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May 03, 2018

Ronald Myers, Ph.D.

Senior Science Editor, *Journal of Visualized Experiments*

Dear Dr. Myers,

On behalf of my coauthors, I am pleased to submit our manuscript entitled "Method of normothermic *ex vivo* heart perfusion in working mode: assessment of cardiac function and metabolism" to be considered for publication in *Journal of Visualized Experiments* as a method article.

We believe this manuscript would be of interest to the readers/viewers of JoVE because:

- *Ex vivo* heart perfusion can be considered as a novel method for preservation, monitoring, and potentially improving of the donated hearts
- Introducing a reliable, reproducible research method/apparatus for *ex vivo* heart perfusion will be beneficial for the researchers in this field.

This manuscript is not under consideration for publication elsewhere, nor has it been previously published. All authors have read and approved the manuscript, all relationship with industry have been fully disclosed.

Thank you for your consideration. We look forward to hearing from you.

Sincerely,

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Title:

Normothermic *Ex Situ* Heart Perfusion in Working Mode: Assessment of Cardiac Function and Metabolism

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Keywords:
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Summary:
Normothermic *ex situ* heart perfusion (ESHP), preserves the heart in a beating, semi-physiologic state. When performed in a working mode, ESHP provides the opportunity to perform sophisticated assessments of donor heart function and organ viability. Here, we describe our method for myocardial performance evaluation during ESHP.

Abstract:
The current standard method for organ preservation (cold storage, CS), exposes the heart to a period of cold ischemia that limits the safe preservation time and increases the risk of adverse post-transplantation outcomes. Moreover, the static nature of CS does not allow for organ evaluation or intervention during the preservation interval. Normothermic *ex situ* heart perfusion (ESHP) is a novel method for preservation of the donated heart that minimizes cold ischemia by providing oxygenated, nutrient-rich perfusate to the heart. ESHP has been shown to be non-inferior to CS in the preservation of standard-criteria donor hearts and has also facilitated the

clinical transplantation of the hearts donated after the circulatory determination of death. Currently, the only available clinical ESHP device perfuses the heart in an unloaded, non-working state, limiting assessments of myocardial performance. Conversely, ESHP in working mode provides the opportunity for comprehensive evaluation of cardiac performance by assessment of functional and metabolic parameters under physiologic conditions. Moreover, earlier experimental studies have suggested that ESHP in working mode may result in improved functional preservation. Here, we describe the protocol for *ex situ* perfusion of the heart in a large mammal (porcine) model, which is reproducible for different animal models and heart sizes. The software program in this ESHP apparatus allows for real-time and automated control of the pump speed to maintain desired aortic and left atrial pressure and evaluates a variety of functional and electrophysiological parameters with minimal need for supervision/manipulation.

Introduction:

Clinical relevance

While most aspects of cardiac transplantation have evolved significantly since the first heart transplant in 1967, cold storage (CS) remains the standard for donor heart preservation[1]. CS exposes the organ to a period of cold ischemia that limits the safe preservation interval (4-6 hours) and increases the risk of primary graft dysfunction[2-4]. Due to the static nature of CS, assessments of function or therapeutic interventions are not possible in the time between the organ procurement and transplantation. This is a particular limitation in extended criteria donors including hearts donated after circulatory death (DCD), creating an obstacle for overcoming the considerable gap between demand and the current donor pool[5,6]. To address this limitation, *ex situ* heart perfusion has been proposed as a novel, semi-physiologic method of preserving donated hearts, minimizing exposure to cold ischemia by providing oxygenated, nutrient-rich perfusate to the heart during preservation time[1,7,8].

***Ex situ* heart perfusion**

One of the most frequently used methods for *ex situ* examination of the isolated heart is Langendorff perfusion. In this method, introduced by Oskar Langendorff in 1895, the blood flows into the coronary arteries and out the coronary sinus of the isolated heart, with the heart in an empty and beating state[9,10]. Clinical ESHP in a Langendorff mode with the Transmedics Organ Care System apparatus (OCS) has been shown to be non-inferior to CS in the preservation of standard-criteria donor hearts[1], and has facilitated the clinical transplantation of DCD hearts. [11] However, there are concerns about the ability of the device to evaluate organ viability, as a number of donor hearts initially thought to be transplantable were discarded after perfusion on the OCS[3]. The OCS supports the heart in the Langendorff (non-working) mode, and thus possesses a limited capacity for evaluation of the pumping function of the heart[3,12]. A growing body of evidence suggests that functional parameters offer a better way to assess organ viability, suggesting that assessments of cardiac function may become a reliable tool for the evaluation and selection of hearts for transplantation during ESHP[3,12-14], Furthermore, our studies on *ex situ* perfused porcine hearts suggest that ESHP in working mode provides enhanced functional preservation of the heart during the perfusion interval[15,16].

An ESHP apparatus capable of preserving the heart in a working mode must possess a level of automation to safely and precisely maintain preload, afterload and flow rates. Also, such a system should possess the flexibility to facilitate comprehensive assessments of cardiac function to be undertaken. The ESHP apparatus used here is equipped with custom software that 1) provides and maintains desired aortic (Ao) and left atrial (LA) pressure/flow and 2) provides real-time analysis of functional parameters and visual evaluation of pressure waveforms with minimal need for supervision. Pressure data is acquired with standard fluid-filled pressure transducers, and flow data is acquired with transit-time doppler flow probes. These signals are digitized with a bridge and analog input, respectively. The heart is positioned horizontally with a slight elevation to the great vessels on a soft silicone membrane. The cannulation attachments pass through the membrane, incorporating a compliance chamber for dampening ventricular ejection. The goal of this work is to provide researchers in the field of cardiac transplantation with a protocol for *ex situ* perfusion and evaluation of the heart, under normothermic, semi-physiologic conditions in working mode, in a large mammal (Yorkshire pig) model.

Protocol:

All the procedures in this manuscript were performed in compliance with the guidelines of the Canadian Council on Animal Care and the guide for the care and use of laboratory animals. The protocols were approved by the institutional animal care committee of the University of Alberta. This protocol has been applied in female juvenile Yorkshire pigs between 35-50 kg. All individuals involved in ESHP procedures had received proper biosafety training.

1. Pre-surgical Preparations

1.1. Place the organ chamber properly on the apparatus cart and install the silicon support membrane inside the organ chamber. The Ao, pulmonary artery (PA) and LA connection points can be seen in **Figure 1**.

1.2. Install the ESHP tubing network (represented in **Figure 2A,B**) oxygenator and filter. Attach the heat exchanger water lines and the sweep gas tubing to the oxygenator.

1.3. Place the flow probes for measuring coronary sinus/PA and LA flow on the corresponding tubing.

1.4. Connect the Ao and LA pressure transducers to the representative lines on the circuit.

1.5. Ensure that all the tubing connections are firmly attached and all the stopcocks and luer locks are properly closed on the unattached sites.

1.6. Prime the circuit with 750 mL of modified Krebs-Henseleit buffer (NaCl, 85; KCl, 4.6; $NaHCO_3$, 25; KH_2PO, 1.2; $MgSO_4$, 1.2; glucose, 11; and $CaCl_2$, 1.25 mmol/L) containing 8% albumin. De-air the Ao and LA pumps by positioning the pump outlet above the inlet so that the air leaves the pump chamber (**Figure 3**). The solution typically does not need to be oxygenized before the start of the perfusion.

1.7. Initiate the software after the Ao and LA pumps are de-aired and the circuit is primed.

2. ESHP Software Initialization and Adjustments

Note: The ESHP apparatus used here is equipped with a custom software program to allow control of pump speed in order to achieve and maintain desired LA and Ao pressures. The software also analyzes functional parameters and provides a visual evaluation of pressure waveforms (**Figure 4**).

2.1. To start the ESHP program, click on the program shortcut on the monitor.

2.2. In the "setting" page, click "initialize". The initializing message will appear on the board (**Figure 5**).

2.3. On the same page, zero the flow sensors clicking the "zero LA flow" and "zero PA flow". The message will appear on the board.

2.4. Adjust the height of the pressure transducers to the height of the silicon support**.** To zero the pressure transducers, open the Ao and LA pressure transducers (and any other transducers set to check the pressure) to the atmosphere, then click "zero all pressures" button. The message will appear on the board.

2.5. In the "main" page, increase the Ao pump speed gradually up to the point where flow from the Ao cannula appears in the organ chamber. In the present system, this is achieved with 900-1000 revolutions per minute (RPM).

2.6. Add 750 mL of blood to the perfusate solution to bring the total perfusate volume to 1.5 L (as described in the "*Surgery, harvesting blood, and heart procurement"* section) and then increase the LA pump PRM (800-900 RPM) so that no air remains in the LA cannula or the LA tubing beneath the silicone support membrane.

2.7. After initializing the controlling software and de-airing of the ESHP apparatus, donor heart procurement may proceed.

3. Preparations and Anesthesia

3.1. Administer 20 mg/kg of ketamine intramuscularly for premedication.

3.2. Transfer the pig to the surgical suite and place the pig on the operating table with tabletop heating to maintain normothermia.

3.3. Titrate oxygen flow rate for mask induction according to animal weight and the anesthetic system. For the closed-circle anesthetic circuits the oxygen flow should be 20 - 40 mL/kg.

3.4. Turn on isoflurane to 4 - 5%; after one or two minutes this may be reduced to 3%.

3.5. Evaluate the depth of anesthesia. The pig is in the surgical plane if there is no withdrawal reflex when a pinch is applied between the hooves or along the coronary band (pedal reflex).

3.6. After confirmation of the appropriate depth of anesthesia, proceed to intubation.

3.7. Place the pulse oximeter probe on the tongue (preferred) or ear. The oxygen saturation measured by pulse oximetry should remain above 90%.

3.8. Shave patches of hair off on the left and right elbow regions, and left stifle. Wash off skin oils with soap and water, rinse with rubbing alcohol and dry completely. Place the ECG contacts. Avoid lead wire interference with the surgical site. Connect the leads to the correct locations.

3.9. To maintain the anesthesia, adjust oxygen flow (20 - 40 mL/kg) and inhalant gas rate (1 - 3%). The heart rate should be 80 - 130 beats/min. Respiration rate should be 12 - 30 breaths/min.

3.10. Shave, wash and aseptically prepare the incision site.

4. Blood Collection and Heart Procurement

4.1. Evaluate the anesthesia level every minimum every 5 minutes to confirm the surgical plane (no pedal reflex and no blink reflex, no response to painful stimuli).

