Refinement of Methodology in *Ex-Situ* Lung Perfusion

by

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Abstract

Lung transplantation is the gold-standard treatment for eligible patients with end-stage lung disease; however, there is an inadequate supply of high-quality donor lungs for transplant, resulting in a wait-list mortality of 15-30%. Ex-situ Lung Perfusion (ESLP) has become an established means of increasing the donor pool over the last twenty years. Significant progress has been made to extend ESLP preservation from 4-hours to 12-hours in the pre-clinical realm. Unfortunately, clinically approved ESLP preservation is still limited to 4-6 hours, but this will continue to expand along with clinical familiarity. A few preclinical ESLP protocols, including our own, have managed to achieve 24-hours of continuous preservation with porcine lungs, although only one platform has managed to achieve adequate reliability for successful *in-vivo* transplantation assessment. Xenogeneic cross-circulation ESLP and cyclic ESLP (prolonged cold static preservation [CSP] with brief intermittent periods of ESLP) have successfully achieved total preservation durations of 36-72 hours; however, these approaches deviate from the core tenets of ESLP: 1) isolation of the lungs from other organ systems with vulnerable physiology, and 2) continuous physiologic assessment.

For ESLP to reach its full potential and optimize the pool of donor lungs, we believe that ultra-prolonged *continuous* and *isolated* preservation that allows for increased dwell time of advanced therapeutics is essential (i.e., without cyclic CSP interruption or vulnerable host dependent cross-circulation). A preservation window of 36- to 48 -hours could eliminate global geographic barriers to transplant and organ sharing, allow for optimized donor-recipient matching with more thorough assessment, and enable the expansion of the donor pool by treating and improving poor quality lungs with advanced pathologies. Our lab has previously established that our unique Negative Pressure Ventilation (NPV)-ESLP device produces less lung injury and

inflammation compared to a standard Positive Pressure Ventilation (PPV)-ESLP strategy. Therefore, NPV-ESLP is well-positioned to be the ideal platform for ultra-prolonged ESLP. Hence, the objectives of this thesis are to: 1) Develop a clinically relevant large animal lung transplant model to validate ESLP physiology via *in-vivo* assessment; 2) Systematically investigate key aspects of our current ESLP protocol, including temperature, flow rate, blood-gas management, and perfusate management, for further optimization as demonstrated through improved acute transplant outcomes; 3) Apply our refined protocol to achieve a reliable 24-hour NPV-ESLP transplant model, and push the envelope to achieve 36-hours of continuous, isolated ESLP preservation, which would represent a milestone achievement.

Preface

This thesis is an original work by Keir Andresen Forgie. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Ex-vivo organ perfusion", AUP. 943, Mar. 24, 2014.

Chapter 1 of this thesis is an overview prepared by me.

Chapter 2 of this thesis is a literature review prepared by me.

Chapters 3 and 4 of this thesis has been published in the *Journal of Visualized Experiments.* Keir Forgie performed the experimental design, data collection, laboratory analysis, data analysis, data synthesis, manuscript preparation, manuscript submission and manuscript revisions. Nicholas Fialka performed data collection, data analysis, and manuscript preparation. Mubashir Khan, Sayed Himmat, Sanaz Himmat, Xiao Qi, Max Buchko, Xiuhua Wang, Katie-Marie Buswell, Ryan Edgar facilitated experiments and supported manuscript preparation. Drs Darren H. Freed and Jayan Nagendran participated in research design, data analysis, manuscript preparation and revisions.

For **Chapters 5-9** of this thesis, I participated in the research design directly with Dr Nagendran. I performed the experiments, data analysis, manuscript preparation and revisions. N. Fialka, A. Watkins, K.Du, A. Ribano, S. Himmat, X. Wang, S. Hatami, G. Mainardi Aguiar da Silva, R. Edgar, K. Buswell-Zuk, D. Domahidi participated in the performance of experiments. Dr. D.H. Freed participated in the performance of experiments, data analysis, manuscript preparation and revisions. Dr J. Nagendran participated in research design, data analysis, manuscript preparation and revisions.

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List of Abbreviations

A1AT: alpha-1-antitrypsin

ABG: arterial blood gas

ADP: adenosine diphosphate

AFC: airspace fluid clearance

AMP: adenosine monophosphate

APRV: airway pressure release ventilation

AR: Argon

ARDS: acute respiratory distress syndrome

ATP: adenosine triphosphate

BAL: bronchoalveolar lavage

BCAAs: branched chain amino acids

BDAS: buffered dextran-albumin solution

BOS: bronchiolitis obliterans syndrome

Cdyn: Dynamic compliance

CF: continuous flow

CFTR: cystic fibrosis transmembrane receptor

CFU: colony forming units

CHIP: common hospital ingredient perfusate

CLAD: chronic lung allograft dysfunction

CLES: centralized lung evaluation centre

CO: cardiac output

$CO_{predicted}$: Predicted cardiac output

CHD: continuous hemodialysis

CIT: cold ischemic time (aka CSP)

CPAP: continuous positive airway pressure

CSP: cold static preservation

CT: computed tomography

CXR: chest x-ray

D25: 25% dextrose solution

DCD: Donation after circulatory death

ECD: extended criteria donor

ECMO: extracorporeal membrane oxygenation

EEP: end expiratory pressure

EIP: End inspiratory pressure

ELISA: enzyme-linked immunosorbent assay

ERB: endothelin receptor blocker

ESLP: Ex Situ Lung Perfusion (aka EVLP)

ESOP: Ex Situ Organ Perfusion

ETT: endotracheal tube

EVLP: Ex Vivo Lung Perfusion (aka ESLP)

EVOSS^{TM}: Ex Vivo Organ Support System

FCV: flow-controlled ventilation

FDA: Food and Drug Administration

FFA: free fatty acids

FiO_2: Fraction of inspired oxygen

HBV: hepatitis B virus

HCV: hepatitis C virus

HF: hemofiltration

HGF: hepatocyte growth factor

HIV: human immunodeficiency virus

HREB: human research ethics board

hr or hrs: hour or hours

ICU: intensive care unit

I:E ratio: inspiratory to expiratory ratio

IL: Interleukin (e.g., IL-6, -8, etc.)

iNO: inhaled nitric oxide

IRI: ischemic reperfusion injury

ISHLT: International Society of Heart and Lung Transplantation

ITP: intrathoracic pressure

IU: international units

IFN-ɣ: interferon gamma

LA: left atrium

LAP: left atrial pressure

LbT: light-based therapy

LF: low flow

LIRI: lung ischemic reperfusion injury

LOS: length of stay

LPD: low-potassium dextran-based solution

LS1™: Lung System 1

MAPC: multipotent adult progenitor cells

MF: moderate flow

MPO: myeloperoxidase

MSC: Mesenchymal Stem Cell

NAC: N-acetylcysteine

NAT: Nucleic acid test

NDD: Neurologic Determination Death

NEI: neutrophil elastase inhibitor

NO: nitric oxide

NTH: normothermic

NPV: negative pressure ventilation

NPV-ESLP: negative pressure ventilation ex-situ lung perfusion

OCS™: Organ Care System

OCT: optimum cutting temperature gel

PA: pulmonary artery

PAP: pulmonary artery pressure

PAWP: peak airway pressure

PEEP: positive end expiratory pressure

PDT: photodynamic therapy

PF ratio: partial pressure of oxygen divided by the fraction of inspired oxygen (PaO_2/FiO_2 ratio)

PF: pulsatile flow

PFCOC: perfluorocarbon-based oxygen carrier

PGD: primary graft dysfunction (e.g., PGD 0-3 score)

pH: 7.35-7.45

pH+: 7.45-7.55

PH: pulmonary hypertension

PPV: positive pressure ventilation

PPV-ESLP: positive pressure ventilation ex-situ lung perfusion

pRBC: packed red blood cells

PVR: pulmonary vascular resistance

QOL: quality of life

RR: respiratory rate

RTX: Rituximab

SEM: standard error of the mean

SNLF: subnormothermic low flow

SVC: superior vena cava

T0, T1....T11, T12: Time in hours.

TEM: Transmission Electron Microscope

THAM: tris(hydroxymethyl)aminomethane

TNF: Tumor necrosis factor (e.g., TNF-$\propto$)

TPN: Total Parenteral Nutrition

TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

TV: tidal volume

VCV: volume-controlled ventilation

VILI: Ventilator induced lung injury

V/Q: ventilation/perfusion

WB: whole blood

WIT: warm ischemic time

Xe: Xenon

XPS^{TM}: XVIVO Perfusion System

Chapter 1

Thesis Overview and Research Proposal

BACKGROUND AND OVERALL OBJECTIVES

Lung transplantation is a lifesaving therapy for patients with end-stage lung disease who have no remaining medical or surgical options available. Currently, over 4600 lung transplants are performed globally each year, and patient outcomes have improved significantly since the first successfully single lung transplant in Toronto by Dr Joel Cooper in 1983[1]. Outcomes have improved due to advances made in donor and recipient selection, perioperative/medical management, surgical techniques, and immunosuppression/anti-rejection therapy[2,3]. With improved outcomes, there has been a broadening of indications for lung transplantation and an associated increase in the number of patients listed for the procedure. Unfortunately, the increase in wait-listed patients has not been met with a proportional increase in useable donor lungs. In fact, due to conservative ideal donor acceptance criteria, rates of donor lung utilization remain low, with approximately 85% of offered lungs being rejected for use by transplant programs [4]. The consequence of inadequate acceptable donor lung availability is a high wait-list mortality of 15-20%, which is significantly higher than other solid organ transplants[5,6]. Indeed, lung transplantation is limited by the lowest organ acceptance rate, highest waitlist mortality, and worst long-term outcomes of any solid organ transplant. In contrast to the aforementioned treatment advances that have been made, the gold-standard method for preserving donor lungs, known as cold static preservation (CSP), has remained largely unchanged over the past 30 years.

Cold static preservation involves flushing the donor lungs with a cold preservation solution and storing the explanted lungs in a cooler of ice for transportation to the implanting centre[7]. The purpose of cooling the organ is to arrest/slow cellular metabolism, thereby decreasing the demand for oxygen and nutrients, as well as slowing the production of deleterious cellular by-products caused by ischemia, inflammation, and oxidative stress that can injure the

lungs[8]. The caveat is that beneficial cellular processes are likewise inhibited, which equates to a lost opportunity for improvement in allograft function prior to implantation. Clearly, CSP is not an ideal form of organ preservation, and its safe duration of application for lungs is generally accepted as 6-8 hours before irreparable damage develops in the graft[9]. Furthermore, CSP lacks the ability to assess donor organs during transportation, nor is there an opportunity to intervene therapeutically beyond the application of cold. In turn, there is ambiguous organ function during transport. Despite the limitations, CSP remains the gold-standard for all solid organ preservation due to its simplicity, cost effectiveness, and reliability. The ideal organ preservation system would enable continuous metabolic support and functional organ assessment, limitless preservation duration, permit therapeutic intervention and recuperation, facile management/operation, high reliability and low-cost. A relatively novel method of organ preservation called *Ex-Situ* Lung Perfusion hits many of these targets.

Ex-Situ Lung Perfusion (ESLP) is a method of donor lung preservation that uses a machine to continuously ventilate the organ and perfuse it with a specialized solution to support metabolic function from procurement to implantation[10-13]. Commercial devices allow for assessment of organ function, isolation from hostile donor pathophysiology, reconditioning of injured lungs, prolonged safe preservation, and have increased the useable pool of donor lungs with recipient outcomes equivalent to CSP[10-13]. Furthermore, ESLP has facilitated the safe use of extended criteria donor lungs that do not meet ideal acceptance criteria[10,11,13]. ESLP has also enhanced research opportunities on donor lung physiology and treatment by providing a vehicle for intervention either via the perfusate/vasculature or ventilator/airway[14,15,16]. Clinically, ESLP is approved for 4-6 hours of preservation, although 12-hours of preservation is standard for pre-

clinical research. A few labs, including our own, have reached 24-hours of preservation, but with limited reliability[15,17].

ESLP is relatively nascent, and there is ongoing need for improvement of devices and management protocols for this technology to reach its full potential. One such improvement unique to our lab has been the development of an ESLP device that uses negative pressure ventilation (NPV) as opposed to positive pressure ventilation (PPV)[14]. Where PPV forces air into the lungs to inflate them like a balloon, NPV applies a vacuum force in an air-tight chamber to pull the lungs open and draw air inward. NPV mimics normal physiologic respiration, and in 2018 Nagendran et al demonstrated that NPV-ESLP preservation of pig and human lungs is associated with reduced lung injury, inflammation, and organ weight-gain (edema) compared to PPV-ESLP[14].

The field of ESLP has developed rapidly since the first clinical report in 2001 of non-heart beating donor lungs being procured, assessed, and successfully implanted[18]; however, many aspects of currently published ESLP protocols have not been scientifically investigated for optimization, such as ideal perfusion temperature, flow rate, and pH management, to name a few. Therefore, we propose that for ESLP to reach its full potential, particularly with respect to ultra-prolonged preservation beyond 24-hours, further refinement in our own protocol is required to optimize outcomes and reliability.

Hence, the objectives of this PhD proposal are to: 1) establish a large animal lung transplantation model to validate ESLP outcomes with *in-vivo* evaluation; 2) determine whether hypothermic or subnormothermic perfusion temperatures are superior to normothermic perfusion; 3) determine whether a further reduction in perfusion flow rate below our standard flow rate enhances lung preservation; 4) determine if a strategy of mild permissive alkalosis

results in superior preservation compared to strict pH control; 5) apply the findings from the preceding studies to achieve reliable 24-hour NPV-ESLP with acceptable transplantation outcomes; 6) determine if partial perfusate exchange of our buffered cellular perfusate during 24-hour NPV-ESLP results in superior transplant outcomes; 7) attempt 36-hours of continuous NPV-ESLP to achieve the longest continuous ESLP protocol to date.

Overall Objective: We hypothesize that through the investigation of key aspects of our current NPV-ESLP protocol, we can refine our methods of porcine ESLP to produce a reliable strategy of continuous perfusion up to 36-hours with parameters acceptable for lung transplantation. Key aspects of our current protocol that we will investigate for optimization include ideal perfusion temperature, flow rate, pH status, and perfusate exchange along with the development of a large animal lung transplantation model to validate our *ex-situ* results with *in-vivo* assessment.

Specific Hypotheses:

A. Hypothermic (10ºC) and subnormothermic (32ºC) perfusion may improve preservation of juvenile pig lungs while on NPV-ESLP and post-transplantation compared to normothermic perfusion (38ºC).
B. Reduced perfusion flow at 10% of predicted cardiac output may improve preservation of juvenile pig lungs while on NPV-ESLP and post-transplantation compared to 30% cardiac output.
C. NPV-ESLP with a pH strategy of mild permissive alkalosis may result in superior preservation and transplant outcomes compared to a physiologic pH.

D. NPV-ESLP can preserve juvenile pig lungs for 24-hours with acceptable acute post-transplant outcomes.

E. Partial perfusate exchange during 24-hours of NPV-ESLP may result in improved transplant outcomes compared to a strategy of no perfusate exchange.

F. NPV-ESLP can preserve juvenile pig lungs for 36-hours with parameters acceptable for transplantation.

Overview of Specific Aims

1) Establish a surgical model for left lung transplantation in juvenile pigs to assess NPV-ESLP results *in-vivo* during an acute reperfusion period of 4-hours.
2) Determine whether hypothermic (10ºC) or subnormothermic (32ºC) perfusion of juvenile pig lungs results in superior NPV-ESLP and transplantation outcomes compared to normothermic (38ºC) NPV-ESLP.
3) Determine whether NPV-ESLP of juvenile pig lungs with perfusion flow at 10% of predicted cardiac output results in superior preservation and transplantation outcomes compared to perfusion at 30% predicted cardiac output.
4) Determine whether NPV-ESLP of juvenile pig lungs with a pH strategy of mild permissive alkalosis (7.46-7.55) results in superior preservation and transplantation outcomes compared to physiologic pH control (7.35-7.45).
5) Establish a protocol for 24-hour NPV-ESLP with transplantation using juvenile pig lungs that yields a reliability (>80% success rate) and PGD 0-1 at 4 hours post-reperfusion.

6) Determine whether a strategy of partial perfusate exchange during 24-hours of NPV-ESLP using juvenile pig lungs results in improved preservation and transplant outcomes compared to a strategy without perfusate exchange.
7) Establish a protocol for 36-hour NPV-ESLP using juvenile pig lungs that is reliable (>80% success rate) with physiologic parameters acceptable for transplantation.

Research Approach and Methods

Design and Methodology:

The University of Alberta (UofA) Animal Care and Use Committee approved all methods. NPV-ESLP will be performed using a custom-built device from the UofA that has undergone a phase one clinical trial (NCT03293043)[10] (Figure 1.1). Our protocols for lung procurement and 12-hours of NPV-ESLP have been described in detail[14,19] (Table 1.1 and 1.2, Figure 1.2) and are summarized below.

Standard ESLP Protocol

NPV-ESLP is performed at normothermia, which is 38ºC for pigs. The pulmonary artery (PA) is cannulated and perfused with common hospital ingredient perfusate (CHIP) and autologous packed red blood cells[20]. Piperacillin-Tazobactam (3.375 g), heparin (10 000 IU), and methylprednisolone (500mg) are added to the perfusate. Pressure and flow sensor are mounted on the cannulas to measure pulmonary vascular resistance (PVR). The trachea is connected to a custom ventilator with CPAP and negative pressure ventilation capabilities. The effluent from the pulmonary veins (PV) runs through a de-oxygenator, which is both continuous and in line with a centrifugal pump and the pulmonary artery (PA) cannula, thereby creating a continuous

circuit resembling normal circulation. Antegrade flow is started at 10% of predicted cardiac output (CO; 70ml/kg/min) and gradually increased to 30% based on donor weight. Lung protective ventilation is employed with tidal volumes of 6-8 mL/kg, respiratory rates of 7 bpm, PEEP of 5-8 cmH_2O, and an FiO_2 of 21%. Hourly pH, pCO_2, glucose, electrolyte, bicarbonate, and lactate measurements are collected using a standard arterial blood gas analyzer and corrected to target physiologic parameters. Every two hours, an evaluation is performed by increasing flow to 50% CO and applying mixed sweep gas (89% N_2, 8% CO_2, 3% O_2) through the de-oxygenator for 5-minutes. Perfusate samples are drawn pre- and post-deoxygenator to assess oxygenation step-up (PF ratio). Enzyme-linked immunosorbent assay kits are used to determine perfusate pro-inflammatory cytokine concentrations from samples drawn every 2 hours (tumor necrosis factor-alpha and interleukin-6, R&D Systems, Minneapolis, Minn, United States). Standard lung performance characteristics are regularly assessed. Oxygenation (PF ratio) is calculated as: partial pressure of oxygen to fraction of inspired oxygen ratio (PaO_2/FiO_2, mmHg) – a ratio of ≥ 300 is deemed acceptable for transplantation; total lung pulmonary vascular resistance: [PVR = (PA pressure-LA pressure/flow) x 80 (dynes·s·cm^5)]; Dynamic Compliance = VTe/(Peak inspiratory pressure - PEEP) [VTe = expiratory tidal volume], and percentage weight-gain [(ΔLung Weight)/ lung weight pre-ESLP) x100%].

Standard Data Collection

Our data outputs are well established[14,15,19,20] and are physiologic (continuously recorded and calculated on ESLP software), perfusate based, and transplant based[21]. Increased partial pressure of oxygen to fraction of inspired oxygen ratio (PaO^2/FiO^2), increased lung compliance, decreased pulmonary artery pressures and vascular resistance, decreased post-ESLP and transplant weight

gain (% weight-gain), preserved electrolyte and lactate parameters, and decreased inflammatory markers (IL-6, TNF-alpha) will indicate improved lung function/preservation.

Analysis of Data

Normally distributed continuous variables will be reported as mean +/- SEM and compared with the Students *t*-test or analysis of variance. The Shapiro-Wilk test will be used to assess normality. Non-normally distributed continuous variables will be reported as median (range) and compared using the Mann-Whitney U test. A p-value <0.05 will be considered statistically significant. All statistical analyses will be performed via GraphPad Prism, version 9.3.0 (GraphPad Software, La Jolla, CA, USA).

DETAILS OF SPECIFIC AIMS

Specific Aim 1: Establish a surgical model for left lung transplantation in juvenile pigs to assess NPV-ESLP results *in-vivo* during an acute reperfusion period of 4-hours.

Rationale: Pre-clinically, *in-vivo* transplantation outcomes are an established means of distinguishing performance between different ESLP management strategies[22, 23, 24], and there is currently no validated alternative to transplantation for comparably robust evaluation. Organ performance in a recipient at 100% cardiac output and exposed to the full spectrum of post-transplant physiology is the best means of assessing the quality of ESLP. Our lab has a unique NPV-ESLP device with an established management protocol, but we do not yet have a transplantation model to fulfill the aforementioned research aims — refinement of assessment parameters and *in-vivo* validation of protocol revisions. Therefore, our objective is to establish a

pig lung transplantation model that is easily reproducible and will add another level of data output to support our research findings and conclusions.

Experimental Design: Isolated left lung transplantation without extracorporeal circulation is the standard procedure in pig lung transplantation for pre-clinical research due to key anatomical considerations (e.g., accessory lung lobe) that make bilateral lung transplant without bypass impractical[22, 23, 24]. These anatomical considerations include a right-sided tracheal bronchus and an accessory lung lobe that is ventilated by the right airways but drains into a common pulmonary vein with the left lower lobe[25]. Therefore, we will develop a left lung transplantation protocol for ESLP in a stepwise manner using juvenile pigs (40-50kg) by increasing complexity incrementally. First, we will perform left lung auto-transplantations without ESLP (n=4 pigs). This first phase is included for surgical troubleshooting and optimization. Second, we will perform a donor-recipient left lung transplant with the donor lungs undergoing a short period of NPV-ESLP (4-hours) prior to implantation. This phase will help with the logistics of organizing two separate operating rooms with two large animals. We will optimize the timing and coordination of anticipated and unanticipated tasks. Phase 3 will be an extension of Phase 2 with an NPV-ESLP period of 12-hours between donor explant and recipient implant. Phase 4 will be the continued optimization between experimental projects. The transplant protocol will be further optimized with the end-goal of simplification to create an easily teachable, reproducible, and reliable transplant model that can serve the NPV-ESLP lab for years to come. Final isolated left lung assessment will be performed following sternotomy and clamping of the right hilum with blood samples subsequently drawn from the left pulmonary veins for blood gas analysis.

Expected Results:

We expect to develop a successful left lung pig transplant protocol that will enable us to assess the transplanted lung function post-ESLP. A reperfusion assessment period of 4-hours is achievable and is a standard post-transplant observation period in the field of large animal ESLP research[24]. We anticipate that the recipient blood can be evaluated via ELISA to quantify the concentrations of pro-inflammatory cytokines, similar to our ELISA assessment of ESLP perfusate. We expect post-transplant left lung blood gases to have PF ratios ≥ 300 mmHg. See Figure 1.3 for proposed left lung transplantation model.

Specific Aim 2: Determine whether hypothermic (10^{o}C) or subnormothermic (32^{o}C) perfusion of juvenile pig lungs results in superior NPV-ESLP and transplantation outcomes compared to normothermic (38^{o}C) NPV-ESLP.

Rationale: Ex-situ organ perfusion (ESOP: kidney, liver, lung, heart) attempts to preserve donor organs in a physiologic or semi-physiologic state to support metabolic function at normothermic temperatures and avoid the deleterious side-effects of cold static preservation (CSP)[26,27]. Hypothermia is employed in CSP to slow metabolic function, decrease demand for oxygen, nutrients, and ATP, thereby protecting the organ from some of the ischemic insult during retrieval and transportation[8]. In an effort to combine the beneficial effects of continuous perfusion with the protective attributes of hypothermia, protocols for hypothermic ESOP have been successfully developed in kidney and liver transplantation[28,29]. The application of cold ESLP in experimental research is also growing.

Recently, small animal studies have demonstrated a beneficial effect of both hypothermic (4-10°C)[30,31] and subnormothermic (21-32°C)[32,33,34] perfusion temperatures during ESLP compared to CSP and normothermic perfusion. These studies suggest that cold ESLP results in

reduced circulating inflammatory markers and improved physiologic function following transplantation.

Hypothermic and subnormothermic ESLP have never been studied in a large animal model. We hypothesize that in a juvenile pig model of NPV-ESLP that hypothermic (10ºC) and subnormothermic (32ºC) NPV-ESLP may result in improved lung function and decreased inflammation on ESLP and following transplantation compared to normothermic NPV-ESLP.

Experimental Design:

Eighteen sets of pig lungs will undergo 12-hours of NPV-ESLP with normothermic (38ºC, n=6), subnormothermic (32ºC, n=6), or hypothermic (10ºC, n=6) perfusion temperatures. Cold lungs will be rewarmed to normothermia over 30-minutes at T6 and T12 for physiologic evaluation with 50% CO and deoxygenated with mixed sweep gas, as per our standard protocol (see "Research Approach and Methods", Table 1.1 and 1.2). Cold lungs will be cooled to their respective target temperatures over 30-minutes in between evaluations for periods of preservation. Subnormothermic and hypothermic lungs will be perfused with an acellular perfusate, as reported in small animal models of cold perfusion ESLP[30-34]. Normothermic lungs will be perfused with our standard cellular perfusate so comparison can be made against our existing successful protocol. Three left lungs from each group will be randomly selected for transplantation and assessed *in-vivo* over a four-hour period. Graft function will be evaluated via physiologic parameters, edema formation, and pro-inflammatory cytokine profiles as outlined in, "Research Approach and Methods."

Expected Results:

We expect that hypothermic (10ºC) and subnormothermic (32ºC) perfusion temperatures may result in improved preservation of lungs on NPV-ESLP, in line with small animal models, compared to normothermic (38ºC) NPV-ESLP. This will be demonstrated by improved physiologic parameters on ESLP and post-transplant, including higher PF ratios and reduced percentage weight gain. It is possible that colder temperatures will cause an increase in PVR and reduction in compliance during ESLP; however, we anticipate that the added metabolic protection of cold in the setting of a reduced flow rate of 30% predicted cardiac output (standard for our protocol), will result in an overall improved evaluation performance, particularly at transplantation. We expect that hypothermia and subnormothermia will both result in reduced pro-inflammatory cytokine markers compared to normothermic ESLP due a reduced metabolic rate. It is possible that subnormothermia will outperform hypothermia due to an anticipated balance of beneficial and deleterious effects from the application of cold and the application of continuous ESLP.

Specific Aim 3: Determine whether NPV-ESLP of juvenile pig lungs with perfusion flow at 10% of predicted cardiac output results in superior preservation and transplantation outcomes compared to perfusion at 30% predicted cardiac output.

Rationale: Early protocols in ESLP aimed to mimic physiologic conditions to optimize preservation. The Lund Protocol, developed by Professor Stig Steen, was the first ESLP management strategy to successfully salvage, assess, and recondition donation after cardiac death donor lungs and extended criteria donor lungs for clinical transplantation with excellent outcomes[18,35]. This protocol employed a perfusion flow rate of 100% cardiac output with the goal of preserving lungs for short time-intervals of approximately 4-hours. In 2008, the Toronto

group, using the XVIVO® XPS^{TM} platform, published their protocol for prolonged ESLP lasting 12-hours. This was achieved through several protocol modifications compared to the Lund approach[24]. Notably, perfusion flow rate was reduced to 40% of predicted cardiac output, based on 100ml/kg/min. Likewise, the OCS^{TM} Lung device by Transmedics® employs a perfusion flow of 2-2.5L/min, roughly 50% of resting cardiac output and has achieved reliable preservation up to 24-hours[12,13, 22, 23]. Our unique NPV-ESLP device uses a flow rate of 30% predicted cardiac output, based on 70mL/kg/min, with evaluations performed at 50% CO to improve V/Q matching[10,14,15,19,20]. Indeed, flow rates of 30-50% of predicted cardiac output in ESLP are standard; however, recent literature suggests that reduced perfusate flow may be advantageous.

In a porcine model of PPV-ESLP, post-transplant lung function was significantly better with lower perfusion flow of 20% CO during ESLP compared to 40% CO[36]. That is, donor-lung specific oxygenation, compliance, and edema all markedly improved. It is unknown if additional reduction in flow rate would results in further improvements in edema formation and preservation. There is a paucity of literature specifically investigating outcome differences in relation to varying perfusion flow rates. We hypothesize that reduced perfusion flow at 10% of predicted cardiac output may further improve preservation of juvenile pig lungs on NPV-ESLP and post-transplantation compared to our current 30% cardiac output approach.

Experimental Design:

Twelve sets of pig lungs will undergo 12-hours of NPV-ESLP at 38ºC with 30% CO (n=6) or 10% CO (n=6) perfusion. Three left lungs from each group will be randomly transplanted post-ESLP and assessed *in-vivo* over four-hours. Both groups will be perfused with buffered CHIP perfusate and autologous packed red blood cells, per protocol. Graft function will be evaluated via

physiologic parameters, edema formation, and pro-inflammatory cytokine profiles as outlined in, "Research Approach and Methods."

Expected Results:

Reduced flow NPV-ESLP of 10% predicted cardiac output may result in superior preservation of lungs compared to normothermic ESLP, and this will be demonstrated by reduced percentage weight-gain as a consequence of lower PAP and PVR, improved compliance and ultimately, superior ESLP and post-transplant PF ratios. Furthermore, the improved physiologic outcomes will be associated with reduced concentrations of pro-inflammatory cytokines on ESLP and post-transplantation.

Specific Aim 4: Determine whether NPV-ESLP of juvenile pig lungs with a pH strategy of mild permissive alkalosis (7.46-7.55) results in superior preservation and transplantation outcomes compared to physiologic pH control (7.35-7.45).

Rationale: To replicate a venous blood gas composition during ESLP, a membrane deoxygenator is employed, across which a mixed ($N_2/CO_2/O_2$) or pure sweep gas (CO_2) is applied to target a physiologic perfusate pCO2 and pH[14, 24]. We have previously observed an initial increase in PVR and PAP during juvenile porcine ESLP that coincides with a rise in pro-inflammatory cytokines (TNF-alpha and IL-6) and is exacerbated by the application of sweep CO_2 to target physiologic pH/pCO_2. In our experience, the reactivity of the pulmonary vasculature in juvenile pig lungs is more profound compared to adult human lungs. Increased capillary hydrostatic pressure due to elevated PAP/PVR can worsen pulmonary edema and lung compliance[37], compromising lung performance on ESLP. Clinically, mild permissive alkalosis can be selectively employed to counteract elevated pulmonary vascular resistance (PVR) through vasodilation[38,39,40]. We

hypothesize that mild permissive alkalosis (pH 7.46-55) may improve outcomes in porcine ESLP through improved PVR and PAP stability.

Experimental Design:

Twelve sets of pig lungs will undergo 12-hours of NPV-ESLP at 38ºC with 30% CO. In the Control group (n=6), pure sweep CO_2 flow will be adjusted to target a physiologic pCO_2 (35-45mmHg) and a physiology pH (7.35-7.45). In the Experimental group, mild permissive alkalosis (pH 7.46-55) will be targeted using a reduced sweep CO_2 flow rate resulting in hypocapnia ($PaCO_2$ < 35 mmHg). Three left lungs from each group will be transplanted post-ESLP and assessed *in-vivo* over four-hours. Control and Experimental groups will be perfused with buffered CHIP perfusate and autologous packed red blood cells, per protocol. Graft function will be evaluated with physiologic parameters, edema formation, and pro-inflammatory cytokine profiles as outlined previously.

Expected Results:

Based on preliminary experience, we expect mild permissive alkalosis (pH 7.46-7.55) with hypocapnia ($PaCO_2$ <35mmHg) will result in more reliable NPV-ESLP of juvenile porcine lungs compared to a targeted physiologic pH (7.35-7.45) and pCO_2 (35-45 mmHg). For our purposes reliability is defined as a decreased failure rate of porcine ESLP, meaning more lungs are successfully preserved for the target time point of 12-hours without premature failure due to physiologic parameters that do not meet transplantation requirements. Our transplantation requirements include a final PF ratio ≥ 300 and PAP < 20 mmHg.

Specific Aim 5: Establish a protocol for 24-hour NPV-ESLP with transplantation using juvenile pig lungs that yields a reliability (>80% success rate) and PGD 0-1 at 4-hours post-reperfusion.

Rationale: To date, only one commercially available PPV-ESLP platform (OCS™ Lung, Transmedics®) has achieved reliable preclinical preservation of pig lungs through continuous perfusion and ventilation for 24-hours with acceptable acute outcomes post-transplantation[22,23]. Other centers have achieved 24-hours of continuous ESLP with pig lungs but lack the reliability to attempt transplantation[15,17,41]. Previously, we have achieved 24-hour preservation with good physiologic outcomes[15,41]; however, our success rate (30-50%) has been inadequate to attempt transplantation, and we lacked an established transplantation model.

It is important to develop a protocol for prolonged continuous ESLP preservation to maximize the limited pool of donor lungs, because some lungs will require 24-hours or greater for treatment of underlying pathology, such as aspiration pneumonia, for example. Greater than 24-hour preservation with acceptable transplantation outcomes would also allow for more thorough assessment and the elimination of many logistical limitations to ideal donor-recipient matching. Given our previous success with 24-hour preservation, it is highly likely that we can continue to improve on our protocol to achieve reliability equal to or greater than 80% success. The preceding studies on ideal temperature, flow rate, and pH status will help inform further modifications to our current 24-hour protocol[15]. We will then assess the quality of our preservation *ex-situ* by transplanting the lungs into a recipient pig for *in-vivo* evaluation.

Experimental Design:

In a porcine model, twelve sets of donor lungs will undergo 24-hours of continuous normothermic NPV-ESLP. A cellular perfusate will be used, composed of autologous packed red blood cells (0.5 L) and buffered perfusate (1L), as previously described. The perfusate with be supplemented with infusions of TPN and multivitamins[15]. Specific protocol modifications will be made based on the results of the previously described studies to produce an improved NPV-ESLP protocol. Six left lungs will be transplanted for *in-vivo* assessment over a 4-hour period. Graft function will be evaluated via physiologic parameters, edema formation, and pro-inflammatory cytokine profiles as described earlier.

Expected Results: We expect to achieve reliable 24-hour preservation with a success rate of 80% or greater. We expect our physiologic parameters after 24-hours of NPV-ESLP with our refined protocol to be better than our previous 12-hour and 24-hour ESLP experiments[14,15]. We expect our post-transplant outcomes to be stable with a PF ratio ≥ 300 mmHg on isolated left lung assessment with an hourly weight-gain of 15-20 g/hour. We expect the perfusate pro-inflammatory cytokines (TNF-alpha, IL-6) to initially rise during reperfusion on ESLP, then decline to a steady state after 6-7 hours. We expect the pro-inflammatory cytokines to rise during the acute phase of transplantation reperfusion. Overall, we anticipate that the extent of the inflammatory response will be less than in our earlier experiments of 12-and 24-hours due to improved management.

Specific Aim 6: Determine whether a strategy of partial perfusate exchange during 24-hours of NPV-ESLP using juvenile pig lungs results in improved preservation and transplant outcomes compared to a strategy without perfusate exchange.

Rationale: To achieve reliable ESLP beyond 12- and 24-hours, all aspects of current protocols must be examined for potential refinement. Perfusate management is one such aspect. Some ESLP protocols employ a strategy of intermittent or continuous acellular perfusate replacement during prolonged preservation[17,41]. The addition of fresh perfusate is thought to dilute the concentration of potentially deleterious by-products of prolonged ESLP, such as pro-inflammatory cytokines and continuous lactate production without clearance. This strategy has never been proven advantageous through experimentation compared to other ESLP approaches that do not replace perfusate volume[17,41]. Previous studies have attempted to purify the perfusate by integrating dialysis; however, these studies failed to demonstrate a statistically significant benefit[41,42].

Our ESLP protocol includes the use of a cellular perfusate comprised of buffered perfusate solution and autologous packed red blood cells (pRBC), which has never been studied in a model of perfusate exchange. Red blood cell destruction is a known consequence of cardiopulmonary bypass due to components of the circuit, such as the non-endothelialized surface of PVC tubing, the rotator or centrifugal pump, various filters, and the membrane oxygenator[27]. Ultra-prolonged ESLP with pRBC may require intermittent perfusate exchange to protect the lungs from damaged red blood cells and their components. We hypothesize that a partial perfusate exchange during 24-hour NPV-ESLP may result in superior preservation and transplant outcomes compared to a strategy of no perfusate exchange.

Experimental Design:

Twelve sets of donor lungs will undergo 24-hours of continuous normothermic NPV-ESLP at 38ºC and 30% CO. A cellular perfusate will be used, composed of autologous packed red blood cells

(0.5 L) and buffered perfusate (1L), as previously described. Supplemental nutrition will be provided with infusions of TPN and multivitamins (15). In the Control group (n=6), the perfusate will not be exchanged during ESLP. In the Perfusate Exchange (PE) group (n=6), 500 mL of perfusate will be siphoned off the circuit at the twelfth hour and replaced with 1L of fresh CHIP with autologous pRBC preserved at 4ºC for 12-hours. All medications are re-dosed in the fresh perfusate batch to avoid diluting the initial concentrations. Three left lungs will be randomly selected and transplanted for *in-vivo* assessment over a 4-hour period. Graft function will be evaluated via physiologic parameters, edema formation, and pro-inflammatory cytokine profiles as described in "Research Approach and Methods."

Expected Results: We expect that a strategy of partial perfusate exchange during 24-hours of NPV-ESLP will result in superior physiologic and transplantation outcomes. We anticipate that the removal of perfusate from the circuit after 12-hours and the replenishment with fresh perfusate will result in a lower concentration of circulating pro-inflammatory cytokines in the perfusate at the end of 24-hours of ESLP compared to a strategy of no perfusate exchange.

Specific Aim 7: Establish a protocol for 36-hour NPV-ESLP using juvenile pig lungs that is reliable (>80% success rate) with physiologic parameters acceptable for transplantation.

Rationale: ESLP is approved by the FDA for 4-6 hours duration, although preclinically 12-hours in animal models is routinely achieved[14,20,24]. As previously mentioned, reliable 24-hours of continuous ESLP preservation has been achieved with pig lungs using the Transmedics® OCS-Lung™ [22,23]. Edmonton and Toronto have also published successful 24-hours of continuous ESLP preservation[15,17,42]. Animal models of allogeneic cross-circulation ESLP and preclinical

xenogeneic cross-circulation ESLP have demonstrated safe continuous preservation for 4-days and 24-hours, respectively[43,44]. The Toronto group has recently demonstrated safe preservation of pig lungs for three days with acceptable acute transplantation outcomes by employing a technique of CSP at 10ºC with intermittent ESLP for periodic metabolic reconditioning[45]. The sum of the evidence suggests that lungs can be safely preserved beyond 24-hours with ESLP under the proper conditions.

To maximize the potential of ESLP as a vehicle for donor organ reconditioning and optimization with gene- and cell-based therapies, continuous perfusion of 48-72 hours and longer is needed. Advanced therapies to treat severe pathology and induce immunomodulation/tolerance will require extensive dwell-time with the target tissue in isolation from other vulnerable physiology. To date, there are no published accounts of 36-hours of continuous PPV-ESLP or NPV-ESLP, excluding the small experience of pre-clinical cross-circulation ESLP. Cross-circulation ESLP has numerous bioethical hurdles to overcome before it is a viable option clinically[44]. Therefore, there is a definite need to achieve the research milestone of 36-hours of continuous ESLP. To reiterate, NPV-ESLP is gentler on the donor lung tissue than PPV-ESLP[14]; therefore, NPV-ESLP is likely the superior platform for ultra-prolonged continuous preservation. After completing experiments in the optimization of our protocol across temperature, flow rate, pH-status, and 24-hour preservation with transplantation, we will attempt to achieve 36-hours of NPV-ESLP using juvenile pig lungs.

Experimental Design:

In a porcine model, six sets of donor lungs will undergo 36-hours of continuous normothermic NPV-ESLP. A cellular perfusate will be used, composed of autologous packed red blood cells (0.5

L) and buffered perfusate (1L), as previously described. Infusions of TPN and multivitamins will be supplemented to the perfusate[42]. The previously described studies will guide specific protocol modifications to produce an improved NPV-ESLP protocol. Donor lung function will be evaluated via physiologic parameters, edema formation, and pro-inflammatory cytokine profiles as described earlier.

Expected Results:

We believe we will be successful in our attempts at 36-hour preservation based on two key previous successes: 1) our lab has previously achieved 24-hours of preservation with a reliability of 30-50% and physiologic parameters acceptable for transplantation with PF ratios ≥ 300 mmHg, PAP < 20 mmHg, and dynamic compliance ≥ 30 ml/cmH_20[15,42]; therefore, it is reasonable to anticipate that through protocol refinement we can increase our reliability and duration of preservation, as mentioned earlier; 2) our lab has previously achieved preservation periods of 36-and 42-hours using human donor lungs rejected for transplantation (n=2); therefore, this establishes the feasibility of our objective using NPV-ESLP with pig lungs.

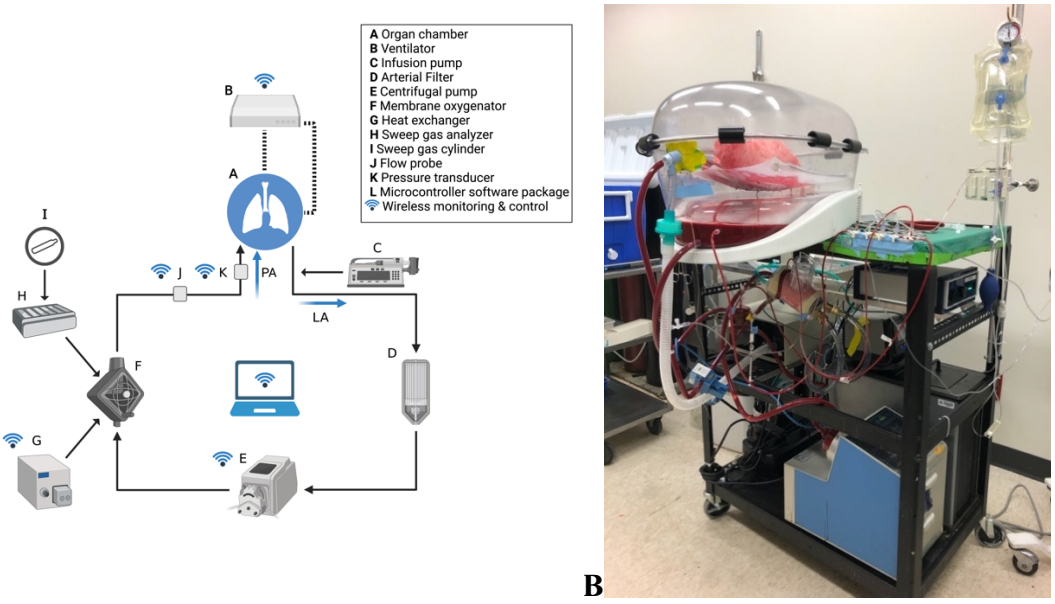
In terms of physiologic outcomes, we expect that PF ratios will be greater than 300 mmHg at the end of perfusion, although these values will possibly decline after the 24-hour time-point due to the progressive development of pulmonary edema and subsequent loss of compliance. The per hour weight gain of the lungs is expected to be consistent with previous experiments of 12- and 24-hours at 15-20 ml/hr. Previously, our data has shown a gradual decrease in PVR and PAP after the first 6-hours of ESLP before reaching a steady state, and we expect that they will not rise significantly beyond 24-hours. We expect TNF-alpha and IL-6

perfusate concentrations to increase during the first 3-6 hours of NPV-ESLP and then decline to a steady-state leading up to 36-hours of preservation.

Significance:

Taken together, the proposed studies will allow us to establish the longest continuous preclinical ESLP protocol published to-date. NPV-ESLP has previously been shown to cause less lung injury and reduced levels of pro-inflammatory cytokine production compared to PPV-ESLP. As such, NPV-ESLP is best suited for ultra-prolonged continuous preservation of donor lungs compared to other commercially available ESLP devices. NPV-ESLP of 36-hours and greater would allow for multiple benefits to the field of lung transplantation: 1) enhanced international sharing of donor organs to optimize the allocation of available lungs; 2) elimination of geographic barriers that pose logistical constraints on the timely allocation of limited donor lungs; 3) increased duration of assessment to allow for optimal donor-recipient matching; 4) elective as opposed to urgent lung transplantation to allow optimal surgical/medical staffing and scheduling; and 5) a vehicle to develop and apply gene- and cell-based therapeutics to donor lungs for advanced reconditioning or creation of "super organs" that evade recipient immune systems. The result will be increased donor organ availability and improved recipient outcomes.

Figures and Tables:



A **B**

Figure 1.1: NPV-ESLP circuit. A) Schematic representation of the circuit with accompanying legend (left). B) Photo of NPV-ESLP circuit (right).

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

	Perfusion time (min)				
	0	10	20	60 (T1)	180 (T3)
Perfusate Temperature (°C)	20°C	32°C	38°C	38°C	38°C
PA flow (% CO; CO = 70 ml/kg/min)	10%	20%	30%	30%	50%
Ventilation mode	PPV (CPAP = 20 cm H_2O)	Initiate NPV Preservation	NPV Preservation	NPV Preservation	NPV Evaluation
Medical gas mixer	None	None	None	100% CO_2	89% N_2, 8% CO_2, 3% O_2
Left atrial pressure	0	0	0	0	0

Table 1.1: Initiation of 12-hour NPV-ESLP Protocol. CO, cardiac output; PA, pulmonary artery; PPV, positive pressure ventilation; NPV, negative pressure ventilation. For preservation mode ventilation parameters see Table 1.2. Beginning at T3, evaluation was conducted serially every 2 hours, for 5 minutes, with PA flow set to 50% CO, medical gas set to 89% N_2, 8% CO_2, 3% O_2, and preservation settings elevated to the ventilation parameters provided in Table 1.2.

NPV Ventilation Strategy

	Ventilation Mode	
	Preservation	Evaluation
Temperature (°C)	38°C	38°C
Pulmonary artery flow	30% of estimated CO; CO = 70 ml/kg/min	50% of estimated CO; CO = 70 ml/kg/min
Ventilation parameters		
Mode	Pressure control	Pressure control
Desired inspiratory tidal volume	4-8 ml/kg	6-10 ml/kg
Inspiratory: Expiratory Ratio	1:1 – 1.5	1:1 – 1.5
Frequency	6 to 10 breaths/min	6 to 10 breaths/min
P_{AWP}	< 25 cm H_2O	< 25 cm H_2O
PEEP	8-12 cm H_2O	5-8 cm H_2O
FiO_2	21%	21%
Pressure parameters		
PAP	< 20 mm Hg	< 25 mm Hg
LAP	0 mm Hg	0 mm Hg
Medical gas mixture	100% CO_2	89% N_2, 8% CO_2, 3% O_2
Medical gas mixture (litres/min) titrated to PCO_2	<35mmHg	35 to 50 mm Hg

Table 1.2: 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 pulmonary arterial circulation.

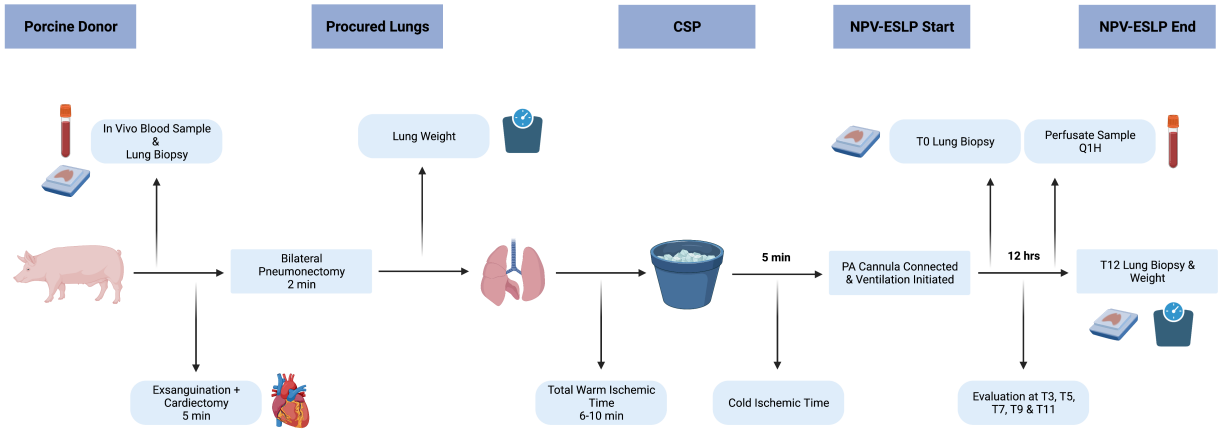


Figure 1.2: NPV-ESLP Protocol. Schematic representation of lung procurement and 12-hour NPV-ESLP run.

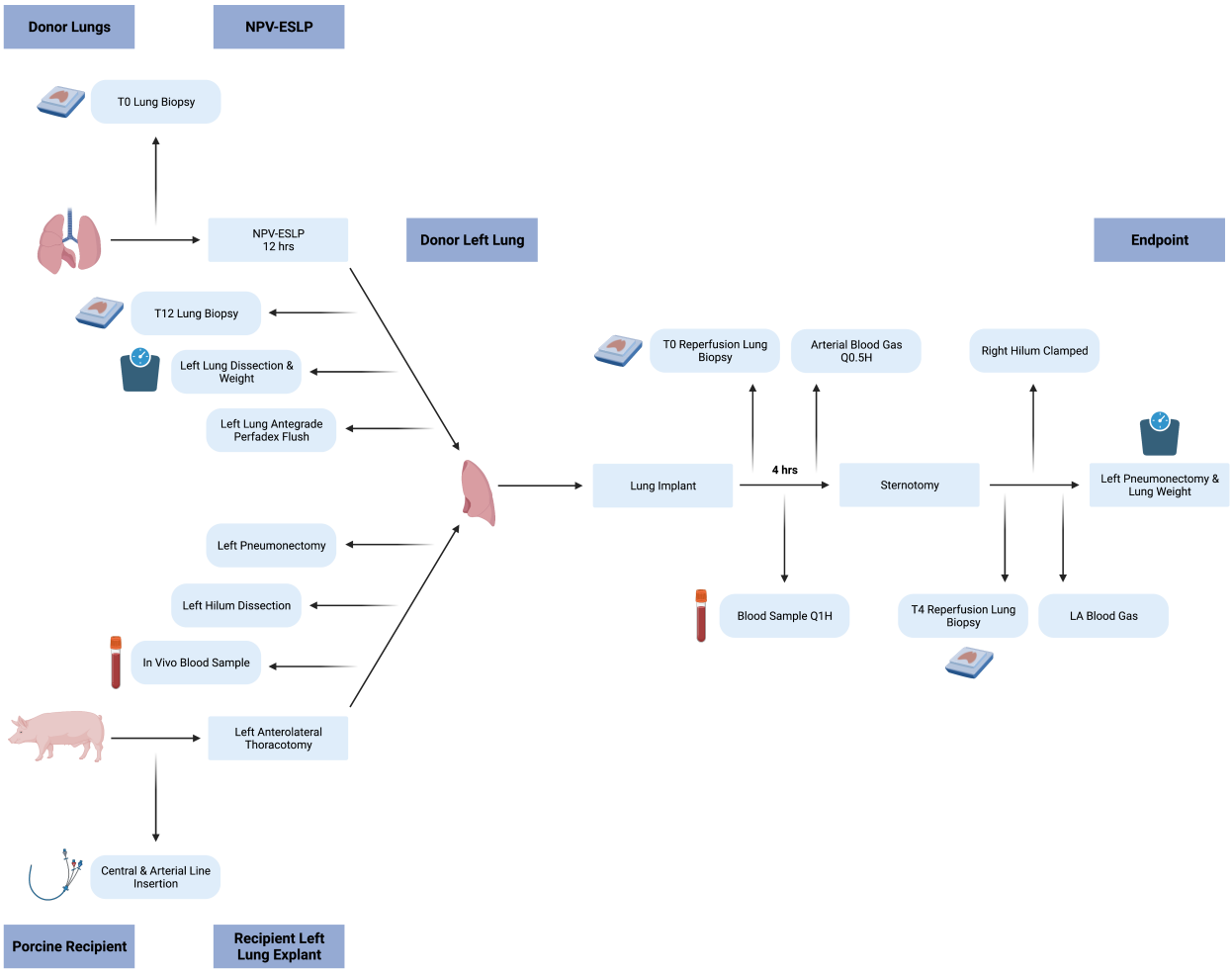


Figure 1.3. Porcine Left lung Transplant Model. Schematic representation of 12-hour NPV-ESLP run followed by left lung transplantation in a Yorkshire pig. ESLP duration can be extended to 24- and 36- hours, as outlined in this proposal.

Chapter 2

ESLP: a Narrative and State-of-the-Art Review

PART 1: Ex-Situ Lung Perfusion: History, Platforms, and Clinical Progress

1. INTRODUCTION

Lung transplantation is a lifesaving therapy for patients with end-stage lung disease. Globally, more than 4600 lung transplants are performed each year. The first successful single lung transplant was performed in Toronto by Dr Joel Cooper in 1983[1], and outcomes have continued to improve due to advances made in donor and recipient selection, perioperative/medical management, surgical techniques, and immunosuppression/anti-rejection therapy [2,3]. As outcomes improved, the number of indications for lung transplantation have expanded resulting in an increased number of patients listed for the procedure. The number of useable donor lungs has not increased proportionately, which limits the number of transplants performed. Indeed, due to conservative ideal donor acceptance criteria, donor lung utilization remains low with approximately 85% of offered lungs being rejected by transplant programs[4]. Consequently, there is a high wait-list mortality of 15-20%, which is significantly higher than other solid organ transplants[5,6]. Currently, the clinical standard for preserving donor lungs is cold static preservation (CSP); however, it is fraught with limitations and has remained largely unchanged over the past 30 years.

Cold static preservation involves flushing donor lungs with a cold preservation solution and storing the explanted lungs in on ice (4ºC) in a standard cooler for transport to the recipient centre[7]. Cooling the organ dramatically decreases the rate of cellular metabolism, thereby decreasing oxygen and nutrient demands. In addition, the production of deleterious cellular by-products from ischemia, inflammation, and oxidative stress is also reduced, which provides some protection to the lungs from injury[8]. The trade-off is that reparative cellular processes are likewise inhibited, which represents a lost opportunity to improve donor lung function prior to

transplant. Furthermore, CSP is a black box for organ function and lacks the ability to assess donor organs during transportation. Likewise, there is no potential to intervene therapeutically beyond the application of cold; therefore, there is ambiguous organ function during transport. Although CSP is simple, reliable, and economical, it is not an ideal form of organ preservation, and its safe duration of application for lungs is generally accepted as 6-8 hours before irreversible damage develops in the graft[9]. Many strategies have emerged to address the inadequate supply of donor lungs and the limitations of CSP, and *Ex-Situ* Lung Perfusion (ESLP) (aka *Ex-vivo* Lung Perfusion; EVLP) has established itself as the most promising solution.

Ex-Situ Lung Perfusion is a machine that preserves lungs and supports metabolic function through continuous mechanical ventilation and pump-driven perfusion with a specialized solution, which enables physiologic support and assessment from procurement to implantation[10-13]. Isolation of lungs from the hostile donor pathophysiology paired with continuous metabolic support allows marginal quality donor lungs to recover, thereby increasing the useable pool of donor lungs[10-13]; furthermore, ESLP of marginal quality lungs results in comparable success rates as healthy lungs preserved with the clinical standard of cold static storage[10-13]. ESLP has enhanced research opportunities on donor lung physiology and treatment by providing a vehicle for intervention through the perfusate/vasculature or ventilator/airway[14,15,16]. Clinically, ESLP is approved for 4-6-hours of preservation, although 12-hours of preservation is standard in pre-clinical research. Select labs have reached 24-hours of continuous ESLP, but with limited reliability[15,17]. This review provides a contemporary summary of our current understanding of ESLP, while incorporating previously underrepresented contributions with negative pressure ventilation (NPV)-ESLP. Part 1 consists of a narrative review, and Part 2 summarizes state-of-the-art ESLP research related to protocol improvements.

2. ISCHEMIC REPERFUSION INJURY, INFLAMMATION, AND PRIMARY GRAFT DYSFUNCTION

Increased risk of Primary Graft Dysfunction (PGD) post-operatively is the primary concern in lung transplant that limits the acceptance of donor lungs with excessive transport time or of marginal quality, so called extended criteria donors (ECD). PGD is an acute lung injury that can occur within the first 72-h post-transplant[18]. It results in diffuse alveolar damage, which manifests clinically as severe hypoxemia and lung edema. Diffuse pulmonary infiltrates on CXR are characteristic. PGD is the leading cause of 30-day mortality post-LTx and affects 11–25% of patients[19]. Furthermore, PGD is associated with an increased the risk for chronic lung allograft dysfunction (CLAD), specifically bronchiolitis obliterans syndrome (BOS)[20]. Indeed, optimal post-operative outcomes are dependent on the prevention of PGD, which is produced by the culmination of donor, preservation, and recipient-related factors; however, ischemic-reperfusion injury (IRI) is thought to be the principal contributor[21-23].

IRI is a paradoxical injury that occurs to organ tissue following the cessation and reintroduction of perfusion and oxygenation. The extent of the injury is proportional to the extent of ischemia, hence the concern with prolonged donor organ transport time. The injury cascade is also more severe in organs with compromised function, which explains the clinical hesitancy with ECD lungs[21,24]. The details of IRI cellular mechanisms are complex and reviewed in detail by Forgie *et al.* [25]. Hallmarks of lung IRI (LIRI) include inflammation, endothelial cell dysfunction, and disruption of the endothelial barrier that can lead to PGD if the injury is sufficiently severe. The risk of LIRI impacts donor organ selection and favours conservative decision making given the limitations of CSP, ultimately resulting in underutilized donor lungs.

Several studies have demonstrated that normothermic ESLP reduces edema, improves alveolar-epithelial tight junction integrity, and provides better metabolic function with improved gas-exchange compared to CSP[26–28]. The principal target of LIRI is the pulmonary vascular endothelium, and increased permeability of the alveolar-capillary barrier is the root cause of pulmonary edema and impaired oxygenation. Therefore, protection of the pulmonary endothelium is essential to prevent complications of IRI leading to PGD. Portable ESLP aims to maintain pulmonary endothelial and epithelial physiology by minimizing episodes of cold ischemia.

3. HISTORY OF ESLP

Over the last twenty years, *Ex-Situ* Organ Perfusion (ESOP) has evolved from a relatively obscure research tool to a well-recognized form of enhanced organ preservation. The desire to artificially perfuse and preserve organs is documented as far back as the 15th century when Leonardo da Vinci first sketched his ideas of isolated organ perfusion. The first model of ESOP that could maintain organs (cat ovary, adrenal gland, thyroid gland, spleen, heart, and kidney) alive outside of the body for more than a few hours was in 1935[29]. Cat thyroid glands were kept alive for 20 days on this device with evidence of tissue proliferation. In 1970, ESLP was described by Jirsch *et al* as a means of safely preserving and evaluating lungs over prolonged distances[30]. In 1987, Hardesty and Griffith published their experience with a custom-made container allowing for the normothermic perfusion and ventilation of heart-lung blocks with successful clinical transplantation in 10 patients[31]. The technology was ahead of its time and foreshadowed many of the achievements to come; however, the device was not adopted for

widespread clinical use given the added cost and complexity relative to CSP, which was highly effective in its own right.

Progress quietly continued and in 2001, Stig Steen *et al*.[32] published a modified approach to ESLP that employed a hyperoncotic perfusate (STEEN Solution™) mixed with erythrocytes, which enabled the transplantation of lungs from a non-heart beating donor after 1-hour of assessment on ESLP. The invention of STEEN Solution™ (XVIVO Perfusion, Goteborg, Sweden) combined with red blood cells allowed for improved preservation of the delicate alveolar-capillary interface compared to earlier approaches, thereby reducing the extent of fluid extravasation and edema formation on ESLP[26,32,33]. In 2005, Steen *et al.* applied their ESLP protocol to a set of human lungs rejected for clinical use, which were later accepted and successfully transplanted after 1-hour of ESLP assessment[33]. Between 2006/7, Steen et al. employed ESLP (1-2 hours) to recondition six sets of lungs from initially rejected donors followed by successful lung transplantation[34]. These accomplishments marked a renewed interest in the field of ESLP, particularly in the area of prolonged preservation; however, preclinical attempts at ESLP for 6-hours duration were unsatisfactory due to rising pulmonary artery pressure and ventilation pressure[35]. Further improvement in ESLP protocol was required to make prolonged treatment of donor lungs feasible.

In 2008, the Toronto group published their preclinical experience with successful 12-hour normothermic ESLP in a porcine model with transplantation following their modified approach[36]. Key modifications included ARDS style low tidal volume ventilation and a reduced perfusion flow rate of 40% cardiac output compared to 100% flow as practiced by Steen *et al*. Clinical translation of the Toronto protocol was achieved in 2011 with successful reconditioning and transplantation of 20 extended criteria donor (ECD) lungs[28]. Rates of PGD at 72-hours were

low (15%) and similar between ECD and CSP ideal donor lungs. These breakthroughs marked an inflection point in the history of ESLP, heralding in a number of competitive devices and clinical trials internationally[10-13,28,37,38]. At present, clinical application of ESLP is approved for 4-6 hours duration across North America, Europe, and Australia[39-42]. Indeed, significant progress has been made in mitigating the limitations that plagued early attempts of ESLP, including progressive injury to the alveolar-capillary barrier with development of pulmonary edema and endothelial dysfunction; however, these issues of circuit related injury remain the primary reason why multi-day continuous ESLP has yet to be achieved. Therefore, there is ongoing need for protocol refinement to optimize ESLP.

4. ESLP PROTOCOLS AND COMMERCIAL PLATFORMS

At present, there are two commercially available PPV-ESLP platforms and four commonly described protocols applied clinically and for research purposes. The commercial devices include: 1) XVIVO Perfusion System (XPS™) (XVIVO Perfusion, Gothenburg, Sweden); 2) Organ Care System (OCS™) Lung (Transmedics, Andover, MA, USA). Earlier devices that are no longer available include the Lung System 1 (LS1™, Vivoline Medical, Sweden) and the Lung Assist™ (Organ Assist B.V., Netherlands), which have both been acquired and discontinued by XVIVO®. The protocols commonly used with these commercial systems include, respectively: 1) Toronto Protocol; 2) OCS™ Lung Protocol. The Lund Protocol was originally developed by Stig Steen and his team in Lund, Sweden, and used most commonly with the Vivoline LS1™. The Lund Protocol is no longer applied clinically; however, it is used for research purposes by select centres. A fourth system in development, pending clinical evaluation and regulatory clearance, uses negative pressure ventilation (NPV-ESLP) and is

called the *Ex-Vivo* Organ Support System (EVOSS^{TM}) by TEVOSOL (Edmonton, AB, Canada) and Bridge to Life (Northbrook, IL, USA). The EVOSS^{TM} device is designed for use with the Edmonton Protocol. The various protocols are summarized in Table 2.1. ESLP management consists of phases: preservation, reconditioning, and evaluation, which are described below.

ESLP Phases: Preservation, Reconditioning, Evaluation

Preservation

Preservation is the phase of ESLP when lung function is maintained with two goals in mind: 1) The clinical goal to provide high quality donor organs to recipients with minimal IRI, and 2) The research goal of extending this maintenance phase indefinitely, which will allow for optimal recipient and donor matching, as well as change the nature of lung transplantation from an emergent procedure to an elective one. General preservation principles exist across ESLP protocols. For example, tidal volumes, peak airway pressure, and respiratory rate are kept low to mitigate barotrauma and ventilator-induced lung injury (VILI). Flow rates (measured as a percentage of cardiac output) are kept at 30-50% to prevent the development of pulmonary edema that can interfere with oxygenation and lung mechanics of ventilation by decreasing compliance. Normothermia is maintained and arterial blood gases are targeted to the physiologic range.

Reconditioning

Reconditioning is the phase of ESLP when the lung function is gradually improved and optimized. This phase occurs concurrently with preservation. The rehabilitation of poor lung function is due to 1) Continuous perfusion and ventilation of lungs that would otherwise be static

in CSP, thereby mitigating effects of ischemia and atelectasis; 2) Isolation of the lungs from the pathologic environment of the donor. Reconditioning will become increasingly distinct from preservation as advanced therapies are developed that can be administered during ESLP to improve lung function. Eventually, ESLP will incorporate targeted therapies for specific insults: infection, pulmonary edema, contusions, and cytokine storms will be treatable and greatly expand the donor pool. Cell- and gene-therapy will also play a role in future reconditioning efforts, perhaps as a means of inducing hibernation or decreasing immunogenicity. Specific examples of targeted therapies to recondition lungs on ESLP already exist and will be covered in Part 2 of this review.

Not all lungs that are preserved on ESLP require reconditioning. Lungs can have excellent function and ESLP is only required for evaluation purposes to assess for possible deconditioning as in the case of lungs donated after circulatory death (DCD). In these instances, ESLP offers an environment that is isolated from the deleterious cascade of death and systemic organ toxicity in the donor.

Evaluation

Evaluation is the phase of ESLP when lungs are assessed on their suitability for transplantation based on their PF ratio (partial pressure of oxygen PaO_2/Fraction of inspired O_2) and other physiologic parameters such as compliance, pulmonary vascular resistance (PVR), and airway pressure. Either an increased perfusion flow rate or FiO_2 along with a deoxygenating sweep gas mixture are employed to challenge the lungs and accurately determine the quality of gas exchange. ESLP is able to provide a more accurate assessment of donor lung suitability

compared to conventional methods due to the absence of confounding factors in the clinical setting.

