Journal of Visualized Experiments

Normothermic Negative Pressure Ventilation Ex-Situ Lung Perfusion: Evaluation of Lung Function and Metabolism.

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62982R3
Full Title:	Normothermic Negative Pressure Ventilation Ex-Situ Lung Perfusion: Evaluation of Lung Function and Metabolism.
Corresponding Author:	Keir Forgie University of Alberta Department of Surgery Edmonton, Alberta CANADA
Corresponding Author's Institution:	University of Alberta Department of Surgery
Corresponding Author E-Mail:	kforgie@ualberta.ca
Order of Authors:	Keir Forgie
	Nicholas Fialka
	Max Buchko
	Sayed Himmat
	Sanaz Hatami
	Xiao Qi
	Xiuhua Wang
	Katie-Marie Buswell
	Ryan Edgar
	Darren Freed
	Jayan Nagendran
Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Medicine
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Edmonton, Alberta, Canada
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	
Please confirm that you have read and agree to the terms and conditions of the video release that applies below:	I agree to the Video Release

TITLE:

Normothermic Negative Pressure Ventilation *Ex Situ* Lung Perfusion: Evaluation of Lung Function and Metabolism

AUTHORS AND AFFILIATIONS:

Keir A. Forgie[1,2], Nicholas Fialka[3], Max Buchko[1,2], Sayed Himmat[4], Sanaz Hatami[4], Xiao Qi[4], Xiuhua Wang[4], Katie-Marie Buswell[5], Ryan Edgar[5], Darren H. Freed[1,2,6,7], Jayan Nagendran[1,2,6,7]*

[1]Division of Cardiac Surgery, Department of Surgery, Faculty of Medicine, University of Alberta
[2]Mazankowski Alberta Heart Institute, Edmonton, AB, Canada
[3]Faculty of Medicine and Dentistry, University of Alberta, Edmonton, AB, Canada
[4]Department of Surgery, Faculty of Medicine, University of Alberta
[5]Ray Rajotte Surgical-Medical Research Institute (SMRI), University of Alberta
[6]Alberta Transplant Institute, Edmonton, AB, Canada
[7]Canadian Donation and Transplantation Research Program, Edmonton, AB, Canada

Email addresses of the authors:

Keir A. Forgie	(kforgie@ualberta.ca)
Nicholas Fialka	(nfialka@ualberta.ca)
Max Buchko	(buchko@ualberta.ca)
Sayed Himmat	(shimmat@ualberta.ca)
Sanaz Hatami	(hatami@ualberta.ca)
Xiao Qi	(xqi3@ualberta.ca)
Xiuhua Wang	(xiuhua@ualberta.ca)
Katie-Marie Buswell	(buswell@ualberta.ca)
Ryan Edgar	(redgar@ualberta.ca)
Darren H. Freed	(dhfreed@ualberta.ca)
Jayan Nagendran	(jayan@ualberta.ca)

*Email address of the corresponding author:

Jayan Nagendran	(jayan@ualberta.ca)

SUMMARY:

This paper describes a porcine model of negative pressure ventilation *ex situ* lung perfusion, including procurement, attachment, and management on the custom-made platform. Focus is made on anesthetic and surgical techniques, as well as troubleshooting.

ABSTRACT:

Lung transplantation (LTx) remains the standard of care for end-stage lung disease. A shortage of suitable donor organs and concerns over donor organ quality exacerbated by excessive geographic transportation distance and stringent donor organ acceptance criteria pose limitations to current LTx efforts. *Ex situ* lung perfusion (ESLP) is an innovative technology that has shown promise in attenuating these limitations. The physiologic ventilation and perfusion of the lungs outside of the inflammatory milieu of the donor body affords ESLP several advantages

over traditional cold static preservation (CSP). There is evidence that negative pressure ventilation (NPV) ESLP is superior to positive pressure ventilation (PPV) ESLP, with PPV inducing more significant ventilator-induced lung injury, pro-inflammatory cytokine production, pulmonary edema, and bullae formation. The NPV advantage is perhaps due to the homogenous distribution of intrathoracic pressure across the entire lung surface. The clinical safety and feasibility of a custom NPV-ESLP device have been demonstrated in a recent clinical trial involving extender criteria donor (ECD) human lungs. Herein, the use of this custom device is described in a juvenile porcine model of normothermic NPV-ESLP over a 12 h duration, paying particular attention to management techniques. Pre-surgical preparation, including ESLP software initialization, priming, and de-airing of the ESLP circuit, and the addition of anti-thrombotic, anti-microbial, and anti-inflammatory agents, is specified. The intraoperative techniques of central line insertion, lung biopsy, exsanguination, blood collection, cardiectomy, and pneumonectomy are described. Furthermore, particular focus is paid to anesthetic considerations, with anesthesia induction, maintenance, and dynamic modifications outlined. The protocol also specifies the custom device's initialization, maintenance, and termination of perfusion and ventilation. Dynamic organ management techniques, including alterations in ventilation and metabolic parameters to optimize organ function, are thoroughly described. Finally, the physiological and metabolic assessment of lung function is characterized and depicted in the representative results.

INTRODUCTION:

Lung transplantation (LTx) remains the standard of care for end-stage lung disease[1]; however, LTx has significant limitations: inadequate donor organ utilization[2] and waitlist mortality of 40%[3], which is higher than any other solid organ transplant[4,5]. Donor organ utilization rates are low (20-30%) due to organ quality concerns. Excessive geographic transportation distance compounded by stringent donor organ acceptance criteria exacerbates these quality concerns. LTx also trails other solid organ transplants in terms of long-term graft and patient outcomes[2]. Primary graft dysfunction (PGD), most often caused by ischemic reperfusion injury (IRI), represents the leading cause of 30-day mortality and morbidity post-LTx and increases the risk for chronic graft dysfunction[6,7]. Efforts to decrease IRI and extend safe transport times are paramount to improve patient outcomes.

Ex situ lung perfusion (ESLP) is an innovative technology that has shown promise in attenuating these limitations. ESLP facilitates the preservation, assessment, and reconditioning of donor's lungs before transplantation. It has exhibited satisfactory short- and long-term outcomes following transplantation of extended criteria donor (ECD) lungs, contributing to an increase in the number of suitable donor lungs for LTx, with organ utilization rates increasing by 20% in some centres[8-10]. Compared to the current clinical standard for LTx, cold static preservation (CSP), ESLP offers several advantages. Organ preservation time is not limited to 6 h, evaluation of organ function is possible before implantation, and due to continuous organ perfusion, modifications can be made to the perfusate that optimizes organ function[11].

The vast majority of current ESLP devices designed for human use utilize positive pressure ventilation (PPV); however, recent literature has indicated that this ventilation strategy is inferior

to negative pressure ventilation (NPV) ESLP, with PPV inducing more significant ventilator-induced lung injury[12-15]. In both human and porcine lungs, NPV-ESLP exhibits superior organ function when compared to positive pressure *ex situ* lung perfusion (PPV-ESLP) across various physiological domains, including pro-inflammatory cytokine production, pulmonary edema, and bullae formation [15]. The homogenous distribution of intrathoracic pressure across the entire lung surface in NPV-ESLP has been suggested as a significant factor underlying this advantage[15,16]. In addition to its pre-clinical benefits, the clinical safety and feasibility of NPV-ESLP have been demonstrated in a recent clinical trial[17]. Utilizing a novel NPV-ESLP device, twelve extended criteria donor human lungs were successfully preserved, evaluated, and subsequently transplanted with 100% 30-day and 1-year survival.

The objective of the present manuscript is to demonstrate a working protocol of our lab's NPV-ESLP device using juvenile porcine lungs under normothermic conditions for 12 h of duration. The surgical retrieval is covered in detail, and our custom software platform's initiation, management, and termination are also described. The strategy for tissue collection and the management of the samples is also explained.

PROTOCOL:

The procedures performed in this manuscript comply with the guidelines of the Canadian Council on Animal Care and the guide for the care and use of laboratory animals. The institutional animal care committee of the University of Alberta approved the protocols. Female juvenile Yorkshire pigs between 35–50 kg were used exclusively. Proper biosafety training was required by all individuals involved in ESLP procedures. A schematic overview of the entire NPV-ESLP experiment is represented in **Figure 1**.

1. Pre-surgical preparations

1.1. Position the organ chamber on the ESLP cart and mount the silicon support membrane (see **Table of Materials**) onto the chamber hooks for suspension.

1.2. Assemble the ESLP tubing, deoxygenator, arterial filter, and centrifugal pump.

1.3. Connect the heat exchanger water lines to the deoxygenator as well as the sweep gas tubing.

1.4. Insert the temperature sensor probe (see **Table of Materials**) into the deoxygenator.

1.5. Secure the pulmonary artery (PA) flow transducer (see **Table of Materials**) onto the PA tubing.

NOTE: The flow transducer uses ultrasound to measure the flow and relay it back to the centrifugal pump.

1.6. Use a three-way stopcock to fasten the PA pressure transducer to the PA cannula.

1.7. Attach all tubing connections firmly to prevent leaks, and close all the stopcocks and Luer locks before adding the perfusate.

1.8. Prime the circuit with 1000 mL of modified common hospital ingredient perfusate (CHIP).

NOTE: CHIP is a custom-made low-cost perfusate with an oncotic measurement of 35 mmHg, comparable to proprietary perfusate solutions[18].

1.9. Initiate the software after the circuit is primed to facilitate de-airing the pump and lines.

NOTE: These steps are associated with **Figure 2** and **Figure 3**.

2. ESLP software initialization, adjustments, and de-airing circuit

2.1. Click on the program shortcut on the monitor to start the ESLP program. Select **Scan**, **Cart 3**, **Connect,** then **NPV program** followed by **Initiate Software**.

2.2. On the **Main** page, once the circuit is primed, increase the flow RPMs to 900 to drive air out of the circuit and demonstrate perfusate flow through the PA cannula with a steady stream of fluid.

2.3. Add 3.375 g piperacillin-tazobactam, 10,000 units of heparin (10,000 U/1.5L perfusate = 6.66 U/L), and 500 mg of methylprednisone to the circuit.

2.4. Take an arterial blood gas (ABG) sample of the perfusate for reference purposes.

2.5. On the **Main** page, turn **CPAP** up to 20 cm H_2O (max) and turn it on to check the function. Turn off once the operation is confirmed.

2.6. On the **Main** page, turn **EIP** to -5 cm H_20 and turn it on to check the function. Turn off once the process is confirmed.

2.7. On the **Settings** page, turn on the heater (click **Start Heater)** and confirm the function. Change temperature set point on the monitors and confirm a congruent change on the heater monitor on the cart. Turn off once the operation is ensured.

NOTE: The ESLP apparatus used here is equipped with a custom software program (**Figure 4**). The program allows control of pump speed and ventilation parameters to achieve and maintain desired PA Flow, continuous positive airway pressure (CPAP), end-expiratory pressure (EEP), end-inspiratory pressure (EIP), respiratory ratio (RR), and inspiratory: expiratory (I:E) ratio. The software computes functional parameters and pressure-volume loops. **Table 1** lists all monitoring parameters provided by the software.

