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REDUCED FLOW EX VIVO LUNG PERFUSION TO REHABILITATE DONATION AFTER CARDIAC DEATH LUNGS

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Abstract

Background: Current ex vivo lung perfusion (EVLP) protocols aim to achieve perfusion flows of 40% of cardiac output or more. We hypothesized that a lower target flow rate during EVLP would improve graft function and decrease inflammation of donation after circulatory death (DCD) lungs.

Methods: A porcine DCD and EVLP model was utilized. Two groups (n=4/group) of DCD lungs were randomized to target EVLP flows of 40% (High Flow) or 20% (Low Flow) predicted cardiac output based on 100mL/min/kg. At the completion of 4 hours of normothermic EVLP using Steen solution, left lung transplantation was performed, and lungs were monitored during 4 hours of reperfusion.

Results: After transplant, left lung-specific pulmonary vein PO₂ was significantly higher in the Low Flow group at 3 and 4 hours of reperfusion (3-hour: 496.0±87.7 vs. 252.7±166.0 mmHg, p=0.017; 4-hour: 429.7±93.6 vs. 231.5±178 mmHg, p=0.048). Compliance was significantly improved at 1 hour of reperfusion (20.8±9.4 vs. 10.2±3.5 ml/cm H₂O, p=0.022) and throughout all subsequent time points in the Low Flow group. After reperfusion, lung wet-to-dry weight ratio (7.1±0.7 vs. 8.8±1.1, p=0.040) and IL-1β expression (927±300 vs. 2070±874 pg/ng protein, p=0.048) were significantly reduced in the Low Flow group.

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Conclusions: EVLP of DCD lungs with low flow targets of 20% predicted cardiac output improves lung function, reduces edema and attenuates inflammation after transplant. Therefore, EVLP for lung rehabilitation should use reduced flow rates of 20% predicted cardiac output.

Introduction

Although the number of lung transplants performed each year continues to rise, the increase in lung transplant volume has failed to keep up with the growing waitlist size.¹ Modifications in lung allocation systems, utilization of extended criteria donors, and improvements in donor recovery protocols have all broadened the reach of this important intervention.²⁻⁴ Of the emerging innovations intended to expand the donor pool, ex vivo lung perfusion (EVLP) has become one of the more viable options to evaluate and recover high-risk donor organs with similar outcomes to transplantation with standard criteria lungs.⁵

Lung evaluation is an important component of EVLP, but the true potential of this technology is the ability to repair lungs and improve graft function. The feasibility of this paradigm has been demonstrated in limited experimental settings, but no large-scale reliable technique for rehabilitation has yet to be realized.⁶⁻⁹ There are a number of EVLP platforms and protocols that have demonstrated promising initial results.¹⁰⁻¹² One of the best studied, both in the clinical and laboratory settings, is that utilized by the Toronto group, which relies on an acellular perfusate, closed left atrium and perfusions flows of 40% of cardiac output.¹⁰ This perfusion strategy may be best suited for rehabilitation when compared to other protocols that rely on higher flow rates because lung stability can be maintained for longer durations of EVLP.^{13, 14} Given the apparent advantages of diminished hydrostatic forces on the pulmonary vasculature, further reduction in perfusion flows may enhance graft rehabilitation, but a safe lower limit on perfusion flows has not been experimentally evaluated in a large animal model. However, perfusion at 20% of cardiac output has been utilized successfully in rat models of isolated lung perfusion.¹⁵

The purpose of this study was to examine the effect of low perfusate flow on lung evaluation and subsequent post-transplant graft function. We sought to understand the implications of this potential modification as a platform intended for graft repair. We hypothesized that a lower flow target would result in improved lung function and reduced edema compared to standard parameters, which target 40% of predicted cardiac output.

Materials and Methods

Animals and Study groups

The University of Virginia's Animal Care and Use Committee approved this study. All procedures were performed in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the National Institute of Health's 1996 Guide for the Care and Use of Laboratory Animals. Mature, farm-raised swine of both sexes (weight, 24–32kg) were used for this study. Animals were randomized to one of two groups (n=4/group): an experimental group where EVLP perfusion flow rate targeted 20% of predicted cardiac output (Low Flow), or a control group where EVLP

perfusion flow rate was maintained at 40% predicted cardiac output (High Flow). The surgical team was blinded to group assignment.