Transplant respirologists and surgeons rely on clinical factors to decide on the suitability for transplant, including chest x-rays, bronchoscopy, PF ratios, and open-chest inspection; however, underlying pathology such as atelectasis, aspiration, edema, and labile blood pressures can interfere with accurate lung evaluation, producing a falsely low PaO_2. This is a major contributor to the rejection of lungs that are otherwise healthy and would benefit from isolation and reconditioning on ESLP prior to evaluation.

ESLP serves to bolster the judgement of surgeons by delaying assessment at the recipient hospital until the lungs have been evaluated under more physiologic conditions with controlled perfusion and ventilation. Currently, PaO_2 and P/F ratios are the functional parameters of ESLP that best predict lung function and are most used for decision-making. That said, two clinical trials have suggested that oxygenation and lung compliance are the most important variables to consider[43,44]. The Toronto ESLP group have supported this claim with pre-clinical findings that oxygenation alone does not predict donor lung function post-transplantation. Instead, compliance, pulmonary vascular resistance, and airway pressure tend to worsen in response to injury on ESLP before any decrease in PaO_2[45]. Here, we see the advantage of ESLP to enhance our understanding of organ evaluation.

Ventilation

Currently, there are two distinct ventilation strategies within the field of ESLP: positive pressure ventilation (PPV) and negative pressure ventilation (NPV). PPV refers to the standard ICU ventilation strategy where the lungs are ventilated by forcing air into the lungs via the

endotracheal tube (ETT) to inflate them, similar to inflating a balloon. NPV aims to be more physiologic and expands the lungs by applying a uniform negative pressure to the pleural surface of the lungs. This is akin to the negative intrathoracic pressure of inspiration and mirrors the historical Iron Lung, which was used to treat Polio survivors in the 1940-50's. The alveoli are pulled open while low pressure air is delivered through the ETT and drawn into the lungs. In 2018, Aboelnazar *et al* demonstrated that NPV-ESLP was superior to PPV-ESLP with respect to reduced inflammation, bullae formation, and accumulation of edema[14].

Perfusates

Cellular versus acellular perfusate composition is another distinguishing features across ESLP strategies[34,46,47]. Cellular perfusates refer to perfusates that contain cellular components such as packed red blood cells (pRBC) and albumin or autologous whole blood, whereas acellular perfusates lack cellular components. Overtime, the terminology has been simplified in the research literature to differentiate between blood (cellular) vs non-blood (acellular) supplemented perfusates. There is evidence in favor of both approaches. The evidence behind cellular based perfusates is that they are better able to mimic physiologic conditions[41,48-50]; however, RBCs are prone to destruction overtime and can exacerbate lung edema and tissue injury[36]. Acellular perfusates are not susceptible to destruction, which makes them theoretically more desirable for prolonged runs of ESLP. Cellular based perfusate is also more demanding in terms of resource utilization due to dependence on autologous blood or a blood bank, which is another disadvantage. This topic is covered in more detail in Part 2 of this review.

Indeed, there are many possible strategies and protocols within the realm of ESLP. As such, several companies have developed with this industry, each with a unique combination of

approaches and platforms. Below is an introduction to the various key players in the industry of ESLP: 1) Toronto Protocol (XPS™), 2) Lund Protocol (Vivoline LS1™), 3) Organ Care System (OCS™) Lung, and 4) Edmonton Protocol (Ex-Vivo Organ Support System, EVOSS™).

Positive Pressure Ventilation Platforms

Toronto Protocol

The Toronto group uses a unique ESLP strategy that involves PPV, an XVIVO perfusion system (XPS™), and an acellular perfusate. The circuit contains standard components including an inflow line, outflow line, a leukocyte filter, a membrane deoxygenator with sweep gas, a centrifugal pump, a heat exchanger, flow-probe, and a hard-shell perfusate reservoir. They also employ a synthetic left atrial cuff sewn to the pulmonary veins that drain blood in a closed system ("closed left atrium") and allows direct pressure measurements through a pressure line. Manual adjustment of the perfusate reservoir height allows for targeted left atrial pressures (LAP) between 3-5 mmHg. The acellular perfusate is STEEN Solution™ (XVIVO Perfusion, Gotenborg, Sweden), developed at Lund University by Stig Steen *et al.*[30]. This buffered extracellular (low K+) solution is designed to minimize lung tissue edema through the colloid action of added human albumin. It also incorporates dextran-40 to protect the endothelium by mitigating complement and cell-mediated injury. This helps prevent platelet aggregation and coagulation within the pulmonary vasculature[26,48]. Ten thousand IU of heparin, 500 mg of cefazolin, and 500mg of solumedrol are also added to the perfusate.

Once the circuit is primed and the lungs connected to the circuit and ventilator, perfusion is initiated at 10% of calculated cardiac output (CO) based on the donor body weight. Over the first hour, CO is progressively increased up to 40% and maintained for the remainder of the run

(predicted CO 100ml/kg/min). Perfusion target parameters include LAP of 3-5 mmHg, a pulmonary artery pressure (PAP) of 10-15mmHg, and a peak airway pressure (Pawp) of 25mmHg during ventilatory recruitment manoeuvres. Along with the progressive, incremental increase in CO, temperature is gradually increased to normothermia (37 °C).

Ventilation is commenced at a temperature of 32°C. A protective ventilation strategy is employed with tidal volumes of 6-8 ml/kg, a respiratory rate of 7 breaths per minute, PEEP of 5 cm H_2O, and FiO_2 of 21%. During evaluation, FiO_2 is increased to 100%. A standard gas mixture (86%N_2, 8% CO_2, 6% O_2) is connected to the membrane deoxygenator to provide sweep gas throughout ESLP. A physiologic PCO_2 between 35-40 mmHg is targeted using the sweep gas[28,36, 45,48,51]. Compared to OCS™ Lung and EVOSS™, XPS™ is a stationary ESLP device and is typically managed from a designated operating room. Table 2.1 summarizes Toronto's ESLP protocol.

Lund Protocol

Although the Lund Protocol is no longer applied clinically, it is important for its research application and historical relevance having paved the way for all subsequent protocols. The Lund protocol was previously paired with the Vivoline LS1™ ESLP device, but an equivalent platform can be fashioned from individual components including an ICU-ventilator, a hard-shelled lung chamber, and a cellular perfusate. The ESLP components include: an inflow line, outflow line, a leukocyte/arterial filter, a membrane deoxygenator with sweep gas, a roller pump, a heat exchanger, flow-probe, a PaO_2 sensor, temperature sensor, a hard-shell lung chamber with perfusate reservoir, and a cellular perfusate. The Lund Protocol has an open left atrial (LA) system that drains freely; therefore, the left atrial pressure (LAP) is maintained at 0 mmHg[30].

The cellular perfusate is a combination of STEEN Solution™ mixed with donor packed red blood cells (pRBC) for a target hematocrit of 14%[30]. Additional perfusate additives include 0.5 g Imipenem, 20 IU insulin, 10000 IU heparin, and isotonic trometamol buffer for a temperature adjusted pH of 7.4[27].

The circuit is primed, and the lungs are connected to the circuit and ventilator. Perfusion is initiated at low flow (50-100mL/min) to target a PAP less than or equal to 20 mmHg at a temperature of 25°C[26,48]. The temperature of the perfusate is gradually increased to warm the lungs to a temperature of 32°C. LAP is maintained at zero mmHg to prevent the development of pulmonary edema. At 32°C, ventilation is commenced at 3ml/kg, and increased by 1L/min/degree until a target temperature of 37°C is reached. At normothermia, a tidal volume of 5-8 ml/kg is achieved with a PEEP of 5 cm H_2O. Sweep gas mixture entering the oxygenator membrane is titrated to target a pCO_2 of 4.5-5 kPA. A ventilator FiO_2 of 50% is used during preservation and 100% during evaluation. Respiratory rate is increased gradually from 5 to 15-20 breaths per minute. Perfusion flow is simultaneously increased with warming to reach full flow at normothermia (5-6L/min or 100% cardiac output)[30] (Table 2.1).

Organ Care System™ Lung

The Organ Care System (OCS™) Lung by Transmedics is the only FDA approved portable ESLP device for standard and extended criteria donor lungs. Similar to other platforms, OCS™ Lung contains a hard-shell lung chamber, a membrane gas exchanger with built in heater-cooler, a hard-shelled perfusate reservoir, and a ventilator. The OCS™ Lung uses a pulsatile pump and does not have a leukocyte filter. The set up includes open LA drainage, similar to the Lund Protocol. The OCS™ Lung uses a cellular perfusate along with pRBCs to

target a hematocrit of 15-25%. The perfusate was initially Perfadex® (XVIVO Perfusion AB, Goteborg, Sweden), but has been replaced by a proprietary formula called OCS Solution™ (Transmedics)[48,41]. Both of these solutions are extracellular compositions and contain dextran, i.e., low potassium dextran solutions (LPD). They do not contain human serum albumin, as seen with STEEN Solution™. OCS Solution™ includes glucose as an energy substrate. Perfusate additives include 1 g cefazolin, 200 mg ciprofloxacin, 200 mg voriconazole, 1 g glucose, 500 mg methyl-prednisone, 1-unit vial of multivitamins, 20 IU of insulin, 4 mg milrinone (single dose), and 1.3 mL of tris(hydroxymethyl)aminomethane (THAM) buffer[41]. Perfusion is initiated at a temperature of 32°C and increased to 37°C over ten minutes. Flows are 2-2.5L/min, PAP is targeted at ≤20 mmHg, and LAP 0 mmHg (open LA cuff). Ventilation begins at 34°C using a bellows ventilator with tidal volumes of 6ml/kg, respiratory rate of 10 breaths per minute, PEEP 5-7 cm H_2O, and an FiO_2 of 12%[41,48,51] (Table 2.1).

Negative Pressure Ventilation Platform

Ex-vivo Organ Support System™

EVOSS™ uses the Edmonton Protocol and is a distinct platform compared to the previously described ESLP devices in that it employs negative pressure ventilation as opposed to positive pressure ventilation. This means that the EVOSS™ more closely replicates physiologic respiration — ventilation is achieved by pulling the lungs open with an extra pleural vacuum rather than forcing the lungs to expand by inflating them with air. Aboelnazar *et al* (2018) demonstrated that NPV-ESLP is associated with reduced inflammation, edema, and lung injury compared to PPV, irrespective of use with a cellular or acellular perfusate[14]. Similar to the other ESLP platforms, EVOSS™ has the following components: inflow and outflow cannulas, flow

sensor, arterial line filter, membrane oxygenator with built-in heater-cooler, sweep gas port with standard mixed gas components (89% N_2, 8% CO_2, 3% O_2), and a centrifugal pump. The air-tight combination organ chamber and perfusate reservoir is unique to EVOSS™ along with its custom ventilator. As of 2020, the Edmonton Protocol perfusate is a non-proprietary cellular solution comprised of common hospital ingredients (CHIP) supplemented with pRBCs to produce a desired hemoglobin concentration of 40-50 g/L (12-15%)[52]. CHIP contains human serum albumin and has a similar oncotic composition (32 mmHg) to Krebs-Henseleit buffer solution; however, it does not contain dextran as seen in STEEN Solution™ and OCS Solution™. Other perfusate ingredients include 10,000 IU of heparin, 500 mg methyl prednisone, and 3.375 grams of piperacillin/tazobactam. During the ESLP runs, insulin is infused at a rate of 2 units/hr and a glucose infusion of 1g/hr to target perfusate glucose of 5-7 mmol/L. THAM buffer is bolused intermittently to target a pH of 7.35-7.45.

To begin NPV-ESLP, the circuit is primed with 1.5 litres of perfusate, the lungs are attached via pulmonary artery (PA) cannulation, and endotracheal intubation. Perfusion is initiated at 10% of cardiac output (CO, predicted cardiac output 70ml/kg/min) at 25°C and progressively increased to 30% CO over the first 30-minutes. Temperature is simultaneously increased until normothermia is reached (humans 37°C, pigs 38°C). Perfusion targets include a PAP <20 mm Hg and LAP 0 mm Hg (open left atrium). Lung inflation is initially maintained with CPAP (20 cm H_2O), and NPV is commenced once perfusate temperature reaches 32°C. Ventilation parameters include a respiratory rate (RR) 6-10 breaths per minute, tidal volume (TV) 6-10 mL/kg, PEEP 5-8 cm H_2O, end-inspiratory pressure (EIP) -13 to -15 cm H_2O, end-expiratory pressure (EEP) of 0 cm H_2O, PawP of <25 cm H_2O, and FiO_2 21%. During organ evaluation, CO is increased to 50% and deoxygenating mixed sweep gas is applied to target a

pCO_2 between 35-50 mm Hg. A summary of the EVOSS protocol is included in Table 2.1 along with the previously mentioned ESLP platforms.

5. PIVOTAL TRIALS

Within the last decade, several pivotal trials in ESLP involving various commercial and "in-development" devices have shaped the current landscape. The following is a brief summary of the major trials involving positive pressure and negative pressure ventilation ESLP applied to extended criteria and standard donor lungs.

Extended Criteria donor trials (PPV)

XVIVO (Toronto EVLP System™): ESLP was initially developed to evaluate extended/marginal quality donor lungs and recondition them to acceptable transplantation standards to expand the pool of donor lungs and decrease waitlist mortality. The first large trials that compared clinical outcomes from initially rejected extended donor lung preserved with ESLP to standard donor lungs preserved with cold static preservation were carried out by Toronto using their custom protocol and the XVIVO system. Toronto's first ESLP trial was published in 2011 by Cypel *et al.* known as the HELP trial[28]. This trial was a prospective, non-randomized clinical trial that subjected 23 high-risk donor lungs to 4-hours of ESLP. Twenty lungs were ultimately accepted for transplantation with PaO_2:$FiO_2 \geq 350$ mmHg. Standard criteria lungs that were transplanted during the same time period were used as controls (n=116). There was no significant difference in the primary outcome of primary graft dysfunction (PGD) at 72-hours post-transplant. Furthermore, all secondary outcomes were comparable, including ICU PF ratios, ECMO requirements, bronchial complications, duration of mechanical

ventilation, ICU LOS, and 30-day mortality. From this study, the Toronto group concluded that extended normothermic ESLP of high-risk donors was feasible and safe with acceptable rates of PGD and comparable early outcomes to standard criteria donor lungs preserved via CSP.

The second major trial with the XVIVO system is the ongoing NOVEL trial, which is a prospective, non-randomized, multi-centre clinical trial that recently released its 5-year outcomes. This trial aims to evaluate the short- and long-term outcomes of ESLP performed across 17 centres in the United States. Between 2011-2017, 216 extended criteria lungs were evaluated with ESLP, and 110 (50.9%) were transplanted into recipients. During the same period, 116 standard lungs preserved with CSP were used as controls. The incidence of PGD was higher in the ESLP groups at 24 hours (p=0.003), but not significantly so at 48- or 72-hours post-transplant. Survival was comparable between groups at 1-year (p=0.06), 3-years (p=0.16), and 5-years (p=0.68). Likewise, pulmonary function and QOL were similar, as was the incidence of CLAD. These results provide further evidence that the XVIVO system and Toronto protocol provide a safe means of evaluating and reconditioning high-risk donor lungs to increase the donor pool without compromising outcomes up to 5-years[37].

The Perfusix trial (NCT02234128) was a phase 2, multicentre, open-label study to compare the safety of receiving lungs with extended preservation and assessment from a facility with a centralized lung evaluation system (CLES) by Lung Bioengineering Inc[53]. Extended criteria donor lungs were procured and preserved in the clinical standard format of CSP at 4°C and delivered to Lung Bioengineering Inc. ESLP facility in Silver Spring Maryland where a Certified ESLP Specialist applied the Toronto EVLP System™ for up to 6-hours to assess the lungs for transplantation. Upon acceptance of the lungs by the implanting surgeon, the lungs were cooled to 10°C and transported to the recipient centre under hypothermic storage. CLES

lungs were matched to standard CSP controls (n=49) from each centre (n=7 US centres). Of 115 enrolled recipients, 66 allografts were transplanted from 63 donors after ESLP at the CLES facility. The incidence of PGD 3 at 72-hours was significantly higher in the CLES group, although 30-d and 1-yr survival was comparable to controls. Total preservation time, hospital, and intensive care unit (ICU) length of stay (LOS), and time to first extubation were significantly longer in the CLES group. Overall, the number on transplantations was increased by the use of a CLES facility to evaluate high-risk lungs with similar survival; however, rates of PGD 3 were significantly worse, which is associated with worse long-term outcomes and an increased incidence of CLAD. Long-term follow-up is required to better assess the utility of CLES.

Vivoline LS1™: Vivoline LS1™ was acquired by XVIVO and is no longer commercially available following the unfavourable results of the DEVELOP-UK trial. DEVLOP-UK was a multi-centre, unblinded, non-randomized, non-inferiority observational study to evaluate the clinical and economic effectiveness of ESLP to recondition high-risk lungs using the LS1 platform compared to standard lungs preserved with CSP[54]. Fifty-three high-risk donor lungs were assessed via ESLP with 18 (34%) being accepted for transplantation. 184 recipients received standard donor lungs during the same time period. A non-inferiority analysis was not conducted due to early termination of the study due to poor outcomes in the ESLP group. ESLP recipients required longer ICU ventilation and ICU LOS, had higher rates of PGD3, and significantly greater requirement for ECMO support. Furthermore, ESLP was £35,000 more than standard lungs with CSP due to the ESLP device itself, ECMO support and ICU stay. The study was limited by a small number of donor lungs in the ESLP arm and a protocol change from the Lund protocol to the Toronto protocol partway through the study. Ultimately, the increased

injury and risk of ESLP along with the significant cost associated with using extended criteria donor lungs on ESLP led to early termination of the trial[54].

***Transmedics OCS™ Lung*:** OCS™ Lung by Transmedics is another commercially available ESLP system that has been FDA approved for extended criteria donor lungs. In their pivotal trial EXPAND, the aim was to evaluate their normothermic portable OCS™ Lung on extended criteria donors after circulatory death with an age >55, PF ratio <300 mmHg, and expected ischemic time > 6 hours. This single-arm, pivotal trial included eight transplant centres (USA, Germany, Belgium) using high-risk lungs. A total of 93 lung pairs were accessed with the OCS™ Lung between 2014-2016, with 81 lungs being accepted for transplantation. Two lungs were excluded due to logistic limitations with 79 reconditioned lungs transplanted. The primary composite endpoint of 30-day survival and absence of PGD3 at 72 hours was achieved in 43/79 (54%), which did not meet the predefined performance goal of 65%. 35/79 (44%) had PGD 3 within 72-hours post-transplant. 78/79 (99%) recipients survived 30-days post-transplant. The average number of adverse events per lung, including respiratory failure and major pulmonary infection, was 0.3 events/patient. Although the primary objective was not reached, the OCS-Lung achieved 87% donor lung utilization with good clinical outcomes, thereby expanding the donor pool with otherwise discarded high-risk lungs[13].

Extended Criteria donor trials (NPV):

***Ex-Vivo Organ Support System (EVOSS™)*:** To date, there is only one published trial of NPV-ESLP applied to high-risk donor lungs, which makes it noteworthy. In 2020, Buchko et al. established the clinical safety and feasibility of EVOSS™ technology through a single-center,

prospective cohort trial of NPV-ESLP whereby twelve extended criteria donor lungs were successfully reconditioned and transplanted into recipients, resulting in 100% 30-day and 1-year survival[10]. Recruitment took place between October 2018 to July 2019. Extended criteria donors were procured in the standard fashion with CSP during transportation to the implanting hospital. Lungs were then connected to EVOSS^{TM} for preservation, reconditioning, and evaluation following the Edmonton Protocol. If the lungs were deemed acceptable for transplantation, they remained on the NPV-ESLP until the first recipient lung was explanted. The average ESLP run was 182 min, and total cold ischemic time was approximately 308 min and 359 min for the right and left lungs, respectively. The average total time from donor explant to implantation was 8h 14min and 9h 6min for right and left lungs, respectively. The mean PF ratio was 492 and all organs met the criteria for utilization, including stable hemodynamics and oxygenation after 3 h of NPV-ESLP. In addition to the excellent survival outcomes from this trial, no patients developed PGD scores grade 3 at 72 h or required extracorporeal membrane oxygenation (ECMO) post-operatively. This study demonstrates very promising results for the commercial use of EVOSS^{TM} [10]. To further establish the clinical utility of this technology a multi-center phase-1 clinical trial (RECLAIM) is underway in North America focused on the reconditioning of marginal quality donor lungs using a fully transportable EVOSS^{TM} device with the Edmonton protocol.

Standard Criteria Donors (PPV):

Given the success of ESLP to salvage high-risk donor lungs and expand the donor pool, comparisons of ESLP versus CSP using healthy/standard donor lungs were needed to assess any additional benefit of ESLP technology and indications for use.

Vienna Group: In a single-centre, prospective randomized trial known as the "Vienna Trial", Slama et al. (2017) demonstrated comparable transplantation outcomes between standard criteria donor lungs preserved with ESLP vs CSP. This group used a custom ESLP platform and modified Toronto protocol. Between 2013-2015, 193 lung transplants were performed at the Medical University of Vienna, and 80 lungs from that cohort were included in this trial with 41 in the control group and 39 in the ESLP group. Donor characteristics were comparable between groups. Four lungs were rejected following ESLP due to poor quality (n=2) and technical reasons (n=2). Total ischemic time was greater in the ESLP group, but there was no statistically significant difference in PF ratios, incidence of PGD>1, need for ECMO support, short-term clinical outcomes, need for mechanical ventilation, hospital LOS or 30-day survival between groups. This was the first study to demonstrate that ESLP can safely be used in standard donor lungs[38].

***Transmedics OCS-Lung*:** The INSPIRE trial was a non-inferiority, randomized, controlled, open-label, phase 3 trial that compared standard criteria lungs preserved with ESLP or CSP. The primary endpoint was a composite of absence of PGD3 within 72-hours and 30-day survival with a 4% non-inferiority margin. Between 2011-2014, 320 transplantations were performed with a random allocation to OCS^{TM} Lung (N=151) or CSP (n=169). Non-inferiority was met with regards to the primary composite endpoint; however, a superiority analysis was non-significant. 30-day survival was significantly better in the CSP group (95% vs 100%, p=0.0090), but this difference was lost at 12-months. The incidence of PGD3 within 72 hours was significantly higher in the CSP group (17.7% vs 29.7%, superiority test p=0.015). The primary safety

endpoint also met the non-inferiority cut-off with comparable rates of lung graft-related serious adverse events (p=0.020). In conclusion, the INSPIRE trial met its primary effectiveness and safety endpoints, and longer follow-up is needed to determine if the lower incidence of PGD3 associated with ESLP portends a survival benefit long-term[12].

6. GLOBAL CLINICAL EXPERIENCE

In addition to the aforementioned pivotal trials there are many small cohort publications detailing single-centre experiences worldwide with ESLP. An in-depth analysis of the individual studies is beyond the scope of this review. A recent comprehensive review of the global ESLP practice is available by Possoz *et al.* (2019)[55]. An overview of the world-wide progress, including shared challenges is summarized below.

Globally, the clinical outcomes from smaller studies are congruent with the findings from larger randomized and non-randomized studies. At least 14 countries and 24 centres have published their experience with ESLP applied to extended criteria donor lungs for transplantation [12,28,34,38,39,40,42,43,48,50,54,56-74]. Participating countries include Canada, USA, UK, Spain, Belgium, France, Germany, Austria, Denmark, Sweden, Iran, Italy, Australia, and Brazil. The geographic spread highlights the global interest in this technology. The average conversion rate from ESLP to transplantation from these studies was approximately 81%, resulting in a mean increase in transplantations performed of 21%[55]. These outcomes are highly encouraging and support the aim of ESLP to decrease waitlist mortality.

An ongoing challenge with assessing the global experience is the variability in platforms, protocols, acceptance criteria, and recorded data. The OCS platform and protocol was the most widely reported system used from the previously mentioned studies (24 centres), followed by the

Lund Protocol (6 centres) and lastly the Toronto Protocol (5 centres)[12,28,34,38,39,40,42,43,48,50,54,56-74]. It is common for centres to modify the technique slightly based on their experience or to use a customized platform rather than a commercial one, which limits a direct comparison of results. Furthermore, there is no established assessment criteria for what constitutes an extended criteria donor, or what performance measures signify successful ESLP reconditioning. Given the overall high rate of conversion and successful short-term survival outcomes with low rates of PGD, the worldwide approach is clinically sound despite a lack of standardization. This result likely stems from the decision criteria being closely based on a shared understanding of ideal donor acceptance criteria and adequate donor lung function. Interestingly, there is evidence that commonly used acceptance criteria for ESLP bear no significant impact on transplant outcomes [75]. This is perhaps unsurprising given traditional ideal donor acceptance criteria are not evidence based, but historically rooted in gestalt clinical judgement. Finally, there is variability in the reported outcomes, particularly PGD at 72-hours[56,76]. Incomplete data sets limit direct comparisons between individual centres, which underscores the benefit of multi-centre trials that standardize all of the above approaches.

The sum of the clinical evidence supports the use of ESLP as a safe intervention that can expand the donor pool without clinical consequence; however, ESLP continues to comprise a small percentage of all transplants. As detailed by Possoz *et al*, most reported extended criteria donor lung ESLP outcomes compare favourably against standard criteria lungs and CSP in terms of PGD and short-term survival. This is impressive given ESLP has been associated with prolonged preservation time with lungs starting from worse condition[55]. Similar findings were recently reported by Jawitz et al (2022)[76] after performing the largest US national analysis of ESLP usage to date. Data was extracted from the United Network for Organ Sharing (UNOS)

national database from 2018-2019. After controlling for baseline difference between donor and recipient characteristics, there was no difference in short-term survival or incidence of acute rejection between lungs preserved with ESLP and those managed with CSP. Prior to adjustment, there was a significantly greater incidence of ESLP lungs requiring ICU ECMO support, but this was likely due to the worse starting condition of the marginal quality lungs and not from ESLP itself[76]. These findings further support the safety of ESLP. Encouragingly, prior research has also demonstrated that ESLP transplants have comparable quality of life (QOL) and functional outcomes compared to standard lungs[77]. It is noteworthy that ESLP transplants represent <5% of all annual lung transplants (UNOS database), and this technology is most commonly applied by academic centres[76]. Indeed, there is significant opportunity to expand the application of ESLP.

7. CONCLUSIONS: PART 1

ESLP has transitioned from an obscure research tool to one of clinical translation, but many questions remain about the extent of its utility. Despite significant progress and promising outcomes, global confidence and/or access is lacking as reflected in the low rates of adoption. Ultimately, favourable long-term outcomes are required to galvanize the translation of ESLP into mainstream clinical practice while prioritizing accessibility and ease of operation. Evidence is required that the ESLP-expanded donor pool does not significantly increase rates of chronic lung allograft dysfunction (CLAD) and negatively influence recipient outcomes. To address these concerns ESLP must undergo further protocol refinement and therapeutic enhancement. This topic is covered in detail throughout Part 2 of this review.

PART 2: State-of-the-Art Review

8. ESLP PROTOCOL INNOVATION

Protocol improvements form the backbone of progress in ESLP, and over the past twenty years, many novel ways to improve the preservation, rehabilitation, and treatment of specific lung pathologies through ESLP have been explored with promising results. Isolation of the donor organ from other vulnerable physiology and organ systems is a key advantage for the development of personalized therapies, allowing the application of supratherapeutic doses that would risk systemic complications *in-situ*. A few of these interventions have been translated into the clinical realm. In the following section, therapeutic and protocol advancements are organized by their route of administration to highlight the unique points of interaction available with donor lungs via ESLP. This literature review is limited to large animal models and studies involving human lungs.

9. RESIRATORY THERAPIES

Alternative Ventilation Strategies

ESLP is most commonly performed using positive pressure (PPV) and volume-controlled ventilation (VCV), although a few alternative ventilation strategies have been investigated that may provide superior results. In 2017, Mehaffey *et al.* explored the use of airway pressure release ventilation (APRV) during ESLP compared to standard VCV in a porcine lung injury transplantation model (warm ischemic time [WIT] 2hr, ESLP 4hr, transplant reperfusion [Tx] 4hr) of hypoxic cardiac arrest. Pressure-directed APRV-ESLP resulted in transplanted lungs with superior oxygenation and dynamic compliance compared to standard ESLP. Weight-gain post-ESLP was significantly less with APRV as well. These results suggest that pressure release

ventilation ESLP may be a superior strategy if applied clinically[78]. As previously mentioned in Part 1, Aboelnazar *et al.* (2018)[14] from the Edmonton group published a comparison experiment between PPV-ESLP and their unique NPV-ESLP device (WIT none, ESLP 12hrs). Results demonstrated that negative pressure ventilation was associated with reduced lung injury scores, reduced pro-inflammatory cytokine production, and less edema formation regardless of perfusate composition (cellular vs acellular). In 2020, Ordies *et al*. investigated the application of flow-controlled ventilation (FCV) in a porcine ESLP model (WIT 2hr, ESLP 6hr). Findings supported the feasibility of FVC-ESLP, which produced improved oxygenation, lung compliance, and alveolar recruitment as evidenced by fewer identified CT scan densities[79]. In sum, ventilation strategies focused on reducing VILI have proven beneficial in preclinical ESLP and may help prolong the period of safe preservation and reconditioning of donor lungs.

Intrabronchial Therapies

Exogenous Surfactant

Donor aspiration causing gastric acid pneumonitis is a common complication in NDD (neurologic determination of death) donors that compromises lung function, increases the risk of PGD, and may be amendable to treatment via ESLP. In a pig gastric aspiration lung injury model, Nakajima *et al.* (2017)[16] demonstrated that bronchial lavage on ESLP (cold-ischemic time [CIT] 10hr, ESLP 6hr, Tx 4hrs) followed by artificial surfactant administration resulted in superior outcomes following transplantation compared to groups of 1) no intervention, 2) lavage only, or 3) surfactant only. EVLP with lavage and surfactant resulted in reduced inflammatory mediators (Il-6, IL-1β, IL-8, and secretory phospholipase A2), reduced breakdown of active

surfactant, and overall greater amounts of active surfactant with reduced alveolar surface tension. Superior lung function was confirmed with a four-hour left lung transplantation model.

Inci *et al.* have also established the beneficial effect of surfactant application via ESLP to repair porcine lungs injured by gastric acid aspiration. In 2008, they demonstrated that diluted surfactant lavage during ESLP (CIT 3hr, ESLP 2hr) improved graft function by decreasing the pulmonary vascular resistance, improving oxygenation, decreasing weight-gain, and displayed lower neutrophil counts on BAL[80]. In 2013, Inci *et al*. repeated their experiment in a DCD (donation after circulatory death) pig transplantation model (CIT 1h, ESLP 2hr, Tx 4hr) and found similar results during ESLP with better oxygenation, lower pulmonary artery pressures and less edema formation post-transplant. BAL demonstrated significantly lower levels of pro-inflammatory cytokine (IL-6, IL-1beta), protein, and neutrophils compared to controls[81].

In 2014, Khalife-Hocquemiller *et al.* also demonstrated the benefit of surfactant lavage with ESLP in a slightly modified experiment that selectively injured the left lower lobes of piglets using gastric juice. Adding surfactant lavage localized to the injured lobe immediately prior to ESLP (4hr) normalized oxygenation, pulmonary vascular resistance, and apoptotic-cell percentage[82]. These studies provide strong evidence that exogenous surfactant administration can facilitate ESLP reconditioning in the setting of gastric acid aspiration.

Intrabronchial Gene Therapy

ESLP has the potential to deliver retroviral gene transfection immunomodulatory therapies through the ventilator or perfusion circuit to improve graft quality. In 2009, Cypel *et al.* demonstrated the successful use of gene therapy with the anti-inflammatory cytokine IL-10. Aerosolized delivery of AdhIL-10 (IL-10 gene transfection via human adenovirus) over 12-hours

of ESLP in rejected NDD human donor lungs resulted in improved oxygenation and PVR, reduced inflammatory marker expression, and improved alveolar-blood barrier integrity compared to controls[83].

In 2012, Yeung *et al.* used a porcine model to demonstrate that *ex-vivo* intratracheal administration of adenoviral IL-10 gene therapy to donor lungs via ESLP resulted in less vector-associated inflammation (IL-1β) with superior physiologic function during 12-hour ESLP preservation and post-transplant (4hr) compared to *in-vivo* intratracheal administration[84]. In 2017, Machuca *et al.* used a porcine transplant survival model to establish the pre-clinical safety and therapeutic potential of AdhIL-10 therapy via ESLP. After 6-hours CSP, pig lungs were preserved for 12-hours on ESLP and administered AdhIL-10 therapy intratracheally. A left lung transplantation was performed post-ESLP with a 7-day recovery and survival period. During ESLP and post-transplantation, lungs treated with AdhIL-10 therapy had superior physiologic function, including oxygenation, reduced histologic inflammation scores, and reduced adaptive immune response (reduced donor-specific IFN-gamma-producing lymphocytes) compared to controls (ESLP only, ESLP vector control, CSP control)[85]. Noteworthy, there was no evidence of systemic toxicity in the transplant recipients. Indeed, there is encouraging evidence supporting the efficacy and safety of adenoviral IL-10 gene therapy in the context of ESLP.

Intrabronchial Cell Therapy

Stem cells may provide a beneficial effect in immune modulation during ESLP. Bone-marrow derived cells are well characterized and used for research related to IRI. There two types commonly used: mesenchymal stem cells (MSCs) and multipotent adult progenitor cells (MAPC). In 2017, Martens *et al.* investigated the role of ventilator delivered MAPCs in a

porcine ESLP model (WIT 90min, ESLP 6hrs). Results demonstrated no significant difference in lung function (PVR, compliance, oxygenation, wet-to-dry ratio) between MAPC treated lungs and controls. MAPC treated lungs had significantly lower BAL pro-inflammatory cytokines (TNF-alpha, IL-1beta, INF-gamma) and neutrophils. The authors concluded that although MAPC airway administration did not improve lung function, suppression of the innate immune response may contribute to improved post-transplant lung function[86]. Several studies investigating the role of MSC administered via the perfusate demonstrated greater effects, which are covered later in this review.

β2-adrenergic receptor agonists

Bronchodilator therapies (β2-adrenergic receptor agonists) dilate airways through the relaxation of smooth muscle, and several research groups have investigated the potential therapeutic role of this medication class during ESLP. In 2007, Frank *et al*. performed an experiment to study air space fluid clearance (AFC) using rejected human donor lungs and a custom ESLP device. Specifically, they investigated the effect of a β2-adrenergic receptor agonist (terbutaline) in the context of pulmonary edema. Results demonstrated that administration of terbutaline into a subsegment of the perfused lung increased alveolar fluid clearance more than two-fold from baseline and decreased the weight-gain. This data is encouraging because even though the lungs were injured, the findings show that the respiratory epithelium remained responsive to β2-adrenergic stimulation with resulting improvement in the rate of edema fluid clearance[87].

In 2015, Kondo *et al*. investigated the role of an aerosolized β2-adrenergic receptor agonists (procaterol) during ESLP to attenuate ischemic reperfusion injury (IRI) in a DCD canine preparation (WIT 210 minutes, ESLP 240 minutes). Results of the procaterol inhalation

group showed significantly lower peak airway pressure, pulmonary artery pressure, pulmonary vascular resistance, and wet-to-dry ratio with greater dynamic compliance compared to controls. Furthermore, the procaterol inhalation group showed higher levels of ATP, ADP, and AMP; greater cAMP levels; and greater CFTR gene expression. These findings suggest that ESLP with aerosolized β2-adrenergic receptor agonists helps protect against the deleterious effects of IRI during preservation[88]. In 2017, the same group from Kyoto validated their results using a canine left lung transplantation model (WIT 150min, ESLP 120min, Tx 240min)[89]. After transplantation, significantly improved lung function was noted in the procaterol treated group compared to controls. The procaterol treated group showed significantly better oxygenation, dynamic compliance, and reduced pulmonary vascular resistance post-transplant. The sum of the available evidence suggests that aerosolized β2-adrenergic receptor agonist therapy may be beneficial if translated to clinical ESLP[89].

Inhaled Sphingosine

Sphingosine has been shown to efficiently kill a range of microorganisms, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *epidermidis*, *Escherichia coli* and *Haemophilus influenzae*, and it may be an effective therapeutic to treat donor lung infections in the context of ESLP. In 2021, Carstens *et al.* set out to test the safety of inhaled sphingosine as a broad-spectrum anti-infective agent in a healthy pig model of ESLP (CIT 2hr, ESLP 4hr). Findings established that sphingosine concentrations were significantly increased in bronchial epithelial cells of treated lungs without evidence of local adverse effects nor physiologic dysfunction compared to controls[90]. In 2022, the same group built on their previous research model by testing sphingosine inhalation antimicrobial potency in a lung infection ESLP

preparation (CIT 1 hr, ESLP 3hr). *Pseudomonas aeruginosa* was used for pneumonic infection ($2x10^9$ CFU) and equally distributed throughout the lungs attached to the ESLP ventilator. After 1 hr of infection, sphingosine inhalation via a nebulizer was initiated for 15-minute duration and the lungs remained on ESLP until a total of 3-hours. BAL samples revealed a 6-fold decrease in *P. aeruginosa* CFU after sphingosine administration compared to controls. There were no significant differences in the physiologic performance of lungs between treated and control lungs, and no local adverse effects were noted in the lungs treated with sphingosine[91]. Although the infection time was limited to 1-hour, and only one bacterial strain was tested, this study offers an encouraging proof of concept application that could eventually translate into clinical ESLP with polymicrobial donor lung pneumonias.

Inhaled N-acetylcysteine

N-acetylcysteine (NAC) has been shown to have a beneficial effect on IRI post lung transplant in small and large animal models[92-95], and preliminary research suggests that it may be well suited for ESLP treatment of donor lungs. In 2017, Yamada *et al*. investigated the anti-inflammatory potential of nebulized NAC in a porcine model of ESLP followed by in-vivo transplantation assessment (CIT 24hr, ESLP 2hr, Tx 6hr). Results demonstrated significantly reduced neutrophil activation as evidenced by lower levels of myeloperoxidase (MPO) and lower levels of inflammatory marker NF-$\kappa\beta$ suggesting a reduced expression of pro-inflammatory genes. No significant difference was found across lung function parameters, edema formation, or histologic injury scores between NAC treated and control lungs while on ESLP or post-transplant[95]. This study supports the safety of nebulized NAC during ESLP with subclinical anti-inflammatory effects. Further research is warranted that includes donor and recipient pre-

treatment with NAC as well as longer durations of NAC treatment on ESLP to understand its potential role in preventing PGD.

Therapeutic Gases

Nitric Oxide

In-vitro, high dose nitric oxide (NO) is effective against a variety of microorganisms, including those commonly found in donor lungs. Unfortunately, administration of NO *in-vivo* carries the risk of formation of methemoglobin and toxic nitrogen compounds; however, ESLP provide a unique opportunity to administer NO to donor lungs in isolation using an acellular perfusate, mitigating those risks. In 2021, Michaelsen *et al.* explored the safety of continuous high dose inhaled NO (iNO) during prolonged (12 hr) ESLP using healthy porcine lungs. Findings demonstrated that a continuous administration of iNO at 200 ppm was feasible with maintenance of stable lung physiology, no histologic evidence of injury, and no change in pro-inflammatory cytokines compared to controls. These results are encouraging because they suggest that prolonged, continuous high dose iNO could potentially be used to treat microbial infections in donor lungs on ESLP without causing injury. Further experiments using infected animal lung preparations are needed[96].

Hydrogen gas

Hydrogen gas has therapeutic antioxidant and anti-inflammatory properties that have been applied successfully via ESLP in large animal research preparations. In 2015, Haam *et al*. studied the effects of a 2% hydrogen gas administered during a DCD porcine ESLP model (WIT 1hr, ESLP 4hr). Hydrogen gas treatment during ESLP resulted in significantly lower pulmonary

vascular resistance and pulmonary artery pressure with equivalent oxygenation and compliance compared to controls. Furthermore, hydrogen gas treated lungs had significantly lower levels of pro-inflammatory cytokines (IL-1beta, IL-6, IL-8, TNF-alpha), better histologic lung injury scores, and reduced wet-to-dry ratios[97]. In 2018, the same group sought to validate their findings with a left-lung transplantation model (WIT 1hr, ESLP 4hr, Tx 3hr). During ESLP and post-transplant reperfusion, physiologic parameters and arterial blood gas analysis results were superior in the hydrogen treated group compared to controls. Pro-inflammatory markers were higher in controls, and anti-inflammatory cytokine IL-10 was higher in the hydrogen group. Antioxidants (superoxide dismutase and heme oxygenase-1) were significantly higher in the hydrogen treated lungs as well. Lung injury scores, number of apoptotic cells, and degree of edema formation were all significantly worse in controls[98]. These results are encouraging that hydrogen gas may help mitigate the deleterious pro-inflammatory state of ESLP and improve the function of donor lungs.

Noble Gases

Inhaled noble gases have organoprotective effects that may be beneficially applied to donor lungs during ESLP preservation. Specifically, Argon (Ar) and Xenon (Xe) have been shown *in vitro* and *in vivo* to have antiapoptotic and anti-inflammatory effects in models of brain, myocardium, and kidney injury[99-103]. In 2016, Martens *et al.* set out to investigate the effects of Ar and Xe on IRI during porcine ESLP (WIT 2h, ESLP 6hr). ESLP control lungs were ventilated with 70%N_2/30%O_2 and experimental lungs were ventilated with either 70%Ar/30%O_2 or 70%Xe/30%O_2. In this particular study, ventilation with the noble gases did not result in improved graft function[104]. In 2017, the same group altered their experiment to investigate if a

prolonged exposure to Ar would decrease cold ischemia-reperfusion injury after lung transplantation in a porcine ESLP model. Pigs were pre-conditioned for 6-hours with an Ar gas mixture, cold flushed and stored inflated for 18-hours in the same gas mixture, then preserved on ESLP for 4-hours and ventilated with the identical gas mixture. Final evaluation consisted of 2-hours of ESLP with whole blood perfusion to simulate transplantation. Control lungs followed the same sequence but were ventilated with room air. Evaluation at the end of the experiment did not reveal a significant performance difference between groups. In other organ systems Ar has been shown to have protective effects, but this experiment failed to show a benefit of prolonged Ar ventilation on ESLP preservation[105].

Prone Positioning

Patients with lung injury often benefit from prone positioning in ICU settings and there is growing evidence that prone positioning during ESLP is also beneficial for lung function. In 2019, Niikawa *et al.* investigated the potential benefit of positioning donor pig lungs prone during ESLP versus standard supine positioning (WIT 2hr, CIT 5hr, ESLP 2hrs). Results showed that prone lungs had better oxygenation, static compliance, less weight gain, and lower levels of pro-inflammatory cytokines (IL-1β) compared to supine ESLP lungs. This data suggests that prone positioning during ESLP may reduce IRI and improve lung function[106]. In 2022, this same research group repeated their study using human donor lungs rejected for clinical use (CIT 16h, ESLP 2hr). Human lungs treated in the prone position while on ESLP demonstrated significantly better oxygenation, and reduced weight-gain. Furthermore, upon isolated assessment of upper versus lower lobes within groups, prone ESLP lungs had similar function and pro-inflammatory cytokine concentrations across lung lobes. In contrast, supine lungs had significantly worse

oxygenation with higher pro-inflammatory cytokine levels (TNF-alpha) in the lower lobes[107]. Prone ESLP may help support lower lobes function in clinical ESLP.

In 2019, Watanabe *et al.* provided further evidence in support of prone positioning in ESLP through the use of a DCD porcine model (WIT 3hrs, CIT 6hrs, ESLP 6 hrs). Prone ESLP lungs showed improved lung function, less weight gain, less cell death (fewer TUNEL positive cells), lower histologic lung injury scores, and lower levels of pro-inflammatory cytokines (IL-1β, IL-8) compared to supine ESLP lungs[108]. This is encouraging that a simple adjustment in lung positioning may produce superior ESLP results for injured lungs.

Also in 2019, Ordies *et al*. explored the potential benefit of prone positioning during porcine ESLP given the tendency for fluid to accumulate in the dorsal regions when lungs are supine during ESLP; however, their findings did not identify as dramatic an effect on improved lung function or suppressed inflammation as prior studies. After the experimental run (CIT minimal, ESLP 6 hrs) results showed similar physiologic function between groups, except prone lungs had significantly higher pulmonary vascular resistance. Prone lungs had more homogenous edema formation between ventral and dorsal tissue biopsies compared to supine lungs. Supine lungs had significantly more edema in dorsal lung biopsies compared to ventral biopsies. Overall edema formation did not differ between groups as evidenced by similar CT densities. There was no significant difference in histologic neutrophil infiltration or pro-inflammatory cytokines between groups[109]. The minimal CIT and injury to these experimental lungs may explain the reduced effect difference between groups based on positioning compared to previously described studies. Additional experiments comparing injured to non-injured lung outcomes in the prone position may clarify these conflicting findings.

10. PERFUSION THERAPIES

Alternative Perfusion Strategies

Cellular versus Acellular Perfusion

Perfusate composition is an ongoing topic of debate with the two principal camps being cellular (blood-based) versus acellular (non-blood based). Overall, cellular perfusate appears to provide superior preservation, but the significant limitations on access to blood products along with logistical complications for collecting autologous blood from donors makes acellular perfusates more convenient and affordable. In 2015, Roman *et al*. performed the first head-to-head comparison of blood based ESLP perfusate against the standard acellular approach using a porcine DCD preparation (WIT 30min, CIT 2hrs, ESLP 4hrs). Groups consisted of acellular (STEEN), STEEN-Blood, and Papworth-Blood perfusates. ESLP lung function was similar between groups in terms of oxygenation, compliance, PVR and wet-to-dry ratios. There was no significant difference in the number or types of leukocytes in the leukocyte filters after ESLP. All groups had elevated perfusate pro-inflammatory cytokines; however, only the acellular group had significantly elevated IL-8 on BAL. Cell characteristics between groups were similar on electron microscopy. The authors concluded that there was no significant difference in physiologic, immunologic, or ultrastructural components of cells between perfusate groups[47]. Therefore, Papworth-Blood was deemed a reasonable alternative to other commercial-blood based perfusates.

In 2017, Loor *et al.* compared perfusates of varying compositions using a DCD porcine ESLP (24-hours) model and the OCS™ Lung platform. The groups included 1) isolated packed red blood cells (pRBC 900ml, diluted in OCS Solution™ 1500ml), 2) autologous whole blood (WB 1600 ml with 800ml OCS Solution™), and 3) acellular buffered dextran-albumin solution

(BDAS 2400mL), which was similar to STEEN Solution™. Lungs perfused with BDAS (acellular) did not meet clinically acceptable standards for transplantation at 8-hours of ESLP due to prohibitively elevated PVR, edema, and poor compliance. In contrast, WB and pRBC groups reached 24-hours of ESLP. Only the WB group had acceptable parameters for transplantation (PF ratio >300 mmHg) with significantly better PAP, PVR, dynamic compliance, pH stability, and minimal weight gain. Ultimately, the WB-based perfusate enabled 24-hours of porcine ESLP with stable functional parameters and was superior to pRBC perfusate, which was superior to the acellular solution[110].

In 2018, Sommer *et al.* from the OCS lung group built on their previous work by investigating the role of various perfusates over 24-hour porcine ESLP with transplantation assessment (6-hour). Groups consisted of 1) STEEN Solution™ with pRBC, 2) acellular STEEN Solution™, and 3) low-potassium dextran (LPD) solution with pRBC. STEEN Solution™ with pRBCs produced the best outcomes, and the worst performance was from LPD with pRBC. On ESLP, STEEN with pRBCs had significantly lower PAWP, PVR, wet-to-dry ratio than the two other groups. Oxygenation was similar between groups during ESLP, but STEEN with pRBC had superior survival, oxygenation, PAP, and PVR post-transplant than other groups[111].

Also in 2018, Nader *et al.* from the Edmonton group explored the effect of cellular and acellular perfusate in a porcine ESLP model (12hrs) across NPV and PPV platforms to see whether a particular type of perfusate influenced lung function depending on ventilation strategy[14]. This study was mentioned earlier regarding novel ventilation strategies in ESLP. Both cellular and acellular perfusates produced acceptable physiologic outcomes during ESLP regardless of ventilation strategy. Pro-inflammatory cytokine production (TNF-alpha, IL-6, IL-8) was also similar across perfusates, and both groups had significantly lower cytokine

concentrations with NPV-ESLP. Acellular perfusate resulted in significantly greater weight-gain post-ESLP across ventilation strategies, suggesting the cellular perfusate is superior for mitigating edema formation[14].

Steinmeyer *et al.* (2018) also compared the effects of cellular and acellular perfusate in a large animal model of ESLP (CIT 24hrs, ESLP 12hrs). Cellular perfusate consisted of STEEN Solution™ supplemented with leukocyte depleted autologous pRBC, and acellular perfusate was STEEN Solution™ alone. Parenchyma integrity was well maintained by both ESLP groups. There was no significant difference in physiologic outcomes during ESLP preservation. Cellular ESLP had significantly less edema formation compared to acellular ESLP; however, both groups had low levels of intraalveolar edema formation from a clinically relevant perspective[112].

Continuous versus Pulsatile Flow

Current FDA approved ESLP devices are available with both continuous flow (CF; XPS™) and pulsatile flow (PF; OCS™ Lung), and limited research suggests there is no significant performance difference between either pump strategy. In 2015, Schumer *et al.* compared CF to PF in a porcine ESLP model (CIT 30-45 min, ESLP 12hrs). All lungs maintained acceptable pulmonary function while on ESLP with minimal edema formation. No significant differences in lung function were noticed between groups, including oxygenation, peak airway pressures, or wet/dry weight ratios[113]. A transplant model may have helped distinguish between the perfusion strategy performances as ESLP does not replicate lung function *in-vivo.*

Reduced Flow ESLP

Early protocols in ESLP aimed to mimic physiologic conditions to optimize preservation. The Lund Protocol, developed by Professor Stig Steen, was the first ESLP management strategy to successfully salvage, assess, and recondition DCD lungs and ECD lungs for clinical transplantation with excellent outcomes[32,33]. The Lund protocol employs a flow rate of 100% cardiac output with the goal of preserving lungs for short time-intervals of approximately 4-hours. In 2008, the Toronto group, using the XVIVO XPS™ platform, published their protocol for prolonged ESLP lasting 12-hours. This was achieved through several protocol modifications compared to the Lund approach[36]. Notably, perfusion flow rate was reduced to 40% of predicted cardiac output, based on 100ml/kg/min. Likewise, the OCS™ Lung device by Transmedics® employs a perfusion flow of 2-2.5L/min, roughly 50% of resting cardiac output and has achieved reliable preservation up to 24-hours[12,13,110,111]. Indeed, flow rates of 30-50% of predicted cardiac output (CO) in ESLP are standard; however, recent literature suggests that reduced perfusate flow may be advantageous.

In 2020, Beller *et al.* investigated whether a further reduction in flow rate was beneficial in a porcine model of PPV-ESLP with transplantation (WIT 90 min, ESLP 4hrs, Tx 4hrs). Results demonstrated that post-transplant lung function was significantly better with lower perfusion flow of 20% CO during ESLP compared to 40% CO[114]. That is, donor-lung specific oxygenation, compliance, and edema were all significantly improved. Pro-inflammatory cytokines (IL-1ß, IL-4) were also significantly lower in the 20% flow group. This study calculated CO based on 100ml/kg/min; therefore, a 20% CO is very similar to the perfusion flowrate used by EVOSS™, which is 30%CO calculated at 70ml/kg/min[10,14,15,52,115]. It is

unknown if additional reductions in flow rate would results in further improvements in edema formation and preservation.

Perfusate Additives

Intravascular Steroids

In 2016, Martens *et al.* explored the effect of Methylprednisone (MP) on donor lung function using a DCD porcine ESLP model (WIT 90min, ESLP 6hrs). In the treatment group, 500 mg MP was administered prior to cardiac arrest and during ESLP. Compared to a control group that received no steroids, the MP group demonstrated significantly greater lung compliance, measures of edema and lung consolidation (wet-to-dry and CT density), as well as significantly lower perfusate concentrations of pro-inflammatory cytokines (IL-1β, IL-8, IFN-α, IL-10, TNF-α, and INF-ɣ). BAL cytokines were similar between groups except IFN-ɣ was lower following MP treatment. Oxygenation and PVR were similar between groups. The authors concluded that steroids could attenuate the injury of WIT in DCD lungs if MP is administered prior to arrest and during ESLP[116].

Intravascular Antimicrobials

Broad-spectrum antimicrobials during ESLP can reduce bacterial and fungal loads along with pro-inflammatory cytokines while improving lung function, and the isolated environment of ESLP allows for supratherapeutic doses without concern for systemic toxicity. ESLP antimicrobial therapy is important to protect donor lungs from fungal and bacterial infection during preservation and to reduce transmission to immunosuppressed recipients. In 2014, Andreasson *et al.* investigated the effect of high-dose, empirical, broad-spectrum anti-microbial

agents (meropenem 500mg, amphoteric B 50mg) administered during ESLP (WIT 30-60 min, ESLP 3-6hrs) using human lungs initially rejected for clinical transplantation that required reconditioning. BAL quantification cultures were performed for fungi and bacteria pre- and post-ESLP. Thirteen of 18 lungs were identified as having positive cultures prior to ESLP, and the bacterial load significantly decreased in all cases after perfusion. Notably, yeast colonies increased during ESLP when anti-fungal medication was absent, but the yeast colonies decreased if prophylactic anti-fungal treatment was added. Six lungs met transplant suitability and were transplanted into recipients with 100% survival at discharge and one death at eleven months[117]. High-dose, broad-spectrum, empirical antimicrobials administered during ESLP is an efficacious and safe way to treat donor lung infection.

In 2016, Nakajima *et al.* also investigated the role of broad-spectrum, empiric, antibiotics (ciprofloxacin 400mg or azithromycin 500mg, vancomycin 15mg/kg, and meropenem 2g) during prolonged ESLP (12hrs) using human lungs rejected for clinical transplantation. Control lungs did not receive antibiotics during ESLP. All antibiotic treated lungs (n=8) demonstrated a quantitative decrease in bacterial counts in BAL after ESLP, whereas only two control lungs (n=7) had a similar decrease. Lung function (oxygenation, compliance, PVR) were significantly better in the antibiotic treated lungs. Antibiotic treated lungs had significantly lower levels of perfusate endotoxin at 12 hours compared to control lungs. Endotoxin levels were strongly correlated with pro-inflammatory cytokines (TNF-α, IL-1β) and macrophage inflammatory proteins (1α, 1 β). Therefore, broad-spectrum empiric antibiotics during ESLP effectively reduced BAL bacterial load, perfusate endotoxin levels, inflammation and resulted in superior lung function[118].

In 2018, Zinne *et al.* explored the use of high-dose antibiotics in a porcine ESLP auto-transplantation model to determine the possibility of treating lungs infected with multi-drug resistant organisms using an ESLP platform and supratherapeutic doses of antibiotics (colistin). In their model, severe *Pseudomonas aeruginosa* pneumonia was induced in the left lower lobes (LLL) of Mini-Lewe pigs. Control pigs received no colistin, standard treatment pigs received colistin daily (2mg/kg), and ESLP lungs were perfused with solution containing 200 μg/ml colistin for 2-hours followed by LLL autotransplantation with 4-day follow-up for all groups. Infection-related mortality was 66.7% in control and standard treatment groups versus 33.3% (1 of 6) in the ESLP treatment group. Additionally, the ESLP group had less severe clinical signs of infection during the survival period[119]. ESLP combined with very high-dose antibiotic therapy may serve as a last-resort therapeutic option for patients suffering from incurable multi-drug resistant pneumonia while avoiding systemic toxicity in other organs.

Fibrinolysis of Pulmonary Embolism

Treatment of pulmonary embolism via ESLP with adjunctive fibrinolytics has been successfully reported several times in pre-clinical and clinical settings. In 2007, Inci *et al.* added urokinase to an ESLP circuit in a DCD pig model (WIT 3hr, CIT 1hr, ESLP 90min) to assess its therapeutic potential on microvascular thrombi after cardiac arrest. Findings demonstrated that lungs treated with the fibrinolytic agent had significantly lower pulmonary vascular resistance, better oxygenation, and reduced edema formation compared to DCD control lungs. Histologic examination also revealed that DCD control lungs had perivascular and interalveolar erythrocytes whereas the urokinase treated lungs appeared normal[120]. In 2014, Inci *et al.* repeated their success with a set of human donor lungs that had persistent segmental pulmonary emboli

(NDD donor), which were successfully treated using ESLP (3hrs) and urokinase (100,000 U). The lungs were transplanted with PGD 0 at 48- and 72-hours and reported excellent 6-month follow-up[121].

In 2012, Machuca *et al.* had similar success treating pulmonary embolism (PE) via ESLP (6 hrs) thrombolysis using alteplase (20mg) followed by clinical transplantation. Successful thrombolysis was assumed following gradual improvement in PAP and PVR (34% decrease after 2hrs) along with an 11-fold increase in d-dimer, suggesting ongoing clot breakdown. The patient had excellent lung function in ICU and upon discharge[122].

In 2015, Luc *et al.* reported a case-study sharing their success with alteplase as an adjunct to ESLP for the reconditioning of DCD donor lungs to lyse a PE. The PE was mechanically removed from the distal pulmonary arteries using 5mg of alteplase along with 2.5 hours of ESLP. During ESLP the PF ratio increased from 202 mmHg to 486 mmHg, indicating a decrease in V/Q mismatch correlating with a lysis of embolic burden. Remaining functional lung parameters were acceptable and the lungs were transplanted without cardiopulmonary bypass. The patient's post-operative course was uneventful with PGD 0 at 48-and 72-hours with excellent lung function at 4-month follow-up[123].

In 2017, Liersch-Nordqvist *et al.* published contrasting findings in their study of alteplase administration with ESLP in a porcine DCD model (WIT 1hr, CIT 4hr, ESLP 70 min). Heparin was not added to the DCD model but was part of the ESLP circuit. There were no significant differences between lungs treated with alteplase and control lungs while on ELSP with similar oxygenation, PAP, PVR and weight-gain. Macroscopic appearance of the lungs was similar between groups. In this particular study, the use of alteplase did not appear to have any benefit to the DCD lungs, and ESLP with heparin alone was adequate for reconditioning after an initial

flush and CSP[124]. The authors believe that the short WIT may have been the distinguishing protocol difference to explain the lack of benefit from alteplase, because the microvasculature remains unaffected during that interval compared to longer durations of warm ischemia and topical cooling. Topical cooling prior to CSP may also alter the endothelial function and worsen clot formation.

β2-adrenergic receptor agonists

As discussed earlier, β2-adrenergic receptor agonists induce smooth muscle relaxation to dilate airways, which have proven beneficial in ESLP research when administered via ventilation, and they have also proven beneficial when added to the perfusate. In 2011, Valenza *et al.* demonstrated the ESLP glucose consumption correlated with the extent of pulmonary edema with worse edema resulting in greater glucose consumption[125]. In a follow-up study in 2012, the same team investigated if salbutamol, which is known to increase fluid clearance in the lung, had an effect on porcine ESLP (5 hrs) perfusate glucose concentrations. Salbutamol or a placebo were administered for 180 minutes of ESLP after an initial reperfusion and stabilization period of 120 minutes. Salbutamol treated lungs demonstrated a decreased glucose concentration over time that was greater than placebo treated controls. *β*-agonists treated lungs had significantly lower pulmonary artery pressures, had a significantly less dramatic drop in compliance over time, but oxygenation was comparable between groups. The increased consumption of glucose in the salbutamol treated lungs fit with the author's hypothesis that increased fluid clearance from *β*-agonist treatment causes increased active transport of fluid, resulting in greater amounts of glucose metabolism[126]. The superior lung mechanics and lower

pulmonary artery pressures are desirable functional improvements from salbutamol treatment, which may translate to clinical ESLP.

Adenosine A_{2A} and A_{2B} Agonists

Adenosine is released into the circulation during states of inflammation and induces pro- and anti-inflammatory cascades via four adenosine receptors (A_1, A_{2A}, A_{2B}, A_3). Reece *et al.* (2005)[127] previously established a potential beneficial role of adenosine A_{2A} receptor agonism in acute lung injury, raising the possibility of A_{2A} as a potential intervention for the reconditioning of marginal quality donor lungs. In 2011, Emaminia *et al.* investigated the potential role of a selective adenosine A_{2A} receptor agonist (10 μM CGS21680) during ESLP (CIT 14hrs, ESLP 5hrs) to mitigate acute IRI in porcine lungs. Findings included significantly improved oxygenation, decreased mean airway pressure, improved minute ventilation, decreased lung edema (wet-to-dry ratio), reduced lung tissue INF-ɣ, and reduced histologic evidence of lung injury compared to control lungs[128].

In 2017, Charles *et al.* investigated the role of concomitant adenosine A_{2B} receptor agonism (ATL802) in a porcine DCD model with prolonged warm-ischemic time followed by ESLP and transplantation (WIT 2hr, CIT 4hrs or ESLP 4hrs, Tx 4hrs). Post-transplant, all transplant outcomes were acceptable, but ESLP + ATL802 demonstrated significantly improved dynamic compliance compared to controls. ESLP + ATL802 and ESLP alone had similar oxygenation and pulmonary edema formation, which were significantly better than cold preservation. ESLP + ATL802 had significantly lower levels of pro-inflammatory cytokines in BAL and tissue supernant (IL-12) compared to ESLP alone[129]. Concomitant use of a selective

A_{2A} receptor agonist or an A_{2B} receptor agonist with ESLP may serves as a valuable reconditioning therapeutic to help increase the donor pool.

α_1-Anti-trypsin

Alpha$_1$-Anti-trypsin is used to treat patients with α_1-Anti-trypsin (A1AT) deficiency and has anti-inflammatory properties that can mitigate the injury from ischemic reperfusion in pig and rat lung transplant preparations[130,131]. In 2018, Lin *et al.* investigated the role of concomitant A1AT administration (3mg/ml Zemaira) during porcine ESLP (12hrs) as a means to improve lung quality after CSP (CIT 24hrs). A1AT treated lungs demonstrated significantly lower PAP, PVR, improved airway pressure, dynamic and static compliance, oxygenation capacity, reduced edema, pulmonary cell apoptosis and lower concentrations of perfusate pro-inflammatory cytokines (IL-1α, IL-8)[132].

In 2020, Mariscal *et al.* tested the effect of A1AT during ESLP (12hrs) using human donor lungs that were rejected for clinical transplantation. Lung blocks were divided into left and right lungs to act as simultaneous control and treatment groups on separate ESLP devices. A1AT-treated lungs demonstrated significantly better oxygenation, compliance, lower PAP and PVR compared to controls. Edema formation and perfusate loss were less in the A1AT group, which also demonstrated lower endothelin-1 (vasoconstrictor), and increased tight junction protein (zonula occludens, ZO-1)[133]. These pre-clinical porcine and human outcomes with A1AT are promising for eventual clinical translation.

Neutrophile Elastase Inhibitor

Inflammatory cells, including neutrophils, can exacerbate donor lung injury of donor lungs, and neutrophil elastase inhibitors (NEI) have been shown to protect lung grafts from inflammatory injury when administered during ESLP. In 2015, Harada *et al.* used a porcine ESLP model with transplantation (WIT 2hr, ESLP 4hr, Tx 4hr) to assess lungs treated with 0.5 g of Sivelestat (NEI) versus controls. During ESLP, lungs treated with Sivelestat demonstrated a significant decrease in neutrophil elastase, edema formation, and PVR with improved oxygenation and pulmonary compliance. Post-transplantation, NEI treated lungs had significantly lower PVR. Treated lungs also produced significantly lower concentrations of pro-inflammatory cytokines during ESLP (IL-8, IL-6). These findings suggest the NEI may be a potential option to mitigate the inflammatory response of donor lungs on ESLP[134].

Endothelin Receptor Antagonist

Following brain-death the endothelin-axis is upregulated and causes systemic inflammation, lung injury, and contributes to poor outcomes; however, endothelin receptor blockers are able to attenuate some of this injury, which makes them potential ESLP therapeutics. *In-vivo*, Tezosentan (endothelin receptor blocker, ERB) can cause adverse effects to other vulnerable organs, but an advantage of ESLP is organ isolation that allows for supratherapeutic doses of medications without compromising neighbouring vulnerable physiology. In 2020, Walweel *et al.* investigated whether Tezosentan (400mg) could improve lung function when administered concomitantly in an ovine ESLP (6hr) preparation. ERB treated lungs demonstrated a significant improvement in oxygenation and ventilation without evidence of lung injury. Pro-inflammatory cytokine perfusate concentrations were similar between treated

and control lungs, and edema (wet-dry ratio) formation was comparable. Therefore, the authors believe that Tezosentan could help improve gas-exchange when administered with ESLP[135].

Monoclonal Antibody/Immunotherapy

In 2020, Ku *et al.* performed a pre-clinical investigation on the utility of administering the anti-CD20 monoclonal antibody, Rituximab (RTX 500mg), during ESLP using human lungs rejected for transplant. CD-20 is a marker on the surface of B-cells, and binding of CD-20 with RTX leads to B-cell depletion and can reduce inflammation that compromises allograft function. RTX treatment can target Eptein-Barr virus (EBV) infected B-cells and prevent post-transplant lymphoproliferative disorder. The authors hypothesized that Rituximab-ESLP may lead to a depletion of allograft B-cells, potentially diminishing the recipient adaptive immune response, improve clinical outcomes such as the latent development of EBV, and decrease the incidence of lymphoproliferative malignancies. Perfusion duration varied from 5-12 hours, depending on the quality of the donated lungs that were rejected for clinical transplantation. Results demonstrated that Rituximab was successfully delivered from the ESLP perfusate into the lung tissue and lymph nodes by way of flow cytometric binding assays with evidence of occupied CD20 epitopes. There was significantly less CD20+ binding in lung tissue and lymph nodes of the treated allografts compared to controls suggesting B-cell depletion. There was no significant difference in markers of acute cell injury, pro-inflammatory cytokines, number of TUNEL positive cells (cell death), or lung function, suggesting there are no significant safety concerns. A post-ESLP tissue culture preparation with the addition of human serum complement demonstrated evidence of B-cell depletion as would be expected after Rituximab treatment[136].

