Normothermic Ex Vivo Perfusion Prevents Lung Injury Compared to Extended Cold Preservation for Transplantation


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Treatment of injured donor lungs ex vivo to accelerate organ recovery and ameliorate reperfusion injury could have a major impact in lung transplantation. We have recently demonstrated a feasible technique for prolonged (12 h) normothermic ex vivo lung perfusion (EVLP). This study was performed to examine the impact of prolonged EVLP on ischemic injury. Pig donor lungs were cold preserved in Perfadex® for 12 h and subsequently divided into two groups: cold static preservation (CSP) or EVLP at 37°C with Steen™ solution for a further 12 h (total 24 h preservation). Lungs were then transplanted and reperfused for 4 h. EVLP preservation resulted in significantly better lung oxygenation (PaO$_2$ 531 ± 43 vs. 244 ± 49 mmHg, p < 0.01) and lower edema formation rates after transplantation. Alveolar epithelial cell tight junction integrity, evaluated by zona occludens-1 protein staining, was disrupted in the cell membranes after prolonged CSP but not after EVLP. The maintenance of integrity of barrier function during EVLP translates into significant attenuation of reperfusion injury and improved graft performance after transplantation. Integrity of functional metabolic pathways during normothermic perfusion was confirmed by effective gene transfer and GFP protein synthesis by lung alveolar cells. In conclusion, EVLP prevents ongoing injury associated with prolonged ischemia and accelerates lung recovery.

Key words: Graft dysfunction, lung preservation, lung transplantation, machine perfusion, machine preservation

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Introduction

Lung transplantation (LTx) is a lifesaving therapy for patients suffering from end-stage lung disease. However, the number of patients that could benefit from LTx greatly exceeds the number of donors available. Currently, only 15% of lungs from brain dead donors are considered ideal for transplantation (1); the rest are generally considered unsuitable due to the injury acquired during brain death and ICU-related complications. To avoid posttransplant primary graft dysfunction (PGD, an acute lung injury that usually manifests in the first 72 h after transplantation) (2), clinicians are usually very conservative in donor selection (3–5). This translates into obligate rationing of the procedure and a high mortality for patients on the waiting list (6).

The shortage of healthy lungs for transplantation demands optimal utilization of the available donor pool, and advances in organ preservation and donor management have all sought to increase the rate of organ usage from the current donor pool. While maintaining organ viability using hypothermic preservation has traditionally been an important prerequisite for successful outcomes after LTx (7,8), the inhibition of cellular metabolism induced by protective hypothermia obviates the possibility of substantial reparative processes occurring during organ preservation (9). The ideal method of preservation should facilitate the use of extended criteria organs, initiate reparative processes for established donor organ injury and provide real-time quality assessment prior to transplantation. Machine preservation of solid organs is being proposed as a method that may achieve these goals (10–14).

Ex vivo lung perfusion (EVLP) is not a new concept and has been widely used to study lung function in small animals (15–19). It also has been shown to be a useful technique to briefly (1 to 2 h) evaluate lungs from donation after cardiac death (DCD) (20–22). However, past attempts at prolonged machine preservation have largely failed due to inability to maintain the integrity and normal barrier functions of the vasculature and epithelial beds leading to progressive deterioration in vascular flow and the concurrent development of edema (23–25). We have recently demonstrated successful application of an acellular EVLP technique in maintaining lung viability ex vivo for an extended period of time (26). Following 12 h of normothermic EVLP, healthy
pig lungs were transplanted and demonstrated immediate life-sustaining function (26).

We present here a preclinical study in which we investigated whether EVLP could interrupt or ameliorate hypothermic ischemic injury and improve extended lung preservation. To achieve this, we directly compared standard cold storage in Perfadex® solution alone (our current clinical standard for lung preservation) to normothermic EVLP with Steen™ solution over a 24 h preservation period in a porcine LTx model. We studied the effect of each method on posttransplant lung function, severity of tissue damage (reperfusion injury), intra-alveolar coagulation (thrombin generation) and alveolar epithelial cell tight junction integrity. Finally, as proof of active metabolic function, we examined the ability of EVLP lungs to synthesize an exogenous protein after ex vivo delivery of a reporter gene through the airways.

