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Modified Langendorff Perfusion Method for Extended Perfusion Times of Rodent Cardiac Grafts

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Abstract

Despite important advancements in the diagnosis and treatment of cardiovascular diseases (CVDs), the field is in urgent need of increased research and scientific advancement. As a result, innovation, improvement and/or repurposing of the available research toolset can provide improved testbeds for research advancement. Langendorff perfusion is an extremely valuable research technique for the field of CVD research that can be modified to accommodate a wide array of experimental needs. This tailoring can be achieved by personalizing a large number of perfusion parameters, including perfusion pressure, flow, perfusate, temperature, etc. This protocol demonstrates the versatility of Langendorff perfusion and the feasibility of achieving longer perfusion times (4 h) without graft function loss by utilizing lower perfusion pressures (30-35 mmHg). Achieving extended perfusion times without graft damage and/or function loss caused by the technique itself has the potential to eliminate confounding elements from experimental results. In effect, in scientific circumstances where longer perfusion times are relevant to the experimental needs (i.e., drug treatments, immunological response analysis, gene editing, graft preservation, etc.), lower perfusion pressures can be key for scientific success.

Introduction

The field of cardiovascular research has seen important advancements in the diagnosis and treatment of cardiovascular diseases (CVDs). However, despite the general decrease in incidence and mortality rates, CVDs remain the leading cause of death globally[1,2]. This alarming fact highlights the need for increased research and scientific advancement, which is undoubtedly dependent on the accuracy and predictability of the available research tools. As a result, there is a constant need for innovation, improvement and/or repurposing of the research toolset. For instance, retrograde or Langendorff heart perfusion, a technique available to the field for over a century, can be easily modified

to cover a larger scope of scientific needs and achieve a broader range of applications.

The isolation of the cardiac graft from the rest of the organism during Langendorff perfusion provides an important degree of control over a wide array of experimental parameters, including temperature, circulating solution, coronary perfusion pressures, etc.[3,4,5,6,7]. The manipulation of these parameters facilitates the simulation of a large number of cardiac scenarios that can be leveraged to further scientific advancements[5,8,9,10]. Among these parameters, perfusion pressure is likely the most overlooked experimental setting[11].

During Langendorff, perfusion pressures exhibit a direct correlation with heart rate, peak systolic/diastolic pressures, and oxygen consumption[11]. This correlation provides direct and precise control over the amount of work produced by the cardiac grafts, which can be adjusted to meet individual experimental needs. Despite this valuable control capability, the field has historically gravitated towards the use of higher perfusion pressures (60-80 mmHg), subjecting all cardiac grafts to high work demand irrespective of experimental needs[8,12,13,14,15]. The consequences of this unnecessarily high demand for work arise from the overarching principle that overwork tends to result in premature failure. This seems to be particularly true for cardiac grafts perfused via Langendorff, as the non-physiological nature of this method and the lack of recovery support present *in vivo* seem to exacerbate graft failure. This premature loss of graft function limits perfusion and experimental times significantly. In effect, in circumstances where longer perfusion times are more relevant to the experimental needs (i.e., drug treatments, immunological response analysis, gene editing, graft preservation, etc.), lower cardiac work can be afforded in exchange for increased graft durability.

This protocol demonstrates the feasibility of utilizing lower perfusion pressures (30-35 mmHg) during Langendorff, as well as the significant effect these pose for cardiac graft function over time when compared to higher perfusion pressures (60-80 mmHg). Furthermore, the findings in this manuscript highlight the importance of prioritizing the customization of the wide array of perfusion parameters to better meet experimental needs.

Protocol

This study is conducted following the Institutional Animal Care and Use Committee (IACUC) of Massachusetts General Hospital.

1. System design

1. Assemble the system with the three double-jacketed components, including a bubble trap, a reservoir, an oxygenator, a peristaltic pump, and a water circulator.
2. Attach all jacketed components to one clamp stand. Connect the components in sequence with silicone tubing in two different sequences (**Figure 1A**).
    1. Sequence 1 - Flow pattern of water through the jacket (solid lines in **Figure 1A**):
        1. Connect the outflow of the water circulator to the bottom inlet of the bubble trap jacket using 36 G tubing. This will guarantee that the perfusate is kept at the proper temperature (37 °C) before reaching the heart, as the water will lose heat as it travels through the other components of the system.

