Published in final edited form as:
J Thorac Cardiovasc Surg. 2012 November ; 144(5): 1208–1216. doi:10.1016/j.jtcvs.2012.07.056.

Ex Vivo Rehabilitation of Non-Heart-Beating Donor Lungs in a Preclinical Porcine Model: Delayed Perfusion Results in Superior Lung Function

Daniel P. Mulloy, MD[a,*], **Matthew L. Stone, MD**[a,*], **Ivan K. Crosby, MBBS**[a], **Damien J. LaPar, MD**[a], **Ashish K. Sharma, MBBS**[a], **David V. Webb, MD**[b], **Christine L. Lau, MD**[a], **Victor E. Laubach, PhD**[a], and **Irving L. Kron, MD**[a]

[a]Department of Surgery, University of Virginia Health System, Charlottesville, Virginia

[b]Department of Pathology, University of Virginia Health System, Charlottesville, Virginia

Abstract

Objectives—*Ex vivo* lung perfusion (EVLP) is a promising modality for the evaluation and treatment of marginal donor lungs. The optimal timing of EVLP initiation and potential for rehabilitation of donor lungs with extended warm-ischemic times is unknown. This study compares the efficacy of different treatment strategies for uncontrolled non-heart-beating donor lungs.

Methods—Mature swine underwent hypoxic arrest followed by 60 minutes of no-touch warm-ischemia. Lungs were harvested and flushed with 4°C Perfadex®. Three groups (n=5/group) were stratified according to preservation method: cold-static preservation (CSP: 4 hrs 4°C storage), immediate EVLP (I-EVLP: 4 hrs EVLP at 37°C), and delayed EVLP (D-EVLP: 4 hrs cold storage followed by 4 hrs EVLP). EVLP groups were perfused with Steen solution™ supplemented with heparin, methylprednisolone, cefazolin, and an adenosine 2A receptor agonist. Lungs then underwent allotransplantation and four hours of recipient reperfusion prior to allograft assessment for resultant ischemia-reperfusion injury.

Results—Donor blood oxygenation (PO_2:FiO_2) prior to euthanasia was not different between groups. Oxygenation after transplantation was significantly higher in the D-EVLP group compared to the I-EVLP or CSP groups. Mean airway pressure, pulmonary artery pressure, and expression of IL-8, IL-1β, and TNF-α were all significantly reduced in the D-EVLP group. Importantly, post-transplant oxygenation exceeded acceptable clinical levels only in D-EVLP lungs.

Conclusions—Uncontrolled non-heart-beating donor lungs with extended warm-ischemia can be reconditioned for successful transplantation. The combination of CSP *and* EVLP present in the D-EVLP group was necessary to obtain optimal post-transplant function. This finding, if confirmed clinically, will allow expanded use of non-heart-beating donor lungs.

Corresponding Author: Matthew L. Stone, MD, Department of Surgery, University of Virginia Health System, P.O. Box 801359, Charlottesville, VA 22908, Phone: 434-924-9297, Fax: 434-924-1218, mstone@virginia.edu.
*Equal contributors

Introduction

Lung transplantation is a lifesaving treatment for patients with end-stage pulmonary disease; however, its success is limited by significant donor organ shortages. To address this growing problem, many centers now utilize a limited number of marginal or extended criteria heart-beating (HB) donor lungs. Recently, transplantation of lungs from non-heart-beating (NHB) donors has gained renewed interest as a potential mechanism to alleviate donor organ shortages. NHB donors are classified by Maastricht category according to the circumstances of expiration (I – dead on arrival to the hospital, II – failed resuscitation, III – withdrawal of life support, awaiting cardiac arrest, IV – cardiac arrest in brain-dead donor), and further described as uncontrolled (categories I and II) and controlled (categories III and IV) donors[1]. Unfortunately, several case series using NHB donor lungs for transplantation have shown higher rates of primary graft dysfunction, bronchiolitis obliterans, and mortality in comparison to HB donor lungs, and accurately predicting post-transplantation function of NHB donors lungs has proven difficult in part due to variation in warm ischemic times[2–4].

Ex vivo lung perfusion (EVLP) is a technique of normothermic acellular lung perfusion for both donor lung assessment and rehabilitation *ex vivo*[5,6]. The promise of this technique has been demonstrated in recent human clinical trials with marginal donor lungs (Maastricht III and IV), yet questions remain regarding the optimal timing of EVLP, potential application as a platform for therapeutic delivery, and rehabilitation potential for Maastricht category I and II donor lungs[7,8].

The purpose of this study was to determine, using a preclinical porcine transplant model, if lungs from uncontrolled NHB donors (Maastricht category I) with extended warm ischemic times could be rehabilitated to an acceptable functional status for subsequent successful transplantation. We hypothesized that initiation of EVLP immediately after NHB donor lung explantation would minimize cold ischemic time, allow for rapid initiation of directed donor lung treatment, and result in superior outcomes after lung transplantation when compared to either cold static preservation alone or delayed initiation of EVLP after a period of cold static preservation.