Donor and Procurement Procedure

A porcine DCD and EVLP model was utilized as previously performed by our laboratory with minor modifications as indicated.^{9, 16} Animals were sedated and intubated. General anesthesia was maintained while animals were ventilated with 100% oxygen in order to measure baseline parameters. Intravenous heparin (200IU/kg) (APP Pharmaceuticals, Schaumburg, IL) was administered and the endotracheal tube was clamped to induce hypoxia ultimately leading to cardiac arrest. The animals underwent 90 minutes of no touch warm ischemia.

A median sternotomy was performed and a cardioplegia cannula (Terumo Heart Inc, Ann Arbor, MI) was inserted into the main pulmonary artery. Prostaglandin-E1 (500 mcg) was administered followed by 1.5L of cold Perfadex (XVIVO Perfusion, Inc, Englewood, CO) flush. The initial flush was supplemented with 15,000 IU heparin (APP Pharmaceuticals) and 0.3mL trometamol which was mixed immediately prior to use. The inferior vena cava and superior vena cava were ligated and the left atrium vented. The thoracic cavity was filled with cold slush. At mid inspiration, the trachea was clamped and the heart and lung bloc explanted. Once explanted, the lungs were flushed retrograde with an additional 500mL of Perfadex solution.

EVLP was performed and monitored by an extracorporeal membrane oxygenation specialist (D.T.M) with expertise in clinical EVLP. The EVLP circuit was primed in the standard fashion with 1500mL of Steen Solution (XVIVO perfusion Inc.) supplemented with 500mg cefazolin (Apotex Corp, Weston, FL) 500mg methylprednisolone (Pfizer, New York, NY) and 10,000 IU IU/ml heparin (APP Pharmaceuticals). Perfusion was initiated at a flow of 10% of the maximum flow target for the given protocol and increased relative to the targeted maximum with the goal to achieve full flow at 60 minutes (Table 1). Estimated cardiac output was calculated based on donor weight (100mL/min/kg). Pulmonary artery (PA) pressures were monitored and flow decreased if pressures exceeded 15mmHg in the Low Flow group. Left atrial (LA) pressures were maintained between 3–5mmHg throughout EVLP in both groups. The perfusate was gradually warmed to 37° Celsius. Ventilation was initiated (tidal volume 8mL/kg) with a standard tri-gas mixture once the temperature reached 32° Celsius. At one hour into EVLP, 500mL of perfusate in the reservoir was exchanged with 500mL of fresh Steen solution (XVIVO perfusion Inc.), along with 10,000 additional units of heparin (APP Pharmaceuticals). Additional Steen (500mL) was added at the completion of the two-hour and three-hour time points, and at any point when the reservoir fell below the minimum safe operating level (200mL).

Perfusate samples were collected hourly following a 15-minute challenge with FiO2 increased to 100% for measurement of oxygenation. After 4 hours of EVLP, the lungs were cooled to 32° Celsius and the endotracheal tube clamped to maintain partial inflation. EVLP was terminated and the lungs were flushed antegrade with 500mL cold Perfadex (XVIVO perfusion Inc), and the left lung was separated and prepared for transplantation.

Lung Transplant Procedure

Recipient animals were anesthetized and ventilated similar to donor animals. Vascular access was obtained and left lung transplantation performed as previously described.¹⁷ Lung function was monitored during 4 hours of reperfusion with the left thoracotomy open and suspended from above by placing upward traction on the chest wall retractor to limit chest wall contribution to compliance. Combined total lung compliance was utilized throughout the experiment. Airway pressure measurements and blood gasses (systemic arterial and direct pulmonary vein samples for graft specific oxygenation) were obtained after 15 minutes, 1 hour and hourly thereafter. At 4 hours after reperfusion the left lung was excised and the animal euthanized.

Biochemical Analysis

During EVLP, LA perfusate samples were collected at the same time as those for measurement of oxygenation and immediately stored at -80°C until analysis was performed. Hyaluronan concentration was measured using a commercially available enzyme-linked immunosorbent assay according to the manufacturer's instructions (Biomatik Corporation, Cambridge, Ontario).