4.2. Perform a median sternotomy.

4.2.1. Identify jugulum and xiphoid as landmarks.

4.2.2. Using electrocautery, develop the midline between the landmarks by dividing the subcutaneous tissue and the fascia between the fibers of the pectoralis major muscle.

4.2.3. Mark the midline along the sternal bone with the cautery. Perform sternal osteotomy with an electric or air-powered saw. To prevent creating injuries to the underlying structures (*e.g.* pericardium and brachiocephalic vein, and innominate artery), proceed gradually with the saw.

4.2.4. Retract the sternum gradually, using a sternal retractor. To avoid excessive tension and vascular injury, do not place the retractor too far cranially.

4.2.5. Free the sternopericardial ligaments from the posterior surface of the sternum using cautery.

4.2.6. Open the pericardium with a Metzenbaum Scissor and fix the pericardial edges to the sternum using 1-0 silk suture.

4.3. Extend the midline incision cranially by 2-3 cm and expose the right common carotid artery and internal jugular vein.

4.4. Obtain proximal and distal control of the vessels by encircling the vessels with silk ties (2-0).

4.5. Tie the cranial encircling ties on each vessel.

4.6. Open the anterior 1/3 of each vessel with an 11-blade and then insert a 5-6 F sheath into each vessel. Tie the caudal encircling tie around each vessel to secure the respective sheathes.

4.7. Monitor the arterial and central venous pressures by connecting each sheath to a pressure transducer.

4.8. Deliver 1000 U/kg heparin intravenously.

4.9. Place a 3-0 polypropylene purse-string suture around the right atrial appendage and secure it with a snare.

4.10. Inside the purse-string suture, create a 1 cm incision on the appendage using an 11 blade. Insert a two-stage venous cannula (28/36 FR) inside the incision and position the distal tip in the IVC. Secure the cannula by tieing snare to the venous cannula. Control the outlet of the cannula with a tubing clamp.

4.11. From the two-stage venous cannula placed in the right atrium, collect 750 mL of whole blood from the pig gradually over a period of 15 min into an autoclaved glass container, and simultaneously replace the volume with 1 L of an isotonic crystalloid solution such as Plasmalyte A.

4.12. Add the blood to the perfusion circuit (which has been previously primed with 750 mL Krebs-Henseleit buffer containing 8% albumin) to reach a final volume of 1.5 L of perfusate. The perfusate is a 1:1 combination of Krebs-Henseleit containing 8% albumin solution and whole blood from the donor animal [17].

4.13. Place a cardioplegia needle (14-16 F) in the ascending Ao and secure it with a snare.

4.14. Connect the cardioplegia cannula to the cardioplegia bag and add 100 mL of blood to 400 mL of cardioplegia (St. Thomas Hospital Solution No. 2) to reach a final volume of 500 mL blood cardioplegia.

4.15. Euthanize the pig by exsanguination. If intending to add more blood to the perfusate after starting of the perfusion (according to the aims of the study), collect the blood and add 10-30 U/mL of heparin to it and store it in a glass container or a plastic bag at 4 ˚C for short durations (hours). For longer storage, follow the institutional guidelines.

4.16. Cross-clamp the ascending Ao with an Ao clamp and deliver the cardioplegic solution into the Ao root.

4.17. After delivery of the cardioplegic solution is completed, remove the cross-clamp and perform the cardiectomy.

4.17.1. For ease of attaching the Ao and PA to their representative cannula, partially dissect the ascending Ao from the PA using a Metzenbaum scissors.

4.17.2. Transect the superior and inferior vena cava, leaving roughly 1 cm of length on each.

4.17.3. Separate the heart from the posterior mediastinum by transecting the pulmonary veins.

4.17.4. Excise the heart ensuring all of the Ao arch vessels are procured along with a segment of descending Ao. Preserve up to the PA bifurcation.

4.18. Weigh the empty heart. The amount of weight gain over the *ex situ* preservation interval can be used as a metric for organ edema.

5. Placement of the Heart onto the ESHP Apparatus and Initiation of Perfusion

5.1. Trim excess tissue around the LA with a Metzenbaum scissor and cut between the pulmonary veins to create a common orifice.

5.2. Place a purse-string suture around the LA orifice using a 3-0 polypropylene suture.

5.3. Place the LA cannula into the LA orifice and secure it with a snare (**Figure 6**).

5.4. Suture and close the inferior vena cava with a 3-0 polypropylene suture. Leave the superior vena cava open at the beginning of the perfusion to ensure the right ventricle (RV) remains decompressed until the perfusate warmed and an organized rhythm is achieved.

5.5. Gently squeeze the ventricles to de-air the heart. Place the LA cannula over the magnet embedded in the silicon membrane. Ensure the magnet in the silicone and the corresponding metal ring in the LA cannula are properly engaged.

5.6. Attach the Ao to the Ao cannula embedded in the silicone membrane. Secure the Ao around the cannula with a silk tie. Trim the Ao to achieve a proper lie without tension or kinking.

5.7. Increase the Ao pump speed to 1600 RPM. The remaining air in Ao root will be ejected through the innominate and subclavian branches.

5.8. Connect the Ao purge line to the innominate artery. Secure the connection with a silk tie.

5.9. Snare the left subclavian artery orifice with a silk tie. Secure the closure with a snare and snap. Through the orifice of the subclavian artery, place an introducer sheath (5f). Ensure that the length of the catheter and its orientation is properly adjusted so that it does not interfere with Ao valve function.

5.10. Connect the Ao pressure transducer to the introducer sheath side port.

5.11. Read the Ao pressure on the monitor. Adjust the Ao pump speed to reach a mean pressure of 30 mm Hg. At this point (Time 0), the perfusion will have started in the non-working mode (Langendorff mode) and appearance of a dark deoxygenated perfusate in the PA line is a reflector of reestablishment of coronary flow. Set a timer to follow duration of the perfusion if needed.

5.12. Turn on the heat exchanger and set the temperature to 38 ˚C. The perfusate will warm up to 37-38 ˚C in approximately 10 minutes. For normothermic perfusion of a porcine heart, keep the temperature at 38 ˚C throughout the perfusion.

5.13. Maintain the perfusion in non-working mode for the first hour of the perfusion. Adjust the LA pump speed to maintain the LA pressure at 0 mmHg.

5.14. Once the perfusate temperature is > 34 ˚C, evaluate the heart rhythm and pace or defibrillate as required (5-20 joules). Ensure the heart is completely decompressed before attempting cardioversion.

5.15. Check the dissolved gas status using a blood gas analyzer. Adjust the gas mixture to maintain a pH: 7.35-7.45, arterial partial pressure of carbon dioxide (P_aCO_2): 35-45 mmHg, arterial partial pressure of oxygen (P_aO_2): of 100-150 mmHg, and oxygen saturation (sO_2) ≥ 95%.

5.16. Once the heart is normothermic and in a stable rhythm, ligate the superior vena cava.

5.17. Attach temporary pacemaker leads to the right atrial wall and pace the heart in an AAI mode at 100 beats/min.

5.18. Attach the epicardial electrocardiography electrodes to the surface of the heart.

5.19. Switch to working mode after 1 hour of perfusion in Langendorff mode. For this purpose, enter the desire LA pressure (typically 6-8 mmHg) on the left side of the main page, in the “desired LAP” section of the software, and click on the button to initiate the feedback loop. The activated working mode will appear as a green button, and the LA pump speed will automatically increase and decrease to reach and maintain the desired LA pressure.

5.20. As the heart begins to work, coronary vascular resistance will drop resulting in a low diastolic pressure. Adjust the Ao pump speed to maintain the Ao diastolic pressure of 40 mmHg as afterload during perfusion in the working mode.

6. Metabolic Support during ESHP

Note: Organ perfusion solutions, including Krebs-Henseleit buffer solution, typically contain glucose as the primary energy substrate.

6.1. Check the glucose level (*e.g.* with blood gas analysis) at regular intervals during the perfusion. In accordance with the consumption rates, using a standard infusion pump replace glucose by continuous arterial infusion and/or bolus doses, to maintain an arterial concentration of 6-8 mmol/L of glucose throughout the perfusion.

6.2. Using a separate infusion pump, deliver 2 U/h of insulin to the perfusate throughout the perfusion, changing the rate of insulin infusion according to the aims of the study.

6.3. For β-adrenoceptor stimulation of the heart, deliver 0.08 mcg/min of epinephrine to the perfusate using a standard infusion pump, and continue throughout the perfusion. Alternatively, an infusion of 4 mcg/min of dobutamine may be used.

7. Anti-microbial and Anti-inflammatory Agents

7.1. Add a broad-spectrum antibiotic (*e.g.* 3.375 grams of piperacillin-tazobactam) to the perfusate at the start of perfusion.

7.2. Add anti-inflammatory agents (*e.g.* 500 mg of methylprednisolone) to the perfusate in accordance with the aims of the study, if necessary.

8. Assessment of Function

Note: The ESHP controlling software automatically calculates and records steady-state hemodynamic and functional indices every ten seconds.

8.1. Assessment of steady state systolic and diastolic function

8.1.1. For assessment and recording of the steady state data, through the introducer sheath placed earlier in the subclavian artery, place a fluid-filled pigtail catheter into the left ventricle (LV) while in working mode.

8.1.1.1. Flush the pigtail catheter with saline and place the guide wire inside it.

8.1.1.2. Gently insert the catheter into the Sheath cannula previously placed in the subclavian artery. As soon as it passes through the Ao valve, remove the guidewire slowly and connect the pigtail catheter to the LV pressure line.

8.1.1.3. Follow the LV pressure wave on the monitor. The diastolic portion of the pressure wave will reach zero when the catheter has properly placed inside of the LV. Of note, this step is only

possible in working mode since the Ao valve must be opening normally for the pigtail catheter to be able to enter the chamber. Once the pigtail catheter is placed in the LV and connected to the LV pressure transducer, the LV maximum and minimum rate of pressure change (dP/dT min and dP/dT max) will be recorded automatically.

8.1.2. Determine the myocardial performance by indexing the measured flow on the LA line, for heart mass ($mL \cdot min^{-1} \cdot g^{-1}$), at a given constant LA pressure (8 mmHg), and an Ao diastolic pressure of 40 mm Hg, and a heart rate of 100 $beats \cdot min^{-1}$. The LA pressure equals the cardiac output, assuming there is no Ao insufficiency. Examine the Ao pressure waveform to ensure there is no Ao insufficiency.