Materials and Methods

Animals

The University of Virginia's Institutional Animal Care and Use Committee reviewed and approved all aspects of this study. Humane animal care was observed in accordance with the "Guide for Care and Use of Laboratory Animals" (National Institutes of Health publication no. 85–23, revised 1985).

Study Groups

Mature domestic swine of both sexes (20–38 kg) were randomized throughout the study among 3 different study groups (n=5/group) stratified according to donor lung preservation method. Donor swine from all groups underwent hypoxic arrest followed by 60 minutes of no-touch warm ischemia. The cold-static preservation (CSP) group underwent lung procurement and 4 hours of storage in 4°C Perfadex® (Vitrolife Inc., Denver, CO), a commercially available preservative solution widely used for human lung transplantation. Left donor lungs were subsequently transplanted into size-matched recipients. The CSP group served as the ischemia-reperfusion injury control. The immediate EVLP (I-EVLP) group underwent lung procurement and 4 hours of immediate normothermic EVLP, followed by transplantation. The delayed EVLP (D-EVLP) group received a combination of both strategies and underwent lung procurement, 4 hours of storage in 4°C Perfadex, followed by 4 hours of normothermic EVLP prior to transplantation. After left lung

transplantation, lungs of all animals were perfused *in vivo* for a continuous 4-hour period, after which parameters of lung function and injury were assessed as described below.

Porcine Arrest and Donor Lung Procedure

Donor animals were anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg), ventilated with room air, and intubated. After intubation, anesthesia was maintained for 10 minutes with 3% isoflurane and the lungs were ventilated with 100% fraction of inspired oxygen (FiO_2) using a volume control ventilator (Harvard Apparatus, Boston MA) at a tidal volume of 8 mL/kg, respiratory rate of 14–18 breaths/minute, and a positive end-expiratory pressure (PEEP) of 5.0 cm H_2O. Each swine was placed in the supine position and continuous electrocardiographic monitoring was initiated. After 10 minutes, a baseline arterial blood gas sample was obtained via percutaneous withdrawal from the carotid artery. Following arterial blood gas measurement, the ventilator was disconnected, the endotracheal tube was occluded, and the animal was euthanized via hypoxic arrest. Electrocardiographic activity was monitored until full cessation of electric activity occurred at which point the animal was declared dead. Following expiration, the animal was kept at room temperature for a 60-minute no-touch period. Ventilation was then resumed with per pre-euthanasia settings, and lung harvest was performed using a standard operative technique as previously described[9].

Briefly, donor animals underwent a median sternotomy and pericardiotomy to expose the heart, great vessels, and both lungs. The main pulmonary artery (PA) was cannulated with a cardioplegia cannula (Sarns, Ann Arbor, MI), the PA was cross-clamped proximal to this point, and prostaglandin E1 (10mg/kg) was injected directly into the main PA. The left atrial appendage was incised, the superior and inferior vena cavae were ligated, and antegrade flushing of both lungs was performed with 1.5L of 4°C Perfadex. Due to the prolonged warm ischemic time, clot formation was commonly encountered in the left atrium and pulmonary veins. Heparin (10,000 IU) was added to the Perfadex flush for all animals. During the flush, the left atrium was incised and efforts were made to manually remove as much clot as possible from the atrium and pulmonary veins (Figure 1A). Upon completion of the antegrade flush, both lungs were inflated with 100% FiO_2 to tidal volume and the heart and both lungs were explanted *en bloc*. The heart was then removed with care to preserve a generous atrial cuff and the lungs were retrograde flushed with an additional 500 mL of 4°C Perfadex to remove remaining clot from the pulmonary vasculature. For the CSP and D-EVLP groups, the lungs were placed in a standard preservation bag and stored in 4°C Perfadex. For the I-EVLP group, lungs were placed directly on *ex vivo* perfusion.

Porcine *Ex Vivo* Lung Perfusion

EVLP was performed as described previously[10] and based upon earlier studies by Cypel et al.[6]. Briefly, a funnel-shaped plastic cannula (Vitrolife) was sewn to the left atrial cuff, a plastic cannula (Vitrolife) was secured into the main pulmonary artery (PA), and an 8-0 endotracheal tube with the balloon removed was secured into the trachea (Figure 1B). The EVLP circuit consisted of a bypass centrifugal pump (Medicus, Minneapolis, MN), membrane oxygenator, heat exchanger, venous reservoir (Sorin Group, Arvada, CO), and polyethylene tubing. Lungs were transferred to an XVIVO chamber (Vitrolife), and retrograde flow was initiated through the left atrium to de-air the pulmonary vasculature and flush any remaining clot. The PA cannula was then connected and antegrade flow was commenced at 0.1 L/min. EVLP was performed using acellular Steen solution™ (Vitrolife Inc., Denver, CO), a commercially available preservative solution designed for *ex vivo* lung assessment, supplemented with 10,000 IU heparin (APP Pharmaceuticals, Schaumburg, IL), 500 mg cefazolin (Apotex Corp., Weston, FL), 500 mg methylprednisolone (Pfizer, New York, NY), and 3.0 ng/kg/min. continuous infusion of ATL-1223 (Dogwood