Materials and Methods

Study design

Animals: Yorkshire male domestic pigs (25–35 kg, Reimens Fur Ranch, Kitchener, Ontario) were used under an experimental protocol approved by the Animal Care Committee at the Toronto General Institute. Donor lungs were explanted as previously described (27). The lungs were preserved with cold static preservation (CSP) for 12 h (n = 10), and then randomly divided into two groups, either CSP (n = 5) or EVLP (n = 5) for an additional 12 h (CSP group: total of 24 h of ischemic aerobic hypothermic static preservation; EVLP group: 12 h of ischemic aerobic hypothermic static preservation followed by 12 h of asanguinous aerobic normothermic perfusion preservation). At the end of preservation, the left lung was transplanted into a recipient animal and reperfused for 4 h to evaluate posttransplant lung function in both groups.

EVLP system

The detailed acellular EVLP technique (Figure 1) is described by our group elsewhere (26). In brief, after the lungs were transferred to the XVIVO™ chamber (Vitrolife, Denver, CO), the left atrial (LA) cannula was first connected to the circuit. Flow was initiated slowly in a retrograde fashion to de-air the pulmonary artery (PA) cannula. The PA cannula was then connected to the circuit and anterograde flow started at 150 mL/min with the perfusate (Steen™ Solution, Vitrolife) at room temperature. The temperature of the perfusate was then gradually increased to 37°C. When 32°C was reached (usually over 30 min), ventilation was started and the perfusate flow rate was gradually increased. The flow of gas used to deoxygenate and provide carbon dioxide to the inflow perfusate via a gas exchange membrane was then initiated. We used 40% of the estimated cardiac output (CO) as the maintenance perfusate flow rate to perfuse both lungs (pigs: CO = 100 mL/kg). Mean PA pressures were maintained between 10 and 15 mmHG. The LA pressure was maintained between 3 and 5 mmHG by adjusting the height of the hard-shell reservoir. A protective mode of mechanical ventilation was applied using a tidal volume of 7 mL/kg, at 7 breaths per min, positive end-expiratory pressure (PEEP) of 5 cmH2O and an inspired oxygen fraction (FiO2) of 21%. The lungs were recruited with inspiratory holds to a peak airway pressure (PawP) of 20 cmH2O every hour. pH, pCO2, electrolytes and glucose were maintained at physiologic levels in the perfusate. At the end of 12 h of EVLP, the lung block was cooled down in the circuit to 15°C. Thereafter, perfusion and ventilation was stopped (FiO2 is increased to 50% for lung storage), and the trachea was clamped to maintain the lungs in an inflated state. The lungs were then stored at 4°C in Perfadex® (preimplantation period) until transplantation in a standard sterile organ bag surrounded by ice.

LTX technique in a porcine model

A left thoracotomy was performed through the fourth intercostal space. The pulmonary hilum was dissected and the left azygous vein was carefully elevated from the left atrium and ligated. The inferior pulmonary ligament

Figure 1: Schematic of the EVLP System. The lungs are placed within the XVIVO™ chamber (Vitrolife AB, Gothenburg, Sweden). The perfusate leaves the lungs via the LA cannula and enters the reservoir. From there, the perfusate is pumped using a centrifugal pump into the oxygenator and heat exchanger where it is deoxygenated by a gas mixture (88% N2, 8% CO2 and 6% O2) and warmed to normothermia. The perfusate then passes through a leukocyte filter before reentering the lungs via the PA cannula for oxygenation.
was divided. Both the right and left main pulmonary arteries were carefully dissected. Once the donor lung was ready, the bronchial anastomosis was performed first with a running 4-0 prolene suture. The PA anastomosis was performed next with a continuous 6-0 prolene suture interrupted in two places. The atrial anastomosis was then performed with a running 5-0 prolene suture interrupted in two places. After reimplantation of the transplanted lung at a pressure of 20–25 cmH₂O, the lung was ventilated normally as the PA clamp was removed gradually, and the lung was de-aired through the LA anastomosis.