8.2. Assessment of preload recruitable stroke work (PRSW)

Note: PRSW is the linear relationship between end-diastolic volume and LV stroke work (LVSW) and represents an index for the evaluation of ventricular function, independent of preload, afterload, and size of the ventricle[18,19]. PRSW can be measured with this system in a non-invasive fashion as described below[13].

8.2.1. Remove the pigtail catheter from the LV, since the catheter may induce arrhythmias during PRSW analysis that will negatively affect the accuracy of the results.

8.2.2. On the main page, in the "Capture PVL" section, adjust the desired rate of drop in LA pump speed during the analysis (typically 100-200 RPM) and desired time during which the analysis will take place (typically 10-12 s) (**Figure 4**).

8.2.3. After performing the adjustments mentioned above, click on "Record PVL". The software will automatically exit working mode and gradually reduce LA pump RPM while simultaneously recording LVSW and LA pressure. At the conclusion of data collection, the software will perform linear regression on the newly acquired dataset to yield PRSW. After the ESHP software has completed the analysis, a message will appear on the main page, showing the correlation coefficient of the analysis. Press "OK" if the coefficient (R-value) is desirable (typically > 0.95). The PRSW analysis results will be recorded.

8.2.4. After performing the analysis, to return to perfusion in the working mode, click on "Press To Start Working Mode;" otherwise the software will continue in Langendorff (non-working) mode. The gray button will turn to green indicating a return to working mode. If repeated PRSW analysis is needed, before each new attempt ensure that the LA pressure/flow values return to the previous steady state values.

9. Metabolic Assessment of the *Ex Situ* Perfused Heart

9.1. Assess the metabolic state of the heart and the perfusate during ESHP, using the information obtained from the blood gas analysis of the perfusate samples collected from both Ao (arterial), and PA (venous) lines every 1-2 hours.

9.2. Perform blood gas analysis (every 1-2 hours) to monitor the gas and ionic state of the perfusate. Adjust the gas composition (O_2 and CO_2) and sweep speed to maintain a pH of 7.35-7.45, paO_2 of 100-150 mmHg, and $paCO_2$ of 35-45 mmHg. Adjust and maintain the perfusate ionic concentration of potassium and calcium in the physiologic range during the perfusion (*e.g.* by addition of calcium chloride if needed).

9.3. Use the information obtained from the blood gas analysis and coronary blood flow to calculate metabolic parameters. For example, calculate myocardial oxygen consumption (MVO_2), and LV mechanical efficiency (ME) as follows:

9.3.1. Determine MVO_2 (mL O2 · min^{-1} · 100 g^{-1}) multiplying the coronary blood flow (CBF) by the arterial-venous difference in oxygen content ($CaO_2 – CvO_2$).

MVO_2 = [CaO_2 - CvO_2 (mL O2 · 100 mL^{-1})] × CBF (mL . min^{-1} . 100 g heart mass), where;

Arterial oxygen content (CaO_2) = [1.34 (mL O_2 . g Hb^{-1}) × Hb concentration (g · 100 mL^{-1}) × oxygen saturation (%)] + [0.00289 (mL O_2 · mm Hg^{-1} · 100 mL^{-1}) × PaO_2 (mm Hg)]

Venous oxygen content ($CvO_{2)}$ = [1.34 (mL O_2 · g Hb^{-1}) × Hb concentration (g · 100mL^{-1}) × oxygen saturation (%)] + [0.00289 (mL O_2 · mm Hg^{-1} · 100 mL^{-1}) × PvO_2 (mm Hg)]

9.3.2. Calculate LV mechanical efficiency (ME) as follows:

ME = LVSW (J . $beat^{-1}$) / MVO_2 (J . $beat^{-1}$) where

Stroke work = {mean arterial pressure (mmHg) - LA pressure (mmHg)} × {LA flow (mL . min^{-1})/ heart rate (beats . min^{-1})} × 0.0001334 (J . mL^{-1} . $mmHg^{-1}$), and

MVO_2 (J . $beat^{-1}$) = {MVO_2 (mL . min^{-1})/heart rate (beats . min^{-1})} × 20 (joules . mL^{-1})

10. Removing the Heart from ESHP Apparatus at the End of Perfusion

10.1. Exit the working mode. Bring the LA pump RPM to zero.

10.2. Decrease the Ao pump RPM to zero.

10.3. Remove the pigtail and sheaths.

10.4. Quickly remove all the attachments to the heart.

10.5. Weigh the empty heart to determine the degree of myocardial edema formation.

10.6. Quickly take tissue samples of proper size from the left and right ventricles and place them in optimum cutting temperature (OCT) gel, formalin and/or snap freeze them in liquid nitrogen.

Store the samples for future investigations (OCT and snap frozen samples in a -80 ˚C freezer, formalin-stored samples in a properly sealed container at room temperature).

10.7. Close the program; all the recorded data will be saved.

10.8. Discard the remaining tissue, blood, bioactive materials and used ESHP apparatus components according to institutional protocols.

10.9. Clean the ESHP cart using a sanitizing hard surface cleaner (*e.g.* 70% ethanol) thoroughly.

Representative results

At the start of the perfusion (in non-working mode), the heart will normally resume a sinus rhythm when the temperature of the system and perfusate approaches normothermia. When entering working mode, as the LA pressures are approaching the desired values, ejection on the Ao pressure tracing should be observed and the LA flow (a reflection of cardiac output) should increase gradually. In a Yorkshire pig model (35-50 kg) and a starting heart weight of 180-220 grams, the initial LA flow will be ~2000 mL/min, and this will typically approach ~2750 mL/min during the first hour of perfusion in working mode. **Figure 7** displays trends in Ao pressure (A) as well as LA and pulmonary arterial flow (B) over 12 hours of perfusion.

During ESHP in the physiologic working mode, various metabolic assessments of the heart are also possible. Blood gas analysis/metabolic assessments performed on the perfusate samples obtained during ESHP provide extensive information on the metabolic status of the heart over time (**Tables 1 and 2**) and (**Figure 8A,B**)[20]. In addition to blood gas analysis, perfusate samples can be collected and assessed for different biomarkers such as brain natriuretic peptide and troponin-I; however, it should be noted that ESHP occurs in a closed system, with no exchange of perfusate solution. In the absence of the organs that naturally metabolize/clear these factors (*e.g.* kidneys), the accumulation of biomarkers over time in the perfusate solution is typically observed (**Figure 9**).

Functional assessment of the heart using this platform may include both load-dependent parameters [including myocardial performance (cardiac index, CI), LVSW, maximum and minimum rates of pressure change (dp/dt max and min)], and load-independent parameters (PRSW) (**Table 3**). **Figure 10** demonstrates the evaluation of LV PRSW during a computer-controlled linear reduction in the LA pressure[13]. In our experience with ESHP of >200 porcine hearts and >10 human hearts, the use of an automated ESHP software program has been in association with the development of standard operating procedures resulting in minimal inter- and intra-operator variability in the functional parameters. The ESHP apparatus and software system used here have been designed to maintain the desired pressures and collect the functional parameters with minimal need for manual adjustments, and we have observed an interclass correlation coefficient (ICC) ≥ 0.9 for all of the assessed parameters (*e.g.* LVSW, and dP/dt max and min) that accounts for excellent inter-rater, intra-rater and test-retest reliability. In this system, the electrocardiographic monitoring of the heart during perfusion can also take

place using two electrodes as described in the protocol, providing information on the heart rate and rhythm during perfusion (**Figure 4**).

The assessment of the heart during ESHP may be extended to different imaging modalities. Echocardiography during ESHP can provide additional information on myocardial function (*e.g.* ventricular ejection fraction) and anatomical parameters (**Figures 11 and 12**). Moreover, an assessment of the coronary vasculature is possible with angiographic imaging[21].

Performing a linear regression analysis identifies which parameters best correlated with myocardial performance (cardiac index: $mL \cdot min^{-1} \cdot g^{-1}$) during ESHP. We previously showed that despite the significant variation in the ability of the measured functional parameters to predict myocardial performance, overall, functional parameters exhibit a high correlation with cardiac output. The best functional predictors included systolic stroke work [coefficient of determination (R^2) = 0.759], for systolic function, and minimum dP/dt, (R^2 = 0.738) for diastolic function. Interestingly, metabolic parameters alone show a very limited ability to predict myocardial performance (oxygen consumption: R^2 = 0.28; coronary vascular resistance: R^2 = 0.20; lactate concentration: R^2 = 0.02).[13] Perfusion of the heart in a normothermic working mode offers the opportunity to obtain comprehensive metabolic and functional assessments of the heart during organ preservation. A clinical ESHP device with the ability to support the donor heart in working mode will provide the healthcare team with the opportunity to made decisions about organ viability based on objective data before transplantation.

Figure Legends:

Figure 1: The silicone support membrane for the heart. Support membrane pictured with integrated aortic cannula (A), left atrial cannula (B), and pulmonary artery cannula (C).

Figure 2: The ESHP circuit. (A) Schematic figure of the ESHP circuit. (B) ESHP apparatus used in our setting. A: organ chamber and silicone support membrane, B: reservoir, C: arterial line filter, D: left atrial pump, E: aortic pump, F: membrane oxygenator and heat exchanger, G: gas mixer, H: tube flow sensor, I: pressure sensor, J: stopcock/luer lock.

Figure 3: De-airing the pumps by positioning the pump outlet to a higher level.

Figure 4: Screen shot from the running ESHP software program showing cardiac functional parameters.

Figure 5: Screen shot from the initialized ESHP software program.

Figure 6: The magnetic left atrial cannula secured to the posterior aspect of the left atrium.

Figure 7: Monitoring pressures and flows during the perfusion. (A) Trends in the aortic pressure during 12 hours of ESHP. (B) Trends in the left atrial and pulmonary artery flows during 12 hours of ESHP

Figure 8: Trends over time. (A) Myocardial oxygen consumption and (B) venous lactate concentration during 12 hours of ESHP

Figure 9: Trends over time in perfusate concentration of cardiac troponin-I during 12 hours of ESHP.

Figure 10: Assessment of preload recruitable stroke work a poorly-functioning heart (grey) versus a well-functioning heart (black).

Figure 11: Representative two-dimensional echocardiographic images.

Figure 12: Representative M-mode echocardiographic images.