After EVLP the lungs were separated as noted above. At this point, peripheral sections of the right lung were tied off and three fresh tissue samples (additional samples reserved for wet-to-dry weight ratios) were taken in consistent locations (apex, base and mid-lung), which were flash-frozen in liquid nitrogen and stored at -80°C . Fresh tissue for edema assessment was similarly obtained from the left lung after completion of the 4-hour reperfusion period. Flash-frozen lung samples were homogenized, and the total protein concentration of the supernatant was determined (NanoDrop 2000 Spectrophotometer, Thermo Scientific, Waltham MA). Multiplex enzyme-linked immunoabsorbant assay (Millipore Sigma, Burlington, MA) was performed to measure cytokines, and the cytokine levels were normalized to total protein concentration per sample. The average cytokine level per lung was then compared between groups.

Assessment of Lung Edema

As noted above, fresh lung tissue was collected in both the right lung after EVLP and the left lung at the conclusion of the 4-hour reperfusion period. These samples were weighed then placed in a 56°C vacuum oven (Best Value Vacs, Naperville, IL) until the weight stabilized. Lung wet-to-dry weight ratios were then calculated to quantify lung edema accumulation. The average wet-to-dry weight ratios per lung were then compared between groups.

Differential Counts of Bronchoalveolar Lavage Cells

After fresh tissue was obtained for cytokine analysis and edema assessment, the upper lobe of the left lung was lavaged with 60mL of normal saline. Bronchoalveolar lavage fluid was centrifuged (1500 rotations/minute, 7 minutes, 4°C) and the cell pellet resuspended in phosphate buffered saline with 1% bovine serum albumin to a concentration of 1×10^7 cells/mL. Slides were prepared using a Thermo Shandon CytoSpin 3 Cytocentrifuge, and cells stained using a Kwik-Diff™ differential staining kit (Thermo Fisher Scientific,

Waltham, MA). Cell types were identified and counted under light microscopy in a blinded fashion.

Histology

After obtaining fresh tissue, the lower lobe of the left lung was filled with 10% buffered formalin by gravity instillation into the bronchus. The lungs were submerged in formalin and allowed to fix overnight at 4°C. Peripheral lung tissue was sampled in predefined locations (n=3/lung) and paraffin embedded. Samples were stained with hematoxylin-eosin and then evaluated by a pathologist blinded to group assignment in order to assess lung injury. These were scored on a standard scale of 0–3 using previously reported criteria.¹⁸ After summation of each component, a composite score was obtained (total score range, 0–9).

Statistical Analysis

Student t test was used to determine statistical significance using Prism 7 (GraphPad Software Inc., La Jolla, CA) at a level of $p < 0.05$ set for significance. In addition, a 2-way analysis of variance (ANOVA) accounting for time and individual animal effect was used to confirm these findings. All data is reported as mean $\pm$ standard deviation. For analysis of change in hyaluron concentration over time, a mixed effects model was fit using SAS version 9.4 (SAS Institute, Cary, NC).

Results

Donor Animals

There were no differences between the two groups of donor animals with regard to systemic arterial PaO₂ (496.7 $\pm$ 72.9 vs. 523.5 $\pm$ 56.3 mmHg, $p=0.582$), lung compliance (24.6 $\pm$ 5.8 vs. 24.1 $\pm$ 7.7 mL/cm H₂O, $p=0.909$) or time from endotracheal tube clamp to death (25.3 $\pm$ 14.8 vs. 23.8 $\pm$ 5.1 minutes, $p=0.854$).

Lung Function

During EVLP, there were statistically significant differences in flow rate beginning at 40 minutes into EVLP and persisting throughout the four hours of EVLP (Figure 1A). Significant differences in pulmonary artery pressures were observed at 2 hours, 3 hours, 3.5 hours and 4 hours with the High Flow group demonstrating higher pulmonary artery pressures (Figure 1B). There were no differences in left atrial pressures throughout EVLP (Figure 1B). During EVLP, the left atrial PO₂ increased in the Low Flow group and remained stable in the High Flow group (Figure 2). Oxygenation was significantly higher in the Low Flow group at the end of 4 hours of EVLP (466.4 $\pm$ 71.3 vs. 280.4 $\pm$ 149.1 mmHg, $p=0.035$). Compliance remained stable in both groups throughout EVLP with no significant differences between groups (15.5 $\pm$ 7.4 vs. 7.6 $\pm$ 3.4 mL/cm H₂O) (Figure 3). The calculated pulmonary vascular resistance throughout EVLP is shown in Figure 4.

