heart (right ventricular outflow tract). **Avoid puncturing the coronary vessels**. Once spontaneous beating is observed, initiate pacing at 300 beats per minute. **Required voltage may vary between experiments and rat strains.**

4.5. After 10 min of stabilization, initiate continuous intraventricular pressure measurement

recording. This moment is considered the beginning of the reconditioning and assessment phase (time 0) that will last for 1 h (**Figure 2**). **Reconditioning may be prolonged, but a time-dependent decrease in contractility is expected in all hearts.**

4.6. At the start of reconditioning, collect cardiac effluent dropping from the cardiac veins for 5 min for baseline coronary flow assessment and biochemical analyses. For troponin T repeat every 15 min (times 0, 15, 30, 45 and 60 min). **For other analyses individualization of collection times is needed** (**Figure 2**).

5. End of experience

5.1. Remove the heart from the Langendorff apparatus.

5.2. Using a straight high carbon steel blade (microtome blade or similar), remove the base of the heart (including aorta and pulmonary artery).

5.3. With the right ventricle facing down, cut transverse ventricular slides of 1-2 mm thickness. In one representative section (normally the third) excise the right ventricle and snap freeze the left ventricle. This sample can be used for biochemical analyses.

5.4. Submerge the remaining sections in to freshly prepared 5% 2,3,5-triphenyl-tetrazolium chloride in commercial phosphate buffer saline pH 7.4 for 10 min at 37 °C. Viable tissues are colored red brick.

5.5. Wash twice with phosphate buffer saline pH 7.4 and fix with 10% formalin at 4 °C overnight. Wash twice with phosphate buffered saline pH 7.4 and keep each slice submerged.

5.6. Withdraw excess liquid and weight each slide. Take digital color images of both sides. Use planimetric analyses to calculate percent infarct size and correct for slice and total ventricular weight. **Coloration fades with time. Photos must be taken as soon as possible.**

6. Data analyses

6.1. Save all pressure data in a new file per animal.

6.2. For pressure analyses, select at least 200 pressure cycles per time points. Analyses can be performed off-line (after completion of the experiment) using dedicated software (i.e., LabChart). Common cardiovascular parameters available include: Maximal generated pressure, end diastolic pressure, +dP/dt (steepest slope during the upstroke of the pressure curve, an indicator of ventricular contractile ability), -dP/dt (steepest slope during the downstroke of the pressure curve, an indicator of ventricular relaxation capacity) among others.

NOTE: For troponin analyses, an increase in troponin release at reperfusion is expected. After 1 h of reperfusion in the EVHP system, troponin levels may decrease to baseline, stressing the need

for careful timing in the collection and handling of these samples.

REPRESENTATIVE RESULTS:

Following extubation, blood pressure rapidly drops in a predictable pattern (**Figure 3**). Expected time to death is less than 5 minutes.

Figure 4 shows an average pressure/time curve at the start of reconditioning following 0, 10 and 15 min of WIT. Contractile function will improve over time. The use of short periods of WIT will allow for contractility to return to normal, and morphological damage will not be detectable (**Figure 5** and **Figure 6**).

Proof-of-concept use of a conditioning agent added with the cardioplegia and at the stabilization phase show that the damage generated by 15 min of WIT in this model are amenable to modulation by cardioprotective agents (**Figure 4, Figure 5** and **Figure 6**).

FIGURE AND TABLE LEGENDS:

Figure 1: Required equipment schemas. Minimal requirements for a **(A)** cardioplegia delivery system and a **(B)** Langendorff ex vivo heart perfusion system.

Figure 2: Protocol timeline. Timeline from the moment of extubation until the end of the protocol. In control animals, cardioplegia is initiated without DCD or warm ischemic time.

Figure 3: Intracarotid blood pressure/time plot. Typical evolution of intracarotid blood pressure following extubation. Warm ischemia time stars when the peak systolic blood pressure drops below 30 mmHg, or if asystole or ventricular fibrillation appears, whatever comes first.

Figure 4: Ex vivo average beat-to-beat ventricular pressure time curve. Image derived from analyses of data taken after 10 min stabilization and perfusion (time 0 in figure 2) with or without the use of an experimental pharmacological cardioprotective conditioning agent. Ischemic time refers to warm ischemic times (WIT).

Figure 5: Ex vivo recovery and functional analyses. (A) Continuous ventricular pressure-time curve after 10 min stabilization and perfusion with or without the use of an experimental pharmacological cardioprotective conditioning agent. Arrows show artifacts due to manual modification of EDP. **(B)** Maximum (+dP/dt) and minimum (-dP/dt) rate of pressure change in the LV vs. time plot derived from **(A)** showing a time-dependant improvement in contractility without treatment (green line). Short WIT (red line) or treated (yellow) hearts show a pattern similar to the control group (blue line). Data points are the mean of at least 200 individual beats. Bars show the standard error of the mean of each data point.

Figure 6: 2,3,5-Triphenyl-tetrazolium chloride coloration at the end of experiments. Infarct area observed following diverse warm ischemic times (WIT) and the use of a pharmacological cardioprotective conditioning agent. Brick red: viable tissue. Light yellow: non-viable tissue.

Supplementary Figure 1: Setup photograph. (A) Photograph showing the setup for the cardioplegia delivery system. Numbered equipment corresponds to: cardioplegia container (1), bubble trap (2), pressure sensor and catheter (3), peristaltic pump (4), polygraph connected to the pressure sensor (5) and small animal ventilator (6). **(B)** Photograph showing the setup for the Langendorff ex vivo heart perfusion system. Numbered equipment corresponds to: Perfusate container (1), conditioning agent container (2) and heart chamber (3).

Supplementary Figure 2: Neck dissection. (A) Photography showing the exposed jugular vein (arrow) prior to heparin injection. **(B)** shows the dissected carotid artery (arrow) with the sutures placed for bleeding control.

Supplementary Figure 3: Opening of the atria to prevent recirculation. (A) Photography showing the opening of the left atrial appendage (1). On the background the aorta (2) is clamped above the diaphragm (3). **(B)** Shows the opening of the right atrial appendage (1).

Supplementary Figure 4: Heart procurement. (A) Photography showing the use of curved forceps to separate the aorta (arrow) and the pulmonary artery. **(B)** Photography showing cardiac dissection and procurement. The heart is hold by the aorta using forceps.

DISCUSSION:

The protocol presented here introduces a simple, convenient and versatile model of cardiac DCD, offering the opportunity to assess cardiac functional recovery, tissue damage and the use of post-conditioning cardioprotective agents to improve recovery of donor hearts otherwise discarded for transplantation. Ex vivo heart perfusion systems (EVHP) systems have been optimized to provide a platform for evaluating cardiac function and offer a unique opportunity to deliver and test modified solutions supplemented with post-conditioning pharmacological agents to preserve and repair DCD hearts in small[15] and large animals[16,17] models of cardiac DCD. Nevertheless the protocols are often insufficiently detailed and not always clinically relevant, making clinical translation difficult.

In the realm of DCD models, ex vivo DCD models, like the one described by Sanz[18], lack an agonal phase. By inducing cardiac arrest by stopping mechanical ventilation, the sympathetic nervous system is overactivated, leading to a "catecholamine storm"[19]. This increase in catecholamines modifies the characteristics of the donor organs, and has been linked to a reduced functional status of experimental DCD organs[19]. Additionally, the progressive decline in function prior to asystole leads to right ventricular distension and consequent injury. In our protocol, we have induced circulatory death using a clinically relevant asphyxiation model, which maintains these responses.

Two main in vivo cardiac DCD models are described in the literature: open chest[15] and closed chest[20] models. Cardiac physiology is altered by the open chest approach by reducing the mechanical lung/heart interaction and preload. Furthermore, in open chest procedures, body heat loss is accelerated, further affecting functional outcomes[21]. Therefore it is preferable to maintain a closed chest approach preventing heat loss. Another refinement is to minimize the

variability of time to circulatory death. Kearns et al. reported that time to death (time to non-pulsatile or mean blood pressure less than 30 mmHg) was between 3 to 11 min. In the 10 and 20 min WIT, 40% and 60% of hearts did not recover function, respectively, on an ex vivo working heart apparatus, making data interpretation more difficult[15]. An alternative to reduce the time to circulatory death is to use paralytic agents[20]; nevertheless, some evidence points towards direct cardiac effects of vecuronium, due to its effects on sympathetic and parasympathetic innervation[22]. To increase reproducibility, we elected for tracheal clamping, combined with a precise arterial pressure monitorization, allowing for a more homogeneous agonal time (<5 min). It is known that organ damage starts before the moment of circulatory death; with some authors considering a cut-off systolic blood pressure below 50 mmHg as the beginning of functional WIT[6], explaining the reluctance to transplant organs following a long period form withdraw of life sustaining measures until reperfusion. In this protocol, the WIT definition used follows the current experimental standard[15], nevertheless, further studies are needed to clarify the exact set of hemodynamic parameters that mark the induction of organ damage in order to improve WIT calculation, thus offering better information for clinical practice.