2. Connect the top inlet of the bubble trap jacket to the bottom inlet of the reservoir jacket using the same size tubing.
3. Subsequently, connect the top inlet of the reservoir jacket to the bottom inlet of the oxygenator jacket.
4. Finally, connect the oxygenator's top inlet to the water circulator's inflow.

2. Sequence 2 - Flow pattern of the perfusate through the system (dashed lines in **Figure 1A**)
    1. Attach the luer connectors to both sides of 16 G tubing. Attach the first end to the base of the reservoir and feed it through the peristaltic pump head. Connect the other end to one of the inlets of the silicone coil within the oxygenator.
    2. Connect a second piece of 16 G tubing, fitted with luer lock connectors on both ends, at the second inlet of the silicone coil of the oxygenator to the inlet in the bubble trap with the long protrusion.
    3. Connect a shorter piece of 16 G tubing, fitted with luer connectors, to the unused outlet of the bubble trap to a three-way luer lock valve.
    4. On the opposite side of the three-way valve, connect a piece of 16 G tubing with a second luer valve on the other end. This second valve lies immediately above the reservoir. Connect the opposite side of the valve to more 16 G tubing, followed by the pressure sensor.
    5. Connect a smaller diameter tube (~3.7 mm) to the vertical port of the three-way valve with a connector to the cannula (14 G angiocath).

The perfusate flows from the reservoir to the oxygenator through the bubble trap before recirculating back into the reservoir through the aortic cannula connection.

2. Perfusate preparation

1. Prepare base perfusate, 0.96% Krebs-Henseleit Buffer, 9.915 mM Dextran, 25 mM Sodium Bicarbonate, 1.054 mM Bovine Serum Albumin, 1% Pen Strep, 0.13% Insulin, 0.02% Hydrocortisone, 0.5% Heparin, and 2.75 mM Calcium Chloride and bring to volume with distilled water.

3. Perfusion system setup

1. Connect two 10 mL syringes to the top and side vent ports on the bubble trap.
2. Add the base perfusate (75 mL) to the reservoir. Turn on the peristaltic pump and set the water circulator to 37 °C.
3. Connect an oxygen (95% O_2 and 5% CO_2) line to the third inlet in the oxygenator and oxygenate the perfusate to a minimum pO_2 of 400 mmHg.
4. Secure an injection port to the vertical port of the three-way valve immediately past the bubble trap. Connect a winged infusion needle with a 1 mL syringe to the injection port (used for sampling). Gently tap the injection port or draw perfusate with the 1 mL syringe to clear any bubbles introduced to the circuit.
5. Once the base perfusate is up to temperature and oxygen level, perform an initial reading of the biochemical parameters to ensure correct ion concentration (**Table 1**) and proper oxygenation.

    NOTE: The ion and pH levels must be read after the solution has been brought up to temperature (37 °C) and

has been oxygenated with the proper gas mix (95% O_2, 5% CO_2).

6. Zero the pressure sensor by unclamping the connected tube and allow the perfusate flow through the open sensor and the cannula to equilibrate. Once equilibrated, press the zero button in the sensor box and re-clamp the tubing. Record base pressures prior to the heart's attachment to the system for flows ranging from 1 mL/min to 15 mL/min.
7. Low pressure perfusion
    1. Adenosine drip: Make an initial 20 mM stock of adenosine in the base perfusate. Dissolve the adenosine by placing the tube in a warm water bath and mixing by inversion.
    2. Dilute the stock adenosine further to a concertation of 0.06 mg/mL in base perfusate and add it to a 50 mL syringe.
    3. Attach a winged infusion needle to the 50 mL syringe and connect it to the injection port in the three-way valve. Secure the syringe to a syringe pump and set it to an infusion speed of 166.6 µL/min.
    NOTE: Bubbles are released from the infusion port by lightly tapping or flicking the port.
8. High pressure perfusion:
    1. Packed red blood cells (pRBCs) isolation:
        1. Collect 10-12 mL of whole rat blood via cardiac puncture of a donor rat.
        2. Centrifuge the blood at 2000 x *g* for 10 min.
        3. Remove the plasma and buffy coat layer via pipetting.
        4. Resuspend the pRBCs in perfusate without calcium chloride at a 1:1 ratio (e.g., 5 mL of pRBCs: 5 mL of perfusate) by inversion mixing.
        5. Repeat steps 3.8.1.2-3.8.1.4 twice for a total of 3 washes.
        6. After the last wash, resuspend the cells in perfusate at a 1:1 ratio and add the mixture to the perfusion system, already containing the 75 mL of base perfusate.
    2. Allow the cells to distribute evenly through the system, and measure the hematocrit of the perfusate using a hematology machine. The hematocrit ranges from 5%-7%.