Table 1: A case of the blood gas analysis performed during the *ex situ* heart perfusion. Ca^{2+}, calcium ion; Cl^{-}, chloride ion; Hb, hemoglobin; HCO_3^{-}, bicarbonate ion; K^{+}, potassium ion; Na^{+}, sodium ion; Osm, osmolarity; $paCO_2$, arterial partial pressure of carbon dioxide; paO_2, arterial partial pressure of oxygen; sO_2, oxygen saturation; T1, 1-hour of *ex situ* perfusion (early perfusion); T5, 5-hours of *ex situ* perfusion (mid-perfusion); T11, 11-hours of *ex situ* perfusion (late perfusion)

Table 2: Metabolic parameters calculated using the blood gas analysis data. MVO2, myocardial oxygen consumption; T1, 1-hour of *ex situ* perfusion (early perfusion); T5, 5-hours of *ex situ* perfusion (mid-perfusion); T11, 11-hours of *ex situ* perfusion (late perfusion)

Table 3: A case of Left ventricular functional parameters assessed during *ex situ* heart perfusion. CI, cardiac index; dP/dT max, maximum rate of pressure change; dP/dT min, minimum rate of pressure change; ME, mechanical efficiency; PRSW, preload recruitable stroke work; SW, stroke work; Sys p, systolic pressure*;* T1, 1-hour of *ex situ* perfusion (early perfusion); T5, 5-hours of *ex situ* perfusion (mid-perfusion); T11, 11-hours of *ex situ* perfusion (late perfusion).

Discussion:

Successful perfusion is defined according to the aims of the study; however, this should include uninterrupted ESHP for the desired amount of time and complete collection of the data on cardiac function during the perfusion. For this purpose, a few critical steps in the protocol must be followed.

The heart is an organ with high oxygen and energy demands, and minimizing the ischemic time before cannulation and perfusion is an important principle that must be followed. The process of procurement, mounting the heart on the ESHP apparatus, and initiating perfusion should not exceed 20-30 minutes.

For efficient perfusion and reliable functional assessment, the process of mounting the heart on the apparatus bears critical importance. Proper anatomical alignment of the great vessels plays

an important role in this regard. The heart should be procured with an adequate length of PA and Ao arch branches so that these vessels are not stretched when attached to the representative cannulae**.** From the start of the perfusion, efficient coronary perfusion plays a pivotal role in the protection of the heart during *ex situ* perfusion. After starting of the perfusion in non-working mode, the Ao pressure should be monitored and adjusted on at least 30 mmHg to support the coronary perfusion efficiently. The appearance of a dark deoxygenated perfusate in the PA line is a reflector of the reestablishment of coronary flow. After switching to the working mode, the Ao pressure should be adjusted to 40 mmHg to provide adequate coronary perfusion pressure for the working heart.

Deairing the heart chambers and Ao is essential for successful ESHP. At the time of attaching the LA cannula, squeezing the chambers will help in deairing the heart. Any air remaining inthe LV that is ejected should recirculate through the purge line in the innominate artery, which minimizes the risk of coronary air embolism. However, if substantial air remains in the left heart at the time of switching to the working mode, coronary air embolism is possible leading to a significant decline in myocardial function.

The goal of the presented approach is to provide a reproducible and reliable platform for experimental ESHP studies in large mammal models. Such a system provides the opportunity for perfusion in a physiologic working mode, and for extensive evaluation of the perfused heart. This provides an opportunity to evaluate cardioprotective protocols aimed at resuscitating dysfunctional donor organs. This system facilitates simple and reproducible assessments of cardiac functional parameters alongside metabolic parameters during ESHP, providing objective data that can be used to identify viable organs for transplantation. Such a comprehensive assessment is of particular importance when evaluating extended criteria donated hearts and hearts donated after circulatory death. Moreover, according to our observations in the setting of experimental ESHP, hearts perfused in a working mode display superior preservation of systolic and diastolic function over time compared to hearts preserved in a Langendorff mode and may help extend the safe preservation time.

ESHP in a working mode is an efficient method to preserve the donated heart and assess its viability, yet it is an artificial setting, lacking many of physiologic aspect of the body (*e.g.* real-time hormonal and nutritional balance/support, and free radical scavenging systems). The heart is an organ with sophisticated energy/metabolic demands. Thus, providing consistent, efficient metabolic support to the heart perfused is critically important. We have observed a decline in the function of the *ex situ* perfused heart, particularly during extended perfusion times[22]. Such a decline may be reflective of metabolic inefficiencies affecting the function of working mode-perfused heart. More studies are warranted to characterize the optimal metabolic support for the heart during ESHP. An additional challenge is the complexity of working mode heart perfusion. Despite the enhanced simplicity of ESHP in this system, working mode perfusion should be performed by well-trained personnel.

The ESHP apparatus with the capacity to perform a comprehensive functional and metabolic assessment of the hearts in a large mammal model, offers great potential to develop translational

therapeutic protocols to improve dysfunctional/suboptimal donated hearts. ESHP may serve as a platform to administer therapeutic interventions targeting a wide range of conditions (*e.g.* ischemia reperfusion injury), and evaluate their effects on the metabolic and functional parameters of the perfused heart[12]. Moreover, working mode ESHP may facilitate extension of the safe preservation interval, which may help to overcome geographic limitations of organ donation and facilitate better allocation of donated hearts.

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Disclosures:

DHF holds patents on *ex situ* organ perfusion technology and methods. DHF and JN are founders and major shareholders of Tevosol, Inc.

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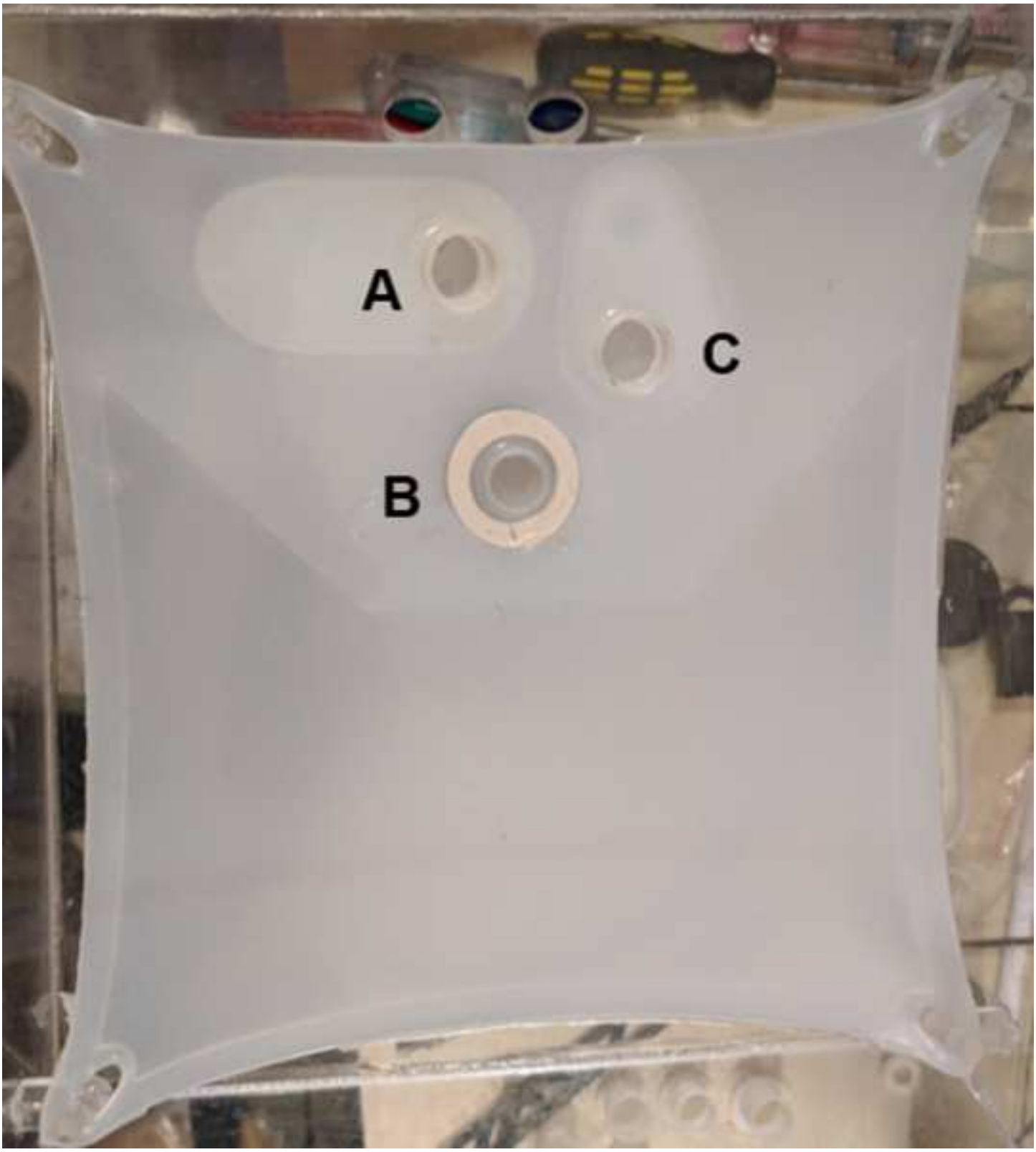