3. Preparations for anesthesia

3.1. Administer ketamine (20 mg/kg) and atropine (0.05 mg/kg) (intramuscular injections) in the operating room as premedication for the donor pig.

3.2. Place the pig supine to maintain normothermia and proceed with mask induction on a heated operating table.

3.3. Titrate oxygen flow in accordance with the animal weight, typically 20-40 mL/kg.

3.4. Administer isoflurane initially at 4–5%. Then reduce to 3% after 1-2 min.

3.5. Evaluate the depth of anesthesia every 5 min. Ensure that the pig has no withdrawal reflex in response to a noxious stimulus.

3.6. Once the correct depth of anesthesia is confirmed, intubate the pig.

3.7. Target an oxygen saturation above 90% by placing a pulse oximeter probe on the tongue (preferred) or ear.

3.8. Adjust the oxygen flow (20–40 mL/kg) and the inhalant gas (1–3%) to maintain the anesthesia level.

3.9. Maintain the ventilator settings at a TV 6-10 mL/kg, respiratory rate of 12-30 breaths/min, PEEP 5 cm H_2O, Peak Pressure 20 cm H_2O.

3.10. Shave and wash using iodine to prepare the incision site.

4. Lung biopsy, exsanguination, and blood collection

4.1. Insert a central line for fluid and heparin administration.

4.1.1. Make a 5-8 cm midline incision with electrocautery centered over the trachea and extending cranially from the sternal notch.

4.1.2. Using the cautery, divide the skin and the subcutaneous fat.

4.1.3. To identify the left or right carotid intravascular bundle lateral to the trachea, divide the midline plane between the strap muscles and separate the connective tissue layers.

4.1.4. Using 2-0 silk ties as vessel loops, obtain distal and proximal control of the jugular vein.

4.1.5. To control the blood flow, tie the cranial encircling tie and retract upwards on the proximal tie.

4.1.6. To accommodate a 7 Fr central line (~1/3 the vessel's circumference, make a small incision in the vein using the Metzenbaum scissors.

4.1.7. Release the tension on the proximal vessel loop simultaneously. Then cannulate the vein, tie it down to secure the cannula in the vein at a depth of 10 cm.

4.1.8. Connect to an IV line of 0.9% Normal Saline after flushing the line with heparin (1 unit/mL). If the pig is intravascularly depleted from dehydration, administer the fluid. Hep-lock any unused ports.

4.2. Perform a median sternotomy

4.2.1. Identify the sternal notch and xiphoid processes as incisional landmarks.

4.2.2. Use electrocautery to make a midline incision that spans the entire sternum (approximately 40-50 cm) and connects the previous incision at the sternal notch to the xiphoid.

4.2.3. Divide the subcutaneous tissue and the fascia between the fibers of the pectoralis major muscle. Cauterize any bleeding vessels to maintain hemostasis.

4.2.4. Use electrocautery to mark the midline along the sternal bone. Use heavy scissors to cut the xiphoid and use a finger to bluntly dissect the pericardium off the posterior table of the sternum to create a palpable space to accommodate the sternal saw.

4.2.5. Apply two towel clips on opposite sides of the sternum at the level of the 4th ribs lateral to the costochondral junction. Purchase the overlying tissue and fascia layer within the towel clips and lift the sternum vertically away from the heart during sternotomy.

4.2.6. Perform the sternotomy with an electric or air-powered saw, teeth up, starting from the xiphoid towards the sternal notch. To prevent injury to the underlying structures (e.g. pericardium and brachiocephalic vein, and innominate artery), proceed gradually with the saw and retract vertically using towel clips.

NOTE: The sternum dives deep posteriorly at the sternal notch, and the saw must be directed posteriorly to complete the sternotomy at that level.

4.2.7. Use cautery to obtain hemostasis of the bleeding sternum.

NOTE: Bone wax can also be employed for this purpose.

4.2.8. Deliver 1,000 U/kg heparin intravenously. Take an *in vivo* blood sample 5 min after heparin administration.

4.2.9. Use a finger to bluntly dissect the pleura off the inner sternum to create space for the sternal retractor.

4.2.10. Insert a sternal retractor with a handle towards the abdomen and retract gradually to expose the mediastinum fully.

4.3. Remove the thymus from the pericardium using a combination of blunt dissection with a finger and electrocautery.

NOTE: It is best to remove the thymus as one large piece rather than small chunks.

4.4. Take a biopsy of the right upper lung lobe for tissue analysis: open the right pleura to expose the right upper lobe. Encircle a $1cm^3$ portion with 0-silk, tie, and excise this portion of the lung using Metzenbaum scissors.

4.4.1. Divide the biopsy into three equal-sized portions, and place one of each in optimum cutting temperature (OCT) gel, formalin, and liquid nitrogen (snap freeze).

4.4.2. Store the OCT and snap-frozen samples in a -80 °C freezer, and store the formalin samples in a 4 °C refrigerator using a properly sealed container.

NOTE: Biopsy samples are stained with hematoxylin-eosin staining to examine the histopathology of lung injury, including interstitial edema, alveolar and interstitial inflammation, interstitial and perivascular neutrophilic infiltrates, and hemorrhage[15].

4.5. Open the pericardium. Tent the pericardium using forceps and make an incision in the midline of the pericardium with Metzenbaum scissors.

4.5.1. Continue this incision cranially to the aortic root, then laterally to expose the superior vena cava (SVC). Complete the pericardiotomy caudally and T-off the incision left and right at the level of the cardiac apex.

4.6. Euthanize the pig by exsanguination. Incise the SVC and insert a Poole tipped suction (see **Table of Materials**) into the lumen, advancing the suction tip to the inferior vena cava (IVC).

NOTE: An incision is made in the anterior wall of the left atrium (LA) to expedite the exsanguination.

4.6.1. Lift the heart apex and incise the LA 1 cm below the coronary sinus using Metzenbaum scissors. At exsanguination, switch from 100% O_2 to room air.

4.7. Collect Whole blood: the Poole tip suction is connected to a cell saver device to collect 1200 mL of whole blood, which is spun down to produce 500 mL of packed red blood cells (pRBC).

NOTE: Sell Saver Protocol Setting: Fill Flow: 300 mL/min, Wash Flow: 100 mL/min, Empty Flow: 150 mL/min, Return Flow: 150 mL/min, Wash Volume: 300 mL, Concentration Flow: 200 mL/min. This will take ~5 min.

5. Cardiectomy

5.1. Perform the cardiectomy: lift the cardiac apex cranially and continue the previous LA incision laterally to transect the coronary sinus where the left hemi-azygous vein joins it.

5.2. Divide the LA by cutting medially across the anterior surface of PA bifurcation.

5.3. Transect the IVC 1 cm above the diaphragm. Connect this incision to the LA by cutting medially.

5.4. Complete the division of the LA by cutting along the top of the right pulmonary artery heading towards the PA bifurcation.

NOTE: This step excludes the right superior pulmonary vein from the posterior LA.

5.5. Lift the IVC cranially and divide the right superior pulmonary vein. Divide the pericardial reflections that coalesce between the main PA and the right atrium (RA)/SVC.

5.6. Put the heart down and transect the SVC. Divide the SVC from the connective tissue layer posteriorly and transect the azygous vein.

5.7. Lift the heart cranially, divide the PA at the level of the pulmonary valve. Partially dissect the Aorta from the PA using Metzenbaum scissors, then transect the ascending Aorta.

NOTE: This completes the cardiectomy.

6. Pneumonectomy

6.1. Perform the pneumonectomy: check that expiratory tidal volume (TVe) is approximately 10 mL/kg. Switch to 2:1 inspiratory: expiratory ratio to achieve this target. If TV remains < 6mL/kg, increase peak pressures and/or PEEP to achieve 8-10 mL/kg target for maximal alveolar recruitment.

6.2. Open the pleura on the pig's left side. Make a horizontal incision along the posterior table of the sternum using Metzenbaum scissors. Make two vertical incisions down the pleura to the phrenic nerve at the superior and inferior borders of the mediastinum.

6.2.1. Excise the pleura by cutting along the phrenic nerve. Repeat this step on the right side. Open and remove the diaphragmatic pleura similarly, using the posterior LA cuff as the inferior border, in a similar manner to the phrenic nerve.

6.3. Divide the pleural attachments from the diaphragm towards the left lower lung lobe. Use a Deaver retractor (see **Table of Materials**) to hold the diaphragm upwards. Divide the inferior pulmonary ligament on the left and continue up towards the hilum.

6.4. Attempt a "no-touch technique" with regards to the lung tissue itself.

NOTE: That is, attempt minimal manual manipulation of the lung to prevent trauma.

6.5. On the right side, divide the IVC and pleural attachments from the diaphragm. Retract the diaphragm upwards using the Deaver retractor. Divide the inferior pulmonary ligament on the right side and continue up towards the hilum.

6.6. Divide the innominate vein and arch vessels to expose the trachea.

6.7. Bluntly dissect the tissue surrounding the trachea. With expiratory tidal volumes (TVe) at approximately 10 mL/kg, clamp the trachea using a tubing clamp at maximal inhalation.

6.8. Transect the trachea and lift the clamped portion upwards for the remaining steps to provide surgical traction.

6.9. Dissect the posterior trachea from the esophagus using blunt dissection with heavy Metzenbaum scissors and a free hand. Divide any remaining pleural attachments, transect the Aorta above and below the left bronchus, and remove the lungs from the chest with a segment of descending Aorta.

6.10. Weigh the lungs with the clamp on and quickly store them in a cooler full of ice. Weight gain during the ESLP run is an indicator of edema formation.

NOTE: This completes the pneumonectomy.

7. Placement of the lungs onto the ESLP apparatus

7.1. Add 500 mL of pRBC to the perfusion circuit (previously primed with 1L of CHIP, step 1.8) to reach a final volume of 1.5 L of perfusate.

NOTE: The hemoglobin concentration is targeted at approximately 50 g/L or a hematocrit of 15%.

7.2. Take photographs of the lungs for data records.

7.3. Biopsy the right middle lung lobe. Encircle a $1cm^3$ portion with 0-silk, tie, and excise this portion of the lung using scissors for tissue analysis as previously described (step 4.4).

7.4. Secure the 3/8, ½ inch tubing adapter to the main pulmonary artery (mPA). Grasp

opposite sides of the mPA using snaps. Insert the adapter with the ½ inch portion into the mPA and hold it in place while an assistant secures the adaptor in position using 0-silk ties.

NOTE: The adaptor should sit 2-3 cm above the PA bifurcation (if the PA has inadequate length, a segment of the donor pig's descending Aorta can be sewn end-to-end onto the mPA for additional length).