Based on this pre-clinical study, high-dose ESLP delivery of RTX appears safe and efficacious at reducing B-cells in the allograft, which may ultimately reduce recipient immunoreactivity.

Perfluorocarbon-based Oxygen Carrier

Due to the scarcity of blood products, there is a desire to develop oxygen carrying blood substitutes that can be used to supplement perfusates and improve *ex-situ* organ preservation. Perfluorocarbons are non-reactive chemicals that dissolve oxygen readily, which is extracted without issue by tissues in exchange of carbon dioxide[137,138]. In 2020, Inci *et al.* investigated the potential role of perfusate instilled perfluorocarbon-based oxygen carrier (PFCOC) as a supplement to STEEN Solution™ in a model of porcine ESLP with transplantation (CIT 24hrs, ESLP 6hrs, Tx 4hrs). During ESLP, PFCOC treated lungs demonstrated comparable physiologic and biochemical markers compared to untreated controls, except perfusate lactate and potassium levels were lower while ATP was significantly higher in the PFCOC group. Transmission electron microscopy (TEM) revealed significantly more lung infiltrates in the control group. Following left lung transplantation, control lungs had significantly greater glucose consumption and higher lactate levels along with greater perfusate flavin mononucleotide (increased mitochondrial dysfunction). Gas-exchange was significantly better in lungs treated with PFCOC. Furthermore, pro-inflammatory cytokines were significantly reduced (IL-8, IL-12), and lung tissue ATP was significantly higher with lower myeloperoxidase (decreased neutrophil activity) compared to controls. Lastly, TEM revealed better tissue preservation and viability[139]. The authors concluded that PFCOC treatment is safe for acellular ESLP and produced superior transplant outcomes.

Total Parenteral Nutrition and Amino Acid Supplementation

ESLP reactivates cellular metabolism resulting in energy substrate consumption, and it has been shown that nutritional supplementation of perfusate with total parental nutrition (TPN) and selective amino acids improves the quality of preservation with superior lung function. In 2019, Buchko *et al.* compared continuous supplementation of TPN and multivitamins to standard ESLP (CIT 0min, ESLP 24hrs) metabolic support (glucose and insulin). TPN treated lungs demonstrated significantly improved oxygenation, and stability of sodium, BCAA, and FFA concentrations compared to controls, which had an increase in BCAAs and a depletion of FFAs suggesting a shift towards proteolysis. Pro-inflammatory cytokine concentrations (TNF-alpha) were significantly lower in the TPN group[15].

In 2021, Takahasi *et al.* investigated potential ESLP protocol refinements to achieve stable 24-hour continuous preservation (CIT 4hrs, ESLP 24hrs), which included continuous infusion of TPN. TPN supplemented lungs achieved significantly longer stable perfusion time, with improved lung function (oxygenation, peak airway pressure, PVR, dynamic compliance, edema formation), reduced pro-inflammatory cytokines (IL-1β, IL-6, IL-8), and fewer TUNEL positive cells (decreased apoptosis) compared to controls[17].

In 2022, Huang *et al.* sought to determine the role of specific amino acids in ESLP (18hrs) nutritional supplementation by continuously administering a compound of L-glutamine and L-alanine (L-alanyl-L-glutamine 4mM q2hr), which are readily consumed for energy production. Amino acid supplemented lungs demonstrated improved oxygenation, dynamic compliance, static compliance, and improved perfusate electrolyte stability. Indeed, two ESLP experiments with L-alanyl-L-glutamine achieved 36-hours of continuous preservation with good

function, further emphasizing the potential benefit of metabolic support and nutritional supplementation to achieve ultra-prolonged ESLP[140].

Intravascular Cell Therapy

As mentioned earlier, stem cells may play a role in immune modulation, which can be applied to ESLP reconditioning. Multiple clinical disorders including myocardial infarction, diabetes, sepsis, and hepatic and acute renal failure have shown therapeutic benefit from the administration of bone marrow-derived multi-potent mesenchymal stem cells (MSCs). In 2009, Lee *et al.* investigated the role of human MSCs as a means of restoring alveolar fluid clearance due to intrabronchial *E.coli* endotoxin (0.1mg/kg) acute lung injury and pulmonary edema while on ESLP[141]. Donor lungs were divided into left and right lungs for separate ESLP devices, and after a stabilization period the lung injury was induced. One hour later, human MSCs were administered followed by three hours of evaluation. Human MSC treated lungs demonstrated improved alveolar fluid clearance, endothelial barrier permeability, and reduced extravascular lung water compared to ESLP alone. The authors concluded that treatment of endotoxin injured lungs with MSC-ESLP is capable of restoring normal fluid balance and endothelial function[141].

In 2016, Mordant *et al.* compared alternate routes (ventilator vs perfusate) for the administration of MSCs during ESLP (CIT 18hrs, ESLP 12hrs) in a porcine lung injury model to assess the effect on concentration levels of growth factors and inflammation. Umbilical cord derived MSCs ($50x10^6$) were administered to lungs via the pulmonary artery or endobronchially. Intravascular deliver was associated with significantly improved retention of MSCs within the pulmonary tissue. There was no significant difference between PVR, oxygenation, or compliance between groups. The administration of MSCs intravascularly was associated with a significant

decrease in pro-inflammatory cytokine IL-8 compared to ESLP alone. The authors concluded that intravascular administration of MSCs appears to be safe and is more effective compared to intrabronchial delivery[142].

In 2019, Nakajima *et al.* explored the application of MSCs ($5x10^6$/kg) administered during porcine ESLP as a means to reduce the deleterious effects of IRI following transplantation (first CIT 24hrs, ESLP 12hrs, second CIT 1hr, Tx 4hrs). MSC treated lungs demonstrated significantly greater levels of hepatocyte growth factor (HGF), and decreased markers of cell death. MSC treated lung tissue showed lower pro-inflammatory IL-18 and INF-gamma concentrations with greater amounts of anti-inflammatory IL-4. Following left-lung transplantation, the MSC group had significantly higher HGF and lower TNF-alpha in lung tissue, less edema formation, and lower lung injury scores compared to ESLP controls without MSC treatment. However, there was no significant difference in overall lung function during ESLP or post-transplant between groups[143]. The authors conclude that MSC mitigated IRI during ESLP resulting in improved markers of IRI post-transplant.

Also in 2020, Nykanen *et al.* investigated the possible application of MSCs genetically engineered to secrete human anti-inflammatory cytokine IL-10 (MSC-hIL-10 cells, $40\text{-}150x10^6$) administered during porcine ESLP (CIT 4 or 24hrs, ESLP 6 or 12hrs, Tx 4hrs) followed by transplantation with either 4hours (n=4) or three-days (n=1) of observation. Human IL-10 was detectable within minutes of MSC-hIL-10 cell administration on ESLP and reached supratherapeutic levels throughout preservation. Following transplantation, hIL-10 was detectable in recipient plasma within 1 hr of reperfusion and remained high for 4-hours before gradually decreasing over three days to low levels[144]. This proof-of-concept study successfully

demonstrated the feasibility of incorporating genetically engineered MSCs as a therapeutic tool for ESLP. Further research into the physiologic effect and long-term fate of MSCs is required.

In 2021, Nykanen built on their previous work by using engineered MSCs (MSC-hIL-10) with ESLP (12 hrs) on human lungs rejected for clinical use due to varying amounts of lung injury. Engineered MSCs were cryopreserved, providing an "off-the-shelf" application with excellent viability. Lung blocks were divided into left and right lungs for separate ESLP devices to produce an experimental MSC arm ($40x10^6$ MSC) and control arm. Lungs treated with MSCs demonstrated rapid and significant increase in IL-10 concentrations in perfusate and lung tissue. Unfortunately, MSC-hIL-10 treatment did not result in a significant changes in lung function between groups, including oxygenation, compliance, PVR, PAP, airway pressure, and wet-to-dry ratio. Furthermore, there was no difference in pro-inflammatory cytokine levels between groups. The authors show that the acidic environment of the injured human lungs contributed to reduced MSC viability *in-vivo*, with acidity and viability being significantly negatively correlated. The authors believe this explains why the IL-10 levels were low at the end of ESLP, signifying reduced MSC viability in the hostile environment. Cryopreserved MSC-hIL-10 can be safely thawed and applied to ESLP to rapidly increase IL-10 levels, but the poor metabolic conditions of injured lungs limit its efficacy[145].

11. CIRCUIT UPGRADES

Light Therapy

An ingenious upgrade to ESLP is the use of light-based therapies (LbT) for the purpose of viral eradication. In 2019, Galasso *et al.* developed a method of LbT incorporated into a ESLP circuit as a means to reduce hepatitis C virus (HCV) viral load and prevent transmission from

HCV positive donor lungs. Nucleic acid test positive (NAT+) human lungs donated to research were split into single lung ESLP runs (CIT unknown, ESLP 9 hrs) with one lung serving as the treatment group and the other as a control with equivalent viral loads. Two forms of LbT were explored and compared to groups of standard ESLP perfusate management and complete circuit exchange. LbT included one ESLP group of germicidal ultraviolet-C (UVC) irradiation and a second ESLP group was treated with photodynamic therapy (PDT) using methylene blue activated by red light irradiation. Circuit exchange and PDT were able to significantly reduce the percentage of viral load compared to controls. UVC did not significantly reduce the percentage of viral load in tissue or perfusate, but on further testing it was established that UVC and PDT both rendered the viral RNA non-functional, effectively reducing HCV infectivity. A follow-up safety experiment was performed with pig lungs treated with LbT on ESLP (CIT 2hr, ESLP 6hrs) with *in-vivo* transplantation assessment (Tx 4hrs). There were no deleterious effects on post-transplant physiology observed in LbT recipients compared to controls[146].

In 2020, Cypel *et al.* built on their previous work with an open-label, single-centre, pilot trial to assess the safety and efficacy of HCV+ donor lungs transplanted into HCV- recipients following UVC-ESLP perfusate inoculation, which resulted in encouraging outcomes. LbT-ESLP was administered along with post-transplantation direct-acting antivirals (DAAs). Eleven HCV+ donor lungs were treated with standard ESLP (4-6hrs), and eleven other HCV+ donors were treated with UVC-ESLP (4-6hrs). Both groups were transplanted into HCV- recipients and compared against 187 HVC- donor transplants acting as controls. UVC-ESLP demonstrated significantly reduced viral load at one-week post-transplant compared to ESLP alone and prevented viral transmission in 2 out of 11 recipients. Survival at 6-months was similar between HCV+ and HCV- donor lung recipients, and 86% of recipients with HCV+ donor lungs were

HVC- at 6-months post-transplant. There were no significant differences in intermediate clinical outcomes between groups. Although, LbT did not prevent the development of HCV in all recipients, there were no adverse effects, and all infected patients were HCV negative after six weeks of DAAs[147]. This is a tremendous proof-of concept paper, which may prove effective in the treatment and prevention of other relevant viruses such as HIV and HBV, further increasing the useable donor pool.

Perfusate Filtration

Leukocyte Filter

Standard ESLP platforms and protocols routinely incorporate a leukocyte filter (LF) into their perfusion circuits; however, there is conflicting evidence whether a leukocyte filter provides added benefit to lung function during prolonged preservation. In 2014, Stone *et al.* performed a porcine ESLP transplant experiment (CIT 2hr, ESLP 3hr, Tx 24hr) to investigate the role of mechanical removal of leukocytes on passenger leukocyte migration. The control group was a left lung transplantation after CSP (2hr) without ESLP. Results demonstrate that ESLP with a LF reduced the transfer of passenger leukocytes into the recipient and donor leukocyte migration to recipient lymph nodes was likewise reduced. Furthermore, recipient T cell infiltration into the donor lung was significantly reduced. ESLP with a LF to remove donor leukocytes resulted in reduced T cell infiltration, the key hallmark of acute rejection, via decreased direct allorecognition and T cell priming[49].

In 2017, Luc *et al.* investigated the effect on lung function of prolonged porcine ESLP (12hrs) with and without a leukocyte filter. In both groups, lung function was stable and acceptable; however, increased concentrations of pro-inflammatory cytokines (TNF-alpha, IL-6)

and leukocytes were present in the perfusate regardless of the presence or absence of a leukocyte filter. The leukocytes filters were examined and through analysis of the washed cells, it was confirmed that the filters do trap leukocytes, but appear to become saturated during prolonged ESLP. Given that there were no performance differences noticed in the group that did not include a leukocyte filter, the Edmonton group concluded that routine incorporation of a LF during prolonged ESLP is not necessary and adds no benefit[148]. It is unknown if intermittent replacement of the LF during prolonged perfusion runs would be beneficial. LFs may not be beneficial over longer durations of ESLP due to saturation of the filter. Alternative means of filtering the perfusate during ESLP should be investigated, particularly in the pursuit of preservation beyond 12-hours.

Continuous Dialysis

Perfusate purification is of growing interest in ESLP research, which has led some groups to investigate the role of dialysis as an adjunct to ESLP perfusion circuits, but studies have reported conflicting or subclinical improvements so far. In 2019, Buchko *et al.* investigated the role of continuous hemodialysis (CHD) in a porcine NPV-ESLP system over 24-hours using a cellular perfusate to assess for the potential of improved ionic homeostasis and functional outcomes. Findings demonstrated that there was no significant difference in the physiologic performance of lungs during ESLP between CHD lungs and controls; however, perfusate sodium and lactate concentrations were significantly lower in the CHD-ESLP group. Although CHD did not improve the function of the lungs, it did improve the perfusate composition without adverse effects over 24-hour NPV-ESLP[149].

In 2019, Wei *et al.* investigated whether a dialyzer could be used to achieve 12-hours of continuous ESLP without the need for periodic fluid replacement, which is required during acellular ESLP. Human lungs that were rejected for clinical transplantation were donated for this research. ESLP-dialysis lungs achieved significantly more stable pH, electrolytes (sodium, potassium), and less circulating lactate compared to standard ESLP controls. Functional parameters were similar between groups for the first 8-hours; however, none of the control lungs (n=4) completed 12-hours of continuous ESLP, while all of the lungs in the ESLP-dialysis group completed 12-hours of preservation. Inflammatory markers were elevated in both groups; however, ESLP-dialysis lungs had significantly higher IL-6 and IL-10 within the first 6 hours of ESLP. There was no significant difference in histologic lung injury scores between groups. A significantly greater number of TUNEL positive cells were found in the perfusate of dialysis ESLP lungs[150]. These findings suggest that ESLP-dialysis may provide more stable preservation to injured lungs and may also cause a degree of subclinical injury.

In 2022, De Wolf *et al.* investigated the role of various perfusate management strategies including no perfusate replacement, partial perfusate replacement (gold standard), adult dialysis filter, and pediatric dialysis filter during porcine ESLP (CIT 1hr, ESLP 4 hrs). The findings were similar to those of Buchko *et al*. (2019)[149]. Adult and pediatric dialysis stabilized the electrolyte and metabolic composition of the acellular perfusate as well as pH status and gas analysis; however, there was no significant difference in lung function. Furthermore, there was no difference between groups in the gene expression profiles related to cell survival, cell proliferation, inflammatory response, cell movement, and inhibition of bleeding. Pediatric dialysis increased IL-10 and IL-6 inflammatory cytokine perfusate concentrations. Overall, strategies of ESLP perfusate management with either periodic replacement or dialysis had no

effect on lung function nor gene expression profiles, but dialysis did stabilize perfusate composition[151]. Thus far, ESLP-dialysis has yet to provide significant improvements in lung function, suggesting that the build-up of dialyzable cell products does not significantly alter the quality of lung preservation on ESLP.

Continuous Hemofiltration

Hemofiltration (HF) is a variation of dialysis that removes solutes via convection as opposed to diffusion and has been applied as an adjunct to ESLP in a large animal model of pulmonary edema (Edema 2hr, CIT 2hr, ESLP 3hr, cellular perfusate) with limited functional benefit to the lungs. In 2016, Nilsson *et al*. demonstrated that HF-ESLP increased the perfusate oncotic pressure by 43% and decreased lung weight gain by 15%. Compliance was significantly better in the HF-ESLP group compared to ESLP alone, although remaining functional parameters were similar between groups[152]. These results may be limited, but they are encouraging. Future research applying HF-ESLP over longer durations of preservation is needed.

Cytokine Filtration and Adsorption

ESLP is associated with a release of pro-inflammatory cytokines and chemokines during IRI, which activates the innate immune system and leads to endothelial dysfunction, capillary leak, and lung injury[25]; therefore, ESLP research into cytokine filtration and adsorption has gained interest, but results are conflicting. In 2010, Kakishita *et al*. explored the application of an adsorbent membrane (Lixelle S-35) as an adjunct to porcine ESLP (12hr) in an attempt to reduce pro-inflammatory cytokines and improve lung function. The findings show that pro-inflammatory cytokines and enzymes (TNF-alpha, IL-8, myeloperoxidase) were significantly

lower in the adsorbent membrane group compared to controls, but lung function and weight-gain were similar[153]. The authors conclude that factors other than pro-inflammatory cytokines may contribute to progressive edema formation and lung injury during prolonged ESLP.

In 2018, Iskender *et al*. explored the safety and efficacy of a cytokine filter (CytoSorb) during porcine ESLP (CIT 24hr, ESLP 12hr) and found functional benefits. The cytokine filter group demonstrated improved airway pressure, dynamic compliance, and post-ESLP xrays with reduced consolidation. A cytokine filter also produced superior electrolyte balance, reduced glucose consumption, lactate production, and lower lactate/pyruvate ratio suggesting less anaerobic metabolism. Furthermore, the adsorbent filter was associated with reduced cytokine expression profile, inflammatory enzyme activity (myeloperoxidase), and less microscopic lung injury. Overall, this study suggests that perfusate filtration through sorbent beads is safe and effective with improved lung physiology and perfusate balance. Additional experiments are required, and certain cytokine filters may be more effective for ESLP purposes[154].

12. CONCLUSIONS: PART 2

Despite the great progress that has been made in lung transplantation and ESLP, there remains significant room for improvement. ESLP could be the technological breakthrough needed to further improve donor supply as well as long-term outcomes through organ modification and immunomodulation to produce ESLP-enhanced lungs. That said, ESLP is still in its infancy as a field of research and treatment modality. This is reflected in the paucity of research supporting fundamental aspects of ESLP management across platforms, including specific studies investigating ideal perfusion temperature, flow rate, method of evaluation, and many others. Efforts to introduce ESLP delivered therapeutics have been covered here, but their

efficacy may be limited by sub-optimized perfusion and ventilation management. Ventilation and perfusion parameters of each platform vary slightly, but the end goals are the same: protection from injury, continuous assessment, reconditioning of function, and extended preservation. There exist many potential areas for methodological improvement in this regard. Identifying the knowledge gaps in ESLP and improving upon the methodology of NPV-EVLP through experimentation is the focus of this doctoral thesis.

Table Legend:

ESLP Platform	TORONTO	LUND	OCS	EVOSS
Portability	Fixed	Fixed	Portable	Portable
Pump	Centrifugal	Roller	Pulsatile	Centrifugal
Initiation Temperature (°C)	32	32	34	32
Preservation Temperature (°C)	37	37	37	37 human/38 pig
Perfusion Parameters	Steen Solution™	Steen Solution™ with pRBC (HCT 14%)	OCS™ Lung Solution with pRBC (15-25%)	CHIP with pRBC (HCT 12-15%)
Flow (CO %)	40	100	2-2.5 L/min	30 pres./ 50 eval
LAP (mm Hg)	3-5 (closed LA)	0 (open LA)	0 (open LA)	0 (open LA)
PAP (mm Hg)	10-15	≤20	≤20	≤20
PAWP (mm Hg)	25	-	-	≤25
Ventilation				
RR (breaths/min)	7	15-20	10	6-10
TV (ml/kg)	6-8	5-7	6	6-10
PEEP (cm H_2O)	5	5	5-7	5-8
FiO_2 (%)	21pres./ 100 eval	50	21	21
pCO_2 (mm Hg)	35-40	35-40	-	35-50

Table 2.1. Comparison between clinical ESLP protocols. ESLP – Ex-Situ Lung Perfusion; EVOSS – Ex-Vivo Organ Support System; OCS – Organ Care System (Transmedics); pRBCs – packed red blood cells; CO – cardiac output; HCT – hematocrit; LAP – left atrial pressure; PAP – pulmonary artery pressure; PAWP – peak airway pressure; RR – respiratory rate; TV – tidal volume; PEEP – positive end-expiratory pressure; FiO_2 – inspired fraction of oxygen; pCO_2 – partial pressure of carbon dioxide.

Chapter 3

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

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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. Particular focus is made to anesthetic and surgical techniques, as well as trouble shooting.

ABSTRACT:

Lung transplantation (LTx) remains the standard of care for end-stage lung disease. A shortage of suitable donor organs, as well as 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 greater 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 has been demonstrated in a recent clinical trial involving extender criteria donor (ECD) human lungs. Herein, we describe the use of this custom device in a juvenile porcine model of normothermic NPV-ESLP over a 12-hour duration, paying particular attention to management techniques. Pre-surgical preparation, including ESLP software initialization, priming and de-airing of the ESLP circuit, as well as 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 initialization, maintenance, and termination of perfusion and ventilation on the custom device. 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 organs utilization [2] and a 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 exacerbate 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 lungs prior to 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 as much as 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 six hours, evaluation of organ function is possible prior to implantation, and due to continuous organ perfusion, modifications can be made to the perfusate that optimize 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 greater 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 major factor underlying this advantage [15, 16]. In addition to its pre-clinical benefits, the clinical safety and feasibility of NPV-ESLP has 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 hours of duration. The surgical retrieval is covered in detail, as well as the initiation, management, and termination of our custom software platform. Our strategy for tissue collection and the management of our samples is also explained.

PROTOCOL:

The procedures performed in this manuscript are in compliance 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 can be seen in Figure 3.1.

1.0. Pre-surgical Preparations

The following steps are associated with Figure 3.2 and 3.3.

1.1. Position the organ chamber on the ESLP cart and mount the silicon support membrane 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 probe into the deoxygenator.

1.5. Secure the pulmonary artery (PA) flow probe onto the PA tubing.

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

1.7. Attached all tubing connections firmly to prevent leaks, and all the stopcocks and luer locks must be closed prior to adding the perfusate.

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].

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

2.0. ESLP Software Initialization, Adjustments, and De-airing Circuit

NOTE: The ESLP apparatus used here is equipped with a custom software program (Figure 3.4,3.5,3.6). 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 3.1 lists all monitoring parameters provided by the software.

2.1. Click on the program shortcut on the monitor to start the ESLP program. Select “scan”, “cart 3”, then “NPV program” followed by “connect”.

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 in the organ chamber.

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.

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 on to check function. Turn off once function confirmed.

2.6. On the “Main” page, turn EIP to -5 cmH_20 and turn on to check function. Turn off once function confirmed.

2.7. On the “Settings” page, turn on the heater and confirm function. Change temperature set point on the monitors and confirm a congruent change on the heater monitor on the cart. Turn off once function confirmed.

3.0. Preparations and Anesthesia

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

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

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

3.4. Administer isoflurane at 4–5% and reduce to 3% after one or two minutes.

3.5. Evaluate the depth of anesthesia: ensure the pig has no withdrawal reflex in response to noxious stimulus. Repeat every 5 minutes.

3.6. Intubate the pig once the correct depth of anesthesia is confirmed.

3.7. Place a pulse oximeter probe on the tongue (preferred) or ear and target an oxygen saturation above 90%.

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

3.9. The ventilator settings are set at: respiratory rate of 12-30 breaths/minute, TV 6-10 ml/kg, PEEP 5 cmH2O, Peak Pressure 20 cmH2O.

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

4. Lung biopsy, Exsanguination, and Blood Collection

4.1.0. Insert central line for fluid and heparin administration.

4.1.1. Use electrocautery to make a 5-8 cm midline incision centered over the trachea and extending cranial from the sternal notch.

4.1.2. Divide the skin and subcutaneous fat using cautery.

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

4.1.4. Obtain proximal and distal control of the jugular vein using 2-0 silk ties as vessel loops.

4.1.5. Tie the cranial encircling tie and retract upwards on the proximal tie to control the flow of blood.

4.1.6. Make a small incision in the vein using Metzenbaum scissors to accommodate a 7Fr central line (approximately 1/3 the circumference of the vessel).

4.1.7. Simultaneously release tension on the proximal vessel loop and cannulate the vein, then tie down to secure the cannula in the vein at a depth of 10cm.

4.1.8. Flush the line with heparin and connect to an IV line of 0.9% Normal Saline. Administer fluid if the pig is intravascularly depleted from dehydration. Hep-lock any unused ports.

4.2.0. Perform a median sternotomy.

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

4.2.2. Use electrocautery to make a midline incision that 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 bluntly dissect the pericardium off the posterior table of the sternum to create a space for the sternal saw.

4.2.5. Apply two towel clips on opposite sides of the sternum at the level of the 4^{th} ribs lateral to the costochondral junction. Purchase the overlying tissue and fascia layer within the towel clips, so the sternum can be lifted 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. 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. 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 five minutes after heparin administration.

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

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

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

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 scissor. Continue this incision cranially to the aortic root, then laterally to expose the 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 superior vena cava (SVC) and insert a Poole tipped suction into the lumen, advancing suction to the level of the cardiac inferior vena cava (IVC). An incision is made in the anterior wall of the left atrium (LA) to expediate the exsanguination. 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 to collect 1200 ml of whole blood that is spun down to produce 500 ml of packed red blood cells (pRBC). This will take approximately 5 minutes.

5.0 Cardiectomy

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

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

5.3. Transect the IVC 1 cm above 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. 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. This completes the cardiectomy.

6.0 Pneumonectomy

6.1. Perform the Pneumonectomy: check that tidal volumes are approximately 10 ml/kg. Switch to 2:1 inspiratory: expiratory ratio to achieve this target. If TV remain <6ml/kg, increase peak pressures and/or PEEP to achieve 8-10ml/kg target for maximal alveolar recruitment.

6.2. Open the pleura on the pig's left side and excise the pleura down to the phrenic nerve. Open and remove the diaphragmatic pleura. Perform the same steps on the right side.

6.3. Divide pleural attachments from diaphragm towards the left lower lung lobe. Use a Deaver retractor 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. 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 TV at approximately 10 ml/kg, the trachea is clamped 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. This completes the pneumonectomy.

7.0 Placement of the Lungs onto the ESLP Apparatus

See Figure 3.7 for a photographic depiction of the following steps.

7.1. Add 500 ml of pRBC to the perfusion circuit (which has been previously primed with 1L of CHIP) to reach a final volume of 1.5 L of perfusate. 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 lung using scissors for tissue analysis as previously described.

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. The adaptor should sit 2-3 cm above the PA bifurcation (if the PA has inadequate length, a segment of the 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). 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. 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.

8.0. Initiation of Perfusion and Ventilation

See Table 3.2 for initiation of the protocol. Table 3.3 details the two modes of NPV-ESLP employed.

8.1. On the "Settings" page, start the heater-cooler and set the temperature to 38 degrees Celsius. The pig's weight is entered as well to calculate cardiac output (flow).

8.2. On the "Main" page, set the CPAP to 20 cmH_20 and click start. 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. 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. The line is now appropriately zeroed, and the pump is now flowing 10% of calculated cardiac output.

8.4. Draw 10 ml of perfusate for centrifugal analysis and draw a “time zero” (T0) ABG as well.

8.5. Once the lungs have been perfused for 10 minutes, flows are increased to 20% of cardiac output.

8.6. When the perfusate temperature reaches 32 degrees Celsius, secure the chamber lid is in place with clamps to create an air-tight seal. Optimally position the lungs prior to 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, 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. Click “Start CPAP” at 20 cmH_2O and unclamp the ventilation tubing. Next, set EEP target to 0 cmH2O, EIP to -1 cm H_20, RR 10, I:E ratio 1:1, and click “Press to Start Vent” to activate negative pressure ventilation. Listen for the vent to change its function then attach the side port ventilation tubing to the chamber. The lungs will compress slightly in response and then begin to reinflate.

8.8. Over the next few breaths, decrease CPAP to 12 cmH_2O while simultaneously increasing the EIP to -9 cmH_2O. Maintain these ventilation parameters for the first hour, then decrease CPAP to 8-10 cmH_2O depending on the alveolar recruitment and increase EIP to -12 to -13 cmH_2O.

8.9. Set peak pressures to 20-21 cmH_2O; however, 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 degrees Celsius, increase the flow to 30% of cardiac output. These are the settings for organ preservation (Table 3.2).

8.11. At hours 3,5,7,9,11 perform an evaluation 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 3.2).

8.12. At every odd hour draw a 10 ml perfusate sample for future analysis. Draw a pre-deoxygenator ABG sample every hour.

8.13. Following 5 minutes of evaluation, draw ABGs from pre- and post-deoxygenator ports (Table 3.3). This completes the placement of lungs on ESLP and initiation of perfusion and ventilation.

9.0 Metabolic Support of the Lung

NOTE: CHIP, along with most other organ perfusion solutions, contain glucose as the primary energy substrate.

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.

9.2. Another infusion pump delivers a continuous infusion of 2 U/h of insulin.

10.0. Heparin, Anti-microbial and Anti-inflammatory Agents

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

10.2. Add 3.375 grams of piperacillin-tazobactam to the perfusate at the start of perfusion, prior to the addition of pRBC.

10.3. Add 500 mg of methylprednisolone to the perfusate at the start of perfusion, prior to the addition of pRBC.

11.0. Assessment of Lung Function

NOTE: The ESLP software automatically calculates and records ventilation and functional indices on a continuous basis.

11.1. There are two modes of ventilation and perfusion employed during a perfusion run, which typically is 12 hours long, although can be extended to 24 hours: Preservation and Evaluation (Table 3.2).

11.2. Preservation Mode: Cardiac output 30%, PEEP 8-12, EEP 0, EIP -10 to -12, Peak Pressure 20-22 cm H2O, RR 6-10, and I:E ratio 1:1-1.5.

11.3. Set peak pressure to match pneumonectomy peak pressure and achieve target TV of 10 ml/kg. Although TVs of 10ml/kg are targeted, generally 6-8ml/kg is attained.

11.4. Every 30 minutes during preservation, preform a recruitment for 30 minutes or less. The duration and extent of recruitment is dependent the TVs reached. If TVs are 8-10 ml/kg, further recruitment is not necessary.

11.5. For recruitment, increase PEEP to 10-12 cmH_2O, decrease RR to 6 breaths/minute, increase Peak Pressures by 2-4 cmH_20 without exceeding 30 cm H_2O (rarely do we exceed 25 cm H_2O),

and change I:E ratio to 1:0.5. Generally, only one or two of these changes are made for each 30-minute interval, with increases in PEEP and Peak Pressure being the most effective.

11.6. At hours 3,5,7,9,11, perform an evaluation of organ function. The main parameter of interest is the PF ratio; however, dynamic compliance and PA pressures are also closely monitored (Figure 3.8).

11.7. During 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/minute via the deoxygenator. This replicates systemic oxygen depletion and occurs over 5 minutes. During this time, decrease PEEP to five cmH_2O, while maintaining peak pressures; therefore, adjust 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.

11.8. Functional Calculations:

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: TV x RR

Dynamic Compliance is calculated by: TV/EIP

P/F ratio is calculated by: PaO2/Fi02, where FiO2 is 21%.

12.0 Metabolic Assessment of the Ex-Situ Perfused Lungs

12.1. The metabolic state of the perfusate acts as a surrogate maker of the state of the lungs and is assessed every hour via ABGs samples and 10 mL perfusate samples taken from the pre-deoxygenator port.

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

12.3 Use PaO_2 as a marker of overall lung function. This is particularly true during phases of evaluation when mixed sweep gas is added to the circuit to simulate systemic deoxygenation and pre vs post deoxygenator gases are compared to assess oxygen step-up by the lungs.

12.4 Target a normal pH (7.35-7.45). Correct acidosis with boluses of THAM buffer. Alkalosis is generally not corrected and does not exceed 7.55. CO_2 sweep can be added to the circuit to correct this to a normal range or if alkalosis exceeds this threshold.

12.5. Treat $PaCO_2$ permissively and is generally in the range of 10- 20 mmHg. These values are interpreted as a sign of satisfactory ventilation.

12.6. 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 3.3 contains sample representative results of ABGs perfusate analysis during a 12-hour run of NPV-ESLP at normothermia and 30% cardiac output using a cellular perfusate (blood + CHIP).

13.0 Terminating Perfusion, Ventilation, and Disconnection the Lungs from ESLP Device

13.1. On the “Setting” page, click “Shutdown Server”

13.2. Remove the lid from the chamber.

13.3. Disconnect the PA adapter from the PA cannula.

13.4. Extubate the trachea.

13.5. Weigh the lungs to determine the amount of edema formation.

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

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.

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

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

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

REPRESENTATIVE RESULTS:

At the beginning of lung perfusion and ventilation (preservation mode), the lungs will normally 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 grams. The achieved tidal volumes (TV) are 6-8 mL/kg and during the first hour of ventilation and perfusion, the TVs will range from 0-6 ml/kg, with the majority in the 4-5 ml/kg range. TVs generally reach 6mL/kg between hours 3-6, and thereafter may continue to increase, but generally stabilize in the 6-8ml/kg range. Likewise, compliance will begin at 0-10 ml/mmHg within the first hour, and occasionally higher. Between hours 3-6, compliance is 10-20ml/mmHg and stabilizes along with the TVs, being interrelated parameters. As pulmonary artery flow is gradually increased from 10 to 30 % of cardiac output, the PAP will gradually rise. Within the first hour this is typically 10+/-2 mmHg and rises slightly over the course of the 12-hour run to a range of 12+/-2 mmHg. During evaluation with flows of 50% of cardiac output, PAP can be much higher at 15-20 mmHg. Pulmonary vascular resistance

(PVR)will rise gradually over the course of ESLP. Figure 3.8 displays trends in PAP, dynamic compliance, and PVR over 12 hours of perfusion and ventilation. All these parameters can be affected by the specific ESLP experimental protocol employed.

During evaluation mode of ESLP, which occurs at hours 3,5,7,9,11 during a 12-hour run, an upward trend in LA PaO_2 is observed (Table 3.4). Evaluation mode lasts for five minutes. It consists of dropping PEEP to 5 cmH2O 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 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 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 3.8 demonstrates a typical trend for the PF ratio at the evaluation time points of 5 and 11 hrs over the course of 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 3.4). Prior to evaluation modes, 10 ml perfusate samples are 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 example. For this reason, we generally see a continual increase in these markers over time with ESLP. The tissue biopsies are likewise helpful for biomarker labelling and visualization as well as histologic assessment of tissue integrity. Edema formation is another important index of inflammation associated with endothelial permeability. Figure 3.8 demonstrates a typical weight gain of 30% at the end of 12-hours 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. Assessment occurs over a 4-hour duration prior to euthanasia via exsanguination. Our lab's transplantation protocol for *in-vivo* assessment is currently under review by JoVE (See Chapter 4).

The PF ratio is the foremost functional assessment parameter of ESLP and human lung transplantation, and this NPV-ESLP technology has successfully been employed in a clinical trial with 100% 30-day and 1-yr 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.

DISCUSSION:

There are a number of critical surgical steps along with troubleshooting needed to ensure a successful ESLP run. Juvenile porcine lungs are extremely delicate compared to adult human lungs, so the procuring surgeon must be extremely careful when handling porcine lungs. It is critical to attempt a "no-touch" technique when dissecting out the lungs to avoid causing trauma and atelectasis. "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 induction tidal volumes to maximize the probability of a successful NPV-ESLP run. Any lost compliance during procurement is very difficult 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 induction tidal 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 TVs 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 free hand while simultaneously lifting upward from the clamped trachea to provide countertraction. 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 compliance initially during NPV-ESLP and do not develop any compliance improvement as measured by the software within the first hour, it is highly unlikely 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 be used to lengthen the PA via end-to-end anastomosis.

There are also several critical steps and trouble-shooting methods that are needed during the operation of the NPV-ESLP apparatus to achieve a successful perfusion. The process of procurement, mounting the lungs on the NPV-ESLP apparatus, and initiating perfusion/ventilation should not exceed 20–30 minutes. Longer periods of ischemia decrease the probability of a successful run. It is critical that the lungs are position on the silicone support membrane such that neither the PA cannula nor the ET tube interfere 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 if need be. Likewise, blood-soaked gauze can also serve to plug tears that cannot be surgically repaired. It is better to avoid an injury than attempt a repair of the lung parenchyma as the lung is very difficult to sew without causing further injury. When initiating ventilation, it is critical that the lungs remain inflated, so CPAP must begin at 20 cmH_20 prior to unclamping the trachea or ventilation tubing. If the lungs

deflate, they will struggle, and any lost alveolar recruitment prior to initiation of ventilation will be very difficult to regain during NPV-ESLP, resulting in a slower recovery. When initiating perfusion, it is critical that the pressure transducer is zeroed correctly, and that 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 perfusion of lung tissue. It is critical to maintain PEEP above 12 for the first hour of ventilation and not to 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 cmH_2O at the time of procurement to achieve TVs of 10 ml/kg, then anything less than 25 cmH_2O will be unlikely to 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 to only report the PaO_2 when calculating P:F ratios; however, the PA PaO_2 is informative because it describes the oxygen step-up that is occurring due to lung oxygenation. This is a better descriptor than P:F ratio alone. During preservation, 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 especially informative for 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. Invivo transplantation is surgically demanding compared to the organ procurement operation with many possible complications that can result in loss of the transplanted lung. As such both ESLP and subsequent transplantation are resource expensive 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[23]. 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 open through higher pressures directed down the airways. One of the other major 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 further 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-d and 1-year survival and

zero incidences of PGD grade 3 [17]. A multi-centre trial is the next step for this device as it moves towards commercial development. With regards to research applications, there is pre-clinical evidence the NPV-ESLP is superior to PPV-ESLP [15], and NPV-ESLP holds promise of becoming the superior 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 introduction of therapies that previously lacked a route of administration to donor 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 has been designed to require minimal manual adjustments.

FIGURE AND TABLE LEGENDS:

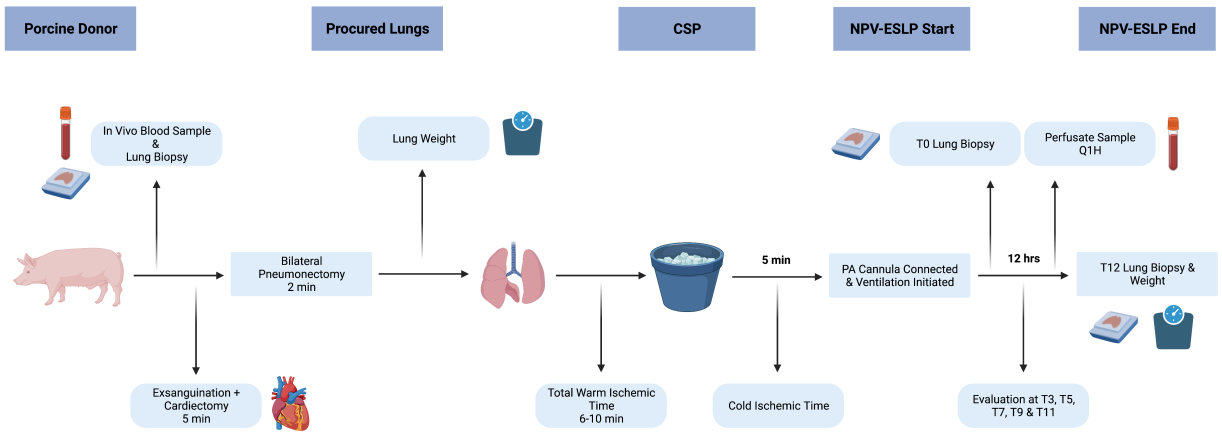

Figure 3.1: NPV-ESLP Protocol. Schematic representation of lung procurement and 12-hour NPV-ESLP run.

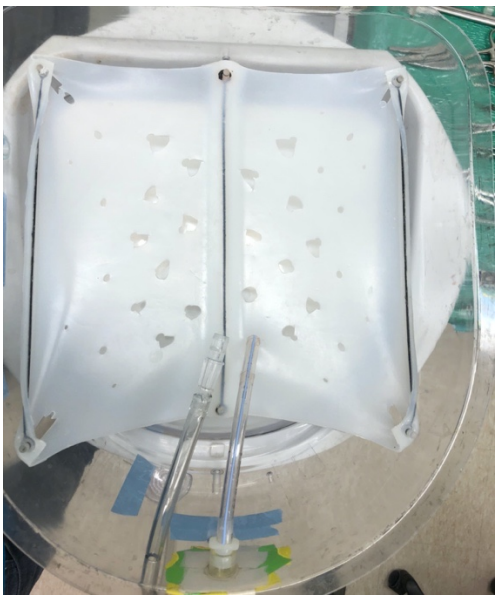

Figure 3.2: Silicone support membrane for the lungs suspended in hard-shell ESLP reservoir. Support membrane pictured with endotracheal tube (centre) and pulmonary artery cannula (left).

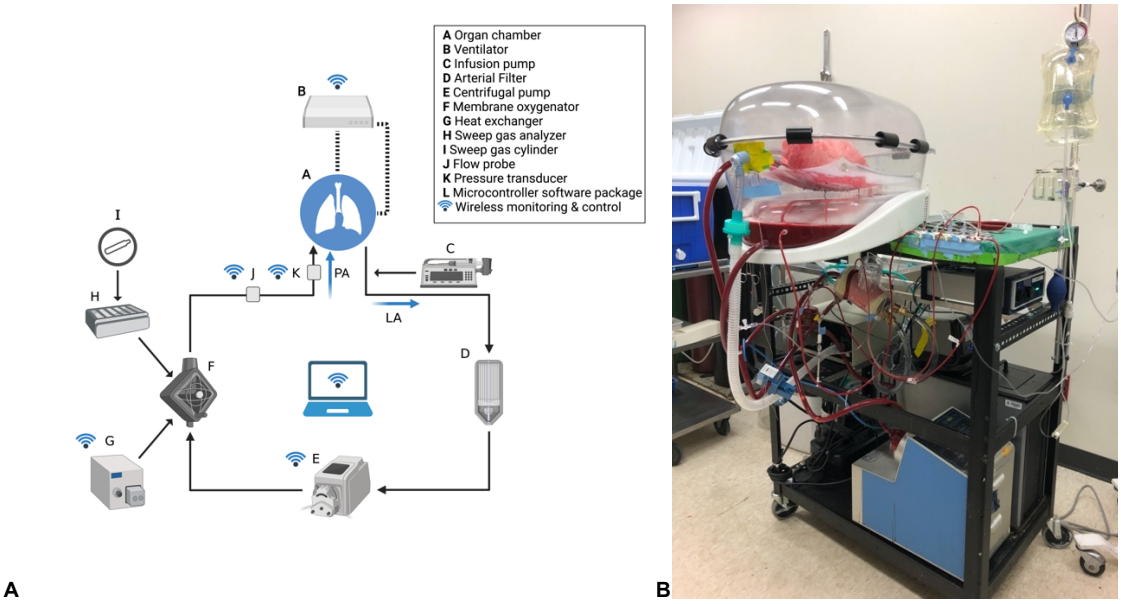

Figure 3.3: NPV-ESLP circuit. A) Schematic representation of the circuit with accompanying legend (left). B) Photo of NPV-ESLP circuit (right).

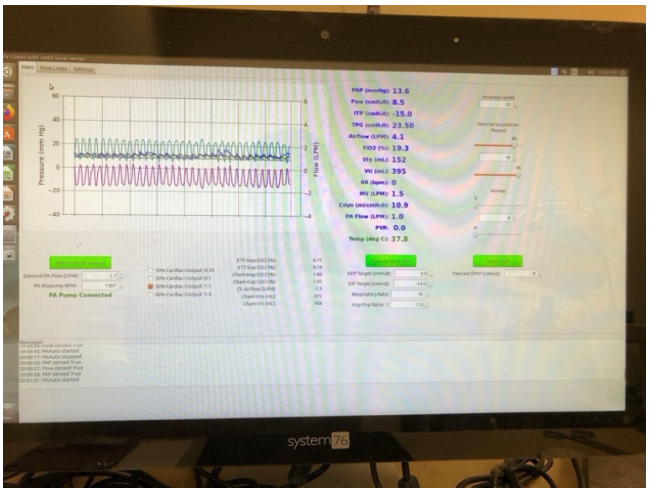

Figure 3.4: Screen Shot from "Main" Screen of NPV-ESLP software program.

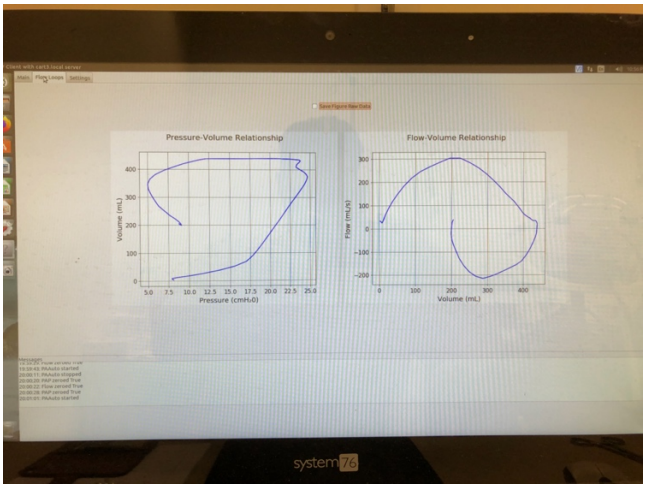

Figure 3.5: Screen Shot from "Flow-Loops" Screen of NPV-ESLP software program.

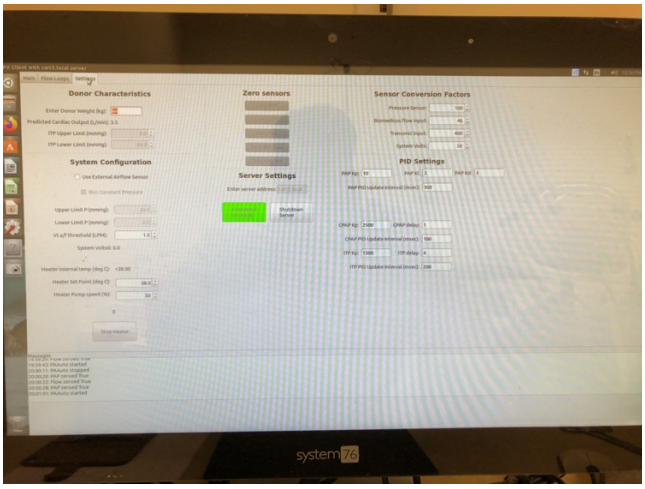

Figure 3.6: Screen Shot from "Settings" Screen of NPV-ESLP software program.

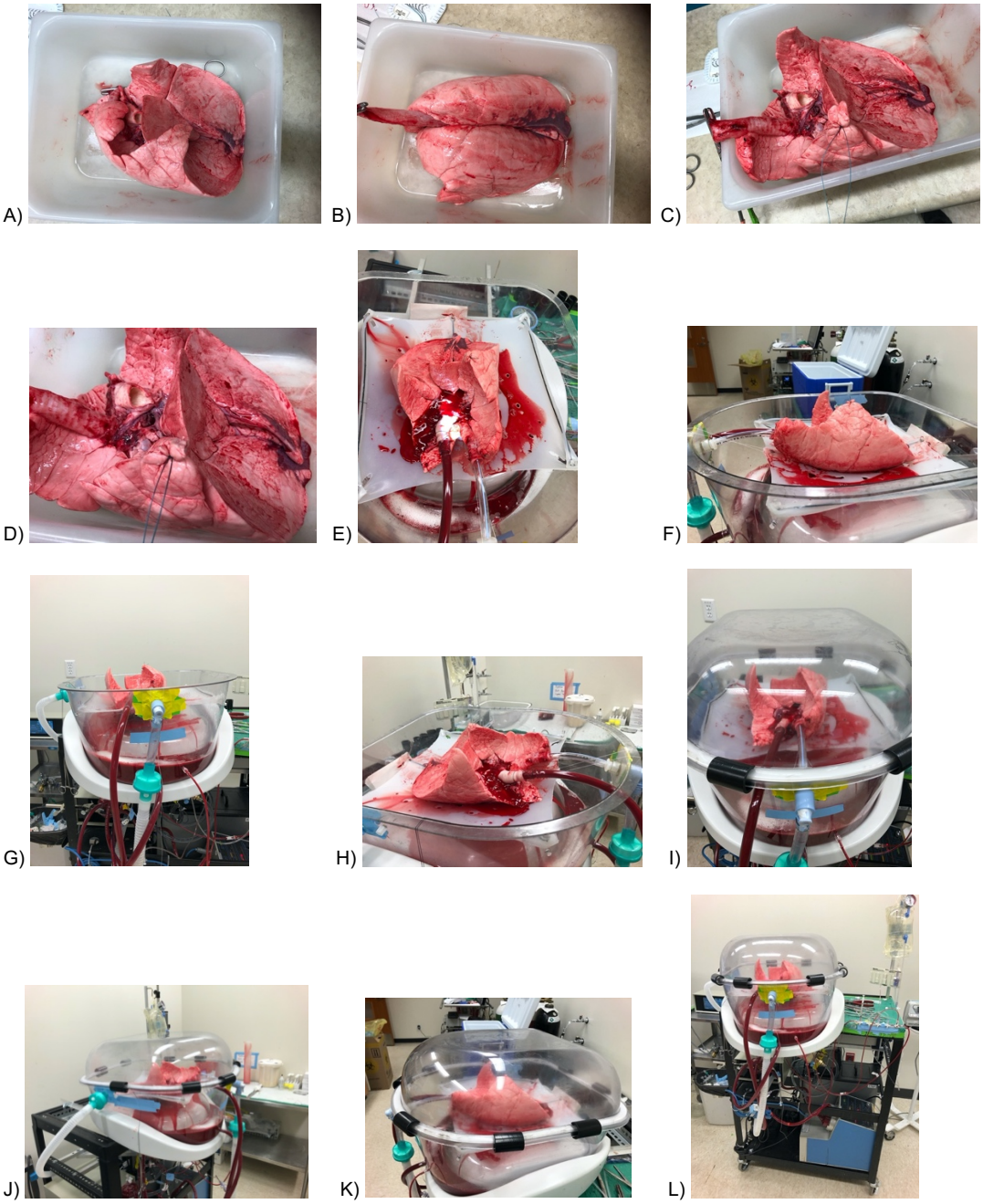

Figure 3.7: Lungs connected to NPV-ESLP circuit. A) Anterior Donor Lungs Pre-ESLP B) Posterior Donor Lungs Post-ESLP. C, D) Tissue biopsy of right middle lung lobe E) Lungs connected to ESLP circuit F) Demonstrated positioning of lungs on silicone support G) Front view of ESLP device demonstrating starting 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.

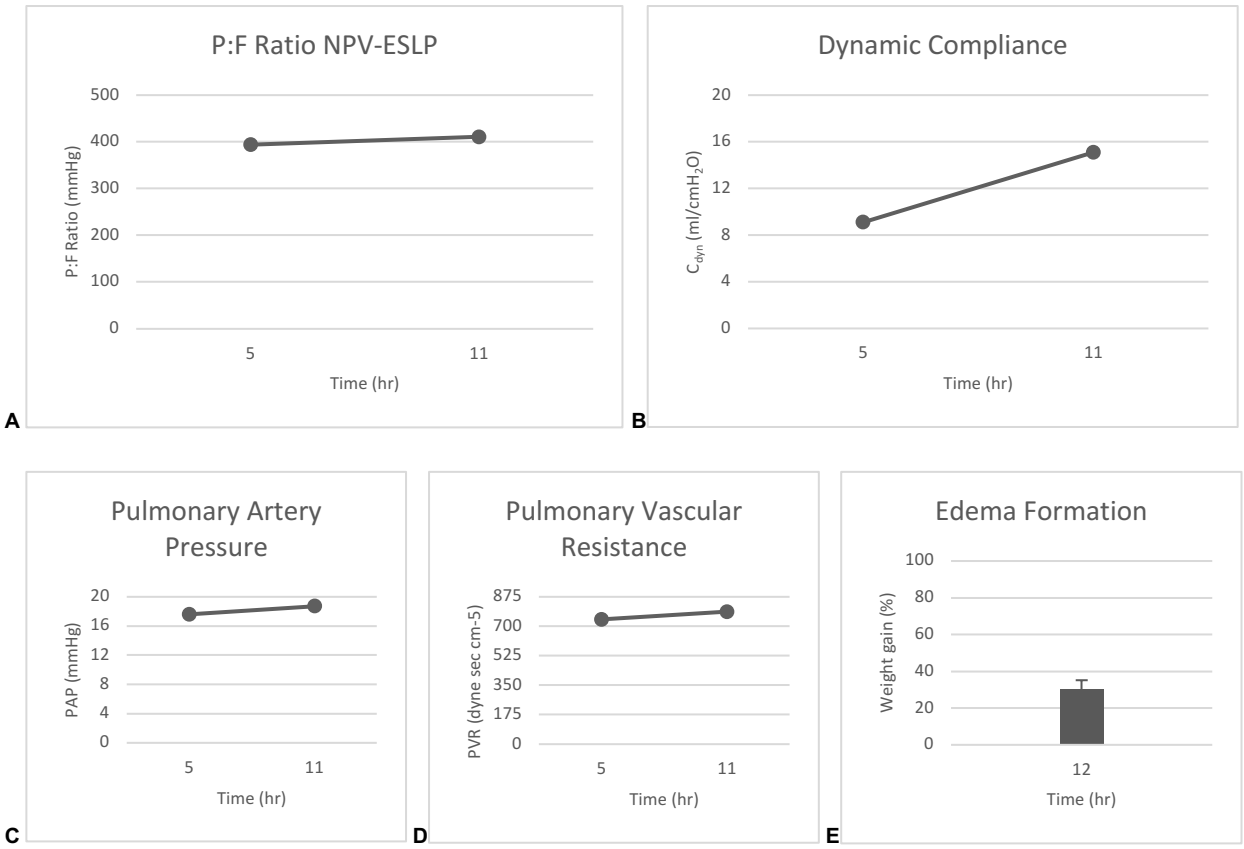

Figure 3.8: Functional Parameters during Evaluation modes over 12-hours of NPV-ESLP: A) P:F Ratio, PaO_2:FiO_2 ratio; B) Compliance; C) PAP, pulmonary artery pressure; D) PVR, pulmonary vascular resistance; E) Weight Gain.

Recorded Interface Parameters	Abbreviations	Units
1. Vascular Parameters:		
Left Atrial Pressure	LAP	mm Hg
Pulmonary Artery Pressure	PAP	mm Hg
Pulmonary Vascular Resistance	PVR	Dynes*s/cm^5
Pulmonary Arterial Flow	PA Flow	Litres/min (LPM)
2. Airway Parameters:		
Airway Pressure	P_{aw}	cm H_2O
Intrathoracic Pressure	ITP	cm H_2O
Transpulmonary Gradient	TPG	cm H_2O
Airflow	Airflow	Litres/min (LPM)
Fraction of Inspired Oxygen	FiO_2	Percentage (%)
Continuous Positive Airway Pressure	CPAP/PEEP	cm H_2O
3. Ventilation Parameters:		
Expiratory Tidal Volume	Vt_e	ml
Inspiratory Tidal Volume	Vt_i	ml
Respiratory Rate	RR	breathe/min (bpm)
Inspiratory: Expiratory Ratio	I:E	N/A
Minute Ventilation	MV	Litres/min (LPM)
Dynamic Compliance	C_{dyn}	ml/cm H_2O

Table 3.1: Recorded monitoring chart parameters.

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

	Perfusion time (min)				
	0	10	20	60 (T1)	180 (T3)
Perfusate Temperature (°C)	20°C	32°C	38°C	38°C	38°C
PA flow (% CO; CO = 70 ml/kg/min)	10%	20%	30%	30%	50%
Ventilation mode	PPV (CPAP = 20 cm H_2O)	Initiate NPV Preservation	NPV Preservation	NPV Preservation	NPV Evaluation
Medical gas mixer	None	None	None	None	89% N_2, 8% CO_2, 3% O_2
Left atrial pressure	0	0	0	0	0

Table 3.2: Initiation of 12-hour NPV-ESLP Protocol. CO, cardiac output; PA, pulmonary artery; PPV, positive pressure ventilation; NPV, negative pressure ventilation. For preservation mode ventilation parameters see Table 2. Beginning at T3, evaluation was conducted serially every 2 hours, for 5 minutes, with PA flow set to 50% CO, medical gas set to 89% N_2, 8% CO_2, 3% O_2, and preservation settings elevated to the ventilation parameters provided in Table 3.2.

NPV Ventilation Strategy

	Ventilation Mode	
	Preservation	Evaluation
Temperature (°C)	38°C	38°C
Pulmonary artery flow	30% of estimated CO; CO = 70 ml/kg/min	50% of estimated CO; CO = 70 ml/kg/min
Ventilation parameters		
Mode	Volume control	Volume control
Desired inspiratory tidal volume	6-10 ml/kg	6-10 ml/kg
Inspiratory: Expiratory Ratio	1:1 – 1.5	1:1 – 1.5
Frequency	6 to 10 breaths/min	6 to 10 breaths/min
P_{AWP}	< 25 cm H_2O	< 25 cm H_2O
PEEP	8-12 cm H_2O	5-8 cm H_2O
FiO_2	21%	21%
Pressure parameters		
PAP	< 15 mm Hg	< 20 mm Hg
LAP	0 mm Hg	0 mm Hg
Medical gas mixture	-	89% N_2, 8% CO_2, 3% O_2
Medical gas mixture (litres/min) titrated to PCO_2	-	35 to 50 mm Hg

Table 3.3: 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 pulmonary arterial circulation.

		Preservation			Evaluation			
Arterial Blood Gases (21% FiO2)	**Invivo Recipient**	**T0 Left Atrium**	**T5 Left Atrium**	**T11 Left Atrium**	**T5 Pulmonary Artery**	**T5 Left Atrium**	**T11 Pulmonary Artery**	**T11 Left Atrium**
Blood Gas Values								
pH		7.42	7.44	7.56	7.35	7.36	7.44	7.48
pCO_2 (mmHg)		12.9	7.2	9.1	13.4	12.0	14.9	12.7
pO_2 (mmHg)		133	102	124	56.5	82.7	53.8	86.4
Oximetry Values						**P/F ratio: 393.8**		**P/F ratio: 411.4**
Hb (g/dL)		5.9	6.5	5.6	6.1	6.0	5.6	5.4
sO_2 (%)		99.0	98.4	98.9	85.5	95.1	87.6	97.8
Electrolyte Values								
K^+ (mmol/L)		4.6	4.1	4.9	4.2	4.1	5.0	5.0
Na^+ (mmol/L)		148	166	164	164	165	164	164
Ca^{2+} (mmol/L)		2.39	2.04	1.81	2.15	2.13	1.88	1.86
Cl^- (mmol/L)		105	110	111	108	110	111	113
Osm (mmol/kg)		301.9	337.4	330.9	334.0	334.6	331.5	331.2
Metabolite values								
Glucose (mmol/L)		5.5	5.0	3.2	5.4	5.2	3.0	2.9
Lactate (mmol/L)		1.1	9.5	14.6	8.5	9.0	14.3	14.4
Acid Base status								
$Hco3^-$ (mmol/L)		8.3	4.8	8.2	7.2	6.7	9.8	9.3

Table 3.4: Blood gas analysis performed during 12 hours of ESLP. Ca^+, calcium ion; Cl^-, chloride ion; Hb, hemoglobin; HCO_3^-, bicarbonate ion; K^+, potassium ion; Na^+, sodium ion; Osm, osmolarity; $paCO_2$, arterial partial pressure of carbon dioxide; paO_2, arterial partial pressure of oxygen; sO_2, oxygen saturation; P/F ratio, paO_2/FiO_2 ratio.

Chapter 4

Left Lung Transplantation in a Juvenile Porcine Model for ESLP

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SUMMARY:

This protocol describes a juvenile porcine model of orthotopic left lung allotransplantation designed for use with ESLP research. Focus is made on anesthetic and surgical techniques, as well as critical steps and troubleshooting.

ABSTRACT:

Lung transplantation is the gold-standard treatment for end-stage lung disease, with over 4,600 lung transplantations performed worldwide annually. However, lung transplantation is limited by a shortage of available donor organs. As such, there is high waitlist mortality. *Ex-situ* lung perfusion (ESLP) has increased donor lung utilization rates in some centers by 15–20%. ESLP has been applied as a method to assess and recondition marginal donor lungs and has demonstrated acceptable short- and long-term outcomes following transplantation of extended criteria donor (ECD) lungs. Large animal (*in vivo*) transplantation models are required to validate ongoing *in vitro* research findings. Anatomic and physiologic differences between humans and pigs pose significant technical and anesthetic challenges. An easily reproducible transplant model would permit the *in vivo* validation of current ESLP strategies and the preclinical evaluation of various interventions to reduce lung ischemia-reperfusion injury, a major cause of primary graft dysfunction – the foremost cause of morbidity and mortality post lung transplantation. Herein, this protocol describes a porcine model of orthotopic left lung allotransplantation. This includes anesthetic and surgical techniques, a customized surgical checklist, troubleshooting, modifications, and the benefits and limitations of the approach.

INTRODUCTION:

Lung transplantation is the preeminent long-term treatment for end-stage lung disease. Over 4,600 lung transplantations are performed worldwide annually[1]. However, lung transplantation currently has significant limitations. For one, the necessity for organs continues to eclipse available donors. Despite rates of lung transplantation increasing every year since 2012 due to the combined effects of more candidates being listed for transplant, an increase in the number of donors, and improved use of recovered organs, the transplant waitlist mortality has not decreased significantly[2]. Organ quality concerns represent another major limitation, with reported organ utilization rates as low as 20-30%[3-5]. Finally, the trends in the post-operative outcomes of lung transplantation are less than satisfactory, with long-term graft and patient outcomes still lagging that of other solid organ transplantations[2].

An emerging technology, *ex-situ* lung perfusion (ESLP), has the potential to mitigate these limitations. ESLP has been increasingly applied as a method to assess and recondition marginal donor lungs and has demonstrated acceptable short- and long-term outcomes following transplantation of extended criteria donor (ECD) lungs[6-10]. Consequently, ESLP has increased utilization rates in some centers by 15–20%[6-11].

Proper ESLP research requires the *in vivo* validation of *in vitro* findings; however, there is limited literature on porcine lung transplantation models for ESLP[12-15]. Furthermore, available literature provides inadequate detail regarding anesthetic management of Yorkshire pigs for lung transplantation, which can be highly unstably hemodynamically[12-15]. Establishing an easily reproducible model would permit the *in vivo* validation of current ESLP strategies and the preclinical evaluation of various interventions to reduce lung ischemia-reperfusion injury. The objective of the present study is to describe a porcine model of orthotopic left lung

allotransplantation for use with ESLP. The protocol includes descriptions of the anesthetic and surgical techniques, a custom surgical checklist, and details regarding the troubleshooting experience and protocol modifications. The limitations and benefits of the left lung porcine transplantation model have also been discussed in this work. This manuscript does not outline the retrieval process of porcine lungs in 35-50 kg Yorkshire pigs, nor does it cover the establishment and termination of ESLP. This protocol exclusively addresses the recipient transplantation operation.

PROTOCOL:

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

1. Pre-surgical preparations/preparations and anesthesia

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

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

1.3. Titrate oxygen flow rate according to animal weight and the anesthetic system.

NOTE: Oxygen flow should be 20 - 40 mL/kg.

1.4. Administer isoflurane at 4–5% and reduce to 3% after 1-2 min.

1.5. Evaluate the depth of anesthesia, ensure the pig has no withdrawal reflex in response to a noxious stimulus. Repeat every 5 min.

1.6. Intubate the pig once the correct depth of anesthesia is confirmed. Use a custom 10-inch, flat blade laryngoscope and size 9 or 10 endotracheal tubes for pigs 40-50 kg.

1.7. Place a pulse oximeter probe on the tongue (preferred) or ear and target an oxygen saturation above 90%.

1.8. To maintain the anesthesia, adjust oxygen flow (20–40 mL/kg) and inhalant gas rate (1–3%).

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

NOTE: Although TVs are targeted as high as 10 mL/kg, 6-8 mL/kg are achieved. Figure 4.1 provides a schematic overview of the negative pressure ventilation (NPV)-ESLP for the transplant protocol applied in the lab.

1.10. Shave, wash and aseptically prepare the incision site using iodine.

NOTE: Following sedation with Ketamine/Atropine, the analgesic regime involves administering 3mg/kg Ketamine IV q 1 h (range 1-3 mg/kg depending on patient parameters) and Hydromorphone 0.05 mg/kg I M q 2h. Any longer duration between doses results in breakthrough pain response, such as elevated heart rate and abnormal breathing patterns/ abdominal muscle movement.

2. Insertion of central venous and arterial lines

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

NOTE: Central line is also used to administer steroids, antibiotics, vasopressors, and inotropes. See Figure 4.2A for line positioning.

2.1.1 Use electrocautery to make a 5-8 cm midline incision centered over the trachea and extend cranially from the sternal notch.

2.1.2 Divide the skin and subcutaneous fat using cautery.

2.1.3 Divide the midline plane between the strap muscles, then divide the connective tissue layers to identify the left or right carotid intravascular bundle lateral to the trachea.

2.1.4 Obtain proximal and distal control of the jugular vein using silk ties (size 2-0) as vessel loops.

2.1.5 Tie the cranial encircling tie and retract upwards on the proximal tie to control blood flow.

2.1.6 Make a small incision in the vein using Metzenbaum scissors (see Appendices: Table of Materials) to accommodate a two-port, 7 Fr central line (~1/3 the vessel's circumference).