After transplantation, by direct pulmonary vein sampling, left lung specific oxygenation was initially similar between groups; however, these diverged throughout the 4-hour reperfusion period with significantly higher PO₂ in the Low Flow group at 3 hours (496.0 $\pm$ 87.7 vs. 252.7 $\pm$ 166.0 mmHg, $p=0.017$) and 4 hours (429.7 $\pm$ 93.6 vs. 231.5 $\pm$ 178 mmHg, $p=0.048$) of

reperfusion (Figure 5A). By 2-way ANOVA, EVLP flow target and time after transplant were both statistically significant factors, accounting for 54.1% ($p<0.001$) and 7.2% ($p=0.030$) of the variability respectively, while the impact of the individual animal was not statistically significant ($p=0.161$). Although compliance was initially similar after transplantation (15 minutes: 19.5 ± 7.7 vs. 12.7 ± 0.8 ml/cm H₂O, $p=0.133$), the Low Flow group demonstrated significantly improved compliance 1 hour after transplantation (20.8 ± 9.4 vs. 10.2 ± 3.5 ml/cm H₂O, $p=0.022$), a difference that persisted throughout the remainder of the reperfusion period (Figure 5B). By 2-way ANOVA, EVLP flow target was a statistically significant factor, accounting for 45.8% ($p<0.001$) of the variability, while the impact of the time after transplant and the individual animal were not statistically significant ($p=0.645$ and $p=0.053$, respectively).

There was no difference in lung wet-to-dry weight ratios between the Low Flow and High Flow groups in samples obtained immediately following EVLP (6.6 ± 0.4 vs. 7.1 ± 1.1 , $p=0.172$; Figure 6). However, in samples obtained at the end of post-transplant reperfusion there was significantly less edema in the Low Flow group (7.1 ± 0.7 vs. 8.8 ± 1.1 , $p=0.040$; Figure 6).

Biochemical Analysis

Unsurprisingly, compared to post-EVLP tissue, there was higher expression of IL-1 β , IL-4, IL-6, and IL-8 in post-transplant tissue in both groups (Figure 7). In post-transplant lung tissue, expression of IL-1 β and IL-4 in the Low Flow group was significantly reduced when compared to that in the High Flow group (Figure 7).

There was an increase in the concentration of hyaluronan, a constituent of the vascular endothelial glycocalyx, in the perfusate buffer throughout the EVLP period for both groups (Figure 8). There was a trend towards a greater increase in hyaluronan in the High Flow group, however this did not reach statistical significance ($p=0.068$).

Lung Injury Severity Score and Bronchoalveolar Cell Profile

In the bronchoalveolar lavage fluid, there were significantly fewer neutrophils as a percentage of the total cell population in the Low Flow group compared to the High Flow group ($43.3\pm14.6\%$ vs. $75.3\pm3.6\%$, $p=0.005$). There were no significant differences in lung injury severity scores between the Low Flow and High Flow groups, although in general, the scores were lower in the Low Flow group (Figure 9).

Discussion

The current study demonstrated the safety and utility of targeting lower perfusion flow rates during EVLP and its impact on post-transplant graft function in a clinically relevant porcine model of EVLP and transplant of DCD lungs. Critical elements of post-transplant lung function, as measured by lung specific oxygenation, compliance and edema accumulation, were all markedly improved with the use of lower perfusion flow during EVLP. In addition, we demonstrate that this may involve the preservation of endothelial integrity (lower wet-to-dry weight ratio) and attenuation of inflammation (reduced IL-1 β expression). This is further

supported by reduced neutrophil infiltration into the alveolar space and evidence of improved glycocalyx preservation.