7.5. Place the lungs supine on the silicone support membrane and connect them to the ESLP device.

7.6. Place a second tubing clamp on the trachea near the location of the tracheal bronchus. Remove the more distal clamp and intubate the trachea with the endotracheal tube (ETT).

7.6.1. Secure the ETT in position using two zip-ties. Clamp the ventilation line using a tubing clamp and release the proximal clamp from the trachea.

NOTE: The lungs stay inflated if this is done correctly and there are no air leaks.

7.7. Connect the PA adapter to the PA line and de-air the mPA. Start the timer for perfusion.

NOTE: See **Figure 5** for a photographic depiction of the steps.

8. Initiation of perfusion and ventilation

8.1. On the **Settings** page, click **Start heater** and set the temperature to 38 °C. Enter the pig's weight as well to calculate cardiac output (flow).

8.2. On the **Main** page, set the CPAP to 20 cm H_20 and click **Start CPAP**. When ventilation begins, unclamp the ventilation line.

8.3. Zero the arterial pressure sensor. Clamp the PA line above the pressure sensor with a tubing clamp. Open the sensor to room air, click **ZERO PAP** and **Zero Bld Flow** on the **Settings** page, then confirm the readings are zeroed on the **Main** page.

8.3.1. Close the pressure sensor stopcock to read the line pressure, open the line to the PA cannula, select **10% cardiac output** on the main page, click **Return to PA Manual** (button turns green), then unclamp the PA line.

NOTE: The line is now appropriately zeroed, and the pump is now flowing 10% of the calculated cardiac output.

8.4. Draw 10 mL of perfusate for centrifugal analysis and draw a **time zero** (T0) ABG.

8.5. Once the lungs have been perfused for 10 min, increase the flow to 20% of the cardiac

output.

8.6. When the perfusate temperature reaches 32 °C, secure the chamber lid in place with clamps to create an air-tight seal. Optimally position the lungs before placing the lid. Repair any air leaks with size 6-0 prolene on BV-1 needles.

8.7. With the lid secure, clamp the ventilation tubing is and turn off CPAP. On the **Settings** page, click **Zero ITP**, **Zero Paw**, **Zero Air Flow**, then confirm readings are zeroed on the **Main** page.

8.7.1. Click **Start CPAP** at 20 cm H_2O and unclamp the ventilation tubing. Next, set EEP target to 0 cm H_2O, EIP to 1 cm H_20, RR 10, I:E ratio 1:1, and click **Press to Start Vent** to activate negative pressure ventilation.

8.7.2. Listen to the vent change its function, then attach the side port ventilation tubing to the chamber.

NOTE: The ventilator begins its respiratory cycle in exhalation. The lungs will compress slightly if the side port is attached during an exhale. It is preferable to wait and listen for inhalation, then connect the side port to maximize recruitment.

8.8. Over the next few breaths, decrease CPAP to 12 cm H_2O while simultaneously increasing the EIP to -9 cm H_2O. Maintain these ventilation parameters for the first hour, then reduce CPAP to 8-10 cm H_2O depending on the alveolar recruitment and increase EIP to -12 to -13 cm H_2O.

8.9. Set peak pressures to 20-21 cm H_2O.

NOTE: If higher pressures were required at the time of pneumonectomy, then that becomes the target peak pressure.

8.10. When the perfusate temperature reaches 35 °C, increase the flow to 30% of cardiac output.

NOTE: These are the settings for organ preservation (**Table 2**).

8.11. At 3, 5, 7, 9, 11 h, evaluate with flows of 50% of cardiac output and the addition of mixed sweep gas (89% N_2, 8% CO_2, 3% O_2) added to the deoxygenator at 0.125 L/min to simulate systemic oxygen utilization (**Table 2**).

8.12. At every odd hour during preservation mode, draw a 10 mL sample of perfusate for future analysis. Draw a pre-deoxygenator 1 ml ABG sample every hour.

8.13. Following 5 min of evaluation mode, draw ABGs from pre-and-post-deoxygenator ports (**Table 3**).

NOTE: This completes the placement of lungs on ESLP and initiation of perfusion and ventilation. See **Table 2** for initiation of the protocol. **Table 3** details the two modes of NPV-ESLP employed.

9. Metabolic Support of the Lung

9.1. Check the perfusate glucose level every hour via ABG analysis. Target glucose at 3-6 mmol/L and titrate according to consumption rates using a standard infusion pump for continuous glucose infusion and bolus doses as needed.

NOTE: Another infusion pump delivers a continuous infusion of 2 U/h of insulin. CHIP, along with most other organ perfusion solutions, contains glucose as the primary energy substrate.

10. Heparin, anti-microbial, and anti-inflammatory agents

10.1. Add 10,000 units of heparin to the perfusate at the start of perfusion before the addition of pRBC.

10.2. Add 3.375 g of piperacillin-tazobactam to the perfusate at the start of perfusion before adding pRBC.

10.3. Add 500 mg of methylprednisolone to the perfusate at the start of perfusion before adding pRBC.

11. Assessment of lung function

11.1. Employ the two distinct modes of ventilation and perfusion during an ESLP run: preservation and ventilation.

NOTE: Please see Preservation and Evaluation (**Table 2**). Preservation Mode: Cardiac output 30%, PEEP 8-12, EEP 0, EIP -10 to -12, Peak Pressure 20-22 cm H_2O, RR 6-10, and I:E ratio 1:1-1.5. ESLP runs are typically 12 h long, although they can be extended to 24 h.

11.2. Set peak pressure to match pneumonectomy peak pressure and achieve a target TV of 10 mL/kg.

NOTE: Although TVe of 10mL/kg is targeted, generally 6-8mL/kg is attained.

11.3. Every 30 min during preservation, perform recruitment for 30 min or less.

NOTE: The duration and extent of recruitment are dependent on the TVe reached. If TVe are 8-10 mL/kg, further recruitment is not necessary.

11.4. For recruitment, increase PEEP to 10-12 cm H_2O, decrease RR to 6 breaths/min, increase

Peak Pressures by 2-4 cm H_20 without exceeding 30 cm H_2O (rarely do we exceed 25 cm H2O), and change I:E ratio to 1:0.5.

NOTE: Generally, only one or two of these changes are made for each 30 min interval, with the increase in PEEP and Peak Pressure being the most effective.

11.5. At 3, 5, 7, 9, 11 h, evaluate organ function.

NOTE: The main parameter of interest is the PF ratio; however, dynamic compliance and PA pressures are closely monitored (**Figure 6**).

11.6. During the evaluation, increase cardiac output to 50% while a mixed sweep gas (89% N_2, 8% CO_2, 3% O_2) is added to the circuit at a flow rate of 0.125 L/min *via* the deoxygenator.

NOTE: This replicates systemic oxygen depletion and occurs over 5 min. During this time, decrease PEEP to 5 cm H_2O while maintaining peak pressures, increasing EIP accordingly. Keep RR at 10 bpm and set I:E to either 1 or 1.5 depending on whether the lungs appear to be air trapping or not.

11.7. Perform the Functional calculations for Pulmonary Vascular Resistance, Minute Ventilation, Dynamic Compliance, and P/F ratio.

NOTE: Pulmonary Vascular Resistance can be calculated by: [(PAP – LAP)/CO] x 80, where LAP (left atrial pressure) is 0 mmHg because of the design of an open LA drainage system.
Minute Ventilation is calculated by: TVexpiratory x RR
Dynamic Compliance is calculated by: TVexpiratory/EIP
P/F ratio is calculated by: PaO2/Fi02, where FiO2 is 21%.
The ESLP software automatically calculates and records ventilation and functional indices continuously.

12. Metabolic assessment of the *ex situ* perfused lungs

12.1. Assess the metabolic state of the perfusate every hour *via* ABGs, which act as a surrogate marker of the state of the lungs. Collect 10 mL of the perfusate from the pre-deoxygenator port for future analysis.

NOTE: Blood gas analysis also serves to monitor the gas and ionic state of the perfusate.

12.2. Use PaO2 as a marker of overall lung function.

NOTE: This is particularly true during evaluation phases when mixed sweep gas is added to the circuit to simulate systemic deoxygenation. Pre vs. post deoxygenator gases are compared to assess oxygen step-up by the lungs.

12.3. Target a normal pH (7.35-7.45)—correct acidosis with boluses of tris-hydroxymethyl aminomethane (THAM) buffer (see **Table of Materials**).

NOTE: Alkalosis is generally not corrected and does not exceed 7.55. CO_2 sweep can be added to the circuit to correct this to normal or if alkalosis exceeds this threshold.

12.4. Treat $PaCO_2$ permissively and is generally in the range of 10- 20 mmHg.

NOTE: These values are interpreted as a sign of satisfactory ventilation. Electrolytes are not adjusted during ESLP, but they are monitored as part of standard ABG analysis. Lactate will climb during increasing durations of ESLP, and so does potassium. Sodium remains stable (135-145 mmol/L), and calcium is typically low. **Table 3** contains sample representative results of ABGs perfusate analysis during a 12 h run of NPV-ESLP at normothermia and 30% cardiac output using a cellular perfusate (blood + CHIP).

13. Terminating perfusion, ventilation, and disconnection of the lungs from ESLP device

13.1. On the **Setting** page, click **Shutdown Server**.

13.2. Remove the lid from the chamber. Disconnect the PA adapter from the PA cannula.

13.3. Extubate the trachea. To determine the amount of edema formation, weigh the lungs.

13.4. Take a 1 cm^3 tissue biopsy of the accessory lobe and divide it into three pieces as previously described.

13.5. Run the final gas analyses, centrifuge the perfusate samples, and store the tissue biopsies as previously described (step 4.4).

NOTE: Centrifugation settings: Speed, 112 x *g*; acceleration, 9; deceleration, 9; temperature, 4 °C, and time, 15 min duration.

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

13.7. Following institutional protocols, discard the remaining tissue, blood, and bioactive materials.

13.8. Clean the ESLP cart using a sanitizing hard surface cleaner (e.g., 70% ethanol) and place all reusable components in a -20 °C freezer to reduce the growth of bacteria.

REPRESENTATIVE RESULTS:

At the beginning of lung perfusion and ventilation (preservation mode), the lungs will generally have a low pulmonary artery pressure (< 10 mmHg) and low dynamic compliance (< 10 mL/mmHg) as the perfusate warms to normothermia. Yorkshire pigs weighing 35-50 kg typically

results in lungs weighing 350-500 g. During the first hour of NPV-ESLP, the measured expiratory tidal volumes (TVe) are 0-2 mL/kg, and the inspiratory tidal volumes (TVi) are 100-200 mL. TVe generally reaches 4-6 mL/kg within 3-6 h, and after that may continue to increase but naturally stabilize in the 6-8 mL/kg range. TVi will always exceed TVe by 100-200 mL. Likewise, dynamic compliance will begin at 0-10 mL/mmHg within the first hour and occasionally be higher. Between 3-6 h, the dynamic compliance is 10-20 mL/mmHg and stabilizes with the TVe, which are interrelated parameters. The PAP will rise progressively as pulmonary artery flow gradually increases from 10 to 30 % of the cardiac output. Within the first hour, this is typically 10+/-2 mmHg and rises slightly throughout the 12 h run to a range of 12+/-2 mmHg. During an evaluation with flows of 50% of cardiac output, PAP can be much higher at 15-20 mmHg. Pulmonary vascular resistance (PVR) will rise gradually throughout ESLP. **Figure 6** displays trends in PAP, dynamic compliance, and PVR over 12 h of perfusion and ventilation. All these parameters can be affected by the specific ESLP experimental protocol employed.