The infusion of cardioplegic solution at constant physiological pressure and temperature offers a unique opportunity to initiate heart conditioning and tissue protection with any pharmacological agent or by other means. Technical refinements include clamping the thoracic aorta, limiting perfusion to the heart and thereby reducing the amount of solution needed for each essay. Once the heart is on the EVHP system, standardized functional evaluation is necessary. It has been shown that the use of an EVHP system has the potential to improve resuscitation of hearts previously considered not transplantable[23,24]. Interestingly, the clinically available EVHP system evaluates cardiac viability only by using serial lactate measurements[8,23]. Lactate measurements are not related to cardiac performance of DCD hearts[24,25], thus additional measurements to evaluate transplantability are necessary. This experimental setup allows for a complete functional evaluation, including generated pressures and myocardial contractility measurements including +dP/dT and –dP/dT, allowing for a more thorough evaluation of cardiac function before the final transplant decision is made. Additionally, measurements of cardiac troponin, a marker of myocardial damage directly correlated to ischemic infarct size[26], and release kinetics are related to the extent of cardiac ischemia in a Langendorff ischemia/reperfusion system. In particular, with long ischemic times (60 min), troponin levels are maintained after 1 h reperfusion, while LDH and creatinine kinase significantly decrease, and being non related to the extent of cardiac damage[27,28], thus the use of serial troponin measures ensure a complete evaluation of organ viability before transplant. A major confounding variable in cardiac functional evaluation is the heart rate. Spontaneous heart rate is inversely related to length of ischemia[29], and heart rate directly correlates with +dP/dt in isolated rat hearts[30] and in animal models[31]. Interestingly, in recently published work on rodent models of DCD hearts and EVHP conditioning, pacing was not used and cardiac rates were variable and recorded in their protocols[15,18,20]. To maintain physiological heart rate, pacing was used once the heart had recovered rhythmic contraction. The chosen 300 bpm frequency is similar to those of healthy, non-stressed rats[32].

Limitations of this protocol include the use of volatile anesthetic for induction. These agents have been shown to confer ischemic preconditioning[33]. Nevertheless, the short time of inhaled

anesthetic use had no observable effect in this protocol and progressive myocardial dysfunction was still noted with increasing WIT. The use of normothermic cardioplegia can be also viewed as a limitation. Using normothermic cardioplegia allows optimal translation from the in vitro conditions used for the development of pharmacological conditioning agents, since cells are usually maintained at 37 °C. Nevertheless, in this setup cardioplegia temperature can be easily regulated according to the requirements of the investigator. On the other hand, the use of a Langendorff preparation versus a working heart preparation for reconditioning might also be seen as a limitation. The working heart preparation allows for continuous recording of a pressure/volume loop[12,15], with controlled pre and afterload, allowing for complete functional evaluation. The main advantage of a Langendorff preparation is that it maintains a constant aortic and perfusion pressure, especially during initial reperfusion, when generated pressure is minimal. In addition, the evaluation setup is simpler for the Langendorff heart compared to a working heart preparation. Nevertheless, this setup can be converted into a working heart preparation if deem necessary. Alternatively, cardiac reanimation can be performed in situ using normothermic regional perfusion, with cardiac performance being measured directly by the use of a Millar catheter[34], allowing comprehensive hemodynamic and myocardial functional evaluation before organ procurement. In humans, both in situ and ex vivo reconditioning strategies have been described[6], thus the development of both models allows for experimental comparisons that may translate in to optimization of clinical practice. Finally, the small size and high heart rate of this animal model may be considered as a limitation due to the potential technical difficulties observed while performing these experiments, and the inevitable physiological differences between rat and human hearts. If the EVHP evaluation is already standardized, a researcher can be familiarized with this technique by performing as little as 3 experiments. On the other hand, the use of this small animal model allows for convenient screening at a reasonable cost, reserving larger and more costly animal models such as the porcine model, to therapies with high human translational potential.

In conclusion, the protocol described here takes into account the best practices emanating from several groups researching DCD hearts. This protocol grants full control of WIT, allowing for a comprehensive structural and functional evaluation of cardioprotective conditioning treatment strategies in rats. This protocol can be upscaled and transferred to large animal models, allowing to translate research findings to clinical reality and ultimately allowing development of novel therapies increasing the quality and availability of lifesaving organs much needed by patients.

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DISCLOSURES:
The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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Figure 1