**Ex vivo lung function**

Oxygenation capacity: \( \Delta pO_2/FiO_2 = \text{perfusate LA } pO_2 - \text{perfusate PA } pO_2, \text{ mmHg} \) was recorded hourly from 1 to 12 h of EVLP.

**Lung function after transplantation**

Blood-gas analyses were performed every hour during the reperfusion period in samples taken from the transplanted lung's pulmonary veins. Also, at the end of reperfusion period (4 h), the right PA was occluded with a tourniquet in order to measure function of the transplanted lung only. This is considered to be the gold standard test to evaluate pulmonary graft function. PaO₂Pw were measured throughout the reperfusion period. The wet-to-dry (W/D) lung weight ratio (reflecting lung edema) was calculated at the end of the reperfusion period. A lung biopsy collected from the left lower lobe was weighed and then placed in an oven at 85°C for 72 h to dry and then reweighed. The ratio of the lung weight before and after drying was calculated.

**Histopathological assessment of reperfusion injury of the lung**

Lung tissue biopsies were collected 4 h after transplantation from the left lower lobe in all experiments. Biopsies were fixed in 10% buffered formalin for 24 h, embedded in paraffin, sectioned at 5 µm thickness, stained with hematoxylin and eosin and examined for pathological changes under light microscopy. A pulmonary pathologist (DH) evaluated midsagittal slices of lung sections in a randomized and blinded fashion to assess histopathological grading of acute lung injury using the following parameters: interstitial edema, intra-alveolar edema, hemorrhage, cell infiltration and hyaline membrane formation (28). The severity of these findings was graded in a four-point scale as follows: 0, absent; 1, mild; 2, moderate and 3, severe (28). For T-cell infiltration assessment, paraffin-embedded tissue sections (5 µm) were mounted on positively charged microscope slides. Tissue sections were then incubated for 12 h at 60°C, deparaffinized in xylene and rehydrated through decreasing concentrations of alcohol. Antibiotin recovery was then performed with citrate buffer at 95°C and tissue sections were blocked with 10% goat serum dilute in phosphate buffer saline (PBS). After a brief wash in PBS, mouse anti-human CD3 primary antibody (Invitrogen, Carlsbad, CA) was applied at 1:50 dilution and incubated at 4°C overnight. Alexa Fluor 555 goat anti-mouse IgG secondary antibody (Invitrogen) was subsequently applied at 1:200 dilution. Controls were performed by omitting the primary antibody. Quantification was performed blindly by an independent investigator and is shown as a mean of number of CD3+ cells within five representative fields per lung.

**Intra-alveolar coagulopathy after LTx—thrombin antithrombin complexes**

Thrombin antithrombin complexes (TATc) were measured in bronchoalveolar lavage (BAL) fluid collected at 4 h after reperfusion. BAL was collected using a flexible fiber-optic video bronchoscope. A 20 mL aliquot of normal saline was instilled into a left lower lobe segmental bronchus and aspirated immediately with low suction (usual recovery was 10–15 mL). Cell-free supernatants were stored at –80°C until analysis. TATc were measured using an ELISA technique (Behringwerke AG, Marburg, Germany) as per manufacturer’s instructions. TATc levels were normalized by total protein content in the BAL fluid sample.

**Advantages of Normothermic Lung Preservation**

**Tight junction integrity before and after transplantation**

For zona occludens-1 (ZO-1) immunofluorescent staining, cryosections of pig lung tissue collected before and after the preservation period and after transplantation were mounted onto glass slides, fixed in precooled acetone for 5 min at room temperature and stained with anti-ZO-1 FITC-conjugated antibody (Invitrogen) at 1:100 dilution overnight at 4°C. After mounting with Gel/Mount medium (Biomed, Foster City, CA), sections were examined under a fluorescent microscope.

**Ex vivo gene transfer studies**

In another set of experiments, pig lungs \( n = 2 \) were transfected ex vivo with a second-generation (E1, E3 deleted) adenoviral vector (serotype 5) under the control of a cytomegalovirus promoter and containing the reporter green fluorescent protein (GFP) gene (AdCMV-GFP). This gene transfer study was designed to assess maintenance of preserved, active metabolic function during normothermic preservation.