During the evaluation mode of ESLP, which occurs at 3, 5, 7, 9, 11 h during a 12 h run, an upward trend in LA PaO_2 is observed (**Table 4**). The evaluation mode lasts for 5 min. It consists of dropping PEEP to 5 cm H_2O while maintaining peak pressures by increasing EIP in compensation. Flows are increased to 50% of cardiac output, and mixed sweep gas is added *via* the deoxygenator at a flow rate of 0.125 L/min to simulate systemic oxygenation consumption. Generally, PaO_2 from the PA is in the range of 50-60 mmHg, and LA PaO_2 can range from 60-120 mmHg, depending on how well the lungs have responded to the preservation and reconditioning. The absolute step-up value in PaO_2 between pre-and-post-deoxygenator is a better indicator of oxygenation capacity of the lungs, and thereby lung function; however, by convention, PF ratios remain a commonly reported parameter to predict successful transplantation. PF ratio is the LA (pre-deoxygenator) PaO_2/FiO_2 and should be > 300, which is the transplantation cut-off for humans. The FiO_2 is 21% (room air); therefore, the minimum LA PaO_2 required during ESLP is 63 mmHg. **Figure 6** demonstrates a typical trend for the PF ratio at the evaluation time points of 5 and 11 h throughout NPV-ESLP.

Both modes of ESLP benefit from various metabolic assessments, including frequent blood gas analysis, repeat perfusate composition sampling, and tissue biopsies. Perfusate acts as a surrogate indicator of overall lung status; therefore, blood gas analysis of the perfusate provides extensive information on the metabolic state of the lungs (**Table 4**). Before each evaluation, a 10 mL perfusate sample is drawn to be centrifuged and analyzed *via* ELISA for various biomarkers of inflammation, including TNF-alpha, IL-6, and IL-8. These values are informative of the inflammatory state of the lungs and the effects of experimental protocols; however, they need to be interpreted in the context of ESLP as a closed circuit without perfusate replacement/exchange. Thus, these biomarker levels do not benefit from the supportive function of natural metabolizers and physiologic clearance as performed by the liver or kidneys. For this reason, a continual increase in these markers over time with ESLP is observed. The tissue biopsies are likewise helpful for biomarker labeling and visualization and histologic assessment of tissue integrity. Edema formation is another important index of inflammation associated with endothelial permeability. **Figure 6** demonstrates a typical weight gain of 30% at the end of 12 h of NPV-ESLP. Recently, *in vitro* functional assessment of lungs on NPV-ESLP has been

supplemented with confirmatory *in vivo* left lung transplantation into 35-50 kg Yorkshire pigs. *In-vivo* transplanted lung assessment occurs over a 4 h duration before euthanasia *via* exsanguination. The transplantation protocol adopted for *in vivo* assessment using this custom NPV-ESLP device can be found in this Reference[19].

The P:F ratio is the main functional assessment parameter of ESLP and human lung transplantation. This NPV-ESLP technology was successfully employed in a clinical trial with 100% 30 days and 1 year survival[17]. Twelve extended criteria human lungs were successfully preserved and reconditioned on ESLP with subsequent transplantation. There were no incidences of PGD grade 3 and no early mortality. Long-term follow-up is ongoing. Although P:F ratio is the gold-standard functional assessment parameter for transplantation and ESLP, NPV-ESLP also measures PAP, pulmonary vascular resistance, edema formation, and compliance as additional functional outcome measures to help guide preservation and reconditioning of lungs. NPV-ESLP provides comprehensive metabolic and functional evaluations of donor lungs. This technology has proven to be clinically beneficial in the context of extended criteria lungs. The software has been designed to require minimal manual adjustments and has minimal inter-and intra-operator variability.

FIGURE AND TABLE LEGENDS:

Figure 1: NPV-ESLP Protocol. Schematic representation of lung procurement and 12 h NPV-ESLP run.

Figure 2: Silicone support membrane for the lungs suspended in hard-shell ESLP reservoir. Support membrane pictured with an endotracheal tube (centre) and pulmonary artery cannula (left).

Figure 3: NPV-ESLP circuit. (**A**) Schematic representation of the circuit with an accompanying legend (left). (**B**) Photo of NPV-ESLP circuit (right).

Figure 4: Screenshots from NPV-ESLP software program. (**A**) "Main" Screen. (**B**) "Flow-Loops" Screen. (**C**) "Settings" Screen.

Figure 5: Lungs connected to NPV-ESLP circuit. (**A**) Anterior Donor Lungs Pre-ESLP. (**B**) Posterior Donor Lungs Post-ESLP. (**C, D**) Tissue biopsy of right middle lung lobe. (**E**) Lungs connected to ESLP circuit. (**F**) Demonstrated positioning of lungs on silicone support. (**G**) Front view of ESLP device illustrating starting fluid level and lung positioning. (**H**) Lungs connected to the device demonstrating open left atrial drainage. (**I, J, K**) Lid secured on the device chamber. (**L**) The device and lungs are fully connected and functioning in NPV mode.

Figure 6: Functional parameters during evaluation modes over 12 h of NPV-ESLP. (**A**) P:F ratio, PaO_2:FiO_2 ratio. (**B**) Compliance. (**C**) PAP, pulmonary artery pressure. (**D**) PVR, pulmonary vascular resistance. (**E**) Weight Gain.

Table 1: Recorded monitoring chart parameters.

Table 2: Initiation of 12 h NPV-ESLP Protocol. CO, cardiac output; PA, pulmonary artery; PPV, positive pressure ventilation; NPV, negative pressure ventilation. For preservation mode, ventilation parameters, see **Table 3**. Beginning at T3, evaluation was conducted serially every 2 h for 5 min, with PA flow set to 50% CO, medical gas set to 89% N_2, 8% CO_2, 3% O_2, and preservation settings as per the parameters provided in **Table 3**.

Table 3: Modes of NPV-ESLP: Preservation vs. Evaluation. CO, cardiac output; FIO_2, fraction inspired of oxygen; LAP, left atrial pressure; NPV, negative pressure ventilation; PAP, mean pulmonary artery pressure; PAWP, peak airway pressure; PEEP, positive end-expiratory pressure; PCO_2, partial pressure of carbon dioxide in the pulmonary arterial circulation.

Table 4: Blood gas analysis performed during 12 h of ESLP. Ca^+, calcium ion; Cl^-, chloride ion; Hb, hemoglobin; HCO_3^-, bicarbonate ion; K^+, potassium ion; Na^+, sodium ion; Osm, osmolarity; paCO2, arterial partial pressure of carbon dioxide; paO2, arterial partial pressure of oxygen; sO2, oxygen saturation; P/F ratio, paO_2/FiO_2 ratio.

DISCUSSION:

There are several critical surgical steps along with troubleshooting needed to ensure a successful ESLP run. Juvenile porcine lungs are highly delicate compared to adult human lungs, so the procuring surgeon must be cautious when handling porcine lungs. It is critical to attempt a "no-touch" technique to avoid causing trauma and atelectasis when dissecting out the lungs. "No-touch" means using the bare minimum amount of manual manipulation of the lungs during procurement. Recruitment maneuvers while on the ventilator during surgery are far less effective in porcine lungs than human lungs. It is ill-advised to redirect air manually through the alveoli as is often performed with human lungs because this will cause irreparable injury to juvenile porcine lungs. It is critical to clamp the trachea at tidal volumes that match the tidal induction volumes to maximize the probability of a successful NPV-ESLP run. Any lost compliance during procurement is challenging to regain on NPV-ESLP when working with porcine lungs; humans' lungs using NPV-ESLP are more forgiving in this regard. Ideally, clamping the lungs at tidal induction volumes is performed without the need for increased peak pressure; however, compliance does start to drop shortly after warm ischemia, and sometimes higher pressures are needed to maintain recruitment. It is helpful to switch to an I:E ratio of 2:1 after the cardiectomy to maintain and even increase alveolar recruitment slightly with TVe above 10ml/kg prior to initiating the pneumonectomy. Do not flip the lungs medially to dissect the posterior pleural attachments from the esophagus as is commonly performed in human lung retrievals. The posterior pleural attachments must be bluntly dissected using a blind approach, teasing the tissue away from the lungs using a freehand while simultaneously lifting upward from the clamped trachea to provide counter traction. Juvenile porcine lungs that have lost significant compliance at the time of tracheal clamping will struggle to recover on ESLP. If the lungs have 0 dynamic compliance initially during NPV-ESLP and do not develop any dynamic compliance improvement as measured by the software within the first hour, it is doubtful that these lungs will recover their function. This is almost certainly an issue with the surgical explant technique. If insufficient PA

length has been procured, descending Aorta can lengthen the PA *via* end-to-end anastomosis.

Several critical steps and troubleshooting methods are needed during the operation of the NPV-ESLP apparatus to achieve successful perfusion. The procurement process, mounting the lungs on the NPV-ESLP apparatus, and initiating perfusion/ventilation should not exceed 20–30 min. Extended periods of ischemia decrease the probability of a successful run. The lungs must be position on the silicone support membrane such that neither the PA cannula nor the ET tube interferes with the movement of the upper lobes during ventilation. The lungs must be elevated off the hard-shell chamber with the silicone support, but not so high that open LA drainage of blood will result in hemolysis from the force of falling onto the hard-shell reservoir from a distance. Any tears in the lung parenchyma must be identified and oversewn with 6-0 prolene to prevent an air leak. Scrap pleura or pericardium can be helpful to perform a patch repair. Likewise, blood-soaked gauze can also serve to plug tears that cannot be surgically repaired. It is better to avoid an injury than to repair the lung parenchyma as the lung is difficult to sew without causing further damage. The lungs must remain inflated when initiating ventilation, so CPAP must begin at 20 cm H_20 before unclamping the trachea or ventilation tubing. If the lungs deflate, they will struggle. Any lost alveolar recruitment before initiation of ventilation will be tough to regain during NPV-ESLP, resulting in a slower recovery. When initiating perfusion, the pressure transducer must be zeroed correctly. The PA clamp is removed slowly to avoid the undesirable effect of pulmonary over-circulation from excessively high pressures and flow. The main PA must not be kinked due to cannulation as this will produce falsely elevated pressure readings. The PA adapter must not abut the PA bifurcation for this same reason. Both situations can interfere with the perfusion of lung tissue. It is critical to maintain PEEP above 12 for the first hour of ventilation and not drop PEEP below 8 except for evaluation, where a PEEP of 5 is desirable. Peak pressures should match those used at the time of procurement as they are informative regarding the state of lung compliance. For example, if the lungs required a peak pressure of 25 cm H_2O at the time of procurement to achieve TVe of 10 mL/kg, anything less than 25 cm H_2O will unlikely sustain the same amount of alveolar recruitment once on the machine.