4. Cardiac graft procurement preparation

1. Completely prime the perfusion system before initiation of procurement to minimize cold ischemia time.
2. Prepare the surgical tools. Surgical tools include blue pads, surgical tape, silk 5-0 sutures, cotton swabs, saline syringes (50 mL and 10 mL), operating scissors, forceps, micro scissors, micro forceps, Halstead clamp, 30 U of heparin, 16 G tubing for portal flush, 14 G tubing for canulation of heart, 16 G angiocath, modified 14 G angiocath with cuff, pressure sensor, ice bucket with ice, 47 mm Petri dish, gauze.
3. Create a modified cannula by inserting a thin ring of tubing (inner diameter [ID] 0.167 mm, outer diameter [OD] 2.42 mm) on the 14 G cannula, creating a cuff effect.
    1. Remove the needle of the cannula and add a drop of super glue below the ring. Carefully slide the ring to 1/4 inches above the base of the cannula. Allow glue to dry before use.

2. Cut the cannula as close as possible to the cuff at an angle and remove sharp edges.

4. Fill a 60 mL syringe with heparinized saline (0.03 U/mL) for portal vein flush. Connect the syringe to the pressure sensor, followed by the 16 G flushing tube.

5. Connect a 10 mL syringe of heparinized saline (0.03 U/mL) to the 14 G tube. Connect the other end of the tube to the aortic cannula and flush to remove any air bubbles.

6. Place the aortic cannula in a 47 mm Petri dish with gauze and filled with saline. Leave the petri dish on ice until the heart is connected to the perfusion system.

5. Cardiac graft procurement

1. Anesthetize the rats in an anesthetic chamber with 3% isoflurane.

2. Once reflexes are not noticed, remove the rat from the chamber, place it into the surgical space, and deliver continuous isoflurane (3%) via face mask.

3. After toe pinch test, heparinize the animal through the penile vein with 30 U of heparin.

4. Shave the rat across the entire abdomen and upper chest area. Remove the fur shavings from the surgical field. Tape each limb to ensure no movement during surgery.

5. Make a horizontal midline incision in the skin of the lower abdomen, exposing the abdominal muscles.

6. Make a second horizontal midline incision in the abdominal muscles exposing the internal organs.

7. Reveal the sternum, secure it with a hemostat, and retract it cranially to expose the liver and portal vein. Cannulate the portal vein using a 16-gauge angiocath.

8. Attach the 60 mL syringe of heparinized saline to the angiocath and create an incision in the inferior vena cava and abdominal aorta for venting. Flush the full amount of saline through the portal vein.

   NOTE: The flushing pressure should remain around 10 mmHg.

9. Make a horizontal cut in the diaphragm, followed by a proximal cut through the ribs on both sides of the sternum to reveal the thoracic cavity.

10. Remove the heart from the cavity and immediately place it on the Petri dish with saline on ice.

11. Identify the aortic arch, clamp with hemostats, and expose the descending aorta by cleaning any remaining connective tissue.

12. Make a horizontal cut halfway across the descending aorta and cannulate with the 14 G angiocath.

    NOTE: Do not breach the aortic valve with the cannula.

13. Secure the cannula via a suture above the cuff and release the hemostat.

14. Let the heart remain on ice until placed in the perfusion system.

6. Perfusion initiation

1. Set the flow of the peristaltic pump to 1.0 mL/min.

   NOTE: The aortic cannula is always handled at a 90˚ angle with respect to the heart to avoid the introduction of bubbles into the coronaries (**Figure 1B**).