The vector was constructed at the Gene Transfer Vector Core of the University of Iowa College of Medicine, Iowa City, IA. At the beginning of EVLP, the vector was diluted in 10 mL of normal saline. A flexible fiber-optic bronchoscope (Olympus® BF type 1T20, Tokyo, Japan) was inserted through the trachea and a catheter was inserted through the bronchoscope channel, which was used to deliver 1 mL into each segmental bronchus. After vector delivery, a recruitment maneuver to a PawP of 25 cmH₂O was performed and lungs were ventilated with 10 mL/kg of tidal volume and 12 breaths per min for 15 min to allow for homogenous distribution of the vehicle throughout the lung segments. GFP gene transfer efficiency was assessed by immunofluorescent staining of lung tissue biopsies obtained 12 h after ex vivo gene delivery. Cryosections from frozen tissues were prepared at 7µm using a cryostat and mounted onto glass slides. Samples were subjected to air drying for 45 min, fixed in –20°C precooled acetone for 5 min at room temperature and then blocked with 10% goat serum diluted in PBS. Primary antibody (anti-GFP) was incubated overnight at 4°C, then washed and a secondary fluorescent antibody was added for 1 h at room temperature. Nuclei were stained blue under ultraviolet excitation using the nuclear counterstain 4,6-diamidino-2-phenylindole dihydrochloride (Roche Diagnostics GmbH, Mannheim, Germany) applied for 10 min just before the fluorescent mounting media (DAKO). For the detection of GFP expression, anti-GFP antibody (Abcam, Cambridge, UK) at 1:500 dilution was used in conjunction with Alexa Fluor 555 goat anti-rabbit IgG secondary antibody (Invitrogen; 1:200 dilution). Two lungs that were perfused but not transplanted served as controls.

**Statistical analysis**

All data are expressed as mean ± standard error of the mean. For functional data comparison after transplantation between CSP and EVLP two-tailed Student’s unpaired t test was performed. Comparison of acute lung injury scores and T-lymphocyte infiltration between the two groups was performed using the nonparametric Mann-Whitney U test.

**Results**

**Lung function after transplantation**

After 24 h of preservation and 4 h posttransplant reperfusion, the PaO₂/FiO₂ was significantly higher in the EVLP group when compared with CSP group (Figure 2A; EVLP: 531 ± 43 mmHg; CSP: 244 ± 49 mmHg; from blood samples collected from left pulmonary veins; \( p < 0.01 \). Similar PaO₂/FiO₂ results were obtained from arterial blood gases collected after occlusion of the right (contra-lateral) PA (EVLP: 487.6 ± 44.7 mmHg; CSP: 169.8 ± 45.06 mmHg;
Pig lungs were recovered and stored in Perfadex® solution (Vitrolife, Sweden) at 4°C for 12 h (baseline). After this period of CSP, lungs were either maintained in CSP for 12 h or transferred to the EVLP system for an additional 12 h. Lung function was assessed during EVLP and hourly after transplantation. (A) PaO$_2$/FiO$_2$ was significantly higher 4 h after transplantation in the EVLP group when compared with CSP group (*p < 0.01). Note that lung oxygenation function was excellent during EVLP and it was comparable to posttransplant function. (B) The PawP was significantly lower in the EVLP group (**p < 0.001). (C) Lung edema was markedly lower in the EVLP lungs compared with the CSP lungs, as measured using W/D weight ratio of the lung tissue (*p < 0.01).

Lung oxygenation function during and at the end of 12 h of EVLP was excellent and, importantly, reflected posttransplant function in this group (Figure 2A; start of EVLP ΔpO$_2$: 502 ± 35 mmHg; end of EVLP ΔpO$_2$: 494 ± 48 mmHg; and after 4 h of transplantation pO$_2$: 531 ± 43 mmHg). PawP was significantly lower in the EVLP group 4 h after transplantation (Figure 2B; EVLP: 21 ± 0.4 mmHg; CSP: 25.7 ± 0.6 mmHg; p < 0.001). Similarly, the W/D weight ratio of the lungs measured 4 h after transplantation was significantly lower in the EVLP lungs compared with the CSP lungs (Figure 2C; EVLP W/D: 6.2 ± 0.3, CSP W/D: 8.2 ± 0.2; p < 0.01).