2.1.7 Simultaneously release the tension on the proximal vessel loop, cannulate the vein, then tie down to secure the cannula in the vein at a depth of 10 cm.

2.1.8 Flush the line with heparin, connect to an IV line of 0.9% normal saline, and administer fluid if the pig is intravascularly depleted from dehydration.

NOTE: Heparin lock any unused ports.

2.1.9 Administer 500 mg of methylprednisone and 1 g of cefazolin IV.

2.2. Follow the same techniques to cannulate the common carotid artery using a 7 Fr arterial line for accurate blood pressure management.

3. Left lung procurement

3.1. Position the pig in a right lateral decubitus position.

3.2. Perform a left anterolateral thoracotomy (Figure 4.2).

3.2.1. Mark the thoracotomy incision (20 cm) using the following landmarks: use palpation to identify the tip of the left scapula; likewise, identify the xiphoid process inferior to the sternum with palpation. Connect the two as shown in Figure 4.2B.

3.2.2. Inject a total of 10 mL of 0.25% bupivacaine into the incisional line and two rib spaces above and below the incision.

3.2.3. Use electrocautery to dissect the skin, subcutaneous layers, and muscle layers. The latissimus dorsi must be divided. Identify the rib immediately below the incision and cauterize on top of the rib to expose the intercostal muscles while avoiding the intercostal neurovascular bundle.

3.2.4. Use a mosquito hemostat to puncture the intercostal muscles immediately above the rib and then feel inside the chest for adhesions using a finger. Push the lung away using a Yankauer suction or finger (see Appendices: Table of Materials) as you cauterize along the top edge of the rib to extend the thoracotomy.

3.2.4.1. Extend the thoracotomy anteriorly until 1 inch away from the sternum. Extend the thoracotomy posteriorly to the paraspinal muscles.

3.2.5. Insert a Cooley sternal retractor (see Appendices: Table of Materials) to open the thoracotomy wide (10 cm) (Figure 4.2C). Retract the lung to expose the left hemi-azygous vein (Figure 4.2D).

3.2.6. Circumferentially dissect the left hemiazygos vein using Metzenbaum scissors and a fine Lauer. Encircle the vessel with silk ties, then ligate and transect it (Figure 4.2E). Keep a silk tie on the proximal stump for added control.

NOTE: Lauer is a "right angle clamp" or a "celiac clamp" used for tissue dissection.

3.2.7. Dissect out the left pulmonary artery (PA) and left pulmonary veins (PV). Encircle the

veins in silk ties for control (Figure 4.2F).

NOTE: The superior PVs are very small and are suture ligated at their branch points or common trunk, depending on the individual anatomy. The left mainstem bronchus is deep to the PA and LA (left atrium), so occasionally, it cannot be dissected easily until the artery and veins have been clamped and transected (Figure 4.2G).

3.2.8. Administer 5000 units of heparin IV 5 min before clamping the PA.

NOTE: Heparin 5000 units IV is also administered 5 min before unclamping the PA. For every hour after that, 1000 units of IV heparin is administered.

3.2.9. Clamp the PA (DeBakey cross-clamp), left inferior pulmonary vein (Satinsky clamp), and the left bronchus (Spoon Potts clamp) individually (see Appendices: Table of Materials). Decrease tidal volumes to 5 mL/kg once the left bronchus is clamped.

3.2.10. Transect the PA, left inferior pulmonary vein, and the left bronchus. Leave at least 0.5 cm of tissue cuff to sew to. Divide the left inferior pulmonary ligament and remove the left lung.

4. Termination of ESLP, division of left lung, and flushing with electrolyte solution

4.1. Clamp the ventilation tubing at maximal inspiration, terminate perfusion and ventilation, and disconnect the lungs from the ESLP device.

4.2. Weigh the lungs to determine the amount of edema formation.

NOTE: Edema is tissue swelling due to the accumulation of excess fluid.

4.3. Take a tissue biopsy of the accessory lobe, divide it into three equal pieces, and place one piece into each of the following: optimum cutting temperature (OCT) gel, formalin, and snap freeze in liquid nitrogen.

NOTE: This step is typically followed in the author's lab. The samples are then stored for future analysis: OCT and snap-frozen samples are kept in a -80 °C freezer, and formalin-stored samples are placed in a properly sealed container and stored in 4 °C refrigerators. Details of the specific ESLP protocol and tissue analysis are published elsewhere[16].

4.4. Divide the left donor lung from the right lung. Leave 1 cm of donor PA, 1 cm of donor bronchus, and adequate donor LA cuff (~0.5 cm circumferentially) to sew to the recipient LA (Figure 4.2H). Leave the left inferior PV and left superior PVs in continuity with the donor LA wall to facilitate later anastomoses.

4.5. Weigh the left lung.

4.6. Cannulate the donor left PA using a drop sucker connected to an IV line and flush 500 mL of extracellular, low potassium, dextran-based electrolyte preservation solution antegrade through the lung vasculature. Secure the cannula in the PA with a silk tie during the flush, and release when the flush is complete.

NOTE: The steps mentioned pertain to the specific ESLP device utilized for this work and may not be directly applicable to other devices.

5. Left Lung Transplantation

5.1. Insert the donor lung into the recipient's chest, beginning with the lower lobe. Do not force the lung into place.

NOTE: The lower ribcage may need to be lifted upwards to accommodate the donor lung by torquing on the sternal retractor. Ideally, the recipient is a few kilograms larger than the donor to facilitate a size match.

5.2. Perform the bronchial anastomosis first using 4-0 prolene on a TF needle (Figure 4.2I).

NOTE: A running, end-to-end anastomosis works well. Trim any excess length from the two anastomotic ends before sewing to avoid kinking caused by redundant tissue.

5.3. Perform the LA anastomosis second with 6-0 prolene on BV-1 needles using a running, end-to-end anastomosis. Again, trim excess tissue to avoid kinking.

NOTE: The LA is friable and benefits from the small BV-1 needle. Horizontal bites on the donor may be required to purchase adequate tissue and correct the size mismatched caused by sewing the donor IPV and SPV to the recipient IPV/LA opening.

5.4. Incorporate the donor SPVs into the inferior PV and LA anastomosis to allow left upper lung lobe venous drainage (Figure 4.2J).

NOTE: The branch superior pulmonary veins (SPVs) are less than 0.5 cm in diameter. The common SPV trunk is variable in length and is not routinely present, making direct anastomosis between the donor and recipient SPVs a poor option.

5.5. Complete the PA anastomosis with 6-0 prolene on BV-1 needles using a running, end-to-end anastomosis. Again, trim excess tissue to avoid kinking.

5.6. Remove the bronchial clamp and increase TVs to target 10 mL/kg.

5.7. Confirm heparinization, administer a potassium shift (40 mg of furosemide, 10 units of insulin, 100 ml of 25% dextrose solution), open the PA clamp partially, de-air, and tie the PA suture. Completely release the PA clamp after 10 min.

5.8. Meanwhile, de-air the LA, tie the sutures, and remove the LA clamp.

5.9. Take a reperfusion blood gas from the central line and a reperfusion tissue biopsy from the left middle lobe.

NOTE: To take a tissue biopsy, use a size 0-silk tie to encircle a 1 cm portion of the middle lobe apex, tie-down to ensnare the tissue, then cut the isolated portion with Metzenbaum scissors. Divide the biopsy into three equal portions and manage as previously described.

5.10. Perform a left and right lung bronchoscopy to assess the bronchial anastomosis and to suction secretions. Insert a bronchoscope into the endotracheal tube using an adaptor connection.

5.10.1. Connect the scope to suction. Advance the bronchoscope into the left bronchus. Inspect the bronchial anastomosis (Figure 4.2N). Advance the scope down the bronchioles and suction any fluid. Repeat on the right side.

NOTE: Do not allow the oxygen saturation to fall below 90%. If saturations fall below this level, remove the scope and allow the pig a few minutes of uninterrupted ventilation to recover.

5.11. Insert a 20 Fr malleable chest tube (Figure 4.2L), close the thoracotomy in three layers (Figure 4.2M), and prone the pig as soon as the arterial blood gases (ABGs) are stable (Figure 4.2O).

5.12. Monitor the pig over 4 h in the prone position. Perform an ABG analysis every 30 min. Administer 1000 units of heparin every hour after reperfusion.

5.12.1. Take a 10 mL blood sample every hour for centrifugation and enzyme-linked immunosorbent assay (ELISA) analysis of inflammatory markers[16].

NOTE: Centrifugation parameters are detailed later.

6. Isolated Left Lung Assessment

6.1. Position the pig supine and perform a midline sternotomy for final isolated left lung assessment (Figure 4.2P).

6.2. Open the left pleura using Metzenbaum scissors and take a tissue biopsy from the left lower lobe as previously described (NOTE to step 5.9).

6.3. Open the accessory lobe pleura and dissect out the common vein using Metzenbaum scissors.

NOTE: This will be clamped later on.

6.4. Take a blood sample from the LA anastomosis using a 21 G needle. Direct the needle towards the left pulmonary veins and away from the common left atrium or accessory lobe trunk.

6.5. Open the right pleura to create space for the right hilar clamps (see Appendices: Table of Materials). Dissect the right inferior pulmonary ligament up to the hilum. Ensure that a clamp can be placed around the hilum superiorly, inferiorly, and anteriorly.

NOTE: This ensures that the hilum is occluded, and all oxygenation is dependent on the left lung. The right lung will not ventilate at this time, which should be evident by a lack of inflation/deflation with ventilator respirations. The right lower lobe can be lifted out of the chest to accomplish this.

6.6. Clamp the accessory lobe vein using a DeBakey aortic cross-clamp (see Appendices: Table of Materials) to occlude any accessory lobe drainage into the standard LA (Figure 4.2Q).

6.7. Take the following serial blood samples from the left PV anastomosis with a 21 G needle directed towards the left lung: 0 min, 1 min, 2 min, 5 min, and 10 min after clamping.

NOTE: Five samples are taken to monitor for any trend in partial pressure of oxygen (PaO_2) (Figure 4.2R). The PaO_2 should remain relatively stable to represent proper left lung function. Five samples also provide insurance of a quality assessment if there is an issue with clotting of any samples or a problem arises with ABG analysis.

6.8. Transect the anastomoses and remove the left lung. Transect the IVC to expedite exsanguination.

6.9. Weigh the donor lung to assess for edema formation and inspect for overall appearance.

Inspect the PA, bronchus and LA cuff for signs of clot or other pathology within the donor lung and the recipient mediastinum.

6.10. Run the final gas analyses, centrifuge the perfusate samples, and store the tissue biopsies as previously described (NOTE to step 4.3).

NOTE: The centrifugation settings are: 112 x *g*, 9 acceleration, 9 deceleration, 4 °C, and 15 min duration.

REPRESENTATIVE RESULTS:

All of the results are in the context of 4 h of reperfusion following 12 h of NPV-ESLP[16]. During lung explant, there are several clinical outcomes to anticipate (Figure 4.3). Typically, the pig will remain hemodynamically stable following a successful left lung explantation but may require a low dose infusion of phenylephrine (dose range: 2-10 mg/h) due to a vasodilatory response to surgery. Heart rate should target approximately 100-120 bpm, respiratory rate (RR) 8-30 for SpO_2 > 90%, mean arterial pressure (MAP) > 60 mmHg, normothermic (38 °C), and tidal volumes (TVs) are targeted at 5 mL/kg while on one-lung ventilation with peak pressures of 20-24 cm H_2O. During one-lung ventilation, the ventilation volumes were reduced by half to protect the right lung from overinflation. The respiratory rate was increased to target a physiologic end-tidal carbon dioxide level (Figure 4.3). Thus, Figure 4.3 displays typical hemodynamic and ventilatory parameters during critical points of the transplant.

During lung implant, the following results are typical. The left lung will have absorbed fluid during the ESLP run and appear heavier and larger than the explanted lung. For this reason, the recipient should be slightly larger than the donor (2-4 kg), so the thorax can accommodate the somewhat edematous lung. The lung will require gentle pressure to insert into the chest

through the thoracotomy. It is easier to insert the lower lobe first, followed by the upper lobe. The bronchus is a direct end-to-end anastomosis and should be performed first. 4-0 prolene on a TF needle is recommended. The LA cuffs are highly friable but not too difficult to sew due to the redundancy and pliability of the tissue. 6-0 prolene on BV-1 needles work well for the LA anastomosis. The PA is the last anastomosis performed. This vessel can tear easily with little traction. If it tears, it is possible to open the pericardium and move the clamp proximally towards healthy tissue for sewing. Again, a 6-0 prolene on BV-1 needles works well for this anastomosis.

At the time of reperfusion, the following trends were noticed. Once the bronchus is unclamped and TVs are increased back to 10 mL/kg, the left lung will begin to inflate. Although the target was 10 mL/kg for tidal volumes, generally 6-8 mL/kg was attained, which is achieved gradually over the first 2-3 h of reperfusion, depending on the ESLP protocol used and the quality of the implanted lung. Rarely, there can be a small air leak, and this can be remedied with a simple stitch on the anterior wall. The posterior wall is more difficult to repair and will require packing. Great effort should be made to avoid air leaks from the bronchial anastomosis. Upon bronchoscopy, the right lung appears normal, and the left lung is typically edematous. The suture line is inspected, and approximately 50-100 mL of clear fluid is suctioned from the airways. The TV will drop significantly during suctioning from 300 s to 20 s, so this action should be performed quickly to allow the pig to recover. If arterial saturation drops below 90%, the bronchoscopy should be terminated, and the pig is allowed to recover over 1-2 min of ventilation. The first arterial blood gas (ABG) is typically normal because the right lung is functioning well as the left lung recovers.

The proactive administration of furosemide, dextrose, and insulin at the time of reperfusion serves to mitigate a dramatic rise in potassium through intracellular "shifting." The potassium will predictably rise during 60-120 min of reperfusion (Table 4.1). Table 4.1 demonstrates a sample of ABGs over transplantation with 4 h reperfusion following 12 h of normothermic negative pressure ventilation (NPV) ESLP. Approximately two to four "shifts" are required during 4 h reperfusion to keep potassium < 5mmol/L. If the trend is upward and appears as a rapid change between two gases drawn at 30 min intervals, the target is K^+< 4.5mmol/L. "Shifts" include 40 mg of furosemide, 100 mL of 25% dextrose (D25), and 10 units of regular insulin administered as IV push *via* the central line. Occasionally, the pig will require a low dose dobutamine infusion (1.5-5 mcg/kg/min) along with phenylephrine (2-10 mg/h) after 30-60 min of reperfusion to treat a developing vasoplegic response. It is preferable to use phenylephrine in this situation exclusively. Still, occasionally dobutamine is a useful supplemental inotrope to maintain a mean arterial pressure greater than 60 mmHg, mainly if the heart rate is bradycardic.

Upon thoracotomy closure and turning the pig prone, an improvement in ventilation and hemodynamics is demonstrated. The modification can be drastic and occur over 5-10 min, but occasionally the response takes 1 h. Tidal volumes increase as pressure/weight is taken off the right lung, and the left lung continues to ventilate with improved compliance and recruitment. A repeat bronchoscopy can be performed further to clear the airway after a change in position. Over the following 4 h, phenylephrine requirements decrease, TVs approach the target 10 mL/kg, and ABGs stabilize (Table 4.1). To reiterate, if TVs of 10 ml/kg are targeted, typically TVs in the range of 6-8 mL/kg are achieved (Figure 4.3).

At the time of the final isolated left lung assessment, a stable pattern of behavior has been observed. The pig is less tolerant hemodynamically in the supine position for sternotomy and

may require additional vasopressor support. Inspection of the left lung reveals variable degrees of mild hyperemia from ischemic reperfusion injury (IRI). The right lung appears normal. Upon clamping the right hilum, the pig becomes sinus tachycardic (120-140 bpm), and 100% of the cardiac output is diverted to the left lung. Targeted tidal volumes are not decreased at this time as the entire process takes 10 min. The pig remains stable up to the 5 min mark, but the heart can develop ventricular fibrillation between 5-10 min and manual cardiac massage is required to continue perfusing the left lung. The left lung is explanted, weighed, and the anastomoses are inspected for patency. The pig expires rapidly at the time of exsanguination, which coincides with the explantation of the previously transplanted lung.

A successful transplant has predictable findings after the experiment (Table 4.1 and Figure 4.4). Figure 4.4 displays typical P: F ratio changes and edema formation during the transplant protocol. Typically, the left lung will experience an approximate 35% (+/-15%) weight gain; however, residual blood in the circulation contributes to this weight. PF ratios drop by approximately 100 at reperfusion as the left lung is not immediately effective at oxygenation, but this discrepancy improves over 2-3 h. Upon isolated left lung assessment at 4 h, the PF ratio will remain stable or decline slightly. Generally, the isolated left lung gas at 10 min will be similar to the final gas analysis post 12 h ESLP (Table 4.1). However, this is entirely dependent on the ESLP protocol employed, and the extent of IRI incurred. An unsuccessful transplant can be caused by clotting of the LPA, which results in an infarcted lung that does not oxygenate. Likewise, the duration of the transplant surgery can affect the quality of the reperfused lung function. An implantation surgery should take between 30-60 min. Longer operations expose the donor lung to damaging warm ischemic time that exacerbates ischemic reperfusion injury and can confound the results of the experimental ESLP protocol. The specific ESLP protocol of a

given experiment may produce a non-functioning lung that fails to oxygenate after transplantation despite patent anastomoses. Such isolated left lung gases will be very dark in color (deoxygenated) with a low partial pressure of oxygen (PaO_2).

DISCUSSION:

Several critical surgical steps are involved in this protocol, and troubleshooting is needed to ensure successful transplantation and lung assessment. Juvenile porcine lungs are incredibly delicate compared to adult human lungs, so the operating surgeon must be cautious when handling porcine lungs. This is especially true after a 12 h run of ESLP as the organ will have taken on fluid volume and be susceptible to injury from excessive manipulation. Any undue pressure will cause atelectasis or trauma to the experimental lung that will affect assessment results. Likewise, the vascular structures are very delicate in the juvenile pig. It is critical to avoid torsion of the PA clamp as this can cause a tear or dissection of the tissue layers. A tear in the PA will necessitate opening the pericardium to access a more proximal portion of the left PA that can be anastomosed to the implanting lung. A DeBakey vascular clamp has a low profile that fits well in the surgical field, but this instrument can cause injury to the delicate PA if the surgeon is not careful. It is helpful to secure the clamp in position using a silk tie that is snapped to the drapes to prevent dislodgement or torsion. Bronchoscopy of the transplanted lung after unclamping of the bronchial anastomosis is also critical. There is often fluid within the donor lung airway after 12 h of ESLP and transplant. Suctioning this fluid is vital to ensure optimal recovery of left lung function and thereby assessment after 4 h of reperfusion. After bronchoscopy and the first ABG has returned with satisfactory potassium levels, it is critical to insert a chest tube, close the incision, and prone the pig. The pig's hemodynamics and ventilation

are considerably more stable in the prone position, with the ribcage reapproximated. Elevated potassium > 5.5 at this stage risks bradycardic arrest and will require emergent re-opening and manual cardiac massage to support perfusion, which is best avoided. Due to the significant risk of hyperkalemia and bradycardic arrest upon reperfusion, it is critical to perform serial ABGs beginning at reperfusion and recurring every 30 min until the 4 h exsanguination. ABGs give essential readings of oxygenation, partial pressure of carbon dioxide (PCO_2), potassium, and glucose. Monitoring these four components closely and treating them appropriately is vital to a successful experiment. A continuous telemetry reading is also critical to monitor for peaked T waves associated with hyperkalemia and the anticipation of bradycardia. At the final stages of the experiment, it is crucial to clamp the right lung hilum and the accessory lobe before drawing final blood samples from the LA anastomosis. The right hilum supplies blood to the accessory lung lobe, and the accessory lobe drains adjacent to the left inferior pulmonary vein, often *via* a common trunk. The right hilum and accessory lobe need to be clamped separately to ensure no right lung function contributes to the sample LA gases through blood mixing. Drawing the left lung ABG sample from the PV anastomosis or just beyond it is suggested.

Several modifications have been made to this protocol along with significant troubleshooting of the described methods. Initially, it was attempted to perform the implantation *via* median sternotomy; however, the exposure was suboptimal due to the orientation of the pig PA, bronchus, and LA. The approach was successfully performed, but a thoracotomy was attempted on subsequent surgeries for improved exposure. This proved to be a superior surgical approach from a visualization and technical perspective. Another essential modification was developing and implementing a surgical safety/protocol checklist (See Appendices: surgical safety/protocol checklist). There was a significant learning curve for all the team members

involved, and these experiments are resource-intensive. A checklist was developed to guide the communication and document protocol development (See Appendices: surgical safety/protocol checklist). The checklist allowed to systemize and simplify the protocol for faster learning. The heparinization protocol was also modified. Two of the first ten transplants performed suffered from left lung ischemia due to clot formation in the left PA. Initially, 5000 units of heparin IV 5 min was administered before PA clamping and an additional 5000 units 5 min before PA unclamping. Dosing frequency was increased to include 5000 units every hour after PA unclamping, and there have not been any issues with bleeding or PA clotting since adopting this approach. A strategy that utilizes less heparin was developed to control expenses, with a dose of 5000 units IV heparin 5 min before PA clamping and 5 min before partial PA unclamping. This is followed by 1000 unit IV heparin boluses every hour for the remainder of the case. There was no access to ACT analysis, which would be the most accurate means of accessing adequacy of heparinization.

The unclamping of the PA was also modified from a sudden unclamping to an approach that gradually reintroduces full flow to the transplanted lung over 10 min. The left inferior PV and LA cuff remain clamped upon PA unclamping to allow for antegrade de-airing. Full PA flow produced significant pressure on the delicate LA suture lines and considerable pressure within the lung vasculature, which appeared damaging. Prolonged PA unclamping allows for the antegrade de-airing of the LA with a gradual increase in flow as opposed to sudden unclamping and a sudden increase in flow. Prolonged unclamping protects the suture lines and lung endothelium from sudden increase in pressure. Even with ESLP, an ischemic insult to the transplanted lung and cell death contributes to a significant release of potassium into the pig's circulation following ischemic-reperfusion. For managing hyperkalemia proactively, the protocol

was modified to pre-emptively shift potassium at the time of reperfusion by administering furosemide 40 mg IV, 100 mL of 25% dextrose (D25), and 10 units of regular insulin. This maintains target potassium on the ABGs within the first hour of reperfusion, and the pig can be safely proned earlier in the experiment, which helps with graft function. From a hemodynamic perspective, the protocol is modified to use phenylephrine as the predominant vasopressor support. Vasopressin was found to be less effective and was thus abandoned. A low dose drip of dobutamine was occasionally run to increase cardiac output, along with a phenylephrine infusion to maintain blood pressure. Still, dobutamine is used sparingly due to its arrhythmogenic properties. Finally, the assessment of the isolated left lung was modified. After clamping the right lung hilum, the LA gases were initially drawn from the body of the LA after lifting the heart cephalad; however, gas mixing from the accessory lobe drainage into the LA produced falsely high PaO_2 readings. Now, samples are drawn distal to the LA anastomosis line after clamping the right lung and the accessory lobe individually. These samples are taken at 0, 1, 2, 5, and 10 min after clamping the right hilum and are a more accurate representation of the isolated left lung function. Manual cardiac massage may be required between the 5–10 min mark. The most recent protocol improvement pertains to the superior pulmonary vein (SPV) anastomoses. Initially, the recipient SPVs were oversewed due to their small caliber and propensity to clot. Still, the donor's upper lobe occasionally suffered congestion as collateral drainage was variable and inadequate between pigs. To remedy this, the donor SPV and IPV were incorporated into the recipient's IPV/LA anastomosis, eliminating any issue with venous drainage and lung congestion. This protocol will continue to benefit from further modification as experience grows.

There are several limitations with this method of left lung transplantation. The model has only been assessed with a 4 h period, which only considers the transplanted lung function in the

acute post-operative period following 12 h of ESLP. This protocol was designed with the animal's recovery in mind; however, it has yet to be tested in that capacity. The technical operation requires considerable surgical skill and necessitates a trained surgeon or highly independent surgical trainee to perform. There are many opportunities for fatal errors to occur that would compromise the entire experiment, and proper surgical technique is needed to avoid or correct such hazards. The only true assessment of the transplanted lung occurs at the very end of reperfusion. The native right lung is capable of meeting the oxygen requirements of the pig and producing satisfactory ABGs. When the right lung is completely clamped at the hilum, it is prevented from receiving fresh oxygen, fresh deoxygenated blood supply, and oxygenated blood drainage. This is a pivotal moment to determine the transplanted left lung function as 100% of cardiac output is redirected towards the transplanted lung, which becomes solely responsible for systemic oxygenation.

There are multiple benefits of this method concerning existing/alternative methods. After reviewing the literature [12-15], this method is the most detailed and reproducible after an initial learning curve of 1 or 2 pigs in the hands of a junior cardiac surgical trainee or fully qualified surgeon. The operation is straightforward; however, the hemodynamics of the pig (including its susceptibility for lethal arrhythmias) creates a learning opportunity for those accustomed to operating on adult humans, which are more robust from a cardiopulmonary perspective. The methods for isolated left lung functional assessment, although brief, are easy to perform and highly reproducible. In particular, this methodology provides more detail about anesthetic management than is currently available in the literature.

This method is essential and has significant applications for ESLP and lung transplantation research. ESLP is the most crucial development in lung transplantation since the

introduction of antirejection medication, with some centers already benefitting from the increased organ utilization rates afforded by this technology[6-12]. Further advancement in this field of research is needed to decrease waitlist mortality and expand the accessibility of ESLP platforms. *In vitro* analysis with ESLP benefits from the *in vivo* assessment and confirmation of a large animal model. Large animal models that confirm *in vitro* findings are often necessary for clinical research trial approval for developing labs. This method provides a reliable and relatively straightforward transplant method for labs performing ESLP research.

FIGURE AND TABLE LEGENDS:

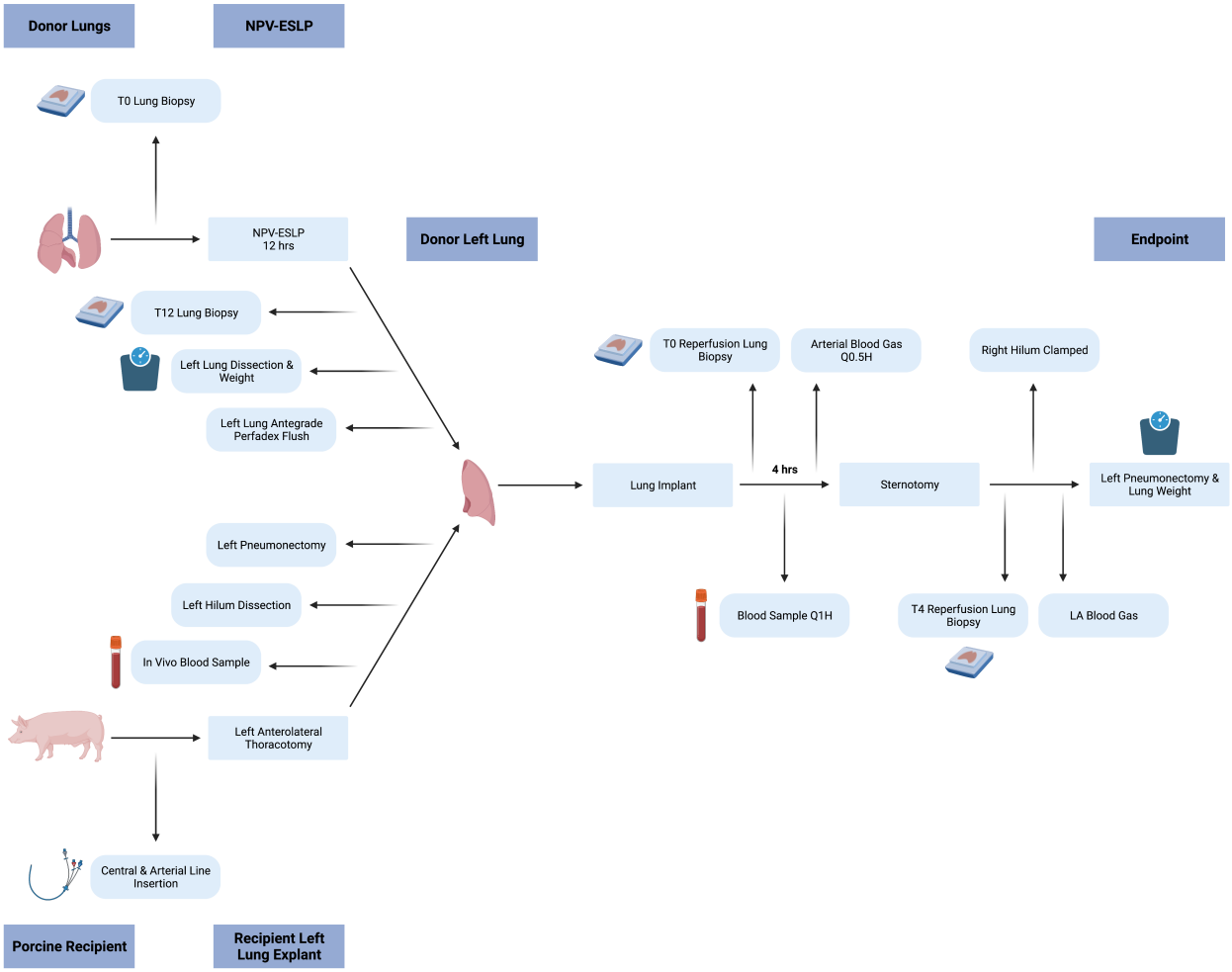
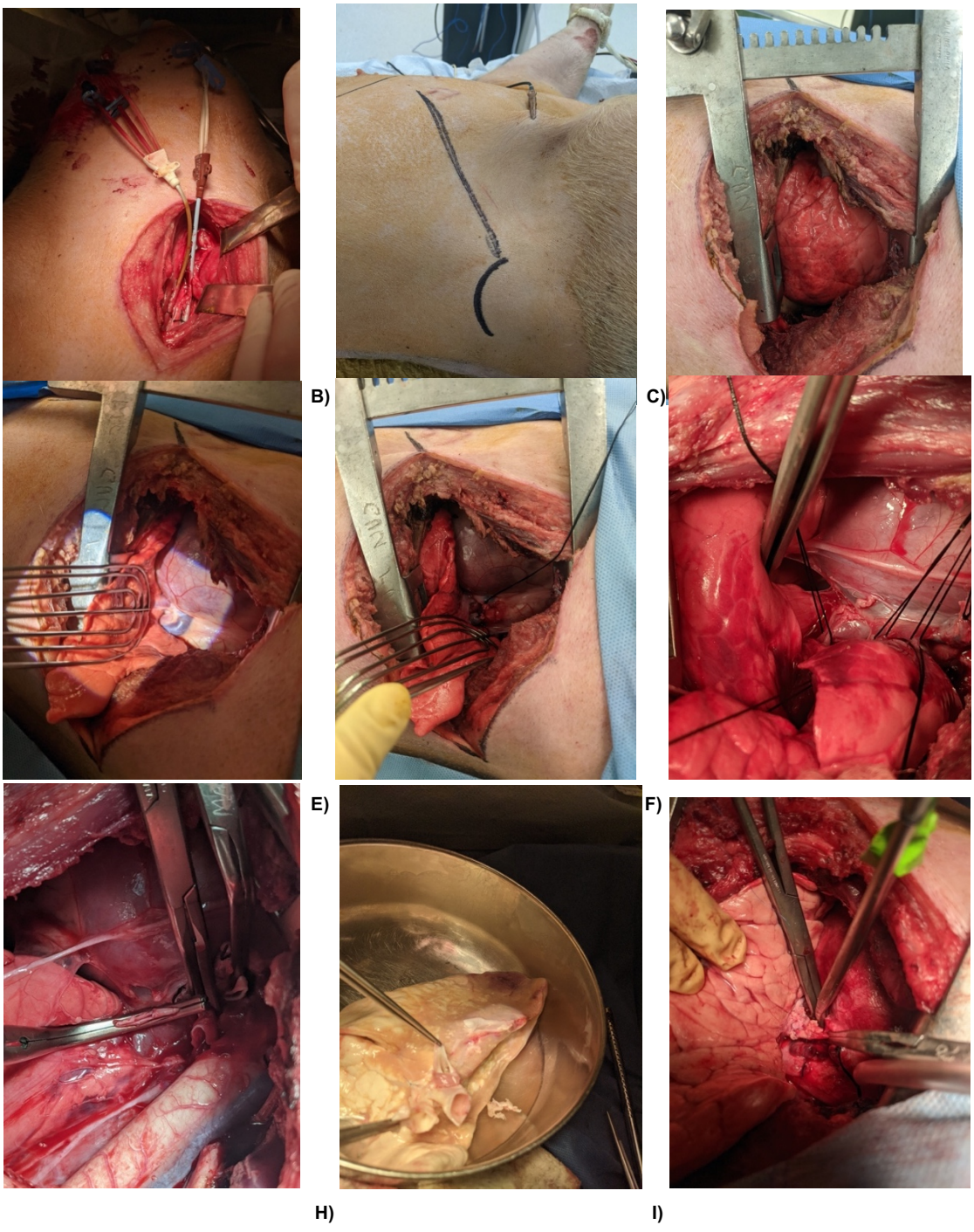


Figure 4.1: Schematic of porcine left lung transplant protocol. Schematic representation of 12 h NPV-ESLP run followed by left lung transplantation in a Yorkshire pig.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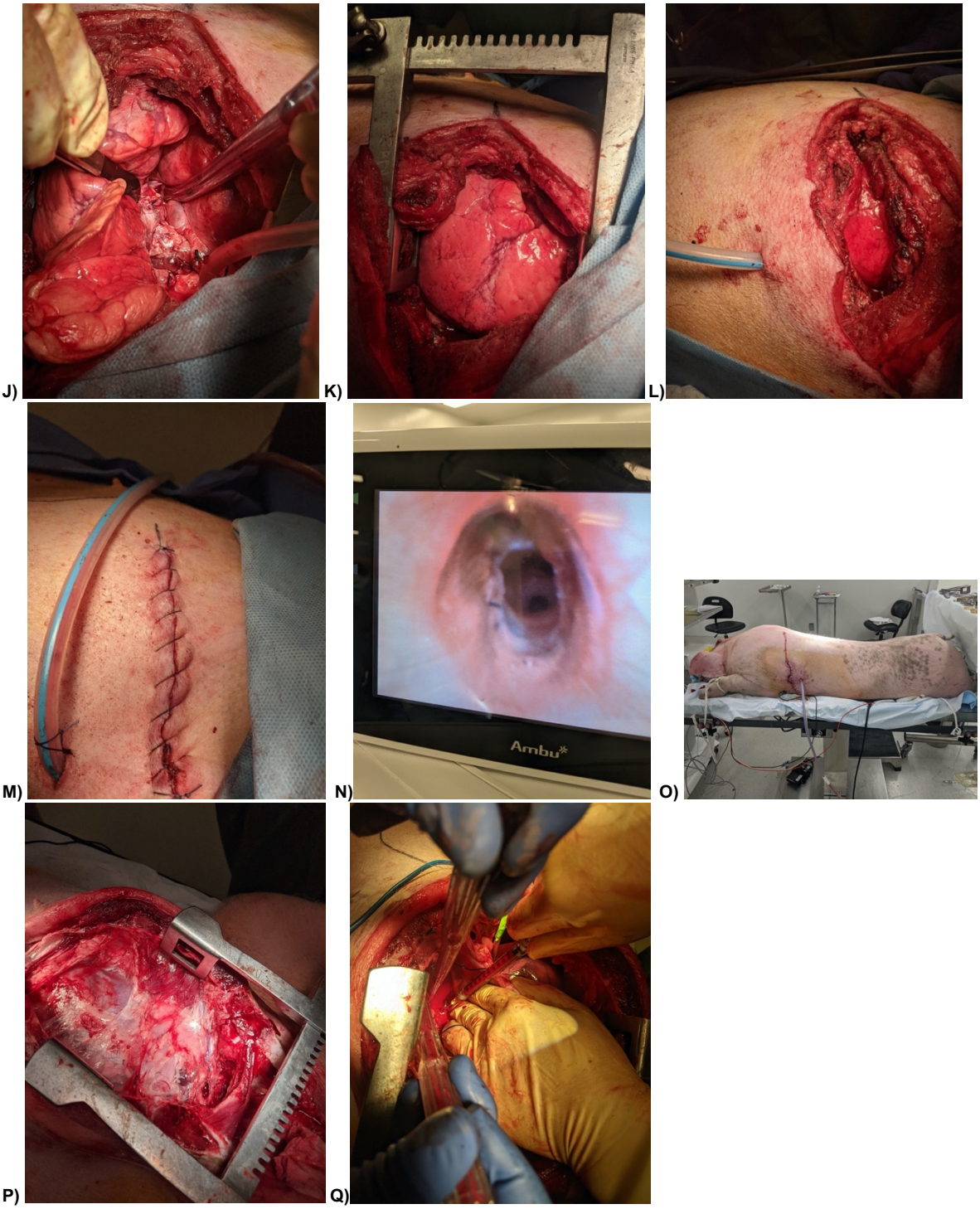
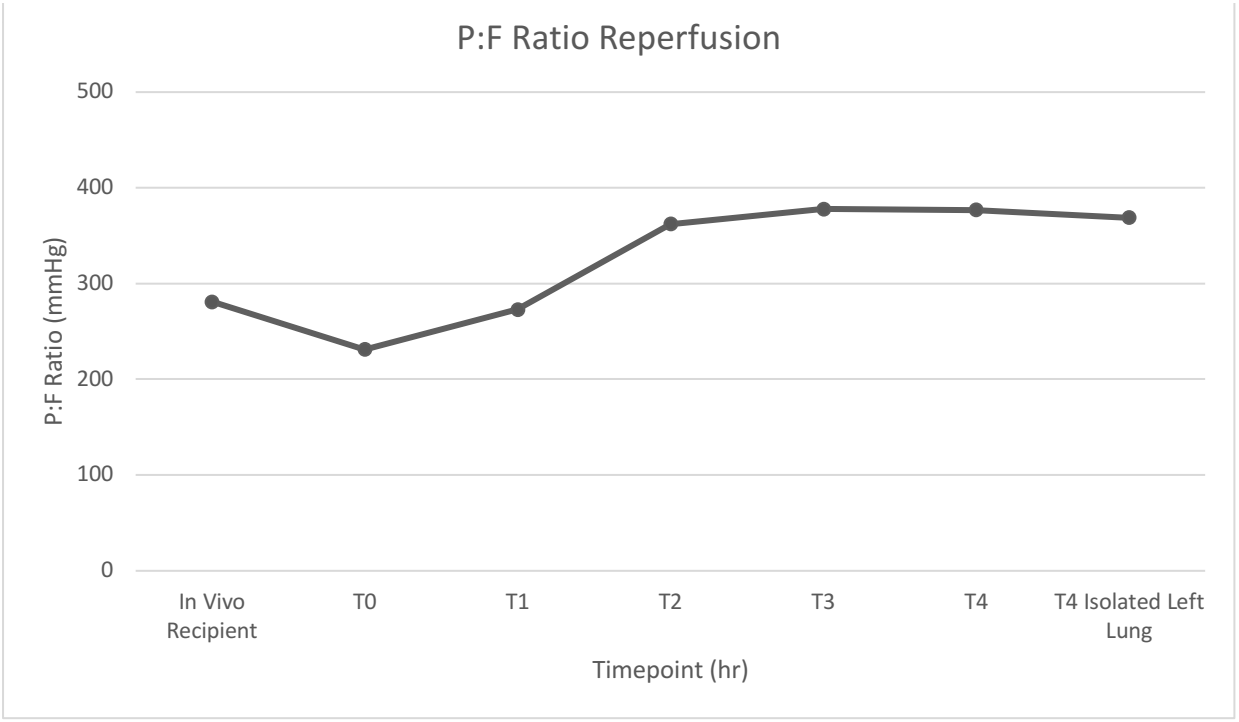


Figure 4.2: Photos of porcine left lung transplant surgery protocol. (**A**) Internal jugular and common carotid line placement. (**B**) Thoracotomy incision. (**C**) Thoracotomy. (**D**) Left Hemi-azygous vein. (**E**) Ligated Left Hemi-azygous vein. (**F**) Isolation of pulmonary veins. (**G**) Clamped left atrial cuff, left bronchus, and left pulmonary artery. (**H**) Left donor lung with pulmonary vein, bronchial and PA cuffs. (**I**) Pulmonary artery anastomosis. (**J**) Left lung transplanted and unclamped. (**K**) Lung repositioned. (**L**) Chest tube positioned. (**M**) Thoracotomy closure. (**N**) Bronchial anastomosis. (**O**) Pig in prone position. (**P**) Sternotomy. (**Q**) Accessory lobe clamped (Right lung clamped, but not shown). (**R**) Left pulmonary vein blood samples were drawn from pulmonary vein anastomosis (bleeding from prior puncture site).

A)  B)  C) 

Figure 4.3: Monitoring and ventilation parameters for porcine left lung transplant surgery. (**A**) Typical parameters for recipient pre-transplant. (**B**) Typical parameters at recipient left lung explant. (**C**) Typical parameters 4 h post left lung donor transplant.



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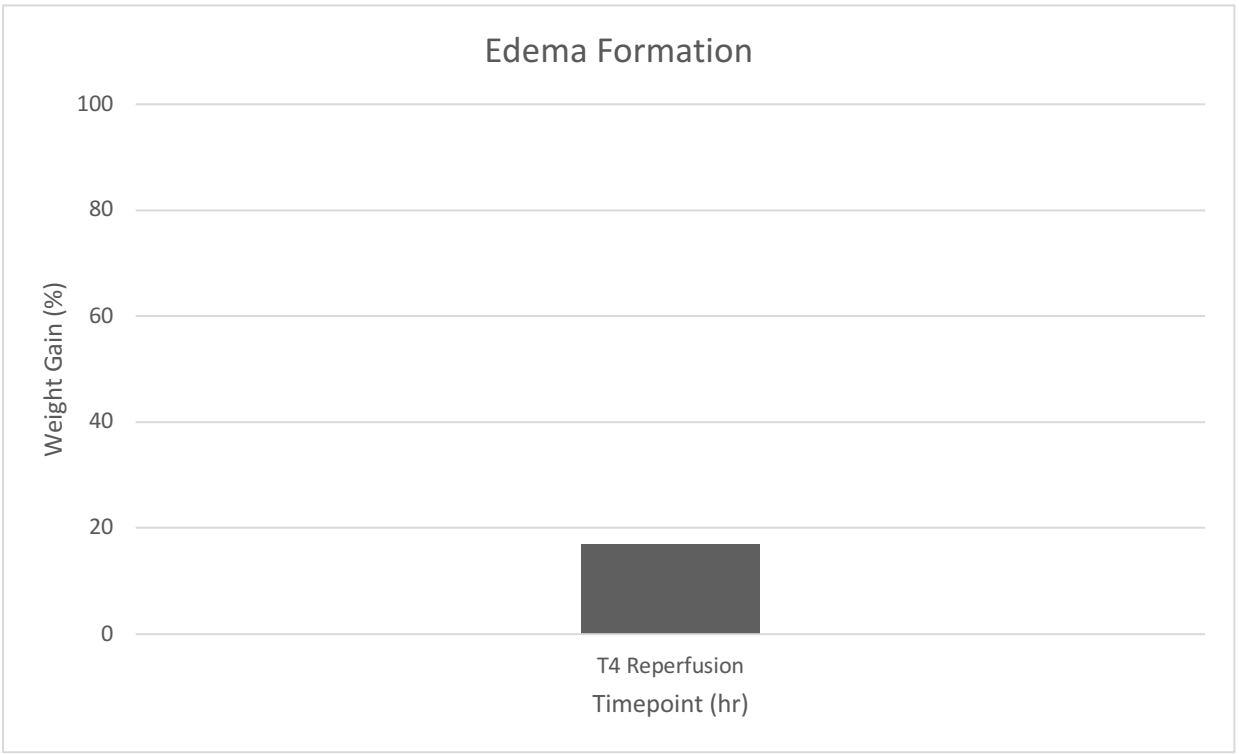

Figure 4.4: PF ratio and weight gain pre-and post-transplant. (**A**) PaO2:FiO2 ratios throughout the transplant. (**B**) Weight gain of left lung throughout transplant after 12 h of NPV-ESLP.

Arterial Blood Gases (100% FiO2)	Invivo Recipient	T0 Reperfusion	T1 Reperfusion	T2 Reperfusion	T3 Reperfusion	T4 Reperfusion	Isolated Left Lung Pre-Clamp	Isolated Left Lung Post-Clamp (10min.)
Blood Gas Values								
pH	7.37	7.33	7.35	7.46	7.52	7.45	7.49	7.485
pCO_2 (mmHg)	51.6	49.1	37.9	33.3	28.6	32.1	29.6	31.0
pO_2 (mmHg)	281	231	273	362	378	377	276	369
Oximetry Values								
Hb (g/dL)	11.5	13.0	12.2	11.9	11.9	12.1	10.6	16.4
sO_2 (%)	99.7	99.5	99.7	99.7	99.9	99.7	99.6	99.8
Electrolyte Values								
K^+ (mmol/L)	4.4	5.1	2.9	3.7	3.8	3.8	4.2	4.5
Na^+ (mmol/L)	142	141	143	145	143	144	141	140
Ca^{2+} (mmol/L)	1.14	0.73	0.77	0.93	0.87	0.57	0.31	0.38
Cl^- (mmol/L)	97	100	106	106	108	110	88	98
Osm (mmol/kg)	290.3	288.9	291.4	293.1	288.6	289.4	284.8	283.9
Metabolite values								
Glucose (mmol/L)	7.0	7.3	5.4	3.8	2.5	2.3	2.6	3.0
Lactate (mmol/L)	0.9	1.3	2.3	1.4	0.9	1.0	1.3	1.5
Acid Base status								
$Hco3^-$ (mmol/L)	29	24.9	20.5	22.6	22.9	21.9	22.3	23.1

Table 4.1: Blood gas analysis performed following left lung transplant post 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; isolated left lung pre-clamp, right hilum open; Isolated left lung post-clamp, 1 min after right hilum clamped.

Chapter 5

Normothermic Perfusion is Superior to Cold Perfusion in Ex-Situ Lung Perfusion

Abstract

Background: Cold Ex-Situ Lung Perfusion (ESLP) has demonstrated improved preservation in small animal ESLP compared to normothermic ESLP and cold static preservation. We hypothesized that cold Negative Pressure Ventilation (NPV)-ESLP would improve graft function in a porcine transplantation model.

Methods: Four perfusate temperatures were examined with 12-hour NPV-ESLP in a large animal transplantation model. Pig lungs were allotted to four groups: (1) Normothermia (38°C, n=6); (2) Profound Hypothermia (10°C, n=6); (3) Moderate Hypothermia (20°C, n=3); (4) Subnormothermia (32°C, n=3). A fifth group Subnormothermic Low-Flow (SNLF) perfusion was examined to assess the effect of reduced cardiac output (CO) with cold perfusion (32°C, 10% CO, n=6).

Results: Only Normothermic and SNLF groups demonstrated acceptable oxygenation after 12-hour NPV-ESLP and were transplanted. All other groups failed prematurely. After 12-hours of ESLP, Normothermic lungs demonstrated significantly greater dynamic compliance compared to SNLF lungs ($p = 0.03$). Edema formation post-ESLP was significantly worse in the SNLF group ($p = 0.01$). There was no significant difference in pulmonary artery pressures after ESLP ($p=0.10$); however, pulmonary vascular resistance was significantly greater in the SNLF ($p=0.04$). Isolated left lung oxygenation 4-hours post-transplant and left lung edema formation were not significantly different between Normothermic and SNLF post-transplant ($p=0.09$). Pro-inflammatory cytokines were significantly greater during SNLF-ESLP (TNF-α, $p<0.05$).

Conclusions: Prolonged Normothermic (38°C) NPV-ESLP is superior to 10°C, 20°C, and 32°C perfusion. Normothermic ESLP of porcine lungs results in superior graft function and reduced inflammation versus SNLF-ESLP.

INTRODUCTION

Lung transplantation for end-stage lung disease is limited by an inadequate number of suitable donor lungs and a consequently high waitlist mortality of 15-40%[1-2]; Ex-Situ Lung Perfusion (ESLP) can help address this discrepancy. Approximately 20% of lungs offered for donation are accepted for transplantation with the remainder deemed unacceptable due to stringent acceptance criteria[2,3]. The current standard for organ preservation is cold static preservation (CSP), which entails flushing the lungs with a cold preservation solution and storing them on ice in a cooler. ESLP offers an improved approach to organ preservation by perfusing and ventilating donor lungs continuously for a more physiologic preservation strategy. Normothermic Negative Pressure Ventilation (NPV)-ESLP is able to expand the donor pool by reconditioning extended criteria donor lungs to acceptable transplant standards[4].

Recently small animal studies have demonstrated a beneficial effect of both hypothermic (4-10°C)[5,6] and subnormothermic (21-32°C)[7,8,9] perfusion temperatures during ESLP compared to CSP and normothermic perfusion. These studies suggest that cold ESLP results in reduced circulating inflammatory markers and improved physiologic function following transplantation.

Hypothermic and subnormothermic ESLP have never been studied in a large animal model. We hypothesized that cold perfusion would result in improved lung function and decreased inflammation on NPV-ESLP with superior transplantation physiology compared to normothermic NPV-ESLP.

MATERIALS AND METHODS

Experimental Protocols:

Experimental protocols were approved by the University of Alberta animal care and use committee (AUP943). All animals received humane care in accordance with the "Principles of Laboratory Animal Care," formulated with the National Society for Medical Research and the National Research Council's Guide for the Care and Use of Laboratory Animals.

Experimental Design:

Four perfusate temperatures were investigated over 12-hour NPV. Pig lungs were allotted to four groups: (1) Normothermia (38°C, n=6); (2) Profound Hypothermia (10°C, n=6); (3) Moderate Hypothermia (20°C, n=3); (4) Subnormothermia (32°C, n=3). A fifth group Subnormothermic Low-Flow (SNLF) perfusion was included to examine the effect of reduced cardiac output (CO) with cold perfusion (32°C, 10% CO, n=6). Temperature groups with fewer than six pigs indicate experiments that were abandoned due to a high failure rate without a promising chance of improved outcomes.

Donor Lung Procurement

Female Yorkshire-Duroc pigs (45 ± 5 kg) were used for all experiments. Donor lung recovery and NPV-ESLP have been previously described in detail[10,11] and are summarized in this text. Animals were sedated with ketamine (20 mg/kg) and atropine sulfate (0.05 mg/kg). The animals were intubated and anesthetized with 1-3% isoflurane. Median sternotomy was performed. The animals were administered heparin directly into the superior vena cava (SVC). *In vivo* wedge biopsies were taken along with blood samples. The SVC was transected and a poole tip suction was inserted into the SVC and right atrium for exsanguination, draining into a cell-saver for blood collection. Cardiectomy was performed, the lungs were dissected free from their

attachments, and the trachea was clamped at a constant airway pressure of 20 cm H_2O. The lungs were removed, weighed, and connected to NPV-ESLP.

Standard Normothermic NPV-ESLP

Additional lung wedge biopsies were taken immediately prior to NPV-ESLP connection. The lungs were connected to the ventilatory circuit by intubating the trachea with a modified endotracheal tube in a manner that maintains lung inflation. The pulmonary artery was connected to the perfusion circuit, and perfusion was initiated at 10% of predicted cardiac output ($CO_{predicted}$ = 70 mL/kg/min). The perfusate temperature was warmed from ambient room temperature to 32℃ over 10-minutes. Once perfusion was established, a continuous positive airway pressure (CPAP) of 20 cm H_2O was applied, and the trachea was unclamped. At 32℃, the air-tight chamber lid was secured and NPV was initiated by simultaneously decreasing CPAP and end-inspiratory pressure (EIP) until a CPAP of 8 cm H_2O and EIP -12 cm H_2O was achieved. The perfusate temperature was gradually warmed to a target of 38℃ within 20-30 minutes of perfusion. Normothermic temperatures for pigs is 38-39.5°C. Every 10 minutes, perfusion flow rate was increased by 10% of CO until a target flow of 30% CO. Lung evaluation (5-minute duration) was performed at hour 5 (T5) and hour 11 (T11) of NPV-ESLP. A medical sweep gas mixture (89% N_2, 8% CO_2, and 3% O_2) was used to deoxygenate the perfusate during evaluation to assess for pre vs post lung oxygenation step-up (Table 5.1). Deviations from the Standard Normothermic NPV-ESLP protocol for specific experimental groups are outlined below.

Profound Hypothermic Group (PH). After connection to ESLP, the lungs were gradually cooled from ambient room temperature (20℃) to 10℃ over 10-minutes. For T5 and T11 evaluations, the lungs were warmed to 38℃ over 30-minutes, evaluated, and cooled to 10℃ over

30-minutes. This rate of temperature change matched the rate of our Standard Normothermic NPV-ESLP initiation protocol at approximately 1℃/minute.

Moderate Hypothermic Group (MH). At ESLP initiation, lungs were maintained at ambient room temperature (20℃) during preservation. At T5 and T11 hour evaluations, the lungs were warmed to 38℃ over a 20-minute period, evaluated, then cooled to 20℃ over 20-mintues.

Subnormothermic Group (SN). Once connected to ESLP, lungs were gradually warmed to 32℃ over 10-minutes and maintained for preservation. Prior to evaluations T5 and T11, lungs were warmed to 38℃ over 10-minutes, evaluated, then cooled to 32℃ over 10-mintues.

Subnormothermic Low-Flow (SNLF). The protocol for the SNLF NPV-ESLP group was identical to that for SN NPV-ESLP except the perfusion flow rate was not increased above 10% of $CO_{predicted}$ during initiation and preservation (Table 5.1).

Perfusate Selection.

Normothermic (38℃) lungs were perfused with 1L common-hospital ingredient perfusate (CHIP) with 500ml autologous packed red blood cells (pRBC)[10] at 30% of predicted cardiac output, as per our standard protocol[11]. Cold ESLP lungs were perfused with 1.5L acellular Perfadex. Perfadex was selected to mirror the approach of cold ESLP in rats by Gloria et al (7). We compared acellular cold perfusion to our standard normothermic cellular perfusate to determine if the previously published acellular cold ESLP outcomes[7,8,9] were superior to our Standard NPV-ESLP methods. Added to the assigned perfusate solutions was 3.375 g of piperacillin-tazobactam, 10,000 IU of heparin, and 500 mg of methylprednisolone. Insulin (2 U/h) was continually infused into the circuit. A dextrose (25%) infusion was titrated to target a perfusate glucose concentrations of 4 to 6 mmol/L.

Left Lung Transplantation

Transplantation was planned for all groups if the lungs successfully reached 12-hours of reliable NPV-ESLP. Three-lungs from each successful ESLP group were transplanted into recipient pigs for *in-vivo* assessment and monitored for 4-hours before a final isolated left lung evaluation followed by euthanasia. Our methods for left lung transplantation have been described in detail[12] and are summarized below.

Animals were sedated as previously described under Donor Lung Procurement with the addition of Hydromorphone 0.05mg/kg. An arterial line and central line were inserted into the carotid artery and jugular vein, respectively. Animals were placed in a modified right-lateral decubitus position. A left thoracotomy was performed, and the donor left lung hilar structures were dissected free. When the donor lung was ready for implant, the recipient left lung structures were clamped and the native lung was discarded. The donor lung was flushed with 0.5L of cold (4°C) extracellular, low-potassium, dextran-based solution to remove debris and ESLP perfusate remnants. The donor lung was implanted, and the recipient was monitored for 4-hours. After 4-hours, a sternotomy was performed, the right lung hilum and accessory lobe were clamped to obstruct perfusion and ventilation, enabling isolated left lung assessment. An isolated left lung blood sample was drawn at 10-minutes to evaluate the transplanted lung function. Animals were subsequently euthanized, and the left lung was explanted, inspected for gross pathology, and weighed.

Physiologic Evaluation

Continuous assessment of physiologic parameters was performed during ESLP, including pulmonary artery pressure (PAP), pulmonary vascular resistance (PVR), dynamic compliance (Cdyn), and the ratio of partial pressure of oxygen in the perfusate to the fraction of inspired

oxygen (PF ratio). During evaluation on ESLP and post-transplant, PF ratios were calculated from the left atrium perfusate. Lung weight was recorded at the following timepoints: post-explant, T0, T12. Left lung weight was recorded pre- and post-transplant. Biopsies were taken at similar timepoints. Edema formation was estimated using percentage of weight gain (weight gain [%] = {[End weight - Start weight]/Start weight}x 100%).

Perfusate Cytokine Analysis

Perfusate samples were collected every two hours during ESLP. Perfusate concentrations of pro-inflammatory cytokines tumor necrosis factor ∝ (TNF-∝) and interleukin-6 (IL-6) were determined using enzyme-linked immunosorbent assay kits (R&D systems, Minneapolis, MN).

Statistical Analysis

GraphPad Prism Software (Version 9; GraphPad Software Inc, La Jolla, California) was used for statistical analysis. Unpaired Student *t* tests were performed to compare normally distributed continuous variables between groups. Normality was assessed using the Shapiro-Wilk test. Results are expressed as mean ± standard error. $P < .05$ was considered statistically significant.

RESULTS

Physiologic Function during NPV-ESLP

Only Normothermic and SNLF groups demonstrated acceptable oxygenation after 12-hour NPV-ESLP and were transplanted. All other groups failed prematurely due to excessive pulmonary edema, low compliance, elevated PVR, and poor oxygenation. Results are shown as Normothermic versus SNLF (mean ± SEM). There was no significant difference in PF ratio at the final evaluation T11 ($p = 0.06$; Figure 5.1A). The average PF ratio at T11 was 454.20 ± 40.85 in the Normothermic groups and 409.70 ± 16.10 in the SNLF group. The PF ratios were above 300 for each individual run within the two groups, which met one of our criteria for transplantation.

Dynamic compliance (Cdyn) did not differ significantly between Normothermic and SNLF at T5 ($p=0.41$), but Cdyn was significantly higher in the Normothermic group by the end of ESLP ($p=0.03$; Figure 5.1B). The average Cdyn was 21.38 ± 2.28 in the Normothermic group and 12.65 ± 3.30 in the SNLF group.

Pulmonary artery pressures (PAP) were not significantly different between groups at T5 ($p=0.30$) or T11 ($p=0.10$; Figure 5.1C). At T11, the average PAP was 10.89 ± 2.28 in the Normothermic group and 16.13± 3.16 in the SNLF group. Both groups demonstrated mean PAP under 20 mmHg, which met criteria for transplantation.

There was no significant difference in PVR between the Normothermic group and SNLF group at T5 ($p=0.09$), but PVR was significantly higher in the SNLF group at T11($p =0.04$; Figure 5.1D). At T11, the average PVR was 438.60 ± 97.97 in the Normothermic group and 775.60 ± 148.20 in the SNLF group.

Weight gain was significantly greater in the SNLF group by T12 ESLP (P=0.01; Figure 5.1E). The average weight gain (%) was 29.42 ± 5.72 in the Normothermic group and 67.39 ± 12.50 in the SNLF group.

Oxygenation and Weight-Gain Post-Transplantation

All recipient animals demonstrated stable oxygenation during the post-transplant period while on two-lung ventilation. After 4-hours of reperfusion, isolated left lung assessment was performed as described under Left Lung Transplantation. There was no significant difference in isolated left lung PF ratios between groups (p= 0.88; Figure 5.2A); the average PF ratio was 300.70 ± 52.26 in the Normothermic group and 191.30 ± 46.20 in the SNLF group. Similarly, there was no significant difference in the left lung weight gain following 4-hours of transplant reperfusion (p=0.35; Figure 5.2B). The average percentage weight gain of the transplanted lung was 29.63 ± 7.23 in the Normothermic group and 42.84 ± 15.80 in the SNLF group.

Cytokine Profiles during NPV-ESLP

As demonstrated in our earlier experiments[13,14], TNF-∝ perfusate concentration levels increased during the first few hours of perfusion and then decreased over the following time on ESLP. The SNLF group demonstrated a more dramatic rise in TNF-∝, and concentrations levels were significantly higher from T3-T9 compared to the Normothermic group ($p<0.05$; Figure 5.3A). In contrast, IL-6 perfusate concentrations levels were not significantly different during ESLP between groups, except at T7, which was greater in the normothermic group (p=0.04; Figure 5.3B).

DISCUSSION

Our study investigated the effects of cold perfusion NPV-ESLP in a large animal model with transplantation assessment compared to our standard protocol of Normothermic NPV-ESLP; however, we were unsuccessful at achieving reliable preservation to our target time of 12-hours using cold temperatures. Cold perfusion ESLP at 10℃ and 20℃ resulted in massive pulmonary edema within a few hours. ESLP at 32℃ failed prematurely for the same reasons, but the effects were slower to develop and often failed after the first evaluation at T5. From this experience we concluded that cold ESLP was not a viable option for prolonged preservation. The negative effect of cold ESLP was so drastic that we stopped trials at 20℃ and 32℃ after three sets of failed lungs to avoid unnecessary waste of animal preparations.

The principal issue limiting successful cold ESLP appeared to be elevated PVR due to vasoconstriction, which resulted in higher PAP and hydrostatic pressure leading to fluid extravasation and pulmonary edema; therefore, we attempted additional experiments using with decreased flow rate from 30% to 10% at 32℃ (Subnormothermic Low-Flow, SNLF) to evaluate the effect of reduced flow in cold ESLP. This modification allowed us to achieve 12-hours of ESLP at 32℃. We maintained our standard evaluation parameters at T5 and T11 to allow for accurate comparison and assessment of the lungs. This included warming the SNLF group to 38℃ and increasing flowrate to 50% CO for 5-minutes while administering a de-oxygenation sweep gas. Evaluation at normothermic temperatures during ESLP enables direct comparison between groups and allows for a more physiologic assessment.

Physiological parameters in the SNLF group during ESLP and following transplantation demonstrated either no-significant difference or were significantly worse compared to the Normothermic group. In the transplantation comparison, there was no significant difference in

PF ratios or transplanted lung weight gain between groups; however, failure to detect statistical significance is likely due to our small transplant sample size (n=3 per group). The pro-inflammatory cytokine profiles in the SNLF group were either significantly worse (TNF-∝) or largely similar to the Normothermic group (IL-6). Therefore, the sum of our evidence suggests that Normothermic NPV-ESLP is a superior preservation strategy compared to SNLF preservation at 32℃.

Our standard protocol uses a cellular perfusate with pRBC[10,11,13,14]; however, we used an acellular perfusate for the cold ESLP groups because that was the strategy employed in previous small animal literature[7,8,9], which we sought to approximate. We selected Perfadex as the perfusate for the cold perfusion groups because it has a higher oncotic pressure (57 mmHg)[15] than our in-house CHIP[10] (32 mmHg) perfusate, and we hoped the added oncotic pressure would offset the absence of pRBC that contribute to a colloid effect in our standard protocol. Perfadex was also successfully employed by Gloria *et al.* in a small animal model of ESLP at 25-30℃ (7); therefore, it was established as a reasonable perfusate choice for cold ESLP.

Our data suggest that cold perfusion NPV-ESLP is inferior in a porcine model compared to our standard normothermic perfusion temperature. It appears that the pulmonary endothelium and respiratory epithelium are both negatively affected by cold perfusion, and this manifests as worse physiologic function with reduced compliance, increased PVR, increased edema formation, and greater inflammation. The increased weight gain in the SNLF group, despite reduced perfusion flow rates with a higher oncotic pressure perfusate, suggests greater endothelial injury with increased capillary leak and lung water accumulation due to cold temperature. SNLF lungs also demonstrated lower Cdyn during ESLP, which suggests a deleterious effect on the lung parenchyma and/or surfactant biophysical properties that increased

surface tension[8,9]. This problem was compounded by the gradual development of pulmonary edema, further restricting compliance. Finally, PVR was elevated in the SNLF group, and this is likely due to pulmonary vasoconstriction in response to cold perfusion as well as the mathematical consequence of reduced cardiac output, where PVR = 80[(mean PAP - left atrial pressure)/CO]. Our system uses an open left atrium, so left atrial pressure is always 0 mmHg; therefore, a reduced flowrate increases PVR.

Our results contrast with previous studies of rat ESLP with subnormothermic perfusion that showed superior preservation based on physiologic function, reduced histologic injury, and reduced inflammation[7,8,9]. In 2021, Gloria et al[7] demonstrated in a rat transplant model of positive pressure ventilation (PPV)-ESLP that subnormothermic perfusion at 25℃ resulted in less TNF-∝ release and reduced histologic lung injury compared to normothermic PPV-ESLP. Also published in 2021, Arni et al[8] investigated the effect of various subnormothermic temperatures in a rat model of PPV and concluded that 28℃ is non-inferior to normothermia regarding physiologic function on ESLP with less pro-inflammatory cytokine generation and greater lung tissue ATP content. In a follow-up study[9], rat lungs were perfused for 4-hours at either 28℃ or 37℃ with PPV-ESLP then transplanted, and the subnormothermic preservation demonstrated improved Cdyn and PVR along with lower levels of pro-inflammatory cytokines. In sum, these papers suggest that subnormothermic ESLP is non-inferior or superior to normothermic preservation. Certainly, the contrasting outcomes between these studies and our own could be explained in part by considerably shorter ESLP duration (4- vs 12-hours) and transplant reperfusion (2- vs 4-hours), ventilation differences (PPV vs NPV), specific temperature difference for subnormothermic perfusion (25℃, 28℃, 30℃ vs 32℃), perfusate

differences (STEEN vs Perfadex), and species differences (rat/"small animal" vs pig/"large animal").

Our paper is the first large animal model for NPV-ESLP with transplantation that investigated the use of perfusion temperatures other than normothermia. Our findings suggest that normothermic perfusion should remain the clinical standard[4,16,17,18] given that porcine models better approximate human physiology compared to small animal models. Our study highlights the important research principle of investigating interesting small animal research findings through follow-up research with larger animal preparations.

Study Limitations

Our study has several limitations. Porcine lungs are not a perfect substitute for human lungs; however, they are widely used in preclinical ESLP research due to the comparable size and function. Our study is limited by a small sample size, particularly with our transplant experiments, but this number reflects other preclinical studies[19,20,21]. Likewise, we used a relatively short time-point for transplantation assessment, and this was also based off previous pre-clinical ESLP studies[19,20,21]. Variables other than temperature differed between groups, such as perfusate composition and flow rate. As previously explained, these protocol modifications were made to reflect previous studies and achieve reproducible 12-hour preservation at reduced temperature. Had flow rate not been reduced, none of the cold perfusion experiments would have reached transplantation. This observation strengthens our conclusions by highlighting the difficulty of perfusing at cold temperatures and the severity of its negative effects on lung function. It must also be recognized that the two episodes of re-warming cold lungs at T5 and T11 for evaluation at 50% CO with a physiologic temperature may have contributed to lung injury. Evaluating at normothermia was not performed in previous studies of cold ESLP[7,8,9];

however, cold evaluation would not provide an accurate assessment of physiologic performance for transplant. Rewarming and cooling of the lungs is typical at the conclusion of clinical ESLP prior to implant without known detriment. Cold perfusion is beneficial in some organs during ex-situ perfusion[22-27] but was detrimental in our study specific to juvenile porcine lungs. A study looking specifically at cold perfusion with our standard cellular perfusate would be of interest.

CONCLUSIONS

In conclusion, NPV-ESLP performed at normothermic temperature results in superior lung function and reduced levels of inflammation in a pre-clinical porcine model compared to cold perfusion. A lower perfusion flow rate was required during ESLP at subphysiologic temperature to prevent premature graft failure (<12 hours).

Figure and Table Legends

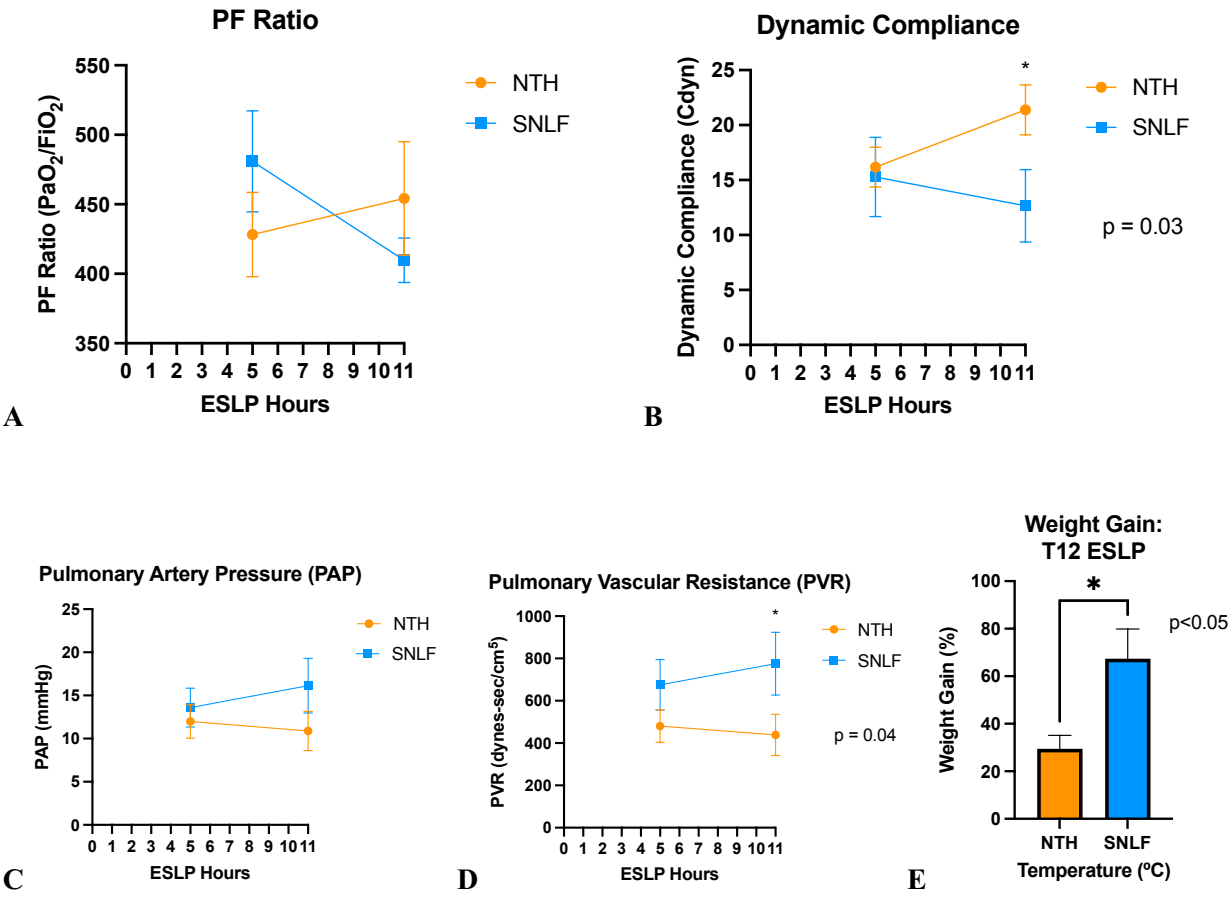

Figure 5.1. Physiologic function during NPV-ESLP: Normothermic (NTH) vs Subnormothermic Low-Flow (SNLF). A, There was no significant difference in PF ratio between temperature groups at T11 evaluation. B, Dynamic compliance was significantly lower in the SNLF group at T11 evaluation ($P<0.05$). C, Pulmonary artery pressure (PAP) were not significantly between groups at T5 or T11 evaluation. D, Pulmonary vascular resistance (PVR) was significantly higher in the SNLF group at T11 evaluation ($P<0.05$). E, Weight gain (%) was significantly higher in the SNLF group at T12 ($P<0.05$). Normothermic = 38℃ and 30% cardiac output; Subnormothermic Low-Flow = 32℃ and 10% cardiac output.

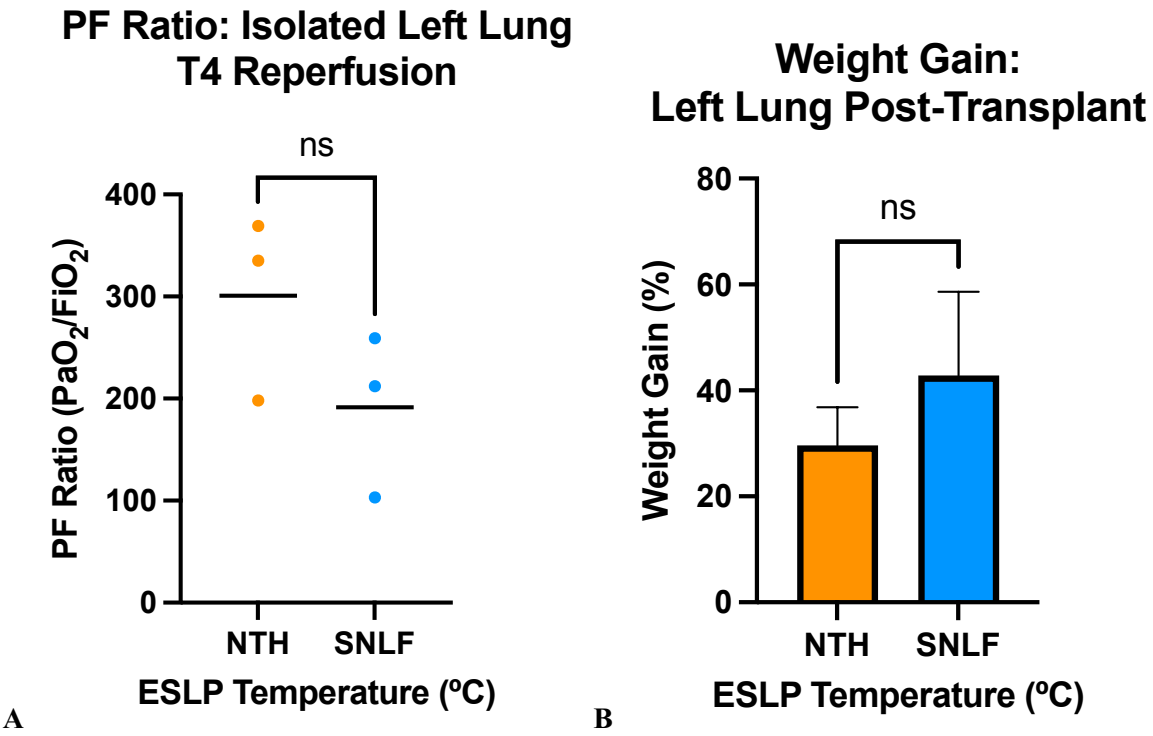

Figure 5.2. Oxygenation and weight gain post-transplantation: Normothermic (NTH) vs Subnormothermic Low-Flow (SNLF). A, There was no significant difference in PF ratio between temperature groups at T4 reperfusion following 10-minutes of isolated left lung evaluation (p=0.09). B, There was no significant difference in weight gain (%) of the left lung pre- vs post-transplant between temperature groups (p=0.24). Normothermic (NTH) = 38℃ and 30% cardiac output; Subnormothermic Low-Flow (SNLF) = 32℃ and 10% cardiac output.

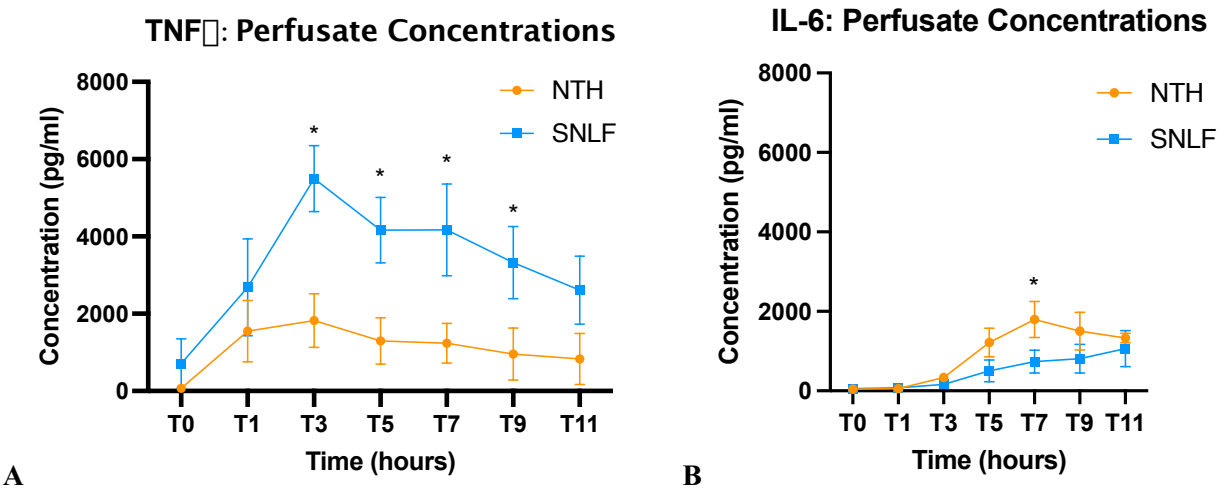

Figure 5.3. Cytokine Profiles during NPV-ESLP: Normothermic (NTH) vs Subnormothermic Low-Flow (SNLF). A, The SNLF group demonstrated a greater rise in TNF-∝ during ESLP with significantly higher concentrations from T3-T9 compared to the NTH group (38℃, *P<0.05). B, IL-6 perfusate concentrations levels were not significantly different between groups during ESLP, except at T7, which was greater in the NTH group (*P<0.05). Normothermic = 38℃ and 30% cardiac output; Subnormothermic Low-Flow = 32℃ and 10% cardiac output.

Table 5.1. NPV-ESLP Perfusion and Ventilation Strategy: Normothermic vs Subnormothermic Low-Flow			
12-hour Protocol	Normothermic (NTH)	Subnormothermic Low-Flow (SNLF)	NTH and SNLF
Perfusion Parameters	Preservation		Evaluation
Temperature	NTH 38℃	SNLF 32℃	38℃
Perfusate Flow	30% predicted CO (CO = 70mL/kg/min)	10% predicted CO (CO = 70mL/kg/min)	50% predicted CO (CO = 70mL/kg/min)
Pulmonary Artery Pressure	< 20 mmHg	< 20 mmHg	< 25 mmHg
Left Atrial Pressure	0 mmHg	0 mmHg	0 mmHg
Medical Gas Mixture (89%N_2, 8%CO_2, 3% O_2)	-	-	0.125L/min
Ventilation Parameters			
Mode	Pressure Control	Pressure Control	Pressure Control
Target Tidal Volume	6-8 ml/kg	6-8 ml/kg	10 ml/kg
End-Inspiratory Pressure	- 10 to -12 cmH_2O	- 10 to -12 cmH_2O	-15 cm H_2O
Inspiratory: Expiratory Ratio	1:1-1.2	1:1-1.2	1:1
Respiratory Rate	6-8	6-8	10
Peak Airway Pressure	< 25 cmH_2O	< 25 cmH_2O	$\leq$ 25 cmH_2O
PEEP	8	8	5
FiO_2	21%	21%	21%

Adapted from Forgie et al.[11]

Legend: CO, cardiac output; ESLP, ex-situ lung perfusion; PEEP, positive end expiratory pressure; FiO_2, fraction of inspired oxygen.

Chapter 6

Moderate-Flow Perfusion is Superior to Low-Flow Perfusion in Ex-Situ Lung Perfusion

Abstract

Background: Full-flow perfusion during prolonged ex-situ lung perfusion (ESLP) results in unacceptable pulmonary edema formation. Clinical ESLP at 30-50% predicted cardiac output (CO) supports acceptable physiologic outcomes; however, progressive pulmonary edema still develops. Lower flow rates may provide equivalent physiologic preservation with less edema formation due to reduced hydrostatic pressures. We report our results of Moderate-Flow (MF; 30% CO) vs. Low-Flow (LF; 10% CO) negative pressure ventilation (NPV)-ESLP with transplantation.

Methods: Twelve pig lungs underwent 12-hours of NPV-ESLP with 30% or 10% CO (n= 6/group). Three left lungs per group were transplanted post-ESLP and assessed *in-vivo* over 4-hours. Lung function was assessed by physiologic parameters, weight-gain, and pro-inflammatory cytokine profiles.

Results: Results are MF vs. LF (mean ± SEM). All lungs demonstrated acceptable oxygenation post-ESLP (454.2 ± 40.85 vs 422.7 ± 31.68, p = 0.28); however, after transplantation MF lungs demonstrated significantly better oxygenation (300.7 ± 52.26 vs 141.9 ± 36.75, p = 0.03). There was no significant difference in compliance after ESLP (21.38 ± 2.28 vs 16.48 ± 2.34, p = 0.08); however, PAP (10.89 ± 2.28 vs 21.11 ± 0.93, p = 0.06) and PVR (438.60 ± 97.97 vs 782.20 ± 162.20, p = 0.05) were significantly higher in the LF group. Weight gain (%) post-ESLP and post-transplant was similar between groups (29.42 ± 5.72 vs 24.17 ± 4.42, p=0.24; 29.63 ± 7.23 vs 57.04 ± 15.78, p=0.09). TNF-α and IL-6 were significantly greater throughout LF ESLP.

Conclusions: Prolonged NPV-ESLP with transplantation suggest that MF produces superior lung function compared to LF.

Introduction

Lung transplantation is the gold standard treatment for end-stage lung disease; however, considerable limitations exist, including an inadequate supply of high-quality donor lungs[1-4]. Approximately 80% of lungs offered for donation are rejected for transplantation due to stringent organ acceptance criteria[2-5]. This is significantly higher than other solid organ transplants. Although estimates vary, the supply-demand mismatch has resulted in a high waitlist mortality of 15-40%[1-4].

Cold static preservation (CSP) is the current clinical standard for organ preservation, flushing the lungs with a cold preservation solution and storing them on ice in a cooler, but it has several limitations. CSP does not allow for physiologic assessment during preservation or prior to transplantation; there is no opportunity for pharmacologic optimization of the donor lungs; and the preservation time is limited to 6-8 hours[5,6,7]. The benefit of CSP stems from its simplicity and the application of cold, which arrests metabolism thereby limiting the development of deleterious cellular by-products. Unfortunately, this process also prevents beneficial metabolic function from healing the lungs.

Portable ex-situ lung perfusion (ESLP) offers a superior means of preserving donor lungs by perfusing and ventilating them continuously at normothermic temperatures from retrieval to implantation. This results in a more physiologic preservation strategy with the added benefit of continuous assessment and the potential to treat various lung pathologies to improve organ function. Clinically, normothermic negative pressure ventilation (NPV) and positive pressure ventilation (PPV)-ESLP have demonstrated that marginal quality donor lungs can be reconditioned to acceptable transplant standards, thereby increasing the pool useable donor lungs [8,9,10,11].

Clinically, ESLP flow rates of 30-50% of predicted cardiac output (CO) are standard [8,9,10,11], and perfusion above 30-50% CO result in unacceptable pulmonary edema over prolonged ESLP [12,13,14]. Subphysiologic flow rates of 30-40% do not appear to cause clinically significant ischemic injury during prolonged ESLP, and the reduced hydrostatic pressure results in less pulmonary edema over time [12,13,14]. It is unknown if further reductions in flow rate would results in further reductions in edema formation or improved preservation. Indeed, there is a paucity of literature specifically investigating outcomes in relation to varying perfusion flow rates [12,13,14]. Recent literature in small and large animal models suggests that reduced perfusate flow may be advantageous [12,13,14]. Therefore, we sought to determine if further reductions in ESLP flow may produce non-inferior or superior ESLP preservation and transplantation outcomes using a large animal model.

Methods

The University of Alberta animal care and use committee approved all experimental protocols (AUP943). Animals received humane care in accordance with the “Principles of Laboratory Animal Care” as dictated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institute of Health (NIH Publication No. 86-23, revised 1996). Methods are compliant with the ISHLT Ethics statement.

Experimental Design

Twelve female domestic pigs (45 ± 5 kg) were allocated to two perfusion flow groups based on predicted cardiac output (CO; 70mL/kg/min)(Table 6.1): 30% (Moderate-Flow/Control) and 10% (Low Flow/Experimental Group) and perfused for 12-hours on NPV-ESLP. All lungs were

perfused at normothermia (38℃ for pigs) and perfused with 1L common-hospital ingredient perfusate (CHIP) with 500ml autologous packed red blood cells (pRBC)[15]. The following medications were added to the perfusate: 3.375 g of piperacillin-tazobactam, 10,000 IU of heparin, and 500 mg of methylprednisolone. Insulin (2 U/h) was continuously infused into the circuit. A dextrose (25%) infusion was titrated to target a perfusate glucose concentrations of 4-6 mmol/L. Three left lungs from each group were subsequently transplanted into recipient pigs and monitored for 4-hours. The right lung was clamped, and the transplanted left lung was evaluated in isolation for oxygenation capacity followed by euthanasia. Our methods for donor organ procurement, NPV-ESLP, and left lungs transplantation have been described in detail[16,17] and are summarized below.

Donor Lung Procurement

Animals were sedated with ketamine (20 mg/kg), and atropine sulfate (0.05 mg/kg). Animals were intubated and anesthetized with 1-3% isoflurane. Median sternotomy was performed. Heparin was administered directly into the superior vena cava (SVC). *In vivo* wedge biopsies were taken along with blood samples. The SVC was transected and a poole tip suction was inserted into the SVC and right atrium for exsanguination, draining into a cell-saver for blood collection. Cardiectomy was performed, the lungs were dissected free from their attachments, the trachea was clamped at a constant airway pressure of 20 cm H_2O. The lungs were removed, weighed, and connected to NPV-ESLP.

Moderate-Flow NPV-ESLP

Lung wedge biopsies were taken immediately prior to NPV-ESLP connection. The trachea was intubated with a modified endotracheal tube in a manner that maintains lung inflation, connecting them to the NPV-ESLP. The pulmonary artery was connected to the perfusion circuit, and perfusion was initiated at 10% of predicted cardiac output. The perfusate temperature was warmed from ambient room temperature to 32℃ over 10-minutes. Once perfusion was established, a continuous positive airway pressure (CPAP) of 20 cm H_2O was applied, and the trachea was unclamped. At 32℃, the air-tight chamber lid was secured and NPV was initiated by simultaneously decreasing CPAP and end-inspiratory pressure (EIP) until a CPAP of 8 cm H_2O and EIP -12 cm H_2O was achieved. The perfusate temperature was gradually warmed to a target of 38℃ within 30-minutes of perfusion. Every 10-minutes, perfusion flow rate was increased by 10% of CO until a target flow of 30% CO. Lung evaluation (5-minute duration) was performed at hour 5 (T5) and hour 11 (T11) of NPV-ESLP. A medical sweep gas mixture (89% N_2, 8% CO_2, and 3% O_2) was used to deoxygenate the perfusate during evaluation and assess for pre- versus post-lung oxygenation step-up (Table 6.1).

Low-Flow NPV-ESLP

The protocol for the Low-Flow NPV-ESLP group was identical to that for Moderate Flow NPV-ESLP except the perfusion flow rate was not increased above 10% CO during initiation and preservation. The two groups were evaluated at T5 and T11 using 50% of CO over a 5-minute duration to improve V/Q mismatching and more accurately assess physiologic function (Table 6.1).

Left Lung Transplantation

The animals were sedated with Ketamine 20mg/kg, Hydromorphone 0.05mg/kg, and Atropine 0.05mg/kg. An arterial line and central line were inserted into the carotid artery and jugular vein, respectively. Animals were placed in a modified right-lateral decubitus position. A left thoracotomy was performed, and the donor left lung hilar structures were dissected free. When the donor lung was ready for implant, the recipient left lung structures were clamped and the native lung was discarded. The donor lung was flushed with 0.5L of extracellular, low-potassium, dextran-based solution (4°C) to remove debris and ESLP perfusate remnants while cooling the lung. The donor lung was implanted, and the recipient was monitored for 4-hours. After 4-hours, a sternotomy was performed, the right lung hilum and accessory lobe were clamped to obstruct perfusion and ventilation, enabling isolated left lung assessment. An isolated left lung blood sample was drawn at 10-minutes to evaluate the transplanted lung function. Animals were subsequently euthanized, and the left lung was explanted, inspected for gross pathology, and weighed.

Physiologic Evaluation

During ESLP, physiologic parameters were continuously assessed, including pulmonary artery pressure (PAP), pulmonary vascular resistance (PVR), dynamic compliance (Cdyn), and the ratio of partial pressure of oxygen in the perfusate to the fraction of inspired oxygen (PF ratio, PaO_2/FiO_2). During evaluation on ESLP and post-transplant, PF ratios were calculated from the left atrium perfusate/blood sample. Lung weight was recorded at the following timepoints: post-explant, T0, T12. Left lung weight was recorded pre- and post-transplant. Tissue biopsies were

taken at similar timepoints. Edema formation was estimated using percentage of weight gain (weight gain [%] = {[End weight - Start weight]/Start weight}x 100%).

Perfusate Cytokine Analysis

Perfusate samples were collected every two hours during ESLP. Perfusate concentrations of pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-∝) and interleukin-6 (IL-6) were determined using enzyme-linked immunosorbent assay kits (R&D systems, Minneapolis, MN).

Statistical Analysis

GraphPad Prism Software (Version 9; GraphPad Software Inc, La Jolla, California) was used for statistical analysis. Unpaired Student *t* tests were performed to compare normally distributed continuous variables between groups. Normality was assessed using the Shapiro-Wilk test. Results are expressed as mean ± standard error. $P < 0.05$ was considered statistically significant.

Results

NPV-ESLP: Physiologic Function

All results are presented as Moderate-Flow vs. Low-Flow and mean ± SEM. Moderate-Flow and Low-Flow groups demonstrated stable and acceptable oxygenation during 12-hours of ESLP. There was no significant difference in PF ratio at T5 (p=0.15) and at the final evaluation T11 (p= 0.28; Figure 6.1A). The average PF ratio at T11 was 454.20 ± 40.85 in the Moderate-Flow group and 422.70 ± 31.63 in the Low-Flow group. All lungs preserved on ESLP had PF ratios above 300 at T11, which met our criteria for transplantation.

Dynamic compliance did not differ significantly between groups at T5 (p=0.42) and T11 (p=0.08; Figure 6.1B). The average Cdyn at T11 was 21.38 ± 2.28 in the Moderate-Flow group and 16.48 ± 2.34 in the Low-Flow group.

Pulmonary artery pressures were significantly lower in the Moderate-Flow group at T5 (p=0.001) and T11 ESLP (p=0.002; Figure 6.1C). At T11, the average PAP was 10.89 ± 2.28 in the Moderate-Flow group and 21.11 ± 0.93 in the Low-Flow group. Pulmonary vascular resistance (PVR) was significantly greater in the Low-Flow group compared to the Moderate-Flow group at T5 (p=0.003) and T11 of ESLP (p=0.05; Figure 6.1D). The average PVR at T11 was 438.60 ± 97.97 in the Moderate-Flow group and 782.20 ± 162.20 in the Low-Flow group.

Weight gain did not differ significantly between groups by T12 ESLP (p=0.24; Figure 6.1E). The average weight gain (%) was 29.42 ± 5.72 in the Moderate-Flow group and 24.17 ± 4.42 in the Low-Flow group.

NPV-ESLP: Pro-Inflammatory Cytokine Profiles

Similar to our previous experiments[18,19], TNF-∝ perfusate concentration levels increased during the first three hours of perfusion and then slowly decreased over the remaining duration of ESLP

in both groups (Figure 6.2A). The Low-Flow group demonstrated a more dramatic rise in TNF-∝ with concentrations levels significantly higher from T1-T3 ($p<0.05$; Figure 6.2A) and T5-T11 ($P<0.01$; Figure 6.2A) compared to the Moderate-Flow group. Likewise, IL-6 perfusate concentrations levels were also significantly greater in the Low-Flow group during ESLP at T1 ($P<0.05$; Figure 6.2B), T3 ($P<0.001$; Figure 6.2B), and from T5-11 ($P<0.01$; Figure 6.2B) compared to the Moderate-Flow group. In contrast to TNF-∝, IL-6 continued to increase until T7 in the Moderate-Flow group and T9 in the Low-Flow group.

Oxygenation and Weight Gain Post-Transplantation

In the post-transplant monitoring period, all animals demonstrated stable oxygenation while on two-lung ventilation. After 4-hours of reperfusion, isolated left lung assessment was performed as previously described. Moderate-Flow ESLP resulted in an average isolated left lung PF ratio that was significantly higher than the Low-Flow group ($P= 0.03$; Figure 6.3A). The average PF ratio was 300.70 ± 52.26 in the Moderate-Flow group and 141.9 ± 36.75 in the Low-Flow group. There was no significant difference in left lung weight gain following 4-hours of transplant reperfusion ($P=0.09$; Figure 6.3B). The average weight gain of the transplanted lung was 29.63 ± 7.23 in the Moderate-Flow group and 57.04 ± 15.79 in the Low-Flow group.

Discussion

Our study investigated the effects of Moderate-Flow versus Low-Flow normothermic NPV-ESLP in a large animal transplant model. The Moderate-Flow group is our pre-existing protocol with 30% flow [8-11], and 30-50% flow is standard across other published clinical protocols [8-11]. Physiological parameters in the Low-Flow group during ESLP and following transplantation demonstrated either no-significant difference or were significantly worse compared to the Moderate-Flow group. Post-transplantation, the Low-Flow group demonstrated significantly worse oxygenation in the isolated left lung. Furthermore, pro-inflammatory cytokine profiles in the Low-Flow group were significantly greater (TNF-∝ and IL-6), demonstrating a more profound inflammatory response. The sum of our evidence suggests that Moderate-Flow NPV-ESLP is a superior preservation strategy compared to Low-Flow perfusion. Furthermore, our data highlights the importance of *in-vivo* transplantation assessment.

On ESLP, Moderate-Flow and Low-Flow lungs demonstrated similar physiologic performance, except Low-Flow lungs had higher PAP and PVR throughout ESLP. This observation can be explained by increased pulmonary vasoconstriction and endothelial dysfunction in response to 10% CO as well as the mathematical consequence of reduced flow, where PVR = 80[(mean PAP - left atrial pressure)/CO]. Interestingly, the ischemic-reperfusion injury after transplantation was greater in the Low-Flow group once exposed to full cardiac output as evidenced by significantly worse oxygenation. This suggests that the Low-Flow group experienced an ESLP-induced injury that was not noticeable on the ESLP machine using our standard physiologic parameters and evaluation protocol. This explanation is substantiated by the significantly greater concentrations of pro-inflammatory cytokines in the Low-Flow perfusate. The use of *in-vivo* transplantation assessment is important when attempting to differentiate

between ESLP management strategies that are highly similar, and transplantation should become more common in pre-clinical research as protocols undergo further refinement.

We chose to compare our standard perfusion flow rate of 30% CO to a much lower 10% CO for two reasons: 1) Beller et al.[14] demonstrated improved preservation on PPV-ESLP at 20% CO compared to 40% CO, suggesting that reduced hydrostatic pressure from lower flow was beneficial for lung grafts; and 2) A flow of 10% CO had a protective effect in our previous experiments using subnormothermic perfusion (32℃) compared to 30%CO at subnormothermic temperatures [20]; therefore, it was justifiable to assess the physiologic effect of 10% CO during normothermic ESLP. Looking ahead, it is important to compare perfusion flow rates of 30%, 40%, and 50% CO during NPV-ESLP to further refine our protocol. Given that these flow rates have all been proven safe clinically[8-11], comparison experiments will likely require ESLP durations of 24-hours or longer to allow sufficient time for physiologic divergence.

There are important similarities and differences worth noting between our study and that of Beller et al[14]. Like Beller et al., we choose an absolute flow difference of 20% between our Experimental and Control groups believing this difference was adequate to reveal a performance divergence during 12-hours of ESLP. Unlike Beller et al. who performed PPV-ESLP for 4-hours, which is a common duration clinically[8-11], we performed NPV-ESLP for 12-hours. Both studies included *in-vivo* transplantation assessment after ESLP. We selected a prolonged ESLP strategy because our lab is focused on improving the application of ESLP for safe prolonged continuous preservation, and we believe 12-hour ESLP will become more common clinically to address geographic and logistical barriers to lung transplantation.

Another important difference between the protocol of Beller et al. and our lab was how cardiac output was calculated. In our protocol, we calculate $CO_{predicted}$ based on a 100% CO of

70mL/kg/min. In contrast, Beller at al. calculated $CO_{predicted}$ based on 100% CO of 100mL/kg/min. Due to this discrepancy, the flow rate by Beller et al. at 20% CO is very similar to our flow rate at 30% CO. Likewise, a flow of 40% CO by Beller et al. approximates a 50% CO by our protocol. From this observation, we suggest that the study by Beller et al. provides a relative comparison of 30% vs 50% CO using our calculations. From this extrapolation, we believe that ESLP with 30% CO is the best supported perfusion flow rate in the literature based on studies directly comparing flow rates in porcine models. As mentioned previously, a direct comparison of 30% and 40% CO is warranted but would likely require prolonged preservation (>24-hours) to distinguish any statistical significance in performance due to the small absolute difference between flows.

Our study is the first large animal model of prolonged NPV-ESLP with transplantation that investigated the utility of Moderate-Flow versus Low-Flow perfusion. Our data suggest that Moderate-Flow perfusion NPV-ESLP is superior to Low-Flow perfusion in a porcine model. The comparable PF ratios during ESLP is possibly due to the increased "dwell-time" of low-flow perfusate through the pulmonary vasculature, which allows maximum oxygen uptake. Oxygen diffusion in a healthy lung model across the pulmonary capillary bed should be virtually instantaneous (0.25 sec) given the high-solubility of oxygen and the thin alveolar-capillary interface[21]. Normally, oxygen transfer is perfusion limited; however, if the diffusion interface is impaired due to endothelial dysfunction or interstitial edema, oxygen transfer becomes diffusion limited. Additional "dwell time" of the perfusate within the capillary bed can enhance oxygen diffusion. It is also possible that local changes in the vascular beds of dependent lobes optimize V/Q matching, which mitigates perfusion limitations to oxygenate transfer. Of note, ESLP PVR and PAP were significantly higher in the Low-Flow group, and this suggests increased

endothelial dysfunction due to ischemia. The worse performance of Low-Flow lungs post-transplant is likely due to profound endothelial injury from inadequate perfusion of non-dependent areas while on ESLP. These injured vascular beds are revealed once full cardiac output and elevated hydrostatic pressures are applied continuously *in-vivo*, resulting in a worse ischemic-reperfusion injury compared to Moderate-Flow lungs. ESLP evaluation is performed at 50% CO to improve V/Q matching, but the short duration of 5-minutes does not sufficiently stress the injured vasculature and lung parenchyma to produce irreparable injury, thus permitting a successful 12-hours of ESLP.

Limitations

Our study has several limitations worth mentioning. This study has a small n value for the number of transplantations performed; however, this number is reflective of other preclinical studies[22, 23, 24]. Similarly, the post-transplantation reperfusion and isolated lung assessment periods are relatively short for post-operative assessment; however, this duration was also based on previous pre-clinical ESLP studies[22, 23, 24]. Only two perfusion flow rates were compared, 30% vs 10% CO; therefore, our conclusions are not definitive regarding optimal flowrates for prolonged ESLP. Further study investigating other CO (%) flows would provide valuable information for ESLP protocol refinement, particularly with perfusion of 24-hours and beyond.

Conclusions

In conclusion, NPV-ESLP performed at Moderate-Flow (30% CO) results in superior lung preservation and transplant function with reduced levels of pro-inflammatory cytokine production compared to Low-Flow (10% CO) NPV-ESLP.

Figure and Table Legends

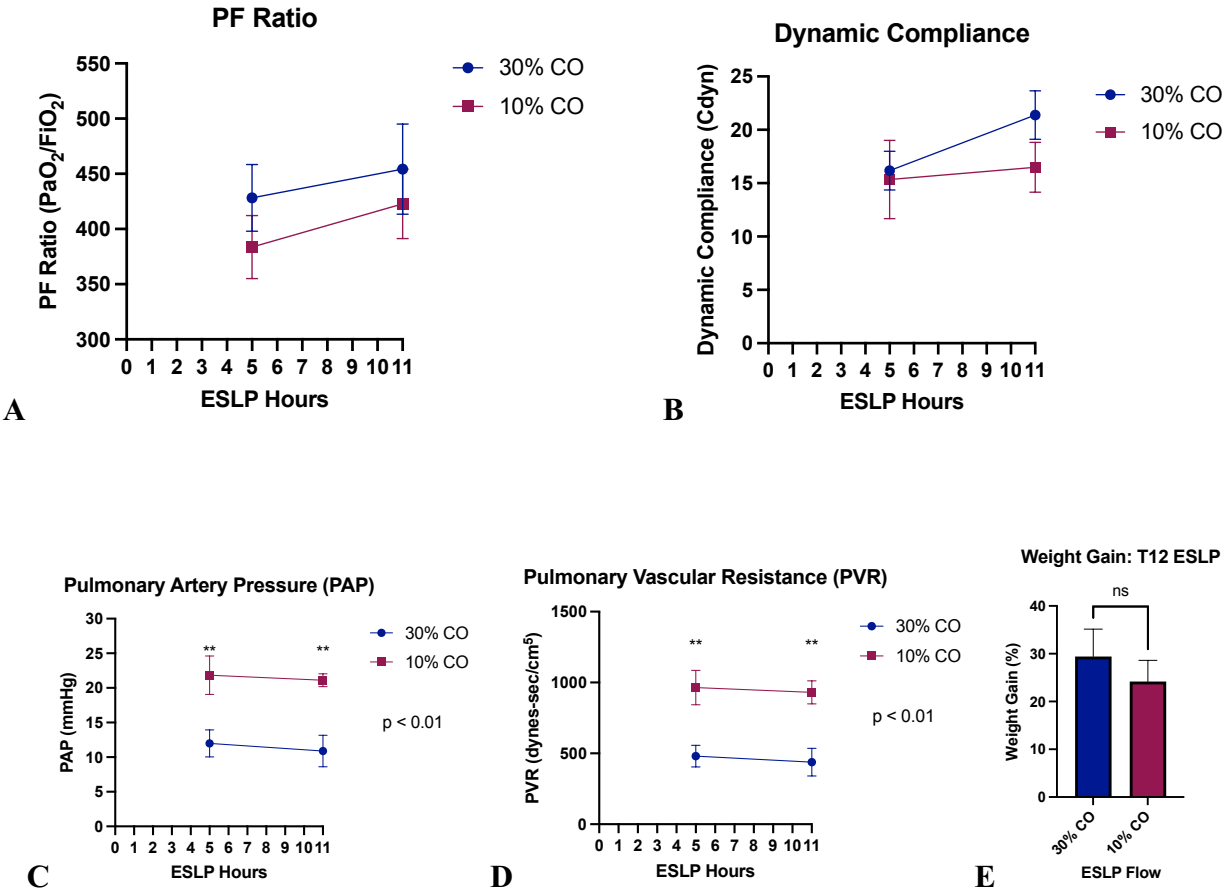

Figure 6.1. Physiologic function during NPV-ESLP: moderate flow (30% CO) vs low flow (10% CO). A, There was no significant difference in PF ratios between flow groups at T5; or T11 evaluations. B, Dynamic compliance was comparable in the two group at T5 and T11 evaluations as well. C, Pulmonary artery pressures were significantly lower at T5 (**p=0.001) and T11(**p=0.002) evaluations in the moderate flow group. D, Pulmonary vascular resistance was significantly higher in the low flow group at T5 (**p=0.003) and T11 evaluations (**p=0.002). E, Weight gain (%) did not differ significantly between flow group at T11 evaluation.

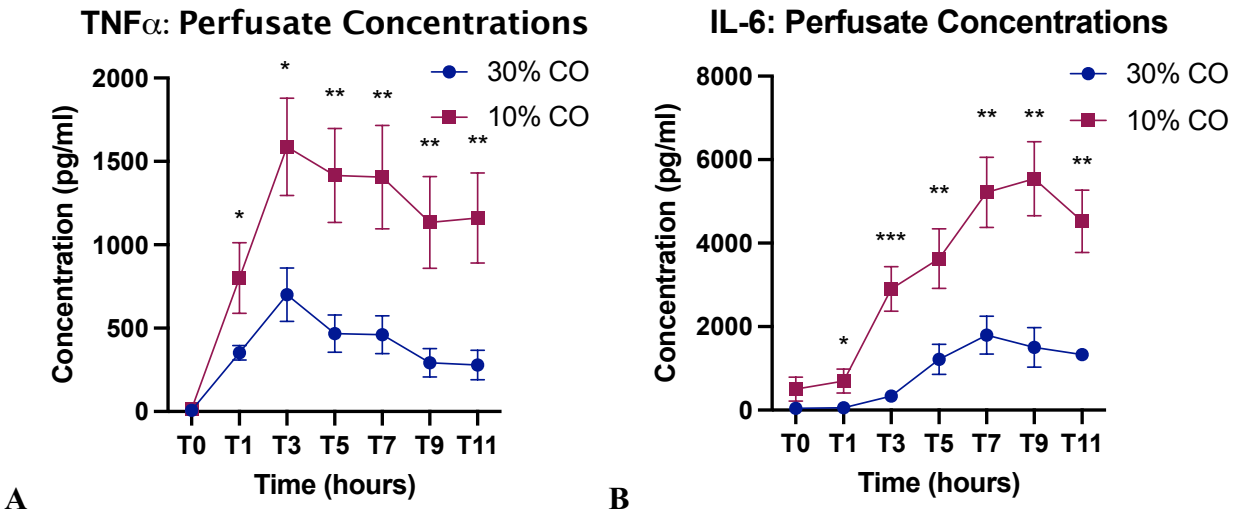

Figure 6.2. Cytokine Profiles during NPV-ESLP. A, The Low-Flow (10% CO) group demonstrated a greater rise in TNF-∝ during ESLP with significantly higher concentrations from T1-T3 (*P<0.05) and T5-T11 (**P<0.01) compared to the Moderate-Flow (30% CO) group. B, IL-6 perfusate concentrations levels were also significantly greater in the Low-Flow group during ESLP at T1 (*P<0.05), T3 (***P<0.001), and T5-T11 (**P<0.01) compared to the Moderate-Flow group.

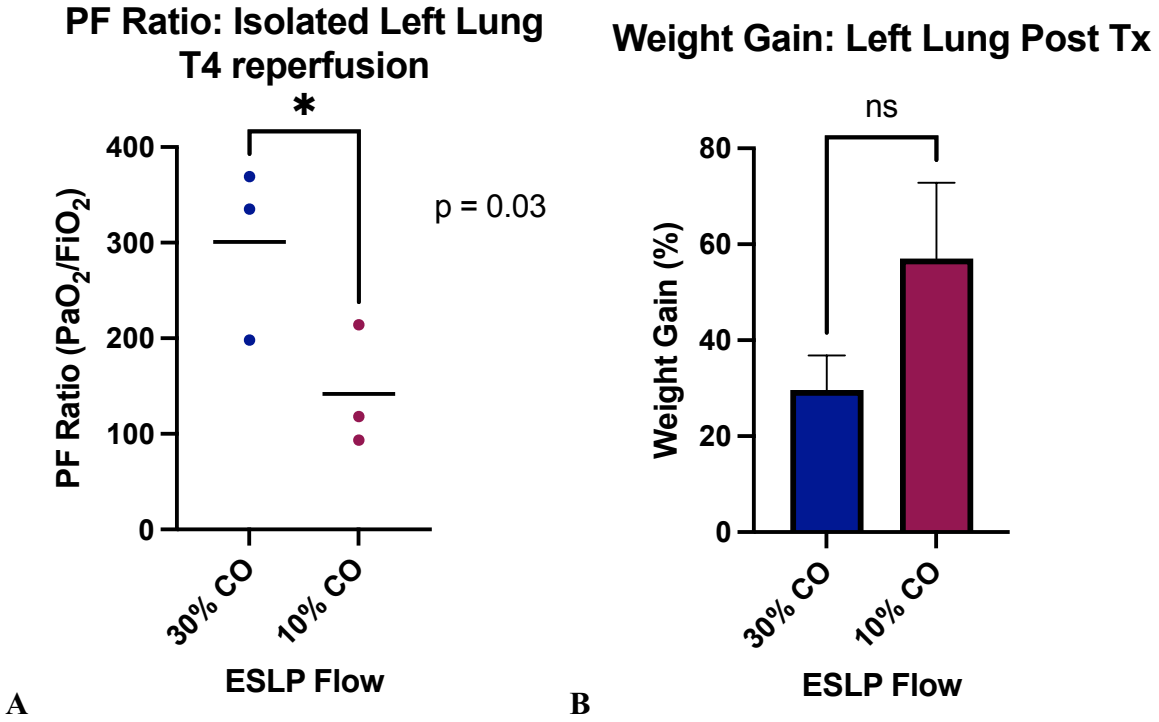

Figure 6.3. Oxygenation and weight-gain post-transplantation. A, The PF ratio of the Moderate-Flow group (30% CO) was significantly greater than the Low-Flow group (10% CO) group at T4 reperfusion following 10-minutes of isolated left lung evaluation (p=0.03). B, There was no significant weight gain (%) difference in the transplanted left lung pre- vs post-transplant between groups (p=0.09). CO, cardiac output; PF ratio, PaO_2/FiO_2; T4, fourth hour of reperfusion.

Table 6.1. NPV-ESLP Perfusion and Ventilation Strategy: Moderate-Flow vs Low-Flow			
Perfusion Parameters	Preservation		Evaluation
Temperature	38℃	38℃	38℃
Perfusate Flow	30% predicted CO (CO = 70mL/kg/min)	10% predicted CO (CO = 70mL/kg/min)	50% predicted CO (CO = 70mL/kg/min)
Pulmonary Artery Pressure	< 20 mmHg	< 20 mmHg	< 25 mmHg
Left Atrial Pressure	0 mmHg	0 mmHg	0 mmHg
Medical Gas Mixture (89%N_2, 8%CO_2, 3% O_2)	-	-	0.125L/min
Ventilation Parameters			
Mode	Pressure Control	Pressure Control	Pressure Control
Target Tidal Volume	6-8 ml/kg	6-8 ml/kg	10 ml/kg
End-Inspiratory Pressure	- 10 to -12 cmH_2O	- 10 to -12 cmH_2O	-15 cm H_2O
Inspiratory: Expiratory Ratio	1:1-1.2	1:1-1.2	1:1
Respiratory Rate	6-8	6-8	10
Peak Airway Pressure	< 25 cmH_2O	< 25 cmH_2O	$\leq$ 25 cmH_2O
PEEP	8	8	5
FiO_2	21%	21%	21%

Adapted from Forgie et al.[16]

Legend: CO, cardiac output; ESLP, ex-situ lung perfusion; PEEP, positive end expiratory pressure; FiO_2, fraction of inspired oxygen.

Chapter 7

Mild Permissive Alkalosis Improves Outcomes in Porcine Negative Pressure Ventilation Ex-Situ Lung Perfusion

Abstract

Background: Ex-Situ Lung Perfusion (ESLP) employs a membrane deoxygenator and mixed ($N_2/O_2/CO_2$) or pure sweep gas (CO_2) to target venous blood gas composition with physiologic pCO_2 and pH. Clinically, mild permissive alkalosis counteracts elevated pulmonary vascular resistance (PVR) to improve perfusion. Increase PVR and pulmonary artery pressure (PAP) during ESLP mirrors rising pro-inflammatory cytokines. Increased hydrostatic pressure worsens edema and lung function. We report improved ESLP outcomes using mild permissive alkalosis.

Methods: Twelve juvenile pig lungs underwent 12-hour Negative Pressure Ventilation (NPV)-ESLP with a physiologic pH (Control: pH 7.35-7.45, n=6) or mild permissive alkalosis (pH+: pH 7.45-7.55, n=6) by varying sweep CO_2 delivery. Three left lungs per group were transplanted and assessed over 4-hours.

Results: Five Control lungs failed on ESLP due to high PAPs, low compliance, and poor oxygenation. Repeat Controls (n=6) were performed to attain 12-hours of ESLP. There were no failures in the pH+ group. Results are pH+ vs Control. Oxygenation (PaO_2/FiO_2 454.2 vs 438.2; p=0.37) and dynamic compliance (21.38 vs 22.22 ml/cmH_2O; p=0.41) were stable over 12-hour NPV-ESLP. Mean evaluation pH/pCO_2/HCO_3^- was 7.50/15.6/14.5 vs 7.41/38.7/24.7. Control lungs required repeat THAM and milrinone boluses on ESLP to prevent acidosis and treat elevated PVR; this was not necessary in the pH+ group. Weight-gain/hour was similar (1.23% vs 1.38%; p=0.37). Mean left lung PF ratios 4-hours post-transplantation were 301 mmHg vs 196 mmHg (p=0.11). Control TNF-α and IL-6 perfusate concentrations were significantly greater.

Conclusions: Mild permissive alkalosis porcine NPV-ESLP demonstrated more reliable preservation with reduced inflammation compared to a physiologic pH strategy.

INTRODUCTION

Ex-Situ Lung Perfusion (ESLP) was designed for short durations of organ assessment and reconditioning to expand the donor pool, and the field quickly advanced towards longer durations of preservation to extend that aim[1-4]. In 2001, Steen et al. achieved the first clinical 4-hour ESLP[1]. By 2008, preclinical ESLP had reached 12-hours[3]. In 2017, 24-hours of continuous porcine ESLP with acceptable transplant outcomes was achieved with OCS™ Lung [5,6]. However, progress in continuous ultra-prolonged preservation has plateaued as other platforms failed to reach this milestone.

Dogmatic ESLP protocols that include untested practices may explain the lack of progress in ultra-prolonged ESLP. Indeed, insufficient studies investigate fundamental aspects of ESLP including ideal perfusion temperature[7], flowrate[8,9], and blood gas management. Such protocol elements are controllable variables that form the basis of outcomes. Optimized perfusion and ventilation are paramount to achieve ultra-prolonged ESLP.

ESLP protocols recommend physiologic blood gas targets[1-4], and alternative targets have not been tested, although deviations in blood gas composition are occasionally applied clinically to improve outcomes[10-12]. For example, elevated pulmonary vascular resistance (PVR) is common in congenital heart disease, and mild permissive alkalosis is employed by some intensive care centres to induce protective pulmonary vasodilation[11]. We have previously observed an initial increase in PVR and pulmonary artery pressure (PAP) during porcine ESLP that mirrors a rise in pro-inflammatory cytokines. Increased PAP and hydrostatic pressure can worsen edema and lung compliance[13]; therefore, mild permissive alkalosis (pH 7.46-55) may improve outcomes in porcine ESLP by preventing a deleterious rise in PVR that leads to premature graft failure.

METHODS

Animals

The University of Alberta animal care and use committee approved all protocols (AUP943). Animals received humane care following “Principles of Laboratory Animal Care” by the National Society for Medical Research and "Guide for the Care and Use of Laboratory Animals" by the Institute of Laboratory Animal Resources, published by the National Institute of Health (NIH Publication No. 86-23, revised 1996). Methods are compliant with the ISHLT Ethics statement.

Experimental Design

Twelve female domestic pigs (45 ± 5 kg) were allocated to two pH groups based on sweep CO_2 delivery: physiologic pH (Control: pH 7.35-7.45, n=6) and mild permissive alkalosis (pH+: pH 7.46-7.55, n=6). Lungs were perfused for 12-hours of normothermic (38℃ for pigs) Negative Pressure Ventilation (NPV)-ESLP using 1L common-hospital ingredient perfusate, 500ml autologous packed red blood cells[14], Piperacillin-tazobactam (3.375g), heparin (10000 IU), and methylprednisolone (500mg). Insulin (2 U/h) and dextrose (25%) were infused to target a perfusate glucose concentrations of 4-6 mmol/L. Three left lungs per group were transplanted into recipient pigs and monitored for 4-hours before a final isolated left lung assessment. Donor organ procurement, NPV-ESLP, and lung transplantation have been described in detail[15,16] and are summarized below.

Donor Lung Procurement

Animals were sedated, intubated, and anesthetized. Median sternotomy was performed followed by heparinization. *In-vivo* wedge biopsies and blood samples were collected. A poole tip suction was inserted into the right atrium for exsanguination, collecting into a cell-saver. Following cardiectomy, the lungs were dissected free, and the trachea clamped at a continuous positive airway pressure (CPAP) of 20 cmH_2O. Lungs were removed, weighed, and connected to NPV-ESLP.

12-hour NPV-ESLP

Lung biopsies were collected prior to NPV-ESLP. The trachea was intubated with lungs inflated and connected to NPV-ESLP with CPAP 20 cmH_2O. The pulmonary artery was connected to the circuit and perfused at 10% cardiac output (CO). Perfusate was warmed from 20℃ to 32℃ over 10-minutes. At 32℃, the air-tight chamber lid was secured and NPV was initiated by simultaneously decreasing CPAP and end-inspiratory pressure (EIP) by 1 cmH_2O every 2-minutes until a CPAP of 8 cmH_2O and EIP -12 cmH_2O was achieved. Perfusate was warmed to 38℃ within 30-minutes of perfusion. Every 10-minutes, flowrate increased by 10% CO until a target flow of 30% CO. Lung evaluation (5-minute duration) was performed at hour 5 (T5) and hour 11 (T11) of NPV-ESLP.

Perfusate Gas Management

Exogenous pure sweep CO_2 was applied to the perfusate in both groups during preservation. The Control group received a higher rate of sweep CO_2 administered to target a perfusate pH of 7.35-7.45 and pCO_2 of 35-45 mmHg. The pH+ group had a lower rate of sweep CO_2 administered to

target a pH of 7.46-7.55 and pCO_2 <35 mmHg. Groups were evaluated at T5 and T11 using 50% cardiac output to improve V/Q matching while administering mixed gas (89% N_2, 8% CO_2, 3% O_2) at a rate of 0.125L/min to deoxygenate the circuit and assess pre- vs post-lung oxygenation (Table 7.1).

Left Lung Transplantation

Animals were sedated, intubated, lined, and placed in a modified right-lateral decubitus position. Following left thoracotomy, hilar structures were dissected. At implantation, the recipient left lung was clamped and discarded. The donor left lung was flushed with 0.5L of extracellular, low-potassium, dextran-based solution and implanted. After 4-hours of monitoring, a sternotomy was performed, the right lung was clamped, and the left lung function evaluated. An isolated left lung blood sample was drawn at 10-minutes. Animals were euthanized, the left lung was explanted, inspected for gross pathology, and weighed.

Physiologic Evaluation

ESLP software continuously monitored PAP, PVR, dynamic compliance (Cdyn), and the ratio of partial pressure of oxygen in the perfusate to the fraction of inspired oxygen (PF ratio). During evaluation on ESLP and of the isolated transplanted lung, PF ratios were calculated from the left atrium perfusate/blood sample. Lung weight was recorded post-explant, T0, T12, and pre-and post-transplant. Edema was estimated using percentage weight gain (weight gain [%] = {[End weight - Start weight]/Start weight}x 100%).

Perfusate Cytokine Analysis

Perfusate samples were collected every two hours. Pro-inflammatory cytokines tumor necrosis factor ∝ (TNF-∝) and interleukin-6 (IL-6) concentrations were determined using enzyme-linked immunosorbent assay kits (R&D systems, Minneapolis, MN).

Statistical Analysis

GraphPad Prism Software (Version 9; GraphPad Software Inc, La Jolla, California) was used for statistical analysis. Unpaired Student *t* tests were performed to compare normally distributed continuous variables between groups. Normality was assessed using the Shapiro-Wilk test. Results are expressed as mean ± standard error. $P < .05$ was considered statistically significant.

RESULTS

NPV-ESLP: Physiologic Function

Five sets of control lungs failed before 8-hours ESLP due to excessively high PVR and PAPs. Repeat attempts in the Control group were performed with additional doses of buffer (THAM) and vasodilator (milrinone instead of nitroglycerin) to attain six lung blocks that successfully reached 12-hours of preservation. All six lungs in the pH+ group successfully completed 12-hours of ESLP without any early failures. The following results pertain to lungs that completed 12-hours of ESLP.

Control and pH+ groups demonstrated stable and acceptable oxygenation (PF ratio>300) throughout preservation. PF ratios did not differ significantly between groups at T5 or T11 evaluations (p=0.37; Figure 7.1A). The average PF ratio at T11 was 438.20 ± 24.71 in the Control group and 485.60 ± 46.03 in the pH+ group. Similarly, dynamic compliance did not

differ significantly between groups (p=0.41; Figure 7.1B). The average Cdyn at T11 was 21.57 ± 2.46 in the Control group and 22.35 ± 2.12 in the pH+ group.

Pulmonary artery pressures were similar between groups at T5 and T11 (p>0.05, Figure 7.1C). At T11, the average PAP was 14.12 ± 2.77 in the Control group and 9.58 ± 1.69 in the pH+ group. Pulmonary vascular resistance was significantly higher in the Control group at T5 (p<0.05, Figure 7.1D). By T11, PVR was similar between groups (p>0.05, Figure 7.1D). The average PVR at T11 was 557.90 ± 98.81 in the Control group and 373.2 ± 68.93 in the pH+ group. Control lungs required repeat THAM and milrinone boluses on ESLP to prevent acidosis and treat elevated PVR; this was not necessary in the pH+ group. Both groups demonstrated PAP equal to or less than 20 mmHg at each evaluation point, which met one of our acceptance criteria in our lab for transplantation. Weight gain did not differ significantly between groups by T12 (p=0.37; Figure 7.1E). The average weight gain (%) was 24.54 ± 3.55 in the Control group and 24.40 ± 3.34 in the pH+ group.

Perfusate gas readings were stable in both groups throughout ESLP. In the Control group, the average pH was 7.37 ± 0.03 at T5 and 7.45 ± 0.03 at T11. In the pH+ group the average pH was 7.50 ± 0.01 at T5 and 7.56 ± 0.03 at T11. The partial pressure of carbon dioxide was 38.97 ± 3.78 at T5 and 38.42 ± 0.73 at T11 in the Control group. In the pH+ group, pCO_2 was 12.20 ± 1.98 at T5 and 15.80 ± 4.21 at T11. Bicarbonate was 23.05 ± 2.31 at T5 and 26.33 ± 2.07 at T11 in the Control group. In the pH+ group, bicarbonate was 13.31 ± 0.86 at T5 and 17.77 ± 1.84 at T11.

NPV-ESLP: Pro-Inflammatory Cytokine Profiles

TNF-∝ perfusate concentrations increased early in the perfusion run during the first 3-hours and progressively decreased thereafter (Figure 7.2A). Concentrations of TNF-∝ were significantly greater in the Control group at T1 and T3 ($p<0.05$), but all other time points were similar ($p>0.05$). IL-6 perfusate concentrations were significantly greater in the Control group for most of ESLP (T3-T11; Figure 7.2B). IL-6 progressively increased to T11 in the Control group but peaked at T7 in the pH+ group and progressively decreased.

Oxygenation and Weight Gain Post-Transplantation

All animals demonstrated stable oxygenation on two-lung ventilation during 4-hours of reperfusion and during isolated left lung assessment (Figure 7.3A). Isolated left lung gases were drawn 10-minutes after clamping the right lung. There was no significant difference between groups in their respective isolated left lung PF ratios ($p=0.12$; Figure 7.3A). The average PF ratio was 195.70 ± 52.92 in the Control group and 300.70 ± 52.26 in the pH+ group. In addition, there was no significant difference in the left lung weight gain following 4-hours of transplant reperfusion ($P=0.12$; Figure 7.3B). The average percentage weight gain of the transplanted lung was 41.13 ± 3.99 in the Control group and 29.63 ± 7.23 in the pH+ group.

Discussion

Our study investigated the effects of physiologic pH versus mild-permissive alkalosis on transplant outcomes in a preclinical model of normothermic NPV-ESLP. Published protocols in clinical human and preclinical porcine ESLP have advocated targeting normal pH and pCO_2 to optimize preservation[1-4]. In clinical practice, pH and pCO_2 are often manipulated to achieve specific physiologic aims, such as vasodilation of pulmonary vasculature[10-12] or vasoconstriction of cerebral vasculature[17]. Prior to this study, our group observed a possible benefit to mild permissive alkalosis during porcine ESLP resulting in lower PAP and PVR, a reduced requirement for intermittent vasodilators, and more reliable preservation. This is the first study to directly compare a strategy of mild permissive alkalosis to physiologic pH in a porcine transplantation model of ESLP.

Our primary experimental finding was that porcine lungs preserved on NPV-ESLP with a physiologic pH target achieved via a physiologic pCO_2 through exogenous sweep CO_2 administration were far more likely to fail before reaching the target 12-hour time-point. Five sets of double lung blocks developed high PVR and PAP that would not respond adequately to standard doses of vasodilators (nitroglycerin) and subsequently developed pulmonary edema during evaluation at 50% cardiac output. Edema worsened lung mechanics and gas exchange, leading to ESLP failure. Through repeated attempts and the introduction of milrinone to manage elevated PVR, six lungs were able to reach 12-hours of preservation with physiologic pH and pCO_2 throughout ESLP. These lungs did not differ in their physiologic performance compared to the mild permissive alkalosis group. Likewise, after transplantation, there was no significant difference in PF ratios or weight gain between groups. Milrinone provided more stable hemodynamic values of PAP and PVR compared to nitroglycerin, and this is in line with

previous research comparing the efficacy of the two medications in the management of acute pulmonary hypertension [18]. The administration of additional milrinone boluses in the Control group to achieve target PAP and PVR parameters for successful 12-hour ESLP ultimately masked any significant difference in hemodynamics between groups.

Pro-inflammatory cytokines TNF-alpha and IL-6 perfusate concentrations were significantly higher in the physiologic pH group compared to the mild permissive alkalosis group at evaluation points T1-3 and T3-11, respectively. Exogenous high-concentration (100%) CO_2 may directly or indirectly enhance the production of TNF-∝ and IL-6 during the initial inflammatory storm from surgery, warm-ischemia, and ESLP. Studies have established clinically and preclinically the association between increased pro-inflammatory cytokines and the development of acute and chronic pulmonary hypertension[19]. Ventilatory hypercapnia is known to induce pulmonary inflammation in a dose-dependent manner with worse pathophysiology and lung injury from higher concentrations of CO_2[20,21,22]. Hypercapnia has also been shown in a mouse model to enhance cytokine mediated cell injury by amplifying nitric oxide inflammatory processes[23]. Although Control lungs in our study were not hypercapnic, the rapid rise in perfusate pCO_2 may produce a similar pathologic effect of inflammation amplification. Perfusate pCO_2 was increased over a 30-minute period from zero to 35-45 mmHg using high concentration (100%) CO_2. In the setting of ESLP inflammation, the sudden change in pCO_2 may explain enhanced production of inflammatory cytokines and PVR compared to the pH+ group. Further research into the potential mechanism between circulating levels of CO_2, pro-inflammatory cytokines, and PVR is needed to substantiate this explanation.

Our findings resemble those by Morray et al[24]. We found that in the context of a normoxic postoperative inflammatory state (ESLP and warm ischemia), targeting physiologic

pCO_2 and pH can result in elevated mPAP, whereas decreasing pCO2 to target mild alkalosis can decrease mPAP. In the study by Morray et al, pediatric patients with pulmonary hypertension (PH) post-CPB and surgical correction for acyanotic CHD were treated with varying levels of inspired CO_2 concentrations while monitoring the effect on mPAP. Patients were ventilated with 100% FiO_2 to achieve normoxia and eliminate the influence of hypoxic pulmonary vasoconstriction. Acidosis and hypercarbia were avoided. The study begins with a state of hypocapnic alkalosis (7.50-7.60) to treat elevated PAP; inspired CO_2 is gradually increased to target physiologic pCO2 and pH with a significant rise in mPAP from baseline; then, inspired CO_2 is gradually decreased back to a mild alkalotic state of 7.50-7.60 and mPAP drops significantly. This study does not isolate/identify whether it is low CO_2 or mild alkalosis that is causing vasodilation, which is also a limitation of our study, but it supports our observation of the therapeutic effect of normoxic hypocarbic mild alkalosis to treat inflammatory PH.

A counter argument to the use of mild permissive alkalosis is provided by evidence that hypocapnic alkalosis can induce and exacerbate lung injury[25]. Laffey et al demonstrated in an isolated perfused rabbit lung model that severe alkalosis (pH >7.7) over 3-hours resulted in worse physiologic lung function and increased weight gain via increased vascular permeability compared to a physiologic pH. Furthermore, severe hypocapnic alkalosis resulted in worse lung function and ischemic-reperfusion injury following a short period of warm-ischemia, which is highly relevant to transplantation. Interestingly, they demonstrated a dose-dependent relationship to the degree of hypocapnic alkalosis and the extent of ill-effects. Further limitations of the therapeutic effects of alkalosis come from literature in persistent pulmonary hypertension of the newborn, which reports deleterious effects of prolonged alkalosis (2-3 days) such as bronchopulmonary dysplasia, increased pulmonary infiltrates, and increased O_2 requirements[26,27].

Our study differs in keyways from these reports: 1) we specifically maintained a mild degree of alkalosis, avoiding severe alkalosis; 2) 12-hour ESLP is significantly shorter than the time interval in which irreversible lung injury has been observed in pediatric patients treated with hyperventilation alkalosis[26,27]; 3) Our ability to employ mild permissive alkalosis to increase the reliability of prolonged ESLP without a decrease in physiologic performance suggests the risks and benefits of mild permissive alkalosis can be strategically managed.

One possible strategy to optimize the application of mild permissive alkalosis is to delay targeting physiologic pH and pCO_2 until after the initial pro-inflammatory cytokine storm caused by surgery, warm-ischemia, and ESLP initiation has subsided. Once ESLP porcine lungs have stabilized on the machine as demonstrated by a persistent lowering of PAP and PVR, then sweep CO_2 can be increased gradually to target a physiologic pH. This strategy may optimize cellular function, including surfactant production and alveolar fluid clearance[28]. Indeed, further research is needed to clarify the optimal application of pH manipulation during ESLP.

Future experiments in our lab will focus on the application of mild permissive alkalosis with juvenile porcine ESLP to achieve reliable 24-hour continuous preservation. The increased reliability provided by mild permissive alkalosis ESLP with our porcine model and the simplicity of its application may improve preservation and transplantation outcomes up to 24-hours. It is unknown if preservation periods beyond 12-hours in the alkalotic range are well tolerated during ESLP and in the acute post-transplant period. As mentioned earlier, mild permissive alkalosis may have a limited window of therapeutic benefit, beyond which significant damage to organ function results.

Limitations:

The number of transplantations performed per groups is small (n=3); however, this sample size matches other preclinical studies[29,30,31]. Likewise, post-transplant reperfusion and isolated lung assessment was based on previous experiments[29,30,31], which are relatively short time-points. Our study does not elucidate the mechanism behind improved reliability of ESLP with mild permissive alkalosis. Hyperventilation affects pCO_2 and pH concurrently; therefore, it is difficult to determine their respective roles in altering PAP and PVR. Animal studies report incongruent results regarding the effects of CO_2 on pulmonary vasculature, and the disagreement may stem from methodological differences, including species and age[32-36]. Metabolic alkalosis may also induce vasodilation[37], so whether hypocarbia or alkalotic pH is responsible for the salutary effects on PAP is unclear.

Conclusions

Mild permissive alkalosis porcine NPV-ESLP demonstrated more reliable preservation and reduced inflammation compared to a physiologic pH strategy. These findings suggest that mild alkalosis may protect porcine lungs with highly reactive vasculature while on ESLP via vasodilation, which decreases iatrogenic injury and increases the reliability of preservation.