There are a few limitations of this method that are worth considering. As previously mentioned, the convention in ESLP literature is only to report the PaO_2 when calculating P:F ratios[8,9,10,11,15,17,18]; however, the PA PaO_2 is informative because it describes the oxygen step-up occurring due to lung oxygenation. This is a better descriptor than P:F ratio alone. When the sweep gas is not running, the machine essentially acts as one large shunt that recirculates blood through the lungs for repeated laps of oxygenation. For this reason, preservation mode ABGs are not particularly informative for the oxygenation capacity of the lungs but are very valuable for the metabolic profile. This is why mixed-gas sweep during evaluation is so important and why demonstrated deoxygenation of the post deoxygenator line is critical. Another limitation is the necessity of an *in vivo* model for accurate assessment of lung function post-ESLP. *In vivo* transplantation is surgically demanding compared to the organ procurement operation, with many possible complications resulting in the loss of the transplanted lung. As such, both ESLP and subsequent transplantation are expensive resource endeavors and possess steep learning curves.

There are several advantages of this NPV-ESLP technology compared to currently available models. Pre-clinical studies comparing NPV-ESLP to PPV-ESLP have shown that NPV is a superior form of ventilation[15]. This is most likely because NPV is a more physiologic method for ESLP. NPV replicates the negative intrathoracic pressure environment of the thorax to induce lung expansion by evenly distributing the force across the pleural surface. PPV induces greater barotrauma as it forces the lungs to open through higher pressures directed down the airways. One of the other significant advantages of this NPV-ESLP device is that it is designed to be entirely portable. Portability allows for the virtual elimination of warm ischemic time as the device can accompany transplant teams to the donor center. Ischemic time is directly related to the extent of lung ischemic reperfusion injury (LIRI) and subsequent development of primary graft dysfunction (PGD), the major cause of death and morbidity post lung transplantation. Therefore, any effort to decrease ischemia should translate into improved post-transplantation outcomes. Reducing ischemic time also allows for the procurement of lungs from distant geographic locations. This is because transport time becomes less of a concern for the development of LIRI and PGD, thereby increasing the availability of donor organs that otherwise would have been rejected.

This device and the described methods have useful clinical and research applications. As previously mentioned, the prototype of this device has already been used for a successful clinical trial of extended criteria donor lungs for transplantation with 100% 30 days and 1-year survival and zero incidences of PGD grade 3[17]. A multi-center trial is a next step for this device as it moves towards commercial development. Regarding research applications, there is pre-clinical evidence that NPV-ESLP is superior to PPV-ESLP[15]. NPV-ESLP holds the promise of becoming the exemplary device, which will drive further research using this technology. The application of ESLP in the lab setting has the advantage of continuous monitoring of organ function, immediate feedback upon the introduction of novel treatment modalities, isolation of the lungs from other organ systems for testing therapeutics, and a vehicle for the delivery of therapies that previously lacked a route of administration to donor's lungs. In this sense, its application in translational research for lung transplantation is unparalleled. This particular device with an automated ESLP software program is easy to use, results in minimal inter-and intra-operator variability in functional parameters, and is designed to require minimal manual adjustments.

ACKNOWLEDGMENTS:
This research was funded on behalf of The Hospital Research Foundation.

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

REFERENCES:
1. Chambers, D. C., et al. The international thoracic organ transplant registry of the international society for heart and lung transplantation: Thirty-fifth adult lung and heart-lung transplant report-2018; focus theme: Multiorgan transplantation. *The Journal of Heart and Lung Transplantation.* **37** (10), 1169-1183 (2018).

2. Valapour, M., et al. OPTN/SRTR 2017 annual data report: Lung. *American Journal of Transplantation*. **19** (Suppl 2), 404-484 (2019).
3. Chambers, D. C., et al. The registry of the international society for heart and lung transplantation: Thirty-fourth adult lung and heart-lung transplantation report-2017; focus theme: Allograft ischemic time. *The Journal of Heart and Lung Transplantation.* **36** (10), 1047-1059 (2017).
4. Klein, A. S., et al. Organ donation and utilization in the united states, 1999-2008. *American Journal of Transplantation.* **10** (4 Pt 2), 973-986 (2010).
5. Singh, E., et al. Sequence of refusals for donor quality, organ utilization, and survival after lung transplantation. *The Journal of Heart and Lung Transplantation.* **38** (1), 35-42 (2019).
6. Bhorade, S. M., Vigneswaran, W., McCabe, M. A., Garrity, E. R. Liberalization of donor criteria may expand the donor pool without adverse consequence in lung transplantation. *The Journal of Heart and Lung Transplantation*. **19** (12), 1199-1204 (2000).
7. Snell, G. I., Griffiths, A., Levvey, B. J., Oto, T. Availability of lungs for transplantation: Exploring the real potential of the donor pool. *The Journal of Heart and Lung Transplantation.* **27** (6), 662-667 (2008).
8. Cypel, M., et al. Normothermic ex vivo lung perfusion in clinical lung transplantation. *The New England Journal of Medicine.* **364** (15), 1431-1440 (2011).
9 . Wallinder A, et al. Early results in transplantation of initially rejected donor lungs after ex vivo lung perfusion: A case-control study. *European Journal of Cardio-Thoracic Surgery.* **45** (1), 40-45 (2014).
10. Cypel, M., et al. Experience with the first 50 ex vivo lung perfusions in clinical transplantation. *The Journal of Thoracic and Cardiovascular Surgery.* **144** (1), 1200-1206 (2012).
11. Buchko M. T., et al. Total parenteral nutrition in ex vivo lung perfusion: Addressing metabolism improves both inflammation and oxygenation. *American Journal of Transplantation.* **19** (12), 3390-3397 (2019).
12. Andreasson, A. S. I, et al. Profiling inflammation and tissue injury markers in perfusate and bronchoalveolar lavage fluid during human ex vivo lung perfusion. *European Journal of Cardio-Thoracic Surgery.* **51** (3), 577-586 (2017).
13. Sadaria, M. R., et al. Cytokine expression profile in human lungs undergoing normothermic ex-vivo lung perfusion. *The Annals of Thoracic Surgery.* **92** (2), 478-484 (2011).
14. Ricard, J. D., Dreyfuss, D., Saumon, G. Ventilator-induced lung injury. *European Respiratory Journal*. **42**, 2s-9s (2003).
15. Aboelnazar, N. S., et al. Negative pressure ventilation decreases inflammation and lung edema during normothermic ex-vivo lung perfusion. *The Journal of Heart and Lung Transplantation*. **37** (4), 520-530 (2018).
16. Lai-Fook, S. J., Rodarte, J. R. Pleural pressure distribution and its relationship to lung volume and interstitial pressure. *Journal of Applied Physiology.* **70** (3), 967-978 (1991).
17. Buchko, M. T., et al. Clinical transplantation using negative pressure ventilation ex situ lung perfusion with extended criteria donor lungs. *Nature Communications*. **11** (1), 5765 (2020).
18. Buchko, M. T., et al. A low-cost perfusate alternative for ex vivo perfusion. *Transplantation Proceedings*. **52** (10),2941-2946 (2020).
19. Forgie, K. A., et al. Left lung orthotopic transplantation in a juvenile porcine model for ESLP. *The Journal of Visualized Experiments*. Accepted (2021).

Figure 1

Click here to access/download;Figure;Revisions_JoVE NPV_Figure 1.psd

Figure 1: NPV-ESLP Protocol. Schematic representation of lung procurement and 12-hour NPV-ESLP run.

Figure 2

Click here to access/download;Figure;Revisions_JoVE NPV_Figure 2.psd

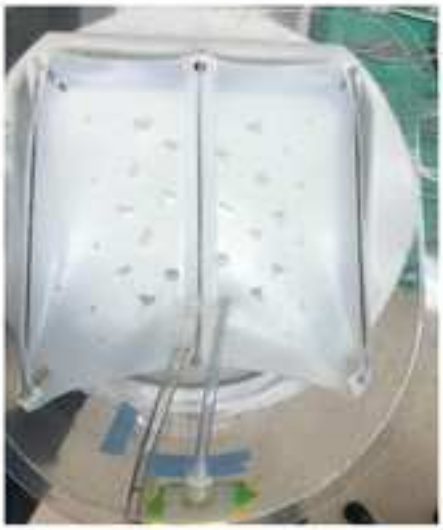

Figure 2: Silicone support membrane for the lungs suspended in hard-shell ESLP reservoir. Support membrane pictured with endotracheal tube (centre) and pulmonary artery cannula (left).

Figure 3

Click here to access/download;Figure;Revisions_JoVE NPV_Figure 3.psd

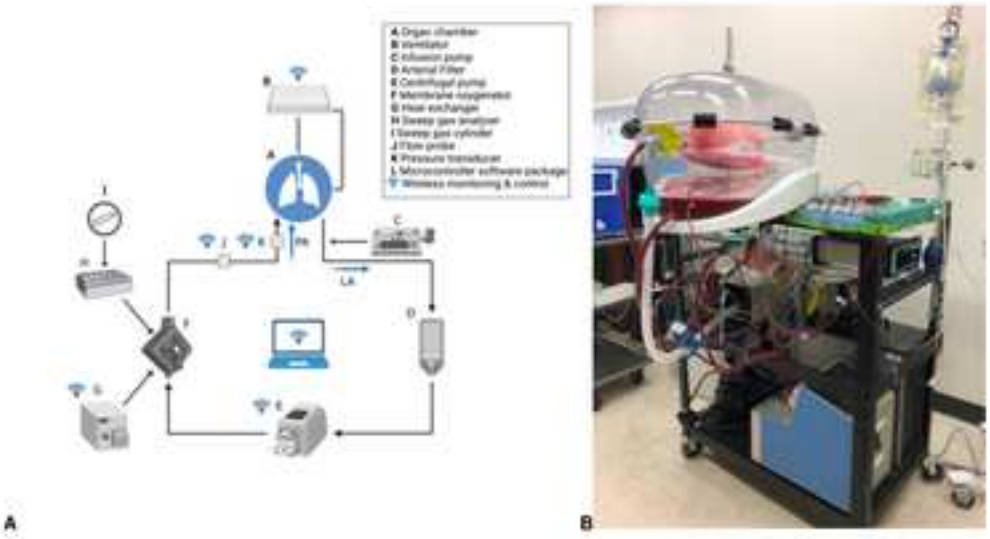

Figure 3: NPV-ESLP circuit. A) Schematic representation of the circuit with accompanying legend (left). B) Photo of NPV-ESLP circuit (right).