Acute lung injury after LTx
Lungs preserved using EVLP had significantly lower acute lung injury scores when compared with the CSP group (Figure 3A; EVLP: 3.5 ± 1.3 mmHg, CSP: 8 ± 0.4 cmH$_2$O; p = 0.02). Since recipient T cells have been previously shown to be a contributing component during the early phases of reperfusion injury (29), we also studied infiltration of CD3$^+$ T-lymphocytes into the lung tissue after transplantation. The number of CD3$^+$ T-lymphocytes was significantly less in the lung tissue of the EVLP group (Figure 3B; EVLP: 26.0 ± 7.3 mmHg, CSP: 62.6 ± 16 cmH$_2$O CD3$^+$ cells per field; p = 0.04).

Intra-alveolar coagulopathy
TATc in the BAL obtained 4 h after transplantation were significantly less in EVLP group (Figure 4; EVLP: 3.06 ± 1.99 mmHg, CSP 72.85 ± 18.57 ng/mg; p = 0.02).

Alveolar blood barrier integrity—ZO-1 cellular distribution
Normal lung tissue as well as lungs preserved with CSP for 12 h demonstrated intact ZO-1 subjunctional cytoplasmic plaques on immunostaining (Figure 5A). However, at the end of 24 h of CSP and 4 h after transplantation, ZO-1 staining was completely lost in the CSP group (Figure 5B). In contrast, lungs subjected to 12 h of EVLP maintained intact peripherally localized ZO-1 staining at the end of the 24 h preservation and after transplantation (Figure 5C).

Ex vivo gene transfer studies
Transbronchial GFP gene transfer was achieved during EVLP as demonstrated by intense and diffuse distribution of the transgene product along the alveolar walls by 12 h after ex vivo transbronchial gene delivery (Figure 6A). Samples from nontransfected EVLP lungs showed no GFP expression (Figure 6B).

Discussion
This study is the first demonstration that EVLP at normothermia provides improved extended lung preservation over CSP alone. Carrel and Lindbergh described the concept of ‘culture of whole organs’ in 1935 (30), but until recently lung perfusion systems were primarily used to study lung physiology in animals. Generally, experimental work in isolated lung perfusion systems has shown progressive deterioration of lung function. Interventions aimed at improving lung function generally have partially improved the inexorable downward slope of lung function over time.
Improved circuit technologies and the development of a lung specific perfusion solution (Steen™ solution) led to the revisitation of the EVLP concept in the 21st century, specifically as a tool to briefly evaluate lungs from DCD (20–22,31). More recently, a case series (n = 6) of successful transplantation after ex vivo assessment (2 h EVLP) of initially unsuitable human donor lungs was reported (32). Extension of normothermic ex vivo perfusion beyond the timeframe for evaluation and into the timeframe needed for preservation and repair of poorly functioning lungs is the next level of lung preservation that we strive to achieve.

The design of our study mirrors clinical practice where lungs recovered for transplantation would require an intermediate period of aerobic cold ischemic preservation (herein simulated as 12 h) to be transported back to the transplant center. Taking advantage of the ability to rein-state an active metabolic state, injured lungs could then be assessed, treated and reassessed in the EVLP system and a decision for transplantation suitability could be made. Thus, we examined whether the EVLP system could safely extend the preservation period up to further 12 h at a near physiologic conditions. The conventional CSP for the entire 24 h served as a control.

To determine the effects of prolonged EVLP after a period of ischemia, three critical features of acute lung injury (33) were studied: (1) alveolar blood barrier integrity (ZO-1 tight junction protein); (2) inflammation (cellular infiltration); and (3) intra-alveolar coagulation (TATc).