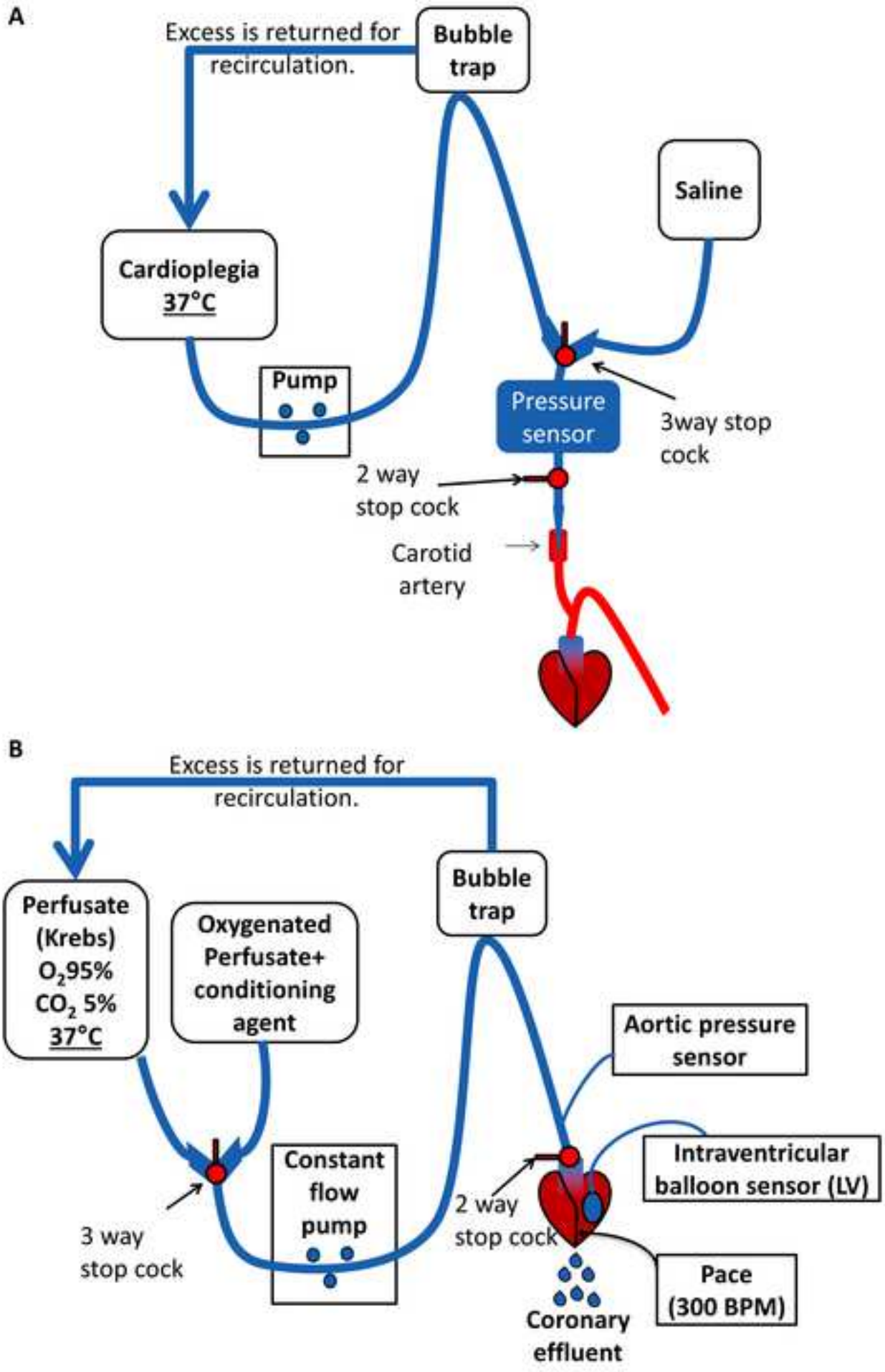

Figure 2

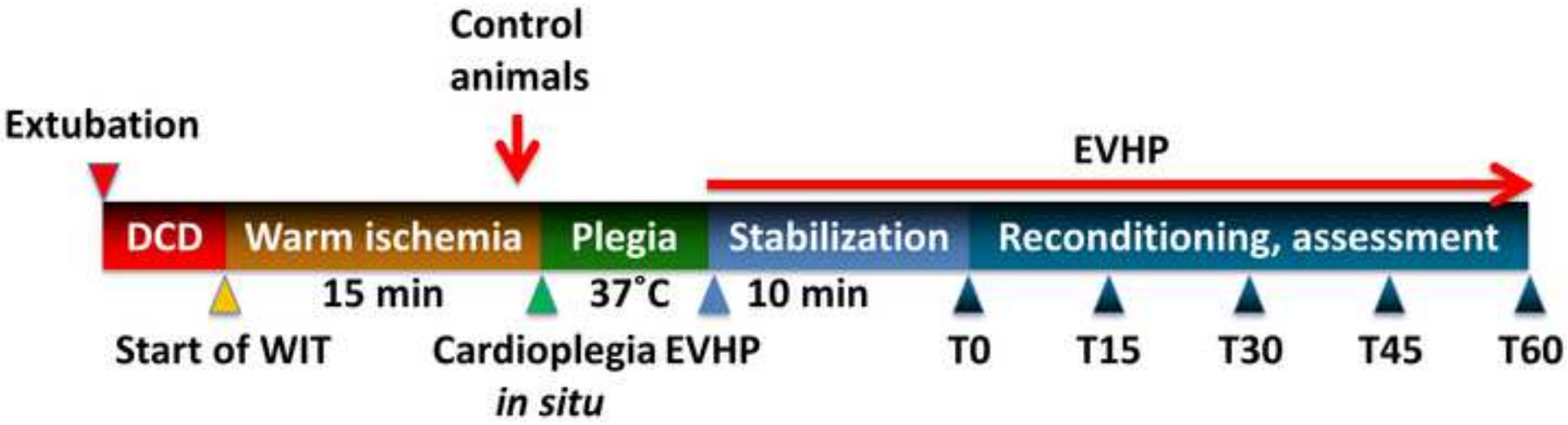

Figure 3

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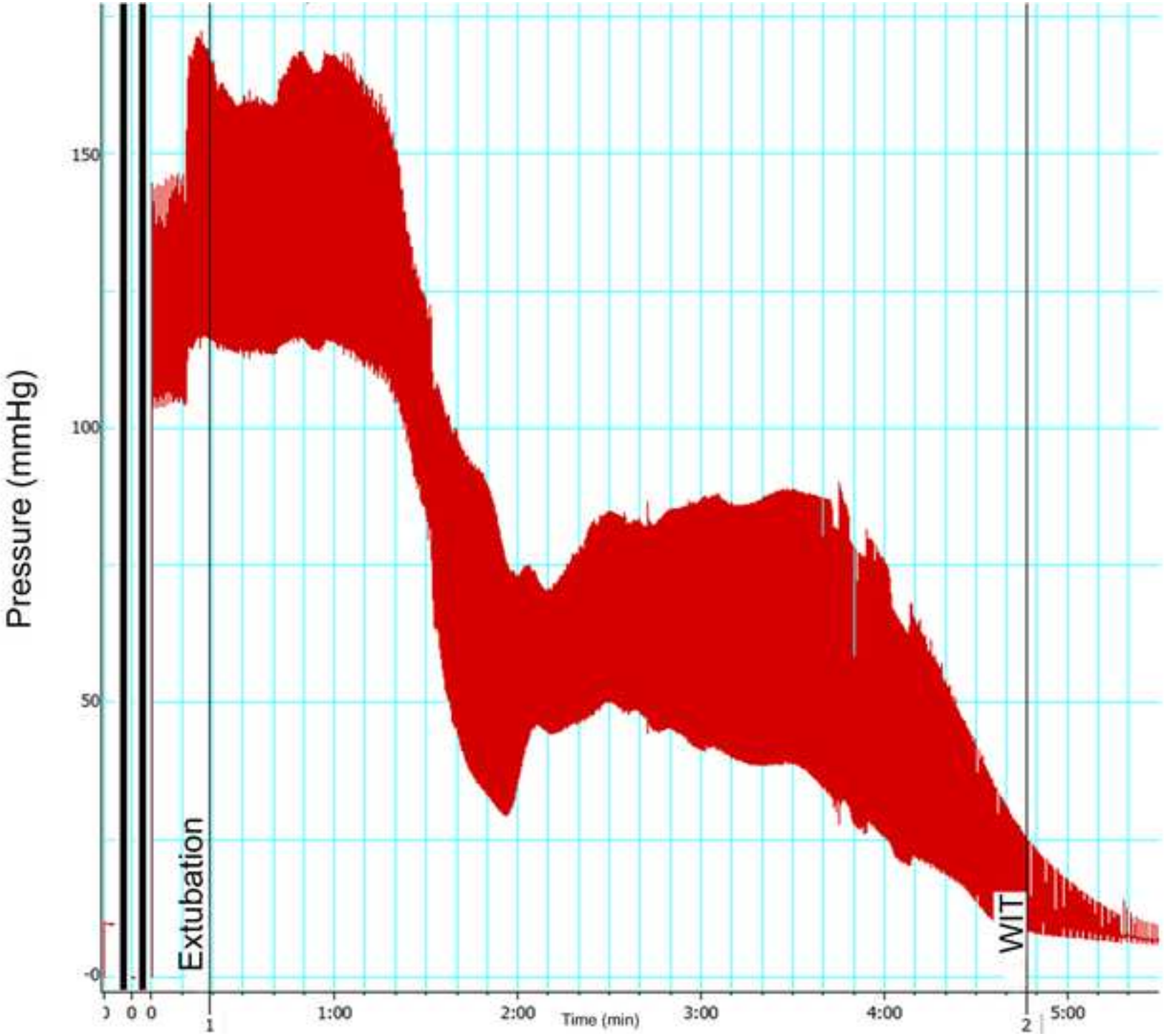

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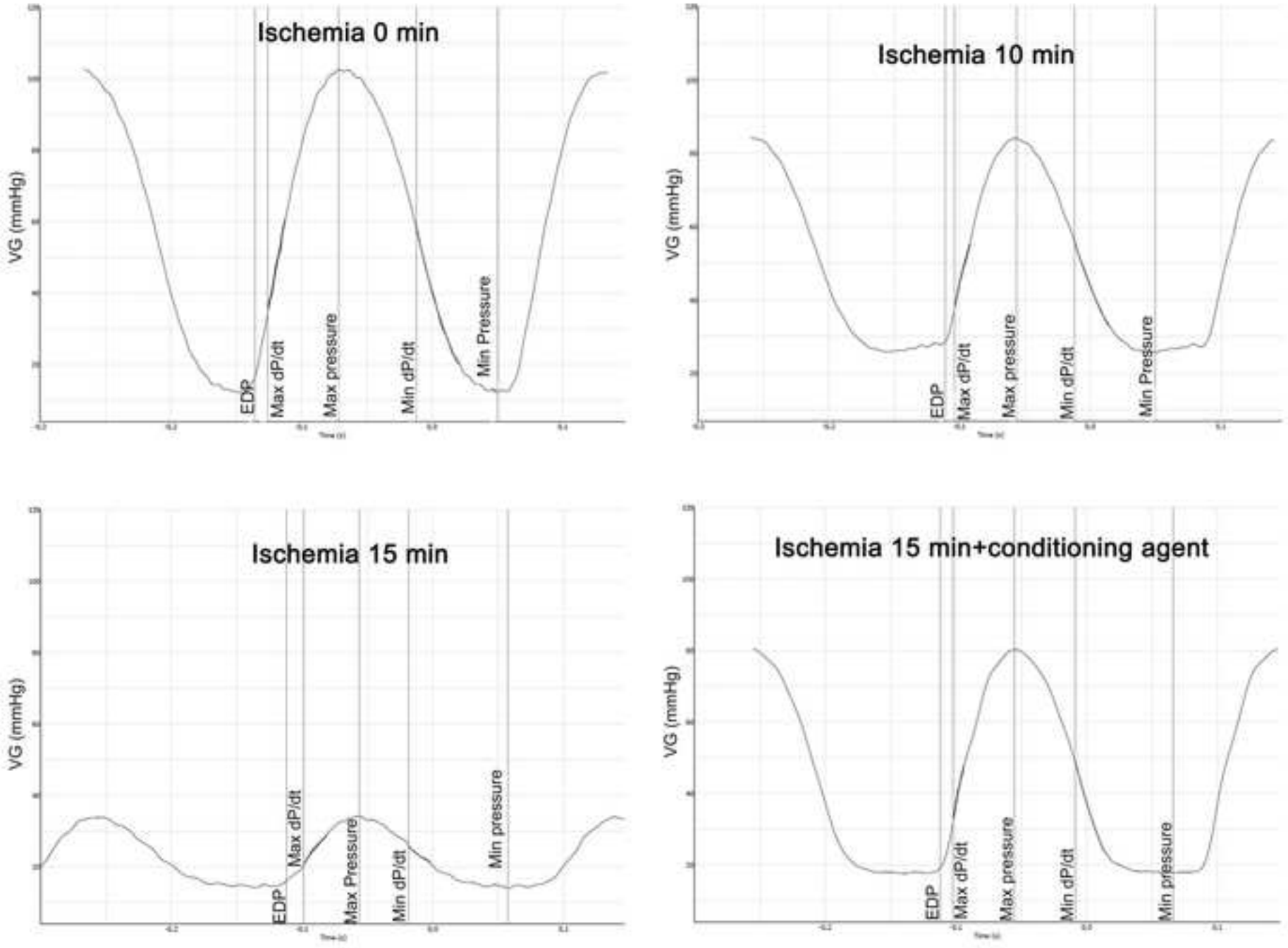