While the importance of left atrial pressure has been experimentally demonstrated, variation in the inflow pressure and perfusate flow targets has been less rigorously examined with contemporary EVLP platforms.¹⁹ One study into alterations in pulmonary vasculature flow, which utilized an isolated rat perfusion model, demonstrated that extravascular fluid accumulation did not occur until capillary pressure exceeded 25mmHg.²⁰ In this study, this threshold was critical for edema formation and was independent of pulmonary flow. A more recent study by Okamoto et. al, which revisited the potential impact of different flow parameters on graft function, compared a low flow acellular method (low flow in Okamoto et. al. corresponds to our High Flow group) to a high flow cellular method of EVLP.¹⁴ While different perfusion solutions and other between-group differences in the study by Okamoto et. al. make separating out the impact of specific protocol parameters such as perfusate flow challenging, the authors do note that perfusion at too high a flow rate may lead to graft damage as a result of excessive hydrostatic forces leading to increased fluid permeability. In addition, they note that lower flows permit longer periods of EVLP, which would be more suitable for lung rehabilitation rather than simply graft assessment as EVLP is often used clinically. In evaluating lower flow systems, there is concern for hypoperfusion of certain capillary beds and therefore overestimation of on-EVLP graft function by P/F ratio alone. Importantly, in addition to assessment of lungs during EVLP, the present study also assesses lungs after transplantation and we found the most meaningful improvement in function (oxygenation and compliance) following transplantation and a period of reperfusion.

The improvement in graft function observed in the Low Flow group likely involves attenuation of pulmonary vascular inflammation and maintenance of the endothelial barrier. As evidence of these mechanisms, we demonstrated significantly lower levels of IL-1 β , which is thought to play an important role in the activation of inflammation in ischemia reperfusion injury.²¹ In addition, elevation of this cytokine in EVLP perfusate in the DEVELOP-UK study was found to be the strongest predictor of both in-hospital and one-year survival.²² Further, an *in vitro* study of conditioned pulmonary endothelial cells demonstrated that IL-1 β neutralizing antibodies could reverse neutrophil adhesion and adhesion molecule expression, suggesting a specific and pathogenic role of this proinflammatory cytokine in neutrophil-pulmonary endothelium interactions during EVLP.²² In other settings, IL-1 β has been repeatedly confirmed as a key mediator of lung inflammation, specifically lung ischemia-reperfusion injury.^{23–26}

A key component of the vascular endothelium is the glycocalyx, a polyanionic layer on the luminal side of endothelial cells comprised of membrane bound proteoglycans, glycosaminoglycans and glycoproteins that together regulate endothelial permeability, mediate interactions with circulating cells and facilitate mechanotransduction.^{27, 28} Hyaluronan, a component of the endothelial glycocalyx, has been shown to be especially important in preventing the accumulation of tissue edema and is vital for appropriate mechanotransduction and shear induced nitric oxide synthesis for vascular regulation.^{29–31} In the setting of EVLP, endothelial function is known to be important for graft outcomes and other glycocalyx breakdown components have been examined as markers of organ quality

during EVLP.³² Similarly, elevated circulating levels of hyaluronan in donor blood was recently shown to correlate with clinical markers of organ acceptability.³³ Lower flow induces less shear stress and therefore may enable better regulation of the vasculature and endothelial protection during EVLP.

The precise mechanism of enhanced lung preservation with EVLP when compared to traditional cold static preservation is not completely understood.³⁴ However, maintenance of the alveolar capillary barrier is thought to be one of the means by which EVLP may preserve lung function.³⁵ Reduction in the perfusion rate, as was performed in the present study, decreases afferent hydrostatic pressure and appears to attenuate many downstream deleterious effects. While excellent results have been achieved with EVLP that targets 40% of predicted cardiac output, the potential benefits of EVLP have not yet been completely reached. An intended goal of EVLP is to expand the donor pool, and in order to continue to progress towards this goal, the transplant community needs to embrace EVLP as a rehabilitative platform rather than merely an assessment tool. Alternative ventilation strategies, attenuation of ischemia-reperfusion injury, modulation of innate immunity, and targeted protein and gene therapy will all be necessary components of successful lung repair; but this must be accomplished on a lung protective platform such as EVLP, which may benefit from lower perfusion flow rates.^{6, 7, 9, 34, 36, 37} Moving forward, these results should be applied in future laboratory and clinical experimental designs. We believe the next logical place to incorporate these findings is in the rehabilitation of lungs that are extremely high risk or have shown early signs of deterioration when initiated on EVLP. Perhaps a reduction in perfusion flow will allow transplant teams the opportunity to rehabilitate lungs that otherwise would have been destined to be rejected through the rigid adherence to standard perfusion parameters.