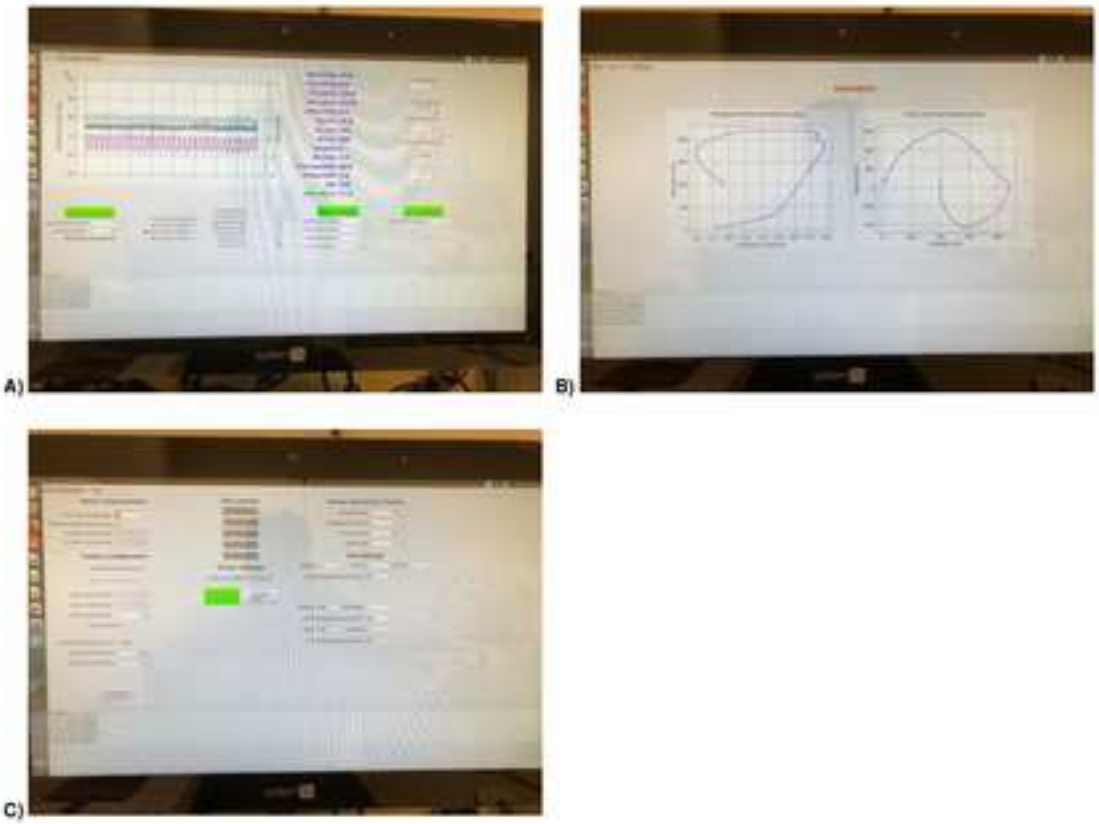

Figure 4: Screen shots from NPV-ESLP software program. A) "Main" Screen. B) "Flow-Loops" Screen. C) "Settings" Screen.

Figure 5

Click here to access/download;Figure;Revisions_JoVE NPV_Figure 5.psd

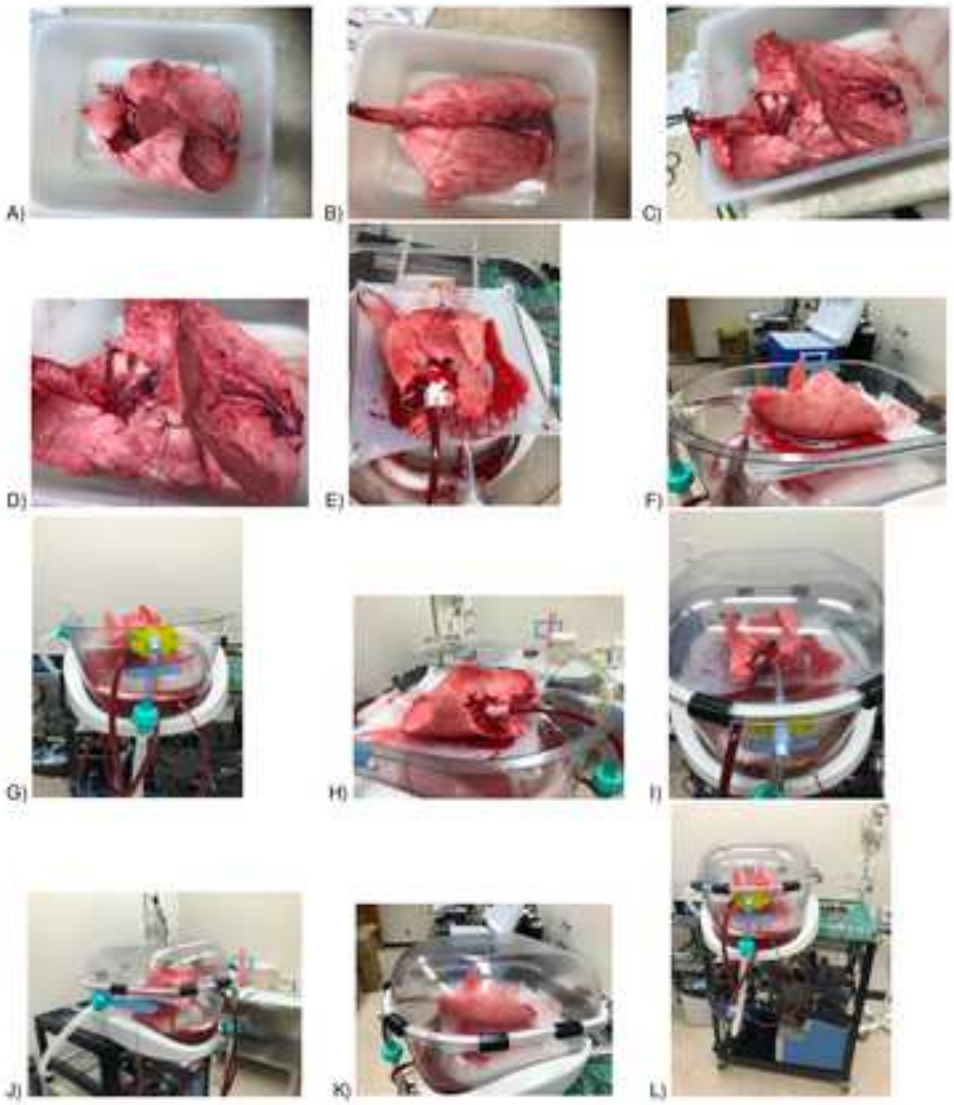

Figure 5: Lungs connected to NPV-ESLP circuit. A) Anterior Donor Lungs Pre-ESLP B) Posterior Donor Lungs Post-ESLP. C, D) Tissue biopsy of right middle lung lobe E) Lungs connected to ESLP circuit F) Demonstrated positioning of lungs on silicone support G) Front view of ESLP device demonstrating starting fluid level and lung positioning H) Lungs connected to device demonstrating open left atrial drainage I, J, K) Lid secured on the device chamber L) Device and lungs fully connected and functioning in NPV mode.

Figure 6

Click here to access/download;Figure;Revisions_JoVE NPV_Figure 6.psd

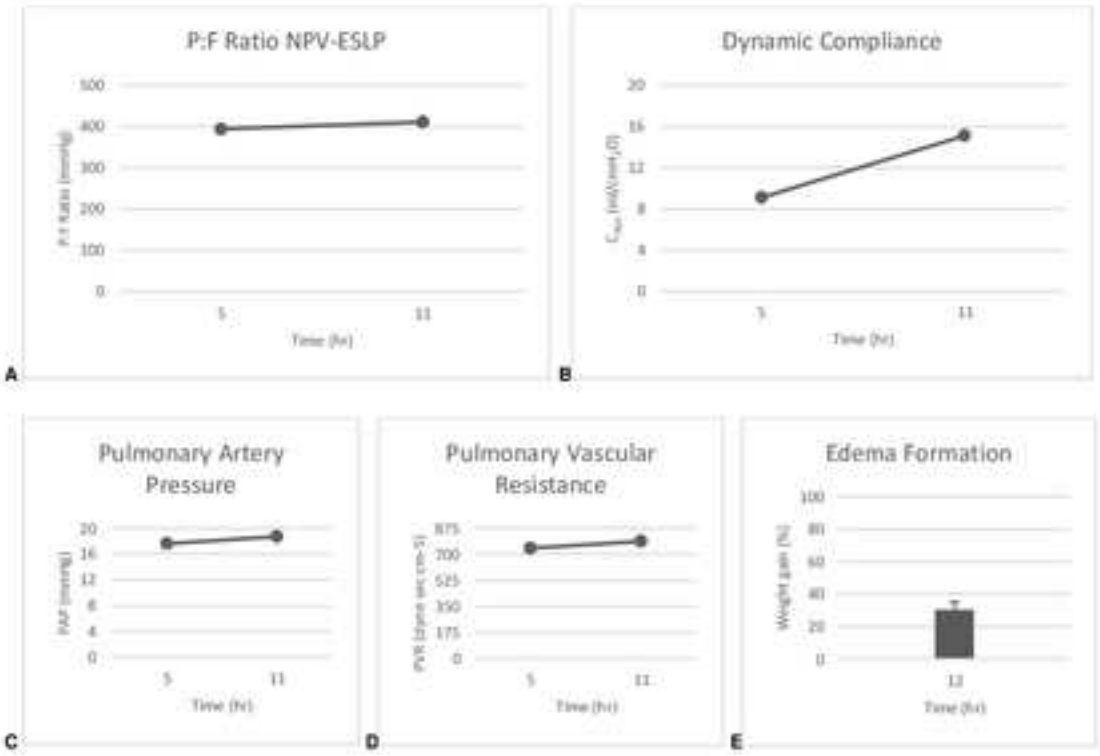

Figure 6: Functional Parameters during Evaluation modes over 12-hours of NPV-ESLP: A) P:F Ratio, PaO_2:FiO_2 ratio; B) Compliance; C) PAP, pulmonary artery pressure; D) PVR, pulmonary vascular resistance; E) Weight Gain (mean ± SE).