Figure 5

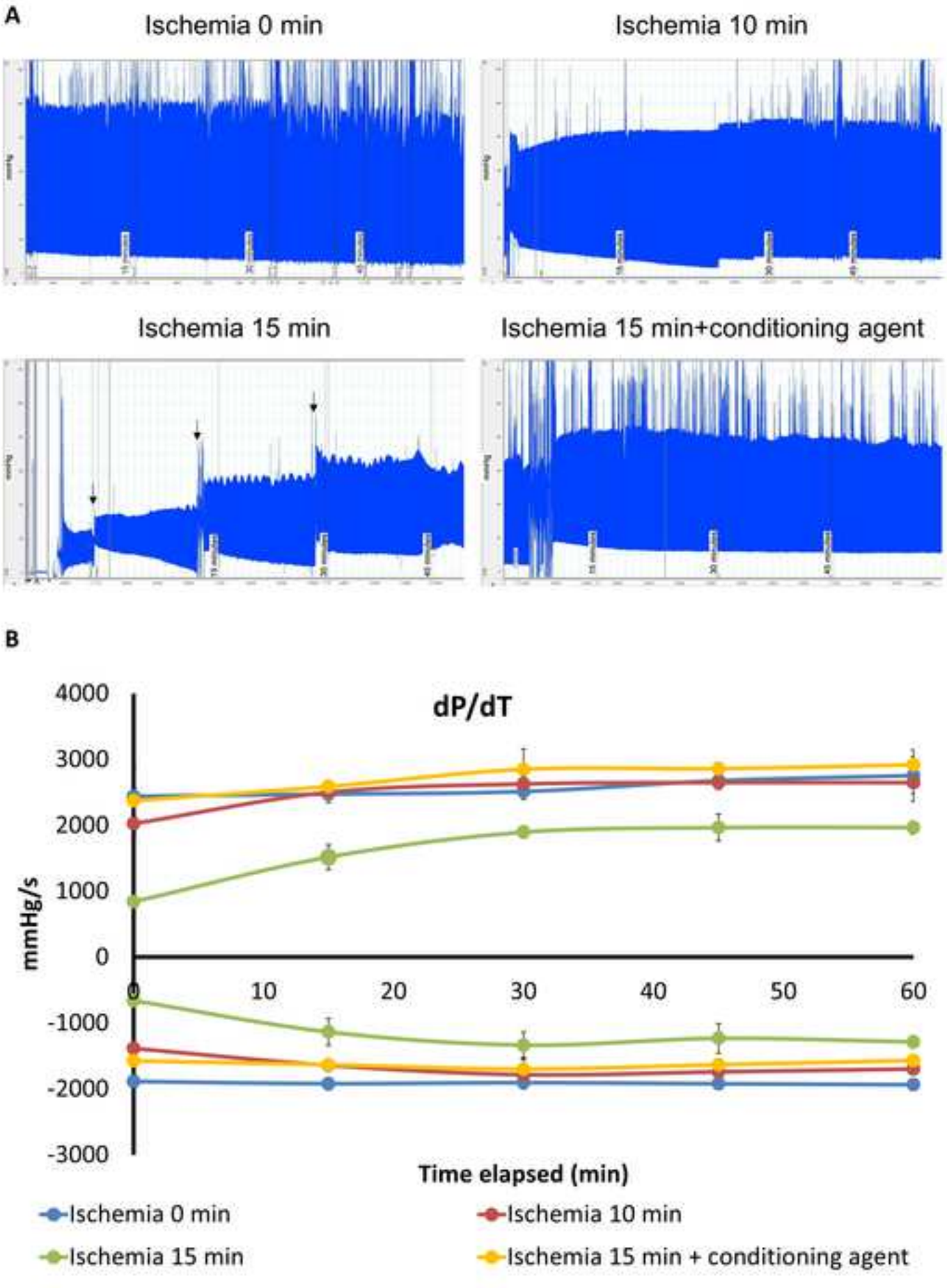

Figure 6

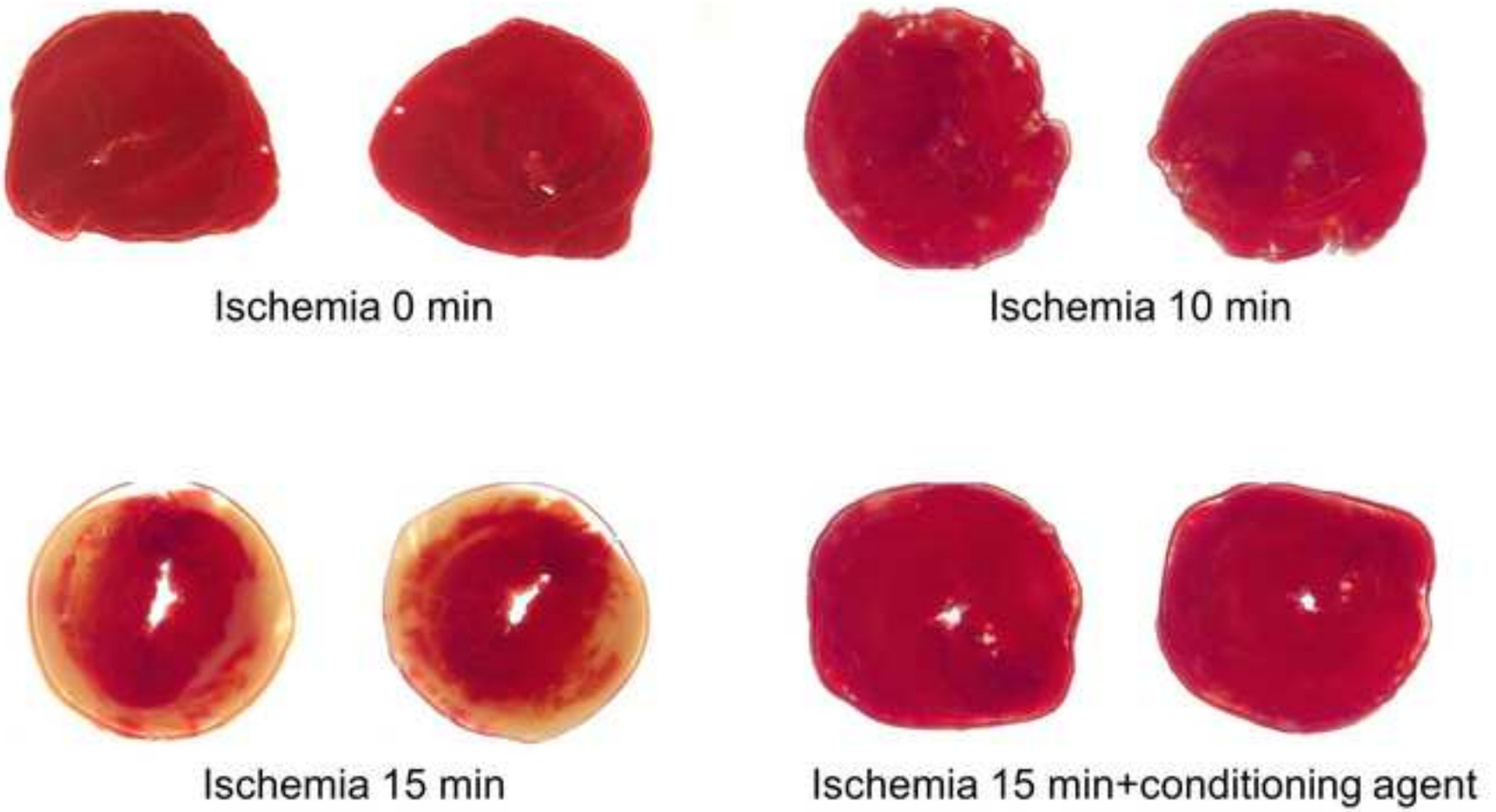

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number
0,9% Sodium Chloride. 1L bag	Baxter	
14G 2" I.V catheter	Jelco	4098
2,3,5-Triphenyltetrazolium chloride	Milipore-Sigma	T8877
22G 1" I.V catheter	BD	383532
Adson Dressing Fcp, 4 3/4", Serr	Skalar	50-3147
Alm Self-retaining retractor 4x4 Teeth Blunt 2-3/4"	Skalar	22-9027
Bridge amp	ADinstruments	FE221
Calcium chloride	Milipore-Sigma	C1016
D-(+)-Glucose	Milipore-Sigma	G8270
DIN(8) to Disposable BP Transducer	ADinstruments	MLAC06
Disposable BP Transducer (stopcock)	ADinstruments	MLT0670
dPBS	Gibco	14190-144
Eye Dressing Fcp, Str, Serr, 4"	Skalar	66-2740
Formalin solution, neutral buffered, 10%	Milipore-Sigma	HT501128
Heating Pad	Sunbean	756-CN
Heparin sodium 1000 UI/mL	Sandoz	
Hydrochloric Acid 36,5 to 38,0%	Fisher scientific	A144-500
Ketamine	Bimeda	
LabChart	ADinstruments	
Left ventricle pressure balloon	Radnoti	170404
Lidocaine HCl 2% solution	AstraZeneca	
Magnesium Chloride ACS	ACP Chemicals	M-0460
Micro pressure sensor	Radnoti	159905
Pacemaker	Biotronik	Reliaty
pH bench top meter	Fisher scientific	AE150
Physiological monitor	Kent Scientific	Physiosuite
Plasma-Lyte A	Baxter	
Potassium Chloride	Milipore-Sigma	P4504
Potassium Chloride 2 meq/ml	Hospira	
PowerLab 8/30 Polygraph	ADinstruments	
Silk 2-0	Ethicon	A305H