2. Weigh the heart with the cannula before attaching the heart to the system.

   NOTE: The aortic cannula should be completely free of any air bubbles.

3. Attach the cannula to the connector in the system and start a timer.

4. Once the heart has a full contraction, increase the flow by 0.2 mL/min increments while closely watching the pressures.
5. Stop increases in flow when the desired pressures are reached or until a minimum of 3.5 mL/min is reached.
    1. Low pressure perfusion
        1. For pressures between 30-35 mmHg, use a flow of 4.5 mL/min.
        2. Start the adenosine syringe pump.
    2. High pressure perfusion
        1. For pressures between 70-80 mmHg, use a minimum flow of 5.0 mL/min.
        2. Start the adenosine syringe pump.

7. Intraventricular balloon:

1. Attach a small (50 µL) latex balloon to a balloon catheter (2 mm diameter, 15 cm long) with a tapered tip (1.4 mm diameter).
2. Connect the catheter to a pressure sensor via a luer lock connector, and secure the entire setup to a clamp stand.
3. Fill the balloon/catheter/pressure sensor with approximately 200 µL of saline via a syringe attached to the top end of the pressure sensor, and remove bubbles from inside the sensor, catheter, and balloon.
4. Calibrate the pressure sensor using a sphygmometer.
5. Make a small horizontal incision above the left atrium. Deflate the balloon by drawing the syringe at the top of the pressure sensor and inserting it into the left ventricle.
6. Initiate data acquisition and inflate the balloon until diastolic pressures read 0 mmHg.

8. Sampling

1. Collect heart rate, aortic flow, and coronary pressures after the first 20 min of perfusion and every hour thereafter.

9. End/cleanup

1. At the end of the perfusion, remove the heart from the system and weigh it for edema estimation.
2. Cut the apex of the heart via a circumferential cut and flash freeze in liquid nitrogen for post-perfusion analyses.
3. Cut a circumferential piece of the heart for histological imaging and staining. Dispose of the rest of the heart and canula.
4. Rinse all the components of the system by adding copious quantities of deionized (DI) water into the reservoir and running the peristaltic pump. Collect the water in an external bucket.
5. Repeat step 9.4 two to three times.
6. Rinse all sample ports and pressure sensor tubing thoroughly.
7. Fill the reservoir with 600 mL of DI water and 3 mL of laboratory-grade detergent pump throughout the system.
8. Turn off the water heater, oxygen tank, and peristaltic pump.

Representative Results

Hearts from adult male Lewis rats (250-300 g body weight) were harvested and perfused at high (70-80 mmHg) or low (30-35 mmHg) perfusion pressures (n = 3 per group). The effects of perfusion pressure on overall cardiac function and

health were determined by collecting heart rate, edema, and left ventricular function.

A clear correlation between heart rate and perfusion pressures was determined (**Figure 2**). Heart rate was statistically higher in high-pressure hearts when compared to low-pressure hearts for all time points, except the first one (60 min, **Figure 2A**,**B**). Interestingly, low-pressure hearts seem to undergo a period of adjustment at the beginning of perfusion, where it took about 30 min for the heart rate to stabilize and reach the levels that were maintained through the rest of the perfusion (**Figure 2A**). A large difference in left ventricular pulse pressure (LVPP) was also observed between the groups, with the LVPP of high-pressure hearts being statistically higher than low-pressure hearts at every timepoint (**Figure 3B**). This sustained high demand for work resulted in a progressive loss of function in high-pressure hearts with a statistical decrease in LVPP seen after 2 h of perfusion (**Figure 3A**,**B**). Alternatively, no loss of function was present in hearts perfused with low pressures, with LVPP remaining unchanged throughout perfusion time (**Figure 3A**,**B**). Similar to LVPP, high-pressure hearts exhibited higher cardiac muscle contraction (dP/dt_{max}) and relaxation (dP/dt_{min}) throughout the perfusion time when compared to low-pressure hearts (**Figure 3C**,**D**). In accordance, high-pressure hearts underwent a progressive loss of contractility and relaxation capacity, with both parameters being statistically higher 1 h into the perfusion time when compared to the last hour of perfusion. Differently, cardiac muscle contractility and relaxation capabilities were comparably low in the low-pressure group and remained unchanged over 4 h of perfusion time (**Figure 3C**,**D**). In addition to the functional effects, high perfusion pressures over extended periods of time also exacerbate interstitial fluid retention within the cardiac grafts, leading to edema. This edema was semi-quantified in percent weight change and resulted in high-pressure hearts having statistically higher weight gain when compared to hearts perfused at low pressures (**Figure 2C**).