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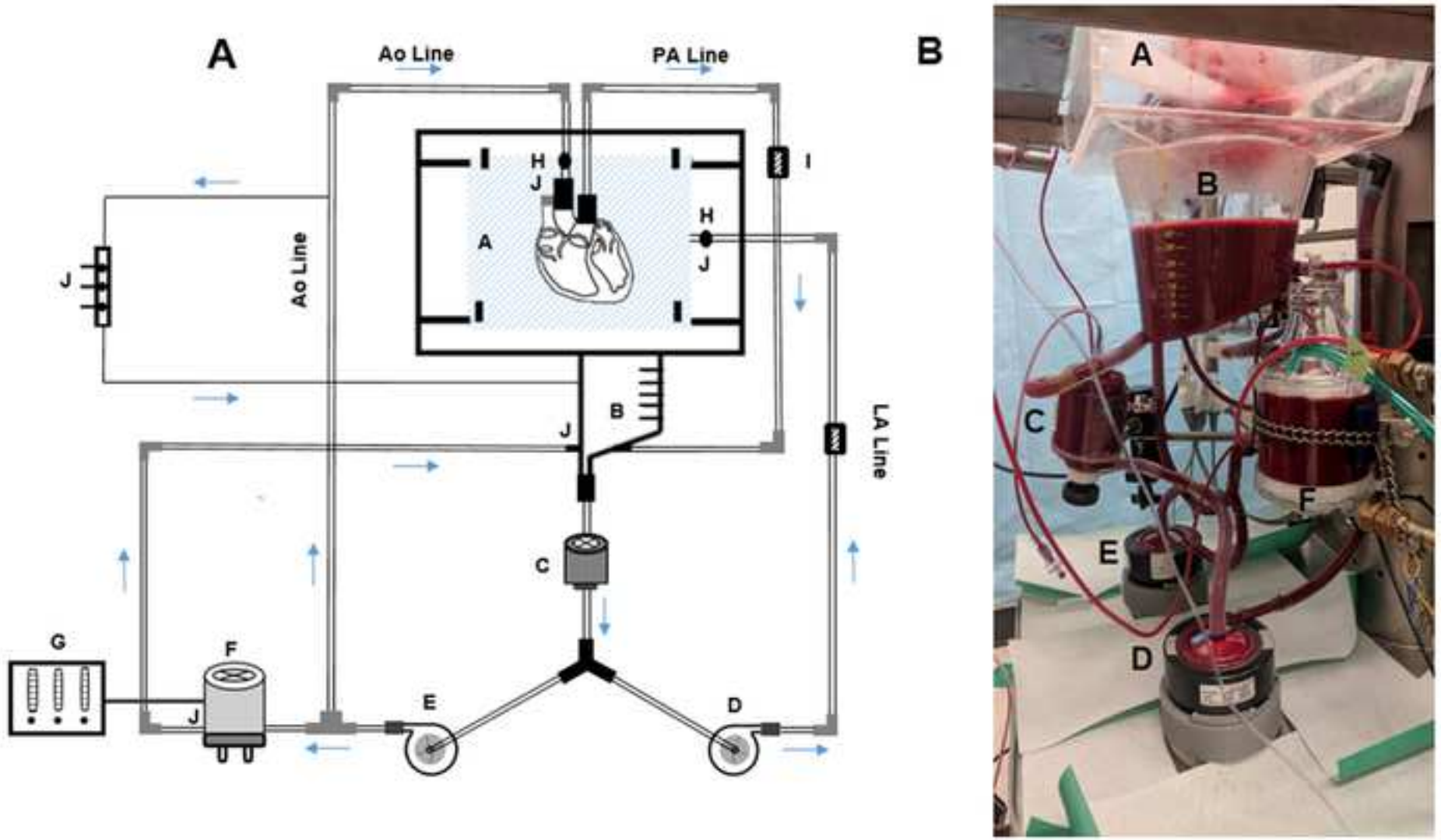

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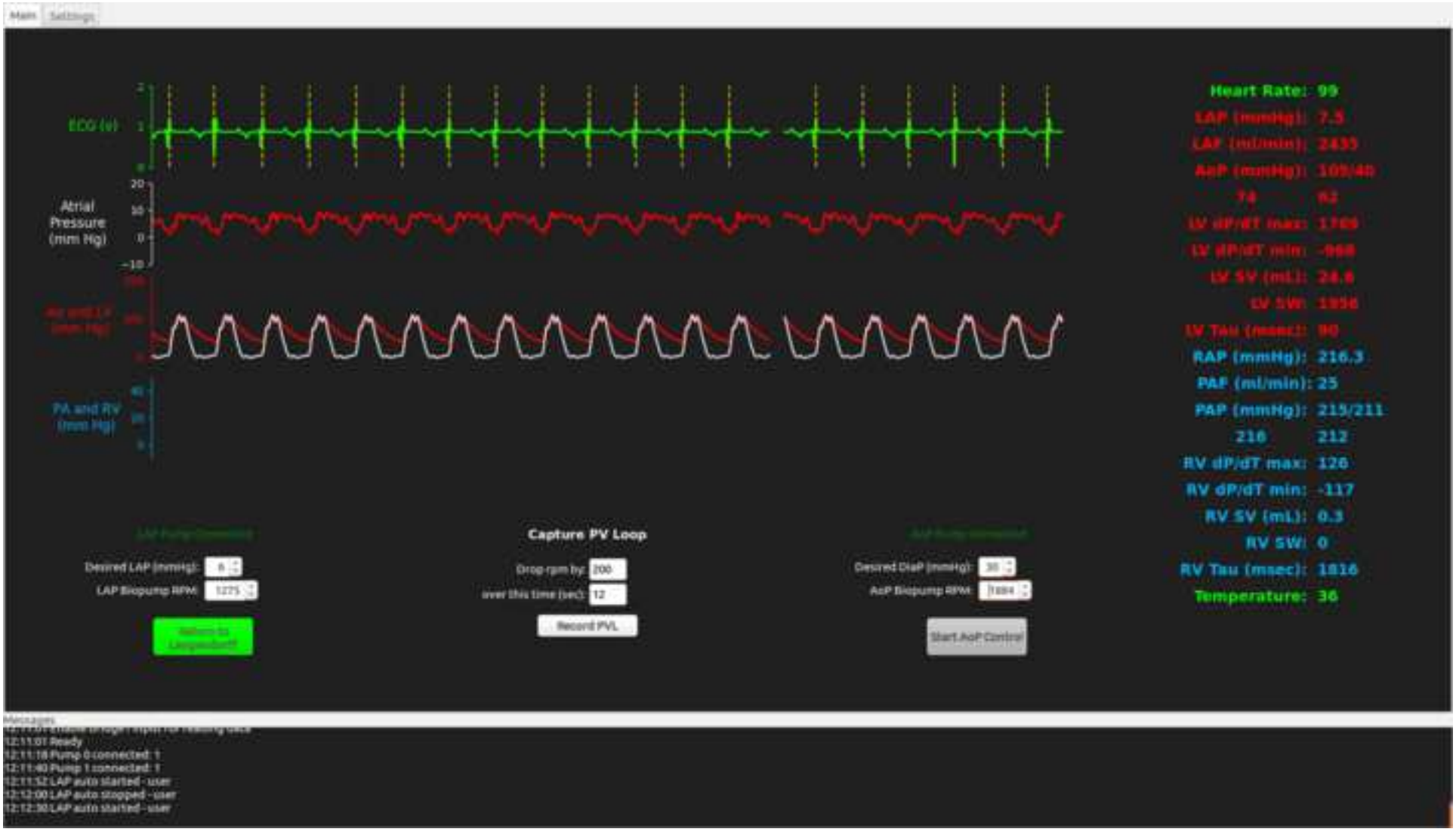

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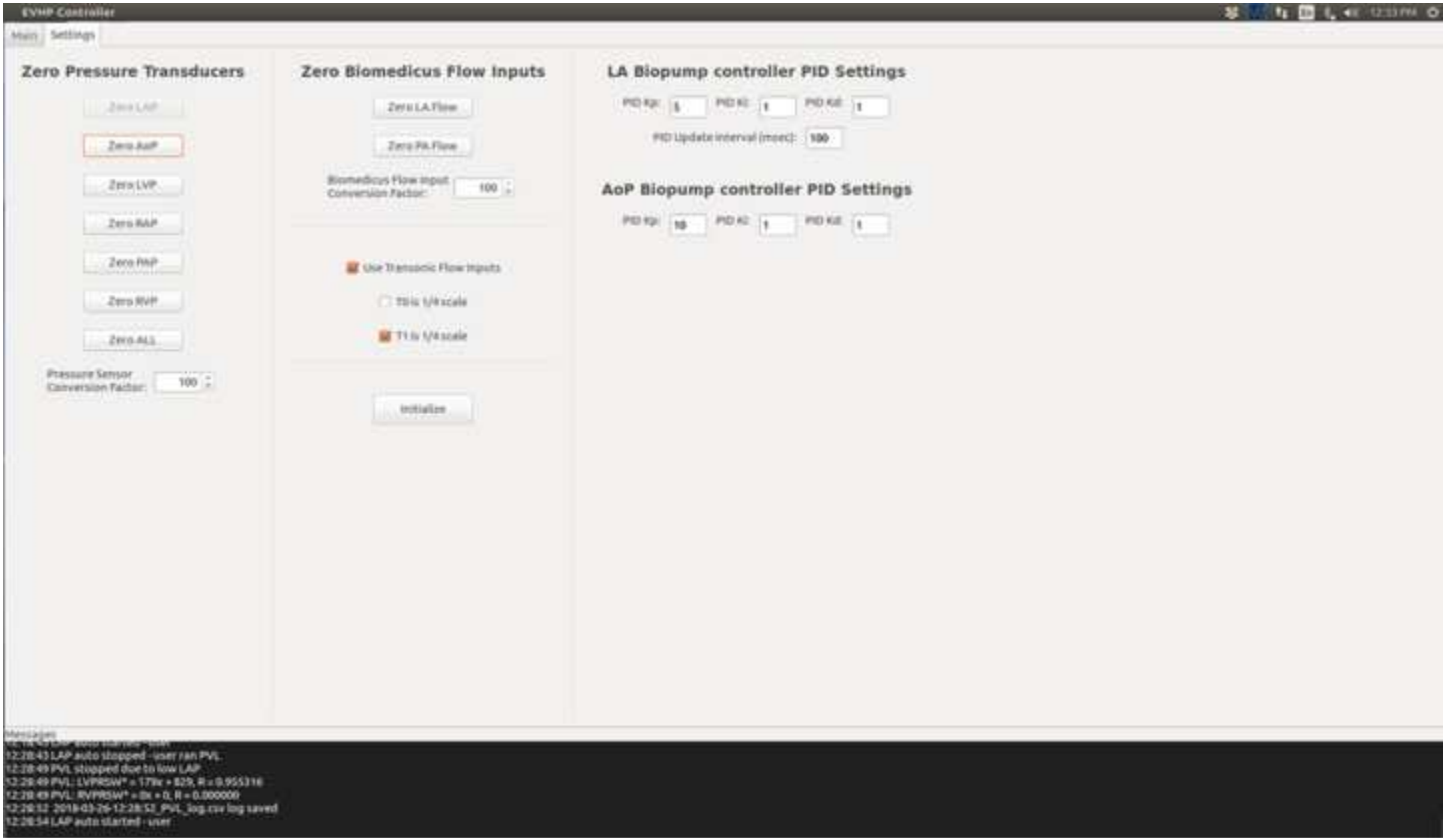