Silk 5-0	Ethicon	A302H
Small animal anesthesia workstation	Hallowell EMC	000A2770
Sodium bicarbonate	Milipore-Sigma	S5761
Sodium Chloride	Milipore-Sigma	S7653
Sodium Hydroxide pellets	ACP chemicals	S3700
Sodium phosphate monobasic	Milipore-Sigma	S0751
Stevens Tenotomy Sciss, Str, Delicate, SH/SH, 4 1/2"	Skalar	22-1240
Tissue slicer blades	Thomas scientific	6727C18
Tuberculin safety syringe with needle 25G 5/8"	CardinalHealth	8881511235
Veterinary General Surgery Set	Skalar	98-1275
Veterinary Micro Set	Skalar	98-1311
Working Heart Rat/Guinea Pig/Rabbit system	Radnoti	120101BEZ
Xylazine	Bayer	

Comments/Description

Electrolyte solution for flushing in the modified Langendorff system.
To act as endotracheal tube.
Vital coloration
I.V catheter with extension tube that facilitates manipulation for carotid catheterization
Additional forceps for tissue manipulation
Tissue retractor used to maintain the chest open.
Bridge amp for intracarotid blood pressure measurement
$CaCl_2$ anhydrous, granular, ≤7.0 mm, ≥93.0% Part of the Krebs solution
D-Glucose ≥99.5% Part of the Krebs solution
Adapter cable for link between bridge amp and pressure transducer
Pressure transducer for intracarotid blood pressure measurement
Electrolyte solution without calcium or magnesium.
Additional forceps for tissue manipulation
Fixative solution

For systemic anticoagulation
Diluted 1:1 for pH correction
Anesthetic. 100 mg/ml
Control software for the Powerlab polygraph, allowing off-line analyses. Version 7, with blood pressure and PV loop modules enabled
In latex. Size 4.
Antiarrhythmic for the cardioplegic solution
$MgCl_2$+$6H_2O$ ≥99.0% Part of the Krebs solution
Micro pressure sensor and amplifier connected to the intraventricular balloon
Set to generate a pulse each 200 ms for a heart rate of 300 bpm.

For continuous monitoring of rodent temperature and saturation during the procedure
Electrolyte solution used as base to prepare cardioplegia
KCl ≥99.0% Part of the Krebs solution
Part of the cardioplegic solution
Electronic polygraph
Suture material for Langendorff apparatus

Suture material for carotid
Small animal ventilator
$NaHCO_3$ ≥99,5% Part of the Krebs solution
NaCl ≥99.5% Part of the Krebs solution
Diluted to 5N (10 g in 50 ml) for pH correction
NaH_2PO_4 ≥99.0% Part of the Krebs solution
Small scisors for atria and cava vein opening
Straight carbon steel blades for tissue slicing at the end of the protocol
For heparin injection
Surgery instruments including disection scisors and mosquito clamps
Surgery instruments with microscisors used for carotid artery opening
Modular working heart system modified for the needs of the protocol. Includes all the necesary tubbing, water jacketed reservoirs and valves
Sedative. 20 mg/ml

s, including 2 and 3 way stop cock

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Author(s):	Henry Aceros, Leyla Joulali, Mélanie Borie, Roberto Vanin Pinto Ribeiro, Mitesh Vallabh Badiwala, Shant der Sarkissian, Nicolas Noiseux.

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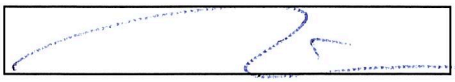
Signature:	 Date: February 01, 2019

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Montreal, March 26, 2019

Dr Philip Steindel, PhD.

Review Editor, JOVE.

Subject: Answer to reviewers. Submission JoVE59789.

Dear Dr. Steindel,

We greatly appreciate the editorial review of our manuscript. We have responded in detail to each of the reviewers' comments and made the necessary changes to the manuscript.

To facilitate review, the changes in the text are marked in red.

Reviewer 1:

Major Concerns:

None

Minor Concerns:

My suggestions for the authors would be:

1. **If authors could present some actual pictures of the EVHP machine/ system, it would be of great benefit for other researchers for reproducibility.**

Response: A picture of each setup was added as supplementary figure 1.

2. **Similarly, actual pictures of heart procurement could be helpful to understand the procedure.**

Response: Pictures of the major procurement steps were added as supplementary figure 2 to 4.

3. **Although authors have mentioned use of anaesthetics in the limitation section, other factors such as high heart rate and small size of animals need to be mentioned in this section.**

Response: In the limitations paragraph the following text has been added:

"*Finally, the small size and high heart rate of this animal model may be considered as a limitation due to the potential technical difficulties observed while performing these experiments, and the inevitable physiological differences between rat and human hearts. If the EVHP evaluation is already standardized, a researcher can be familiarized with this technique by performing at as little as 3 experiments. On the other hand, the use of this small animal model allows for convenient screening at a reasonable cost, reserving larger and more costly animal models such as the porcine model, to therapies with high human translational potential.*"

4. **Figure 5b could be explained in detail**

Response: The figure 5 legend now reads:

Figure 5: Ex vivo recovery and functional analyses. (A) *Continuous ventricular pressure-time curve after 10 minutes stabilization and perfusion with or without the use of an experimental pharmacological cardioprotective conditioning agent. Arrows show artifacts due to manual modification of EDP.* ***(B)*** *Maximum (+dP/dt) and minimum (-dP/dt) rate of pressure change in the LV vs. time plot derived from* ***(A)*** *showing a time-dependant improvement in contractility without treatment (green line). Short WIT (red line) or treated (yellow) hearts show a pattern similar to the control group (blue line). Data points are the mean of at least 200 individual beats. Bars show the standard error of the mean of each data point.*

5. **More emphasis needs to be given on functional assessment of the DCD heart. Mere mentioning of dP/dT is insufficient from readers point of view. Try to dedicate a paragraph for this purpose.**

Response: In the subheading data analysis, paragraph 6.2 now reads:

"*6.2 For pressure analyses, select at least 200 pressure cycles per time points. Analyses can be performed off-line (after completion of the experiment) using dedicated software (i.e. LabChart). Common cardiovascular parameters available include: Maximal generated pressure, end diastolic pressure, +dP/dt (steepest slope during the upstroke of the pressure curve, an indicator of ventricular contractile ability), -dP/dt (steepest slope during the downstroke of the pressure curve, an indicator of ventricular relaxation capacity) among others.*"

Also, the fourth paragraph of the discussion was changed as follows:

"The infusion of cardioplegic solution at constant physiological pressure and temperature offers a unique opportunity to initiate heart conditioning and tissue protection with any pharmacological agent or by other means. Technical refinements include clamping the thoracic aorta, limiting perfusion to the heart and thereby reducing the amount of solution needed for each essay. Once the heart is on the EVHP system, standardized functional evaluation is necessary. It has been shown that the use of an EVHP system has the potential to improve resuscitation of hearts previously considered not transplantable[23,24]. Interestingly, the clinically available EVHP system evaluates cardiac viability only by using serial lactate measurements[8,23]. Lactate measurements are not related to cardiac performance of DCD hearts[24,25], thus additional measurements to evaluate transplantability are necessary. This experimental setup allows for a complete functional evaluation, including generated pressures and myocardial contractility measurements including +dP/dT and –dP/dT, allowing for a more thorough evaluation of cardiac function before the final transplant decision is made. Additionally, measurements of cardiac troponin, a marker of myocardial damage directly correlated to ischemic infarct size[26], and whose release kinetics are related to the extent of cardiac ischemia in a Langendorff ischemia/reperfusion system, in particular, with long ischemic times (60 minutes), troponin levels are maintained after 1h reperfusion, while LDH and creatinine kinase significantly decrease, and being non related to the extent of cardiac damage[27,28], thus the use of serial troponin measures ensure a complete evaluation of organ viability before transplant. A major confounding variable in cardiac functional evaluation is the heart rate. Spontaneous heart rate is inversely related to length of ischemia[29], and heart rate directly correlates with +dP/dt in isolated rat hearts[30] and in animal models[31]. Interestingly, in recently published work on rodent models of DCD hearts and EVHP conditioning, pacing was not used and cardiac rates were variable and recorded in their protocols[15,18,20]. To maintain physiological heart rate, pacing was used once the heart had recovered rhythmic contraction. The chosen 300 bpm frequency is similar to those of healthy, non-stressed rats[32]."

Finally, since in larger models the working heart preparation is going to be essential for full evaluation, in the limitations paragraph the following phrase was added:

"*Nevertheless, in this setup cardioplegia temperature can be easily regulated according to the requirements of the investigator. On the other hand, the use of a Langendorff preparation versus a working heart preparation for reconditioning might also be seen as a limitation. The working heart preparation allows for continuous recording of a pressure/volume loop[12,15], with controlled pre and afterload, allowing for complete functional evaluation.*"

6. **Evaluation of Injury to heart in terms of troponin T and infarct size could be discussed in bit more details.**

Response: In the data analysis the following subheading was added:

"6.3 For troponin analyses, an increase in troponin release at reperfusion is expected. After 1 h of reperfusion in the ESHP system, troponin levels may decrease to baseline, stressing the need for careful timing in the collection and handling of these samples."