This study is limited in that it is a preclinical, porcine model with an inherent variability associated with farm-raised animals. The model utilized a prolonged warm ischemia period, which results in severe lung injury. The results of the study may not be applicable to grafts with minimal injury, which are not as primed for a robust ischemia reperfusion-injury. However, the intent was to evaluate this platform for the rehabilitation and repair of organs. In addition, a prolonged cold static preservation time was not used, which may limit the clinical relevance, as this is a standard component of clinically utilized EVLP protocols. Finally, we assessed post-transplant graft function using a combination of direct pulmonary vein sampling and total lung compliance. While pulmonary vein sampling is a direct measure of graft oxygenation, it is never clinically assessed in human subjects and results may be subject to ventilation perfusion mismatch. Total lung compliance is a measure that combines the properties of both the transplanted and native recipient lung. While these factors cannot be completely controlled, they should have a similar impact on both the control and study groups.

In conclusion, in a preclinical porcine DCD model of EVLP and lung transplantation, we demonstrate dramatic improvements in post-transplant graft function with a low flow perfusion target of 20% of predicted cardiac output. The mechanism by which this clinical improvement occurs is likely through a decrease in capillary hydrostatic forces, attenuation of vascular inflammation and preservation of the endothelial-alveolar barrier. In order to

realize the full potential of EVLP for lung repair, a lung protective strategy with lower flows should be the foundation for EVLP.

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Figure 1:

Flow and pressure parameters during ex vivo lung perfusion (EVLP). **(A)** Perfusate flow expressed as a percentage of predicted cardiac output. **(B)** Pulmonary Artery and left atrial pressures during EVLP. * $p < 0.05$. LA, Left Atrium; PA, Pulmonary Artery.

Figure 2:

Left atrial oxygenation measured during ex vivo lung perfusion (EVLP). Inset shows the individual measurements at the conclusion of EVLP. * $p < 0.05$. EVLP, ex vivo lung perfusion; LA, left atrium.

Figure 3:

Total lung compliance measured during ex vivo lung perfusion (EVLP). There were no significant differences between the two groups throughout the perfusion period.

Figure 4:
Calculated pulmonary vascular resistance during during ex vivo lung perfusion (EVLP).
There were no significant differences between the two groups throughout the perfusion period.

Figure 5:
Post-reperfusion oxygenation (A) and compliance (B) during the 4-hour reperfusion period after transplantation. *p<0.05.

Figure 6:
Lung wet-to-dry weight ratios in post-ex vivo lung perfusion (EVLP) tissue and post-reperfusion lung tissue.

Figure 7:
Lung tissue expression of cytokines in the High Flow and Low Flow groups at the conclusion of ex vivo lung perfusion (EVLP) and at the end of reperfusion.

Figure 8:
Hyaluronan concentration in EVLP perfusate throughout the normothermic ex vivo lung perfusion period.

Figure 9:

Lung injury severity scores in histology specimens after reperfusion. Neutrophils per high powered field (score of 0,<5; 1, 6 to 10; 2, 11 to 20; and score of 3,>20), alveolar edema (score of 0,<5%; 1, 6%–25%; 2, 26%–50%; and 3,>50%), and interstitial infiltration (score of 0, none; 1, minimal; 2, moderate; and 3, severe). HPF, High Powered Field; PMN, Polymorphonuclear cells.

Table 1:

Flow targets during the first 60 minutes for Low Flow EVLP and High Flow EVLP protocols.

	EVLP Time (minutes)	0	10	20	30	40	50	60
	Percent of Target	10%	10%	20%	30%	50%	80%	100%
Low Flow (20% predicted cardiac output)	Flow Rate Target (ml/kg)	2	2	4	6	10	16	20
	For 30kg donor (ml)	60	60	120	180	300	320	600
High Flow (40% predicted cardiac output)	Flow Rate Target (ml/kg)	4	4	8	12	20	32	40
	For 30kg donor (ml)	120	120	240	360	600	960	1200