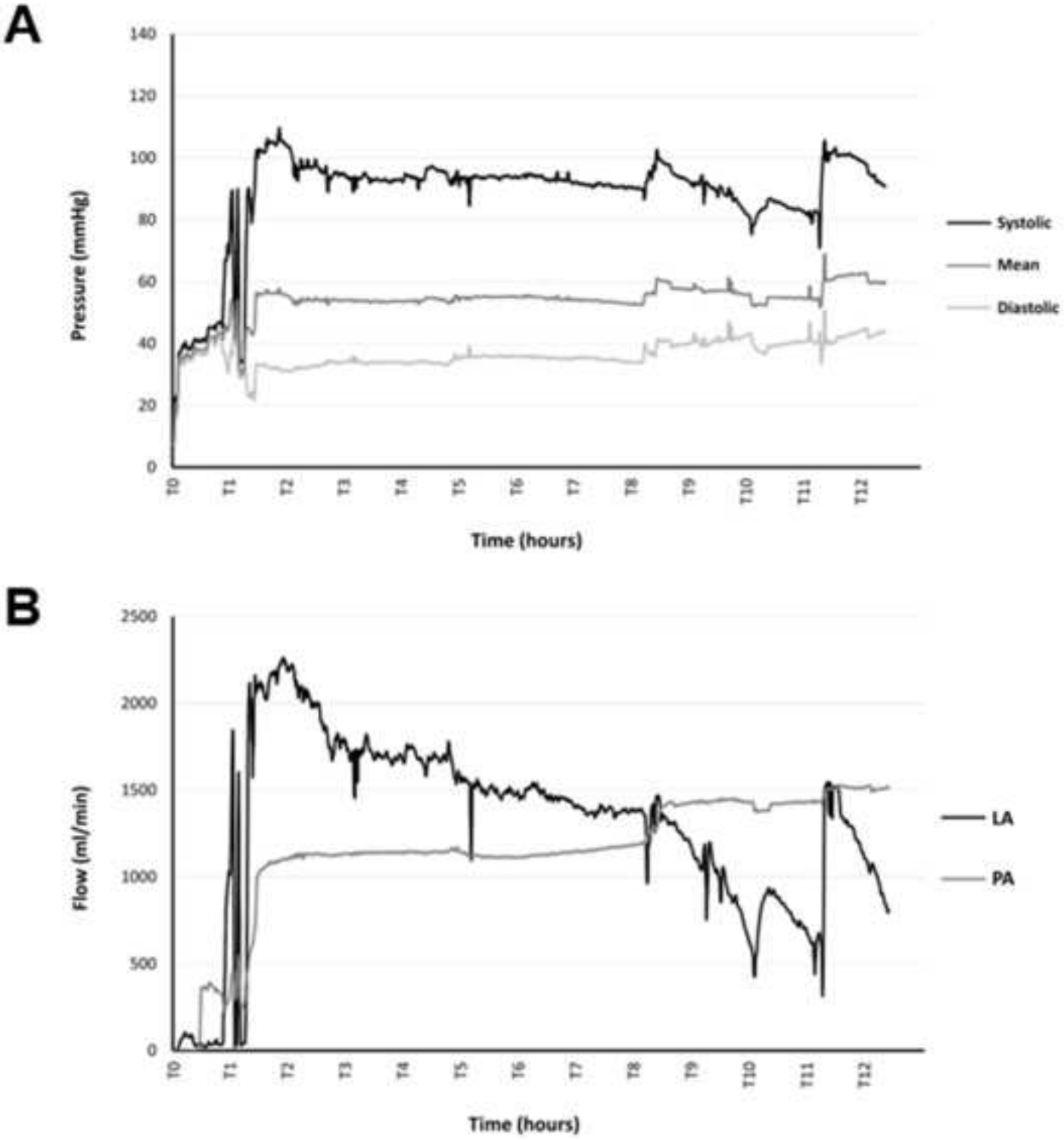

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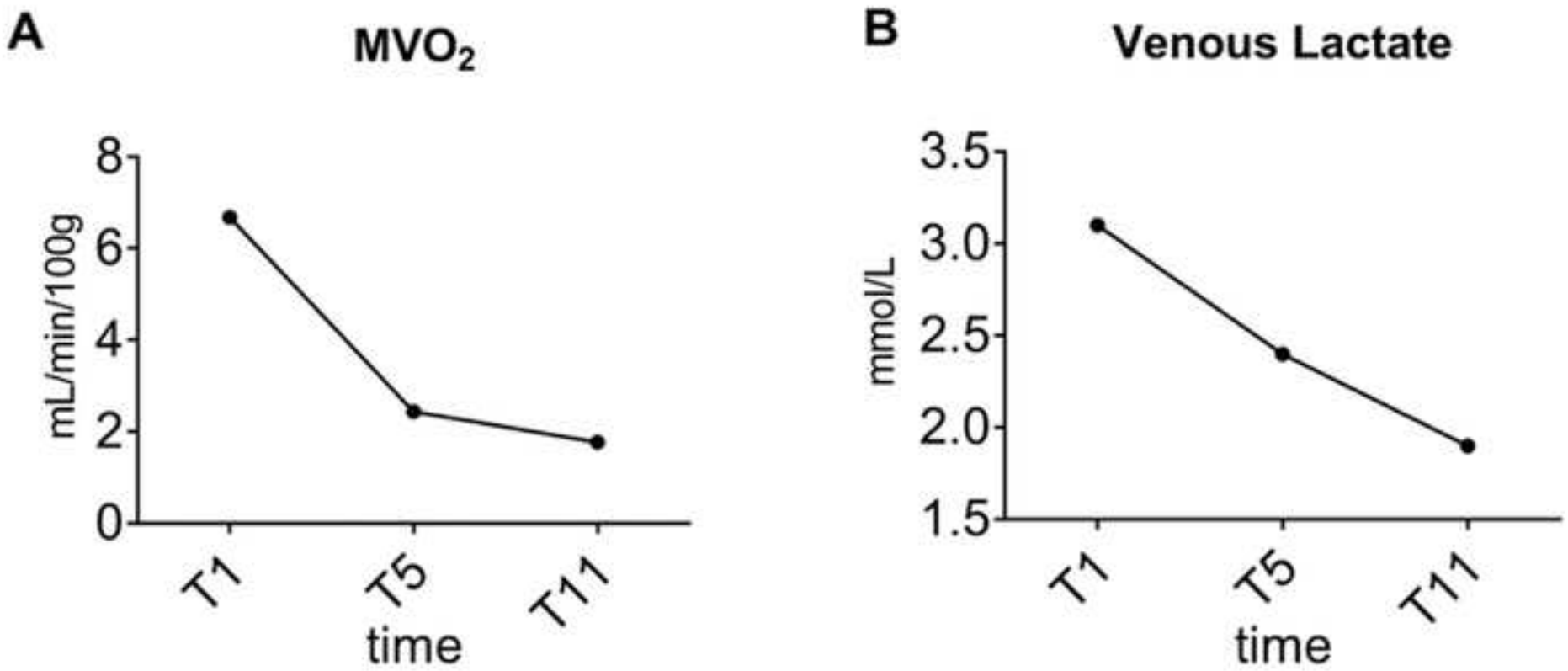

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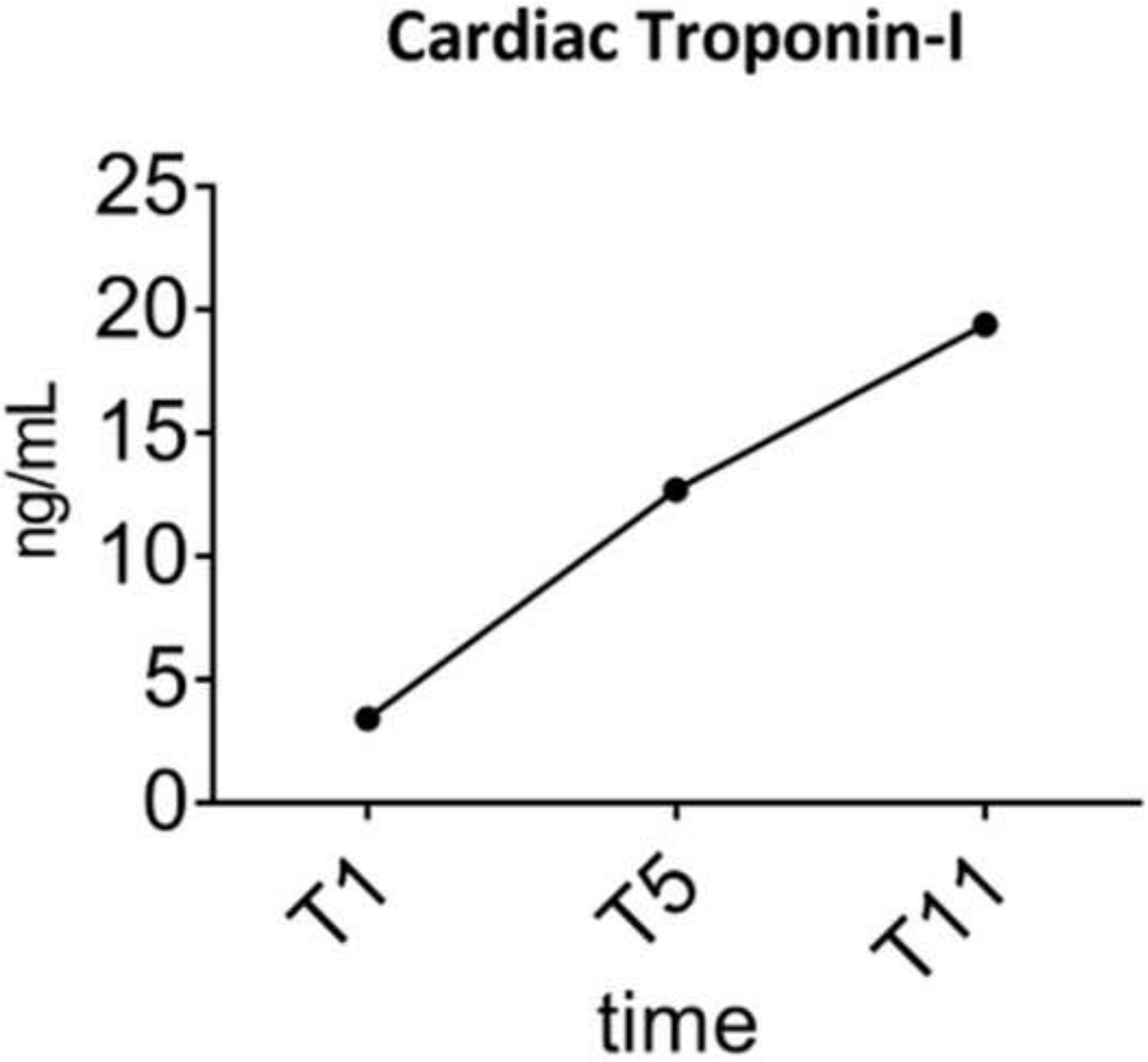