The changes made in the fourth paragraph of the discussion also address this point.

7. **Can authors provide their future plans with this model?**

This model is to be used in our laboratory for the evaluation of different pharmacological approaches to cardioprotection added with the cardioplegia and at the first moments of EVHP. Those plans include evaluation and combination of pharmacological conditioning with non-pharmacological strategies based on collaborations with other research groups. The timing of the intervention (medication on the cardioplegia only, on the first minutes of EVHP, on the whole experience), and the use of other strategies, including cold vs. warm cardioplegia, different cardioplegia solutions and different reconditioning times are to be evaluated. The best cardioprotective conditions will be upscaled to porcine and finally human models.

Once again, we thank you for your thoughtful suggestions.

Reviewer 2:

Major Concerns:

No major concerns

Minor Concerns:

1. **In section 1. Preliminary preparations, subheading 1.2, the authors comment on a 'wrong pH' in the cardioplegic solution. This comment requires clarification as it has previously been shown in both large and small animal models that a slightly acidic pH may in fact be cardioprotective.**

Response: We agree with the reviewer that initial reperfusion with a slightly acidic solution is considered cardioproctective. Nevertheless, the cardioplegic solution described has a tendency to move towards a more extreme pH level (7 or less) following the addition of KCl and corrections need to be made before use. To clarify the safe range that we have used in our experiences the text has been changed:

"*This model is highly sensitive to pH. A wrong pH correction (outside the 7.3-7.4 physiological range) or pH unstable solutions may compromise the experiment or provide unreliable data.*"

2. **The authors should adopt the "ex situ" nomenclature rather than "ex vivo" as it more accurately reflects that the perfused organ is out of a deceased donor, not a live subject.**

Response: The reviewer raises a point about the correct use of the terms *ex vivo* and *ex situ*. Accessing pubmed on March 20, no articles were found using the search terms "ex situ" AND "Langendorff", while 302 are found using "ex vivo" AND "Langendorff". Similarly, "ex vivo" AND "DCD" gives 110 articles, while "ex situ" AND "DCD" finds 15 articles, "ex vivo" and "heart" gives 4873 articles, while "ex situ" and "heart" only finds 44 articles. Finally, the only currently clinically used cardiac perfusion system (TransMedics OCS Heart) also uses the *ex vivo* terminology on its webpage (accessed on March 20 2019. http://www.transmedics.com/wt/redirect/heart_preservation_med.html), as well as a Tevosol, a Canada-based company developing *ex vivo* organ perfusion systems (http://www.tevosol.com/ex-vivo-heart-liver-lung-perfusion/). Based on this information, we decided to maintain the use of the *ex vivo* terminology, in order to follow the literature standard and facilitate the finding of the article in databases.

3. **Secondly, there is reference to "cardiac death" which should be changed to "circulatory death".**

Response: We agree with the reviewer; the text has been reviewed accordingly. Now it reads circulatory death.

4. **Thirdly, the authors may wish to briefly discuss/contrast this approach to DCD heart reperfusion and evaluation with an NRP model described by Ali et al in J Transl Med Feb 2014.**

Response: The following text has been added to the discussion, limitations paragraph:

"Alternatively, cardiac reanimation can be performed in situ using normothermic regional perfusion, with cardiac performance being measured directly by the use of a Millar catheter[34] allowing comprehensive hemodynamic and myocardial functional evaluation before organ procurement. In humans, both in situ

and ex vivo reconditioning strategies have been described[6], thus the development of both models allows for experimental comparisons that may translate in to optimization of clinical practice."

Reference 34 is the article that the reviewer suggested.

Once again, thank you for your kind suggestions. We hope to have addressed them adequately.

Reviewer 3.

Major Concerns:

1. **It is defined in Lines 164-165 that WIT starts "When peak systolic blood pressure drops below 30 mmHg, asystole or ventricular fibrillation (whatever comes first) is observed". However, I think generally WIT starts much earlier either at the time of extubation or when BP becomes below 80 mmHg for example. In fact, when asystole happens, that is the time of "cardiac death" and again WIT starts much earlier. The authors need to clearly define what the WIT is, and when it starts and ends.**

Response: We fully agree with the reviewer in the fact that tissue ischemia begins during the agonal phase at the moment when perfusion pressure is not sufficient to support gaseous and nutrient exchange. Various definitions of warm ischemic time have been proposed and different transplant centres adopt different definitions. More recently, Messer et. al. have defined functional warm ischemia time (WIT) as the period between the moment systolic blood pressure falls below 50 mmHg and reperfusion (Messer S, Page A, Axell R, Berman M, Hernández-Sánchez J, Colah S, et al. Outcome after heart transplantation from donation after circulatory-determined death donors. J Heart Lung Transplant. 2017 Dec;36(12):1311–8.). Alternatively, the Australian group has been using a definition of WIT measured from when SBP falls below 90 mmHg. Extrapolating these definitions to our small animal model, we have opted to consider that functional warm ischemia time starts at the moment of circulatory death or when systolic blood pressure is below 30 mmHg and ends at the moment of cardioplegia, as described by Kearns (Kearns MJ, Miller SD, Cheung A, Bashir J, Wong S, Seidman MA, Boyd JH. A Rodent Model of Cardiac Donation After Circulatory Death and Novel Biomarkers of Cardiac Viability During Ex Vivo Heart Perfusion. Transplantation. 2017 Aug;101(8):e231-e239.), since in this model blood pressure decreases rapidly after extubation, with decrease from the 50 mmHg point to the published 30 mmHg within 1 minute, thus making little difference in the final warm ischemia time recorded. To avoid misinterpretation the step 3.1 was changed as follows:

"*3.1 Turn off the ventilator and extubate the animal. Using mosquito forceps, clamp the trachea. This moment is considered as the start of the agonal phase. Start counting the functional warm ischemic time (WIT) when the peak systolic blood pressure drops below 30 mmHg, or if asystole or ventricular fibrillation appears, whatever comes first (Figure 3). Damage extent should be proportional to WIT. Experiments are needed to optimize WIT time according to animal strain, sex and weight chosen. In control animals, immediately after carotid vascular access is secured, cardioplegia is injected and the heart is procured as described in the next step (Figure 2). The start of perfusion with cardioplegia is considered as the end of WIT.***"**

The discussion was also modified. At the end of the 3rd paragraph the following text was added:

"To increase reproducibility, we elected for tracheal clamping, combined with a precise arterial pressure monitorization, allowing for a more homogeneous agonal time (<5 minutes). It is known that organ damage starts before the moment of circulatory death; with some authors considering a cut-off systolic blood pressure below 50 mmHg as the beginning of functional WIT[6], explaining the reluctance to transplant organs following a long period form withdraw of life sustaining measures until reperfusion. In this protocol, the WIT definition used follows the current experimental standard[15], nevertheless, further studies are needed to clarify the exact set of hemodynamic parameters that mark the induction of organ damage in order to improve WIT calculation, thus offering better information for clinical practice."

2. **Related to above, in Lines 265-266, it is defined that "Cardiac death is considered when maximal blood pressure is below 30 mmHg or if no pulsatile activity is observed", and generally I do not think "cardiac death" is the start time for WIT, and please clarify the "definition of WIT" and "definition of cardiac death".**

Response: The text was modified as follows:

Figure 3: Intracarotid blood pressure/time plot. *Typical evolution of intracarotid blood pressure following extubation. Warm ischemia time stars when the peak systolic blood pressure drops below 30 mmHg, or if asystole or ventricular fibrillation appears, whatever comes first.*

3. **Furthermore, as long as heart is beating, then it is not considered as cardiac death (at least in clinical human care practice settings), and please discuss whether "maximal blood pressure is below 30 mmHg" can be considered as cardiac death. This may only apply for animal experimental settings, to obtain homogenous data, but again this will not apply for clinical human care settings. As we can see in Figure 3, even after"maximal blood pressure is below 30 mmHg", heart is still generating pulse pressure, and is beating, so this is not cardiac death yet.**

Response: We agree with the reviewer that the definition of cardiac death vs. WIT was not clear. The text changes in response to the previous comments address this situation.

4. **In Lines 250-251, it is described as "If insufficient WIT is allowed, contractility will return to normal, and morphological damage will not be detectable". Does "insufficient WIT" mean "shorter WIT"? I think it is because in Lines 165-166, it is stated as "Damage extent should be proportional to WIT". Please clarify. "insufficient" may not be the best word to describe this condition.**

Response: The text was modified. It now reads *"Figure 4 shows an average pressure/time curve at the start of reconditioning following 0, 10 and 15 minutes of WIT. Contractile function will improve over time. The use of short periods of WIT will allow for contractility to return to normal, and morphological damage will not be detectable (Figure 5 and 6)."*

Minor Concerns:

1. **Line 170. It is described as "median thoracotomy" and is this actually "median sternotomy"?**

Response: The text has been changed as suggested.

Once again, thank you for your kind suggestions.