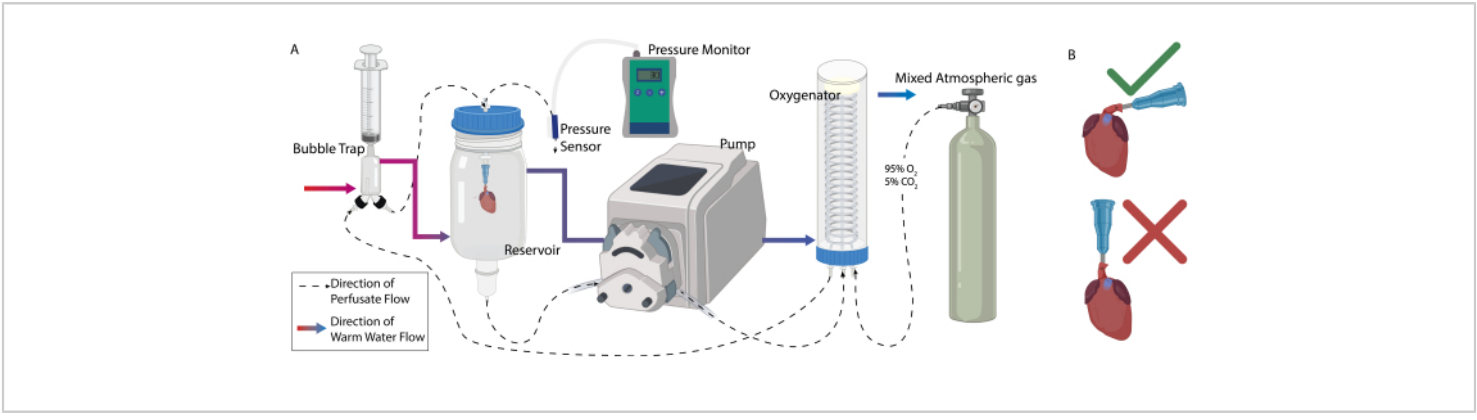

Figure 1: Perfusion system setup. (**A**) Overall perfusion setup. Dashed lines represent the order in which the components of the system were connected to optimize perfusate circulation. Solid, colored lines represent the order in which the components were connected to optimize perfusate temperature. (**B**) The proper way of handling the heart after cannulation to avoid emptying of the catheter and introducing air into the coronaries. Please click here to view a larger version of this figure.

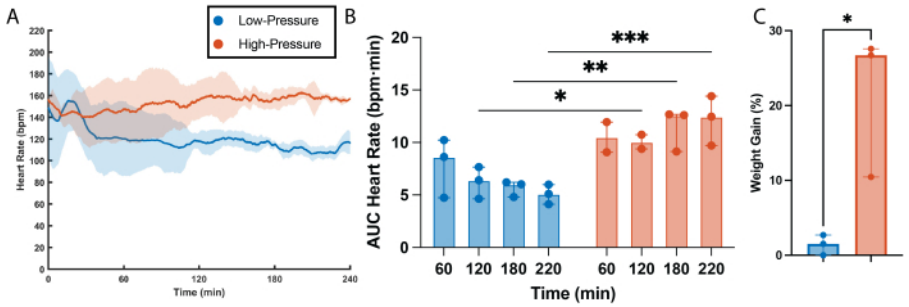

Figure 2: Effects of pressure on heart rate and edema. (**A**) Heart rate obtained from the intraventricular balloon measurements. The solid line is the median of the experimental groups. The shaded area is the interquartile range. (**B**) Area under the curve (AUC) of heart rate data for every hour of perfusion. (**C**) Percent weight gained after 4 h of perfusion at low and high pressures. All data are expressed as median ± interquartile range (IQR). *p < 0.01, **p < 0.05, ***p < 0.001. Please click here to view a larger version of this figure.