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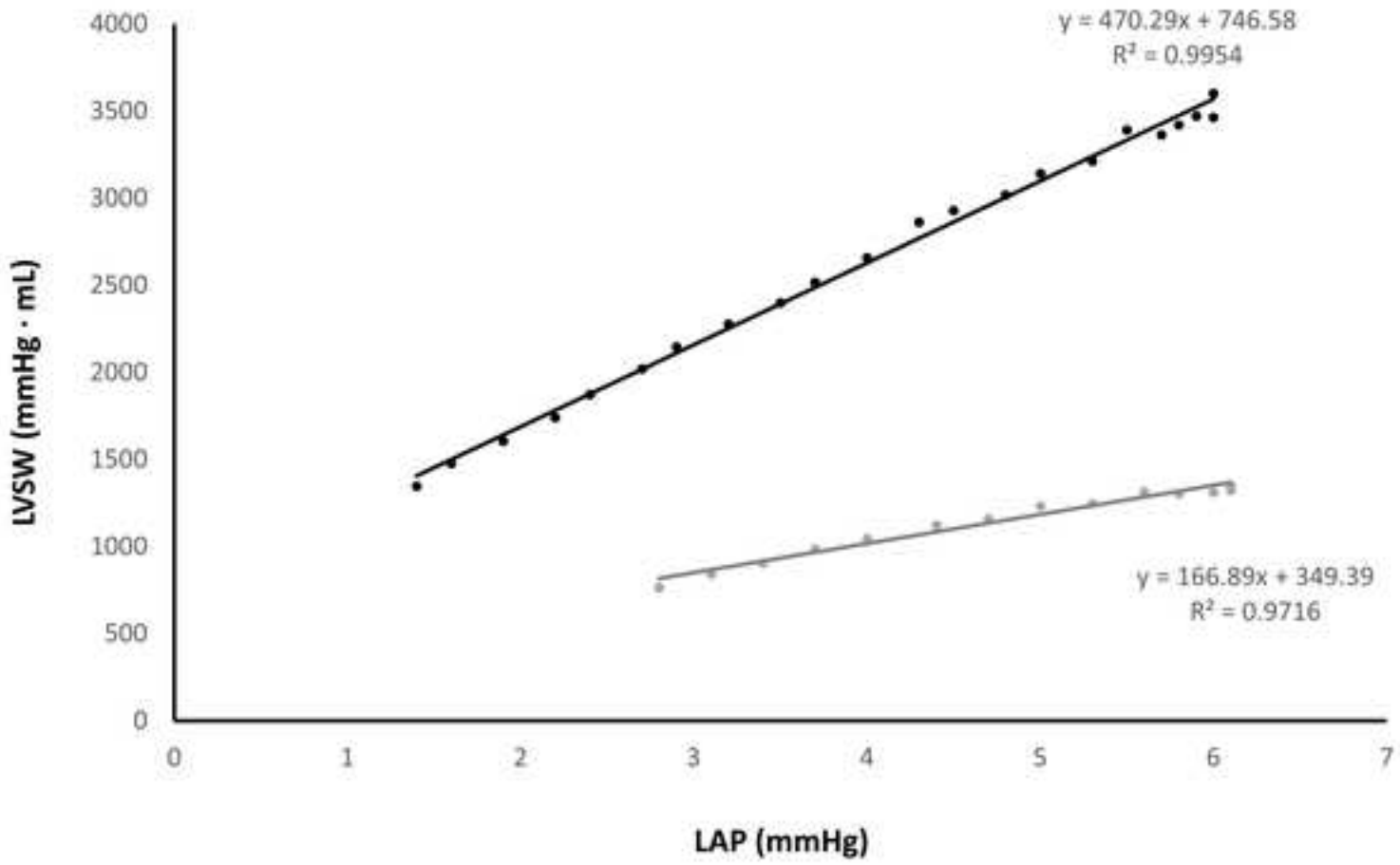

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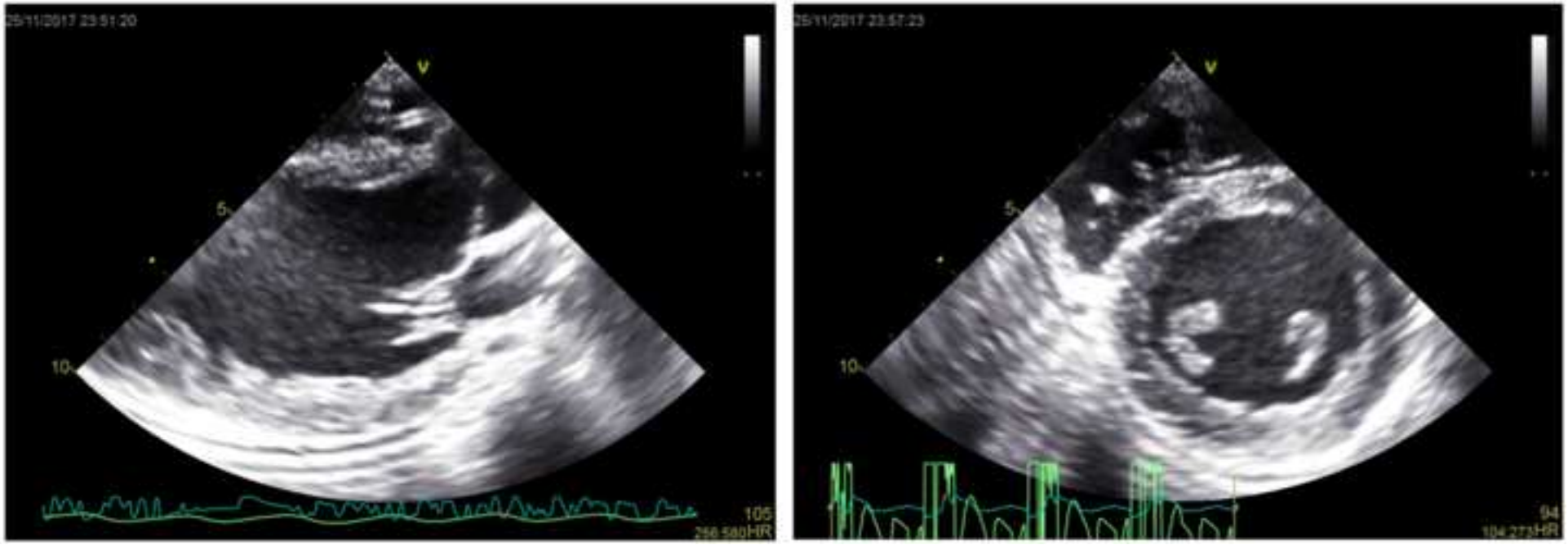

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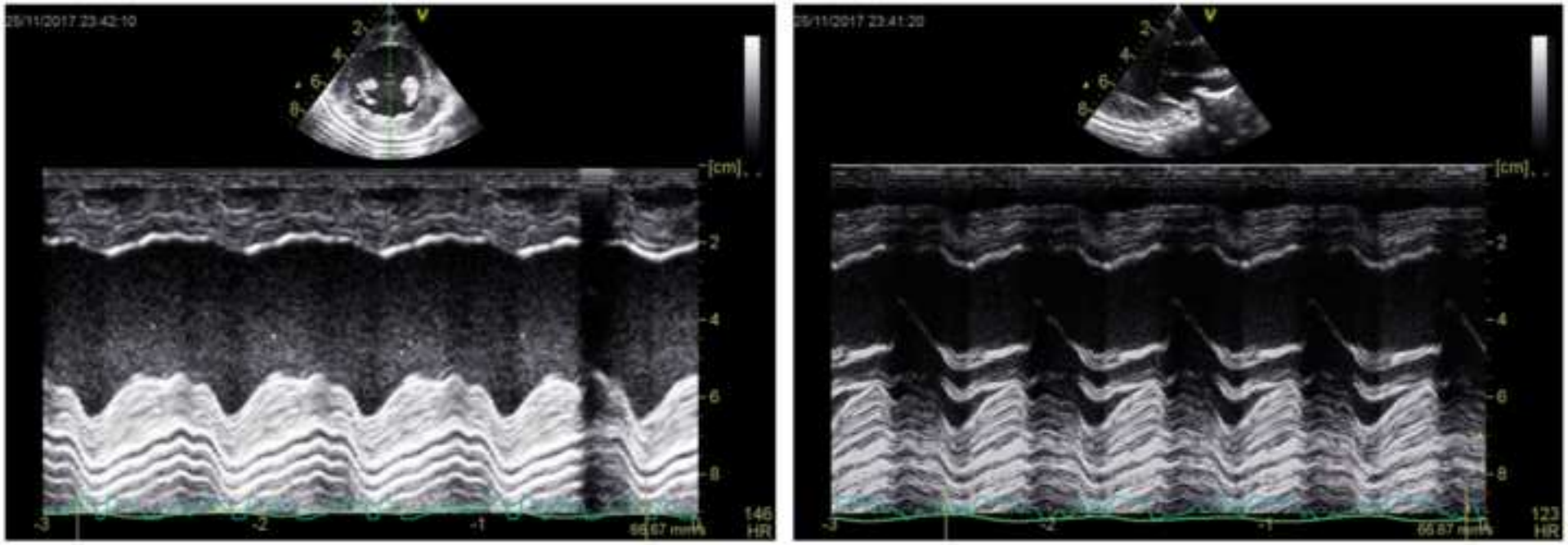

Table 1

	Aortic (arterial) parameters			PA (venous) parameters		
	T1	**T5**	**T11**	**T1**	**T5**	**T11**
Blood Gas values						
pH	7.28	7.44	7.33	7.25	4.42	7.30
pO_2 (mmHg)	123.00	149.00	141.00	44.00	55.40	57.80
pCO_2 (mmHg)	38.00	33.90	42.50	43.00	37.10	46.10
Oximetry Values						
Hb (g/dL)	4.20	4.10	3.90	4.20	4.10	3.90
sO_2 (%)	100.00	100.00	100.00	64.00	95.50	92.00
Electrolyte Values						
K^+ (mmol/L)	4.20	4.60	5.20	4.20	4.60	5.20
Na^+ (mmol/L)	142.00	144.00	149.00	142.00	144.00	149.00
Ca^{2+} (mmol/L)	1.02	1.20	1.40	1.02	1.20	1.40
Cl^- (mmol/L)	107.00	109.00	114.00	107.00	109.00	114.00
Osm (mmol/kg)	291.30	292.50	302.40	291.90	292.90	302.40
Metabolite values						
Glucose (mmol/L)	7.00	5.30	5.10	7.00	5.20	5.00
Lactate (mmol/L)	3.00	2.30	2.00	3.10	2.40	1.90
Acid Base status						
$Hco3^-$ (mmol/L)	17.60	23.10	21.90	18.50	23.70	22.40