Pharmaceuticals, Charlottesville, VA), a selective adensonine 2A receptor (A2AR) agonist. ATL-1223 was included to optimize the rehabilitative potential of EVLP because we have previously established the potent anti-inflammatory effects of A2AR agonism in lung ischemia-reperfusion injury and the potential protective advantages afforded with EVLP-directed A2AR agonism treatment[10–14]. The perfusate was slowly warmed to 37°C over a 30-minute period as the flow was titrated up to the target of 40% of the estimated cardiac output (estimated cardiac output = 100 ml/kg). When the perfusate reached 32°C, ventilation was initiated with room air at a tidal volume of 8 mL/kg, respiratory rate of 8 breaths per minute, and a positive end-expiratory pressure (PEEP) of 5.0 cm H_2O. After initiation of ventilation, a mixture of 6% O_2, 8% CO_2, and 86% N_2 was infused into the membrane oxygenator to de-oxygenate the pulmonary artery perfusate and allow for accurate measurement of lung oxygenation capability. At 1 and 4 hours after initiation of EVLP, the lungs were ventilated with 100% FiO_2 for 10 minutes and a sample of the perfusate was taken from the left atrial return for arterial blood gas analysis. At the conclusion of the 4-hour EVLP period, the lungs were removed from the EVLP circuit and an antegrade flush was performed with 500mL of 4°C Perfadex. The lungs were separated and the right lung was discarded, while the left lung was stored in 4°C Perfadex prior to transplantation.

Porcine Left Lung Recipient Transplantation

Transplantation of the left lung was performed as described previously[9]. Briefly, a left thoracotomy and left pneumonectomy were performed in a size-matched recipient swine after heparin administration (5,000 IU). The donor lung was then brought into the field and the donor-to-recipient left bronchus anastomosis was completed in continuous fashion followed by the donor-to-recipient pulmonary artery anastomosis. A portion of the left atrial appendage was then isolated with a side-biting vascular clamp, the atrium was incised, and the donor atrial cuff was then anastomosed to the recipient atrial appendage in continuous fashion. The vascular and airway clamps were then removed to establish reperfusion and ventilation of the transplanted lung.

Lung Physiology

All transplanted lungs underwent 4 hours of *in vivo* reperfusion. During reperfusion, hourly arterial blood gas measurements were obtained in addition to mean arterial pressure, heart rate, and mean pulmonary artery pressure via a Swan-Ganz catheter. Pulmonary function post-transplantation was additionally evaluated with mean airway pressure measurements obtained utilizing a pressure monitoring line attached to the endotracheal tube. After 3.5 hours, the endotracheal tube was advanced into the left main-stem bronchus and the right main pulmonary artery was occluded by a preplaced vessel loop, thereby establishing isolated perfusion and ventilation of the transplanted left lung that continued for 30 minutes. Upon isolation, ventilator settings were changed to a tidal volume of 5 ml/kg (equivalent to approximately 10 mL/kg on the isolated left lung) and the rate was increased to maintain minute ventilation. Upon conclusion of the 30-minute isolated reperfusion period, additional assessment of the recipient swine and the transplanted donor lung was performed by way of final arterial blood gas, pulmonary function, and PA catheter measurements before explantation of the transplanted lung.

Cytokine Measurement

Bronchoalveolar lavage (BAL) of the upper lobe of the left lung was performed immediately after explantation in all groups using 40 mL normal saline. BAL samples were centrifuged at 1800 rpm for 8 minutes and the supernatant was then stored at −80°C. Quantification of cytokine levels in BAL fluid was assessed using a commercially available porcine cytokine multiplex immunoassay kit (RayBiotech, Norcross, GA).

Histopathology and Lung Injury Severity Score

The lower lobe was fixed in 10% buffered formalin via tracheal inflation to 25 cmH_20. Three tissue specimens were obtained from standardized locations within the lung parenchyma with subsequent paraffin embedding and hematoxylin-eosin staining. Lung sections were blindly assessed by a lung pathologist and graded according to total neutrophil counts per high power field, the extent of alveolar edema, and the degree of interstitial infiltration. A score on a scale of 0–3 was assigned for each section using previously reported criteria[9]: neutrophils per high powered field (score: 0=<5, 1=6–10, 2=11–20, 3=>20), alveolar edema (score: 0=<5%, 1=6–25%, 2=26=50%, 3=>50%), and interstitial infiltration (score: 0=none, 1=minimal, 2=moderate, 3=severe), and a composite score was obtained by summation of these three criteria (0–9). The average of the three sample values for each variable was obtained for group comparisons.

Statistical Analysis

All experimental methodology was designed to test the null hypothesis that no significant differences in the degree of injury would be observed despite different preservation strategies. Independent, pairwise group comparisons were performed utilizing the unpaired Student's *t* test. Experimental results are reported as mean ± standard deviation. Significance was defined as $p<0.05$.

Results

Lung Function

Although we hypothesized that I-EVLP lungs would function better after transplantation, we instead observed that lung function was significantly improved in the D-EVLP group when compared to either CSP or I-EVLP (Figure 2). