Interventions to Improve the Conduct of *Ex Vivo* Lung Perfusion

by

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Abstract

Lung transplant remains the gold standard therapy for end-stage lung disease. As medical therapy continues to improve, though, the number of patients reaching stable end-stage lung disease continues to increase [1]. Despite increasing need for suitable lung grafts, the number of donors remains stable [1]. One of the major issues regarding organ availability is organ utilization. Currently, only 30% of lungs offered for donation are actually transplanted [2]. *Ex vivo* lung perfusion (EVLP) is a developing technology, whereby the lungs are harvested and connected to a circuit which continually perfuses and ventilates the lungs. This allows the lungs to be reconditioned and evaluated outside the inflammatory milieu of the donor's body. EVLP has been shown to improve utilization rates and allows for transplantation of marginal or extended criteria donor lungs [3–6]. Despite its expanding clinical use, several key characteristics of machine perfusion remain untested. The objective of this thesis is to develop strategies for the improvement of EVLP. These include the development of a low-cost perfusate alternative, the development of a system for perfusate dialysis in EVLP to improve ionic homeostasis in prolonged EVLP, extension of preservation times beyond current thresholds, and to determine whether metabolic supplementation improves graft function in EVLP. The results of this thesis may be used to reduce costs and improve access to EVLP, as well as, elucidate the requirements of donor lung grafts during machine perfusion, especially during prolonged preservation. By continuing to optimize and improve EVLP, there lies the potential to further increase utilization rates among the current donor pool and improve outcomes in patients with end-stage lung disease.

Preface

This thesis is an original work by Max Buchko. 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.

For Chapter 2 of this thesis, D.H. Freed and J. Nagendran, and myself participated in manuscript preparation and revisions.

For Chapter 3 and 4 of this thesis, I participated in research design, the performance of experiments, data analysis, manuscript preparation and revisions. S. Himmat and N. Aboelnazar participated in research design and the performance of experiments. C.J. Stewart and S. Hatami participated in the performance of experiments. P. Dromparis participated in the performance of experiments and data analysis. B. Adam participated in data analysis, manuscript preparation and revisions. D.H. Freed participated in research design, performance of experiments, data analysis, manuscript preparation and revisions. J. Nagendran participated in research design, data analysis, manuscript preparation and revisions.

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List of Abbreviations

ANOVA, analysis of variance

ARDS, acute respiratory distress syndrome

APRV, airway pressure release ventilation

ATP, adenosine triphosphate

BCAA, branch chain amino acid

BPM, breaths per minute

BSA, body surface area

Cdyn, dynamic compliance

Cstat, static compliance

CHD, continuous hemodialysis

CHIP, common hospital ingredient derived perfusate

CO, cardiac output

DBD, donation after brain death

DCD, donation after circulatory determination of death

ECD, extended criteria for donation

EIP, end inspiratory pressure

EVLP, *ex vivo* lung perfusion

ET-1, endothelin-1

FFA, free fatty acid

FiO_2, fraction of inspired oxygen

H&E, hematoxylin-eosin

Hct, hematocrit

ICU, intensive care unit

IL, interleukin

IRI, ischemia-reperfusion injury

IU, international units

KHB-Alb, Kreb's Henseleit Buffer with 8% serum albumen

LA, left atirum

LAP, left atrial pressure

KHB-Alb, Krebs-Henseleit buffer with albumin derived perfusate

NPV, negative pressure ventilation

OCS, Organ Care System

P:F Ratio, arterial oxygen tension over fraction of inspired oxygen

PaO_2, partial pressure of oxygen or arterial oxygen tension

Paw, peak airway pressure

PGD, primary graft dysfunction

Pplat, plateau pressure

PPV, positive pressure ventilation

PRBC, packed red blood cells

PA, pulmonary artery

PAP, pulmonary artery pressure

PEEP, positive end expiratory pressure

PPN, positive pressure ventilation

PVR, pulmonary vascular resistance

SaO2, oxygen saturation

SE, standard error

TCA, tricarboxylic acid

TLC, total lung capacity

TG, triglyceride

TNF-α, tumor necrosis factor- α

TPGi, transpulmonary gradient upon inhalation

TPN, total parenteral nutrition

VC, volume control

VILI, ventilator induced lung injury

Chapter 1: Thesis Overview

Background

Lung transplant remains the primary therapy for end-stage lung disease. Currently, the number of patients with end stage lung disease continues to increase; however, the overall rate of donation has remained stagnant. Compounding this discrepancy, is fact that only about one-third of organs offered for donation are transplanted due to concerns of donor graft function. This has left a substantial shortage of donor lungs leading to increasing mortality on the wait-list for lung transplantation.

Traditionally, lungs have been preserved using cold static storage. Organs are flushed with a cold low potassium dextran solution and stored on ice until they can be implanted. Cold static preservation has several limitations, though. Lungs preserved for longer than six hours have a significantly higher risk of primary graft dysfunction, which is the leading cause of early mortality in lung transplantation [7]. Furthermore, while static, organ function can not be assessed prior to reimplantation. Therapies for organ resuscitation cannot be performed as there is neither circulatory or ventilatory flow in the lungs.

Ex vivo lung perfusion (EVLP) is an emerging technology, whereby the lungs are harvested and connected to a circuit which continually perfuses and ventilates the lungs. This allows the lungs to be reconditioned and evaluated outside the inflammatory milieu of the donor's body. The use of EVLP has allowed for the resuscitation and subsequent transplantation of extended criteria or marginal donor lungs with similar results to standard criteria donors[3,5,6,8,9]. In doing so, multiple programs across the world have increased the overall donor pool available to their patients.

A significant amount of research has already gone into developing the current EVLP systems and protocols. Despite this fact, a several key aspects of EVLP lack optimization. Much of the potential benefits of EVLP remain unrealized due to key barriers preventing the widespread

adoption of this technology. By addressing these barriers and improving our understanding of the requirements of an organ on machine perfusion the sooner we may transition from the theoretical benefits of EVLP to the real benefits of EVLP and the more organs that will be suitable for transplantation.

Hypothesis

By optimizing contemporary EVLP protocols to address cost, stable time on machine perfusion, electrolyte homeostasis, and metabolism, we hope to improve donor graft function. Improved graft performance may increase the total number of grafts suitable for transplantation, thereby increasing to donor pool and decreasing the overall burden of end-stage lung disease.

Objectives

The objectives described are displayed in Figure 1.

1. Provide an overview of how EVLP is currently performed and what aspects of machine perfusion have already been examined (Chapter 2).
2. Develop a low cost perfusate alternative with similar efficacy to conventional perfusates to reduce the cost of EVLP and improve access (Chapter 3).
3. Develop a model for extended EVLP up to 24 hours (Chapter 4).
4. Investigate whether the addition of continuous hemodialysis (CHD) of the perfusate during EVLP may improve overall graft function (Chapter 5).
5. Investigate whether metabolic support with conventional total parenteral nutrition (TPN) during EVLP would improve overall graft function (Chapter 6).
6. Investigate the metabolic requirements of lung grafts undergoing machine perfusion including oxidation of key substrates such as lipids and amino acids (Chapter 6).

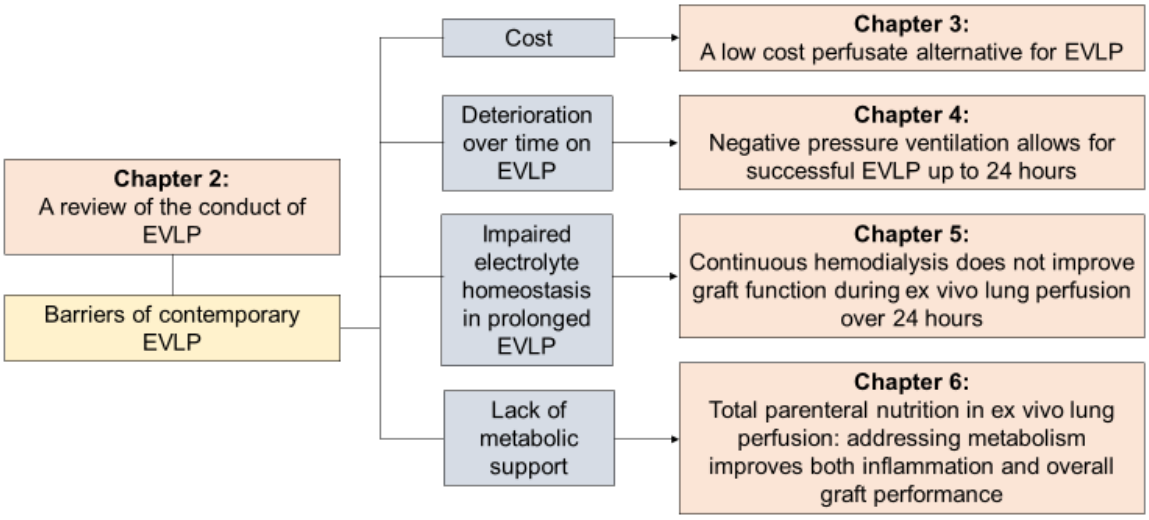

Figure 1. Thesis overview

Chapter 2: A review of the conduct of *ex vivo* lung perfusion

Introduction

Hardesty and Griffith described the first concept of ex-vivo lung perfusion (EVLP) in 1987[10]. It would take until 2001, though, for Steen *et al* to describe its utility in evaluating graft function in donation after circulatory determination of death (DCD) donors before it gained traction within the transplant community[11]. Later, in 2011, the Toronto lung transplant program published their landmark trial, *Normothermic Ex-vivo Lung Perfusion in Clinical Lung Transplantation*[4]. This trial demonstrated the safety of using EVLP to screen marginal grafts and demonstrated an increase in the utilization rates of offered lungs. Contemporary utilization rates are relatively low, ranging from 15-20%, and therefore any means of increasing organ utilization has the potential to significantly increase the number of transplants performed[4,12]. Since then, several groups have published their results using EVLP, establishing it as a safe and efficacious therapy. Furthermore, with its more widespread adoption, new utilities continue to be found. For example, size mismatch can be corrected on EVLP allowing for post-correction testing for air leaks, size measurement, and assessment prior to implantation[13]. Other techniques such as split-lung or lobar transplantation may be used to increase the donor pool in pediatric patients, which has the highest wait-list mortality due to donor shortage[14].

The objectives of ex-vivo organ perfusion are similar to most aspects of medicine. We look to diagnose problems, in this case with donor lungs. We intend to treat problems once identified. Finally, we aim to maintain normal physiologic function for as long as possible. Each paper discussed will touch on one or more of these key objectives. Other aspects of EVLP, such as clinical trial results and resuscitative interventions are not discussed within the scope of this article.

Mainstream protocols and platforms

Currently, three significant EVLP methodologies have emerged as a foundation for the majority of studies. These are the Lund protocol, the Toronto protocol, and the Organ Care System protocol (see Table 1). Furthermore, commercial platforms are in various stages of development to support these protocols. The Lund protocol most commonly uses the LS1 system by Vivoline Medical (Sweden); while, the Toronto protocol commonly uses the XPS system by XVIVO Perfusion (Colorado, USA). The OCS protocol uses the OCS Lung System by Transmedics (Massachusetts, USA). While the general components of these systems are similar, each protocol and subsequent platform varies in terms of flow rate, pump type, ventilation strategy, targeted pressures, perfusion temperature, and perfusate composition[15].

	LUND PROTOCOL	TORONTO PROTOCOL	ORGAN CARE SYSTEM
APPLICATION	Reevaluation, reconditioning, treatment after procurement	Reevaluation, reconditioning, treatment after procurement	Substitute of cold ischemia
TRANSPORTABLE	No	No	Yes
PUMP TYPE	Roller	Centrifugal	Pulsatile
INITIAL FLOW	100 mL/min	150 mL/min	200 mL/min
TARGET FLOW (CO = 3 X BSA)	100% CO	40% CO	2.5 L/min.
VENTILATION MODE	Volume Control	Volume Control	Volume Control
TIDAL VOLUME	3-8 mL/kg (1.5 x PA Flow)	7 mL/kg	6 mL/kg
FREQUENCY	12 bpm	7 bpm	10 bpm
PEEP	5 cmH_2O	5 cmH_2O	5 cmH_2O
FIO_2	50%	21%	21%
PULMONARY ARTERY PRESSURE	< 20 mmHg	< 15 mmHg	< 20 mmHg
LEFT ATRIAL PRESSURE	0 mmHg	3-5 mmHg	0 mmHg
PERFUSATE	Steen Solution + red cells (Hct 10-15%)	Steen Solution	Steen Solution + red cells (Hct 15-25%)
TOTAL PERFUSION TIME	2-7 hr	Up to 12 hr	Transport time
INITIAL REPERFUSION TEMPERATURE	25 C	25 C	32 C
TEMPERATURE UPON INITIATION OF VENTIALTION	32 C	32 C	32 C
TEMPERATURE UPON INITIATION OF EVALUATION	37C	37C	37 C

Bpm, breaths per minute; BSA, body surface area; CO, cardiac output; Hct, hematocrit; PA, Pulmonary Artery; PEEP, positive-end expiratory pressure.

Table 1: Initiation and steady phase in ex-vivo lung perfusion. Reproduced with permission from Sanchez PG, Mackowick KM, and Kon ZN. Current state of ex-vivo lung perfusion. Curr Opin Organ Transplant 2016;21:258–266.

Assessment of lung function

The characteristics of an ideal lung donor have remained relatively constant since the 1980's[16]. These include: donation after brain death (DBD), donor age 20-45 years old, P:F ratio > 350 mmHg, non-smoker, with clear chest radiograph and bronchoscopy, less than 5 days of mechanical ventilation, no growth on gram stain, and potential ischemic time of less than four hours[17]. Due to the relative disparity of donor grafts, though, most programs have become more liberal with regards to organ acceptability and have settled on some form of extended criteria for donation (ECD). There is little agreement; however, on what constitutes ECD[4,18–20]. The emphasis on continually extending the criteria for donation to increase the size of the donor pool, while maintaining safety necessitates a means of evaluating the lungs prior to implantation. For example, earlier studies also often included DCD lungs, categorically, as marginal lungs. However, as DCD outcomes have shown to be equivalent to DBD, the nomenclature is beginning to shift[21,22]. Grafts are increasingly categorized as either acceptable or not acceptable. This is first assessed at the time of harvest. If they are designated as unacceptable, they may be re-assessed after a course of EVLP (see Figure 2.1).

Currently many factors, both objective and subjective, go into the decision of whether to use lungs screened using EVLP. Despite a multitude of factors, a few key variables have become the mainstay of lung assessment. Firstly, there is general consensus that the EVLP ΔPO_2 or P:F Ratio (P:F Ratio=$PO_{2\,LA}$–$PO_{2\,PA}$/FiO2) must be >300-400mmHg, with a stable or increasing trend over time[4,23]. Regarding EVLP, Okamoto *et al* showed that the P:F ratio is not constant at varying FiO_2's[24]. Therefore, it has generally become standardized that lung assessment is to be done at a FiO_2 of 1.0. Interestingly, they also noted that when the P:F ratio at FiO_2 0.21 was greater than the P:F ratio at FiO_2 1.0, transplant suitability was significantly decreased adding another possible marker of lung quality. The same group also showed that P:F ratio is

correlated with peak airway pressure (P_{aw}), plateau pressure (P_{plat}), dynamic compliance (C_{dyn}), and static compliance (C_{stat}) in both pigs and humans[25]. Interestingly, P:F ratio also correlated with pulmonary vascular resistance (PVR) and pulmonary artery pressure (PAP) in porcine models, but not humans. This fact is important in the translation of results from animal research models to human clinical trials. The other emerging significant indicator of lung function is compliance. Both Yeung *et al* and Sanchez *et al* showed that compliance begins to decline before the P:F ratio, making its trend an important early indicator of lung function[26,27]. The acknowledgement of decreasing compliance should provide an impetus for further reparative maneuvers, for example by administering bronchodilators or performing therapeutic bronchoscopy, to best assess whether lungs are truly acceptable or not[28]. Other ancillary tests may include X-rays, bronchoscopy, deflationary maneuvers, and manual inspection and all contribute to the overall decision of whether a lung is useable for transplant or not[4,29].

A developing area in lung assessment is the use of biomarkers to determine lung quality. In a retrospective analysis of perfusate samples, Machuca *et al* showed that both endothelin-1 (ET-1) and big endothelin-1 (Big ET-1) concentrations were capable of predicting whether lungs would be declined or used with suitable initial function. Suitable initial function was defined as Primary Graft Dysfunction Grade (PGD) <3 within the first 72 hours[30]. In the subgroup of utilized lungs, ET-1 and Big ET-1 predicted whether a recipient would have PGD ≥3 in DCD patients, but not DBD patients. In another biomarker study, Andreasson *et al* found strong correlations between survival to discharge and levels of IL-1β and TNFα in perfusate at 30 minutes of EVLP[31]. Impressively, with respect to IL-1β, they found a sensitivity and specificity of 100% for mortality prior to discharge in lungs transplanted with a IL-1β level > 0.1pg/mL adjusted to predicted total lung capacity (TLC). This trend also was noted in an 83% sensitivity and 100% specificity for one-year survival using the same threshold. Immunoassays using bronchoalveolar lavage were not predictive of outcomes.

Because of the known links between inflammation, endothelial cell function, and functional outcomes, the majority of EVLP systems include a leukocyte filter within the constructs of the platform. Luc *et al* have recently challenged the widespread use of a leukocyte filter, though[32]. Their work showed that a leukocyte filter did not affect the number of circulating leukocytes and inflammatory cytokines in perfusate after prolonged EVLP (12 hours). They found the leukocyte filters had likely become saturated quite early in the perfusion and did not contribute to either functional or inflammatory outcomes. In comparison, Iskender *et al* studied the effects of cytokine adsorption device, rather than a filter, that had been incorporated into an EVLP circuit. By removing inflammatory cytokines, non-selectively, they were able to demonstrate improved metabolism, electrolyte homeostasis, compliance, and microscopic assessment of acute lung injury.

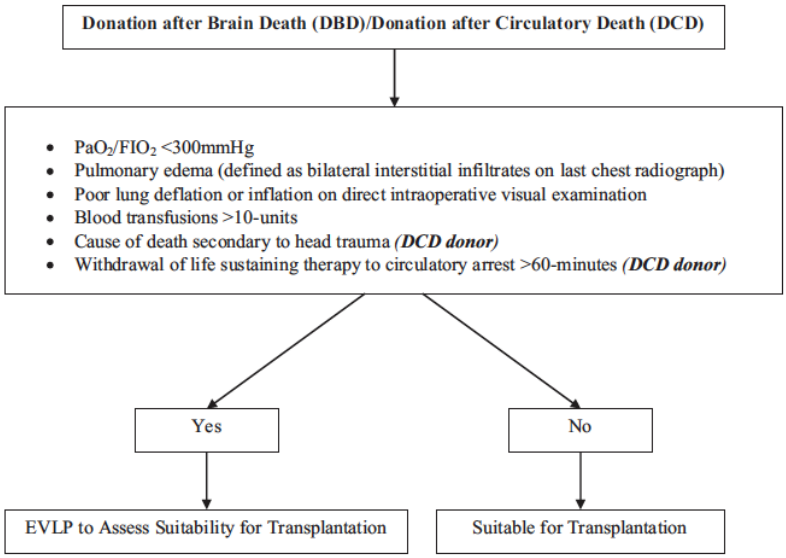

Figure 2.1: Rationale for ex-vivo lung perfusion in nonacceptable donor lungs. Reproduced with permission from Bozso SJ and Nagendran J. Life after death: breathing life into lung transplantation from donation after circulatory death donors. Am J Transplant 2017;10:2507-2508.

Perfusion

To date, the majority of EVLP protocols, except for the OCS Protocol, use Steen solution, at least in some proportion. Steen solution is an acellular solution with a physiologic oncotic pressure[33]. The primary source of oncotic pressure is human serum albumin. It also contains dextran as a scavenging agent, a physiologic electrolyte composition, and glucose as a metabolic energy source. OCS Lung Solution is also an acellular dextran-containing solution with a slightly different electrolyte and glucose composition[34,35]. It does not contain albumin and therefore requires the addition of a blood derivative to increase its oncotic pressure.

Despite the widespread use of these validated solutions, the search for the ideal perfusion strategy and perfusate composition continues to evolve. Countless variables remain unexplored. Furthermore, each individual variable may be dependent on other characteristics of the overall perfusion strategy. Therefore, translation from one methodology and platform to another may not always be applicable. For this reason, we try to identify a general description of the methodology used, when discussing a particular variable.

For example, Linacre *et al* studied the effects of perfusion with an open left atrium (LA)(left atrial pressure (LAP) of 0 mmHg) compared to a closed LA with LAP adjusted to 5 mmHg[36]. For this, they perfused swine lungs with Steen solution using the Toronto protocol. They found that the lungs perfused with an open LA had significantly increased PVR, decreased oxygenation, decreased compliance, and increased pulmonary edema. More impressively 5 out 5 lungs perfused with a closed LA were able to complete 12 hours of EVLP. Contrastingly, 4 out of 5 lungs in the open LA group were unable to complete 12 hours of EVLP due to massive edema and complete loss of perfusate volume.

In contrast, both the OCS and Lund protocols typically use an open left atrial approach and have both achieved satisfactory results. A study by Loor *et a*l highlights this point and makes

another. Using the OCS protocol, they compared whole blood, isolated red blood cells (RBC's), and acellular perfusate solutions[34]. Whole blood perfused swine lungs showed decreased PAP, PVR, and profoundly less edema formation compared to RBC's over a 24-hour EVLP run. The whole blood lungs also had increased dynamic compliance and oxygenation. The acellular, homemade Steen perfused lungs developed such profound edema that they could not complete 12 hours of EVLP. By the end of the run, only whole blood perfused lungs were still suitable for transplantation. The majority of RBC perfused lung degeneration occurred beyond 10 hours of EVLP.

Returning to the Toronto protocol, Becker *et al* evaluated the effects of acellular vs. cellular (isolated RBC) perfusates over 12 hours of EVLP[37]. The only statistically significant variables found were higher PVR and pulmonary artery pressures in the cellular group. All other variables did not reach statistical significance. Therefore, using the Toronto protocol and a closed LA, the use of cellular versus an acellular perfusate may not contribute to profoundly different outcomes, whereas on systems with an open LA, it may be more significant.

A recent collaboration between the University of Gothenburg and the Medical University of Vienna has sought to delineate whether the Lund protocol or the Toronto protocol provides superior results[38]. Ten porcine lungs were perfused by each protocol. Experiments were performed by experienced practitioners of that particular protocol. In the Toronto group, only 7 out of 10 lungs were capable of 5 hours of EVLP. Three of the lungs developed overwhelming pulmonary edema and could not maintain reservoir volumes sufficient to maintain EVLP. In the Lund group, all 10 lungs were capable of undergoing 5 hours of EVLP. Furthermore, the Lund group had lower overall weight gain and increased final compliance compared to the Toronto group. Despite these physiologic differences between groups, markers of ischemia-reperfusion injury, histopathologic assessment of acute lung injury, and inflammatory biomarkers were not different between groups.

Another variable assessed recently is the comparison of variable rates of de-oxygenation and their effect on functional and inflammatory parameters. Noda *et al* showed that in a rat model perfused with Steen solution, both hypoxic and hyperoxic perfusates showed increased inflammatory and apoptotic markers, as well as worse indicators of metabolism compared to those perfused with an oxygen saturation (SaO2) of 40-60%[39]. Lungs perfused with a physiologic SaO2 of 40% had the best functional parameters including PVR, P:F ratios, and compliance, as well as better inflammatory and metabolic profiles.

Ventilation

Ventilation strategies have conventionally utilized a volume-controlled positive pressure ventilation (VC-PPV) mode. However, recent studies are beginning to challenge this generically applied approach to ventilation. Extrapolation from the acute respiratory distress (ARDS) literature is beginning to show that the avoidance of ventilator-induced lung injury (VILI) may improve outcomes. Using the XVIVO platform, with Steen perfusate and the Toronto Protocol, Terragni *et al* looked at outcomes in human patients compared to their respective ventilation stress index on EVLP[40]. The stress index is a mathematical coefficient derived from the rate of change of the P_{AW}-time curve. Stress indices between 0.95 and 1.05 indicate constant compliance during tidal inflation and are considered protective. They found that lungs ventilated with normal protective stress indices had better outcomes including ICU and hospital length of stay, duration of mechanical ventilation, and cytokine expression compared to those that did not. Compliance, itself, showed no correlation with the above outcomes. Similarly, Mehaffey *et al* showed decreased edema, neutrophil infiltration and inflammatory markers including IL-6 and IL-12 using airway pressure release ventilation (APRV-PPV) compared to VC-PPV in swine lungs[41]. Functional parameters including P:F ratio and compliance were not significantly different between APRV-PPV and VC-PPV during the short 4-hour EVLP run; however, they did develop statistical significance by 4 hours post-transplantation favoring APRV-PPV.

Other research efforts aimed at reconditioning lungs are also being investigated with varying levels of success. Hijiya *et al* showed high dose procaterol (a short acting beta agonist) administered by nebulization every 30 minutes over a 2-hour EVLP run slowed degeneration in lung function after warm ischemia in DCD canines[42]. Lungs reconditioned with procaterol had significantly less pulmonary edema, higher P:F ratios, and higher dynamic compliance. Haam *et al* showed that the addition of 2% hydrogen to the EVLP ventilation gas mixture had significant effects on inflammatory cytokine markers (IL-1β, IL-6, IL-8 and TNFα) and pulmonary edema development[43,44]. Although, P:F ratios were not significantly affected, PVR and peak airway pressure were significantly lower in the hydrogen group compared to control group. Similar studies by Martens *et al* using noble gases (Argon and Xenon) had no clinical effect[45].

Another promising development is the use of negative pressure ventilation. Aboelnazar *et al* showed significantly less edema, bullae, and inflammatory cytokines in swine lungs perfused using a custom circuit and negative pressure ventilation over 12 hours compared to traditional positive pressure ventilation[46]. This was noted with both cellular and acellular perfusates. Furthermore, in a trial of rejected human lungs, they noticed an actual drying effect with NPV-EVLP, which had not been noted in the literature.

Conclusion

The use of EVLP offers great promise as it allows for evaluation, preservation, and intervention on lung grafts, especially extended donor criteria lungs, prior to transplantation. Initial results of clinical trials continue to show safety and efficacy in this respect. Because of this, its adoption continues to increase. With continued academic endeavors into the conduct of EVLP, we may continue to increase the number and quality of suitable organs and ultimately improve outcomes in patients with end-stage lung disease.

Chapter 3: A low cost perfusate alternative for *ex vivo* lung perfusion.

Abstract

Normothermic ex-vivo lung perfusion (EVLP) has successfully been used to evaluate and recondition marginal donor lungs; however, multiple barriers continue to prevent its widespread adoption. We sought to develop a common hospital ingredient derived perfusate (CHIP) with equivalent functional and inflammatory characteristics to a standard Krebs–Henseleit buffer with 8% serum albumin derived perfusate (KHB-Alb) to improve access and reduce costs of *ex vivo* organ perfusion. Sixteen porcine lungs were perfused using NPV-EVLP for 12 hours in a normothermic state and were allocated equally to two groups: KHB-Alb vs CHIP. Physiologic parameters, cytokine profiles, and edema formation were compared between treatment groups. Perfused lungs in both groups demonstrated equivalent oxygenation (partial pressure of arterial oxygen/ fraction of inspired oxygen ratio >350 mmHg) and physiologic parameters. There was equivalent generation of tumor necrosis factor-α and IL-6, irrespective of perfusate solution used, when comparing CHIP vs KHB-Alb. Pig lungs developed equivalent edema formation between groups (CHIP: 15.8 ± 4.8%, KHB-Alb 19.5 ± 4.4%, $p>0.05$). A perfusate derived of common hospital ingredients provides equivalent results to standard Krebs–Henseleit buffer with 8% serum albumin based perfusate in NPV-EVLP.

Introduction

Pulmonary transplantation remains a critical therapeutic option for the management of end-stage lung disease. Despite proven benefit, organ utilization rates remain low[4,12]. *Ex vivo* lung perfusion (EVLP) is a developing technology that has been used to evaluate and recondition marginal donor lungs, allowing for a potential increase in the overall donor pool[4]. Multiple barriers, though, including cost, availability, and expertise limit the widespread adoption of EVLP in lung transplantation despite its proven benefit.

Several proprietary perfusion systems have been developed including the XVIVO Perfusion System (XVIVO Perfusion AB, Goteborg, Sweden), the Vivoline LS1 System (Vivoline Medical,

Lund, Sweden), and the Organ Care System Lung (OCS)(Transmedics, Andover MA)[29]. The XVIVO Perfusion system uses acellular Steen solution (Vitrolife, Gothenburg, Sweden), while the Vivoline system uses a mixture of Steen solution and packed red blood cells (pRBC's). The OCS Lung uses a combination of OCS Lung Solution and pRBC's. Steen solution is a dextran and albumin containing solution, which mimics extracellular electrolyte concentrations[34,47]. OCS Lung Solution also is a buffered, extracellular-based solution which contains dextran, but not albumin[34]. Acceptable functional performance in EVLP has been demonstrated up to 12 hours in multiple systems utilizing multiple perfusate compositions in both swine and human experiments[26,34,46,48]. Furthermore, over the course of extended periods of EVLP, there appears to be significant electrolyte derangement, as well as accumulation of lactate causing significant fluxes of key ions such as sodium, calcium, and potassium[48–50]. Despite these large fluxes, lung function and lung fluid volume homeostasis remain stable. We therefore hypothesize that the use of a non-proprietary common hospital ingredient derived perfusate (CHIP) would provide similar results compared to a standard Kreb's Henseleit-derived perfusate solution with 8% serum albumin (KHB-Alb), which was used in initial studies of NPV-EVLP. It would also provide a significant cost reduction with the potential to improve access and utilization of EVLP.

Methods

The experimental protocol was approved by the University of Alberta animal care and use committee. Care was performed in accordance with the "Principles of Laboratory Animal Care," as formulated by the National Society for Medical Research. Eight female domestic pigs (45.9 ± 1.3 kg) were assigned to each treatment group according to the composition of the perfusate solution. Porcine lungs in group 1 were perfused with 1.5L of KHB-Alb. Lungs in group 2 were perfused with 1.5L of CHIP. The perfusate in both groups was supplemented with 0.5L of red blood cell (RBC) concentrate, bringing the final perfusate volume to 2L. The described volumes

for the cellular groups provided a constant desired hemoglobin concentration of 40 to 50 g/liter. Lungs were perfused using negative pressure *ex vivo* lung perfusion (NPV-EVLP) for 12 hours. The NPV-EVLP platform has been described in detail and was primed with 3.375g of Pipercillin-Tazobactam, 10 000 IU of Heparin, and 500 mg of methylprednisolone in addition to the assigned perfusate[46].

Perfusate Composition

Standard KHB-Alb solution and CHIP were made fresh for each experiment. KHB-Alb solution[51–53] contains a similar hyperoncotic pressure and electrolyte composition to the published composition of Steen solution (XVIVO Perfusion, Goteborg, Sweden)[33]. 1L of CHIP solution was comprised of 332mL of 25% Human Serum Albumin (Grifols, Toronto, Canada), 18mL of 8.4% Sodium Bicarbonate (Baxter, Mississauga, Canada), 2.3mL of 50% Dextrose (Baxter, Mississauga, Canada), 0.9mL of 1.29mmol/L Potassium Phosphate (Baxter, Mississauga, Canada), 639.3mL of Plasmalyte-A (Baxter, Mississauga, Canada), and 7.5mL of 100mg/mL Calcium Chloride (Omega, Montreal, Canada). Calcium chloride was added last to avoid the precipitation of calcium carbonate that occurs when calcium chloride is mixed with concentrated sodium bicarbonate.

Autologous blood was washed with 0.9% saline and concentrated with a Sorin Xtra Cell Saver (Sorin Group Canada Inc., Burnaby, Canada) to produce red blood cell concentrates.

Initiation of EVLP

Our protocol for lung procurement and initiation of NPV-EVLP has been described previously[46]. Ketamine (20 mg/kg), atropine sulfate (0.05 mg/kg), and xylazine (0.9mg/kg) were used for induction of anesthesia. Following orotracheal intubation, general anesthesia was maintained with 1-3% isofluorane. The animal was anticoagulated with 40 000 IU of heparin. Median sternotomy was performed, and a 2-stage venous cannula was inserted into the right atrium. In vivo wedge biopsies were taken. The animal was then exsanguinated into a

cell saver (Sorin Xtra) and the left atrial appendage was transected. Cardiectomy was performed. The trachea was then clamped just above the carina at a peak airway pressure of 20 cm H_2O and bilateral pneumonectomy was performed en bloc. The lungs were then transferred to the NPV-EVLP organ chamber.

Upon placement in the chamber, the pulmonary artery was then cannulated and connected to the EVLP circuit. Perfusion was initiated at 10% of predicted cardiac output (70 mL/kg) and 32C. The trachea was intubated with an endotracheal tube. Once the perfusate temperature reached 32C, the trachea was then unclamped, and ventilation was initiated. Perfusate flow was increased in increments of 10% predicted cardiac output every 20 minutes until a flow of 30% cardiac output had been achieved. The perfusate temperature was gradually increased to achieve a temperature of 38C within 40 minutes of perfusion.

Preservation mode ventilation was performed as described in Table 2. A medical gas mixer (89% N_2, 8% CO_2, and 3% O_2) was titrated to keep perfusate pCO_2 between 35 and 50 mmHg. Evaluation was performed every 2 hours, for 5 minutes, by increasing upper end inspiratory pressures to a peak transpulmonary gradient limits of 25 cm H_2O or a peak tidal volume of 10mL/kg, whichever was achieved first.

Table 2. NPV-EVLP perfusion and ventilation strategy.

	Preservation	**Evaluation**
Temperature	38 C	38 C
Pulmonary Artery Flow	30% estimated CO (CO=70 mL/kg/min.)	50% estimated CO (CO=70 mL/kg/min.)
Ventilation Parameters		
Mode	Volume control	Volume control
Desired inspiratory tidal volume	8 mL/kg	10 mL/kg
Frequency	10 bpm	10 bpm
Inspiratory:Expiratory Ratio	1:1.5	1:1.5
Peak TPGi	<21 cm H20	<25 cm H20
PEEP	5 cm H20	5cm H20
FiO2	21%	21%
Pressure Parameters		
PAP	<25 mmHg	<25 mmHG
LAP	0 mmHg	0 mmHg

Inflammatory markers and histopathology

Cytokine profiles for tumor necrosis-α (TNF-α) and interleukin-6 (IL-6) were analyzed using enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, USA). Biopsies were fixed in 10% buffered formalin for 24 hours and embedded in paraffin. 5 μm slices were stained with hematoxylin-eosin. A blinded pulmonary pathologist examined the samples using light microscopy and graded them using an established score for acute lung injury[41,54].

Statistics

All results are expressed as mean ± standard error. All analyses were performed on STATA 15 (StataCorp LLC, College Station, Texas). Normality was assessed using Shapiro-Wilk test. Student's t-test was used to compare normally distributed continuous variables. Wilcoxon rank-sum test was used to compare non-normally distributed continuous variables. Chi-square was used for categorial variables and analysis of variance was used to assess for significance within groups, over time. $p<0.05$ was considered statistically significant.

Results

Lung oxygenation

Porcine lungs perfused with either KHB-Alb and CHIP demonstrated acceptable lung oxygenation as defined by a partial pressure of oxygen / fraction of inspired oxygen ratio (P:F Ratio) > 350 mmHg during EVLP. The groups did not differ significantly at T11 ($p>0.36$) (Figure 3.1B).

Pulmonary vascular resistance and pulmonary artery pressure

There was no statistically significant difference between PVR and PAP between lungs perfused with KHB-Alb and CHIP ($p>0.52$ and $p>1.0$, respectively). Both groups demonstrated a decline in PAP and PVR over time (Figure 3.1C-D).

Dynamic compliance

Both groups demonstrated a significant improvement over time. Lungs perfused with CHIP had a significantly higher dynamic compliance than lungs perfused with KHB-Alb at T11 (CHIP 38.79 ±3.53, KHB-Alb 29.25 ±2.31 [$p>0.04$]) (Figure 3.1A).

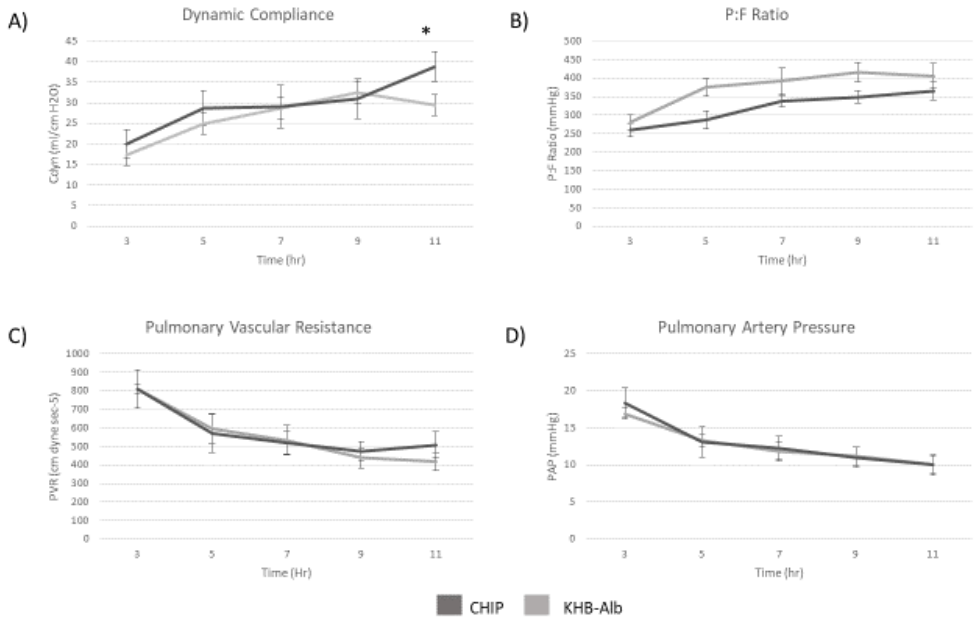

Figure 3.1. **There was no difference in lung physiology between perfusates.** Dynamic compliance, P:F ratio, pulmonary vascular resistance, and pulmonary artery pressure in porcine lungs (A,B,C,D). Results are shown at mean ± SE. Only dynamic compliance (A) was different at T11 between groups (*p<0.05).

Lung Injury

Edema formation as determined between by weight gain was similar between groups over the course of EVLP (CHIP 15.75 ± 4.75%, KHB-Alb 19.51 ± 4.37% [p>0.57]) (Figure 3.3). Histologic scores of lung injury including interstitial edema, hemorrhage, perivascular neutrophil infiltration, and interstitial neutrophil infiltration did not demonstrate a difference between groups (Figure 3.2).

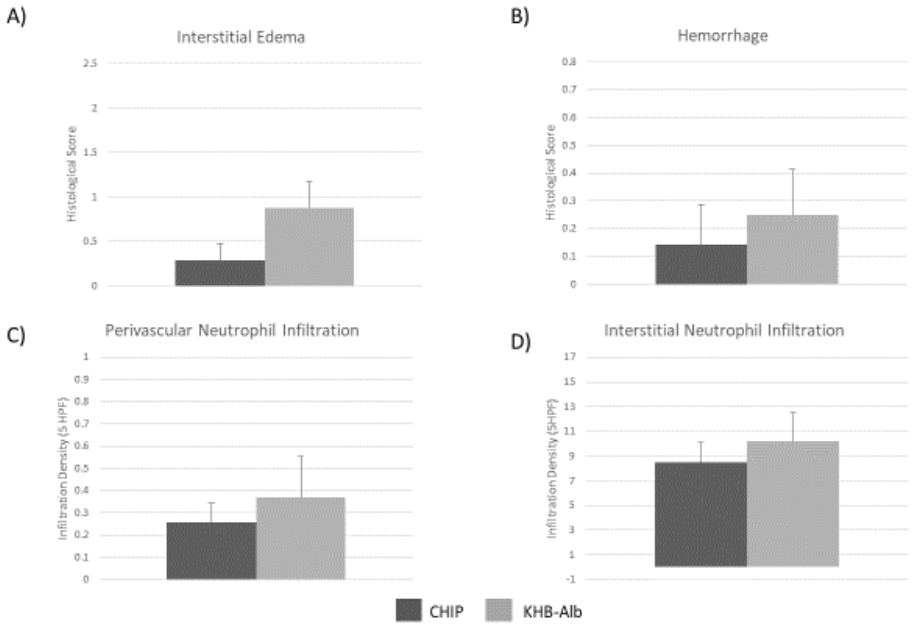

Figure 3.2. **Histologic assessment of lung injury was not different between perfusates.** Histological assessment of interstitial edema, hemorrhage, perivascular neutrophil infiltration, and interstitial neutrophil infiltration (A-D). Results are shown at mean ± SE. No difference was observed between groups ($p>0.05$).

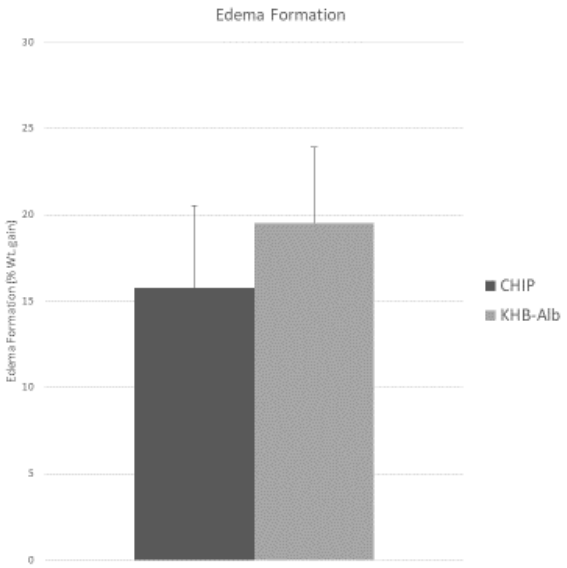

Figure 3.3. **Edema formation was not different between perfusates.** Edema formation as determined by percent weight gain. Results are shown at mean ± SE. No difference was observed between groups (p>0.05).

Inflammatory Cytokines

TNF-α was found to be similar between groups (CHIP: 445±65 pg/mL KHB-Alb: 327±60 pg/mL, p>0.20). IL-6 levels were also found to be similar between groups (CHIP 2859±343 pg/mL, KHB-Alb 2011 ± 344 pg/mL, p>0.10).

Discussion

Our findings suggest that common hospital ingredient derived perfusates can provide equivalent outcomes to current research standard perfusates. Equivalency was shown across multiple aspects, including functional, inflammatory, and histologic parameters.

Ultimately, the goal of preservation using EVLP is to mimic normal physiology. Correspondingly, the basis of the current commercially available perfusates, whether that be Steen solution[33] or OCS Lung Solution[35], is the basic electrolyte composition of human

plasma. This also formed the foundation of Krebs and Henseleit's buffer when they developed it in 1932[55]. Furthermore, the oncotic pressure of a perfusate needed to maintain normal endovascular-interstitial fluid gradients may be derived from either physiologic (albumin) or non-physiologic sources (dextran). The development of CHIP allows for an open source, low-cost, commercially available means of perfusate production that achieves both a physiologic electrolyte composition, as well as suitable oncotic pressure.

As put forth in the American Society of Transplant Surgeons Standards Committee White Paper, by Quintini *et al*, "proactive efforts in all aspects of donation and transplantation affected by ex situ machine perfusion will assure maximum benefits of this technology, minimize unintended consequences and improve access to a scarce and precious resource"[56]. Multiple trials have shown EVLP to be both safe and efficacious in reconditioning marginal lungs[8,57–59]. Despite increasing adoption, cost and availability remain a considerable hindrance to the development of EVLP. For example, a recent report analyzing the cost of the DEVELOP-UK trial showed an estimated overall increase in cost of $47 000 per run for EVLP compared to conventional cold static preservation[60]. In order to achieve widespread adoption of EVLP technology, significant reductions in cost are required before reaching conventional targets of cost-effectiveness.

Currently, the cost of CHIP is $310.72 per run (1.5L) and can be prepared by any hospital pharmacy. In comparison, 1.5L of Steen Solution (XVIVO Perfusion AB, Goteborg, Sweden) is $3885.00. The primary difference between Steen solution and KHB-Alb is the presence of dextran. Dextran has shown to improve lung preservation in cold static preservation[61,62] however, to date no trial has shown its benefit in normothermic perfusion.

Limitations

Our study is limited as it is a pre-clinical study. Further studies, including transplantation of both large animals and humans are required for validation of CHIP. As the source of albumin used in

the groups was from different species, there may be varying levels of cross-sensitization of porcine grafts.

Chapter 4: Negative pressure ventilation allows for successful *ex vivo* lung perfusion up to 24 hours.

Abstract

Negative pressure ventilation *ex vivo* lung perfusion (NPV-EVLP) has been shown to develop less ventilator-induced lung injury than positive pressure ventilation (PPV). We evaluated our current experience with 24-hour EVLP in both human and porcine models to assess its potential for applications requiring prolonged intervention and assessment times. Six porcine and four non-utilized human lungs were perfused using NPV-EVLP for 24 hours in a normothermic state. Physiologic parameters including oxygenation, pulmonary artery pressure, pulmonary vascular resistance, and dynamic compliance were assessed. Edema formation was also determined as a surrogate for lung injury. Both human and porcine lungs demonstrated stable and acceptable oxygenation (partial pressure of arterial oxygen/ fraction of inspired oxygen ratio >350 mmHg) up to 24 hours. Human lungs retained stable dynamic compliance and pulmonary vascular resistance over 24 hours with negligible weight gain (2.7 ± 10.6 % at 24 hours). Porcine lungs demonstrated continued improvement in compliance, pulmonary artery pressure, and pulmonary vascular resistance over the first 12 hours, followed by a stable phase, then a slow decline between hours 19 and 24. These factors contributed to a modest weight gain by 24 hours (38.74 ± 10.4 %) but did not affect oxygenation. Negative pressure ventilation allows for stable functional performance in *ex vivo* lung perfusion up to 24 hours in both human and porcine models.

Introduction

Pulmonary transplantation remains a critical therapeutic option for the management of end-stage lung disease. Despite this fact, organ utilization rates of available grafts remains low[12]. *Ex vivo* lung perfusion (EVLP) is a developing technology that has been used to evaluate and recondition marginal donor lungs, allowing for a potential increase in the overall donor pool[15,29].

In the current clinical spectrum, EVLP has repeatedly demonstrated utility in evaluating marginal lungs[4,6,8,59,63]. EVLP allows for evaluation of donor lungs outside of the inflammatory milieu of the whole donor body. By doing so, utilization rates have increased by as much as 15-20% in some centres[4,58]. EVLP's therapeutic benefit; however, remains more theoretical than a reality. Currently several therapeutic options have been assessed using EVLP. These include, but are not limited to thrombolytics, antibiotics, surfactant therapies, alpha-1-antitrypsin, and gene therapy[48,64–68]. Apart from occasional case reports, though, very few therapies have made the transition from the research realm to the clinical realm. The key limitation to this transition is time. These therapies need time to perform their intended action. Subsequently, more time is needed to ensure the treatment was successful and stable lung function is maintained. Contemporary clinical EVLP protocols are usually limited to six hours, while research protocols are usually limited to 12 hours[23,46,48]. Beyond these times frames, it is often difficult to assess whether declining lung function is a product of a failed intervention or the current limitations of EVLP. Our objective is to develop a stable platform for extended preservation so that the therapeutic potential of EVLP may be realized.

Methods

The experimental protocol was approved by the University of Alberta animal care and use committee. Care was performed in accordance with the "Principles of Laboratory Animal Care," as formulated by the National Society for Medical Research. Lungs from six female domestic pigs (46.7 ± 1.5 kg) were harvested and underwent prolonged *ex vivo* lung perfusion for 24 hours. All porcine lungs were perfused with 1.5L of common hospital ingredient derived perfusate mimicking STEEN solution and 0.5L of red blood cell concentrated (pRBC). The described volumes for the cellular groups provided a constant desired hemoglobin concentration of 40 to 50 g/liter. All solutions were prepared the day prior to each perfusion. The NPV-EVLP platform has been described in detail and was primed with 3.375g of Piperacillin-Tazobactam,

10 000 IU of Heparin, and 500 mg of methylprednisolone in addition to assigned perfusate[46]. Additionally, four non-utilized human lungs were perfused. Two of the runs used an acellular perfusate, one containing 1.5L of our previously mentioned homemade perfusate, while the other utilized 2L of STEEN solution™ (XVIVO Perfusion, Gothenburg, Sweden). Acellular EVLP was performed when donor hemodynamics did not allow for sufficient blood collection prior to pneumonectomy to concentrate and prime the NPV-EVLP circuit. The other two runs were perfused with 1.5L of STEEN solution and 0.5-1.0L of pRBC, titrated to achieve the previously described hemoglobin concentration.

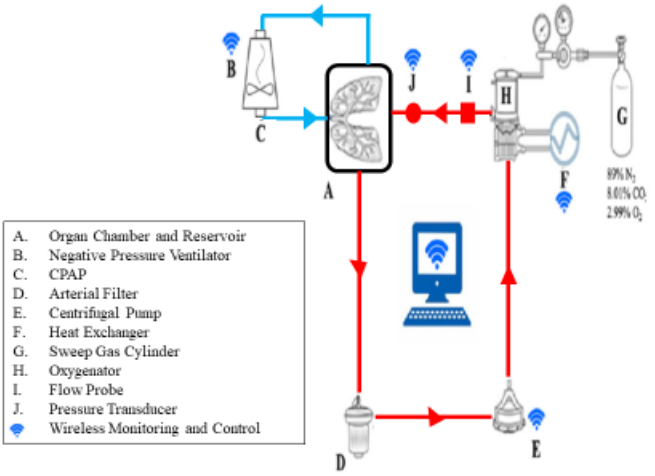

Figure 4.1. **Schematic representation of NPV-EVLP platform.** *Adapted from Aboelnazar NS, Himmat S, Hatami S, et al. Negative pressure ventilation decreases inflammation and lung edema during normothermic ex-vivo lung perfusion. J Hear Lung Transplant. 2018;37:520-530.*

Perfusate Composition

One litre of CHIP solution was comprised of 332mL of 25% Human Serum Albumen (Grifols, Toronto, Canada), 18mL of 8.4% Sodium Bicarbonate (Baxter, Mississauga, Canada), 2.3mL of

50% Dextrose (Baxter, Mississauga, Canada), 0.9mL of 1.29mmol/L Potassium Phosphate (Baxter, Mississauga, Canada), 639.3mL of Plasmalyte-A (Baxter, Mississauga, Canada), and 7.5mL of 100mg/mL Calcium Chloride (Omega, Montreal, Canada).

Red blood cell concentrates were attained by washing autologous whole blood collected during donor exsanguination with 1 litre of 0.9% saline and concentrating using a Sorin Xtra Cell Saver (Sorin Group Canada, Inc. Burnaby, BC, Canada).

Initiation of EVLP

Our protocol for lung procurement and initiation of NPV-EVLP has been described previously[46]. Induction of anesthesia was achieved with Ketamine (20 mg/kg), atropine sulfate (0.05 mg/kg), and xylazine (0.9mg/kg). Endotracheal intubation was performed. General anesthesia was maintained with 1-3% isoflurane. Upon satisfactory anesthesia, median sternotomy was performed. The animal was anticoagulated with 40 000 IU of heparin. A 2-stage venous cannula was inserted into the right atrium. The animal was then exsanguinated. Blood was collected into a cell saver (Sorin Xtra). The left atrial appendage was transected and cardiectomy was performed. The lungs were inflated to a constant pressure of 20 cm H_2O and the trachea was clamped. Bilateral pneumonectomy was performed. The lungs were then transferred to the NPV-EVLP device.

The pulmonary artery was then cannulated and connected to the EVLP circuit. Perfusion was initiated at 10% of predicted cardiac output (70 mL/kg) and 32C. The trachea was intubated and secured to a modified an endotracheal tube. Once the perfusate temperature had reached 32C, continuous positive airway pressure (CPAP) was applied at 20cm H_20. The trachea was then unclamped. Ventilation was initiated by incrementally decreasing the CPAP and increasing end inspiratory pressures (EIP) by 1cm H_20 every 2 minutes until a CPAP of 5cm H_20 and a transpulmonary gradient (TPG=Paw-EIP) of 18cm H_20 had been achieved. Perfusate flow was increased in increments of 10% predicted cardiac output every 20 minutes until a flow of 30%

cardiac output had been achieved. The perfusate temperature was gradually increased to achieve a temperature of 38C within 40 minutes of perfusion.

Preservation mode ventilation was performed as described in Table 2. Perfusate pCO2 was titrated to 35-50mmHg using a medical gas mixer (89% N2, 8% CO2, and 3% O2). Evaluation was performed every 2 hours. Upper end inspiratory pressures were increased to a peak transpulmonary gradient limits of 25cm H_20 or a peak tidal volume of 10mL/kg, whichever was achieved first.

Table 2. NPV-EVLP perfusion and ventilation strategy.

	Preservation	**Evaluation**
Temperature	38 C	38 C
Pulmonary Artery Flow	30% estimated CO (CO=70 mL/kg/min.)	50% estimated CO (CO=70 mL/kg/min.)
Ventilation Parameters		
Mode	Volume control	Volume control
Desired inspiratory tidal volume	8 mL/kg	10 mL/kg
Frequency	10 bpm	10 bpm
Inspiratory:Expiratory Ratio	1:1.5	1:1.5
Peak TPGi	<21 cm H20	<25 cm H20
PEEP	5 cm H20	5cm H20
FiO2	21%	21%
Pressure Parameters		
PAP	<25 mmHg	<25 mmHG
LAP	0 mmHg	0 mmHg

Organ Evaluation

Pulmonary artery pressure (PAP), pulmonary vascular resistance (PVR), dynamic compliance (Cdyn), and the ratio of partial pressure of oxygen in the pulmonary venous blood to the fraction of inspired oxygen (P:F Ratio) were measured during evaluation. Lungs were weighed immediately following pneumonectomy. At 12 hours, the trachea was clamped and the lungs were removed from the circuit and reweighed. They were then returned to the circuit. Following 24 hours of EVLP, they were once again reweighed. Edema, as described by percentage

weight gain over the course of the EVLP run was calculated as weight gain (%) = (End_{weight} – $Start_{weight}$ / $Start_{weight}$) x 100%. In porcine runs, biopsies were taken at T0, T12, and T24. Biopsies were fixed in 10% buffered formalin for 24 hours. They were then embedded in paraffin. 5 µm slices were obtained and stained with hematoxylin-eosin. A blinded pulmonary pathologist examined the samples using light microscopy. They were graded using an established scoring system for acute lung injury[41,54]

Statistics

All results are expressed as mean ± standard error. All analyses were performed on STATA 15 (StataCorp LLC, College Station, Texas). One-way ANOVA was used to compare trends over time. Variables that showed significant improvement during the early phase following reperfusion and recruitment, were subsequently re-assessed with a paired Student's t-test between T11 and T23 to assess for deterioration with prolonged perfusion. $p<0.05$ was considered statistically significant.

Results

Lung oxygenation

P:F Ratio was defined as the ratio of partial pressure of oxygen in the perfusate compared to the fraction of inspired oxygen (P:F Ratio = PaO_2/FIO_2). Both human and porcine lungs demonstrated consistent and stable oxygenation over 24 hours (Figure 4.2A, p=0.99 and Figure 4.3A, p=0.97, respectively). Furthermore, there was no difference in P:F ratios between T11 and T23 in either human or porcine lungs. In humans, the P:F ration was 435 ± 44 mmHg at T11, and 427 ± 50 mmHg at T24 (p=0.68). In porcine lungs, the average P:F Ratio at T11 was 410 ± 45 mmHg. At 24 hours, the average P:F Ratio was 397± 64 mmHg (p=0.88).

Dynamic compliance

Human lungs retained stable compliance over 24 hours (Figure 4.2B, p=0.99). Porcine lung compliance; however, showed significant variation over time (p=0.01). Dynamic compliance improved over the first twelve hours as the lungs were slowly recruited. They then reached a stable plateau phase, which was followed by declining function between hours 19 and 24 (Figure 4.3B). At hour 11 the dynamic compliance was 39.5 ± 4.2 mL/cm H_2O, while at hour 23 the compliance was 31.9 ± 4.5 mL/cm H_2O (p=0.25).

Pulmonary vascular resistance and pulmonary artery pressure

In human EVLP runs PVR remained relatively stable from hour 11 to 23 (Figure 4.2C, p0.42). The PVR at T11 in human lungs was 334 ± 21 dynseccm^{-5} compared to 419 ±107 dynseccm^{-5} at T23. In porcine lungs, the PAP and PVR followed an inverse relationship to the dynamic compliance, over time. Pulmonary artery pressure (PAP) decreased slowly over the first half of the perfusion, reaching a nadir at T11. The PAP then increased slowly from hour 11 to hour 23. Pulmonary vascular resistance showed a similar trend. The changes in PAP and PVR over time, though, were not significant (p=0.19 and p=0.56, respectively).

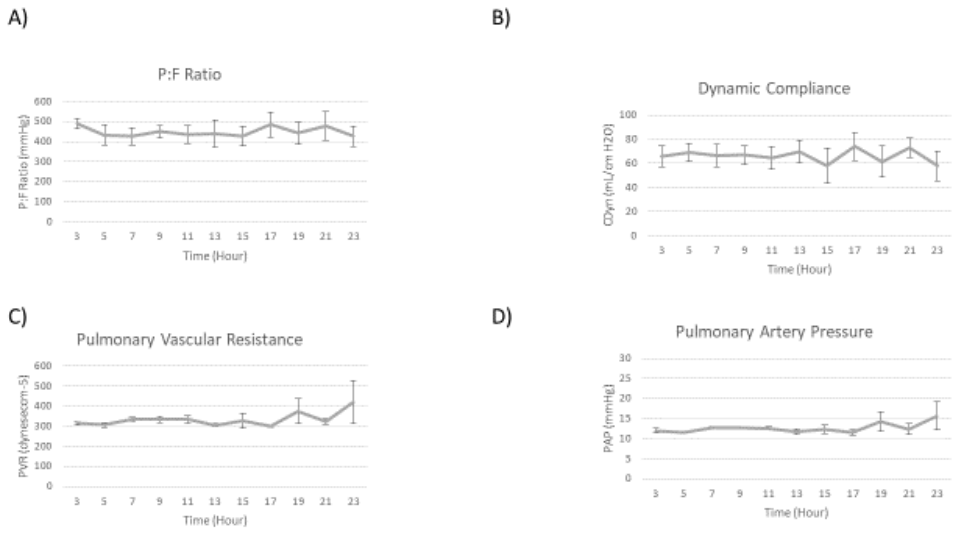

Figure 4.2. **Human lungs demonstrate stable lung physiology over 24 hours.** P:F ratio (A), dynamic compliance (B), pulmonary vascular resistance (C), and pulmonary artery pressure (D) over time. Results are shown as mean ± SE.

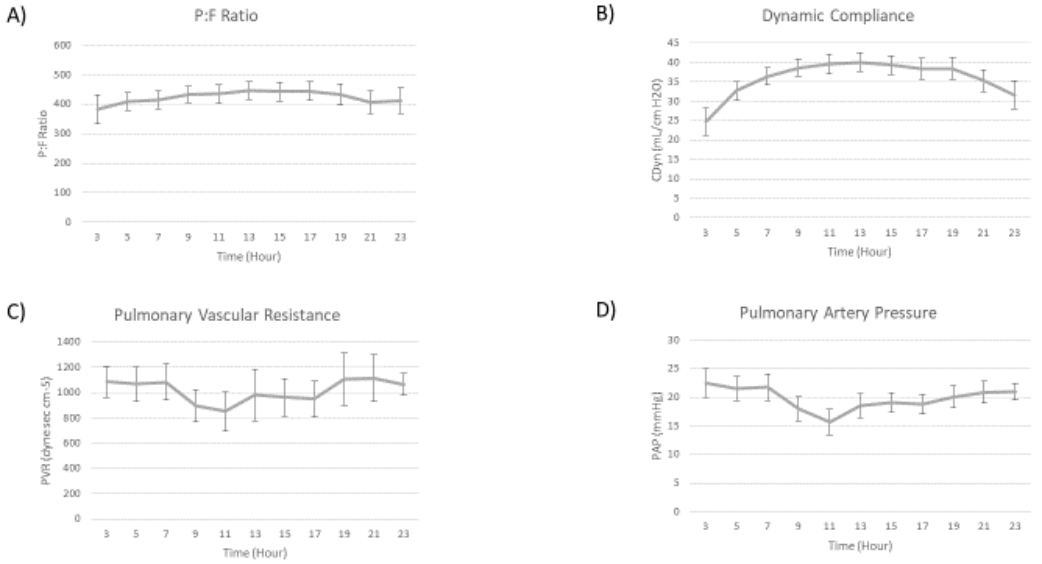

Figure 4.3. **Porcine lung physiology demonstrated improvement over the first 12 hours, followed by a stable plateau phase, and ended with a slow decline over the final 6 hours.** Porcine P:F ratio (A), dynamic compliance (B), pulmonary vascular resistance (C) and pulmonary artery pressure (D) over time. Results are shown as mean ± SE.

Edema Formation

In human EVLP, there was weight loss in all cases at 12 hours (-16.0 ± 4.5%). By the end of the run, edema formation was negligible compared to pre-EVLP weights (2.7 ± 10.6%). The difference in weights between T12 and T24 was found to be non-significant (p=0.09). Porcine lung weight increased over the course of EVLP. Once again, the difference in weight gain between the first and second half of the perfusion was not significantly different (T12 13.1 ± 5.9%, T24 31.8 ± 13.1%, p=0.23).

Histopathology

Histologic scores of lung injury including interstitial edema, hemorrhage, perivascular neutrophil infiltration, and interstitial neutrophil infiltration did not demonstrate a difference between T0, T12, and T24 (Figure 4.4). The presence of low levels of histological evidence of acute lung

injury at T0, demonstrates that minor amounts of lung injury occurred during procurement; however, this did not worsen during the period of EVLP.

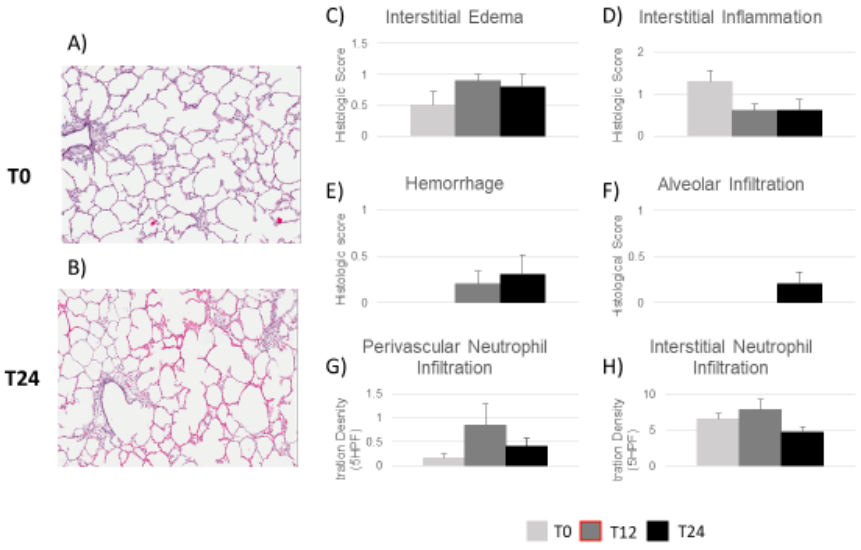

Figure 4.4. **Porcine lungs do not show any histologic evidence of increased lung injury over 24 hours of NPV-EVLP compared to baseline.** Representative photomicrographs of lung tissue at 20x magnification hematoxylin-eosin (H&E) staining prior to (A) and after 24 hours (B) of EVLP and corresponding histopathologic lung injury scores. Assessment included interstitial edema, interstitial inflammation, alveolar inflammation, hemorrhage, perivascular neutrophil infiltration, and interstitial neutrophil infiltration (C-H) at 24 hours of EVLP. Results are shown as mean ± SE. No difference was observed between groups ($p>0.05$).

Discussion

Machine perfusion has been labelled as the “most important advancement in transplant technologies since the development of immunosuppression”[56]. Optimized machine preservation has the potential to remove geographical constraints to transplantation, improve matching for disadvantaged patients, increase organ quality through various interventions, improve disease screening, induce immune tolerance, as well as a plethora of other possibilities[69]. Significant advancements across multiple fields are needed, though, before

the potential of EVLP is fully realized. Integral to the development of all these domains is the aspect of time. Stable, prolonged preservation with normal function is the imperative first step to it all.

Ex vivo machine perfusion carries a significant number of limitations, compared to whole body perfusion. One key limitation involves the aspect of organ repair, both at a cellular level as well as at a tissue level. Efforts are being made at novel therapeutics to mediate ischemic-reperfusion injury processes[70–73]. Macroscopic mechanisms of tissue injury, though, are often overlooked in the literature. For example, perfusates are typically void of platelets and clotting factors in order to prevent coagulation within the circuit. Additionally, circuits are often further anticoagulated with heparin. Any vascular injury, whether from traction injuries or tissue handling during surgical removal, often lack sufficient mediators for prompt and effective repair and containment. Hematoma expansion and tissue involvement may be much more pronounced than *in vivo*. Therefore, the less damage done initially, the better the final product. Similar principles can also be applied to barotrauma. Negative pressure ventilation has been associated with a decrease in ventilator induced lung injury[46,74]. Our previous work highlighted improvements over positive pressure ventilation across multiple domains of lung assessment including inflammatory cytokine parameters, histopathologic assessment of lung injury, and gross bullae formation[46]. Our current study demonstrates that by decreasing lung injury, we can extend the safe preservation time up to 24 hours, especially in human lungs.

Currently there are limited reports of successful EVLP out to 24 hours. The most noted of these works involves the Organ Care System (OCS) Lung System and positive pressure ventilation[34]. Loor *et al* was able to demonstrate acceptable functional performance out to 24 hours. The principle difference between this study and the study by Loor *et al* is the lack of dependency on the use of whole blood, which is often difficult to obtain in a clinical setting. The authors found that a perfusate comprised of OCS Lung solution and whole blood allowed for

stable compliance, oxygenation, and perfusion pressures over 24 hours in a porcine model. Lungs perfused with OCS Lung Solution and packed red blood cells demonstrated acceptable performance over the first 12 hours but developed profound pulmonary edema by hour 24 with negligible oxygenation. This supports the current OCS clinical guidelines for short term perfusion as an assessment and transport tool. However, if a clinical model is to be expanded to other applications requiring longer perfusion, the necessity on whole blood limits translation.

Other methods, such as prolonged cold static storage, followed by a period of normothermic machine perfusion have also been reported to allow for increased safe organ preservation[75]. The drawback of this method is that further interventions often cannot be performed in the non-circulating, cold static state, and therefore a period of prolonged normothermic perfusion is still required if an EVLP platform is to fill an interventional role. For example, Andreasson *et al* showed that an EVLP circuit, primed with high dose broad spectrum antimicrobials was capable of significantly decreasing bacterial loads from 6,900 cfu/mL to 400 cfu/mL after 3-6 hours of EVLP[68]. This ultimately leads to questions such as: how long does it take to achieve perfusate sterility?

Limitations

The study in question demonstrates acceptable functional outcomes in both porcine and human models up to 24 hours. A variety of perfusate compositions were used between human and porcine runs. Further analysis, assessing CHIP vs. STEEN solution is needed to determine the individual effects of these subgroups.

Chapter 5: Continuous hemodialysis does not improve graft function during *ex vivo* lung perfusion over 24 hours.

Accepted

Transplantation Proceedings

2019

Abstract

Extended periods of *ex vivo* lung perfusion (EVLP) lead to several inadvertent consequences including accumulation of lactate and increasing electrolyte concentrations in the perfusate. We sought to determine whether continuous hemodialysis (CHD) of the perfusate would be a suitable modality for improving ionic homeostasis in extended EVLP without compromising functional outcomes. Twelve porcine lungs were perfused using EVLP for 24 hours. Lungs in the treatment group (n=6) underwent continuous hemodialysis of the perfusate. Functional parameters, edema formation and histopathologic analysis were used to assess graft function. Electrolyte and lactate profiles were also followed to assess the efficiency of hemodialysis. Lungs in both treatment and control groups demonstrated stable and acceptable oxygenation to 24 hours. Lungs demonstrated a decrease in compliance over time. There was no difference in oxygenation and compliance between groups. CHD-EVLP lungs had higher pulmonary vascular resistance and pulmonary artery pressures. Despite increased perfusion pressures, weight gain at both 11 and 23 hours was not different between groups. Perfusate sodium and lactate concentrations were significantly lower in the CHD-EVLP group. The addition of continuous hemodialysis to EVLP did not improve graft function up to 24 hours despite improved maintenance of perfusate composition.

Introduction

Ex vivo lung perfusion (EVLP) is a developing technology aimed at evaluating and reconditioning donor lungs. EVLP also provides a theoretical platform for the resuscitation of dysfunctional lungs, thereby increasing the potential donor pool. To increase the therapeutic potential of EVLP, there is need to optimize organ preservation techniques and better mimic the *in vivo* environment.

Negative pressure ventilation (NPV) has been shown to reduce ventilator induced lung injury (VILI) in EVLP[46,76]. Multiples domains of lung injury assessment, including pro-inflammatory cytokine production, bullae formation, and pulmonary edema, were all reduced with NPV compared to positive pressure ventilation (PPV). To extend the length of preservation, an NPV-EVLP platform was, therefore, selected for the model.

For EVLP to be an effective tool, whether diagnostic or interventional, each platform must be able to provide stable function for a prolonged period. As our interventional capabilities increase, the time needed on the circuit will also increase. Time is needed to allow for both the intervention to take effect, as well as to evaluate the organ post-intervention. It has been observed during prolonged machine perfusion, that concentrations of key electrolytes and metabolites such as calcium, potassium, sodium, and lactate increase slowly over time[48–50]. Acellular systems may undergo simple perfusate exchange to correct electrolyte abnormalities; however, definitive evidence supporting the benefit of costly perfusate exchange, has yet to be described. Cellular-based perfusates, though, require a membrane-exchange system for electrolyte correction, if they are to maintain a desired perfusate hemoglobin concentration. We, therefore, sought to develop an easily translatable model to maintain perfusate composition during prolonged EVLP in a cellular-based model.

Methods

The experimental protocol was approved by the University of Alberta animal care and use committee. Care was performed in accordance with the "Principles of Laboratory Animal Care," as formulated by the National Society for Medical Research. Lungs from twelve female domestic pigs (43.8 ± 5.3kg) were recovered and underwent prolonged *ex vivo* lung perfusion for 24 hours. Lungs were allocated equally to two groups: one group receiving continuous hemodialysis (CHD) versus control. CHD lungs had a hemoconcentrator incorporated into the circuit to allow for continuous hemodialysis of the perfusate (Figure 5.1). All porcine lungs were perfused with 1.5L of a low-cost, common hospital ingredient derived perfusate mimicking STEEN solution and 0.5L of red blood cell concentrate (pRBC). The dialysate was comprised of the same components as the perfusate, without albumin, and adjusted to maintain physiologic concentrations in the absence of albumin. The described volumes provided a hemoglobin concentration of 40 to 50 g/liter. Piperacillin-Tazobactam (3.375g), Heparin (10 000IU), and methylprednisolone (500mg) were also added to the perfusate. All solutions were prepared the day prior to each perfusion.

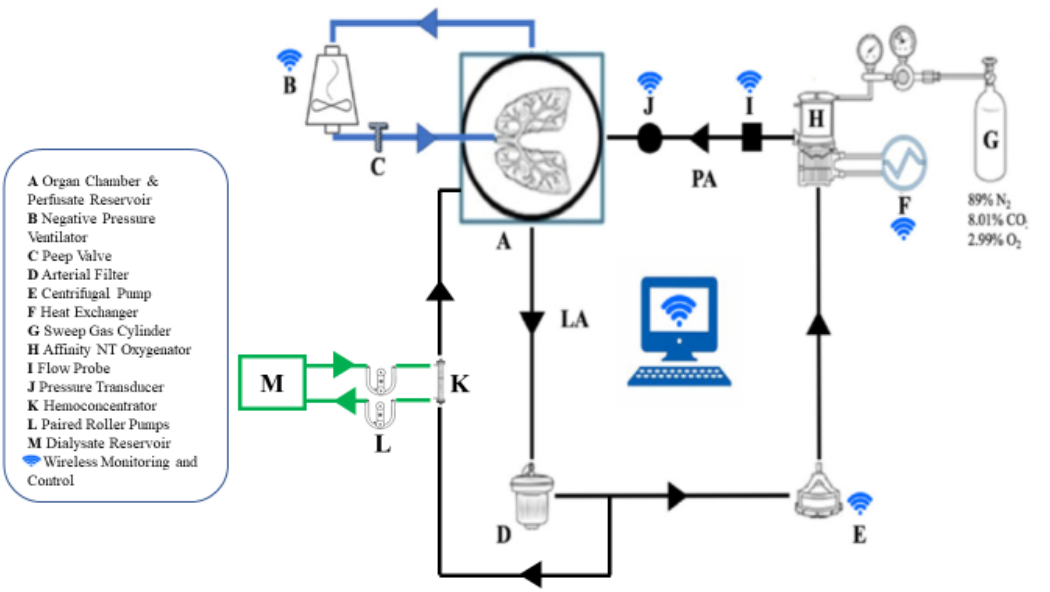

Figure 5.1. **Schematic representation of NPV-EVLP platform with incorporated dialysis circuit.** *Adapted from Aboelnazar NS, Himmat S, Hatami S, et al. Negative pressure ventilation decreases inflammation and lung edema during normothermic ex-vivo lung perfusion. J Hear Lung Transplant. 2018;37:520-530.*

Perfusate and dialysate solutions

Each litre of CHIP was produced from 332mL of 25% Human Serum Albumin (Grifols, Toronto, Canada), 18mL of 8.4% Sodium Bicarbonate (Baxter, Mississauga, Canada), 2.3mL of 50% Dextrose (Baxter, Mississauga, Canada), 0.9mL of 1.29mmol/L Potassium Phosphate (Baxter, Mississauga, Canada), 639.3mL of Plasmalyte-A (Baxter, Mississauga, Canada), and 7.5mL of 100mg/mL Calcium Chloride (Omega, Montreal, Canada).

Each litre of dialysate was made from 37.8mL of sterile water, 938mL of Plasmalyte-A, 0.9mL of 1.29mmol/L Potassium Phosphate, 2.3mL of 50% Dextrose, 18mL of sodium bicarbonate, and 3 mL of 100mg/mL calcium chloride.

Whole blood was collected during exsanguination and washed with 0.9% saline and concentrated with a Sorin Xtra Cell Saver (Sorin Group Canada Inc., Burnaby, Canada) to obtain red cell concentrates.

Initiation of EVLP

Our protocol for lung procurement and initiation of NPV-EVLP has been described previously[46]. In summary, induction of anesthesia was achieved with Ketamine (20mg/kg), atropine sulfate (0.05mg/kg), and xylazine (0.9mg/kg). The animal was intubated, and general anesthesia was maintained with 1-3% isoflurane. Median sternotomy was performed. Heparin (40 000IU) was given and in vivo wedge biopsies were taken. The animal was then exsanguinated into the cell saver via a 2-stage venous cannula inserted into the right atrium. The left atrial appendage was transected and cardiectomy was performed. The trachea was clamped at a constant airway pressure of 20cmH_2O just above the carina. Bilateral pneumonectomy was then performed, and the lungs were transferred to the NPV-EVLP chamber.

T0 biopsies were taken immediately prior to reperfusion. The pulmonary artery was cannulated. The trachea extending beyond the clamp was intubated and secured to a modified endotracheal tube. Reperfusion was initiated at 10% of cardiac output (70mL/kg) and the perfusate was warmed to 32C. Upon reaching a temperature of 32C, the ventilator's continuous positive airway pressure (CPAP) was set to 20cmH_20. The trachea was then unclamped preventing decruitment during the initiation of EVLP.

CPAP and end inspiratory pressures (EIP) were decreased by 1cmH_2O every 2 minutes until both a CPAP of 5cmH_20 and a transpulmonary gradient (TPG=Paw-EIP) 20cmH_2O was reached. Perfusate flow was increased by 10% of predicted cardiac output ever 10 minutes until the flow reached 30%. The perfusate temperature was slowly increased to reach a temperature of 38C within 30 minutes of the initiation of perfusion. For the runs including

hemodialysis, a Maquet BC20 (Rastatt, Germany) hemoconcentrator with fibre surface area of 0.22m^2 was incorporated into the circuit. 2.0L of dialysate was continuously recirculated at 1-1.5 L/hour using a roller pump (Masterflex, Barrington, Illinois) with matched inflow and outflow rates to allow for isovolumic diffusion of small molecules from the perfusate to the dialysate. The dialysate reservoir was drained and exchanged for fresh dialysate every six hours. Perfusate flow through the hemoconcentrator was driven passively by the pressure gradient between pulmonary artery line and the chamber pressure. Specifications for preservation and evaluation mode ventilation are described in Table 1. A medical gas mixer (89% N2, 8% CO2, and 3% O2) was used to target perfusate pCO2 from 35-50 mmHg. Insulin (2IU/100g lung wt/hr) was infused continually. A dextrose (50%) infusion was titrated to maintain perfusate glucose concentrations of 5-10 mmol/L. Evaluation was performed for 5 minutes, every 2 hours.

Organ Evaluation

Physiologic parameters including pulmonary artery pressure (PAP), pulmonary vascular resistance (PVR), dynamic compliance (Cdyn), and the ratio of partial pressure of oxygen in the left atrial blood to the fraction of inspired oxygen (P:F Ratio) were continually recorded. Prior to initiation of EVLP, the lungs were weighed. The lungs were clamped at peak inflation, disconnected from the circuit, and reweighed at T12. Biopsies were taken and reperfusion was initiated. After 24 hours of EVLP, a final set of biopsies were performed, and a final weight was taken. Percentage weight gain [weight gain (%) = (End_{weight} – $Start_{weight}$ / $Start_{weight}$) x 100%] was used as a surrogate for edema formation.

Electrolytes, Cytokine Analysis, and Histopathology

Perfusate electrolytes and lactate were followed every two hours using an ABL800 blood gas analyzer (Radiometer, California, USA). Blood gas analysis of the dialysate was also performed before and after each 6-hour period of CHD. Enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, USA) were used to determine perfusate cytokine concentrations every 2-

hours for tumor necrosis-α (TNF-α) and interleukin-6 (IL-6) and interleukin-8 (IL-8). Dialysate samples taken immediately prior to exchange were also analyzed for the above-mentioned cytokines. Biopsies were fixed in 10% buffered formalin for 24 hours, embedded in paraffin and sliced to a thickness of 5 μm. Segments were stained with hematoxylin-eosin. A blinded pulmonary pathologist graded the specimens using an established score for acute lung injury[41,54].

Statistics

All results are expressed as mean ± standard error. All analyses were performed on STATA 15 (StataCorp LLC, College Station, Texas). Shapiro-Wilk test was used to assess normality. Student's t-test was used to compare normally distributed continuous variables. Wilcoxon rank-sum test was used to compare non-normally distributed continuous variables. Repeated measures analysis of variance was used to assess for significance within groups. $p<0.05$ was considered statistically significant.

Results

Lung oxygenation

In the control group, the average P:F Ratio at 11 hours was 410 ± 45mmHg. In the dialysis group, the P:F ratio was 451 ± 39mmHg. By 24 hours of EVLP, oxygenation was relatively unchanged (noCHD 397 ± 64mmHg, CHD 416 ± 53mmHg)(Figure 5.2B). No difference was detected between groups (p=0.60).

Physiologic Parameters: Dynamic Compliance, Pulmonary Vascular Resistance and Pulmonary Artery Pressures

The PAP and PVR demonstrated similar trends over time in both groups; however, the PAP and PVR were significantly higher in the CHD group than control (p=0.05 and p=0.04, respectively). The PAP and PVR improved over the first half of the perfusion, reaching a nadir around T11.

The PAP at T11 was 12.3 ± 1.6mmHg in the control group and 22.4 ± 4.3mmHg in the CHD group. PAP and PVR, then continued to increase over the second half of the perfusion. By T23, the PAP was 19.9 ± 1.9mmHg in the control group and 25.1 ± 3.3mmHg in the CHD group. The PVR showed similar trends (Figure 5.2C). By the end of the run, PVR was significantly higher in the CHD group (noCHD 896 ± 100dyneseccm^{-5}, CHD 1592 ± 335dyneseccm^{-5}).

There was no difference in dynamic compliance (Cdyn) between groups (p=0.95). At T11 the compliance in the control group was 39.5 ± 4.2 mL/cm H_2O in the control group and 38.4 ± 2.6mL/cmH_2O in the CHD group. By T23 the compliance was 31.9 ± 4.5mL/cmH_2O and 30.2 ± 5.3 mL/cmH_2O in the CHD group. Lungs in both groups exhibited a significant change in compliance over time (p<0.001)(Figure 5.2A).

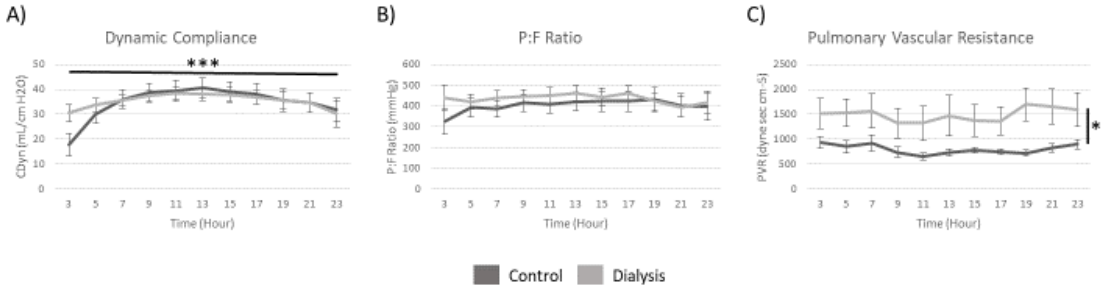

Figure 5.2. **CHD does not improve lung physiology during extended EVLP.** Lungs in both groups exhibited a significant change in compliance over time. Dynamic compliance (A), pulmonary vascular resistance (B), P:F Ratio (C) over time. Results are displayed as mean ± SE. *p<0.05 and ***p<0.001 between groups.

Electrolytes and Metabolites

The concentration of electrolytes increased over time in the control group. For example, sodium had increased to 164.0 ± 2.4mmol/L in the control group at T23. In comparison, sodium slightly decreased initially in the CHD group, until reaching a stable equilibrium with the dialysate bath by T11 (Figure 5.3B). This led to a significant difference in perfusate sodium concentration by the end of the run ($p<0.001$). Lactate increased over time in both groups; however, the rate of increase was significantly higher in the control group (Figure 5.3A), leading to significantly higher perfusate lactate concentrations at T23 (noCHD 12.8 ± 1.5 mmol/L, CHD 9.1 ± 0.5 mmol/L, $p=0.01$). Blood gas analysis of the dialysate showed that electrolyte and lactate concentrations had fully equilibrated with the perfusate by the end of 6 hours of CHD, prior to dialysate exchange.

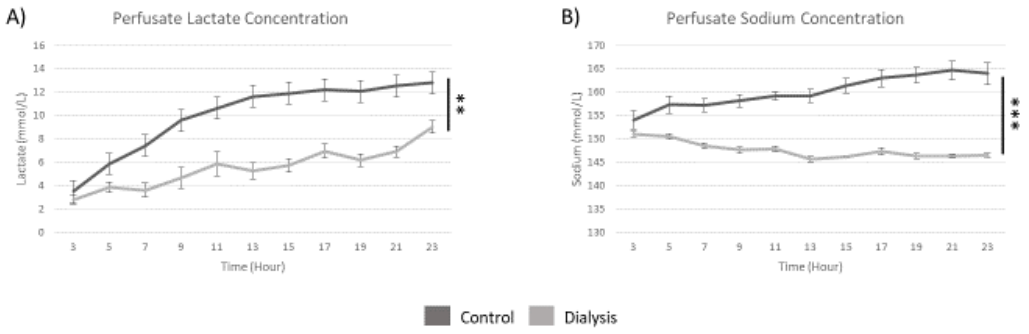

Figure 5.3. **CHD does maintain perfusate electrolyte composition over time.** Perfusate lactate concentration (A) and perfusate sodium concentration (B) over time. Results are displayed as mean ± SE. **p<0.01 and ***p<0.001 between groups.

Cytokine Profile

TNF-α levels increased over the first 5 hours of perfusion then began to decline (Figure 5.4A). TNF-α levels rose faster in the dialysis group with higher values at T1 (noCHD 683 ± 172 pg/mL, CHD 1413 ± 303 pg/mL). TNF-α levels plateaued at a slightly higher level by T5 (noCHD 1221 ± 248 pg/mL, CHD 1601 ± 167); however, ultimately the difference between the two groups did not reach significance over time(p=0.86). Dialysate TNF-α concentrations were highest at the first dialysate exchange and continued to decrease over time (Figure 5.4D).

IL-6 levels did not demonstrate a significant difference between groups throughout the run (0.98)(Figure 5.4B). At T11, perfusate IL-6 concentration was 1842 ± 459 pg/mL in the control group and 2194 ± 496 pg/mL in the CHD group. At T23 IL-6 concentrations had reached 3787 ± 781 pg/mL in the control group and 2962 ± 936 pg/mL in the CHD group. Unexpectedly there was a drop in IL-6 concentrations between T11 and T17, then a substantial rise by T23. IL-6 was undetectable in the dialysate.

Perfusate concentrations of IL-8 remained low in the perfusate of both groups up to T17, then rose sharply to T24 (Figure 5.4C). At T11, the perfusate concentration was 79.7 ± 42.8 pg/mL in the control group and 164.3 ± 113.2 in the CHD group. By T23, it has risen to 2515 ± 887 in the control group and 3019 ± 1142 in the CHD group. The difference between groups was not significant (p0.63). Clearance of IL-8 with dialysis was relatively inefficient. The dialysate concentration at the end of the final exchange was only 10.4% of the total IL-8 concentration.

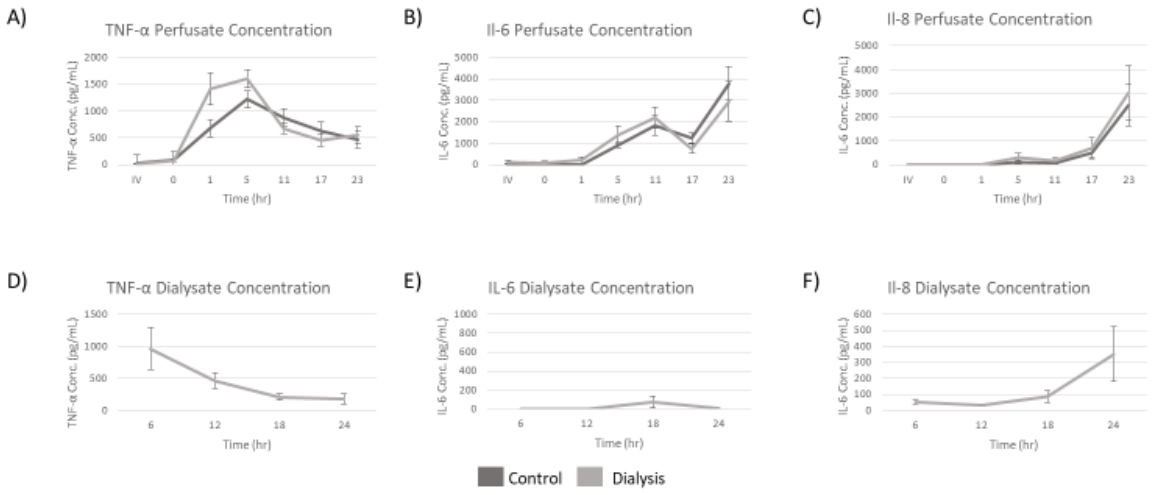

Figure 5.4. **CHD does not decrease pro-inflammatory cytokine perfusate concentrations during extended EVLP.** Perfusate TNF-α, IL-6, and IL-8 concentrations over time (A-C). Dialysate concentrations of TNF-α, IL-6, and IL-8 are also shown (D-F). Results are shown as mean ± SE. A difference in perfusate cytokine concentrations was not observed (p>0.05).

Lung Injury

There was no difference in edema formation between groups (p0.78)(Figure 5.6). By T12, the weight in the control group had increased by 13.1 ± 5.9% and the CHD group had increased by 12.0 ± 3.2%. By the end of the run, lung weight had increased by 31.8 ± 13% in the control group and 39.9 ± 13.1% in the CHD group.

Histopathologic scoring assessed multiple markers of lung injury including interstitial inflammation, interstitial edema, hemorrhage, perivascular neutrophil infiltration, and interstitial neutrophil infiltration. No difference was observed between groups (p>0.05)(Figure 5.5).

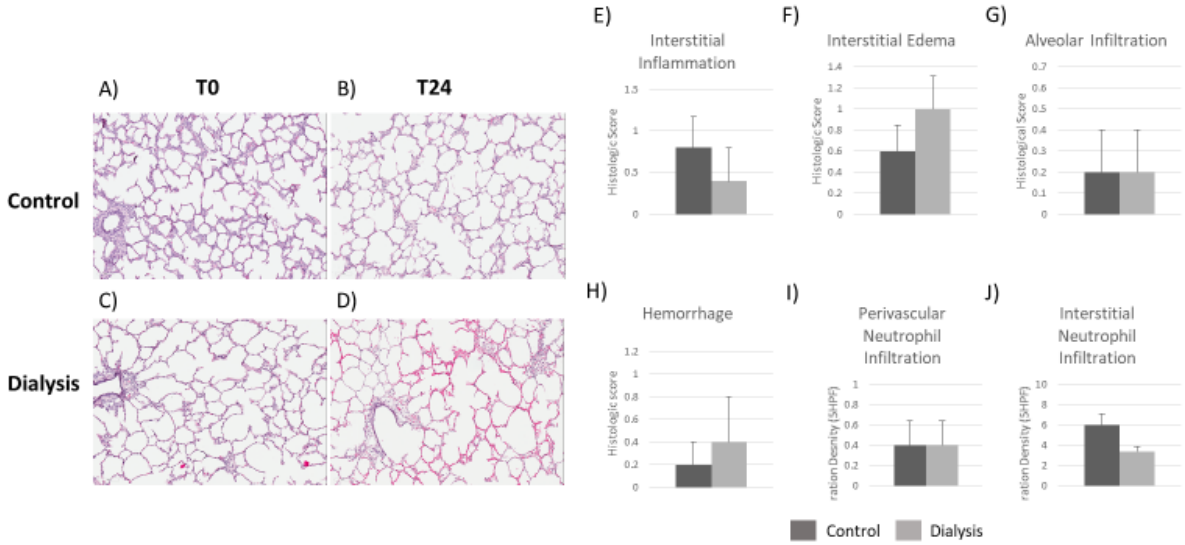

Figure 5.5. **CHD does not reduce histopathologic findings of acute lung injury over time.** Representative photomicrographs of lung tissue at 20x magnification hematoxylin-eosin (H&E) staining prior to (A,C) and after 24 hours (B,D) of EVLP and corresponding histopathologic lung injury scores. Assessment included interstitial edema, interstitial inflammation, alveolar inflammation, hemorrhage, perivascular neutrophil infiltration, and interstitial neutrophil infiltration (E-J) at 24 hours of EVLP. Results are shown as mean ± SE. No difference was observed between groups ($p>0.05$).

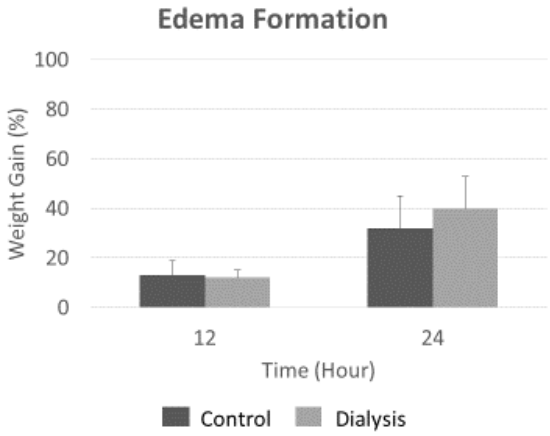

Figure 5.6. **CHD does not affect edema formation.** Edema formation at 12 and 24 hours of NPV-EVLP. Results are shown as mean ± SE. No difference was observed between groups ($p>0.05$).

Discussion

Currently, there are several EVLP platforms with varying methodologies in clinical practice. For example, the XVIVO Perfusion System (XVIVO Perfusion AB, Goteborg, Sweden) typically uses the Toronto Protocol, while the Vivoline LS1 System (Vivoline Medical, Lund, Sweden) uses the Lund Protocol[3,6,15,77]. The Organ Care System Lung (OCS)(Transmedics, Andover MA) uses the OCS Lung Protocol[78,79]. Both the Vivoline LS1 System and the OCS Lung System have shown improved extended preservation with red blood cell based perfusates[34,38].

Our study demonstrates that dialysis with cellular perfusates in machine perfusion can be straightforward, efficient and relatively low-cost. The use of hemodialysis maintained normal perfusate electrolyte concentrations throughout the run. Lactate was also removed from the perfusate leading to a reduction in perfusate lactate at 24 hours.

Despite improved electrolyte homeostasis and a reduction in surrogate markers such as lactate, functional outcomes were not significantly affected by hemodialysis. Pulmonary artery pressures and pulmonary vascular resistance were higher in the dialysis group. Although hemodialysis removes uremic wastes and regulates electrolyte concentrations, it is also capable of affecting several other small molecules, such as nitric oxide and endothelin-1, whose vasoactive effects are capable of changing the equilibrium between vasoconstriction and vasodilation[80,81]. Despite higher perfusion pressures, oxygenation and edema formation were unaffected. Although there was a statistically significant increase in PVR by 24 hours, this may not be clinically significant, at least over 24 hours of EVLP.

The results of this study are consistent with prior studies in the ICU literature looking at inflammatory cytokine clearance by dialysis and ultrafiltration. Van Bommel *et al* demonstrated that cytokine clearance was often from cytokine adherence to the membrane itself, and only when the membrane was saturated, does small amounts of filtration occur[82]. Kellum *et al* showed in septic patients that continuous hemodialysis does not significantly affect circulating levels of inflammatory cytokines[83]. Similar results were shown by Sanchez-Izquierdo *et al* using hemofiltration in trauma patients[84]. Furthermore, they noted that, although, substantial amounts of TNF-α were cleared in the ultrafiltrate, TNF-α levels in plasma still matched control patients.

It is also important to note that compliance is relatively stable over the initial portion of the experiment but begins to decline by hour 18 of EVLP in both groups (see Figure 2A). Previous works have established that a decline in compliance generally precedes a deterioration in oxygenation[26,27]. This study supports that neither uremic metabolite accumulation, nor electrolyte imbalance is the primary driver of the deterioration in compliance over 24 hours of EVLP. Further studies looking at other mechanisms for failure such as metabolism or immunomodulation are needed to extend perfusion significantly beyond 24 hours. For example,

Iskender *et al* showed that cytokine filtration with a CytoSorb membrane significantly improved graft quality over 12 hours of EVLP; however, by the end of the run, perfusate sodium concentrations were significantly above physiologic levels and continuing to climb[49].

Overall, our results do not support significant functional benefit of dialysis during EVLP in the first 24 hours; however, the lack of harm caused by dialysis enables future therapeutic possibilities such as organ banking of lungs. As our capabilities for organ perfusion continue to progress beyond a state where critical metabolite thresholds have been reached, hemodialysis is an effective tool for restoring electrochemical gradients in EVLP.

Limitations

One of the limitations of our study was the use of hemodialysis instead of hemofiltration, which may improve cytokine removal[83]. Prior studies utilizing rapid ultrafiltration, with the aim of significantly increasing oncotic pressure in short-term (< 3 hours) EVLP in order to resuscitate edematous lungs have shown modest results[85]. Hemodialysis; however, was chosen over hemofiltration in our model to avoid fluxes in oncotic pressure that might alter lung fluid balance in prolonged EVLP as ultrafiltrate is removed and replacement solution is added. To ascertain the effect of hemodialysis, itself, and limit the effects other solutes contained within conventional dialysates, we chose a model with a dialysate modelled on the original perfusate. A hemoconcentrator membrane was used for hemodialysis as these are widely available in cardiothoracic surgical suites, where EVLP is typically performed, allowing for easier clinical translation.

Chapter 6: Total parenteral nutrition in *ex vivo* lung perfusion: addressing metabolism improves both inflammation and oxygenation.

Abstract

Ex vivo lung perfusion (EVLP) protocols generally limit metabolic supplementation to insulin and glucose. We sought to determine whether the addition of total parenteral nutrition (TPN) would improve lung function in EVLP. Ten porcine lungs were perfused using EVLP for 24 hours and supplemented with insulin and glucose. In the treatment group (n=5), the perfusate was also supplemented with a continuous infusion of TPN containing lipids, amino acids, essential vitamins and cofactors. Physiologic parameters and perfusate electrolytes were continuously evaluated. Perfusate lactate, lipid and branch chain amino acid (BCAA) concentrations were also analyzed to elucidate how substrates were being utilized over time. Lungs in the TPN group exhibited significantly better oxygenation. Perfusate sodium was more stable in the TPN group. In the control group, free fatty acids (FFA) were quickly depleted, reaching negligible levels early in the perfusion. Alternatively, BCAA in the control group rose continually over the perfusion demonstrating a shift towards proteolysis for energy substrate. In the TPN group, both FFA and BCAA concentrations remained stable at *in vivo* levels after initial stabilization. TNF-α concentrations were lower in the TPN group. The addition of TPN in EVLP allows for better electrolyte composition, decreased inflammation, and improved graft performance.

Introduction

Ex vivo lung perfusion (EVLP) is an emerging technology targeted at evaluating and reconditioning donor lungs. Multiple studies have shown its benefit in the assessment of extended criteria donors, which has led to the expansion of the donor pool[3,4,6,57,59,86].

As early as 1959, Harry Eagle determined 28 essential metabolites required for the metabolic support of multiple cell lines[87]. Since then, numerous alterations and improvements have been made to enhance cell viability across a variety of cell types and compositions[88]. Contemporary clinical perfusates are designed to mimic normal intravascular electrolyte and osmotic composition; however, little attention is paid to metabolic factors in EVLP[33,35]. Generally, EVLP protocols only supplement the perfusate with insulin and glucose during the perfusion[4,89,90]. Otherwise, perfusates are generally void of many of the ingredients considered essential for cellular viability. As we continue to perfuse organs for longer, the inherent metabolic deprivation will also likely become more apparent. Furthermore, organ repair after ischemic reperfusion injury is an energy exhaustive process[91,92]. We, therefore, sought to determine whether enhanced metabolic support, with total parenteral nutrition (TPN) would improve graft function.

Methods

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," as formulated by the National Society for Medical Research. Lungs from ten female domestic pigs (49.2 ± 1.4 kg) were procured and underwent prolonged *ex vivo* lung perfusion for 24 hours. Lungs were allocated equally to two groups: one group receiving total parenteral nutrition (TPN) versus control. All porcine lungs were perfused with 1.5L of a common hospital ingredient derived perfusate comprised of similar ingredients to STEEN solution and 0.5L of red

blood cell concentrate (pRBC). 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 also added to the perfusate. All solutions were prepared the day prior to each perfusion. In the TPN group, the perfusate was continually infused with 0.08 $g/kg_{lungweight}/hr$ of 6.3% amino acid solution and 0.1 $g/kg_{lungweight}/hr$ of 15% lipid solution. Amino acid and lipid solutions were derived from components of PeriOlimel 2.5%E (Baxter Corporation, Missisauga, Canada). In the TPN group, the perfusate was also supplemented with 80mg ascorbic acid, 17mg Niacinamide, 5mg d-Panthenol, 1mg Pyridoxine hydrochloride, 1.4mg Riboflavin, 1.2mg Thiamine, 2 300IU Vitamin A, 400 IU Vitamin D, 7IU Vitamin E, 20mcg Biotin, 5mcg Vitamin B12, 0.2mg Vitamin K_1 and 140mcg Folic acid (supplied as Pediatric Multi-12/K1 Injection, Sandoz Canada Inc, Boucherville, Canada).

Perfusate solution

Each litre of perfusate was produced from 332 mL of 25% Human Serum Albumen (Grifols, Toronto, Canada), 18 mL of 8.4% Sodium Bicarbonate (Baxter, Mississauga, Canada), 2.3 mL of 50% Dextrose (Baxter, Mississauga, Canada), 0.9 mL of 1.29 mmol/L Potassium Phosphate (Baxter, Mississauga, Canada), 639.3 mL of Plasmalyte-A (Baxter, Mississauga, Canada), and 7.5 mL of 100 mg/mL Calcium Chloride (Omega, Montreal, Canada).

Red blood cell concentrates were obtained by washing whole blood collected during exsanguination with 0.9% saline and concentrating it using a Sorin Xtra Cell Saver (Sorin Group Canada Inc., Burnaby, Canada).

Initiation of EVLP

Lung procurement and NPV-EVLP was performed as previously described[46]. The animals were sedated with Ketamine (20 mg/kg), atropine sulfate (0.05 mg/kg), and xylazine (0.9 mg/kg). The animals were then intubated and anesthetized with 1-3% isoflurane. Median

sternotomy was performed. The animals were administered intravenous Heparin (40 000 IU) and a 2- stage venous cannula was inserted into the right atrium to facilitate blood collection during exsanguination. *In vivo* wedge biopsies were taken. The animal was then exsanguinated into the cell saver. The left atrial appendage was transected and cardiectomy was completed. The trachea was clamped just above the carina at a constant airway pressure of 20 cmH_2O. The lungs were then removed and transferred to the NPV-EVLP chamber.

The segment of trachea distal to the clamp was intubated and secured to a modified endotracheal tube. The pulmonary artery was also connected to the NPV-EVLP circuit. T0 biopsies were taken immediately prior to reperfusion. Perfusate flow was initiated at 10% of predicted cardiac output ($CO_{predicted}$ = 70 mL/kg/min). The perfusate was warmed to 32°C. Upon reaching a temperature of 32°C, a continuous positive airway pressure (CPAP) of 20 cmH_20 was applied. Once at target CPAP, the trachea was then unclamped.

Ventilation was initiated by simultaneously decreasing the CPAP and end inspiratory pressures (EIP) by 1 cmH_2O every 2 minutes until a CPAP of 5 cmH_20 and transpulmonary gradient (TPG=Paw-NIP) of 18 cmH_2O was achieved. Perfusate flow was increased by 10% $CO_{predicted}$ ever 10 minutes to a target preservation flow of 30% $CO_{predicted}$. The perfusate temperature was gradually increased to reach a final temperature of 38°C within 30 minutes of reperfusion. A medical gas mixer (89% N_2, 8% CO_2, and 3% O_2) was used to target perfusate pCO_2 from 35-50 mmHg. Lung evaluation (5 minutes duration) was performed every 2 hours. Ventilation and perfusion parameters during preservation and evaluation are summarized in Table 2.

Organ Evaluation

Physiologic parameters including pulmonary artery pressure (PAP), pulmonary vascular resistance (PVR), dynamic compliance (Cdyn), and the ratio of partial pressure of oxygen in the pulmonary venous blood to the fraction of inspired oxygen (P:F Ratio = $Pa_{LA}O_2$ / FiO_2) were continually assessed. The lungs were weighed at T0, T12, and T24. Biopsies were taken at

similar timepoints. Percentage weight gain [weight gain (%) = (End_{weight} – $Start_{weight}$ / $Start_{weight}$) x 100%] was used as a surrogate for edema formation.

Electrolytes, Cytokine Analysis, and Histopathology

Blood gas analysis was performed every two hours using an ABL800 blood gas analyzer (Radiometer, California, USA). Perfusate cytokine concentrations of tumor necrosis-α (TNF-α) and interleukin-8 (IL-8) were determined using enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, USA). Free fatty acid (FFA), branch chain amino acid (BCAA), and triglyceride (TG) perfusate concentrations were determined using colorimetric assay kits (abcam, Cambridge, UK).

Statistics

All results are expressed as mean ± standard error. Analyses were performed on STATA 15 (StataCorp LLC, College Station, Texas). Shapiro-Wilk test was used to assess normality. Student's t-test was used to compare normally distributed continuous variables. Wilcoxon rank-sum test was used to compare non-normally distributed continuous variables. Repeated measures analysis of variance was used to assess for significance within groups, over time. $p<0.05$ was considered statistically significant.

Results

Lung oxygenation

Oxygenation was stable over time in both groups out to 24 hours. Interestingly, TPN significantly improved oxygenation, throughout the NPV-EVLP run (p=0.01)(Figure 6.1). The average P:F ratio at hour 11 was 447 ± 32 mmHg in the control group and 580 ± 9 mmHg in the TPN group. The difference continued to be preserved at hour 23 (noTPN 448 ± 49 mmHg, TPN 565 ± 16).

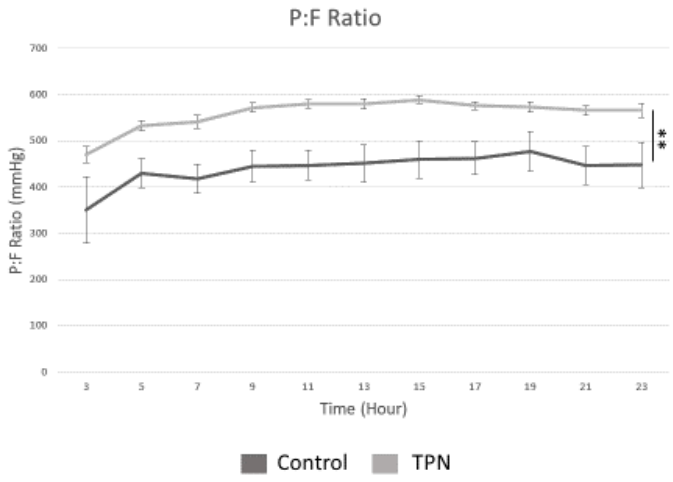

Figure 6.1. **TPN improves oxygenation in EVLP.** P:F ratio over time. Results are displayed as mean ± SE. **p<0.01 between groups.

Physiologic Parameters: Dynamic Compliance, Pulmonary Vascular Resistance, and Edema Generation

In both groups, PVR showed significant variation over time (Figure 6.2A, p=0.01). The PVR decreased initially, reaching a nadir at T11. It then continued to rise slowly throughout the latter half of perfusion. PVR was not significantly different between groups (p=0.15).

There was no difference in Cdyn between groups over time (Figure 6.2B, p=0.89). In both groups the compliance increased initially, then reached a stable plateau phase from T9 to T19. This was followed by a small decline in compliance in the last four hours of perfusion.

There was no significant difference in edema formation between groups at T12. By T12, the weight in the control group had increased by 7.6 ± 2.8%, while the TPN group had increased by

8.5 ± 1.7% (p=0.80). By the end of the run, lung weight had increased by 19.3 ± 4.4% in the control group and 14.7 ± 2.4% in the CHD group (Figure 6.2C, p=0.39).

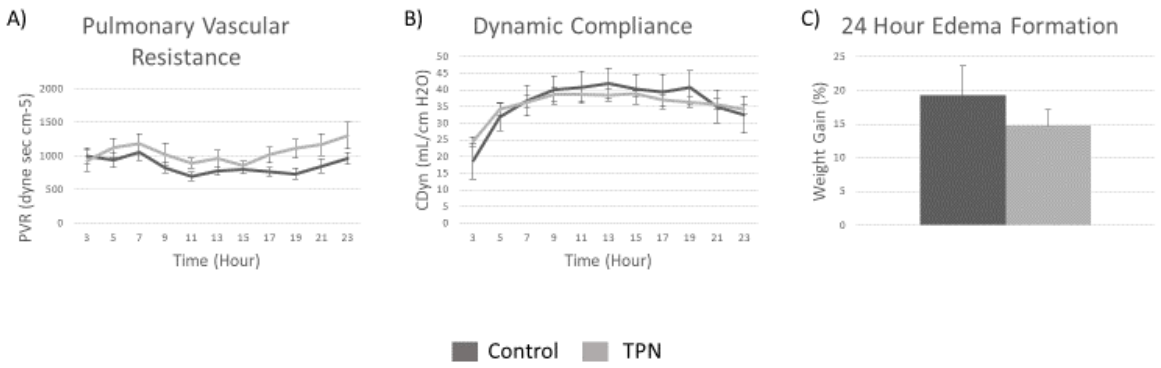

Figure 6.2. **TPN does not significantly affect mechanical characteristics of lung physiology**. Dynamic compliance (A) and pulmonary vascular resistance (B) over time, as well as edema formation at 24 hours of NPV-EVLP (C). Results are shown as mean ± SE. No difference was observed between groups (p>0.05).

Electrolytes and Metabolites

The concentration of sodium was more stable over time in the TPN group compared to the control group (Figure 6.3A, p=0.04), with the sodium concentration reaching 165.0 ± 2.6 mmol/L in the control group. Lactate, alternatively, was not significantly affected the presence of TPN (Figure 6.3B, p=0.43). Final perfusate lactate concentrations were 14.1 ± 1.0 mmol/L in the control group and 12.9 ± 1.9 mmol/L in the TPN group.

Cytokine Profile

As seen in our previous experiments, TNF-α levels rose significantly in the initial part of the experiment then began to recede over time[46]. TNF-α concentrations were significant lower in the in the TPN group over time (Figure 6.3C, p=0.02).

Perfusate concentrations of IL-8 remained low in the perfusate of both groups over the first half of the experiment but had rose significantly by the end of the experiment. IL-8 concentrations were not significantly different between groups (Figure 6.3D, p=0.50).

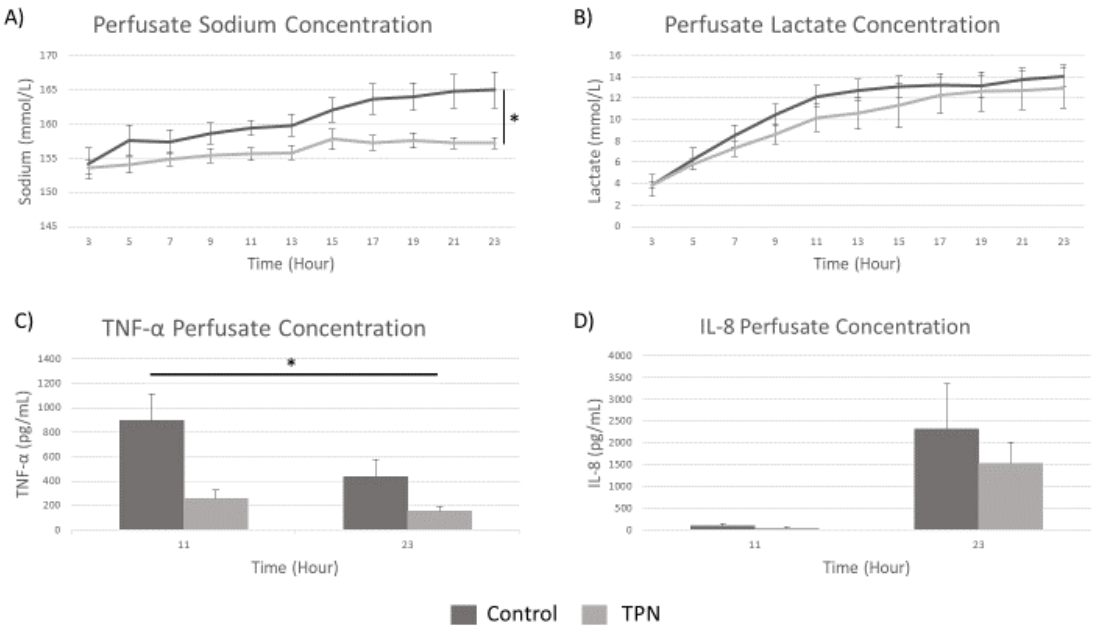

Figure 6.3. **TPN allows for improved electrolyte homeostasis and reduces pro-inflammatory cytokines in EVLP.** Perfusate sodium (A), lactate (B), TNF-α (C) and IL-8 (D) concentrations over time. Results are displayed as mean ± SE. *p<0.05 between groups.

Lipid and Protein Metabolism

As previously stated, our perfusate is comprised of 0.5L of concentrated pRBC and 1.5L of an acellular, albumen containing perfusate. Therefore, FFA, TG, or BCAA were only present in the initial perfusate if they were not readily washed from the RBC during the process of hemoconcentration by the cell saver. As the RBC content in the perfusate was targeted to provide a desired hemoglobin concentration, the relative initial concentration of FFA, TG, and BCAA was determined by the efficiency of clearance by the cell saver of each individual metabolite.

Free fatty acids (FFA) were found in increased levels in the original perfusate compared to *in vivo* samples. In the control group, there was ongoing catabolism of FFA that led to trace amounts by T11 (Figure 6.4A). In the TPN group, the perfusate FFA concentration stabilized, over time, at concentrations similar to *in vivo* plasma concentrations (IV 1.39 ± 0.23 mM, T23 1.99 ± 0.14 mM, $p>0.05$). The difference in perfusate FAA concentration between control and TPN groups was significantly different over time ($p<0.001$).

Triglycerides (TG) appeared to be readily washed from the RBCs by the cell saver, as seen by the difference between in vivo and T0 concentrations. As the control group had no exogenous sources of TG, perfusate TG concentrations remained negligible for the entirety of the perfusion. In the TPN group, plasma TG concentrations increased over the first 5 hours of the perfusion and then plateaued at concentrations similar *in vivo* samples (Figure 6.4B). There was no difference between *in vivo* perfusate TG concentrations and T23 perfusate concentrations (IV 46.9 ± 13.9 mM, T23 105.3 ± 42.6 mM, $p=0.23$).

The concentration of BCAA in the initial perfusate after cell washing was relatively low. In both groups, there was a steady increase in perfusate BCAA concentrations over the first 5 hours. In the TPN group, the BCAA concentration began to plateau following T5. In the control group, the BCAA concentration continued to increase steadily over time. This led to a significantly higher

final concentrations of BCAA in the control group at T23 (Figure 6.4C: noTPN 68.7 ± 5.7 nM, TPN 53.2 ± 1.7 nM, p=0.02). The perfusate concentration in the TPN group at T23 was similar to *in vivo* perfusate BCAA concentrations (IV 46.1 ± 3.4, T23 53.2 ± 1.7, p=0.09).

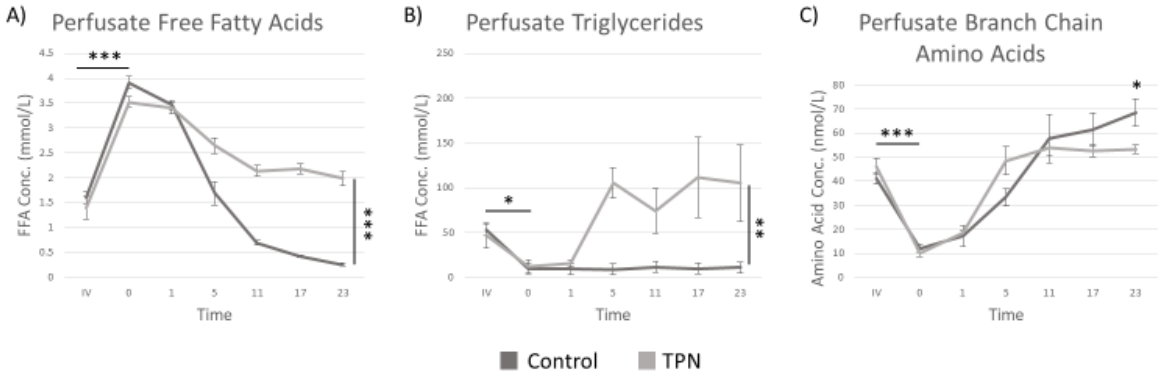

Figure 6.4. **TPN returns perfusate lipid and branch chain amino acid concentrations to near *in vivo* levels.** Perfusate FFA (A), TG (B), and BCAA (C) concentrations over time. Results are shown as mean ± SE. *p<0.05, **p<0.01 and ***p<0.001 between groups.

Discussion

Conceptually, prolonged EVLP can likely be divided into two phases: recovery and preservation. During initial reperfusion there is a significant inflammatory response as a consequence of ischemia reperfusion injury (IRI). The mechanisms of IRI are well documented and several interventions have tried to limit the extent of overall graft damage during the recovery phase[93–95]. These include, but are not limited to, sphingosine-1-phosphate, alpha-1-anti-trpysin, noble gases, and mesenchymal stem cells[43,45,48,71,96]. However, assuming the graft has not suffered an irremediable insult, the organ ultimately reaches a stable plateau in lung function. At this point, the objective is to prolong this preservation phase for as long as possible. Currently there is limited evidence of interventions targeting the preservation phase of organ perfusion.

Our study demonstrates that improved metabolic supplementation with TPN improves graft function in extended EVLP throughout the 24-hour period. The addition of TPN improved oxygenation, decreased inflammatory cytokines, and allowed for improved homeostasis of electrolytes free fatty acids, and branch chain amino acids at near *in vivo* levels.

Interestingly, the addition of TPN improved organ function, immediately from the outset of reperfusion. Given that oxygenation was higher from the initiation of EVLP (Figure 6.1), combined with lower TNF-α concentrations in the perfusate during the initial half of the perfusion, suggests that at least part of the benefit of TPN is from reduced IRI and better endothelial cell integrity. The fact that oxygenation was higher, yet the compliance was unaffected by TPN, also suggests that the benefit was not a consequence of improved mechanical properties of ventilation, but rather oxygen exchange at the endothelial level. Many of the vitamins and cofactors contained in conventional TPN have been implicated in the improvement of ischemia reperfusion injury (IRI). For example, lipid peroxidation has been shown to irreversibly damage cell membranes. Antioxidants such as d-Panthenol, Vitamin C

and E inhibit lipid peroxidation and reduce IRI[97–99]. Vitamin B12 has recently been show to protect against superoxide induced cell injury, which has also been implicated in IRI[100,101]. Niacinamide has been shown to replenish NAD+ pools, improve bio-energetic profiles, and ameliorate consequence of cerebral ischemia[102]. As such, there are multiple mechanisms and pathways to which TPN may have contributed to improved oxygenation throughout 24 hours of EVLP.

Beyond T11, lungs in the control group began to display evidence of substrate depletion, which has the potential to significantly affect the preservation phase of EVLP. The maintenance of physiologic energy substrates is pertinent, as many of the regulators of cellular homeostasis, fluid volume, and membrane integrity are energy dependent. Our results demonstrate that by improving metabolic supplementation, other markers of overall cellular and tissue stability such as electrolyte regulation were improved.

As early as 1976, Longmore *et al* demonstrated that the lung produced lactate continuously and at a near constant rate from glucose, with between 3-10 times more glucose being incorporated into lactate than oxidized to CO_2[103]. The rate of lactate production is dependent on insulin and glucose concentrations and also rises following episodes of ischemia and reperfusion[103,104]. Multiple groups have also shown that lung lactate production is related to the severity of acute lung injury and not tissue hypoxia[105,106]. Therefore, it is not surprising, that in an experimental setting where the insult is the same amongst groups, the insulin concentration is fixed, and the glucose concentration is tightly regulated at a defined concentration, that the lactate produced would be similar amongst groups.

During periods of ischemia, cellular energy metabolism has been shown to transition from primarily utilizing fatty acid oxidation to favouring glycolysis for ATP production[94]. However, Ayene *et al* demonstrated that following reperfusion of ischemic lung tissue there were increases in both lipid and protein oxidation compared to prior to the ischemic insult[107]. Our

results support these findings. Free fatty acids were continually depleted over time in the control group, ultimately reaching negligible levels by the end of the run. Supplementation with TPN maintained perfusate FFA concentrations, allowing for stable levels similar to *in vivo* concentrations over the latter half of the run. Triglycerides were found to be removed by the cell saver, and therefore without addition, perfusate concentrations were negligible over time in the control group. However, the presence of TPN restored them to normal *in vivo* levels.

Branch chain amino acids (BCAA) refer to leucine, isoleucine, and valine. These amino acids, in addition to their role as substrates for protein synthesis, may also be utilized as an energy source. Their catabolism allows for the production of succinyl-CoA and acetyl-CoA, which feed directly into the tricarboxylic (TCA) acid cycle[108]. Mixed amino acid supplementation has been shown to be beneficial in both liver and cardiac recovery from IRI; however, has not been studied in lung[109,110]. We found that the concentration of BCAA in the original perfusate to be very low. In the control group, BCAA were continually released, accumulating in the perfusate over time, likely as a response to nitrogen-starvation induced autophagy. In the TPN group, where both amino acids and lipids were supplemented, there appeared to be a restoration of homeostasis and stabilization of BCAA levels similar to *in vivo,* resulting in significantly lower BCAA perfusate concentrations at the end of the run.

As TPN is relatively inexpensive, further studies aimed at testing the function in a rejected human lung model, as well as transplantation models of both large animal and human lungs are required for ultimate clinical translation of these preclinical findings.

In conclusion, metabolic supplementation with TPN provides improved graft function during prolonged EVLP, even during early phases of the perfusion. By improving graft function, especially with respect to oxygenation during EVLP, we may increase the rate of marginal lung acceptance in EVLP, and ultimately increase the rate of utilization of donor lungs offered for transplantation.

Chapter 7: Thesis Summary

Currently there is a wide disparity between the number of patients with end stage lung disease being listed for lung transplantation and the number of transplants being performed. Unfortunately, this gap is also increasing over time. Finding means to maximize transplantation of the grafts available is essential to reducing this disparity. EVLP provides a platform for the reconditioning and resuscitation of marginal donor grafts so that they lungs may be use to provide similar results to conventional transplantation with standard criteria donors.

This thesis explored several barriers to current EVLP protocols. The first barrier addressed was the development of a low cost perfusate alternative with similar efficacy to currently available perfusates. Cost is an integral factor to both medicine and research. Expanding the use of ELVP to the greater population of patients with end stage lung disease requires significant cost reduction before it is considered cost effective by most health regulatory authorities. Our data demonstrates that EVLP can be safely performed with a perfusate comprised of readily available, common hospital ingredients at substantial savings to either the researcher or the clinician. Further studies validating our perfusate in both large animal transplant and human models is needed, though, before widespread adoption.

The second barrier addressed involved the development of a model for prolonged EVLP. As previously mentioned, current clinical EVLP protocols limit perfusion times to less than six hours. This length of time is suitable for assessment of graft function outside the stressors of a donor body; however, it is not suitable for interventions that may take time to be effective. Our groups had previously shown that negative pressure ventilation in EVLP reduced VILI compared to positive pressure ventilation. This thesis explored the limits of prolonged EVLP using negative pressure ventilation and developed a model for safe 24 hour EVLP which greatly exceeds current protocols.

The third barrier investigated was a lack of mechanisms for metabolic waste clearance and electrolyte homeostasis in current EVLP systems. By incorporating a hemodialysis circuit into the our EVLP systems we were able to demonstrate that both electrolyte homeostasis and lactate clearance could be achieved. This did not affect overall lung function within 24 hours of EVLP. This is especially pertinent and relates to the aspect of cost mentioned above. In showing that CHD did not affect graft outcomes, we are better able to determine what is essential to EVLP capabilities. As we continually optimize EVLP platforms and protocols, though, and can perfuse organs longer, then CHD may be a suitable means of reconditioning cellular based perfusates.

This final barrier explored in this thesis relates to metabolic support of a lung underdoing prolonged EVLP. This thesis demonstrated the supplementation of the perfusate with amino acids, lipids, and essential vitamins and cofactors contained in conventional TPN improved graft quality in EVLP. We also demonstrated lung continually oxidized free fatty acids, resorting to liberation of branch chain amino acids as an energy source during later stages of machine perfusion. Previous studies have focussed on inflammatory aspects of IRI and machine perfusion; however, little has been described on the aspect of metabolism in EVLP. Further studies aimed at determining the precise metabolic requirements of the lung during the different stages following reperfusion are essential before we can entertain the possibility of long term normothermic storage needed in endeavours such as gene therapy or organ banking.

Despite these findings, several limitations exist. Firstly, because the previous studies did not involve transplantation of perfused lungs, we are unable to ascertain that benefits during machine perfusion translate in improved outcomes post-transplantation. Secondly, experiments such as the effects of dialysis and total parenteral nutrition were designed to explore if the intervention in question had a direct beneficial effect or not. Subsequent optimization of protocols is still needed in order to determine the greatest extent of which a particular

intervention might benefit lung function on EVLP. For example, TPN administration rates, as well as ratios of lipid: amino acid: vitamins may be titrated to improve functional outcomes, and experiments with labeled metabolic substrates would confirm the optimal delivery rate as well as provide clarity on lung utilization of these nutrients. Exploration of other modes of dialysis such as hemofiltration or use of other dialysates may allow for greater efficacy during EVLP. Thirdly, deeper understanding of the in-depth mechanisms for the benefits of each particular therapy is needed in order to continue advancing the field. For example, nutrient tracing to determine which cell processes are being utilized would give us a better understanding of how lung metabolism is responding to IRI.

Advances in EVLP will continue to improve our ability to preserve and recondition extended criteria donors. This will continue to increase our rate of utilization of donor lungs, while maintaining currently established safety margins and outcomes. Scientific advances in device innovation, understanding of both metabolic and inflammatory consequences of ischemia-reperfusion injury and prolonged *ex vivo* organ perfusion, and therapies to reverse the deleterious consequences of these states is imperative for continued improvement. Furthermore, improvement in health-systems related variables such as cost, transportability, and expertise are also imperative before widespread adoption and utilization. However, by incrementally addressing the multitude of obstacles surrounding *ex vivo* organ perfusion, we move closer to realizing the full potential of EVLP.

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