Figure and Table Legend:

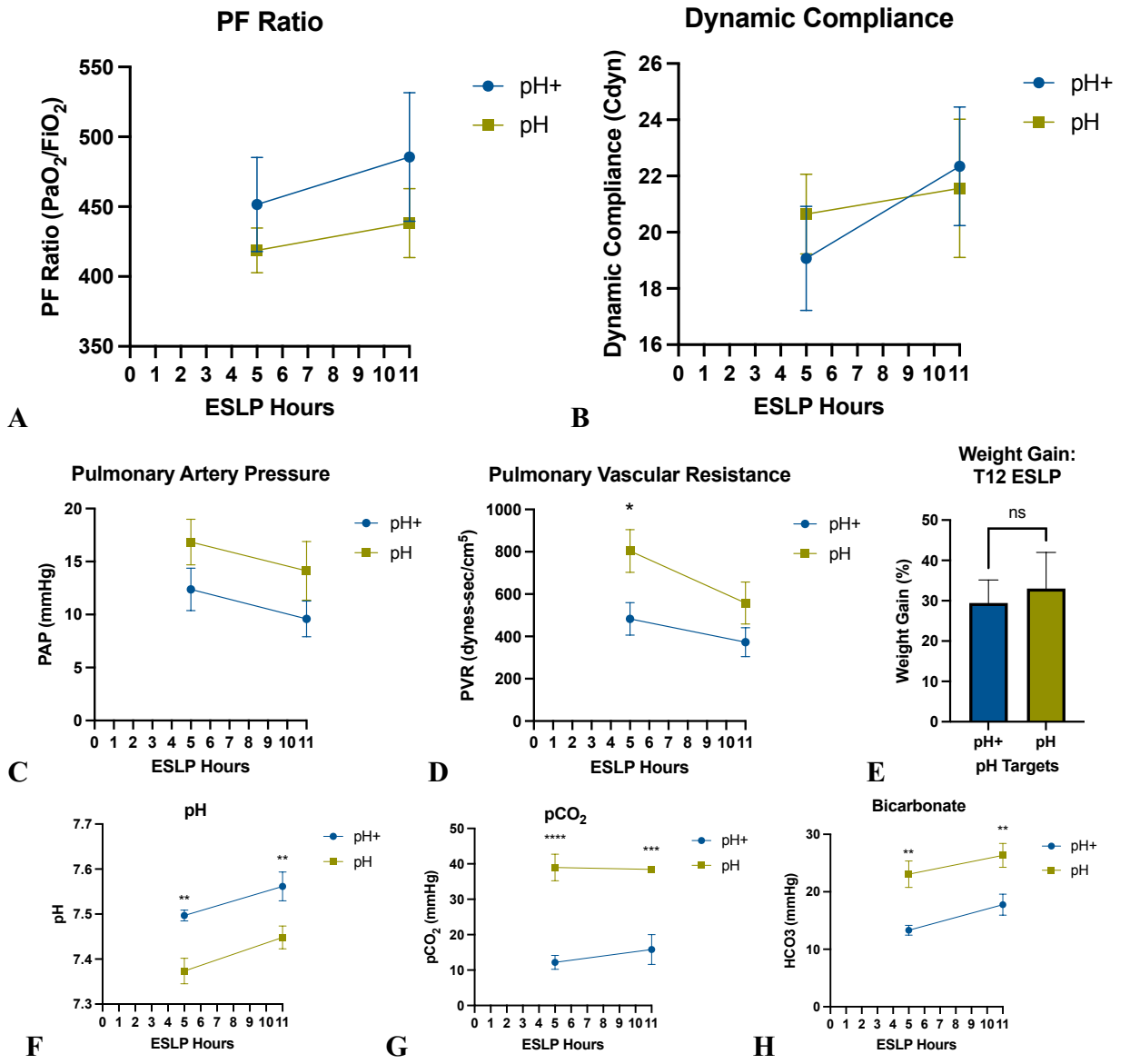

Figure 7.1. Evaluation lung physiology parameters during 12-hours of NPV-ESLP: pH (n=6) vs. pH+ (n=6). PF ratio (PaO_2/FiO_2 Ratio) (A), dynamic compliance (Cdyn) (B), pulmonary artery pressure (PAP)(C), pulmonary vascular resistance (D), percentage weight gain per hour (E), pH (F), pCO_2 (G), Bicarbonate (HCO_3^-; H). Over 12-hours of NPV-ESLP, all lung parameters remained stable, and weight-gain was similar between groups (E; $p>0.05$). There was no significant difference in PF ratio (A; $p>0.05$), dynamic compliance (B; $p>0.05$), or PAP between groups (C; $p>0.05$). PVR was significantly higher in the pH group at T5 ($p<0.05$) compared to the pH+ group, but there was no significant difference at T11 ($p>0.05$). Blood gas parameters were significantly different between groups in accordance with the respective protocols: pH ($p<0.01$), pCO_2 (T5, $p<0.001$; T11 $p<0.0001$), and HCO_3 ($p<0.01$). *NPV-ESLP*, Negative Pressure Ventilation Ex-Situ Lung Perfusion.

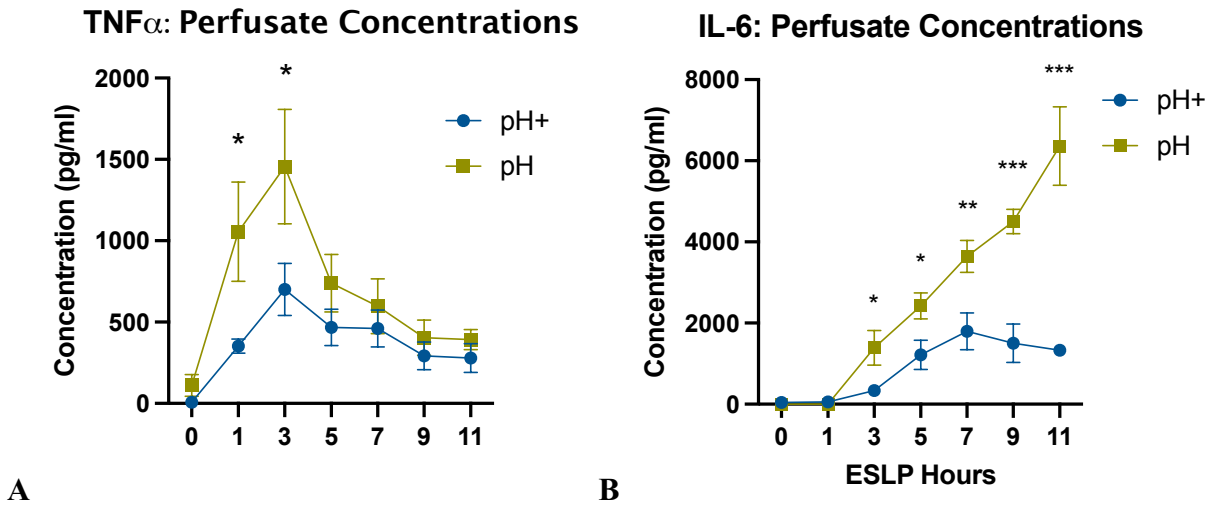

Figure 7.2. Cytokine Profiles during NPV-ESLP. A, TNF-∝ perfusate concentrations were significantly higher in the pH (Control) group at T1-3 compared to the pH+ group ($p<0.05$). Both groups demonstrated an early rise in TNF-∝ that peaked at T3 and decreased thereafter. B, IL-6 perfusate concentrations climbed continually in the pH group and were significantly higher from T3-5 ($p<0.05$), T7($p<0.01$), and T9-11 ($p<0.001$) compared to the pH+ group. The pH+ group demonstrated an increase in IL-6 beginning at T3, peaked at T7 and gradually declined to T11.

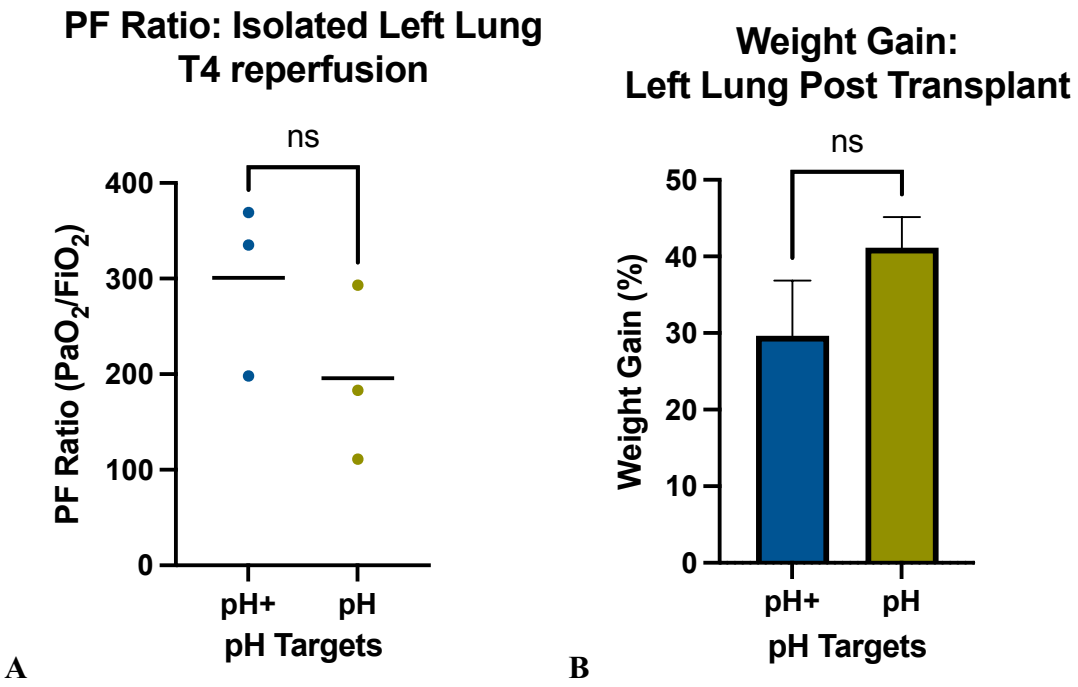

Figure 7.3. Isolated left lung oxygenation and weight-gain post-transplantation. A, there was no significant difference between the PF ratio of the pH group and the pH+ group (p= 0.12). B, no significant difference in weight gain (%) in the transplanted left lung pre- vs post-transplant was demonstrated between groups (p=0.12). CO, cardiac output; PF ratio, PaO_2/FiO_2; T4, fourth hour of reperfusion.

Table 7.1. NPV-ESLP Perfusion and Ventilation Strategy: pH vs pH+				
12-hour Protocol	pH	pH+	pH	pH+
Perfusion Parameters	Preservation		Evaluation	
Perfusate Flow (CO = 70mL/kg/min)	30% CO	30% CO	50% CO	50% CO
PAP (mmHg)	< 20	< 20	< 20-25	< 20-25
LAP (mmHg)	0	0	0	0
Medical Gas Mixture	100% CO_2	100% CO_2	89%N_2, 8%CO_2, 3%O_2 0.125L/min	89%N_2, 8%CO_2, 3%O_2 0.125L/min
pCO_2 target (mmHg)	35-45	<35		
pH target	7.35-.45	7.45-.55	7.35-.45	7.45-.55
Ventilation Parameters				
Mode	Pressure Control	Pressure Control	Pressure Control	Pressure Control
Target Tidal Volume	6-8 ml/kg	6-8 ml/kg	10 ml/kg	10 ml/kg
End-Inspiratory Pressure (cmH_2O)	- 10 to -12	- 10 to -12	-15 cmH_2O	-15 cmH_2O
Inspiratory: Expiratory Ratio	1:1-1.2	1:1-1.2	1:1	1:1
Respiratory Rate	6-8	6-8	10	10
Peak Airway Pressure (cmH_2O)	< 25	< 25	$\leq$ 25	$\leq$ 25
PEEP (cmH_2O)	8	8	5	5
FiO_2	21%	21%	21%	21%

Legend: 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 pulmonary arterial circulation.

Chapter 8

Perfusate Exchange does not Improve Outcomes in *Ex-Situ* Lung Perfusion

Abstract

Background: Reliable 24-hour preservation is required to optimize the rehabilitation potential of ESLP. Other ESLP protocols include fresh perfusate replacement to counteract an accumulation of deleterious by-products. We describe the results of our reliable 24-hour negative pressure ventilation (NPV)-ESLP protocol with satisfactory acute post-transplant outcomes. We also investigate perfusate exchange as a modification to enhance prolonged ESLP.

Methods: Twelve pig lungs underwent 24-hours of NPV-ESLP using 1.5L of cellular perfusate (500mL pRBC and 1L buffered perfusate). The Control (n=6) had no perfusate exchange; the Perfusate-Exchange (PE, n=6) had 500mL replaced after 12-hours of NPV-ESLP with 1000mL fresh perfusate. Three left lungs per group were transplanted.

Results: Results are reported as Control vs PE (mean ± SEM). Both groups demonstrated stable and acceptable oxygenation during 24-hours of ESLP with final PF ratios of 527.5 ± 42.19 and 488.4 ± 35.38 (p=0.25). Final compliance measurements were 20.52 ± 3.59 and 18.55 ± 2.91 (p = 0.34). There were no significant differences in PAP after 24-hours of ESLP (10.02 ± 2.69 vs. 14.34 ± 1.64, p = 0.10), and PVR only differed significantly at T12 (417.6 ± 53.06 vs. 685.4 ± 81.19, p=0.02). Percentage weight-gain between groups was similar (24.32 ± 8.4 and 45.33 ± 7.76, p=0.07). Post-transplant left lung oxygenation was excellent (327.3 ± 14.62 and 313.3 ± 15.38, p=0.28). There was no significant difference in % weight gain of lungs post-transplant (22.20 ± 7.22 vs 14.36 ± 9.96, p=0.28).

Conclusion: Acceptable lung function was maintained during 24-hours NPV-ESLP and post-transplant regardless of perfusate exchange.

INTRODUCTION

Ex-Situ Lung Perfusion (ESLP) is effective at increasing the available pool of donor lungs for transplantation[1]. Clinically, the FDA has approved ESLP for 4–6-hour durations[2-7]. Preclinically, 12-hours of ESLP is routinely published with or without *in-vivo* transplantation data[8-11]. Continuous ESLP preservation of 24-48 hours is necessary to optimize the pool of donor lungs. These timepoints could eliminate geographic barriers and allow for true global organ sharing, improve donor-recipient matching via more thorough assessment, and allow for the treatment of donor lung pathology that requires a prolonged dwell-time with advanced therapies.

Only one ESLP platform has achieved continuous 24-hour preservation with acceptable acute post-transplant outcomes in a porcine model[12,13]. Other ESLP platforms have achieved 24-hours of continuous ESLP using pig lungs but have not achieved sufficient reliability to attempt transplantation and validate the organ quality *in-vivo*[14-16]. Limitations to prolonged continuous ESLP include progressive development of pulmonary edema and iatrogenic injury that result in deterioration of lung mechanics, compliance, and gas exchange.

Perfusate management is an important research topic to improve ESLP fluid balance and extend ESLP beyond 24-hours. Our lab and others have previously investigated the potential role of perfusate purification and dialysis in ESLP[15, 17, 18, 19]; however, the specific applications have been ineffective at mitigating extravascular fluid accumulation in the lungs or present conflicting results. Some protocols intermittently or continuously exchange or add perfusate during ESLP to decrease the concentration of presumed circulating deleterious by-products of organ preservation[8,20]. It is not clear if this strategy is effective at improving organ function or if it significantly reduces any key mediators of tissue injury.

We report a refined protocol for porcine NPV-ESLP using a cellular perfusate with packed red blood cells (pRBC) that can safely preserve donor porcine lungs for 24-hours with acceptable transplantation outcomes. Furthermore, we compare two separate strategies of perfusate management: 1) a control group with no perfusate exchange; 2) a batch perfusate exchange method.

METHODS

Animals

The experimental protocol was approved by the University of Alberta Animal Care and Use Committee. Handling was performed in accordance with the "Principles of Laboratory Animal Care," formulated by the National Society for Medical Research. Lungs from 12 female domestic pigs (45-55kg) were procured and underwent ESLP for 24-hours. Lungs were perfused with 1 L of a common hospital ingredient perfusate (similar ingredients to STEEN) and 0.5 L of red blood cell concentrate (pRBC)[11]. The described volumes provided a hemoglobin concentration of 40 to 50 g/L. Piperacillin-Tazobactam (3.375 g), heparin (10 000 IU), and methylprednisolone (500 mg) were added to the perfusate. All solutions were prepared fresh for each perfusion. TPN was continuously infused into the perfusate with 0.08 g/ kglungweight/h of 6.3% amino acid solution and 0.1 g/kglungweight/h of 15% lipid solution. Amino acid and lipid solutions were derived from components of PeriOlimel 2.5%E (Baxter Corporation, Missisauga, Canada). A multivitamin solution was also added to the perfusate with 80 mg ascorbic acid, 17 mg niacinamide, 5 mg d-panthenol, 1 mg pyridoxine hydrochloride, 1.4 mg riboflavin, 1.2 mg thiamine, 2 300 IU vitamin A, 400 IU vitamin D, 7 IU vitamin E, 20 mcg bio- tin, 5 mcg vitamin

B12, 0.2 mg vitamin K1, and 140 mcg folic acid (supplied as Pediatric Multi-12/K1 Injection, Sandoz Canada Inc., Boucherville, Canada).

Donor Lung Procurement

Our protocol for donor lung procurement and *ex-situ* perfusion management has previously been described in detail[9,10]. Animals were sedated with ketamine (20 mg/kg), and atropine sulfate (0.05 mg/kg). Animals were intubated and anesthetized with 1-3% isoflurane. Median sternotomy was performed. Heparin was administered directly into the superior vena cava (SVC). *In-vivo* wedge biopsies were taken along with blood samples. The SVC was transected and a poole tip suction was inserted into the SVC and right atrium for exsanguination, draining into a cell-saver for blood collection. Cardiectomy was performed, the lungs were dissected free from their attachments, the trachea was clamped at a constant airway pressure of 20 cm H_2O. The lungs were removed, weighed, and connected to NPV-ESLP.

24-hour ESLP

Lung wedge biopsies were taken immediately prior to NPV-ESLP connection. The trachea was intubated and connected to the NPV-ESLP ventilator with a modified endotracheal tube in a manner that maintains lung inflation. Continuous positive airway pressure (CPAP) of 20 cm H_2O was applied, and the trachea was unclamped. The pulmonary artery was connected to perfusion, and the pressure transducer was zeroed to atmospheric pressure. Flow was initiated at 10% of predicted cardiac output ($CO_{predicted}$; 70 ml/kg/min). The perfusate temperature was warmed from ambient room temperature to 32℃ over 10 minutes. Once perfusion was established, the ventilator was re-clamped, zeroed to atmospheric pressure, and unclamped at a CPAP of 15 cm

H_2O without any loss of lung volume. At 32℃, the air-tight chamber lid was secured and NPV was initiated: the negative pressure ventilator limb was secured to the air-tight chamber at the beginning of an inspiratory cycle to pull the lungs open further; end-inspiratory pressure (EIP) is initially set at -8 cm H_2O and CPAP is rapidly decreased to -5 cm H_2O while EIP is decreased to -14 cm H_2O in a stepwise manner under 1-minute. Perfusate temperature was gradually warmed to 38℃ (normothermic for pigs) within 30-minutes of perfusion. Every 10-minutes, perfusion flow rate was increased by 10% of CO to reach 30% CO. Lung evaluation (5-minute duration) was performed every 6-hours of NPV-ESLP. A medical sweep gas mixture (89% N_2, 8% CO_2, and 3% O_2) was used to deoxygenate the perfusate during evaluation to assess for pre- vs post-lung oxygenation. During preservation, a pure CO_2 sweep gas was applied to target a pH 7.45-7.55, tolerating mild permissive alkalosis. Lungs were ventilated using room-air (FiO_2 21%) throughout ESLP. Table 8.1 outlines the ventilator and perfusion parameters during initiation of NPV-ESLP, as described above, and Table 8.2 outlines the maintenance parameters, which were not manipulated beyond the first hour. Table 8.3 outlines the refinements made to our 24-hour protocol compared to our original strategy for 12-hour preservation published in 2018[9].

Perfusate Management

The perfusate was not exchanged during 24-hours ESLP in the Control group (n=6). In the Perfusate Exchange group (PE; n=6), after the T12 evaluation was performed, 500 mL of perfusate was drained from the circuit and replaced with 1L of fresh perfusate of identical composition, including medications and volume of pRBC. Five hundred millilitre is the maximum amount of perfusate that can be drained from the ESLP reservoir without decreasing the fluid level to the point that air is drawn into the circuit, which risks air embolism. Autologous

pRBCs were stored at 4°C, which is considered safe for up to 24-hours according to the cell-saver manufacturer, which is based on the American Association of Blood Banks Standards for perioperative autologous blood collection and administration[21,22].

Left Lung Transplantation

Our protocol for left lung transplantation has been previously described[23]. The animals were sedated with Ketamine 20mg/kg, Hydromorphone 0.05mg/kg, and Atropine 0.05mg/kg. An arterial line and central line were inserted into the carotid artery and jugular vein, respectively. Animals were placed in a modified right-lateral decubitus position. A left thoracotomy was performed, and the donor left lung hilar structures were dissected free. When the donor lung was ready for implant, the recipient left lung structures were clamped and the native lung was discarded. The donor lung was flushed with 0.5L of extracellular, low-potassium, dextran-based solution to remove debris and ESLP perfusate remnants. The donor lung was implanted, and the recipient was monitored for 4-hours. After 4-hours, a sternotomy was performed, the right PA was clamped to obstruct perfusion, followed by an isolated left lung assessment. An isolated left lung blood sample was drawn at 30-minutes to evaluate the transplanted lung function. Animals were subsequently euthanized, and the left lung was explanted, inspected for gross pathology, and weighed.

Physiologic Evaluation

Physiologic parameters were continuously assessed during ESLP. These parameters include pulmonary artery pressure (PAP), pulmonary vascular resistance (PVR), dynamic compliance (Cdyn), and the ratio of partial pressure of oxygen in the perfusate to the fraction of inspired oxygen (PF ratio). During ESLP evaluation and isolated transplanted lung evaluation, PF ratios

were calculated from the left atrium perfusate/blood sample. Pig lung weight was recorded post-explant, T0, and T24, as well as immediate pre-and post-transplant. Edema formation was estimated using percentage of weight gain (weight gain [%] = {[End weight - Start weight]/Start weight}x 100%).

Perfusate Analysis

Perfusate samples were collected every two hours during ESLP for pro-inflammatory cytokine, electrolyte, and lactate analysis. Perfusate concentrations of tumor necrosis factor ∝ (TNF-∝) and interleukin-6 (IL-6) were determined using enzyme-linked immunosorbent assay kits (R&D systems, Minneapolis, MN).

Statistical Analysis

GraphPad Prism Software (Version 9; GraphPad Software Inc, La Jolla, California) was used for statistical analysis. Unpaired Student *t* tests were performed to compare normally distributed continuous variables between groups. Repeated measure ANOVA was used to compare within-groups over time. Normality was assessed using the Shapiro-Wilk test. Results are expressed as mean ± standard error. $P < .05$ was considered statistically significant.

Results

NPV-ESLP: Physiologic Function

Control and Perfusate Exchange (PE) groups demonstrated stable and acceptable oxygenation during 24-hours of ESLP. There was no significant difference in PF ratio at the final evaluation T24 ($p= 0.25$; Figure 8.1A). The average PF ratio was 527.5 ± 42.19 in the Control

group and 488.4 ± 35.38 in the PE group. All lungs preserved on ESLP had PF ratios above 300 at T24 and throughout preservation, meeting one of our acceptance criteria for transplantation.

Dynamic compliance did not differ significantly between groups (p=0.34; Figure 8.1B). The average Cdyn was 20.52 ± 3.59 in the Control group and 18.55 ± 2.91 in the PE group. Likewise, pulmonary artery pressures were not significantly different between groups during ESLP (p=0.10; Figure 8.1C). At T24, the average PAP was 10.02 ± 2.69 in the Control group and 14.34 ± 1.64 in the PE group. Both groups demonstrated mean PAP equal to or less than 20 mmHg at each evaluation point.

The pulmonary vascular resistance was similar between groups except at T12, where PVR was higher in the PE group (p=0.02; Figure 8.1D). Like PAP, PVR progressively decreased throughout preservation after a peak reading at T6. The average PVR at T24 was 357.3 ± 88.37 in the Control group and 576.0 ± 72.28 in the PE group. Weight gain did not differ significantly between groups by T24 of ESLP (p=0.07; Figure 8.1E). The average percentage weight gain was 24.32 ± 8.4 in the Control group and 45.33 ± 7.76 in the PE group.

NPV-ESLP: Perfusate Electrolyte and Lactate Concentrations

Sodium increased over time in the Control group from 151.8 mmol/L (T1) to 160.2 mmol/L (T23). In contrast, the PE group maintained stable sodium levels from 152.7 mmol/L (T1) to 153.8 mmol/L (T23). There were significant differences in sodium levels between groups at T13 (p=0.02), T19 (p=0.04), T21 (p=0.01), and T23 (p=0.008) (Figure 8.2A). Osmolality increased in the Control group over time and was stable in the PE group. There were significant differences in osmolality between groups at T13 (p=0.007), T21 (p=0.02), and T23 (p=0.01) (Figure 8.2B). Potassium increased in both groups over time with no significant difference

between groups throughout preservation ($p>0.05$). Lactate increased over time in both groups and was significantly lower in the PE group at T13 after batch replacement of perfusate (Figure 8.2D, $p=0.04$); however, there was no significant difference between groups from T15-23 (Figure 8.2D, $p>0.05$).

NPV-ESLP: Perfusate Pro-Inflammatory Cytokines Concentrations

There was no significant difference in the concentrations of TNF-∝ between groups from T0-T11 ($p>0.05$). After batch perfusate exchange, the PE group had significantly lower levels of TNF-∝ at T13 ($p= 0.004$) and T15 ($p=0.02$). Thereafter, TNF-∝ levels in both groups declined and there was no significant difference from T17-23 ($p>0.05$) (Figure 8.3A). Similarly, IL-6 perfusate concentrations levels increased in both groups but did not differ between group from T0-T11 ($p>0.05$). Following perfusate exchange, IL-6 levels were significantly lower in the PE group from T13-23 (Figure 8.3B, $p<0.01$).

Oxygenation and Weight-Gain Post-Transplantation

All animals demonstrated stable oxygenation while on two-lung ventilation post-transplant. After 4-hours of reperfusion, isolated left lung assessment demonstrated excellent function (Figure 8.3A). Isolated left lung blood gases were drawn 30-minutes after clamping the right pulmonary artery. There was no significant difference between groups in their respective isolated left lung PF ratios ($p>0.05$; Figure 8.4A). The average PF ratio was 327.3 ± 14.62 in the Control group and 313 ± 15.38 in the PE group. Similarly, there was no significant difference in the left lung weight gain following 4-hours of transplant reperfusion ($P=0.28$; Figure 8.4B). The average

weight gain of the transplanted lung was 22.20 ± 7.22 in the Control group and 14.36 ± 9.96 in the PE group.

DISCUSSION

The primary finding from this study is that NPV-ESLP can reliably preserve porcine lungs for 24-hours with acceptable post-transplant oxygenation. Our platform has previously attained 24-hours of preservation, but reliability was approximately 30-50%. This study employed a refined protocol that demonstrated a high reliability with 100% of lungs (n=12) reaching the 24-hour timepoint. Furthermore, 100% of transplanted lungs (n=6) were capable of sustaining life and produced acceptable PF ratios (>300 mmHg) in the acute post-transplant period.

The secondary finding from this study is that the specific perfusate exchange protocol that we tested did not produce any significant benefit to lung performance either during ESLP or post-transplantation. This is the first ESLP study that investigated the use of perfusate exchange with a cellular perfusate comprised of pRBC. In 2018, our group demonstrated that a cellular perfusate made with pRBCs produces less edema over 12-hours of preservation compared to an acellular perfusate regardless of ventilation strategy (NPV or positive pressure ventilation)[9]. pRBCs reduce perfusate viscosity compared to whole blood, improves workflow with other procurement teams, and maximizes availability[12].

Our perfusate exchange protocol resulted in a significant decrease in the concentrations of pro-inflammatory cytokines within the perfusate; however, reduced perfusate levels of TNF-∝ and IL-6 did not result in improved physiologic performance over 24-hours of ESLP or post-

transplant. The lack of hypothesized benefit from perfusate exchange may be due to an overestimation of the deleterious effect of TNF-∝ and IL-6 on donor lungs during 24-hour ESLP.

Perfusate exchange also resulted in more stable sodium concentrations and osmolality, as well as a transient decrease in lactate. Although sodium and osmolality were more stable in the PE group, functional outcomes did not differ, suggesting that these parameters are not the primary drivers of organ deterioration during ultra-prolonged ESLP. Lactate was significantly lower immediately following perfusate exchange, but this difference was transient, and lactate levels steadily rose over ESLP with similar concentrations between groups by T23. This observation is unsurprising, as lactate is known to rise during prolonged ESLP, and it is thought to be related to physiologic production by the lung in the absence of systemic clearance[24]. Importantly, elevated lactate levels during clinical ESLP have not been associated with inferior outcomes post-transplant.

Previous work by our group investigating ESLP perfusate purification via continuous hemodialysis also demonstrated improved sodium and lactate profiles without associated improvement in physiologic performance[15]. These findings corroborate the idea that deterioration in perfusate electrolyte composition and elevated lactate are not significant drivers of ESLP deterioration. Pro-inflammatory cytokines, perfusate electrolyte shifts, and rising lactate have been presumed to be dangerous to prolonged ESLP, but that is not supported by our findings.

Our successful 24-hour ESLP with transplantation results are similar to those achieved by OCS Lung™ [12,13], which employs a PPV platform, an open left atrium, and a blood-based perfusate. The OCS Lung™ device is clinically operated with pRBCs, but their study demonstrated improved outcomes using whole blood[12]. Our clinical application mirrors our

preclinical strategy by incorporating pRBC in the perfusate[7]. In 2018, we demonstrated that NPV-ESLP results in less tissue injury and reduced concentrations of pro-inflammatory cytokines than PPV-ESLP[9]; therefore, we believe our device is best suited for attaining preservation periods beyond the 24-hour milestone.

Preservation periods with ESLP beyond 24-hours have been achieved via xenogeneic cross-circulation ESLP[25,26] and with the strategic use of CSP at 10°C combined with cyclic ESLP[27]. Both of these discoveries represent milestone achievements in ESLP research; however, each approach contains select deviations away from the core tenets of ESLP: 1) isolation of the donor organ from vulnerable physiology, 2) continuous assessment of organ function, and 3) opportunity for prolonged treatment. Xenogeneic cross-circulation ESLP highlights the importance of absent physiologic processes contributing to organ deterioration in isolated ESLP. Cross-circulation borrows the full physiologic spectrum of support when maintaining lungs, but that supportive physiology is at risk during ESLP and will limit the use of supratherapeutic doses of medications, such as antibiotics, steroids, and immunosuppression, for donor lung treatment. Furthermore, xenogeneic cross-circulation has clear logistical and ethical limitations that may prevent wide-spread adoption. Cyclic ESLP capitalizes on the strengths of CSP (simplicity, cost-effectiveness, and low edema formation) and ESLP (mitochondrial and metabolic support) while minimizing respective weaknesses (lack of evaluation during CSP vs progressive ESLP edema formation). In light of current ESLP limitations, we believe that there remains significant improvement to be made in the application of prolonged continuous ESLP, especially with a NPV platform.

To optimize the pool of available donor lungs, we need to achieve prolonged continuous ESLP of 24-48 hours. ESLP of this duration will allow for the elimination of geographic barriers

and true global organ sharing, ideal donor-recipient matching with more thorough assessment, and the treatment of diverse lung pathologies via advanced cell and gene-based therapies. Arguably, xenogeneic cross-circulation and intermittent CSP-ESLP could achieve the first two aforementioned aims, but prolonged continuous ESLP is likely required for advanced treatment. Cell- and gene-based therapies that can improve lung function will require prolonged dwell time with the target tissue in isolation from connected vulnerable physiology that could limit effectiveness. As a next step in this direction, we will attempt to achieve the milestone of 36-hours of continuous NPV-ESLP.

Limitations

This study has several limitations. First, this study has a small n value for the number of transplantations performed; however, this number is reflective of other preclinical studies[28-30]. Similarly, the post-transplantation reperfusion and isolated lung assessment periods are relatively short, and this was also based off previous pre-clinical ESLP studies[28-30]. Longer duration follow-up is needed to establish safety of 24 hr ESLP. The specific volume and frequency of the perfusate replacement strategy used in our experimental group (PE) was based predominantly on practicality: 1) we removed the maximum amount of perfusate that could be safely siphoned-off the reservoir, as explained earlier, and 2) the replacement perfusate volume was selected based on the addition of one unit (250-300mL) of pRBC while maintaining target Hgb (40-50g/L). In sum, we selected a highly conservative strategy for perfusate exchange that could translate easily to the clinical realm, which may have limited our ability to demonstrate a significant effect. Further study investigating other perfusate management strategies, including more aggressive batch or continuous perfusate replacement and possibly hemofiltration is warranted.

Conclusions

In conclusion, we demonstrate feasibility of 24-hour NPV-ESLP with acceptable transplantation outcomes in the acute post-operative period. Furthermore, we observed no significant advantage with a strategy of perfusate exchange in terms of physiologic performance on ESLP or post-transplant.

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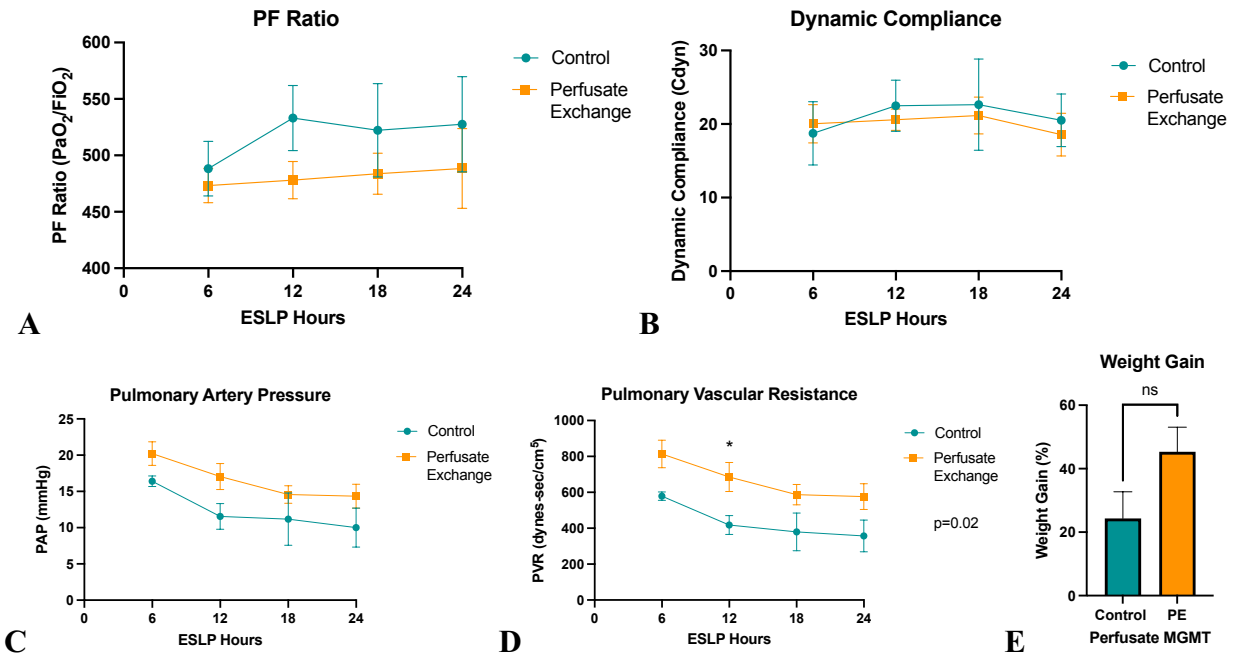

Figure 8.1. Lung physiology parameters during 24-hours NPV-ESLP: Control (n=6) vs. BR (n=6). PF ratio (PaO_2/FiO_2 Ratio) (A), dynamic compliance (Cdyn) (B), pulmonary artery pressure (PAP)(C), pulmonary vascular resistance (D), and percentage weight gain per hour (E). Over 24-hours of NPV-ESLP, all lung parameters remained stable, and weight-gain was similar between groups. *NPV-ESLP*, Negative Pressure Ventilation *Ex-Situ* Lung Perfusion.

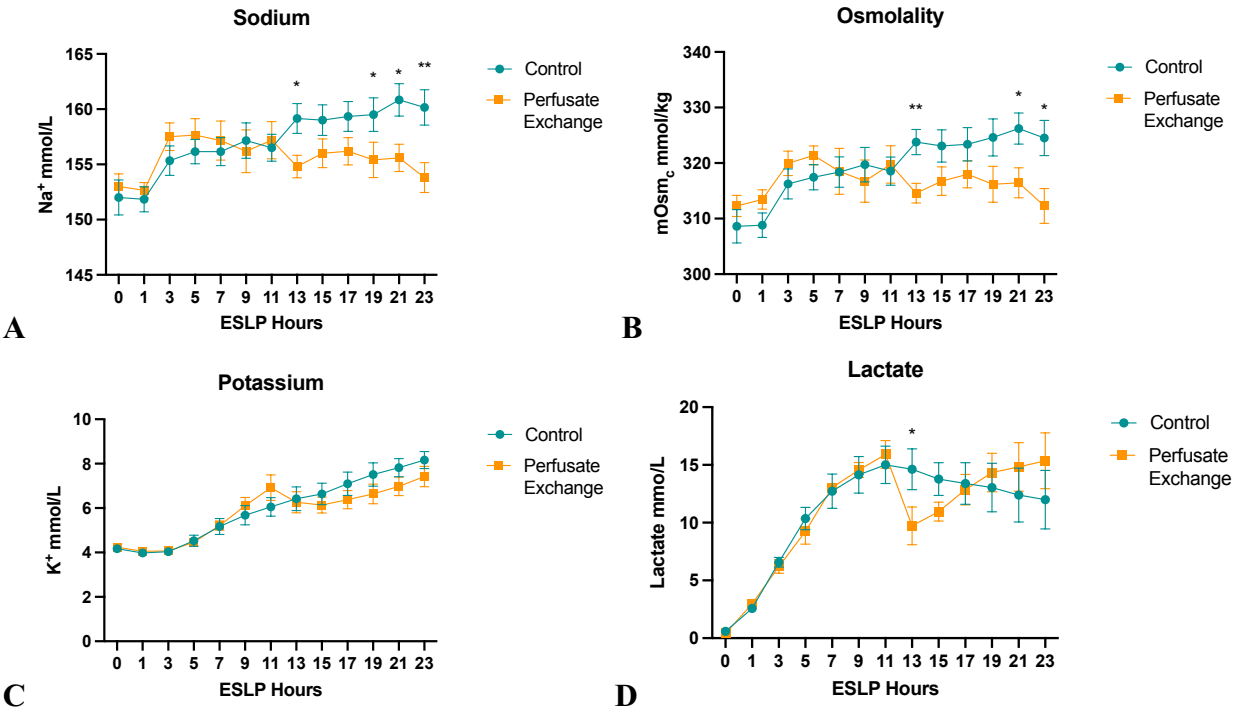

Figure 8.2. NPV-ESLP Perfusate Electrolyte and Lactate Concentrations. Perfusate sodium concentration (A), perfusate osmolality (B), perfusate potassium concentration (C), and perfusate lactate concentration (D) over time. Results are displayed as mean ± SEM. *p<0.05, **p<0.01 between groups.

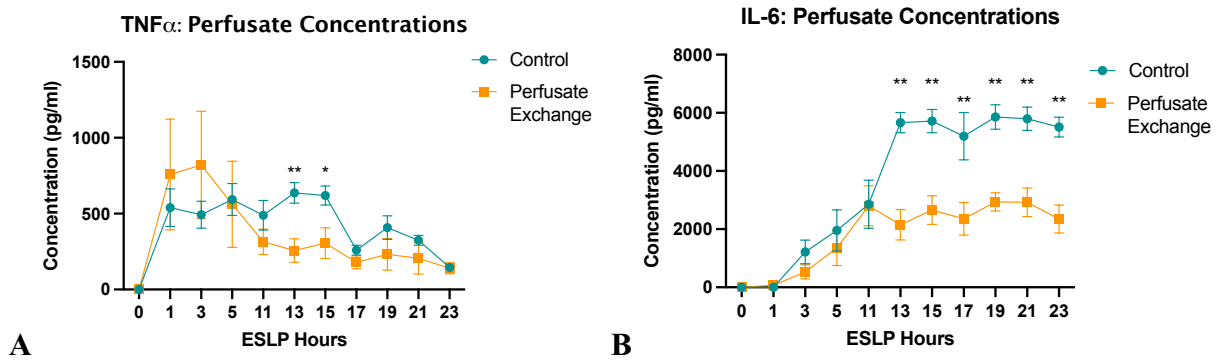

Figure 8.3. Pro-Inflammatory Cytokine Profiles during NPV-ESLP. TNF-∝ perfusate concentrations (A), and IL-6 perfusate concentrations (B) over time. Results are displayed as mean ± SEM. *p<0.05, **p<0.01 between groups.

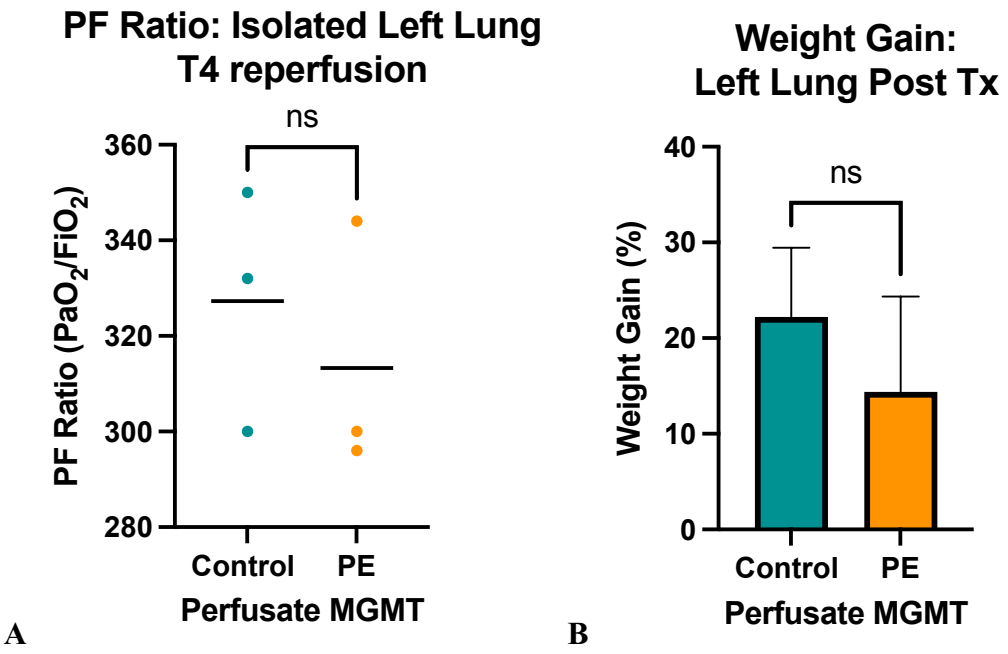

Figure 8.4. Isolated left lung oxygenation and weight-gain post-transplantation. A, there was no significant difference between the PF ratio of the Control group and the Perfusate Exchange group (PE) (p=0.28). B, no significant difference in weight gain (%) in the transplanted left lung pre- vs post-transplant was demonstrated between groups (p=0.28). CO, cardiac output; PF ratio, PaO_2/FiO_2; T4, fourth hour of reperfusion; PE, Perfusate Exchange.

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

	Perfusion time (min)				
	0	10	20	60 (T1)	360 (T6)
Perfusate Temperature (^oC)	20^{o}C	32^{o}C	38^{o}C	38^{o}C	38^{o}C
PA flow (% CO; CO = 70 ml/kg/min)	10%	20%	30%	30%	50%
Ventilation mode	PPV (CPAP = 20 cm H_2O)	Initiate NPV Preservation	NPV Preservation	NPV Preservation	NPV Evaluation
Medical gas mixer	None	None	None	100% CO_2	89% N_2, 8% CO_2, 3% O_2
Left atrial pressure	0	0	0	0	0

Table 8.1. Initiation of 24-hour NPV-ESLP Protocol. CO, cardiac output; PA, pulmonary artery; PPV, positive pressure ventilation; NPV, negative pressure ventilation. For preservation mode ventilation parameters see Table 8.2. Beginning at T6, evaluation was conducted serially every 6 hours, for 5-minutes, 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 ventilation parameters provided in Table 8.2.

NPV-ESLP Strategy: Preservation vs Evaluation

	NPV-ESLP Mode	
	Preservation	Evaluation
Perfusion Parameters		
Temperature (^oC)	38^oC	38^oC
Pulmonary artery flow (CO = 70 ml/kg/min)	30% of estimated CO	50% of estimated CO
PAP	<15-20 mmHg	<15-20 mmHg
LAP (open LA)	0 mmHg	0 mmHg
Medical gas mixture	100%CO_2	89%N_2,8%CO_2,3%O_2
Medical gas mixture (L/minute) titrated to PCO_2	<35 mmHg	<35 mmHg
pH target	7.45-7.55	7.45-7.55
Ventilation parameters		
Mode	Pressure control	Pressure control
Desired inspiratory tidal volume	3-8 ml/kg	3-8 ml/kg
Inspiratory: Expiratory Ratio	1:1 – 1.5	1:1 – 1.5
Frequency (breaths/min)	7	7
P_{AWP}	< 15-20 cm H_2O	< 15-20 cm H_2O
PEEP	5 cm H_2O	5 cm H_2O
FiO_2	21%	21%

Table 8.2. 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 pulmonary arterial circulation.

Porcine NPV-ESLP

	NPV-ESLP Protocol Refinement	
	12-hour ESLP (2018)	24-hour ESLP (2022)
Perfusion Parameters		
Temperature (°C)	38°C	38°C
Pulmonary artery flow	30% CO Preservation	30% CO Preservation
(CO = 70 ml/kg/min)	50% CO Evaluation	50% CO Evaluation
PAP	<20-25 mmHg	<15-20 mmHg
LAP (open LA drainage)	0 mmHg	0 mmHg
Medical gas mixture (89%N_2,8%CO_2,3%O_2)	Evaluation: mixed Preservation: mixed	Evaluation: mixed Preservation:100%CO_2
Medical gas mixture (litres/min) titrated to		
pCO_2	35-50 mmHg	<35 mmHg
pH target	7.35-7.45	7.45-7.55
Ventilation parameters		
Mode	Pressure control	Pressure control
Inspiratory tidal volume	6-10 ml/kg	3-8 ml/kg
Respiratory Rate	7-12 breaths/min	7 breaths/min
Inspiratory: Expiratory Ratio	1:1 – 1.5	1:1 – 1.5
P_{AWP}	< 20-25 cm H_2O	< 15-20 cm H_2O
PEEP	7 cm H_2O	5 cm H_2O
FiO_2	21%	21%
Buffered Cellular Perfusate		
Hyperoncotic with HSA	32-35 mmHg	32-35 mmHg
pRBC	Hct 12-15%	Hct 12-15%
Nutritional Supplementation	25% Dextrose (D25)	D25, TPN, multivitamins
Antibiotics	Piperacillin-Tazobactam	Piperacillin-Tazobactam
Steroids	Methylprednisone	Methylprednisone
Heparin	10,000 IU	10,000 IU
Perfusate Management	No Perfusate Exchange	No Perfusate Exchange vs Perfusate Exchange (TBD)

Table 8.3. 24-hour NPV-ESLP protocol refinement. 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 pulmonary arterial circulation; HSA, human serum albumin; pRBC, packed red blood cells; Hct, hematocrit; TPN, total parenteral nutrition; D25, 25% Dextrose; TBD, to be determined.

Chapter 9

Negative Pressure Ventilation Ex-Situ Lung Perfusion Successfully Preserves Porcine Lungs and Rejected Human Lungs for 36-hours

Abstract

Background: Continuous Ex-Situ Lung Perfusion (ESLP) beyond 24-hours is required to optimize the extended criteria donor pool through cell- and gene-based therapies. Preclinically, continuous ESLP for 24-hours is the longest duration achieved in large animal models and rejected human lungs. Here, we present the results of our 36-hour Negative Pressure Ventilation (NPV)-ESLP protocol applied to porcine and rejected human lungs.

Methods: Five sets of donor lungs from domestic pigs (45-55kg) underwent 36-hours of NPV-ESLP. Two sets of human lungs rejected for clinical transplant were donated to research and preserved on NPV-ESLP for 36-hours. Graft function was assessed via physiologic parameters, edema formation, and cytokine profiles.

Results: Porcine and human lung function was stable during 36-hours of NPV-ESLP with mean PF ratios throughout preservation of 473 ± 11.79 and 554.7 ± 13.26, respectively (mean±SEM). In porcine lungs, mean compliance (Cdyn) during ESLP was 33.96 ± 2.18, pulmonary artery pressure (PAP) 13.03 ± 0.53, and pulmonary vascular resistance (PVR) 481.20 ± 21.86. In human lungs, mean Cdyn was 82.68 ± 3.54, PAP 6.00 ± 0.33, and PVR 184.00 ± 9.71. Average percentage weight-gain was 34.47 ± 13.22 in porcine lungs and 116.3 ± 6.65 in rejected human lungs.

Conclusions: NPV-ESLP can preserve porcine lungs and human lungs for 36-hours with acceptable physiologic function. Greater weight-gain in the human lungs is likely due to prolonged ischemic time prior to ESLP and use of an acellular perfusate. Continuous 36-hour NPV-ESLP could support therapies for endothelial protection and mitigate fluid accumulation.

INTRODUCTION

Lung transplantation is the gold-standard treatment for end-stage lung disease and outcomes have improved drastically over the past forty years[1]. Concurrently, the indications for transplantation have expanded along with donor waitlists. Unfortunately, waitlist mortality for lung-transplantation is highest of any solid organ transplant, ranging from 15-20%[2,3]. To meet the increasing demand for high-quality donor lungs, innovative technologies such as *Ex-Situ* Lung Perfusion (ESLP) have garnered significant interest as a means to preserve, recondition, evaluate, and salvage donor lungs that would otherwise be discarded.

ESLP has undergone tremendous development over the last twenty years and is an established means of increasing the pool of donor lungs[4]. The application of ESLP has evolved from short-term evaluation to prolonged preservation with reconditioning[4]. Clinically, ESLP is approved for 4-6 hours[5-10]. Pre-clinically, ESLP is routinely performed for 12-hours, and a few devices have achieved 24-hours of preservation with porcine models[11-18]. Creative protocol modification, including cyclic/intermittent ESLP with optimized cold static preservation (CSP) and xenogeneic cross-circulation, have achieved preclinical preservation periods of 72- and 48-hours, respectively[19-21]. These are exciting developments for the future of ESLP research and practice; however, continuous isolated ESLP preservation remains an important target to expand the donor pool.

Gene- and cell-based therapeutics along with other medications administered via the ventilator or perfusate during ultra-prolonged ESLP represent the next frontier in ESLP research with the potential to produce superior organs that improve long-term outcomes[22-24]. These therapies will require continuous "dwell-time" with the target tissue in isolation from other

vulnerable physiology, which are limitations of cyclic CSP-ESLP and xenogeneic cross-circulation.

Negative Pressure Ventilation (NPV)-ESLP has been shown preclinically to cause less lung injury and inflammation compared to Positive Pressure Ventilation (PPV)-ESLP[12]; therefore, NPV-ESLP is likely the superior approach to attain reliable continuous preservation for ultra-prolonged duration. The primary aim of this study was to develop a reliable protocol of continuous NPV-ESLP for 36-hours with excellent physiologic maintenance.

METHODS

Animals

The University of Alberta Animal Care and Use Committee approved the experimental protocol. Handling was performed in accordance with the "Principles of Laboratory Animal Care," by the National Society for Medical Research. Lungs from 5 female domestic pigs (45-55kg) were procured and underwent 36-hours ESLP. Lungs were perfused with 1.5 L common hospital ingredient derived perfusate (CHIP) comprised of ingredients similar to STEEN solution and 0.5 L of red blood cell concentrate (pRBC). Hemoglobin concentration was 40-50 g/L. Piperacillin-Tazobactam (3.375 g), heparin (10,000 IU), and methylprednisolone (500 mg) were added to CHIP. Solutions were prepared fresh for each perfusion. TPN was continuously infused into the perfusate: 0.08 g/ kglungweight/h of 6.3% amino acid solution and 0.1 g/kglungweight/hour of 15% lipid solution. Amino acid and lipid solutions were derived from components of PeriOlimel 2.5%E (Baxter Corporation, Missisauga, Canada). Multivitamin solution was added to CHIP: 80 mg ascorbic acid, 17 mg niacinamide, 5 mg d-panthenol, 1 mg pyridoxine hydrochloride, 1.4 mg riboflavin, 1.2 mg thiamine, 2300 IU vitamin A, 400 IU vitamin D, 7 IU vitamin E, 20 mcg bio-

tin, 5 mcg vitamin B12, 0.2 mg vitamin K1, and 140 mcg folic acid (Pediatric Multi-12/K1 Injection, Sandoz Canada Inc., Boucherville, Canada).

Human Lungs

Human lungs (n=2, 70-80 kg donor) were donated for laboratory research due to a lack of eligible recipients. This protocol was approved by the University of Alberta Human Research and Ethics Board (HREB). One lung block had whole autologous blood (780 ml) added to 1L CHIP. The second lung block used an acellular composition (2L CHIP). Otherwise, perfusate composition was similar to porcine ESLP.

Donor Lung Procurement and Ultra-Prolonged ESLP

Our donor lung procurement and NPV-ESLP have been described in detail[12,13]. To initiate NPV-ESLP, the trachea was intubated in a manner that maintains lung inflation. Continuous positive airway pressure (CPAP) of 20 cmH_2O was applied, and the trachea was unclamped. The pulmonary artery was connected to the perfusion circuit, and the pressure transducer was zeroed to atmospheric pressure. Perfusion was initiated at 10% of cardiac output ($CO_{predicted}$; 70 ml/kg/min). Perfusate temperature was warmed from 20℃ to 32℃ over 10-minutes. Once perfusion was established, the ventilator was re-clamped, zeroed to atmospheric pressure, and unclamped at a CPAP of 15 cmH_2O without loss of lung volume. At 32℃, the air-tight chamber lid was secured and NPV was initiated: the negative pressure ventilator limb was secured to the air-tight chamber at the beginning of an inspiratory cycle to inflate the lungs; end-inspiratory pressure (EIP) was initially set at -8 cmH_2O and CPAP was rapidly decreased to -5 cmH_2O while EIP was decreased to -14 cmH_2O in a stepwise manner under 1-minute. Perfusate temperature

was warmed to 38℃ over 20-minutes. Every 10-minutes, flowrate was increased by 10% CO until a target flow of 30% CO. Lung evaluation (5-minute duration) was performed every 6-hours. A deoxygenating sweep gas (89% N_2, 8% CO_2, and 3% O_2) was used to assess pre- vs post-lung perfusate oxygenation. During preservation, a pure CO_2 sweep gas was applied to target a pH 4.45-7.55, tolerating mild permissive alkalosis. Lungs were ventilated using room-air (FiO_2 21%) throughout ESLP. Table 9.1 outlines ventilator and perfusion parameters during NPV-ESLP initiation. Table 9.2 outlines maintenance parameters, which were not manipulated beyond 60-minutes. Table 9.3 highlights refinements to our 36-hour protocol compared to our strategy for 12-hour ESLP from 2018[12].

Physiologic Evaluation

During ESLP, physiologic parameters were continuously assessed, including pulmonary artery pressure (PAP), pulmonary vascular resistance (PVR), dynamic compliance (Cdyn), and the ratio of partial pressure of oxygen in the perfusate to the fraction of inspired oxygen (PF ratio). Pig lungs were weighed post-explant, T0, and T36. Human lungs were weighed pre- and post-ESLP. Edema formation was estimated by percentage weight-gain (weight gain [%] = {[End weight - Start weight]/Start weight}x 100%).

Perfusate Cytokine Analysis

Perfusate samples were collected every 6-hours. Perfusate concentrations of pro-inflammatory cytokines tumor necrosis factor ∝ (TNF-∝) and interleukin-6 (IL-6) were determined using enzyme-linked immunosorbent assay kits (R&D systems, Minneapolis, MN). Human perfusate samples were processed by Eve Technologies (Calgary, AB).

Statistical Analysis

GraphPad Prism Software (Version 9; GraphPad Software Inc, La Jolla, California) was used for statistical analysis. Unpaired Student *t*-tests were performed to compare normally distributed continuous variables between groups. Normality was assessed using the Shapiro-Wilk test. Results are expressed as mean ± standard error. $P < 0.05$ was considered statistically significant.

RESULTS

36-hour Pig NPV-ESLP: Physiologic Function

Figure 9.1 shows pig NPV-ESLP physiologic lung function results. Physiologic parameters remained stable throughout 36-hour ESLP. The parameters (mean ± SEM) at 6-hours and 36-hours of NPV-ESLP were: PF ratio 442.50 ± 37.30 and 432.30 ± 50.17; Cdyn 33.90 ± 5.84 and 25.04 ± 5.221; PAP 14.31 ± 1.00 and 11.95 ± 1.58; PVR 531.90 ± 36.17 and 437.30 ± 43.67. All lungs preserved on NPV-ESLP had PF ratios above 300 mmHg at 36-hours, PAPs were under 20 mmHg, and mean Cdyn of 25. Figure 9.3 shows a representative set of pig lungs from the sample pre-ESLP (a) and post-ESLP (b). Gross appearance is well preserved. Average percentage weight gain was 34.47 ± 13.22 (Figure 9.3C).

36-hour Human NPV-ESLP: Physiologic Function

Figure 9.2 shows NPV-ESLP physiologic lung function results for two human lung blocks donated for research. Throughout 36-hours of preservation, the physiologic parameters remained stable. Parameters (mean ± SEM) at 6-hours and 36-hours of NPV-ESLP were: PF ratio 604.80 ± 9.53 and 559.50 ± 11.90; Cdyn 76.45 ± 4.65 and 71.25 ± 7.35; PAP 4.80 ± 0.50 and 6.05 ± 2.25;

PVR 175.30 ± 5.73 and 215.80 ± 13.15. Both lung blocks had PF ratios above 500 mmHg at 36-hours, PAPs were under 10 mmHg, and Cdyn >60, which are acceptable criteria for transplantation. Average percentage weight gain was 116.3 ± 6.65 (Figure 9.3D). Greater weight-gain was evident in the group perfused without blood, which is in-line with our previous observations[12].

36-hour Pig NPV-ESLP: Pro-Inflammatory Cytokine Profiles

TNF-∝ perfusate concentration levels increased during the first six hours of perfusion with a peak mean reading of 715.00 ± 51.06 pg/mL and then slowly decreased over the remaining time on ESLP to 147.10 ± 31.53 pg/mL. IL-6 perfusate concentrations levels were increased until T12 with a peak mean measurement of 5513.00 ± 458.7 pg/mL and then decreased to 4192.00 ± 307.2 pg/mL at T36 (Figure 9.4)

36-hour Human NPV-ESLP: Pro-Inflammatory Cytokine Profiles.

TNF-∝ perfusate concentration levels increased over the first twelve hours of ESLP with a peak mean reading of 261.20 ± 107.30 pg/mL and remained elevated over the remaining time on ESLP with a final reading of 232.3 ± 116.4 pg/mL. IL-6 perfusate concentrations levels continued to increase until T30 with a mean peak reading of 5621.00 ± 1843.00 pg/mL and a final measurement of 4514 ± 1809 pg/mL at T36 (Figure 9.4).

42-hour Human and Pig NPV-ESLP: Physiologic Function

Figures 9.5A and 9.5B (Supplemental Figures) show NPV-ESLP physiologic lung function results for one porcine lung block (9.5A) and one human lung block (9.5B). Both groups reached

42-hours of preservation with PF ratios above 300 mmHg; however, the human lungs had a drop in oxygenation over the final 6-hours. Dynamic compliance was stable in both groups over 42-hours. Porcine PAP and PVR demonstrated variability throughout preservation whereas human lungs were stable, which is congruent with our anecdotal experience (Figures 9.5A&B, panels C&D).

DISCUSSION

Our study demonstrates that NPV-ESLP can successfully preserve pig and human lungs for 36-hours of continuous preservation. Normothermic NPV-ESLP with reduced flow, nutritional supplementation, and the strategic use of very low tidal volume ventilation and mild-permissive alkalosis allows for stable *ex-situ* organ function for 36-hours. We also share preliminary evidence that NPV-ESLP over 40-hours is possible (Supplemental Figures 9.5A&B). The significant improvement in our protocol reliability and extended duration of preservation is due to maintenance strategies that avoid iatrogenic injury to allow reconditioning.

Lung Protective Perfusate

1. *Buffered Hyperoncotic Cellular Perfusate*. Lungs are perfused with a non-proprietary buffered hyperoncotic cellular perfusate with a physiologic electrolyte composition: Common Hospital Ingredient Perfusate (CHIP)[15]. A hyperoncotic pressure (32-35 mmHg vs blood plasma 25 mmHg) is achieved by incorporating human serum albumin, which helps shift the starling equation in favour of intravascular fluid retention and counteracts the inflammatory extravasation of fluid into the interstitial space during ESLP. A hyperoncotic pressure is achieved by incorporating human serum albumin into the perfusate composition. CHIP is supplemented with pRBC to attain a 12-15% HCT and a

Hgb 40-50 g/L. pRBCs enhance perfusate buffering capacity, increases oxygen carrying capacity, and act as a colloid. Hemodilution minimizes viscosity and improves perfusion at reduced flowrates. Lungs readily produce ATP via anaerobic metabolism, which leads to elevated lactate during ESLP without hepatic clearance[25], and buffering helps counter potential acidosis.

2. *Nutritional Supplementation.* For prolonged ESLP of 24-36 hours, our protocol includes nutritional supplementation with TPN and multivitamins. The physiologic advantage of nutritional supplementation during ESLP beyond glucose and its requisite hormone insulin is supported by two studies in ESLP[14,26]. Cellular reparative processes are energy exhaustive and prolonged ESLP benefits from the supply of metabolic building blocks through macronutrients and vitamins.

Lung Protective Perfusion

1. *Moderate Perfusion Flow.* Our protocol employs a perfusion flowrate of 30% predicted cardiac output based on an ideal CO of 70mL/kg/min. Flows of 100% CO result in excessive levels of pulmonary edema and limit preservation to 4-6 hours[11,27]. Reduced flowrates have long been used in clinical and preclinical ESLP[7-18]. We investigated a further reduction in flowrates (10% CO), but inferior transplant results were obtained[28]. Beller et al. compared the outcomes of ESLP in porcine lungs perfused with 20% and 40% CO for 100ml/kg/min with 20% perfusion producing superior preservation[29]. A flow of 20% CO at 100mL/kg/min is similar to our 30% CO at 70ml/kg/min.
2. *Normothermia:* We compared the performance of lungs preserved at normothermic, hypothermic, and subnormothermic ESLP, and superior results were attained with

normothermic perfusion[30]. Small animal models of ESLP and other organs have reported beneficial outcomes from cold perfusion, but that was not our experience when similar cold temperatures were applied to NPV-ESLP in a large animal model. Cold perfusion increases PVR due to smooth muscle vasoconstriction[31,32]. An increase in PVR is associated with increased hydrostatic pressure and a shift towards greater fluid extravasation causing pulmonary edema. Edema leads to mechanical dysfunction of the lungs with reduced compliance and gas exchange. Therefore, normothermic perfusion appears to be the ideal temperature for NPV-ESLP.

3. *Mild Permissive Alkalosis.* During porcine ESLP the pulmonary vasculature is exquisitely sensitive to the addition of sweep CO_2 to titrate pH and pCO_2 levels, particularly early in the ESLP run. In our experience, a strategy of mild permissive alkalosis (target pH 7.45-7.55) via reduced sweep CO_2 (target $pCO_2<35$) results in more reliable preservation and reduced PVR, PAP, and pro-inflammatory cytokines (manuscript in preparation). The mechanism for this beneficial effect requires ongoing investigation. Mild alkalosis may induce a vasodilatory effect that protects the lungs from elevated PVR in the face of inflammation[33-36]. A dramatic rise in pCO_2 at ESLP initiation/reperfusion via sweep CO_2 may amplify rising pro-inflammatory cytokines that increase PVR and PAP[37-39]. Notably, human lungs do not demonstrate the same reactivity in response to targeting a physiologic pH and pCO_2. This observation may be explained by interspecies differences and/or a consequence of using juvenile pig lungs.
4. *Open Left Atrial Drainage.* Open versus closed left atrial (LA) drainage with ESLP is a matter of debate[40]; however, an open LA drainage approach is used by our platform and OCS™ Lung, which have both achieved 24-hour preservation[14,16,17,18] and excellent

clinical outcomes[8,9,10]. An advantage of NPV is that intrathoracic pressure (ITP) exerts radial traction on the pulmonary vasculature to support low pressure drainage. Concerns about endothelial injury from repeated vascular collapse or a lack of retrograde LA pressure have not manifested clinically[10,40].