Conclusion:

Thank you very much for considering our manuscript for publication. We have addressed the reviewer's questions and made the proper modifications according to their suggestions.

In summary, we feel that the reviewers' thoughtful suggestions have significantly improved the quality of our submission. We look forward to hearing your thoughts and are at your disposal to address future comments.

Sincerely,

_____

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Supplemental Figure 1

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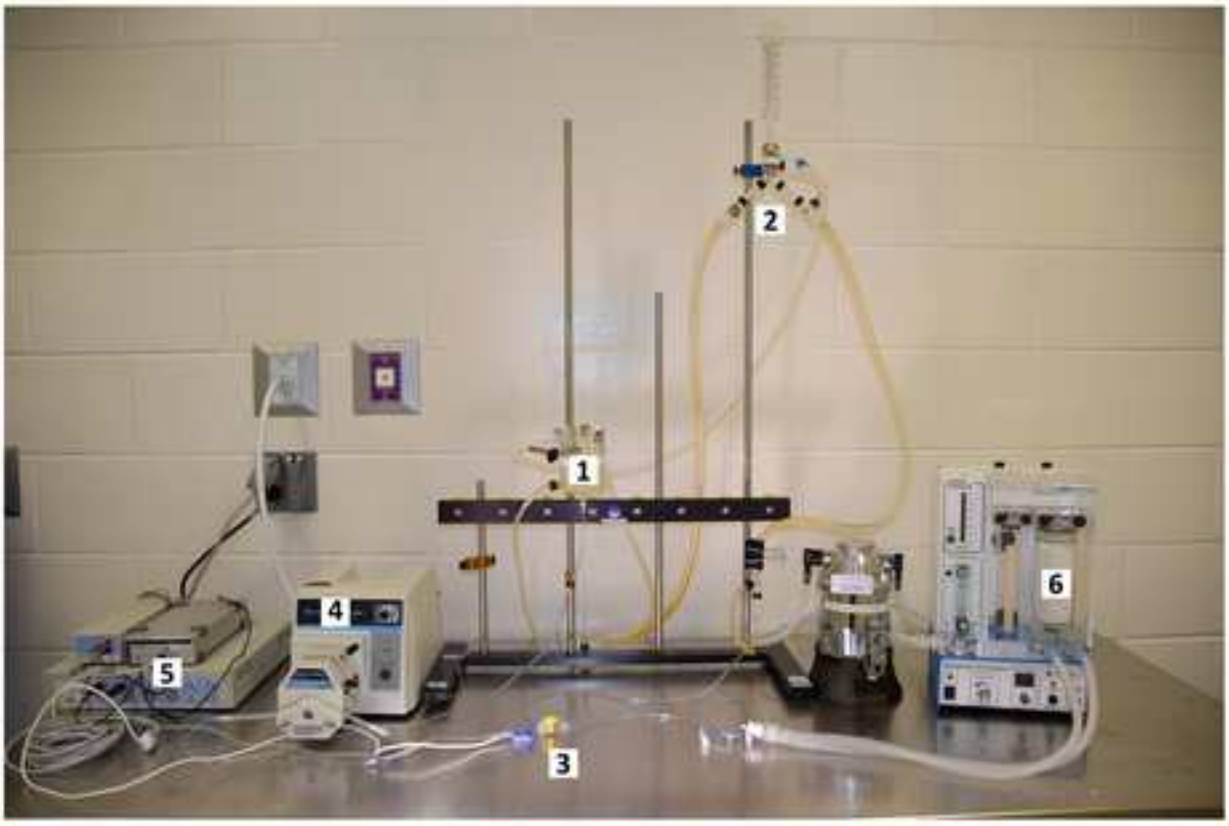

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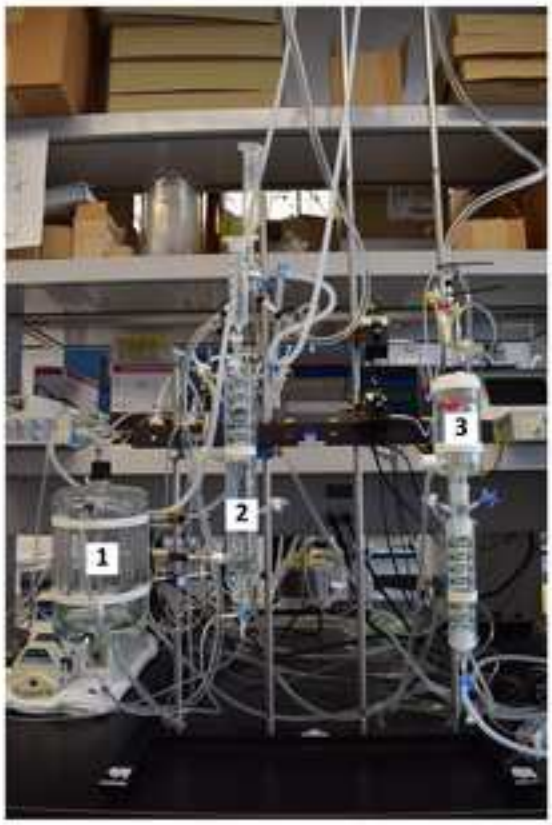

Supplemental Figure 2

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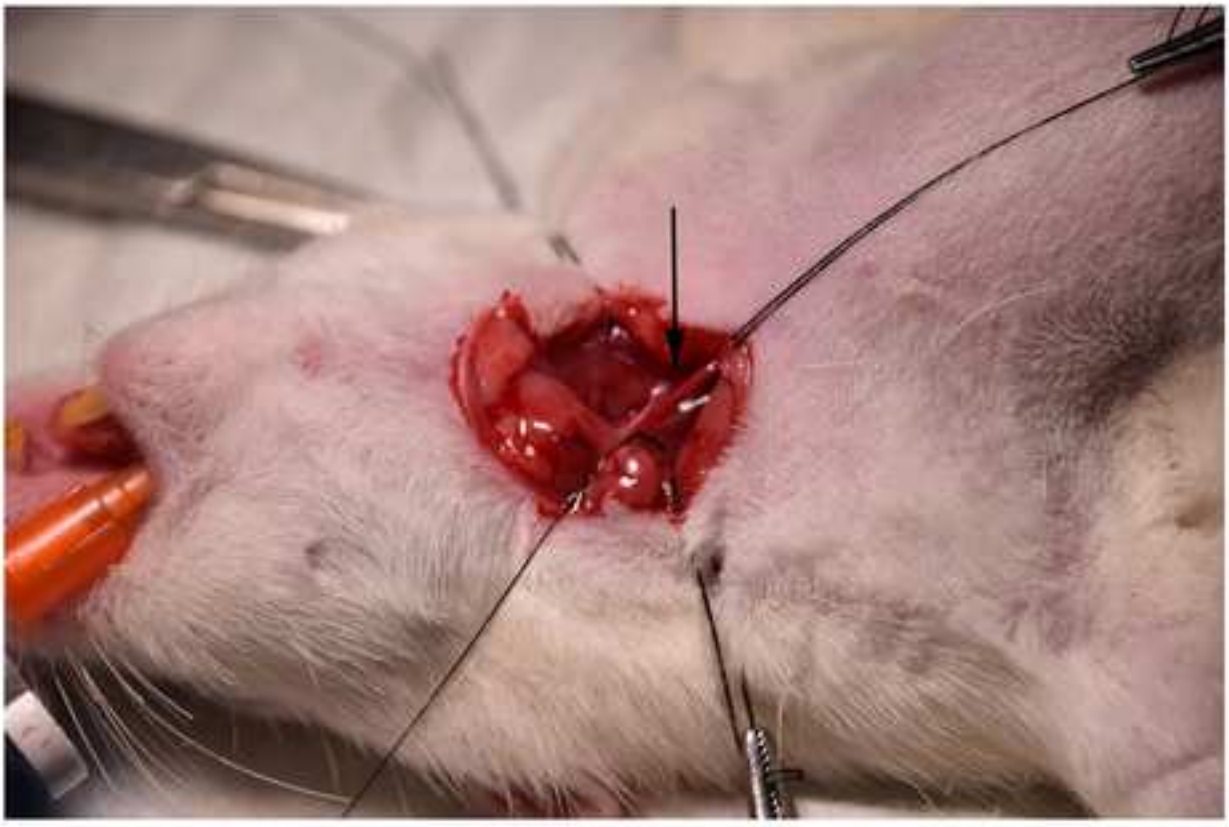