Table 1

Recorded Interface Parameters	Abbreviations
1. Vascular Parameters:	
Left Atrial Pressure	LAP
Pulmonary Artery Pressure	PAP
Pulmonary Vascular Resistance	PVR
Pulmonary Arterial Flow	PA Flow
2. Airway Parameters:	
Airway Pressure	P_{aw}
Intrathoracic Pressure	ITP
Transpulmonary Gradient	TPG
Airflow	Airflow
Fraction of Inspired Oxygen	FiO_2
Continuous Positive Airway Pressure	CPAP/PEEP
3. Ventilation Parameters:	
Expiratory Tidal Volume	Vt_e
Inspiratory Tidal Volume	Vt_i
Respiratory Rate	RR
Inspiratory: Expiratory Ratio	I:E
Minute Ventilation	MV
Dynamic Compliance	C_{dyn}

Units
mm Hg
mm Hg
Dynes s/cm^5
Litres/min (LPM)
cm H_2O
cm H_2O
cm H_2O
Litres/min (LPM)
Percentage (%)
cm H_2O
mL
mL
breathe/min (bpm)
N/A
Litres/min (LPM)
mL/cm H_2O

Initiation of *Ex-vivo* Lung Perfusion (Negative Pressure Ventilation)

	Perfusion time (min)		
	0	**10**	**20**
Perfusate Temperature (°C)	20 °C	32 °C	38 °C
PA flow (% CO; CO = 70 mL/kg/min)	10%	20%	30%
Ventilation mode	PPV (CPAP = 20 cm H_2O)	Initiate NPV Preservation	NPV Preservation
Medical gas mixer	None	None	None
Left atrial pressure	0	0	0

60 (T1)	**180 (T3)**
38 °C	38 °C
30%	50%
NPV Preservation	NPV Evaluation
None	89% N_2, 8% CO_2, 3% O_2
0	0

Table 3 Click here to access/download;Table;Table 3.xlsx

NPV Ventilation Strategy

	Ventila
	Preservation
Temperature (°C)	38 °C
Pulmonary artery flow	30% of estimated CO; CO = 70 mL/kg/min
Ventilation parameters	
Mode	Volume control
Desired inspiratory tidal volume	6-10 mL/kg
Inspiratory: Expiratory Ratio	1:1 – 1.5
Frequency	6 to 10 breaths/min
P_{AWP}	< 25 cm H_2O
PEEP	8-12 cm H_2O
FiO_2	21%
Pressure parameters	
PAP	< 15 mm Hg
LAP	0 mm Hg
Medical gas mixture	-
Medical gas mixture (litres/min) titrated to PCO_2	-

ation Mode
Evaluation
38 °C
50% of estimated CO; CO = 70 mL/kg/min
Volume control
6-10 mL/kg
1:1 – 1.5
6 to 10 breaths/min
< 25 cm H_2O
5-8 cm H_2O
21%
< 20 mm Hg
0 mm Hg
89% N_2, 8% CO_2, 3% O_2
35 to 50 mm Hg

Table 4

		Preservation			
Arterial Blood Gases (21% FiO2)	***In vivo* Recipient**	**T0 Left Atrium**	**T5 Left Atrium**	**T11 Left Atrium**	**T5 Pulmonary**
Blood Gas Values					
pH	7.4	7.42	7.44	7.56	7.35
pCO2 (mmHg)	47.7	12.9	7.2	9.1	13.4
pO2 (mmHg)	299	133	102	124	56.5
Oximetry Values					
Hb (g/dL)	11.2	5.9	6.5	5.6	6.1
sO2 (%)	100	99	98.4	98.9	85.5
Electrolyte Values					
K+ (mmol/L)	4.5	4.6	4.1	4.9	4.2
Na+ (mmol/L)	141	148	166	164	164
Ca2+ (mmol/L)	0.99	2.39	2.04	1.81	2.15
Cl- (mmol/L)	97	105	110	111	108
Osm (mmol/kg)	287	301.9	337.4	330.9	334
Metabolite values					
Glucose (mmol/L)	4.2	5.5	5	3.2	5.4
Lactate (mmol/L)	1.2	1.1	9.5	14.6	8.5
Acid Base status					
HCO_3^- (mmol/L)	28.1	8.3	4.8	8.2	7.2

Evaluation		
T5 Left Atrium	**T11 Pulmonary**	**T11 Left Atrium**
7.36	7.44	7.48
12	14.9	12.7
82.7	53.8	86.4
P/F ratio: 393.8		**P/F ratio: 411.4**
6	5.6	5.4
95.1	87.6	97.8
4.1	5	5
165	164	164
2.13	1.88	1.86
110	111	113
334.6	331.5	331.2
5.2	3	2.9
9	14.3	14.4
6.7	9.8	9.3

Rebuttal Letter NPV-ESLP for Revisions 3

All requested revisions have been completed.

Thank you.

Keir Forgie

Separate Rebuttal Document NPV-ESLP JoVE

Dear Dr. Forgie,

Your manuscript, JoVE62982 "Normothermic Negative Pressure Ventilation Ex-Situ Lung Perfusion: Evaluation of Lung Function and Metabolism.," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually.

Your revision is due by **Aug 02, 2021**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision". Please note that the corresponding author in Editorial Manager refers to the point of contact during the review and production of the video article.

Best,

Vidhya Iyer, Ph.D.
Review Editor
JoVE
vidhya.iyer@jove.com
617.674.1888
Follow us: Facebook | Twitter | LinkedIn
About JoVE

_____

Please revise the manuscript to thoroughly address the reviewers' concerns and all the editorial comments. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

We have proofread the manuscript and believe all spelling and grammar issues have been fixed. All abbreviations are defined at first use. If there are any specific issues remaining, please identify them by line and we will gladly address them.

We use the Canadian/British spelling for "centre". Sentences that were originally written in point-form have been re-written into full-sentences.

2. Please provide an email address for each author.

Keir A Forgie	kforgie@ualberta.ca
Nicholas Fialka	nfialka@ualberta.ca
Max Buchko	buchko@ualberta.ca
Sayed Himmat	shimmat@ualberta.ca
Sanaz Hatami	hatami@ualberta.ca
Xiao Qi	xqi3@ualberta.ca
Xiuhua Wang	xiuhua@ualberta.ca
Katie-Marie Buswell	buswell@ualberta.ca
Ryan Edgar	redgar@ualberta.ca
Darren H. Freed	dhfreed@ualberta.ca
Jayan Nagendran	jayan@ualberta.ca

3. Please revise the following lines to avoid overlap with previously published work (even if it's your own paper): 111, 113, 115-117, 128, 152-153, 160, 163, 189, 270-271, 413-416.

The following text has been added/edited:

Line 111: "1.1. Position the organ chamber on the ESLP cart and mount the silicon support membrane onto the chamber hooks for suspension."

Line 113: "1.3. Connect the heat exchanger water lines to the deoxygenator as well as the sweep gas tubing."

Line 115-117: "1.5. Secure the pulmonary artery (PA) flow probe onto the PA tubing. 1.6. Use a three-way stop-cock to fasten the PA pressure transducer to the PA cannula. 1.7. Tubing connections must be firmly attached to prevent leaks, and all the stopcocks and luer locks need to be closed prior to adding the perfusate.

Line 128:" The software computes functional parameters and pressure-volume loops."

Line 152-153: "3.3. Titrate oxygen flow in accordance to animal weight, typically 20 - 40 mL/kg."

Line 160: "3.8. Maintain the anesthesia by adjusting oxygen flow (20–40 mL/kg) and inhalant gas (1–3%)."

Line 163: "3.10. Prepare the incision site: shave and wash using iodine."

Line 189: "4.2.4. Use electrocautery to mark the midline along the sternal bone"

Line 270-271: " Any weight gain over the ESLP run is used as an indicator of edema."

Line 413-416: " Divide the biopsy into three equal sized portions, and place one of each in optimum cutting temperature (OCT) gel, formalin, and liquid nitrogen (snap freeze). Store the OCT and snap frozen samples in a -80 °C freezer, and store the formalin samples in a 4 °C refrigerator using a properly sealed container."

4. To avoid confusion, please ensure that the corresponding author is the same in the manuscript and Editorial Manager.

Thank you for pointing this out. Dr. Jayan Nagendran MD/PhD in the primary investigator and should be the corresponding author. Dr. Keir Forgie MD is a PhD student, responsible for most of the writing and experiments. Keir will be able to provide the timeliest communication, which is why he was listed as the corresponding author in the Editorial software. If congruence is required, please use Dr Jayan Nagendran as the corresponding author.

5. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), but before punctuation.

Reference numbers now appear as superscripts.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Protocol sections 1-13 are now in the imperative tense.
There are no hoods used in this protocol. There is no additional safety information to add.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

The "how" questions are answered. Enough detail is included in each step to supplement the actions seen in the video.

8. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.

Line spacing is single. A one-line space has been included between each protocol step.

3 pages of the protocol have been highlighted for filming.

9. Please discuss all figures in the Representative Results. However, for figures showing the experimental setup, please reference them in the Protocol.

Figure 8 and Table 4 are discussed in the Representative Results section.

All other figures and tables relate to the experimental set up and are referenced in the protocol.

10. Please include a section “Figure and Table Legends” to follow the representative results section (before the discussion section) and include the figure legends in this legends section.

A section titled "Figure and Table Legends" is now included. It now contains the figure and table legends.

11. Please check the figures—you have two figures labeled as Figure 3. Upload all figures as individual files in the correct order.

We have corrected the figure numbers.

12. Consider making the table in Figure 6 an actual table in .xls format and upload it separately.

Figure 6 is now labelled as Table 1. The other tables and figures have had their numbering adjusted.

13. Please upload all tables as individual .xls files.

Tables have been uploaded as individual .xls files.

14. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include

volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.

References now appear as directed. Reference 14 does not have an available issue number.

15. Please sort the Materials Table alphabetically by the name of the material.

The Materials Table has been sorted alphabetically.

_____

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this study on negative pressure ventilation for ex-situ lung perfusion to preserve donor lungs for transplantation, the autors describe their procedure in a porcine model. It is important to note that this ventilation mode has already been tested in humans, in a recent clinical trial with positive results, and that the procedure has been well described elsewhere. Nontheless, reports on this technique are still scarce, and single center experiences like this are of great interest.

Thank you for your comments. It is true that we have described this protocol in some detail elsewhere; however, this manuscript provides significantly more detail for the purpose of anyone who wishes to emulate our research. It also includes protocol updates that are not reflected in detail in any of our previous publications.

Major Concerns:

None

Minor Concerns:

None

Reviewer #2:

Manuscript Summary:

This manuscript presents the detailed protocol for normothermic negative pressure ex-situ lung perfusion (ESLP) using a custom device in a porcine model. The manuscript is well written, clearly presented, and protocol is detailed. Lung transplantation is limited by the availability of suitable organs, and mortality is high on the waiting list. Ex vivo lung perfusion (EVLP), or ex situ (ESLP), utilizes complex pump and ventilator system to perfuse and ventilate the lungs in order to recondition and assess lung function to improve transplantability. Many protocols have been described and translated into clinical applications and trials. Conventional EVLP systems rely on positive pressure ventilation, but recent work suggest that negative pressure ventilation (NPV) is superior to reduce barotrauma and injury. Clinical safety and feasibility of a custom NPV-ESLP has been demonstrated, with 100% 30-days and 1-year survival. The manuscript presents the detailed protocols for NPV-ESLP in a pig lungs model, for a 12 hours duration. List of materials

detailed.
Conflict of interest is disclosed, DHF and JN are founders and major shareholders of Tevosol, organ chamber apparatus.