5. *"ESLP-ARDS".* Initiation of ESLP is followed by a predictable rise in PVR and PAP along with a drop in compliance over the first three hours, which may be associated with a drop in oxygenation. These perturbations are typically transient. After 6-7 hours, the lungs return to normal function as the initial inflammatory response subsides. Previously, we have treated rising PAP with THAM or nitroglycerin; however, a small dose of milrinone delivered within the second hour when PAP rises above 15 mmHg is effective at mitigating a further rise in PAP. Milrinone is a potent pulmonary vasodilator with a long half-life, and other ESLP protocols recommend its application[41]. Preventing increased hydrostatic pressure in the pulmonary vasculature due to rising PVR guards against the development of early onset pulmonary edema, which can compromise lung mechanics and result in premature failure. A decrease in dynamic compliance is managed by ventilating at very low tidal volumes as explained below to avoid iatrogenic injury from barotrauma or volutrauma. As the lungs recover, classic lung protective tidal volumes of 6-8ml/kg are accepted.

Lung Protective Ventilation

1. *Negative Pressure Ventilation.* Our lab uses a unique NPV-ESLP device developed by Dr Darren Freed and Dr Jayan Nagendran. In 2018, Nader et al.[12] demonstrated that NPV-ESLP causes less lung injury, inflammation, and weight-gain regardless of a

cellular/acellular perfusate strategy compared to PPV-ESLP. Given that NPV-ESLP is gentler on lung tissue, we believe it is the ideal platform for ultra-prolonged continuous preservation.

2. *Very Low Tidal Volume (VLTV) ventilation*. NPV mimics physiologic respiratory mechanics and is highly effective at recruiting lung alveoli, even with very low-tidal volumes (VLTV 3-5 mL/kg). VLTV ventilation avoids undue stress on the lung parenchyma, and NPV maintains mean intra-alveolar pressures at PEEP, which is significantly lower than intra-alveolar pressures during PPV (PEEP + Pressure Support (PS) above PEEP). We employ a strategy of VLTV ventilation in the early phase of ESLP when the inflammatory response begins to peak and lung compliance decreases. VLTV-NPV avoids iatrogenic injury, and as the inflammatory response subsides, compliance improves, and classic lung protective ventilation volumes (6-8 mL/kg) are targeted. Our target tidal volumes are dictated by Peak Transpulmonary Pressure (TPP), which we strive to keep at 15 cmH_2O or less.
3. *Peak Inspiratory Pressure and mean Intra-alveolar Pressure.* During NPV-ESLP, Peak Inspiratory Pressure (PIP) is synonymous with peak Transpulmonary Pressure (TPP). NPV effectively maintains alveolar recruitment, and low TPP can be targeted. In NPV the mean alveolar pressure is PEEP, which is kept low at 5 cmH_2O. The Intrathoracic Pressure (ITP), or End-Inspiratory Pressure (EIP) on the ventilation software, is generally maintained around -11 cmH_2O. Therefore, peak TPP (TPP = PEEP - ITP) is in the vicinity of 15 cmH_2O. In contrast, during PPV with a PEEP of 5 cmH_2O and PS of 10 cmH_2O the peak TPP will also be 15 cmH_2O, but mean intra-alveolar pressure is much

higher. NPV recruits collapsed alveoli with lower peak TPP and much lower mean intra-alveolar pressure, protecting delicate alveolar epithelial cells.

Interspecies differences on ESLP

In our experience, human lungs are significantly more robust on ESLP in terms of tissue strength and ability to withstand physiologic derangement compared to juvenile porcine lungs, which is the large animal model of choice in ESLP research. As such, optimal ESLP management strategies can differ slightly between species. Recognizing these differences is important because progress in clinical translation of ESLP findings is limited by the success attained via large animal research.

Limitations

Our study has limitations. This study has a small sample size for both pig and human lungs; however, this number is reflective of other preclinical studies[42,43,44]. Our study would benefit from transplantation post-ESLP for *in-vivo* assessment, and this will be pursued in a future study with an expanded sample.

Conclusions

In conclusion, we have established a reliable protocol for ultra-prolonged continuous preservation up to 36-hours. We have preliminary evidence that NPV-ESLP can achieve continuous preservation exceeding 40-hours. The application of ultra-prolonged NPV-ESLP will enable further collaboration into the application of advanced therapies. =

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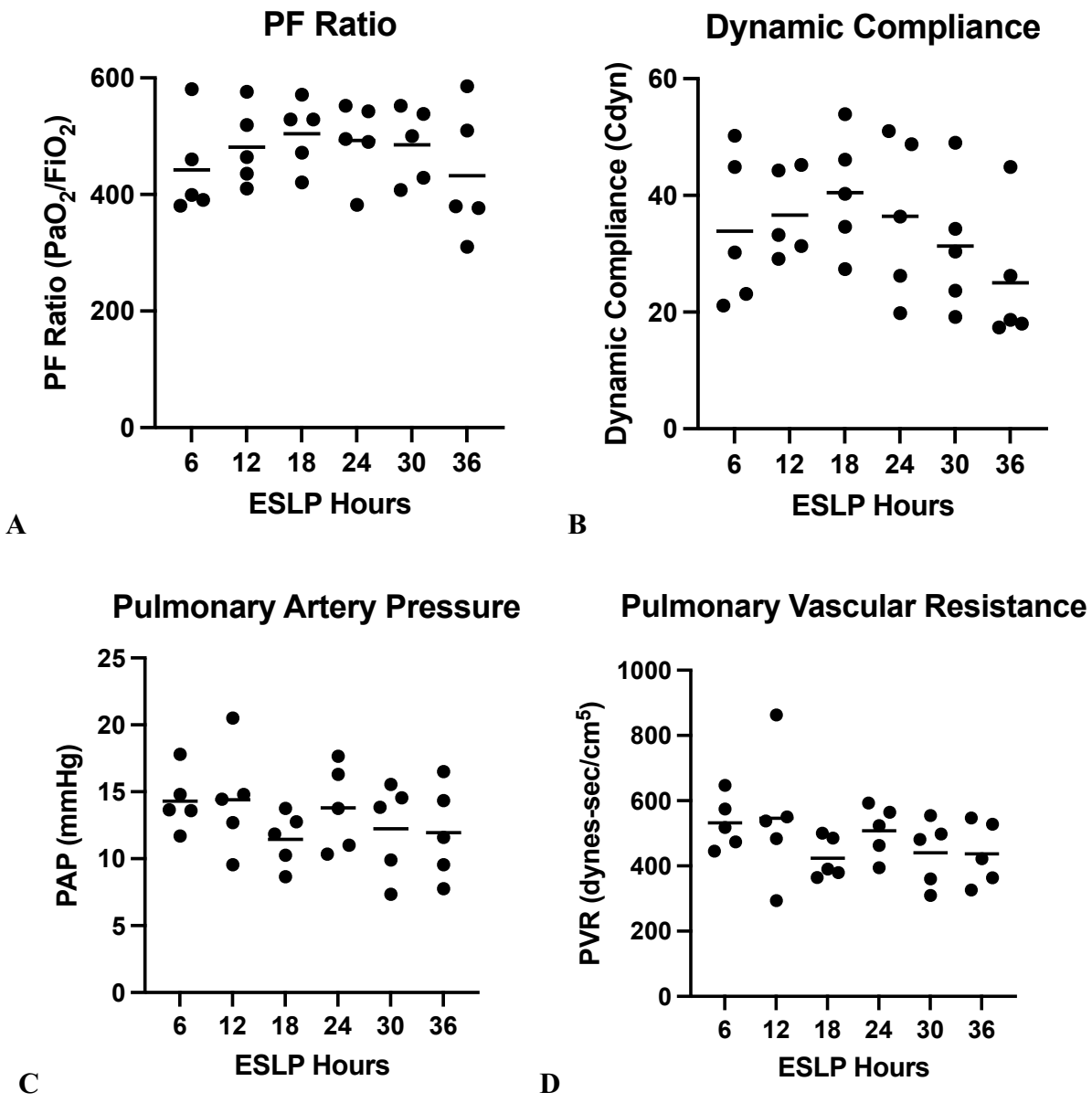


Figure 9.1. Pig lung physiology parameters during 36-hours NPV-ESLP. PF ratio (PaO_2/FiO_2 Ratio) (A), dynamic compliance (Cdyn) (B), pulmonary artery pressure (PAP)(C), and pulmonary vascular resistance (PVR) (D). Over 36-hours of NPV-ESLP, lung parameters remained stable in all groups. Oxygenation and dynamic compliance improved gradually up to hour 18 to 24 and then experienced a gradual decline in performance. PAP and PVR demonstrated a gradual and modest decrease over the duration of ESLP with both parameters being greatest at the during the initial 12 hours. *NPV-ESLP*, Negative Pressure Ventilation Ex-Situ Lung Perfusion.

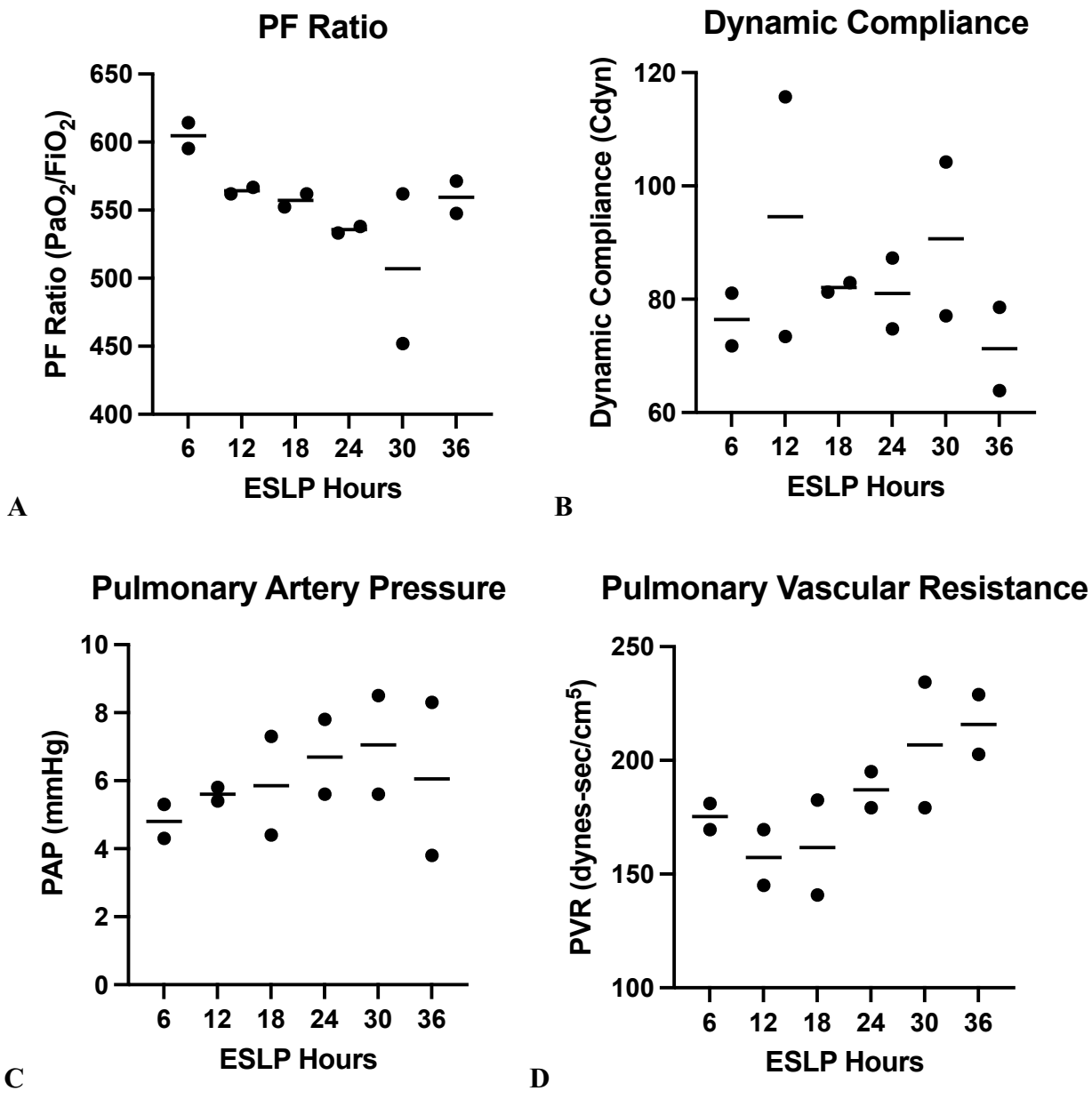
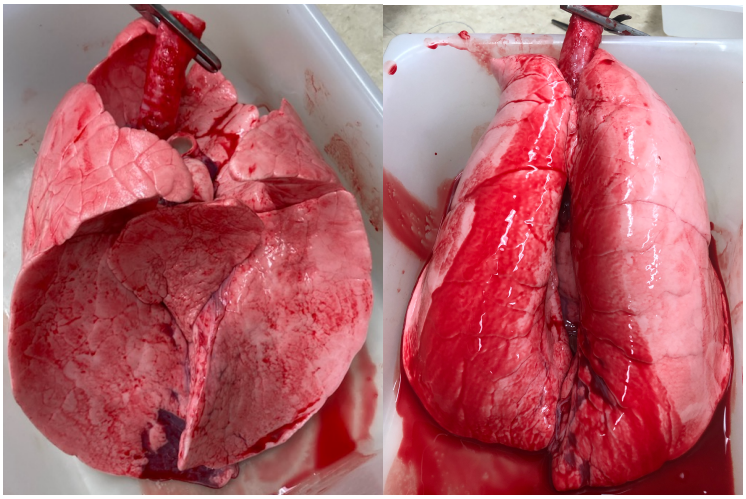


Figure 9.2. Human lung physiology parameters during 36-hours NPV-ESLP (n=2). PF ratio (PaO_2/FiO_2 Ratio) (A), dynamic compliance (Cdyn) (B), pulmonary artery pressure (PAP)(C), and pulmonary vascular resistance (PVR) (D). Lung parameters were stable over 36-hours of NPV-ESLP. PF ratios ranged from 451.9-614.3 mmHg. Cdyn ranged from 63.9-115.7 mL/cmH_2O. PAP ranged from 3.8-8.5 mmHg. PVR ranged from 145.0-234.5 dynes-sec/cm^5. *NPV-ESLP*, Negative Pressure Ventilation Ex-Situ Lung Perfusion.

A



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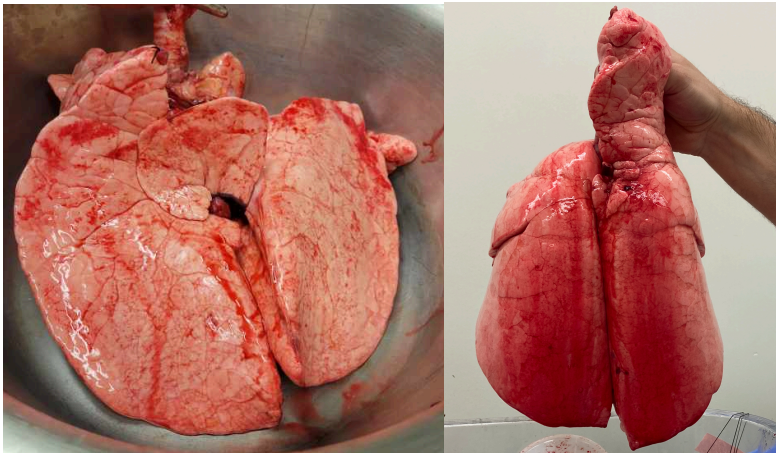

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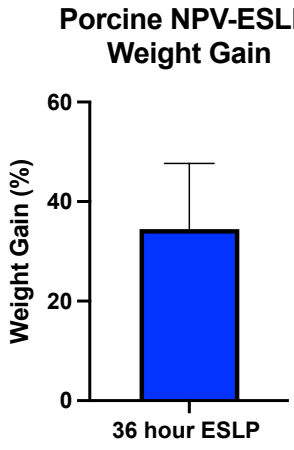


Figure 9.3. Representative gross images of the ventral surface of porcine lungs. Pre-ESLP (A), Post-36-hour NPV-ESLP (B), percentage weight-gain per hour (C, n=5 pigs), and percentage weight-gain per hour (D, n=2 human). All data points are plotted as dots. Bar graph denotes mean hourly percentage weight-gain +/- SEM. *NPV-ESLP*, Negative Pressure Ventilation Ex-Situ Lung Perfusion.

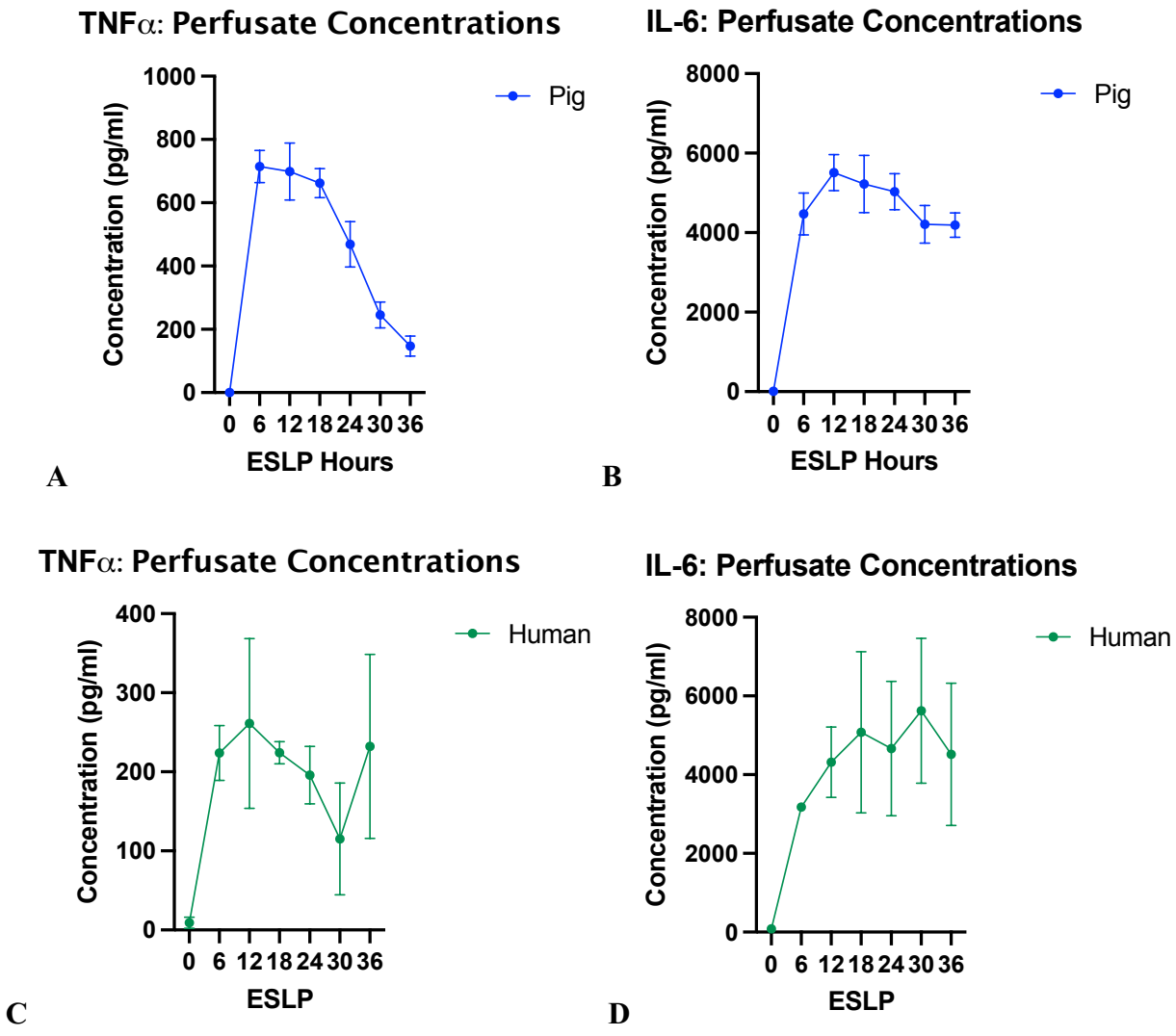


Figure 9.4. Pro-inflammatory cytokine concentrations in ESLP perfusate. TNF-alpha Pig (A), IL-6 Pig (B), TNF-alpha Human (C), and IL-6 Human (D). Results are presented as mean +/- SEM. *P<0.05 denotes statistical significance. IL, Interleukin; *TNF-alpha*, tumor necrosis factor alpha *NPV-ESLP*, Negative Pressure Ventilation Ex-Situ Lung Perfusion.

Initiation of Ex-vivo Lung Perfusion (Negative Pressure Ventilation)					
	Perfusion time (min)				
	0	10	20	60 (T1)	360 (T6)
Perfusate Temperature (°C)	20°C	32°C	38°C	38°C	38°C pig 37°C human
PA flow (% CO; CO = 70 ml/kg/min)	10%	20%	30%	30%	50%
Ventilation mode	PPV (CPAP 20 cmH_2O)	Initiate NPV Preservation	NPV Preservation	NPV Preservation	NPV Evaluation
Medical gas mixer	None	None	None	100% CO_2	89% N_2, 8% CO_2, 3% O_2
Left atrial pressure	0	0	0	0	0

Table 9.1. Initiation of 36-hour NPV-ESLP Protocol. CO, cardiac output; PA, pulmonary artery; PPV, positive pressure ventilation; NPV, negative pressure ventilation. For preservation mode ventilation parameters see Table 2. Beginning at T6, evaluation was conducted serially every 6 hours, for 5 minutes, 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 ventilation parameters provided in Table 2.

NPV-ESLP Strategy: Preservation vs Evaluation

	NPV-ESLP Mode	
	Preservation	Evaluation
Perfusion Parameters		
Temperature (^oC)	38^oC	38^oC
Pulmonary artery flow (CO = 70 ml/kg/min)	30% of estimated CO	50% of estimated CO
PAP	<15-20 mmHg	<15-20 mmHg
LAP (open LA)	0 mmHg	0 mmHg
Medical gas mixture	100% CO_2	89% N_2,8%CO_2,3%O_2
Medical gas mixture (L/minute) titrated to PCO_2	<35 mmHg	<35 mmHg
pH target	7.45-7.55	7.45-7.55
Ventilation parameters		
Mode	Pressure control	Pressure control
Desired inspiratory tidal volume	3-8 ml/kg	3-8 ml/kg
Inspiratory: Expiratory Ratio	1:1 – 1.5	1:1 – 1.5
Frequency	6 to 10 breaths/min	6 to 10 breaths/min
P_{AWP}	< 15-20 cmH_2O	< 15-20 cmH_2O
PEEP	5 cmH_2O	5 cmH_2O
FiO_2	21%	21%

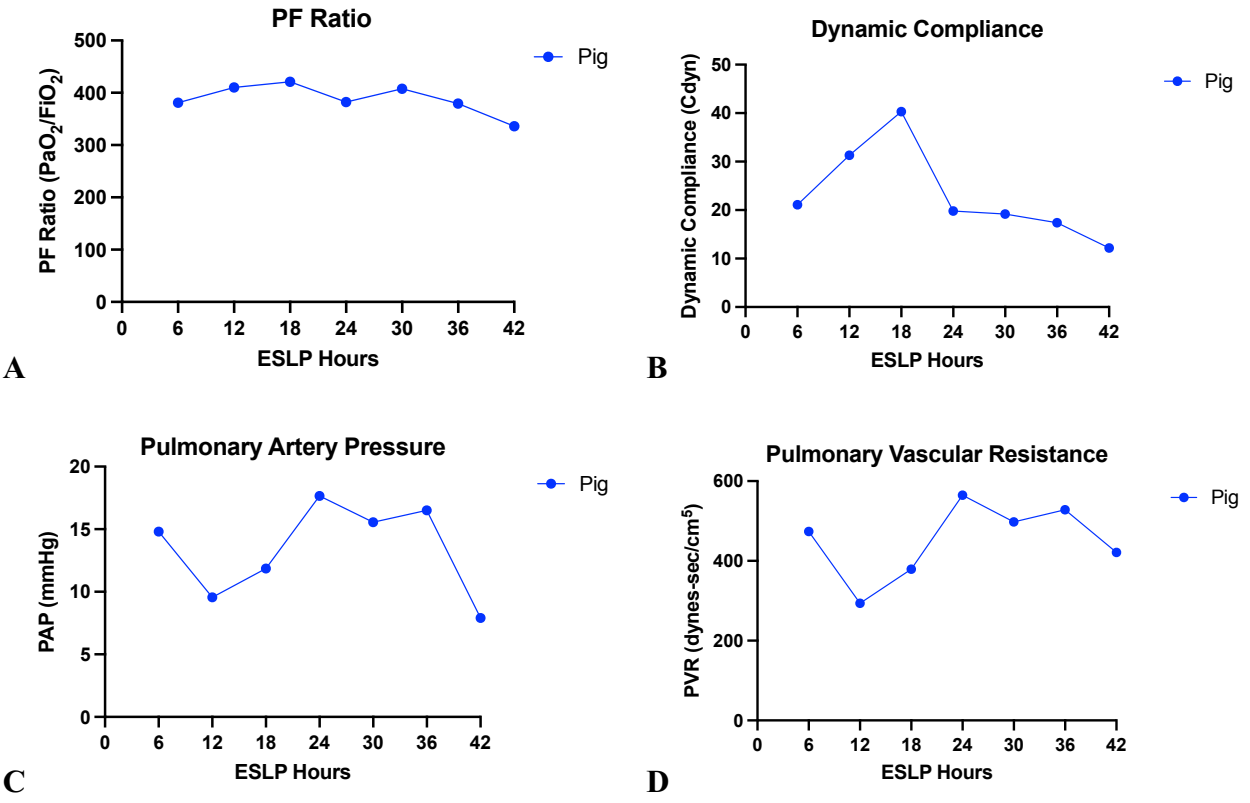
Table 9.2. Modes of NPV-ESLP: Preservation vs Evaluation. CO, cardiac output; FIO_2, fraction inspired of oxygen; LAP, left atrial pressure; NPV, negative pressure ventilation; PAP, mean pulmonary artery pressure; PAWP, peak airway pressure; PEEP, positive end-expiratory pressure; PCO_2, partial pressure of carbon dioxide in pulmonary arterial circulation.

Porcine NPV-ESLP

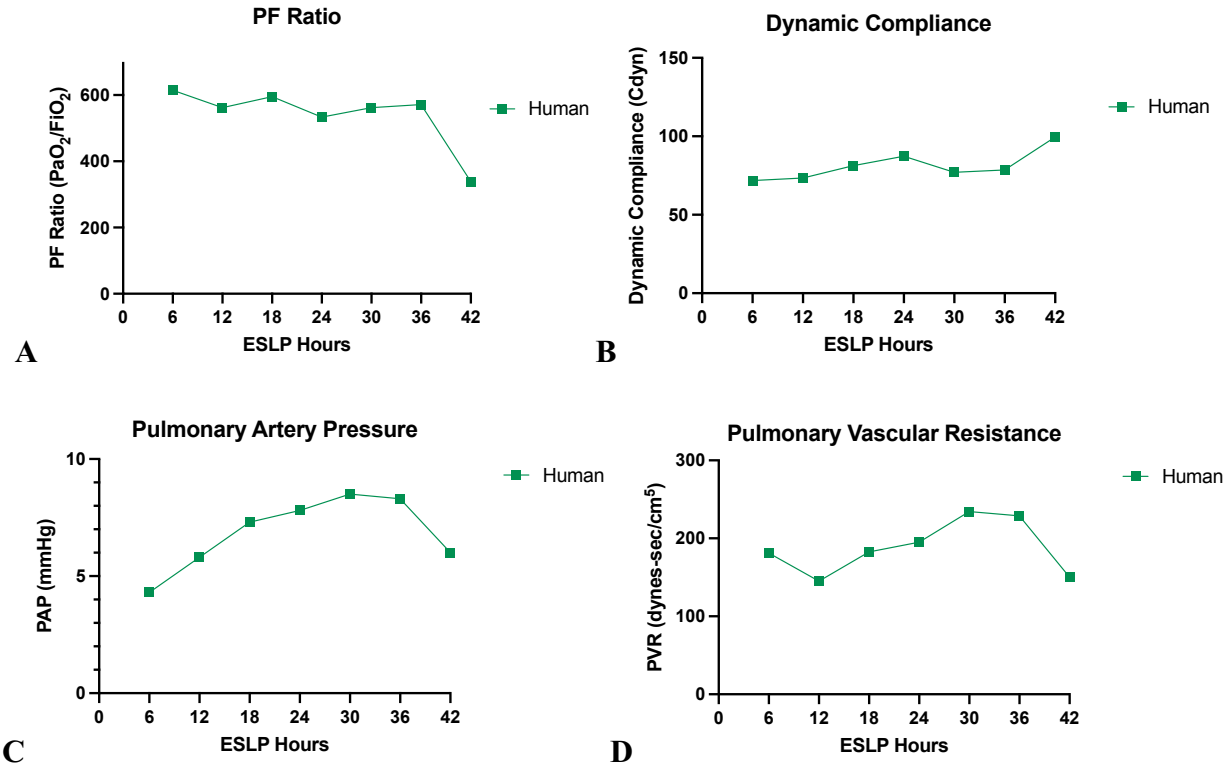
	NPV-ESLP Protocol Refinement	
	12-hour ESLP (2018)	36-hour ESLP (2022)
Perfusion Parameters		
Temperature (ºC)	38ºC	38ºC
Pulmonary artery flow	30% CO Preservation	30% CO Preservation
(CO = 70 ml/kg/min)	50% CO Evaluation	50% CO Evaluation
PAP	<20-25 mmHg	<15-20 mmHg
LAP (open LA drainage)	0 mmHg	0 mmHg
Medical gas mixture (89%N_2,8%CO_2,3% O_2)	Preservation &	Evaluation only
Medical gas mixture (litres/min) titrated to	Evaluation	
pCO_2	35-50 mmHg	<35 mmHg
pH target	7.35-7.45	7.45-7.55
Ventilation parameters		
Mode	Pressure control	Pressure control
Inspiratory tidal volume	6-10 ml/kg	3-8 ml/kg
Respiratory Rate	7-12 breaths/min	7 breaths/min
Inspiratory: Expiratory Ratio	1:1 – 1.5	1:1 – 1.5
P_{AWP}	< 20-25 cmH_2O	< 15-20 cmH_2O
PEEP	7 cmH_2O	5 cmH_2O
FiO_2	21%	21%
Buffered Cellular Perfusate		
Hyperoncotic with HSA	32-35 mmHg	32-35 mmHg
pRBC	Hct 12-15%	Hct 12-15%
Nutritional Supplementation	25% Dextrose (D25)	D25, TPN, multivitamins
Antibiotics	Piperacillin-Tazobactam	Piperacillin-Tazobactam
Steroids	Methylprednisone 10,000 IU	Methylprednisone 10,000 IU
Heparin		
Perfusate Management	No Perfusate Exchange	No Perfusate Exchange

Table 9.3. 36-hour NPV-ESLP protocol refinement. 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 pulmonary arterial circulation; HSA, human serum albumin; pRBC, packed red blood cells; Hct, hematocrit; TPN, total parenteral nutrition; D25, 25% Dextrose.

Supplemental Figures:



Supplemental Figure 9.5A. Lung physiology parameters during 42-hours porcine NPV-ESLP (n=1). PF ratio (PaO_2/FiO_2 Ratio) (A), dynamic compliance (Cdyn) (B), pulmonary artery pressure (PAP)(C), pulmonary vascular resistance (D). Pig lungs demonstrated stable PF ratios over 42-hours of ESLP. Dynamic compliance peaked at 18-hours and then decreased to baseline levels thereafter. PAP and PVR show greater variability over time. This observation highlights the reactivity of porcine lungs compared to human lungs that we have noticed in the lab (Supplemental Figure 5b). *NPV-ESLP*, Negative Pressure Ventilation Ex-Situ Lung Perfusion.



Supplemental Figure 9.5B. Lung physiology parameters during 42-hours human NPV-ESLP (n=1). PF ratio (PaO_2/FiO_2 Ratio) (A), dynamic compliance (Cdyn) (B), pulmonary artery pressure (PAP)(C), pulmonary vascular resistance (D). Over 42-hours of NPV-ESLP, human lung parameters remained stable, except for a decrease in the final PF ratio over the last 6-hours of preservation. *NPV-ESLP*, Negative Pressure Ventilation Ex-Situ Lung Perfusion.

Ch 10:

Thesis Summary and Future Directions

Thesis Summary

The number of lives saved by lung transplantation is limited by an inadequate supply of high-quality donor lungs. ESLP was developed to salvage lungs in the DCD setting and has evolved into the preservation of lungs from NDD donors. Available clinical platforms have enabled the prolonged preservation, continuous assessment, and reconditioning of marginal quality lungs to enhance the donor pool. The next horizon for this technology is continuous prolonged preservation in the clinical realm beyond what can be safely achieved with the current clinical standard of cold static storage. This next era will include further advancements in the specific treatment of donor lung pathologies that were previously untreatable. The application of therapeutics will include immunomodulatory approaches to dampen the deleterious immune response of recipients towards grafts and potentially reduce the incidence of PGD and CLAD, thereby enhancing long-term survival.

This thesis investigated several aspects of NPV-ESLP protocol refinement with the aim of enhancing the quality and duration of preservation up to 36-hours. A secondary aim of this thesis was to progress in a logical manner such that the chapters can serve as an instruction manual for incoming graduate students with each component of the various protocols clearly defined and justified with objective data. The hope is that this documentation will serve as a "how-to" manual and flatten the learning curve of these complex experiments.

This thesis begins with a comprehensive review of ESLP preclinical and clinical research to establish both content and context for subsequent chapters. Next, detailed method chapters outline our initial standard ESLP protocol and transplant operation applied throughout the

remaining chapters. Specific protocol modifications are explained in successive chapters to outline the maturation of our approach.

The subsequent three chapters investigate temperature, flow rate, and blood gas management over 12-hour NPV-ESLP. First, we established that normothermic NPV-ESLP is superior to cold perfusion ESLP, and colder temperatures benefit from a reduced perfusion flow rate to compensate for the increased PVR and decreased lung compliance. Second, we investigated whether further reductions in perfusate flow at normothermia are similarly protective as seen in cold perfusion, and we determined that a further reduction in flow results in endothelial dysfunction and worse IRI at transplantation manifested as a higher score of PGD. Third, we studied the potentially beneficial role of mild permissive hypocarbic alkalosis during ESLP and demonstrated an increased reliability in preservation of 12-hours with a simplification of active management.

The last two chapters involve the application of these protocol refinements to achieve reliable 24-hour and 36-hour NPV-ESLP. The fourth experiment investigates the effect of perfusate exchange during 24-hour ESLP and demonstrated no significant effect on physiologic performance during preservation or following transplantation compared to no perfusate management strategy. The final experiment tests the quality of our refined protocol by pursing the maximum duration of porcine lung preservation up to 36-hours, which is further supported by a similar endpoint achieved using discarded human lungs. This is a milestone achievement in preclinical continuous preservation using ESLP. NPV-ESLP has previously been shown by our lab to result in reduced lung injury, reduced inflammation, and less edema formation compared to PPV-ESLP. Our ability to achieve 36-hours of continuous ESLP preservation with excellent

physiologic outcomes is supportive of NPV-ESLP as the ideal platform for ultra-prolonged preservation, and this further opens the door for targeted therapeutic intervention.

Future investigations with NPV-ESLP should focus on further refinement of the standard protocol to establish objective evidence for the following steps: evaluating lung using 50% CO versus 30% and initiating ventilation at 32°C versus normothermia. Furthermore, it would be interesting to assess the temperature inside the lung chamber during ESLP >24 hours due to the potential to induce fever through a green-house effect, explore the role of ultrafiltration for perfusate management in ESLP >36 hours, transplantation of lungs after 36-hour NPV-ESLP to assess *in-vivo* performance, and post-transplant survival observation of 3–7-day duration to better assess for PGD score. Given additional opportunity to research NPV-ESLP, these are the questions and achievements that I would pursue. Ultimately, I believe the full potential for the therapeutic role of ESLP depends upon ongoing refinement of the protocol as well as extension of safe-continuous preservation to allow for more advanced donor organ manipulation. This will allow for the optimization of the donor pool and the maximization of lives saved through lung transplant.

REFERENCES

Chapter 1

Thesis Overview and Research Proposal

1. van der Mark SC, Hoek RAS, Hellemons ME. Developments in lung transplantation over the past decade. Eur Respir Rev. 2020;29:190132.

2. Leard LE, Holm AM, Valapour M, et al. Consensus document for the selection of lung transplant candidates: An update from the International Society for Heart and Lung Transplantation. JHLT, 2021; 40(11): 1349-1379.

3. Courtwright A, Cantu E. Evaluation and management of the potential lung donor. Clin Chest Med. 2017; 38: 751-759

4. Punch JD, Hayes DH, Laporte FB, McBride V, Seely MS. Organ donation and utilization in the United States, 1996-2005. Am J Transplant. 2007;7(SUPPL. 1): 1327-1338.

5. Valapour M, Lehr CJ, Skeans MA, et al. OPTN/SRTR 2019 Annual Report: Lung. Am J Transplantation. 2021;21: 441-520.

6. Canadian Institute for Health Information. *Annual Statistics on Organ Replacement in Canada: Dialysis, Transplantation and Donation, 2009 to 2018*. Ottawa, ON: CIHI; 2019.

7. Keshavjee SH, Yamazaki F, Cardoso PF, McRitchie DI, Patterson GA, Cooper JD. A method for safe twelve-hour pulmonary preservation. *J Thorac Cardiovasc Surg.* 1989;98(4):529-534.

8. Guibert EE, Petrenko AY, Balaban CL, et al. Organ preservation current concepts and new strategies for the next decade. Transfus Med Hemother 2011;38:125–142.

9. Chambers DC, Cherikh WS, Goldfarb SB, et al. The International Thoracic Organ Transplant Registry of the International Society for Heart and Lung Transplantation: Thirty-fifth adult lung and heart-lung tranplant report — 2018; Focus theme: Multiorgan Transplantation. J Hear Lung Transplant. 2018.

10. Buchko MT, Boroumand N, Cheng JC, et al. Clinical transplantation using negative pressure ventilation ex situ lung perfusion with extended criteria donor lungs. Nature Communications. 2020; https://doi.org/10.1038/s41467-020-19581-4

11. Sanchez PG, Cantu E, Hartwig M, et al. The NOVEL study: A multi-center clinical trial studying the safety of Ex Vivo Lung Perfusion. JHLT. 2020; 39(4); s110. https://doi.org/10.1016/j.healun.2020.01.977

12. Warnecke G, Van Raemdonck D, Smith MA, et al. Normothermic ex-vivo preservation with the portable Organ Care System Lung device for bilateral lung transplantation (INSPIRE): a randomized, open-label, non-inferiority, phase 3 study. Lancet Respir Med. 2018; 6: 357-67.

13. Loor G, Warnecke G, Villavicencio MA, et al. Portable normothermic ex-vivo lung perfusion, ventilation, and functional assessement with the Organ Care System on donor lung use for transplantation from extended-criteria donors (EXPAND): a single-arm, pivotal trial. Lancet Respir Med. 2019;7:975-84.

14. Aboelnazar N, Himmat S, Hatami S, et al. Negative pressure ventilation decreases inflammation and lung edema during normothermic ex-vivo lung perfusion. JHLT. 2018; 37:520-530.

15. Buchko MT, Catherine JS, Hatami S, et al. Total parenteral nutrition in ex vivo lung perfusion: Addressing metabolism improves both inflammation and oxygenation. AJT. 2019;19:3390-3397.

16. Nakajima D, Liu M, Ohsumi A, et al. Lung lavage and surfactant replacement during ex-vivo lung perfusion for treatment of gastric acid aspiration-induced donor lung injury. JHLT. 2017;36:577-585.

17. Takahashi M, Cheung H, Watanabe T, et al. Strategies to prolong homeostasis of ex vivo perfused lungs. JTCVS. 2020; 161(6): 1963-73.

18. Steen S, Sjoberg T, Pierre L, et al. Transplantation of lungs from a non-heart-beating donor. Lancet. 2001; 357: 825-29.

19. Forgie KA, Fialka N, Bucko MT, et al. Normothermic negative pressure ventilation ex-situ lung perfusion: evaluation of lung function and metabolism. Journal of Visualized Experiments. 2022;180: doi:10.3791/62982.

20. Buchko MT, Himmat S, Aboelnazar NS, et al. A low-cost perfusate alternative for ex vivo lung perfusion. Transplantation Proceedings 2020;52(10):2941-2946.

21. Forgie KA, Fialka N, Mubashir K, et al. Left lung orthotopic transplantation in a juvenile porcine model for ESLP. Journal of Visualized Experiments. 2022;180: doi: 10.3791/62979.

22. Loor G, Howard BT, Spratt JR, et al. Prolonged EVLP using OCS Lung: Cellular and Acellular Perfusates. *Transplantation.* 2017; 101(10): 2303-2311.

23. Sommer W, Salman J, Avsar M, et al. Prediction of transplant outcome after 24-hours ex vivo lung perfusion using the Organ Care System in a porcine lung transplantation model.

24. Cypel M, Yeung JC, Hirayama S, et al. Technique for prolonged normothermic Ex Vivo Lung Perfusion. JHLT. 2008; 27(12): 1318-1325.

25. Judge EP, Hughes JM, Egan JJ, et al. Anatomy and bronchoscopy of the porcine lung: a model for translational respiratory medicine. Am J Resp Cell and Mol Biol. 2014; 51(3):334-43.

26. Weissenbacher A, Vrakas G, Nasralla D, Ceresa C. The future of organ perfusion and re-conditioning. Transplant International. 2019;32:586-597.

27. Hatami S, Hefler J, and Freed DH. Inflammation and oxidative stress in the context of extracorporeal cardiac and pulmonary support. Frontiers in Immunology. 2022; 13: https://doi.org/10.3389/fimmu.2022.831930

28. Jochmans I, O'Callaghan JM, Pirenne J, Ploeg RJ. Hypothermic machine perfusion of kidneys retrieved from standard and high-risk donors. Transplant International. 2015;28:665-76.

29. Schlegel A, Muller X, Dutkowski P. Hypothermic machine preservation of the liver: state of the art. Current Transplantation Reports. 2018;5:93-102.

30. Nakajima D, Chen F, Yamada T, Sakamoto J, Osumi A, Fujinaga T, et al. Hypothermic machine perfusion ameliorates ischemia-reperfusion injury in rat lungs from non-heart-beating donors. Transplantation. 2011;92:858-63.

31. Nakajima D, Chen F, Okita K, Motoyama H, Hijiya K, Ohsumi A, et al. Reconditioning lungs donated after cardiac death using short-term hypothermic machine perfusion. *Transplantation.* 2012;94:999-1004.

32. Gloria JN, Yerxa J, Kesseli SJ, et al. Subnormothermic ex vivo lung perfusion attenuates graft inflammation in a rat transplant model. *The Journal of Thoracic and Cardiovascular Surgery*. 2021; S0022-5223(21)00165-3.

33. Arni S, Maeyashiki T, Opitz I, Inci I. Subnormothermic ex vivo lung perfusion attenuates ischemia reperfusion injury from donation after circulatory death donors. *PLoS ONE*. 2021; 16(8) e0255155.

34. Arni S, Maeyashiki T, Citak N, et al. Subnormothermic ex vivo lung perfusion temperature improves graft preservation in lung transplantation. *Cells*. 2021; 10: 748. https://doi.org/10.3390/cells10040748

35. Steen S, Ingemansson R, Eriksson L, et al. First human transplantation of a nonacceptable donor lung after reconditioning ex vivo. Ann Thorac Surg. 2007; 83: 2191-5.

36. Beller JP, Byler MR, Money DT, et al. Reduced-flow ex vivo lung perfusion to rehabilitate lungs donated after circulatory death. *J Heart Lung Transplant.* 2020;39:74-82.

37. Chamarthy MR, Kandathil A, Kalva SP. Pulmonary vascular pathophysiology. Cardiovasc Diagn Ther 2018;8(3):208-213.

38. Mahdi M, Joseph NJ, Hernandez DP, Crystal GJ, Baraka A, Salem MR. Induced hypocapnia is effective in treating pulmonary hypertension following mitral valve replacement. Anesth. 2011; 21(2):259-70.

39. Chang AC, Zucker HA, Hickey PR, Wessel DL. Pulmonary vascular resistance in infants after cardiac surgery: Role of carbon dioxide and hydrogen ion. Crit Car Med. 1995;23:568-574.

40. Fox WW, Duara S: Persistent pulmonary hypertension in the neonate: Diagnosis and management. J Pediatr 1983; 103:505-514.

41. De Wolf J, Glorion M, Jouneau L, et al. Challenging the ex vivo lung perfusion procedure with continuous dialysis in a pig model. Transplantation. 2022; 106(5):979-87.

42. Buchko MT, Himmat S, Stewart CJ, et al. Continuous hemodialysis does not improve graft function during ex vivo lung perfusion over 24 hours. Transplantation Proceedings;51:2022-28.

43. Hozain AE, Tipograf Y, Pinezich MR, et al. Multiday maintenance of extracorporeal lungs using cross-circulation with conscious swine. JTCVS. 2020;159(4):1640-1653.

44. O'Neill JD, Guenthart BA, Hozain AE, Bacchetta M. Xenogeneic support for the recovery of human donor organs.

45. Ali A, Nykanen AI, Beroncal E, et al. Successful 3-day lung preservation using a cyclic normothermic ex vivo lung perfusion strategy. Lancet 2022;83:104210. https://doi.org/10.1016/j.ebiom.2022.104210

Chapter 2

ESLP: a Narrative and State-of-the-Art Review

1. van der Mark SC, Hoek RAS, Hellemons ME. Developments in lung transplantation over the past decade. Eur Respir Rev. 2020;29:190132.

2. Leard LE, Holm AM, Valapour M, et al. Consensus document for the selection of lung transplant candidates: An update from the International Society for Heart and Lung Transplantation. JHLT, 2021; 40(11): 1349-1379.

3. Courtwright A, Cantu E. Evaluation and management of the potential lung donor. Clin Chest Med. 2017; 38: 751-759

4. Punch JD, Hayes DH, Laporte FB, McBride V, Seely MS. Organ donation and utilization in the United States, 1996-2005. Am J Transplant. 2007;7(SUPPL. 1): 1327-1338.

5. Valapour M, Lehr CJ, Skeans MA, et al. OPTN/SRTR 2019 Annual Report: Lung. Am J Transplantation. 2021;21: 441-520.

6. Canadian Institute for Health Information. *Annual Statistics on Organ Replacement in Canada: Dialysis, Transplantation and Donation, 2009 to 2018*. Ottawa, ON: CIHI; 2019.

7. Keshavjee SH, Yamazaki F, Cardoso PF, McRitchie DI, Patterson GA, Cooper JD. A method for safe twelve-hour pulmonary preservation. *J Thorac Cardiovasc Surg.* 1989;98(4):529-534.

8. Guibert EE, Petrenko AY, Balaban CL, et al. Organ preservation current concepts and new strategies for the next decade. Transfus Med Hemother 2011;38:125–142.

9. Chambers DC, Cherikh WS, Goldfarb SB, et al. The International Thoracic Organ Transplant Registry of the International Society for Heart and Lung Transplantation: Thirty-fifth adult lung and heart-lung tranplant report — 2018; Focus theme: Multiorgan Transplantation. J Hear Lung Transplant. 2018.

10. Buchko MT, Boroumand N, Cheng JC, et al. Clinical transplantation using negative pressure ventilation ex situ lung perfusion with extended criteria donor lungs. Nature Communications. 2020; https://doi.org/10.1038/s41467-020-19581-4

11. Sanchez PG, Cantu E, Hartwig M, et al. The NOVEL study: A multi-center clinical trial studying the safety of Ex Vivo Lung Perfusion. JHLT**. 2020**; 39(4); s110. https://doi.org/10.1016/j.healun.2020.01.977

12. Warnecke G, Van Raemdonck D, Smith MA, et al. Normothermic ex-vivo preservation with the portable Organ Care System Lung device for bilateral lung transplantation (INSPIRE): a randomized, open-label, non-inferiority, phase 3 study. Lancet Respir Med. 2018; 6: 357-67.

13. Loor G, Warnecke G, Villavicencio MA, et al. Portable normothermic ex-vivo lung perfusion, ventilation, and functional assessement with the Organ Care System on donor lung use for transplantation from extended-criteria donors (EXPAND): a single-arm, pivotal trial. Lancet Respir Med. 2019;7:975-84.

14. Aboelnazar N, Himmat S, Hatami S, et al. Negative pressure ventilation decreases inflammation and lung edema during normothermic ex-vivo lung perfusion. JHLT. 2018; 37:520-530.

15. Buchko MT, Catherine JS, Hatami S, et al. Total parenteral nutrition in ex vivo lung perfusion: Addressing metabolism improves both inflammation and oxygenation. AJT. 2019;19:3390-3397.

16. Nakajima D, Liu M, Ohsumi A, et al. Lung lavage and surfactant replacement during ex-vivo lung perfusion for treatment of gastric acid aspiration-induced donor lung injury. JHLT. 2017;36:577-585.

17. Takahashi M, Cheung H, Watanabe T, et al. Strategies to prolong homeostasis of ex vivo perfused lungs. JTCVS. 2020; 161(6): 1963-73.

18. De Perrot, M.; Weder, W.; Patterson, G.A.; Keshavjee, S. Strategies to increase limited donor resources. *Eur. Respir. J.* 2004, *23*, 477–482.

19. Snell, G.I.; Griffiths, A.; Levvey, B.J.; Oto, T. Availability of lungs for transplantation: Exploring the real potential of the donor pool. *J. Heart Lung Transplant.* 2008, *27*, 662–667.

20. 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. J. Heart Lung Transplant. 2000, 19, 1199–1204.

21. Botha, P.; Trivedi, D.; Weir, C.J.; Searl, C.P.; Corris, P.A.; Dark, J.H.; Schueler, S.V. Extended donor criteria in lung transplantation: Impact on organ allocation. J. Thorac. Cardiovasc. Surg. 2006, 131, 1154–1160.

22. De Perrot, M.; Liu, M.; Waddell, T.K.; Keshavjee, S. Ischemia-reperfusion-induced lung injury. Am. J. Respir. Crit. Care Med. 2003, 167, 490–511.

23. Suzuki, Y.; Cantu, E.; Christie, J.D. Primary graft dysfunction. Semin. Respir. Crit. Care Med. 2013, 34, 305–319.

24. Lardinois, D.; Banysch, M.; Korom, S.; Hillinger, S.; Rousson, V.; Boehler, A.; Speich, R.; Weder, W. Extended donor lungs: Eleven years experience in a consecutive series. Eur. J. Cardiothorac. Surg. 2005, 27, 762–767.

25. Forgie KA, Fialka N, Freed DH, Nagendran J. Lung transplantation, pulmonary endothelial inflammation, and *ex-situ* lung perfusion: a review. *Cells* 2021;10,1417. https://doi.org/10.3390/cells10061417

26.Steen,S.;Liao,Q.;Wierup,P.N.;Bolys,R.;Pierre,L.;Sjoberg,T.Transplantation of lungs from non-heart-beating donor safter functional assessment ex vivo. Ann. Thorac. Surg. **2003**, 76, 244–252.

27. Cypel M, Keshavjee S. The clinical potential of ex vivo lung perfusion. *Expert Rev Respir* Med; 2012,6,27–35.

28. Cypel M., Yeung J.C., Liu,M., Anraku,M.; Chen,F.; Karolak,W.; Sato,M.; Laratta,J.; Azad,S.; Madonik,M.; et al. Normothermic ex vivo lung perfusion in clinical lung transplantation. *N. Engl. J. Med.* 2011, *364*, 1431–1440.

29. Carrel A, Lindbergh CA. THE CULTURE OF WHOLE ORGANS. Science 1935;81:621-3.

30. Jirsch DW, Fisk RL, Couves CM. Ex vivo evaluation of stored lungs. Ann Thorac Surg 1970;10:163-8.

31. Hardesty, R.L.; Griffith, B.P. Autoperfusion of the heart and lungs for preservation during distant procurement. *J. Thorac. Cardiovasc. Surg.* 1987, *93*, 11–18

32. Steen S, Sjöberg T, Pierre L, et al. Transplantation of lungs from a non-heart-beating donor. Lancet 2001;357:825-9.

33. Steen S, Ingemansson R, Eriksson L, et al. First human transplantation of a nonacceptable donor lung after reconditioning ex vivo. Ann Thorac Surg 2007;83:2191-4.

34. Ingemansson R, Eyjolfsson A, Mared L, et al. Clinical transplantation of initially rejected donor lungs after reconditioning ex vivo. Ann Thorac Surg 2009;87:255-60

35. Erasmus ME, Fernhout MH, Elstrodt JM, et al. Normothermic ex vivo lung perfusion of non-heart- beating donor lungs in pigs: from pretransplant function analysis towards a 6-h machine preservation. Transpl Int 2006;19:589-93.

36. Cypel M, Yeung JC, Hirayama S, et al. Technique for prolonged normothermic ex vivo lung perfusion. J Heart Lung Transplant 2008;27:1319-25.

37 (NOVEL) Sanchez P, Chan E, Davis R, et al. Normothermic *Ex Vivo* Lung Perfusion (NOVEL) As An Assessment of Extended/Marginal Donor Lungs: A Prospective Multi-Center Clinical Trial. The Journal of Heart and Lung Transplantation, Vol 41, No 4S, April 2022.

38 Slama A, Schillab L, Barta M, et al. Standard donor lung procurement with normothermic ex vivo lung perfusion: A prospective randomized clinical trial. J Heart Lung Transplant 2017**;**36:744-53.

39. Aigner C, Slama A, Hötzenecker K, et al. Clinical ex vivo lung perfusion--pushing the limits. Am J Transplant 2012;12:1839-47.

40. Sage E, Mussot S, Trebbia G, et al. Lung transplantation from initially rejected donors after ex vivo lung reconditioning: the French experience. Eur J Cardiothorac Surg 2014;46:794-9.

41 Warnecke G, Moradiellos J, Tudorache I, et al. Normothermic perfusion of donor lungs for preservation and assessment with the Organ Care System Lung before bilateral transplantation: a pilot study of 12 patients. Lancet **2012**;380:1851-8.

42. Zhang ZL, van Suylen V, van Zanden JE, et al. First experience with ex vivo lung perfusion for initially discarded donor lungs in the Netherlands: a single-centre study. Eur J Cardiothorac Surg 2019;55:920-6.

43. Cypel M, Yeung JC, Machuca T, et al. Experience with the first 50 ex vivo lung perfusions in clinical transplantation. *J Thorac Cardiovasc Surg.* 2012;144(5):1200-1206.

44. Sanchez PG, Davis RD, D'Ovidio F, et al. The NOVEL Lung Trial One-Year Outcomes. *The Journal of Heart and Lung Transplantation.* 2014;33(4):S71-S72.

45. Yeung JC, Cypel M, Machuca TN, et al. Physiologic assessment of the ex vivo donor lung for transplantation. *JHLT* 2012;31(10):1120-1126.

46. Becker S, Steinmeyer J, Avsar M, et al. Evaluating acellular versus cellular perfusate composition during prolonged ex vivo lung perfusion after initial cold ischaemia for 24 hours. *Transpl Int.* 2015;29(1):88-97.

47. Roman M, Gjorgjimajkoska O, Neil D, et al. Comparison between cellular and acellular perfusates for ex vivo lung perfusion in a porcine model. J Heart Lung Transplant. 2015;34(7):978-987.

48. Van Raemdonck D, Neyrinck A, Cypel M, Keshavjee S. Ex-vivo lung perfusion. *Transpl Int.* 2014;28(6):643-656.

49. Stone JP, Sevenoaks H, Sjoberg T, Steen S, Yonan N, Fildes JE. Mechanical removal of dendritic cell-generating non-classical monocytes via ex vivo lung perfusion. *J Heart Lung Transplant.* 2014;33(8):864-869.

50. Wallinder A, Ricksten SE, Hansson C, et al. Transplantation of initially rejected donor lungs after ex vivo lung perfusion. *J Thorac Cardiovasc Surg.* 2012;144(5):1222-1228.

51. Machuca TN, Cypel M, Keshavjee S. Advances in lung preservation. *Surg Clin North Am.* 2013;93(6):1373-1394.

52. Buchko MT, Himmat S, Aboelnazar NS, Stewart CJ, Hatami S, Dromparis P, Adam B, Freed, DH, Nagendran J. A low-cost perfusate alternative for ex vivo lung perfusion. *Transplantation Proceedings* (**2020**); XX: 1-6.

53 (PERFUSIX) Mallea JM, Hartwig MG, Keller CA, et al. Remote ex vivo lung perfusion at a centralized evaluation facility. JHLT 2022; 41(12): 1700-1711.

54. DEVELOP-UK: Fisher A, Andreasson A, Chrysos A, et al. An observational study of Donor Ex Vivo Lung Perfusion in UK lung Transplantation: DEVELOP-UK. Health Technology Assessment 2016; 20(85): DOI: 10.3310/hta20850

55. Possoz J, Neyrinck A, Raemdonck DV. Ex vivo lung perfusion prior to transplantation: an overview of current clinical practice worldwide. *J Thoracic Disease.* 2019; 11(4): 1635-1650. From Possoz:

56. Shafaghi S, Najafizadeh K, Sheikhy K, et al. The First Experience of Ex-Vivo Lung Perfusion (EVLP) in Iran: An Effective Method to Increase Suitable Lung for Transplantation. Int J Organ Transplant Med 2016;7:219-27.

57. Abdalla LG, Braga KA, Nepomuceno NA, et al. Ex vivo lung perfusion in Brazil. J Bras Pneumol 2016;42:95-8.

58. Pêgo-Fernandes PM, de Medeiros IL, Mariani AW, et al. Ex vivo lung perfusion: early report of Brazilian experience. Transplant Proc 2010;42:440-3.

59. Van Raemdonck D, Neyrinck A, Rega F, et al. Machine perfusion in organ transplantation: a tool for ex-vivo graft conditioning with mesenchymal stem cells? Curr Opin Organ Transplant 2013;18:24-33.

60. Wallinder A, Riise GC, Ricksten SE, et al. Transplantation after ex vivo lung perfusion: A midterm follow-up. J Heart Lung Transplant 2016;35:1303-10.

61. Wallinder A, Ricksten SE, Silverborn M, et al. Early results in transplantation of initially rejected donor lungs after ex vivo lung perfusion: a case-control study. Eur J Cardiothorac Surg 2014;45:40-4; discussion 44-5.

62. Henriksen IS, Møller-Sørensen H, Møller CH, et al. First Danish experience with ex vivo lung perfusion of donor lungs before transplantation. Dan Med J 2014;61:A4809.

63. Fildes JE, Archer LD, Blaikley J, et al. Clinical Outcome of Patients Transplanted with Marginal Donor Lungs via Ex Vivo Lung Perfusion Compared to Standard Lung Transplantation. Transplantation 2015;99:1078-83.

64. Bozso S, Vasanthan V, Luc JG, et al. Lung transplantation from donors after circulatory death using portable ex vivo lung perfusion. Can Respir J 2015;22:47-51.

65. Luc JGY, Jackson K, Weinkauf JG, et al. Feasibility of Lung Transplantation from Donation After Circulatory Death Donors Following Portable Ex Vivo Lung Perfusion: A Pilot Study. Transplant Proc 2017;49:1885-92.

66. Koch A, Pizanis N, Olbertz C, et al. One-year experience with ex vivo lung perfusion: Preliminary results from a single center. Int J Artif Organs 2018;41:460-6.

67. Nilsson T, Wallinder A, Henriksen I, et al. Lung transplantation after ex vivo lung perfusion in two Scandinavian centres. Eur J Cardiothorac Surg 2019;55:766-72

68. Lindstedt S, Hlebowicz J, Koul B, et al. Comparative outcome of double lung transplantation using conventional donor lungs and non-acceptable donor lungs reconditioned ex vivo. Interact Cardiovasc Thorac Surg 2011;12:162-5.

69. Valenza F, Citerio G, Palleschi A, et al. Successful Transplantation of Lungs From an Uncontrolled Donor After Circulatory Death Preserved In Situ by Alveolar Recruitment Maneuvers and Assessed by Ex Vivo Lung Perfusion. Am J Transplant 2016;16:1312-8.

70. Valenza F, Rosso L, Gatti S, et al. Extracorporeal lung perfusion and ventilation to improve donor lung function and increase the number of organs available for transplantation. Transplant Proc 2012;44:1826-9.

71. Boffini M, Ricci D, Bonato R, et al. Incidence and severity of primary graft dysfunction after lung transplantation using rejected grafts reconditioned with ex vivo lung perfusion. Eur J Cardiothorac Surg 2014;46:789-93.

72. Zeriouh M, Sabashnikov A, Mohite PN, et al. Utilization of the organ care system for bilateral lung transplantation: preliminary results of a comparative study. Interact Cardiovasc Thorac Surg 2016;23:351-7.

73. Zych B, Popov AF, Stavri G, et al. Early outcomes of bilateral sequential single lung transplantation after ex- vivo lung evaluation and reconditioning. J Heart Lung Transplant 2012;31:274-81.

74. Schiavon M, Faggi G, Rebusso A, et al. Extended
criteria donor lung reconditioning with the organ care system lung: a single institution experience. Transpl Int 2019;32:131-40.

75. Okahara S, Levvey B, McDonald M, et al. Common criteria for ex vivo lung perfusion have no significant impact on posttransplant outcomes. Ann Thorac Surg. 2021;111:1156-63.

76. Jawitz OK, Raman V, Becerra, et al. Lung transplantation after ex vivo lung perfusion early outcomes from a US national registry. Annals of Surgery. 2022; 275(5): 1006-1012.

77. Tikkanen JM, Cypel M, Machuca TN, et al. Functional outcomes and quality of life after normothermic ex vivo lung perfusion lung transplantation. J Heart Lung Transplant. 2015;34:547–556.

78. Mehaffey JH, Charles EJ, Sharma AK, et al. Airway pressure release ventilation during ex vivo lung perfusion attenuates injury. *JTCVS* 2017; 153(1): 197-204.

79. Ordies S, Orlitova M, Heigl T, et al. Flow-controlled ventilation during EVLP improves oxygenation and preserves alveolar recruitment. *ICMx* 2020; 8:70. https://doi.org/10.1186/s40635-020-00360-w

80. Inci I, Ampollini L, Arni S, et al. Ex vivo reconditioning of marginal donor lungs injured by acid aspiration. J Heart Lung Transplant 2008;27:1229-36.

81. Inci I, Hillinger S, Arni S, et al. Reconditioning of an injured lung graft with intrabronchial surfactant instillation in an ex vivo lung perfusion system followed by transplantation. J Surg Res 2013;184:1143-9.

82. Khalife-Hocquemiller T, Sage E, Dorfmuller P, et al. Exogenous surfactant attenuates lung injury from gastric-acid aspiration during ex vivo reconditioning in pigs. *Transplantation* 2014;97:413-418.

83 Cypel, M, Liu, M, Rubacha, M, Yeung, JC, Hirayama, S, Anraku, M *et al*. (2009). Functional repair of human donor lungs by IL-10 gene therapy. *Sci Transl Med* **1**: 4ra9

84. Yeung JC, Wagnetz D, Cypel M, et al. *Ex vivo* adenoviral vector gene delivery results in decreased vector-associated inflammation pre- and post-lung transplantation in the pig. *Molecular Therapy* 2012; 20(6): 1204-1211.

85. Machuca TN, Cypel M, Bonato R, et al. Safety and efficacy of *Ex vivo* donor lung adenoviral IL-10 gene therapy in a large animal lung transplant survival model. *Human Gene Therapy* 2017; 20(90): 757- 765. DOI: 10.1089/hum.2016.070

86. Martens A, Ordies S, Vanaudenaerde BM, et al. Immunoregulatory effects of multipotent adult progenitor cells in a porcine ex vivo lung perfusion model. *Stem Cell Research & Therapy* 2017; 8:159. DOI 10.1186/s13287-017-0603-5

87. Frank JA, Briot R, Lee JW, et al. Physiological and biochemical markers of alveolar epithelial barrier dysfunction in perfused human lungs. *Am J Physiol Lung Cell Mol Physiol* 2007;293:L52-9.

88. Kondo T, Chen F, Ohsumi A, et al β2-adrenergic agonists inhalation during ex vivo lung perfusion attenuates lung injury. *Ann Thorac Surg* 2015;100:480-486.

89. Hijiya K, Chen-Yoshikawa TF, Kondo T, et al. Bronchodilator Inhalation During Ex Vivo Lung Perfusion Improves Posttransplant Graft Function After Warm Ischemia. *Ann Thorac Surg* 2017;103:447-53.

90. Carstens H, Kalka K, Verhaegh R, et al. Inhaled sphingosine has no adverse side effects in isolated ventilated and perfused pig lungs. Nature 2021; 11:18607. https://doi.org/10.1038/s41598-021-97708-3

91. Carstens H, Kalka K, Verhaegh R, Schumacher F, Soddemann M, Wilker B, et al. (2022) Antimicrobial effects of inhaled sphingosine against Pseudomonas aeruginosa in isolated ventilated and perfused pig lungs. PLoS ONE 17(7): e0271620. https://doi.org/10.1371/journal.pone.0271620

92. Inci I, Zhai W, Arni S, Hillinger S, Vogt P, Weder W. N- acetylcysteine attenuates lung ischemia-reperfusion injury after lung transplantation. Ann Thorac Surg. 2007;84:240e246. discussion 246.

93. Inci I, Erne B, Arni S, et al. Prevention of primary graft dysfunction in lung transplantation by N-acetylcysteine after prolonged cold ischemia. J Heart Lung Transplant. 2010;29:1293e1301.

94. Erne BV, Jungraithmayr W, Buschmann J, Arni S, Weder W, Inci I. Effect of N-acetylcysteine on acute allograft rejection after rat lung transplantation. Ann Thorac Surg. 2013;95:1021e1027.

95. Yamada Y, Iskener Ilker, Arni S, et al. Ex vivo treatment with inhaled N-acetylcysteine in porcine lung transplantation. *Journal of Surgical Research* 2017; 218:341-347

96. Michaelsen, V.S.; Ribeiro, R.V.P.; Ali, A.; Wang, A.; Gazzalle, A.; Keshavjee, S.; Cypel, M. Safety of Continuous 12-Hour Delivery of Antimicrobial Doses of Inhaled Nitric Oxide during Ex Vivo Lung Perfusion. *J. Thorac. Cardiovasc. Surg.* 2021, *3*, 841–849.