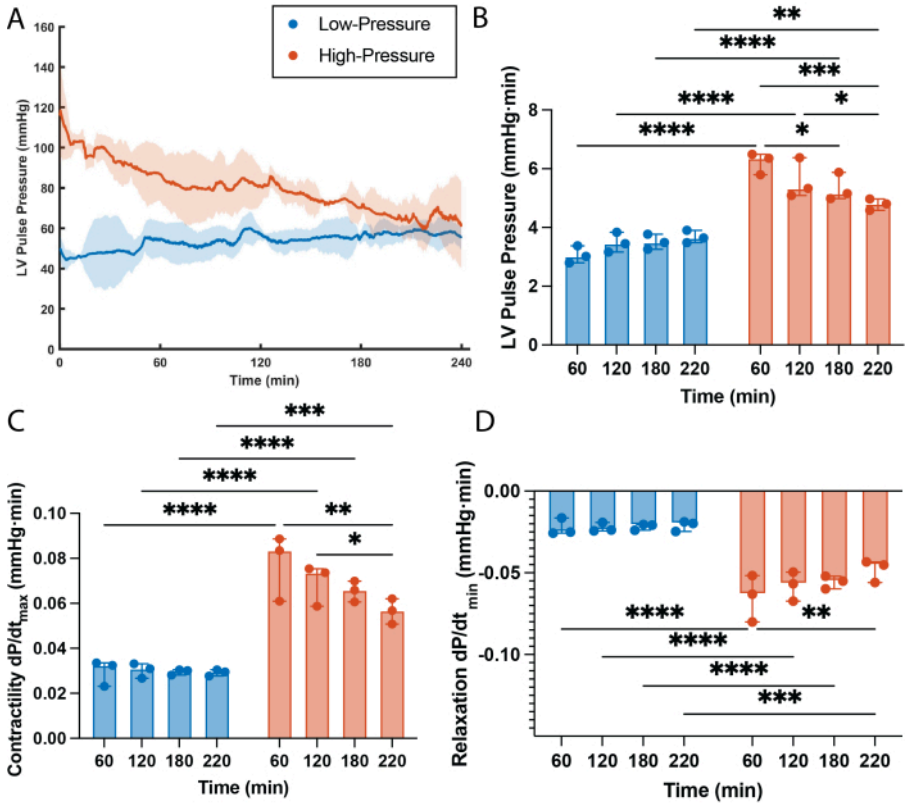

Figure 3: Effects of pressure on left ventricular function. (**A**) Maximum systolic pressure plotted over time, denoted as left ventricular pulse pressure (LVPP). The solid line is the median of the experimental groups. The shaded area is the interquartile range. (**B**) The area under the LVPP curve (AUC) for every hour of perfusion. (**C**) Cardiac muscle contractility quantified from the maximum derivative of the pressure pulse. (**D**) Cardiac muscle relaxation quantified from the minimum derivative of the pressure pulse. All data are expressed as median ± interquartile range. *p < 0.01, **p < 0.05, ***p < 0.001, ****p < 0.0001. Please click here to view a larger version of this figure.

Ion	Concentration (mmol/L)
Na^+	135–145
K^+	<6.00
Ca^{+2}	1.0–1.3
Cl^-	96–106

Table 1: Acceptable range of ion concentration in the perfusate.