The pulmonary barrier has three compartments: blood, interstitium, and the alveolar space. Acute lung injury after transplantation is characterized by a diffuse inflammatory process with damage to the alveolar cell barrier together with leukocyte infiltration resulting in extravasation of vascular fluid (33). In concert with a break in the alveolar epithelial cell barrier, the filling of alveolar spaces (via paracellular gaps) by fluid and inflammatory cells leads to hypoxemia and respiratory failure. We thus wanted to determine the ability of EVLP to maintain the integrity of blood-gas alveolar barrier (34). To evaluate this, ZO-1 was studied. This cytoplasmic scaffolding protein of the junctional complex links the actin cytoskeleton to tight junction proteins such as occludin and maintains epithelial barrier integrity, keeping the air space dry for efficient gas exchange (34–36). The cytoplasmic dispersion of ZO-1 can be used as an injury marker, and its reassocation with cellular tight junctions is indicative of recovery of cytoskeletal integrity (37).
ZO-1 degradation during ischemia was previously demonstrated in kidneys (38) and in alveolar epithelial cells subjected to hypoxia in vitro (39). In our studies, ZO-1 integrity was evident in CSP lungs up to 12 h of ischemia. However, by prolonging CSP to a 24 h period, degradation of ZO-1 in the areas of cell–cell contact occurred. In contrast, transferring the lungs to the EVLP system after 12 h of CSP prevented ZO-1 degradation, suggesting that normothermic perfusion of the donor lungs prevents ongoing ischemic damage to cell tight junction. The mechanism by which EVLP better protects ischemia-induced ZO-1 injury still remains unknown and further studies are required to shed light on this finding.

Histological markers of reperfusion injury reflected by lung edema, hemorrhage and leukocyte infiltration was also significantly attenuated in the EVLP group. Previous studies have shown that T cells infiltrate lung grafts within 1 h of reperfusion and mediate lung injury within the subsequent 12 h (29). In this study, we found that T-cell infiltration was decreased in EVLP lungs. In addition to less inflammation, there was significant attenuation of intra-alveolar coagulation in perfused lungs as represented here by decreased TATc in the BAL. While hypercoagulability has not been thoroughly explored in reperfusion injury after LT, in patients with other forms of acute lung injury, intra-alveolar activation of the coagulation cascade with the deposition of fibrin along the injured alveolar surface has been well described (40,41). One recent hypothesis is that the exposure of alveolar epithelial cells to proinflammatory mediators decreases alveolar epithelial activation of protein C, which is an endogenous natural anticoagulant responsible for balancing levels of intra-alveolar thrombin (42). Interestingly, a clinical study showed that lower postoperative protein C and higher PAI-1 (antifibrinolytic protease) plasma levels were associated with PGD after LT (43).

Although simple and effective, CSP has its limitations in terms of preservation length and ability to maintain a viable organ devoid of ischemic injury. Despite this, good donor lungs do tolerate up to 12 h of aerobic cold ischemia, and thus we do not believe that in the near future, warm machine preservation will be required for transportation, which would add to the overall complexity of the procedure. Rather, we envision that EVLP should be used as a platform to treat and repair injured donor lungs once they arrive in the respective transplant center. By mimicking the lung’s natural physiological environment and by providing oxygen and other substrates necessary for active metabolism, normothermic lung perfusion may offer the next step in lung preservation. Pretransplantation treatment of donor lungs such as the use of pharmacologic agents to reduce pulmonary edema and inflammation or the use of gene therapy to better prepare the organ to deal...
with the reperfusion and subsequent immunologic insults will be the major goals of the future. To that end, we have shown here that in contrast to the known inefficient gene transfer efficiency during cold preservation (44–46), EVLP provided sufficient metabolic function for effective gene transfer and protein production along the alveolar walls. While this represents a proof of concept, the small number of ex vivo gene transfer experiments \( n = 2 \) dictates that further studies to examine the efficiency and characterization of gene transfer during EVLP are required.

In conclusion, EVLP attenuates ongoing injury associated with prolonged ischemia. We have demonstrated that the addition of 12 h normothermic EVLP provides superior preservation to CSP alone for a 24 h period. EVLP lungs demonstrated significantly better function, decreased edema, attenuated histological reperfusion injury and better preserved tight junctions of alveolar epithelial cells. This study represents the experimental foundation for the first clinical trial using normothermic EVLP, which is currently being carried out at our institution.

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