97. Haam S, Lee S, Paik HC, Park MS, Song JH, Lim BJ et al. The effects of hydrogen gas inhalation during ex vivo lung perfusion on donor lungs obtained after cardiac death. Eur J Cardiothorac Surg 2015;48:542–7.

98. Haam S, Lee JG, Paik HC, et al. Hydrogen gas inhalation during ex vivo lung perfusion of donor lungs recovered after cardiac death. J Heart Lung Transplant. 2018;37(10):1271–1278. doi:10.1016/j.healun.2018.06.007 [PubMed: 30100327]

99. Coburn M, Rossaint R. Argon in the fast lane: noble gases and their neuroprotective effects. Crit Care Med 2012;40:1965.

100. Ma D, Hossain M, Pettet GKJ, et al. Xenon preconditioning reduces brain damage from neonatal asphyxia in rats. J Cereb Blood Flow Metab 2006;26:199.

101. Pagel PS. Cardioprotection by noble gases. J Cardiothorac Vasc Anesth 2010;24:143.

102. Irani Y, Pype JL, Martin AR, et al. Noble gas (argon and xenon)-saturated cold storage solutions reduce ischemia- reperfusion injury in a rat model of renal transplantation. Nephron Extra 2011;1:272.

103. Rizvi M, Jawad N, Li Y, Vizcaychipi MP, Maze M, Ma D. Effect of noble gases on oxygen and glucose deprived injury in human tubular kidney cells. Exp Biol Med (Maywood) 2010; 235:886.

104. Martens A, Montoli M, Faggi G, et al. Argon and xenon ventilation during prolonged ex vivo lung perfusion. *Journal of Surgical Research* 2016; 201: 44-52.

105. Martens A, Ordies S, Vanaudenaerde BM, Verleden SE, Vos R, Verleden GM, Verbeken EK, Van Raemdonck DE, Claes S, Schols D, Chalopin M, Katz I, Farjot G, Neyrinck AP. A porcine ex vivo lung perfusion model with maximal argon expo- sure to attenuate ischemia-reperfusion injury. Med Gas Res. 2017;7(1):28-36.

106. Niikawa H, Okamoto T, Ayyat KS, et al. The protective effect of prone lung position on ischemia-reperfusion injury and lung function in an ex vivo porcine lung model. *JTCVS* 2019; 157(1): 425-433.

107. Niikawa H, Okamoto T, Ayyat KS, et al. Prone ex vivo perfusion protects human lungs from reperfusion injury. *Artificial Organs* 2022;46:2226-2233.

108. Watanabe Y, Galasso M, Watanabe T, et al. Donor prone positioning protects lungs from injury during warm ischemia. Am J Transplant 2019;19:2746-55.

109. Ordies S, Frick AE, Claes S, et al. Prone positioning during ex vivo lung perfusion influences regional edema accumulation. *Journal of Surgical Research* 2019; 239:300-308.

110. Loor G, Howard BT, Spratt JR, et al. Prolonged EVLP using OCS Lung: cellular and acellular perfusates. *Transplantation* 2017;101(10):2303–2311. doi:10.1097/TP.0000000000001616.

111. Sommer W, Salman J, Avsar M, et al. Prediction of transplant outcome after 24-hour ex vivo lung perfusion using the Organ Care System in a porcine lung transplantation model. *Am J Transplant.* 2019;19:345–355.

112. Steinmeyer J, Becker S, Avsar M, et al. Cellular and acellular ex vivo lung perfusion

preserve functional lung ultrastructure in a large animal model: a stereological study. *Respiratory Research* 2018;19:238 https://doi.org/10.1186/s12931-018-0942-5

113. Schumer EM, Zoeller KA, Linsky PL, et al. Feasibility study of pulsatile left ventricular assist device for prolonged ex vivo lung perfusion. *Ann Thorac Surg* 2015; 99:1961-8.

114. Beller JP, Byler MR, Money DT, et al. Reduced-flow ex vivo lung perfusion to rehabilitate lungs donated after circulatory death. *J Heart Lung Transplant.* 2020;39:74-82.

115. Forgie KA, Fialka N, Bucko MT, et al. Normothermic negative pressure ventilation ex-situ lung perfusion: evaluation of lung function and metabolism. Journal of Visualized Experiments. 2022;180: doi:10.3791/62982.

116. Martens A, Boada M, Vanaudenaerde BM, et al. Steroids can reduce warm ischemic reperfusion injury in a porcine donation after circulatory death model with *ex vivo* lung perfusion evaluation. *Transplant International* 2016;29:1237-1246.

117. Andreasson A, Karamanou DM, Perry JD, et al. The effect of ex vivo lung perfusion on microbial load in human donor lungs. *J Heart Lung Transplant* 2014;33:910-6.

118. Nakajima D, Cypel M, Bonato R, et al. *Ex vivo* perfusion treatment of infection in human donor lungs. *American Journal of Transplantation* 2016;16:1229-1237.

119. Zinne N, Krueger M, Hoeltig D, et al. Treatment of infected lungs by ex vivo perfusion with high dose antibiotics and autotransplantation: a pilot study in pigs. *PLoS ONE* 2018;13(3): e0193168. https:// doi.org/10.1371/journal.pone.0193168

120. Inci I, Zhai W, Arni S, et al. Fibrinolytic treatment improves the quality of lungs retrieved from non-heart-beating donors. *J Heart Lung Transplant* 2007;26:1054-60.

121. Inci I, Yamada Y, Hillinger S, et al. Successful lung transplantation after donor lung reconditioning with urokinase in ex vivo lung perfusion system. *Ann Thorac Surg* 2014;98:1837-8.

122. Machuca TN, Hsin MK, Ott HC, et al. Injury-specific ex vivo treatment of the donor lung: pulmonary thrombolysis followed by successful lung transplantation. *Am J Respir Crit Care Med* 2013;188:878-80.

123. Luc JG, Bozso SJ, Freed DH, et al. Successful repair of donation after circulatory death lungs with large pulmonary embolus using the lung organ care system for ex vivo thrombolysis and subsequent clinical transplantation. *Transplantation* 2015;99:e1-2.

124. Liersch-Nordqvist A, Fakhro M, Pierre L, et al. The impact of alteplase on pulmonary graft function in donation after circulatory death — an experimental study. *Annals of Medicine and Surgery* 2017;22:1-6.

125. Valenza F, Rosso L, Pizzocri M, et al. The consumption of glucose during ex vivo lung perfusion correlates with lung edema. Transplant Proc 2011;43:993-6.

126. Valenza F, Rosso L, Coppola S, et al. β-adrenergic agonist infusion during extracorporeal lung perfusion: effects on glucose concentration in the perfusion fluid and on lung function. J Heart Lung Transplant 2012;31:524-30.

127. Reece TB, Ellman PI, Maxey TS, et al. Adenosine A2A receptor activation reduces inflammation and preserves pulmonary function in an in vivo model of lung transplantation. *J Thorac Cardiovasc Surg* 2005;129:1137– 43.

128. Emaminia A, LaPar Damien, Zhao Y, et al. Adenosine A_{2A} agonist improves lung function during Ex Vivo Lung Perfusion. *Ann Thor Surg* 2011; 92:1840-6.

129. Charles EJ, Mehaffey JH, Sharma AK, et al. Lungs donated after circulatory death and prolonged warm ischemia are transplanted successfully after enhanced ex vivo lung perfusion using adenosine A2B receptor antagonism. J Thorac Cardiovasc Surg 2017;154:1811-20.

130. Iskender I, Sakamoto J, Nakajima D, et al. Human alpha(1)-antitrypsin improves early post-transplant lung function: Pre-clinical studies in a pig lung transplant model. J Heart Lung Transpl 2016;35:913-21.

131. Gao W, Zhao J, Kim H, et al. alpha1-Antitrypsin inhibits ischemia reperfusion-induced lung injury by reducing inflammatory response and cell death. J Heart Lung Transplant 2014;33:309-15.

132. Lin H, Chen M, Tian F, et al. Alpha1-Antitrypsin improves function of porcine donor lungs during ex-vivo lung perfusion. *J Heart Lung Transplant* 2018;37:656-666.

133. Mariscal A, Nykanen A, Tikkanen J, et al. Alpha 1 antitrypsin treatment during human ex vivo lung perfusion improves lung function by protecting lung endothelium. J Heart Lung Transplant 2020;39:S71-2.

134. Harada M, Oto T, Otani S, et al. A neutrophil elastase inhibitor improves lung function during ex vivo lung perfusion. *Gen Thorac Cardiovasc Surg* 2015; 63:645-651.

135. Walweel K, Skeggs K, Boon AC, et al. Endothelin receptor antagonist improves donor lung function in an ex vivo perfusion system. *J Biomed Sci* 2020; 27:96.

136. Ku T, Ribeiro R, Ferreira VH, et al. *Ex-vivo* delivery of monoclonal antibody (Rituximab) to treat human donor lungs prior to transplantation. *EBioMedicine* 2020;60:102994.

137. Riess, J.G. Oxygen carriers ("blood substitutes")—Raison d'etre, chemistry, and some physiology. *Chem. Rev.* 2001, *101*, 2797–2920. [CrossRef]

138. Cabrales, P.; Intaglietta, M. Blood substitutes: Evolution from noncarrying to oxygen- and gas-carrying fluids. *Asaio J.* 2013, *59*, 337–354. [CrossRef]

139. Inci I, Arni S, Iskender I, et al. Functional, metabolic, and morphologic results of ex vivo donor lung perfusion with a perfluorocarbon-based oxygen carrier nanoemulsion in a large animal transplantation model. *Cells* 2020;9:2501. doi:10.3390/cells9112501

140. Huang L, Hough O, Vellanki RN, et al. L-alanyl-L-glutamine modified perfusate improves human lung cell functions and extend porcine ex vivo lung perfusion. *Journal Heart Lung Transplant* 2023; 42(2): 183-195.

141. Lee JW, Fang X, Gupta N, et al. Allogeneic human mesenchymal stem cells for treatment of E. coli endotoxin-induced acute lung injury in the ex vivo perfused human lung. *PNAS* 2009;106(38): 16357–16362

142. Mordant P, Nakajima D, Kalaf R, et al. Mesenchymal stem cell treatment is associated with decreased perfusate concentration of interleukin-8 during ex vivo perfusion of donor lungs after 18-hour preservation. *J Heart Lung Transplant* 2016;35:1245-54.

143. Nakajima D, Watanabe Y, Ohsumi A, et al. Mesenchymal stromal cell therapy during ex vivo lung perfusion ameliorates ischemia-reperfusion injury in lung transplantation. *J Heart Lung Transplant* 2019;38:1214-23.

144. Nykanen A, Mariscal A, Estrada C, et al. Engineered mesenchymal stromal cell therapy during human lung ex vivo lung perfusion is affected by lung acidosis. *J Heart Lung Transplant* 2020;39:S178.

145. Nykanen A, Mariscal A, Duong A, et al. Engineered mesenchymal stromal cell therapy during human lung *ex vivo* lung perfusion is compromised by acidic lung microenvironment. *Molecular Therapy: Methods and Clinical Development* 2021; 23: 184-197.

146. Galasso M, Feld JJ, Watanabe Y, et al. Inactivating hepatitis C virus in donor lungs using light therapies during normothermic ex vivo lung perfusion. *Nature Communications* 2019; 10:481. https://doi.org/10.1038/s41467-018-08261-z

147. Cypel M, Feld JJ, Galasso M, et al. Prevention of viral transmission during lung transplantation with hepatitis C-viraemic donors: an open-label, single-centre, pilot trial. *Lancet Respir Med* 2020;8:192–201

148. Luc GY, Aboelnazar NS, Himmat S, et al. A leukocyte filter does not provide further benefit during *ex vivo* lung perfusion.

149. Buchko M, Himmat S, Stewart CJ, et al. Continuous hemodialysis does not improve graft function during ex vivo perfusion over 24 hours. *Transplantation Proceedings* 2019; 51:2022-2028.

150. Wei D, Gao F, Yang Z, et al. Ex vivo lung perfusion purification for human donor lungs following prolonged cold storage. *Ann Transl Med* 2020; 8(3):38. http://dx.doi.org/10.21037/atm.2019.10.17

151. De Wolf J, Glorion M, Jouneau L, et al. Challenging the ex vivo lung perfusion procedure with continuous dialysis in a pig model. *Transplantation* 2022;106(5):979-987.

152. Nilsson T, Hansson C, Wallinder A, et al. Hemofiltration in ex vivo lung perfusion-a study in experimentally induced pulmonary edema. J Thorac Cardiovasc Surg 2016;151:570-5.e1.

153. Kakishita T, Oto T, Hori S, et al. Suppression of inflammatory cytokines during ex vivo lung perfusion with an adsorbent membrane. *Ann Thorac Surg* 2010;89:1773-81.

154. Iskender I, Cosgun T, Arni S, et al. Cytokine filtration modulates pulmonary metabolism and edema formation during ex vivo lung perfusion. J Heart Lung Transplant 2017. [Epub ahead of print]. doi: 10.1016/ j.healun.2017.05.021.

Chapter 3

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

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), 1169-83 (2018).

2. Valapour, M., et al. OPTN/SRTR 2017 annual data report: Lung. *American Journal of Transplantation*. 19 (Suppl 2), 404-84 (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), 1047-59 (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), 973-86 (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), 1199-204 (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), 662-7 (2008).

8. Cypel, M., et al. Normothermic ex vivo lung perfusion in clinical lung transplantation. *The New England Journal of Medicine.* 364(15), 1431-40 (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), 40-5 (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), 1200-6 (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), 577-86 (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), 478-84 (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), 520-30 (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), 967-78 (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).

Chapter 4

Left Lung Transplantation in a Juvenile Porcine Model for ESLP

1. Chambers, D.C., Cherikh, W.S., Goldfarb, S.B., 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*: *the official publication of the International Society for Heart Transplantation.* 37(10), 1169-1183 (2018).

2. Valapour, M., Lehr, C.J., Skeans, M.A., et al. OPTN/SRTR 2017 annual data report: Lung. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons*. 19 (Suppl 2), 404-484 (2019).

3. Klein, A.S., Messersmith, E.E., Ratner, L.E., et al. Organ donation and utilization in the united states, 1999-2008. *American journal of transplantation: official journal of the American Society of Transplantation and the American Society of Transplant Surgeons.* 10(4 Pt 2), 973-86 (2010).

4. Kotecha, S., Hobson, J., Fuller, J., et al. Continued successful evolution of extended criteria donor lungs for transplantation. *The Annals of thoracic surgery*. 104(5), 1702-1709 (2017).

5. Singh, E., Schecter, M., Towe, C., 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. Cypel, M., Yeung, J.C., Liu, M., et al. Normothermic ex vivo lung perfusion in clinical lung transplantation. *.N Engl J Med.* 364(15),1431-40 (2011).

7. Wallinder, A., Ricksten, S-E., Silverborn, M., 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 : official journal of the European Association for Cardio-thoracic Surgery*. 45(1), 40-44 (2014).

8. Sage, E., Mussot, S., Trebbia, G., et al. Lung transplantation from initially rejected donors after ex vivo lung reconditioning: The french experience. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery*. 46(5), 794-9 (2014).

9. Valenza, F., Rosso, L., Gatti, S., et al. Extracorporeal lung perfusion and ventilation to improve donor lung function and increase the number of organs available for transplantation. *Transplantation proceedings.* 44(7), 1826-9 (2012).

10. Fildes, J.E., Archer, L.D., Blaikley, J., et al. Clinical outcome of patients transplanted with marginal donor lungs via ex vivo lung perfusion compared to standard lung transplantation. *Transplantation.* 99(5), 1078-83 (2015).

11. Cypel, M., Yeung, J.C., Machuca, T., et al. Experience with the first 50 ex vivo lung perfusions in clinical transplantation. *The Journal of thoracic and cardiovascular surgery.* 144(5), 1200-6 (2012).

12. Clark, S.C., Sudarshan, C.D., Khanna, R., et al. A new porcine model of reperfusion injury after lung transplantation. *Laboratory Animals.* 33, 135-142 (1999).

13. Karimi, A., Cobb, J.A, Staples, E.D., et al. Technical pearls for swine lung transplantation. *Journal of Surgical Research.* 171, e107-1111 (2011).

14. Kruger, M., Zinne, N., Biancosino, C., et al. Porcine pulmonary auto-transplantation for *ex vivo* therapy as a model for new treatment strategies. *Interactive CardioVascular and Thoracic Surgery.* 23, 358-366 (2016).

15. Mariscal, A., Caldarone, L., Tikkanen, J., et al. Pig lung transplant survival model. *Nature Protocols.* 13, 1814-1828 (2018).

16. Aboelnazar, N.S., Himmat, S., Hatami, S., et al. Negative pressure ventilation decreases inflammation and lung edema during normothermic ex-vivo lung perfusion. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation*. 37(4), 520-530 (2018).

Chapter 5

Normothermic Perfusion is Superior to Cold Perfusion in Ex-Situ Lung Perfusion

1. Valapour M., Lehr C.J., Skeans M.A., et al. OPTN/SRTR 2019 Annual Data Report: Lung. *American Journal of Transplantation*. 2021; 21(S2):441-520.

2. Israni A. K., Zaun D., Bolch C., et al. OPTN/SRTR 2015 Annual Data Report: Deceased Organ Donation. *American Journal of Transplantation*. 2017;17(S1):503-542.

3. Munshi L, Keshavjee S, Cypel M. Donor management and lung preservation for lung transplantation. *The Lancet Respiratory Medicine*. 2013;1(4):318-328.

4. Buchko MT, Boroumand N, Cheng JC, et al. Clinical transplantation using negative pressure ventilation ex situ lung perfusion with extended criteria donor lungs. *Nature Communications.* 2020: 11: 5765.

5. Nakajima D, Chen F, Yamada T, Sakamoto J, Osumi A, Fujinaga T, et al. Hypothermic machine perfusion ameliorates ischemia-reperfusion injury in rat lungs from non-heart-beating donors. Transplantation. 2011;92:858-63.

6. Nakajima D, Chen F, Okita K, Motoyama H, Hijiya K, Ohsumi A, et al. Reconditioning lungs donated after cardiac death using short-term hypothermic machine perfusion. *Transplantation.* 2012;94:999-1004.

7. Gloria JN, Yerxa J, Kesseli SJ, et al. Subnormothermic ex vivo lung perfusion attenuates graft inflammation in a rat transplant model. *The Journal of Thoracic and Cardiovascular Surgery*. 2021; S0022-5223(21)00165-3.

8. Arni S, Maeyashiki T, Opitz I, Inci I. Subnormothermic ex vivo lung perfusion attenuates ischemia reperfusion injury from donation after circulatory death donors. *PLoS ONE*. 2021; 16(8) e0255155.

9. Arni S, Maeyashiki T, Citak N, et al. Subnormothermic ex vivo lung perfusion temperature improves graft preservation in lung transplantation. *Cells*. 2021; 10: 748. https://doi.org/10.3390/ cells10040748

10. Buchko MT, Himmat S, Nader A, et al. A low-cost perfusate alternative for ex vivo lung perfusion. *Transplantation Proceedings*. 2020;52(10):2941-2946.

11. Forgie KA, Fialka N, Buchko M, et al. Normothermic Negative Pressure Ventilation Ex-Situ Lung Perfusion: Evaluation of Lung Function and Metabolism. *Journal of Visualized Experiments*

12. Forgie KA, Fialka N, Khan M, Buchko MT, Himmat S, Hatami S, Qi X, Wang X, Buswell KM, Edgar R, Domahidi D, Freed DH, Nagendran J. Left lung transplantation in a porcine model of NPV-ESLP: evaluation of lung function with four-hour survival. *The Journal of Visualized Experiments*. (180), e62979, doi:10.3791/62979 (2022).

13. 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: The Official Publication of the International Society for Heart Transplantation.* 2018; 37 (4): 520-530.

14. Buchko M. T., et al. Total parenteral nutrition in ex vivo lung perfusion: Addressing metabolism improves both inflammation and oxygenation. *American Journal of Transplantation.* 2019; 19 (12): 3390-3397.

15. Wierup P, Liao Q, Bolys R, et al. Lung edema formation during cold perfusion: Important differences between rat and porcine lung. *The Journal of Heart and Lung Transplantation.* 2005; 24: 379-85.

16. Sanchez P.G., Chan E.G., Davis R.D., et al. Normothermic ex vivo lung perfusion (Novel) as an assessment of extended criteria donor lungs: a prospective multi-centre clinical trial. *The Journal of Heart and Lung Transplantation.* 2022; 41(4), S40-S41.

17. Loor G, Warnecke G, Villavicencio MA, et al. Portable normothermic ex-vivo lung perfusion, ventilation and functional assessment with the Organ Care System on donor lung use for transplantation from extended-criteria donors (EXPAND): a single-arm, pivotal trial. *The Lancet*. 2019; 7 (11): 975-984.

18. Warnecke G, Van Raemdonck D, Smith MA, et al. Normothermic ex-vivo preservation with the portable Organ Care System Lung device for bilateral lung transplantation (INSPIRE): a randomised, open-label, non-inferiority, phase 3 study. *The Lancet.* 6(5): 357-367.

19. Ali A, Nykanen A, Brambate E, et al. Successful transplantation of porcine lungs following 3 days of preservation using a modified cold static method paired with intermittent normothermic ex vivo lung perfusion (EVLP). *The Journal of Heart and Lung Transplantation.* 2022; 39 (4): S353.

20. Egan TM, Haithcock B, Ali MH, et al. A Large Animal Model of Ex-Vivo Lung Perfusion as a Platform to Deliver Therapeutic Agents to Lungs Before Lung Transplant [abstract]. *Am J Transplant.* 2020; 20 (suppl 3).

21. Galasso M, Feld JJ, Watanabe Y, et al. Inactivating hepatitis C virus in donor lungs using light therapies during normothermic ex vivo lung perfusion.

22. Fontes P, Lopez R, van der Plaats A, Vodovotz Y, Minervini M, Scott V, et al. Liver preservation with machine perfusion and a newly developed cell-free oxygen carrier solution under subnormothermic

con- ditions. Am J Transplant. 2015; 15(2):381–94. Epub 2015/01/24. https://doi.org/10.1111/ajt.12991 PMID: 25612645; PubMed Central PMCID: PMC5024042.

23. Eshmuminov D, Becker D, Bautista Borrego L, Hefti M, Schuler MJ, Hagedorn C, et al. An integrated perfusion machine preserves injured human livers for 1 week. Nat Biotechnol. 2020; 38(2):189–98. Epub 2020/01/15. https://doi.org/10.1038/s41587-019-0374-x PMID: 31932726; PubMed Central PMCID: PMC7008032.

24. Obara H, Morito N, Matsuno N, Yoshikawa R, Gouchi M, Otani M, et al. Optimum Perfusate Volume of Purified Subnormothermic Machine Perfusion for Porcine Liver Donated After Cardiac Death. Transplant Proc. 2018; 50(9):2830–3. Epub 2018/11/08. https://doi.org/10.1016/j.transproceed.2018.03.055 PMID: 30401406.

25.Vairetti M, Ferrigno A, Rizzo V, Richelmi P, Boncompagni E, Neri D, et al. Subnormothermic machine perfusion protects against rat liver preservation injury: a comparative evaluation with conventional cold storage. Transplant Proc. 2007; 39(6):1765–7. Epub 2007/08/19. https://doi.org/10.1016/j. transproceed.2007.06.002 PMID: 17692606.

26. JM, Spetzler VN, Goldaracena N, Louis KS, Selzner N, Selzner M. Technique of subnormother- mic ex vivo liver perfusion for the storage, assessment, and repair of marginal liver grafts. J Vis Exp. 2014;(90):e51419. Epub 2014/08/26. https://doi.org/10.3791/51419 PMID: 25145990; PubMed Central PMCID: PMC4672992.

27. Sun Q, Jiang L, Richard-Mohamed M, Ruthirakanthan A, et al. Renal Protection Against Ischemia Reperfusion Injury: Hemoglobin-based Oxygen Carrier-201 Versus Blood as an Oxygen Carrier in Ex

Vivo Subnormothermic Machine Perfusion. Transplantation. 2020; 104 (3):482–9. Epub 2019/10/01. https://doi.org/10.1097/TP.0000000000002967 PMID: 31568396.

Chapter 6

Moderate-Flow Perfusion is Superior to Low-Flow Perfusion in Ex-Situ Lung Perfusion

1. Chambers DC, Cherikh WS, Goldfarb SB, 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. J Heart Lung Transplant 2018;37:1169-83.

2. Valapour M, Lehr CJ, Skeans MA, et al. OPTN/SRTR 2017 annual data report: Lung. Am J Transplant 2019;19 Suppl 2:404-84.

3. Valapour M., Lehr C.J., Skeans M.A., et al. OPTN/SRTR 2019 Annual Data Report: Lung. *American Journal of Transplantation*. 2021; 21(S2):441-520.

4 Chambers DC, Yusen RD, Cherikh WS, 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. J Heart Lung Transplant 2017;36:1047-59.

5. Munshi L, Keshavjee S, Cypel M. Donor managment and lung preservation for lung transplantation. *Lancet Respir Med.* 2013;1:318-328.

6. Machuca TN, Cypel M, Keshavjee S. Advances in lung preservation. *Surg Clin North Am.* 2013; 93:1373-1394.

7. Van Raemdonck D. Thoracic organs: Current preservation technology and future prospects; part 1: *Lung Curr Opin Organ Transplant*. 2010;15:150-155.

8. Buchko MT, Boroumand N, Cheng JC, et al. Clinical transplantation using negative pressure ventilation ex situ lung perfusion with extended criteria donor lungs. *Nat Commun* 2020;11:5765.

9. Cypel M, Yeung JC, Liu M, et al. Normothermic ex vivo lung perfusion in clinical lung transplantation. *N Engl J Med*. 2011; 364:1431-1440.

10. Cypel M, Yeung JC, Machuca T, et al. Experience with the first 50 ex vivo lung perfusions in clinical transplantation. *J Thorac Cardiovasc Surg* 2012; 144:1200-1206.

11. Loor G, Warnecke G, Villavicencio MA, et al. Portable normothermic ex-vivo lung perfusion, ventilation, and functional assessment with the Organ Care System on donor lung use for transplantation from extended-criteria donors (EXPAND): a single-arm, pivotal trial. *Lancet Respir Med.* 2019;7:975-84.

12. Noda K, Shigemura N, Tanaka Y, et al. Successful prolonged ex vivo lung perfusion for graft preservation in rats. *Eur J Cardiothorac Surg.* 2014;45:e54-60.

13. Okamoto T, Wheeler D, Farver CF, McCurry KR. Transplant suitability of rejected human donor lungs with prolonged cold ischemia time in low flow acellular and high flow cellular ex vivo lung perfusion systems. Transplantation 2019;103:1799-808.

14. Beller JP, Byler MR, Money DT, et al. Reduced-flow ex vivo lung perfusion to rehabilitate lungs donated after circulatory death. *J Heart Lung Transplant.* 2020;39:74-82.

15. Buchko MT, Himmat S, Nader A, et al. A low-cost perfusate alternative for ex vivo lung perfusion. *Transplantation Proceedings*. 2020;52(10):2941-2946.

16. Forgie KA, Fialka N, Buchko M, et al. Normothermic Negative Pressure Ventilation Ex-Situ Lung Perfusion: Evaluation of Lung Function and Metabolism. *Journal of Visualized Experiments*. 2022;180: doi:10.3791/62982.

17. Forgie KA, Fialka N, Khan M, et al. Left lung transplantation in a porcine model of NPV-ESLP: evaluation of lung function with four-hour survival. *The Journal of Visualized Experiments*. 2022;180, e62979, doi:10.3791/62979.

18. 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: The Official Publication of the International Society for Heart Transplantation.* 2018; 37 (4): 520-530.

19. Buchko M. T., et al. Total parenteral nutrition in ex vivo lung perfusion: Addressing metabolism improves both inflammation and oxygenation. *American Journal of Transplantation.* 2019; 19 (12): 3390-3397.

20. Forgie KA, Fialka N, Freed DH, Nagendran J. Normothermic Perfusion is Superior to Subnormothermic Perfusion in a Juvenile Porcine Transplantation Model of Ex-Situ Lung

Perfusion (published abstract). *JHLT.* April 2022; 41(4): S255.
DOI:10.1016/j.healun.2022.01.624

21. West JB, Luks AM. Diffusion: How gas gets across the blood-gas barrier. In: West's respiratory physiology: the essentials, 11th ed. Hong Kong, China: Wolters Kluwer; 2021:31-44.

22. Ali A, Nykanen A, Brambate E, et al. Successful transplantation of porcine lungs following 3 days of preservation using a modified cold static method paired with intermittent normothermic ex vivo lung perfusion (EVLP). *The Journal of Heart and Lung Transplantation.* 2022; 39 (4): S353.

23. Egan TM, Haithcock B, Ali MH, et al. A Large Animal Model of Ex-Vivo Lung Perfusion as a Platform to Deliver Therapeutic Agents to Lungs Before Lung Transplant [abstract]. *Am J Transplant.* 2020; 20 (suppl 3).

24. Galasso M, Feld JJ, Watanabe Y, et al. Inactivating hepatitis C virus in donor lungs using light therapies during normothermic ex vivo lung perfusion. *Natu Commun.* 2019; 10:481. https://doi.org/10.1038/s41467-018-08261-z

Chapter 7

Mild Permissive Alkalosis Improves Outcomes in Porcine Negative Pressure Ventilation Ex-Situ Lung Perfusion

1. Steen S, Sjoberg T, Pierre L, et al. Transplantation of lungs from a non-heart-beating donor. Lancet. 2001; 357: 825-29.

2. Steen S, Ingemansson R, Eriksson L, et al. First human transplantation of a nonacceptable donor lung after reconditioning ex vivo. Ann Thorac Surg. 2007; 83: 2191-5.

3. Cypel M, Yeung JC, Hirayama S, et al. Technique for prolonged normothermic Ex Vivo Lung Perfusion. JHLT. 2008; 27(12): 1318-1325.

4. Aboelnazar N, Himmat S, Hatami S, et al. Negative pressure ventilation decreases inflammation and lung edema during normothermic ex-vivo lung perfusion. JHLT. 2018; 37:520-530.

5. Loor G, Howard BT, Spratt JR, et al. Prolonged EVLP using OCS Lung: Cellular and Acellular Perfusates. *Transplantation.* 2017; 101(10): 2303-2311.

6.Sommer W, Salman J, Avsar M, et al. Prediction of transplant outcome after 24-hours ex vivo lung perfusion using the Organ Care System in a porcine lung transplantation model. *Am J Transplant.* 2019; 19:345-355.

7. Forgie KA, Fialka N, Freed DH, Nagendran J. Normothermic Perfusion is Superior to Subnormothermic Perfusion in a Juvenile Porcine Transplantation Model of Ex-Situ Lung Perfusion. *JHLT.* 2022; 41(4): S252. https://doi.org/10.1016/j.healun.2022.01.624

8. Beller JP, Byler MR, Money DT, et al. Reduced-flow ex vivo lung perfusion to rehabilitate lungs donated after circulatory death. *J Heart Lung Transplant.* 2020;39:74-82.

9. Forgie KA, Fialka N, Freed DH, Nagendran J. Moderate flow is superior to low flow perfusion in a juvenile porcine transplantation model of ex-situ lung perfusion. *JHLT.* 2022; 41(4): S252. http://dx.doi.org/10.1016/j.healun.2022.01.1774

10. Mahdi M, Joseph NJ, Hernandez DP, Crystal GJ, Baraka A, Salem MR. Induced hypocapnia is effective in treating pulmonary hypertension following mitral valve replacement. Anesth. 2011; 21(2):259-70

11. Chang AC, Zucker HA, Hickey PR, Wessel DL. Pulmonary vascular resistance in infants after cardiac surgery: Role of carbon dioxide and hydrogen ion. Crit Car Med. 1995;23:568-574.

12. Fox WW, Duara S: Persistent pulmonary hypertension in the neonate: Diagnosis and management. J Pediatr 1983; 103:505-514.

13. Chamarthy MR, Kandathil A, Kalva SP. Pulmonary vascular pathophysiology. Cardiovasc Diagn Ther 2018;8(3):208-213.

14. Buchko MT, Himmat S, Aboelnazar NS, et al. A low-cost perfusate alternative for ex vivo lung perfusion. Transplantation Proceedings 2020;52(10):2941-2946.

15. Forgie KA, Fialka N, Bucko MT, et al. Normothermic negative pressure ventilation ex-situ lung perfusion: evaluation of lung function and metabolism. Journal of Visualized Experiments. 2022;180: doi:10.3791/62982.

16. Forgie KA, Fialka N, Khan M, et al. Left lung transplantation in a porcine model of NPV-ESLP: evaluation of lung function with four-hour survival. *The Journal of Visualized Experiments*. (180), e62979, doi:10.3791/62979 (2022).

17. Godoy DA, Seifi A, Garza D, Lubillo-Montenegro S, Murillo-Cabezas F. Hyperventilation therapy for control of posttraumatic intracranial hypertension. *Frontiers in Neurology.*2017;8:250.

18. Kim JS, Nam MH, Do YS, et al. Efficacy of milrinone versus nitroglycerin in controlling pulmonary arterial hypertension induced by intravenous injections of absolute ethanol in anesthetized dogs. *J Vasc Interv Radiol* 2010;21:882-887.

19. Scott TE, Kemp-Harper BK, Hobbs AJ. Inflammasomes: A novel therapeutic target in pulmonary hypertension. *British Journal of Pharmacology.* 2019; 176: 1880-1896.

20. Abolhassani M, Guais A, Chaumet-Riffaud P, Sasco AJ, Schwartz L. Carbon dioxide inhalation causes pulmonary inflammation. *Am J Physiol Lung Cell Mol Physiol.* 2009; 296: L657-665.

21. Thom SR, Bhopale VM, Hu JP, Yang M. Inflammatory responses to acute elevations of carbon dioxide in mice. *J Appl Physiol.* 2017; 123: 297-302.

22. Ding H, Li Y, Li X, Liu X, Chen S, Liu M, Zeng H. Treatment with 7% and 10% CO_2 enhanced expression of IL-1Beta, TNF-alpha, and IL-6 in hypoxic cultures of human whole blood. *Journal of International Medical Research.* 2020; 48(4):1-12.

23. Lang JD, Chumley P, Eiserich JP, et al. Hypercapnia induces injury to alveolar epithelial cells via a nitric oxide-dependent pathway. *Am J Physiol Lung Cell Mol Physiol; 2000; 279:L994-1002.*

24. Morray JP, Lynn AM, Mansfield PB. Effect of pH and PCO_2 on pulmonary and systemic hemodynamics after surgery in children with congenital heart disease and pulmonary hypertension. *The Journal of Pediatrics.* 1988; 113(3): 474-479.

25. Laffey JG, Engelberts D, Kavanagh BP. Injurious Effects of Hypocapnic Alkalosis in the Isolated lung. *Am J Respir Crit Care Med.*2000;162: 399-405.

26. Fox WW, Duara S. Persistent pulmonary hypertension in the neonate: Diagnosis and management. *The Journal of Pediatrics*. 1983; 103(4): 505-514.

27. Sosulski R, Fox WW. Hyperventilation therapy for persistent pulmonary hypertension of the neonate and occurrence of a transition phase. *Pediatr Res.* 1982;16: 309A.

28. Myrianthefs PM, Briva A, Leucona E, et al. Hypocapnic but not metabolic alkalosis impairs alveolar fluid reabsorption. Am J Respir Crit Care Med 2005;171:1267–71.

29. Ali A, Nykanen A, Brambate E, et al. Successful transplantation of porcine lungs following 3 days of preservation using a modified cold static method paired with intermittent normothermic ex vivo lung perfusion (EVLP). *The Journal of Heart and Lung Transplantation.* 2022; 39 (4): S353.

30. Egan TM, Haithcock B, Ali MH, et al. A Large Animal Model of Ex-Vivo Lung Perfusion as a Platform to Deliver Therapeutic Agents to Lungs Before Lung Transplant [abstract]. *Am J Transplant.* 2020; 20 (suppl 3).

31. Galasso M, Feld JJ, Watanabe Y, et al. Inactivating hepatitis C virus in donor lungs using light therapies during normothermic ex vivo lung perfusion. *Natu Commun.* 2019; 10:481. https://doi.org/10.1038/s41467-018-08261-z

32. Hyde RW, Lawson WH, Forster RE. Influence of carbon dioxide on pulmonary vasculature. *J Appl Physiol.* 1964; 19:734-744.

33. Barer GR, Howard P, McCurrie JR. The effect of carbon dioxide and changes in blood pH on pulmonary vascular resistance in cats. *Clin Sci.* 1967; 32: 361-376.

34. Stroud RC, Rahn H. Effect of O_2 and CO_2 tensions upon the resistance of pulmonary blood vessels. *Am J Physiol.* 1953; 172:211-221.

35. Peters RM. Effect of unilateral carbon dioxide breathing on pulmonary blood flow. *Am J Physiol.* 1957; 191: 399-403.

36. Viles PH, Shepherd JT. Evidence for a dilator action of carbon dioxide on pulmonary vessels of the cat. *Circ Res.* 1968; 22: 325-332.

37. Fike CD, Hansen TN. The effect of alkalosis on hypoxia-induced pulmonary vasoconstriction in lungs of newborn rabbits. *Pediatric Research.* 1989; 25(4): 383-388.

Chapter 8

Perfusate Exchange does not improve outcomes in *Ex-Situ* Lung Perfusion

1. Ali A, Cypel M. Ex-vivo lung perfusion and ventilation: where to from here? *Curr Opin Organ Transplant*. 2019; 24:297-304.

2. Steen S, Ingemansson R, Eriksson L, et al. First human transplantation of a nonacceptable donor lung after reconditioning ex vivo. Ann Thorac Surg. 2007; 83: 2191-5.

3. Steen S, Sjoberg T, Pierre L, et al. Transplantation of lungs from a non-heart-beating donor. Lancet. 2001; 357: 825-29.

4. Sanchez PG, Cantu E, Hartwig M, et al. The NOVEL study: A multi-center clinical trial studying the safety of Ex Vivo Lung Perfusion. JHLT. 2020; 39(4); s110. https://doi.org/10.1016/j.healun.2020.01.977

5. Warnecke G, Van Raemdonck D, Smith MA, et al. Normothermic ex-vivo preservation with the portable Organ Care System Lung device for bilateral lung transplantation (INSPIRE): a randomized, open-label, non-inferiority, phase 3 study. Lancet Respir Med. 2018; 6: 357-67.

6. Loor G, Warnecke G, Villavicencio MA, et al. Portable normothermic ex-vivo lung perfusion, ventilation, and functional assessement with the Organ Care System on donor lung use for

transplantation from extended-criteria donors (EXPAND): a single-arm, pivotal trial. Lancet Respir Med. 2019;7:975-84.

7. Buchko MT, Boroumand N, Cheng JC, et al. Clinical transplantation using negative pressure ventilation *ex-situ* lung perfusion with extended criteria donor lungs. Nature Communications. 2020; https://doi.org/10.1038/s41467-020-19581-4

8. Cypel M, Yeung JC, Hirayama S, et al. Technique for prolonged normothermic Ex Vivo Lung Perfusion. JHLT. 2008; 27(12): 1318-1325.

9. Aboelnazar N, Himmat S, Hatami S, et al. Negative pressure ventilation decreases inflammation and lung edema during normothermic ex-vivo lung perfusion. JHLT. 2018; 37:520-530.

10. Forgie KA, Fialka N, Bucko MT, et al. Normothermic negative pressure ventilation *ex-situ* lung perfusion: evaluation of lung function and metabolism. Journal of Visualized Experiments. 2022;180: doi:10.3791/62982.

11. Buchko MT, Himmat S, Aboelnazar NS, et al. A low-cost perfusate alternative for ex vivo lung perfusion. Transplantation Proceedings 2020;52(10):2941-2946

12. Loor G, Howard BT, Spratt JR, et al. Prolonged EVLP using OCS Lung: Cellular and Acellular Perfusates. *Transplantation.* 2017; 101(10): 2303-2311.

13. Sommer W, Salman J, Avsar M, et al. Prediction of transplant outcome after 24-hours ex vivo lung perfusion using the Organ Care System in a porcine lung transplantation model. *Am J Transplant.* 2019; 19:345-355.

14. Buchko MT, Catherine JS, Hatami S, et al. Total parenteral nutrition in ex vivo lung perfusion: Addressing metabolism improves both inflammation and oxygenation. AJT. 2019;19:3390-3397.

15. Buchko MT, Himmat S, Stewart CJ, et al. Continuous hemodialysis does not improve graft function during ex vivo lung perfusion over 24 hours. Transplantation Proceedings;51:2022-28

16. Takahashi M, Cheung H, Watanabe T, et al. Strategies to prolong homeostasis of ex vivo perfused lungs. *JTCVS.* 2020, 161(6): 1963-1973. https://doi.org/10.1016/j.jtcvs.2020.07.104

17. De Wolf J, Glorian M, Journeau L, et al. Challenging the ex vivo lung perfusion procedure with continuous dialysis in a pig model. *Transplantation.* 2022; 106(5): 979-987.

18. Wei D, Gao F, Yang Z, et al. Ex vivo lung perfusion with perfusate purification for human donor lung following prolonged cold storage. *Ann Transl Med.* 2020; 8(3): 38. doi: 10.21037/atm.2019.10.17

19. Hough O, Gao X, Yang C, et al. Addition of dialysis to ex-vivo lung perfusion maintains homeostasis and stability of donor lungs: a proof of concept study. *JHLT.* 2021; 40(4) supplement, S69. https://doi.org/10.1016/j.healun.2021.01.1909

20. Schumer EM, Zoeller KA, Linsky PL, et al. Feasibility study of pulsa- tile left ventricular assist device for prolonged ex vivo lung perfu- sion. *Ann Thorac Surg*. 2015;99(6):1961-1967

21. Sorin Xtra Operators Manual: Software version 2.0. (page 18, Table 1-1). https://www.perfusion.com/wp-content/uploads/2021/03/CP_MIR_LLA_20077_021_US_hi.pdf

22. American Association of Blood Banks. Standards for Perioperative Autologous Blood Collection and Administration. 4th Edition. Bethesda, MD. 2010 Reference Standard 5.1.8A (Handling, Storage, and Expiration of Perioperative Autologous Red Cell Blood Products).

23. Forgie KA, Fialka N, Khan M, et al. Left lung transplantation in a porcine model of NPV-ESLP: evaluation of lung function with four-hour survival. *The Journal of Visualized Experiments*. (180), e62979, doi:10.3791/62979 (2022).

24. Koike T, Yeung JC, Cypel M, et al. Kinetics of lactate metabolism during acellular normothermic ex vivo lung perfusion. *JHLT*. 2011; 30(12): 1312-1319.

25. O'Neill JD, Guenthart BA, Hozain AE, Bacchetta M. Xenogeneic support for the recovery of human donor organs.

26. Ali A, Nykanen A, Brambate E, et al. Successful transplantation of porcine lungs following 3 days of preservation using a modified cold static method paired with intermittent normothermic ex vivo lung perfusion (EVLP). *The Journal of Heart and Lung Transplantation.* 2022; 39 (4): S353.

27. Ali A, Nykanen AI, Beroncal E, et al. Successful 3-day lung preservation using a cyclic normothermic ex vivo lung perfusion strategy. Lancet 2022;83:104210. https://doi.org/10.1016/j.ebiom.2022.104210

28. Ali A, Nykanen A, Brambate E, et al. Successful transplantation of porcine lungs following 3 days of preservation using a modified cold static method paired with intermittent normothermic ex vivo lung perfusion (EVLP). *The Journal of Heart and Lung Transplantation.* 2022; 39 (4): S353.

29. Egan TM, Haithcock B, Ali MH, et al. A Large Animal Model of Ex-Vivo Lung Perfusion as a Platform to Deliver Therapeutic Agents to Lungs Before Lung Transplant [abstract]. *Am J Transplant.* 2020; 20 (suppl 3).

30. Galasso M, Feld JJ, Watanabe Y, et al. Inactivating hepatitis C virus in donor lungs using light therapies during normothermic ex vivo lung perfusion. *Natu Commun.* 2019; 10:481. https://doi.org/10.1038/s41467-018-08261-z

Chapter 9

Negative Pressure Ventilation Ex-Situ Lung Perfusion Successfully Preserves Porcine Lungs and Rejected Human Lungs for 36-hours

1. van der Mark SC, Hoek RAS, Hellemons ME. Developments in lung transplantation over the past decade. *Eur Respir Rev*. 2020;29:190132.

2. Valapour M, Lehr CJ, Skeans MA, et al. OPTN/SRTR 2019 Annual Report: Lung. Am J Transplantation. 2021;21: 441-520.

3. Canadian Institute for Health Information. *Annual Statistics on Organ Replacement in Canada: Dialysis, Transplantation and Donation, 2009 to 2018*. Ottawa, ON: CIHI; 2019.

4. Ali A, Cypel M. Ex-vivo lung perfusion and ventilation: where to from here? *Curr Opin Organ Transplant*. 2019; 24:297-304.

5. Steen S, Ingemansson R, Eriksson L, et al. First human transplantation of a nonacceptable donor lung after reconditioning ex vivo. Ann Thorac Surg. 2007; 83: 2191-5.

6. Steen S, Sjoberg T, Pierre L, et al. Transplantation of lungs from a non-heart-beating donor. Lancet. 2001; 357: 825-29.

7. Sanchez PG, Cantu E, Hartwig M, et al. The NOVEL study: A multi-center clinical trial studying the safety of Ex Vivo Lung Perfusion. JHLT. 2020; 39(4); s110. https://doi.org/10.1016/j.healun.2020.01.977

8. Warnecke G, Van Raemdonck D, Smith MA, et al. Normothermic ex-vivo preservation with the portable Organ Care System Lung device for bilateral lung transplantation (INSPIRE): a randomized, open-label, non-inferiority, phase 3 study. Lancet Respir Med. 2018; 6: 357-67.

9. Loor G, Warnecke G, Villavicencio MA, et al. Portable normothermic ex-vivo lung perfusion, ventilation, and functional assessement with the Organ Care System on donor lung use for transplantation from extended-criteria donors (EXPAND): a single-arm, pivotal trial. Lancet Respir Med. 2019;7:975-84.

10. Buchko MT, Boroumand N, Cheng JC, et al. Clinical transplantation using negative pressure ventilation ex situ lung perfusion with extended criteria donor lungs. Nature Communications. 2020; https://doi.org/10.1038/s41467-020-19581-4

11. Cypel M, Yeung JC, Hirayama S, et al. Technique for prolonged normothermic Ex Vivo Lung Perfusion. JHLT. 2008; 27(12): 1318-1325.

12. Aboelnazar N, Himmat S, Hatami S, et al. Negative pressure ventilation decreases inflammation and lung edema during normothermic ex-vivo lung perfusion. JHLT. 2018; 37:520-530.

13. Forgie KA, Fialka N, Bucko MT, et al. Normothermic negative pressure ventilation ex-situ lung perfusion: evaluation of lung function and metabolism. Journal of Visualized Experiments. 2022;180: doi:10.3791/62982.

14. Buchko MT, Catherine JS, Hatami S, et al. Total parenteral nutrition in ex vivo lung perfusion: Addressing metabolism improves both inflammation and oxygenation. AJT. 2019;19:3390-3397.

15. Buchko MT, Himmat S, Aboelnazar NS, et al. A low-cost perfusate alternative for ex vivo lung perfusion. Transplantation Proceedings 2020;52(10):2941-2946.

16. Buchko MT, Himmat S, Stewart CJ, et al. Continuous hemodialysis does not improve graft function during ex vivo lung perfusion over 24 hours. Transplantation Proceedings;51:2022-28

17. Loor G, Howard BT, Spratt JR, et al. Prolonged EVLP using OCS Lung: Cellular and Acellular Perfusates. *Transplantation.* 2017; 101(10): 2303-2311.

18. Sommer W, Salman J, Avsar M, et al. Prediction of transplant outcome after 24-hours ex vivo lung perfusion using the Organ Care System in a porcine lung transplantation model.

19. Hozain AE, Tipograf Y, Pinezich MR, et al. Multiday maintenance of extracorporeal lungs using cross-circulation with conscious swine. JTCVS. 2020;159(4):1640-1653.

20. O'Neill JD, Guenthart BA, Hozain AE, Bacchetta M. Xenogeneic support for the recovery of human donor organs.

21. Ali A, Nykanen AI, Beroncal E, et al. Successful 3-day lung preservation using a cyclic normothermic ex vivo lung perfusion strategy. Lancet 2022;83:104210. https://doi.org/10.1016/j.ebiom.2022.104210

22. Prasad NK, Pasrija C, Talaie T, Krupnick AS, Zhao Y, Lau CL. Ex vivo lung perfusion: Current achievements and future directions. *Transplantation.* 2021; 105(5) 979-985.

23. Niroomand A, Hirdman G, Olm F, Lindstedt S. Current status and future perspectives on machine perfusion: a treatment platform to restore and regenerate injured lungs using cell and cytokine adsorption therapy. *Cells*. 2022; 11, 91. https://doi.org/ 10.3390/cells11010091

24. Iske J, Hinze CA, Salman J, Haverich A, Tullius SG, Ius F. The potential of ex vivo lung perfusion on improving organ quality and ameliorating ishchemia reperfusion injury. *Am J Transplant.* 2021; 21: 3831-3839.

25. Koike T, Yeung JC, Cypel M, et al. Kinetics of lactate metabolism during acellular normothermic ex vivo lung perfusion. *J Heart Lung Transplant*. 2011;30:1312-9.

26. Takahashi M, Cheung H, Watanabe T, et al. Strategies to prolong homeostasis of ex vivo perfused lungs. *JTCVS.* 2020, 161(6): 1963-1973. https://doi.org/10.1016/j.jtcvs.2020.07.104

27. Okamoto T, Wheeler D, Farver C, McCurry K. Transplant suitability of rejected human donor lungs with prolonged cold ischemia time in low-flow acellular and high-flow cellular ex vivo lung perfusion systems. *Transplantation.* 2019; 103(9): 1799-1808.

28. Forgie KA, Fialka N, Freed DH, Nagendran J. Moderate flow is superior to low flow perfusion in a juvenile porcine transplantation model of ex-situ lung perfusion. *JHLT.* 2022; 41(4): S252. http://dx.doi.org/10.1016/j.healun.2022.01.1774

29. Beller JP, Byler MR, Money DT, et al. Reduced-flow ex vivo lung perfusion to rehabilitate lungs donated after circulatory death. *J Heart Lung Transplant.* 2020;39:74-82.

30. Forgie KA, Fialka N, Freed DH, Nagendran J. Normothermic Perfusion is Superior to Subnormothermic Perfusion in a Juvenile Porcine Transplantation Model of Ex-Situ Lung Perfusion. *JHLT.* 2022; 41(4): S252. https://doi.org/10.1016/j.healun.2022.01.624

31. Rubini A. Effect of perfusate temperature on pulmonary vascular resistance and compliance by arterial and venous occlusion in the rat. Eur J Appl Physiol 2005;93:435–9.

32. Suzuki S, Sugita M, Sadafumi O, et al. Difference in the effects of low temperatures on the tension of human pulmonary artery and vein ring segments. Respiration 2000;67:189–93.

33. Chamarthy MR, Kandathil A, Kalva SP. Pulmonary vascular pathophysiology. Cardiovasc Diagn Ther 2018;8(3):208-213.

34. Mahdi M, Joseph NJ, Hernandez DP, Crystal GJ, Baraka A, Salem MR. Induced hypocapnia is effective in treating pulmonary hypertension following mitral valve replacement. Anesth. 2011; 21(2):259-70

35. Chang AC, Zucker HA, Hickey PR, Wessel DL. Pulmonary vascular resistance in infants after cardiac surgery: Role of carbon dioxide and hydrogen ion. Crit Car Med. 1995;23:568-574.

36. Fox WW, Duara S: Persistent pulmonary hypertension in the neonate: Diagnosis and management. J Pediatr 1983; 103:505-514.

37. Scott TE, Kemp-Harper BK, Hobbs AJ. Inflammasomes: a novel therapeutic target in pulmonary hypertension. *British Journal of Pharmacology.* 2019;176: 1880-1886.

38. Thom SR, Bhopale VM, Hu JP, Yang M. Inflammatory responses to acute elevations of carbon dioxide in mice. *J Appl Physiol.* 2017;123: 297-302.

39. Ding H, Li Y, Li X, Liu X, Chen S, Liu M, Zeng H. Treatment with 7% and 10% CO_2 enhanced expression of IL-1*B*, TNF-*a*, and IL-6 in hypoxic cultures of human whole blood. *Journal of International Medical Research.* 2020; 48(4): 1-12.

40. Linacre V, Cypel M, Machuca T, et al. Importance of left atrial pressure during ex vivo lung perfusion. *JHLT*. 2016; 35:808-814.

41. Hassanein WH, Fattah IA, Lezberg P, Khayal TI, Havener R, AB-Delazim A. Organ care solution for ex-vivo machine perfusion of donor lungs. WIPO/PCT. (18.10.2012). International Publication Number: WO 2012/142487 A1.

42. Ali A, Nykanen A, Brambate E, et al. Successful transplantation of porcine lungs following 3 days of preservation using a modified cold static method paired with intermittent normothermic ex vivo lung perfusion (EVLP). *The Journal of Heart and Lung Transplantation.* 2022; 39 (4): S353.

43. Egan TM, Haithcock B, Ali MH, et al. A Large Animal Model of Ex-Vivo Lung Perfusion as a Platform to Deliver Therapeutic Agents to Lungs Before Lung Transplant [abstract]. *Am J Transplant.* 2020; 20 (suppl 3).

44. Galasso M, Feld JJ, Watanabe Y, et al. Inactivating hepatitis C virus in donor lungs using light therapies during normothermic ex vivo lung perfusion. *Natu Commun.* 2019; 10:481. https://doi.org/10.1038/s41467-018-08261-z

Appendices

Materials List: NPV-ESLP

Name of Material/Equipment	Company	Catalog Number	Comments/Description
ABL 800 FLEX Blood Gas Analyzer	Radiometer	989-963	
Adult-Pediatric Electrostatic Filter HME - Small	Covidien	352/5877	
Arterial Filter	SORIN GROUP	01706/03	
Backhaus Towel Clamp	Pilling	454300	
Bovine Serum Albumin	MP biomedicals	218057791	
Biomedicus Pump	Maquet	BPX-80	
Cable Ties – White 12”	HUASU International	HS4830001	
Calcium Chloride	Fisher Scientific	C69-500G	
Cooley Sternal Retractor	Pilling	341162	
CUSHING Gutschdressing Forceps	Pilling	466200	
Debakey-Metzenbaum Dissecting Scissors	Pilling	342202	
Debakey Straight Vascular Tissue Forceps	Pilling	351808	
D-glucose	Sigma-Aldrich	G5767-500G	
Endotracheal Tube 9.0mm CUFD	Mallinckrodt	9590E	Cuff removed
Flow Transducer	BIO-PROBE	TX 40	
Infusion Pump	Baxter	AS50	
Inspire 7 M Hollow Fiber Membrane Oxygenator	SORIN GROUP	K190690	
Intercept Tubing Connector 3/8" x 1/2"	Medtronic	6013	

Intercept Tubing 1/4" x 1/16" x 8'	Medtronic	3108	
Intercept Tubing 3/8" x 3/32" x 6'	Medtronic	3506	
MAYO Dissecting Scissors	Pilling	460420	
Medical Carbon Dioxide Tank	Praxair	5823115	
Medical Oxygen Tank	Praxair	2014408	
Medical Nitrogen Tank	Praxair	NI M-K	
Organ Chamber	Tevosol		
PlasmaLyte A	Baxter	TB2544	
Poole Suction Tube	Pilling	162212	
Potassium Phosphate	Fischer Scientific	P285-500G	
Scale	TANITA	KD4063611	
Silicon Support Membrane	Tevosol		
Sodium Bicarbonate	Sigma-Aldrich	792519-1KG	
Sodium Chloride 0.9%	Baxter	JB1324	
Sorin XTRA Cell Saver	SORIN GROUP	75221	
Sternal Saw	Stryker	6207	
Surgical Electrocautery Device	Kls Martin	ME411	
TruWave Pressure Transducer	Edwards	VSYPX272	
Two-Lumen Central Venous Catheter 7fr	Arrowg+ard	CS-12702-E	
Vorse Tubing Clamp	Pilling	351377	
Willauer-Deaver Retractor	Pilling	341720	
Yankauer Suction Tube	Pilling	162300	
0 ETHIBOND Green 1X36" Endo Loop 0	ETHICON	D8573	
	ETHICON	SA77G	

2-0 SILK Black 12" x 18" Strands			

Materials List: Left Lung Transplant

Name of Material/Equipment	Company	Catalog Number	Comments/Description
ABL 800 FLEX Blood Gas Analyzer	Radiometer	989-963	
Adult-Pediatric Electrostatic Filter HME - Small	Covidien	352/5877	
Allison Lung Retractor	Pilling	341679	
Arterial Filter	SORIN GROUP	01706/03	
Backhaus Towel Clamp	Pilling	454300	
Bovine Serum Albumin	MP biomedicals	218057791	
Biomedicus Pump	Maquet	BPX-80	
Bronchoscope			
Cable Ties – White 12”	HUASU International	HS4830001	
Calcium Chloride	Fisher Scientific	C69-500G	
Cooley Sternal Retractor	Pilling	341162	
CUSHING Gutschdressing Forceps	Pilling	466200	
Debakey-Metzenbaum Dissecting Scissors	Pilling	342202	
DeBakey Peripheral Vascular Clamp	Pilling	353535	
Debakey Straight Vascular Tissue Forceps	Pilling	351808	
D-glucose	Sigma-Aldrich	G5767-500G	
Drop sucker			
Endotracheal Tube 9.0mm CUFD	Mallinckrodt	9590E	Cuff removed
Flow Transducer	BIO-PROBE	TX 40	
Infusion Pump	Baxter	AS50	
Inspire 7 M Hollow Fiber Membrane Oxygenator	SORIN GROUP	K190690	
Intercept Tubing Connector 3/8" x 1/2"	Medtronic	6013	
Intercept Tubing 1/4" x 1/16" x 8'	Medtronic	3108	
Intercept Tubing 3/8" x 3/32" x 6'	Medtronic	3506	

MAYO Dissecting Scissors	Pilling	460420	
Medical Carbon Dioxide Tank	Praxair	5823115	
Medical Oxygen Tank	Praxair	2014408	
Medical Nitrogen Tank	Praxair	NI M-K	
Mosquito Clamp	Pilling	181816	
Harken Auricle Clamp			
Organ Chamber	Tevosol		
PlasmaLyte A	Baxter	TB2544	
Poole Suction Tube	Pilling	162212	
Potassium Phosphate	Fischer Scientific	P285-500G	
PERFADEX Plus	XVIVO	19811	
Satinsky Clamp	Pilling	354002	
Scale	TANITA	KD4063611	
Silicon Support Membrane	Tevosol		
Sodium Bicarbonate	Sigma-Aldrich	792519-1KG	
Sodium Chloride 0.9%	Baxter	JB1324	
Sorin XTRA Cell Saver	SORIN GROUP	75221	
Sternal Saw	Stryker	6207	
Surgical Electrocautery Device	Kls Martin	ME411	
TruWave Pressure Transducer	Edwards	VSYPX272	
Two-Lumen Central Venous Catheter 7fr X2	Arrowg+ard	CS-12702-E	
Vorse Tubing Clamp	Pilling	351377	
Willauer-Deaver Retractor	Pilling	341720	
Yankauer Suction Tube	Pilling	162300	
0 ETHIBOND Green 1X36" Endo Loop 0	ETHICON	D8573	
0 PDS II CP-1 2x27”	ETHICON	Z467H	
1 VICRYL MO-4 1x18”	ETHICON	J702D	
2-0 SILK Black 12" x 18" Strands	ETHICON	SA77G	
4-0 PROLENE Blue TF 1x24”	ETHICON	8204H	
6-0 PROLENE Blue BV 2x30”	ETHICON	M8776	
21-Gauge Needle			

Surgical Checklist Left Lung Transplant:

Swine Left Lung Transplant Surgical Safety Checklist (Recipient Operation)		
Before Induction of Anesthesia	***Before Skin Incision***	***Before Team Leaves Operating Room***
Supplies/Equipment: ☐ Change CO_2 crystals ☐ Fill Isoflurane ☐ O_2 on, Ensure adequate O_2 ☐ 2 suctions rtg, plus 3rd spare rtg ☐ Tool table, saw, drapes, cautery, lap sponges, silk ties, triple lumen catheter ☐ Tray for dissection ☐ Surgivet Monitor, incl ART line BP ☐ Table plugged in, heat on ☐ ETT tray ☐ Defibrillator rtg ☐ IV pump ☐ Fluid bags- Main bag on IV pump, Bolus bag on slam bag ☐ Syringe pumps set up, dose rates to be inputted confirmed, know dose range ☐ Container of Saline w/ 60cc syringe for flush ☐ Hep/Saline Bottle/Bag ☐ Various needles/syringes ☐ Blood gas syringes x6 rtg ☐ Cadaver bags x2 ☐ Perfadex Flush rtg ☐ Blake Chest tube ☐ Bronchoscope w/ ETT adapter, Fog off, Flush ☐ 7Fr double lumen in Carotid **Animal Prep:** ☐ Prepare Anesthesia sheets (Donor/Recipient) ☐ Ensure enough drug, check expiry ☐ Intubate with 8.5mm or larger ETT ☐ Clean/ iodine spray ☐ Sternotomy vs Thoracotomy ☐ Drape animal ☐ Jugular IV line (2ml/kg/hr) ☐ ART line BP (carotid or heart) **Premed Doses:** ☐ Ketamine 17mg/kg IM ☐ 3mg/kg IV q 1hr (range 1-3mg/kg) ☐ Hydromorphone 0.05mg/kg q2hr ☐ Atropine 0.04mg/kg IM ☐ Naloxone 0.01mg/kg IV if needed **MGMT Parameters:** **Vitals** ☐ MAP 60-70 mmHg ☐ HR 90-110 ☐ Temp 37.5-39 **Ventilation:** ☐ RR: 15-20 ☐ TV: 6-10 mL/kg ☐ I/E: 1:2 ☐ Peak Pressure: 20-30 cm H_20	**Anticipated Critical Events:** **Predraw:** ☐ Dextrose 100mL ☐ Insulin R 10 units ☐ Calcium Gluconate ☐ Cephazolin and Methyl Pred. ☐ Bupivacaine 10ml 0.5% ☐ Furosemide 40mg **To Surgeon:** ☐ What are the critical or non-routine steps? ☐ How long will the case take? ☐ What is the anticipated blood loss? **To Veterinary Technicians:** ☐ Any specific concerns? **Have Abx and Steroids been administered after central line insertion?** ☐ Cefazolin 1 g IV ☐ Methlyprednisone 500mg IV **Intercostal nerve block prior to skin incision** ☐ Bupivacaine 5 mL – incision ☐ Bupivacaine 5 mL – Intercostal block **Heparin 5000 units IV** 1) ☐ Prior to PA clamp 2) ☐ Prior to PA unclamp + 40mg Lasix **Bronchus Clamp** ☐ decrease TV to 5 ml/kg ☐ increase RR to 30 ☐ increase peak pressure to 25-30 ☐ maintain end-tidal CO_2 35-55 (40) mmHg ☐ I:E = 1:1 **Prone Pig** ☐ T1 after reperfusion with good ABG ☐ Close chest ☐ ECG leads **PA Unclamp** ☐ Lasix 40mg IV **Infusion Rates: MAP<60, HR<90** ☐ **Total IV fluid rate:** 70-100 ml/hr ☐ Phenylephrine 2-10 ml/min ☐ Dobutamine 1.5-5 mcg/kg/min ☐ Norepinephrine 0.01-0.3mcg/kg/min ☐ Vasopressin 0.5-2 ml/hr (0.1-0.4 u/hr)	**Technician Verbally Confirms:** ☐ Review Equipment Problems ☐ Review New Protocol and any planned changes ☐ Debrief: What went well? What can be improved? ☐ Return Checklist to Surgeon **ABGs** ☐ arterial access x1 ☐ at reperfusion ☐ q30minutes after reperfusion ☐ Isolated Pulm. Vein sample at T4 **10cc blood samples** ☐ Invivo with arterial access ☐ T0 at reperfusion ☐ T1 ☐ T2 ☐ T3 ☐ T4 **Tissue Samples** ☐ Invivo – auto transplant only. ☐ T0 Reperfusion ☐ T4 Reperfusion **Weigh Lungs** ☐ Explant/pre ESLP ☐ Post ESLP ☐ Left lung pre-implant: _____ ☐ Left lung post exsanguination: _____ ***Common Troubleshooting Algorithm:*** ☐ Rapid Afib – 50mg Amiodarone IV bolus, repeat PRN ☐ Tachycardia ☐ assess pain ☐ assess depth of anesthetic ☐ assess drips ☐ Hypotension MAP <60 – 250 ml fluid bolus ☐ HR <90 – Dob or NE ☐ HR >90 – Phenyl or Vaso ☐ Hyperkalemia K>5.0-5.5 ☐ Furosemide 40mg IV bolus ☐ 100 ml D25 with 10u Insulin ☐ Hyperkalemia K>5.5 -6.0 ☐ Furosemide 60mg IV bolus ☐ 100 ml D25 with 10u Insulin ☐ Hyperkalemia K>6.0 – 6.5 ☐ Furosemide 80mg IV bolus ☐ 100 ml D25 with 10u Insulin ☐ 1-gram Calcium

Surgical Safety Checklist for Left lung transplantation. Rtg, ready to go; hr, hour.