Discussion

Langendorff perfusion is an extremely pliable technique that allows impressive tailoring and adjustment to meet a wide array of experimental needs. This tailoring is permitted by the significant adjustability of most perfusion parameters, including perfusion pressures. Due to the retrograde nature of Langendorff, perfusion pressures are equivalent to coronary perfusion pressures, which play an essential role in cardiac function. Coronary perfusion pressures (CPP) are known to directly control cardiac work, as a wide array of cardiac indices (i.e., left ventricular pressure, contractility (dP/dt_{max}), wall tension, ventricular stiffness) are directly proportional to CPP[16,17,18]. Historically, the field has utilized perfusion pressures, and in effect CPP, between 60 mmHg and 80 mmHg in an attempt to mimic physiological conditions[5,8,15,19,20,21]. However, the non-physiological nature of retrograde *ex vivo* machine perfusion, in combination with the high demand for work, leads to an over-time loss of cardiac function (**Figure 3**). Alternatively, lower perfusion pressures (30-35 mmHg), despite not accurately replicating the physiological conditions of rat hearts *in vivo*, inherently decrease cardiac work demand and achieve extended perfusion times (4 h) without the over-time loss of function (**Figure 3**), and decreased graft edema (**Figure 2C**). The use of lower perfusion pressures, although it signifies a deviation from physiological CPP, seems to provide important advantages over the use of physiological perfusion pressures, as the elimination of existent technique-dependent loss of function during Langendorff perfusion improves the technique into a more accurate and predictable model system with significant potential to advance cardiovascular research. Particularly, the research areas that benefit and/or require extended perfusion times to reach scientific relevance (i.e., drug treatments, immunological response analysis, gene editing, normothermic graft preservation, etc.) are becoming increasingly important in the battle against CVDs.

Langendorff perfusion is undisputable an essential tool for the field of cardiovascular research. Therefore, along with the significant benefits this scientific technique poses to the research community, it comes with an important level of scientific complexity. In effect, there are several critical steps within this protocol that require careful standardization, primarily to avoid cardiac graft damage prior to, during, and immediately after initiating perfusion. The first chance of graft damage is inconspicuous during the portal vein flush. This flush with heparinized saline aims to remove as much whole blood as possible from the cardiac graft with a double purpose. First, it serves as a way of euthanasia via exsanguination. Second, it minimizes the chances of coagulation within the cardiac graft during retrieval, cannulation, and transportation, as rat whole blood is known to have extremely short clothing times[22,23]. However, after hundreds of successful cardiac perfusions, it became apparent that the pressure applied to the rat organism during flushing is of utter importance, with the ideal flush pressure being around 10 mmHg. Higher portal vein flush pressures seem to result in damage to the vasculature of the cardiac graft, leading to increased vascular resistance ($R = \frac{Perfusion\ Pressure}{Perfusion\ Flow}$). Higher vascular resistance in effect results in target perfusion pressures being reached at lower flow rates. This imbalance between pressure and coronary flow is conveyed in the produced left ventricular pulse pressure (LVPP), resulting in significant variability.

The next instance of possible cardiac graft damage is during the connection of the graft to the system via the introduction of air bubbles into the coronaries. Air bubbles can be easily

introduced by mishandling the cannulated heart (**Figure 1B**) or improper bubble removal from the perfusion system upstream of the bubble trap[24]. Due to the retrograde nature of this setup, any introduction of air will result in cardiac air embolism, leading to ischemic insults, fibrillation, and, very commonly, graft death. Finally, the last critical step to ensure protocol success occurs during the initiation of perfusion. Differently from the large majority of manuscripts that report utilizing Langendorff as a technique, the initiation of perfusion in this protocol is performed at relatively low flows (1 mL/min) with incremental increases (+0.2 mL/min), which warrant complete control over perfusion pressures[5,8,15,19,20,21]. This incremental increase in flow, and therefore pressure, is critical as abrupt changes in pressure irreversibly increase vascular resistance and alter the delicate flow/pressure balance.

High vascular resistance in pressure-controlled Langendorff perfusions is very consequential, as target perfusion pressures are reached at lower flows, and grafts result under-perfused. The large reliance on this perfect balance between flow and pressure is likely the largest limitation of this protocol, as any prior graft damage, intentional (i.e., extended cold preservation, warm ischemia insult, myocardial infarction, etc.) or unintentional, leads to increased vascular resistance. In effect, this protocol is particularly useful for research where the experiment starts after the initiation of perfusion (i.e., drug treatments, immunological response analysis, gene editing, normothermic graft preservation, etc.) but not prior. This limitation is a perfect example of one Langendorff not fitting all purposes and special care should be taken to tailor perfusion parameters to better meet experimental needs.

Disclosures

SNT has patent applications relevant to this study and serves on the Scientific Advisory Board for Sylvatica Biotech Inc., a company focused on developing organ preservation technology. All competing interests are managed by the MGH and Partners HealthCare in accordance with their conflict-of-interest policies.

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