Supplemental Figure 3

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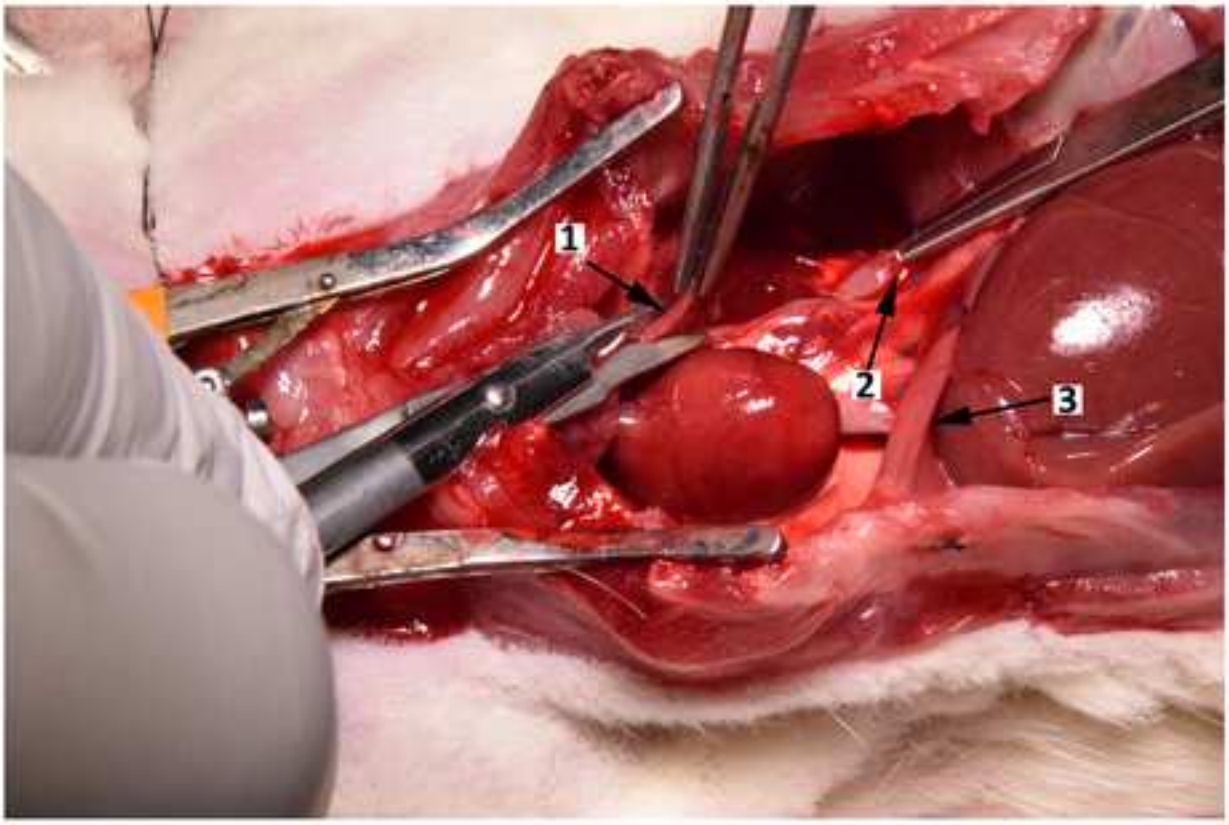

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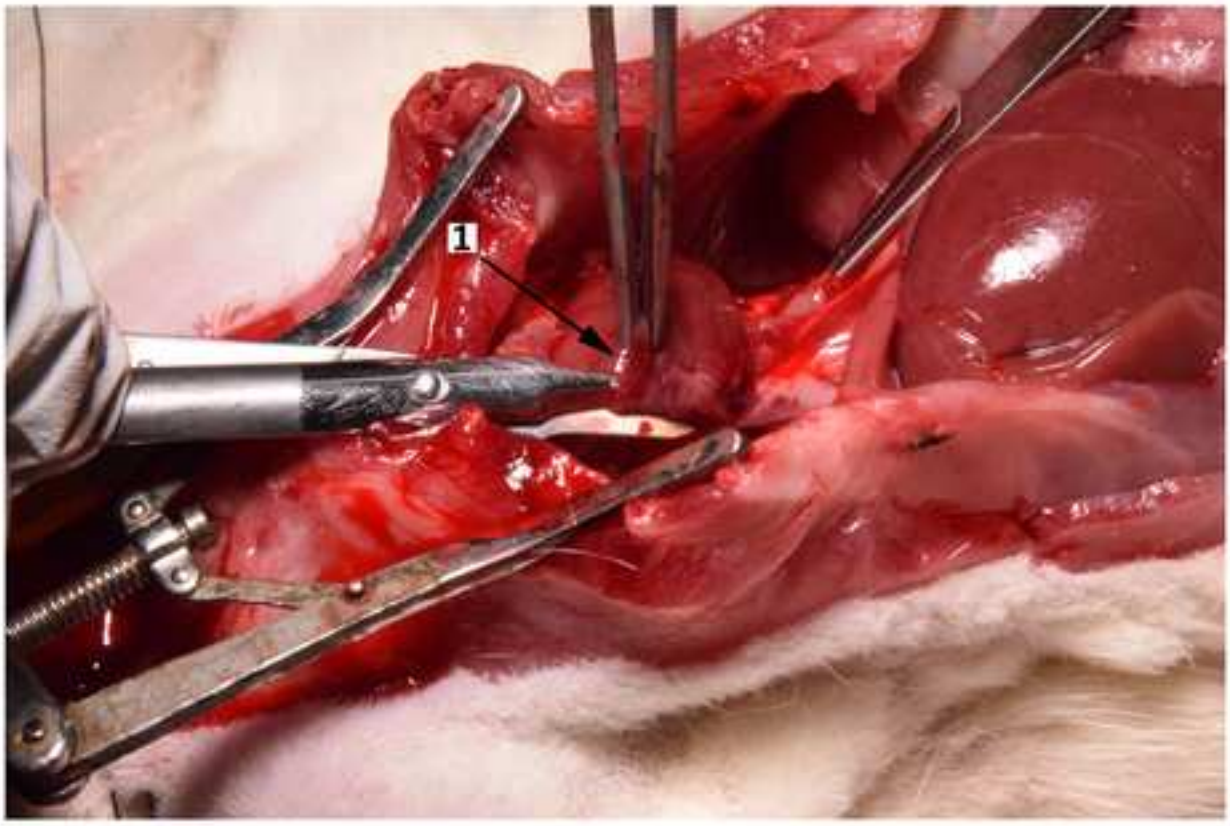

Supplemental Figure 4

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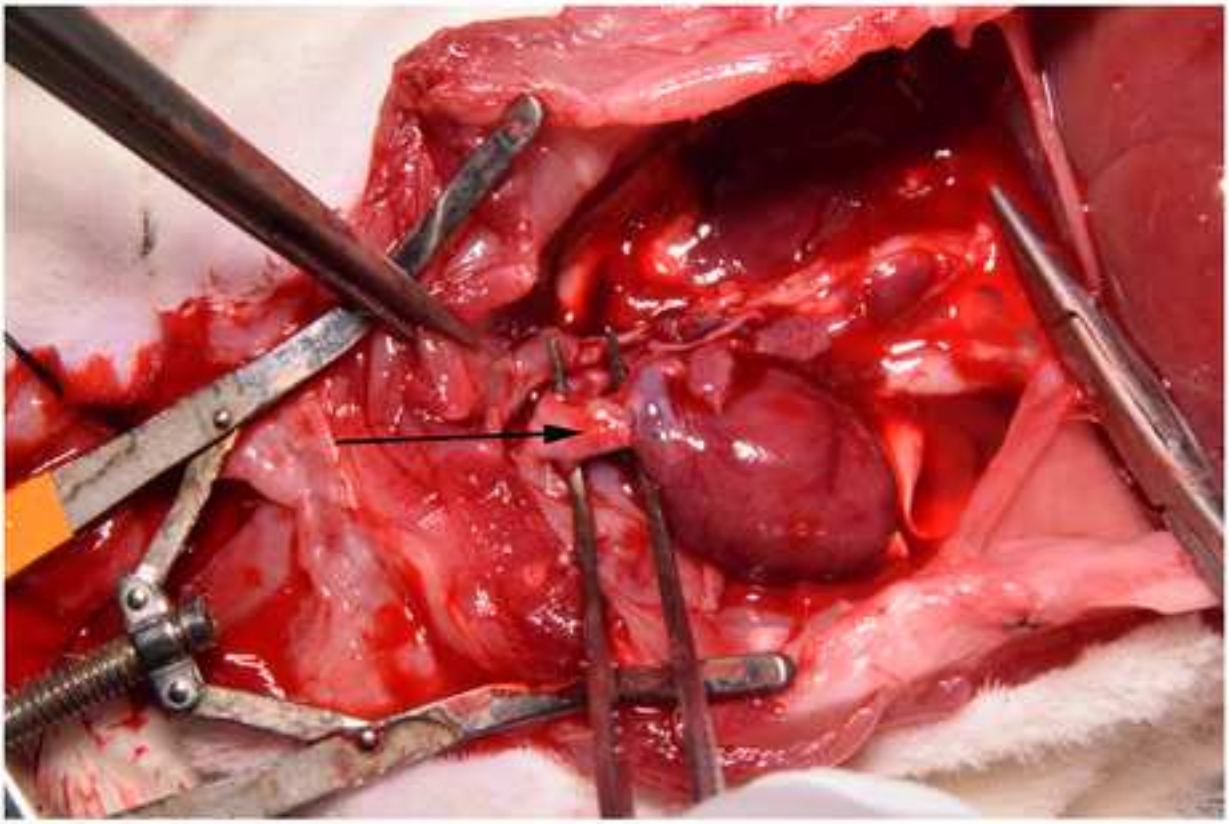

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