Minor Concerns:
Why is ex situ LP instead of ex vivo LP has been used in the manuscript?

In previous papers, we used the terminology of ex-vivo lung perfusion (EVLP) and we also previously used ex-vivo heart perfusion (EVHP). Since then, the terminology has been updated to reflect the fact that we are not taking organs from "living" animals or human donors. These donors are NDD or DCD; therefore, the ethically correct terminology is "ex-situ". In the most recent publications on this topic, ESLP and ESHP are now used.

Is there any perfusion of the lungs (preservation solution such as Perfadex) in the pulmonary artery before harvesting?

No, we do not flush the lungs with Perfadex prior to pneumonectomy, although that is common practice with human retrievals. The primary reason for this is financial. Perfadex is very expensive, and we reason that because the donor pigs are given a large dose of heparin 1000 units/kg and the lungs are quickly attached to the ESLP machine, which flushes them, in-situ flushing is unnecessary.

What is CHIP solution?

CHIP stands for Common Hospital Ingredient Perfusate. It was developed in our last in 2019 and has been systematically compared to other commonly used proprietary perfusates. The objective was to produce an affordable perfusate using ingredients that are typically available on a crash cart, since perfusates represent a significant barrier to global distribution of ESLP technology due to their high cost.

Reference: Buchko, M.T., Himmat, S., Aboelnazar, N.S., et al. A low-cost perfusate alternative for ex vivo perfusion. Transplantation Proceedings. XX, 1-6 (2020).

pRBC is added, what is the desired hematocrit?

Thank you for pointing this out. Hemoglobin concentration is targeted at approximately 50 g/L or a hematocrit of 15%.

The following lines have been added to the text (line 591-592): " The hemoglobin concentration is targeted between 50 g/L or a hematocrit of 15%."

What is the oncotic pressure of the perfusate and how does it compare with Steen solution?

CHIP contains a similar hyperoncotic pressure and electrolyte composition to KHB with albumin and Steen solution. Our typical oncotic measurement is 30 mmHg

The following lines have been added to the text (line 220-222): "1.8. Prime the circuit with 1000 mL of modified common hospital ingredient perfusate (CHIP) [18]. CHIP is a custom-made low cost perfusate with an oncotic measurement of 30 mmHg, comparable to KHB with albumin and STEEN solution[18]."

Reference: Buchko, M.T., Himmat, S., Aboelnazar, N.S., et al. A low-cost perfusate alternative for ex vivo perfusion. Transplantation Proceedings. XX, 1-6 (2020).

Heparin should be given as U per units of volumes (final concentration) in the ESLP system.

The following text has been added to line 359: "2.3. Add 3.375 g piperacillin-tazobactam, 10,000 units of heparin (10,000 U/1.5L perfusate = 6.66 U/L), and 500 mg of solumedrol to the circuit."

How many biopsies are performed during all the procedure?

Our biopsies are described at these steps 4.3, 7.3, 13.6. We take one *in-vivo* biopsy, one biopsy just prior to ESLP, and one biopsy at the termination of ESLP.

What size?

Steps 4.3 and 7.3 mention a 1 cm^3 size and how to perform the biopsy. This has been added to step 13.6.

What is it used for? Proteins, which biomarkers, staining? Please suggest some markers studied or what to look for.

The biomarkers used are specific to the experimental protocol employed. Generally, our biopsies are used to compare signs of acute lung injury. We rely on our perfusate samples for specific biomarkers of inflammation, such as IL-6, IL-8, TNF-alpha, because the perfusate is in contact with the entire lung surface and gives us a holistic measurement of overall inflammation. The reason we do no use biopsies for this purpose, generally, is because 1cm^3 does not accurately represent the entirety of the lung. Even when sampling in a predictable manner, not all lungs behave the exact same on ESLP due to anatomical variation (i.e. some lungs have more dominant lower lobes vs upper lobes and vice-versa), and this effects the biopsy quality.

We mention our perfusate biomarkers in the discussion, lines 608-611.

The following text has been added to line 865-870: "13.7 Run the final gas analyses, centrifuge the perfusate samples, and store the tissue biopsies as previously described. Centrifugation

settings include: 1600RPM, 9 acceleration, 9 deceleration, 4 degrees Celsius, and 15-minute duration."

The following text has been added to line 482-491:
"4.4. Take a biopsy of the right upper lung lobe for tissue analysis: open the right pleura to expose the right upper lobe. Encircle a 1cm3 portion with 0-silk, tie, and excise this portion of lung using Metzenbaum scissors. Divide the biopsy into three equal sized portions, and place one of each in optimum cutting temperature (OCT) gel, formalin, and snap freeze in liquid nitrogen, respectively. Store the samples for future investigations (OCT and snap frozen samples in a -80 °C freezer, formalin-stored samples in a properly sealed container in 4 °C Celsius refrigerator).

Note: Biopsy samples are stained with hematoxylin-eosin staining to examine the histophathology of lung injury including interstitial edema, alveolar and interstitial inflammation, interstitial and perivascular neutrophilic infiltrates, and hemorrhage (17)."

Lungs are weighted before and after ESLP? What is normal or expected weight gain or loss? In Fig8E, 50% weight gain is reported, this could seem a lot for normal lungs? Is the edema is interstitial or alveolar?

Thank you for point this out. Lungs are weighed after donor explant and after ESLP. Generally, the weight-gain is 20-30% in juvenile porcine lungs, but the range is 15-50%. The example that we included in Fig 8E should not have been used in isolation for our representative results section. This was an oversight on our end, so we appreciate you highlighting this. To remedy this, we have pooled a sample of 6 control runs produced in the past 6 months, which includes the 50% edema formation run. The range of values was 15.7-50%, with a mean of 30%. With a larger sample size, we believe the mean would be closer to 20-25%. Acellular runs (CHIP only) are associated with significantly more edema formation. Please see our paper for a more detailed discussion of perfusate compositions and ventilatory strategy: 15. Aboelnazar, N.S., et al. Negative pressure ventilation decreases inflammation and lung edema during normothermic ex-vivo lung perfusion. The Journal of Heart and Lung Transplantation. 37(4), 520-30 (2018).

Edema formation is both interstitial and alveolar. It is not clear why normal juvenile pig lungs can develop such a wide range of weight gain over 12 hours of ESLP using a consistent protocol. The delicate and sometime unpredictable nature of juvenile porcine lungs is one reason why they are such good models for extended criteria donor lungs. Human lungs in contrast, being more robust, lose weight while on ESLP (15).

Any X-ray? Or imaging to assess the pattern of edema?

We do not have the resources to support the use of portable X-ray machinery in our lab. Previously, we have enlisted the help of a blinded pathologist to assess the character of tissue injury.

We have previously compared the extent of interstitial edema, Alveolar inflammation, interstitial neutrophil infiltration, interstitial inflammation, hemorrhage, and perivascular neutrophil infiltration.

No bronchoscopy performed?

We used to perform BAL on the lungs; however, we stopped doing this because the samples drawn were not particularly informative above and beyond our perfusate and tissue samples, and the requirement to perform a bronchoscopy for suction purposes during ESLP foreshadows an unsuccessful run. That is, if fluid is building up in the larger airways or ETT, the edema is already beyond a point of reconditioning with juvenile porcine lungs. Human lungs are far more robust, but this protocol is specific to how we manage our large animal models.

Reviewer #3:
Manuscript Summary:
This manuscript describes a new technique - normothermic negative pressure ventilation ex-situ lung perfusion - which will be further developed for donor lung assessment, repair and reconditioning.
Overall, this is a paper for protocol description. It is hard for the reviewer to imagine how accurate the protocol is. I focus my review on the preparation of the manuscript

Major Concerns:
1. The Abstract should be the summary of the whole article. Currently, it is only a Background of the research field, introducing the concept of this new technique, not the protocol. It has to be totally re-written.

Thank you for your comment. The Abstract has been totally re-written to better reflect the whole article.

2. Introduction. The rationale of developing negative pressure ventilation (NPV) for EVLP is the foundation of this paper. In the Introduction, most of references cited are not directly related to NPV-EVLP. Please focus on the most relevant publication(s) to demonstrate the beneficial effects of NPV-EVLP and its potential application. This is very important.

Thank you for your comment. We agree with your suggestion to improve the introduction and have removed papers on PPV that were not necessary or immediately relevant. We have also added an additional NPV-ESLP publication from our lab and redirected the focus of the introduction towards NPV-ESLP.

3. In the Protocol, please use proper figures or videos to help readers to understand how the system works.

Figures were included with our manuscript submission. It is unfortunate that you did not have access to them for your review. A video will be produced by JoVE if our manuscript is accepted for publication.

Minor Concerns:
1. Currently, ex vivo organ perfusion (EVOP) and ex vivo lung perfusion (EVLP) is commonly used in the literature. In the original paper (reference #23) published by this group, they also used EVLP. Please consider change ex-situ lung perfusion (ESLP) to EVLP.

Thank you for your comment. You are correct that in our original paper, we used the terminology of ex-vivo lung perfusion (EVLP) and we also previously used ex-vivo heart perfusion (EVHP). Since then, the terminology has been updated to reflect the fact that we are not taking organs from "living" animals or human donors. These donors are NDD or DCD; therefore, the ethically correct terminology is "ex-situ". In the most recent publications on this topic, ESLP and ESHP are now used.

2. Fig 8E. Weight gain >50%? This indicates severe pulmonary edema. Please clarify.

Thank you for point this out. Lungs are weighed after donor explant and after ESLP. Generally, the weight-gain is 20-30% in juvenile porcine lungs, but the range is 15-50%. The example that we included in Fig 8E should not have been used in isolation for our representative results section. This was an oversight on our end, so we appreciate you highlighting this. To remedy this, we have pooled a sample of 6 control runs produced in the past 6 months, which includes the 50% edema formation run. The range of values was 15.7-50%, with a mean of 30%. With a larger sample size, we believe the mean would be closer to 20-25%. Acellular runs (CHIP only) are associated with significantly more edema formation. Please see our paper for a more detailed discussion of perfusate compositions and ventilatory strategy: 15. Aboelnazar, N.S., et al. Negative pressure ventilation decreases inflammation and lung edema during normothermic ex-vivo lung perfusion. The Journal of Heart and Lung Transplantation. 37(4), 520-30 (2018).

Edema formation is both interstitial and alveolar. It is not clear why normal juvenile pig lungs develop such a wide range of weight gain over 12 hours of ESLP using a consistent protocol. The delicate and sometime unpredictable nature of juvenile porcine lungs is one reason why they are such good models for extended criteria donor lungs. Human lungs in contrast, being more robust, lose weight while on ESLP (15).