Table 2

	Time		
Metabolic Parameters	**T1**	**T5**	**T11**
MVO_2 mL/min/100 g	6.68	2.44	1.77
Venous Lactate mmol/L	3.1	2.4	1.9
Venous - Arterial lactate difference mmol/L	0.1	0.1	-0.1
Glucose Utilization g/hr	1.23	0.6	1.14

Table 3

	Time		
Functional Parameters	**T1**	**T5**	**T11**
CI (mL/min/g)	10.26	9.66	7.50
SW (mmHg*mL)	2253	1965	1323
dP/dT max (mmHg/s)	1781	1783	1482
Sys p (mmHg)	128	121	91
ME (%)	6.69	16.85	21.68
PRSW	399	348.38	248.63
dP/dT min (mmHg/s)	-1444	-2350	-844

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Debakey-Metzenbaum dissecting scissors	Pilling	342202	
MAYO dissecting scissors	Pilling	460420	
THUMB forceps	Pilling	465165	
Debakey straight vascular tissue forceps	Pilling	351808	
CUSHING Gutschdressing forceps	Pilling	466200	
JOHNSON needle holder	Pilling	510312	
DERF needle holder	Pilling	443120	
Sternal saw	Stryker	6207	
Sternal retractor	Pilling	341162	
Vorse tubing clamp	Pilling	351377	
MORRIS ascending aorta clamp	Pilling	353617	
Surgical snare (tourniquet) set	Medtronic	CVR79013	
2-0 SILK black 12 X 18" strands	ETHICON	A185H	
3-0 PROLENE blue 18" PS-2 cutting	ETHICON	8687H	
Biomedicus pump drive (modified)	Medtronic	540	Modified to allow remote e
Biomedicus pump	Maquet	BPX-80	
Membrane oxigenator D 905	SORIN GROUP	50513	
Tubing flow module	Transonic	Ts410	
PXL clamp-on flow sensor	Transonic	ME9PXL-BL37SF	
TruWave pressure transducer	Edwards	VSYPX272	
Intercept tubing 3/8" X 3/32" X 6'	Medtronic	3506	
Intercept tubing 1/4" X 1/16" X 8'	Medtronic	3108	
Heated/Refrigerated Bath Circulator	Grant	TX-150	
ABL 800 FLEX Blood Gas Analyzer	Radiometer	989-963	
5F Ventriculr straight pigtail cathter	CORDIS	534550S	
5F AVANTI+ Sheath Introducer	CORDIS	504605A	
Emerald Amplatz Guidewire	CORDIS	502571A	
Dual chamber pace maker	Medtronic	5388	
Defibrilltor	CodeMaster	M1722B	
Infusion pump	Baxter	AS50	
Surgical electrocautery device	Kls Martin	ME411	
Gas mixer	SECHRIST	3500 CP-G	

Medical oxygen tank	praxair	2014408
Cabon dioxide tank	praxair	5823115
Bovine serum albumin	MP biomedicals	218057791

lectronic control of pump speed

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Response to Editor,

We greatly appreciate the comments and helps of the Editor and the opportunity to re-submit the revised manuscript. We have modified the manuscript per the editor's comments.

Editor: Roughly 4 pages are currently highlighted. Please reduce to 2.75, and please also ensure that continuous (or nearly-continuous) narratives are highlighted.

Author's answer: We have reduced the highlighted text. A separate word file for the narrative part has also been uploaded.

Editor: Generally we can't film calculation steps, unless they are being done in a graphical user interface on a computer.

Author's answer: It is not necessary to introduce the calculations in either movie/narrative.

Editor: Some protocol steps need more details; see notes in attached manuscript.

Author's answer: We have added more details to the text and table of the materials according to editor's suggestions.

Editor: Figures 8/9: 'mL', not 'ml'

Author's answer: We thank the editor for pointing out to this typo. We have corrected the figures 8 and 9.

Editor: Table 3: Please check the units for dP/dT-should it be mmHg/s?

Author's answer: We thank the editor for pointing out to this typo. We have corrected the units in tables.

Note: We have also replaced figure 2 A & B with an updated more detailed version (minor additions).

1. ESHP software initialization and adjustments

1.1. After initializing the controlling software and de-airing of the ESHP apparatus, donor heart procurement may proceed.

2. Blood collection and heart procurement

2.1. Perform a median sternotomy.

2.1.1. Open the pericardium with a Metzenbaum Scissor and fix the pericardial edges to the sternum using 1-0 silk suture.

2.2. From the two-stage venous cannula placed in the right atrium, collect 750 mL of whole blood from the pig gradually over a period of 15 min, into an autoclaved glass container, and simultaneously replace the volume with 1 L of an isotonic crystalloid solution such as Plasmalyte A.

2.3. Add the blood to the perfusion circuit (which has been previously primed with 750 mL Krebs-Henseleit buffer containing 8% albumin) to reach a final volume of 1.5 L of perfusate. The perfusate is a 1:1 combination of Krebs-Henseleit containing 8% albumin solution and whole blood from the donor animal [17].

2.4. Place a cardioplegia needle (14-16 F) in the ascending aorta and secure it with a snare.

2.5. Connect the cardioplegia cannula to the cardioplegia bag, to add 100 mL of blood to 400 mL of cardioplegia (St. Thomas Hospital Solution No. 2) to reach a final volume of 500 mL blood cardioplegia.

2.6. Cross-clamp the ascending aorta with an aortic clamp and deliver the cardioplegic solution into the aortic root.

2.7. Excise the heart ensuring all of the aortic arch vessels are procured along with a segment of descending aorta. Preserve up to the pa bifurcation.

3. Placement of the heart onto the ESHP apparatus and initiation of perfusion

3.1. Place a purse-string suture around the LA orifice using a 3-0 polypropylene suture.

3.2. Place the LA cannula into the LA orifice and secure it with a snare (Figure 6).

3.3. Suture and close the inferior vena cava with a 3-0 polypropylene suture. Leave the superior vena cava open at the beginning of the perfusion to ensure the right ventricle (RV) remains decompressed until the perfusate warmed and an organized rhythm is achieved.

3.4. Gently squeeze the ventricles to de-air the heart. Place the LA cannula over the magnet embedded in the silicon membrane. Ensure the magnet in the silicone and the corresponding metal ring in the LA cannula are properly engaged.

3.5. Attach the aorta to the aortic cannula embedded in the silicone membrane. Secure the aorta around the cannula with a silk tie. Trim the aorta to achieve a proper lie without tension or kinking.

3.6. Increase the aortic pump speed to 1600 RPM. The remaining air in aortic root will be ejected through the innominate and subclavian branches.

3.7. Connect the aortic purge line to the innominate artery. Secure the connection with a silk tie.

3.8. Snare the left subclavian artery orifice with a silk tie. Secure the closure with a snare and snap. Through the orifice of the subclavian artery, place an introducer sheath (5f). Ensure that the length of the catheter and its orientation is properly adjusted so that it does not interfere with aortic valve function.

3.9. Connect the Ao pressure transducer to the introducer sheath side port.

3.10. Read the Ao pressure on the monitor. Adjust the aortic pump speed to reach a mean pressure of 30 mm Hg. At this point (Time 0) the perfusion has started in the non-working mode (Langendorff mode) and appearance of a dark deoxygenated perfusate in the PA line is a reflector of reestablishment of coronary flow. Set a timer to follow duration of the perfusion if needed.

3.11. Turn on the heat exchanger and set the temperature to 38 ˚C. The perfusate will warm up to 37-38 ˚C in approximately 10 minutes. For normothermic perfusion of a porcine heart, keep the temperature at 38 ˚C throughout the perfusion.

3.12. Maintain the perfusion in non-working mode for the first hour of the perfusion. Adjust the LA pump speed to maintain the LA pressure at 0 mmHg.

3.13. Check the dissolved gas status using a blood gas analyzer. Adjust the gas mixture to maintain a pH: 7.35-7.45, arterial partial pressure of carbon dioxide (P_aCO_2): 35-45 mmHg, arterial partial pressure of oxygen (P_aO_2): of 100-150 mmHg, and $sO_2 \geq 95$ %.

3.14. Switch to working mode after 1 hour of perfusion in Langendorff mode. For this purpose, enter the desire LA pressure (typically 6-8 mmHg) on the left side of the main page, in the “desired LAP” section of the software, and click on the button to initiate the feedback loop. The activated working mode will appear as a green button, and the LA pump speed will automatically increase and decrease to reach and maintain the desired LA pressure.

4.

Note: The ESHP controlling software automatically calculates and records steady-state hemodynamic and functional indices every ten seconds.

4.1. Assessment of steady state systolic and diastolic function

4.1.1. For assessment and recording of the steady state data, through the introducer sheath placed earlier in the subclavian artery, place a fluid-filled pigtail catheter into the left ventricle (LV) while in working mode.

4.2. Assessment of preload recruitable stroke work (PRSW)

4.2.1. Remove the pigtail catheter from the left ventricle, since the catheter may induce arrhythmias during PRSW analysis that will negatively affect the accuracy of the results.

4.2.2. On the main page, in the "Capture PVL" section, adjust the desired rate of drop in LA pump speed during the analysis (typically 100-200 RPM) and desired time during which the analysis will take place (typically 10-12 sec) (Figure 4).

4.2.3. After performing the adjustments mentioned above, click on "Record PVL". The software will automatically exit working mode and gradually reduce LA pump RPM while simultaneously recording LV stroke work and LA pressure. At the conclusion of data collection, the software will perform linear regression on the newly acquired dataset to yield PRSW. After the ESHP software has completed the analysis, a message will appear on the main page, showing the correlation coefficient of the analysis. Press "OK" if the coefficient (R-value) is desirable (typically > 0.95). The PRSW analysis results will be recorded.

5. Metabolic assessment of the *ex situ* perfused heart

5.1. Assess the metabolic state of the heart and the perfusate during ESHP, with the blood gas analysis of the perfusate samples collected from both Ao (arterial), and Pulmonary artery (venous) lines every 1-2 hours.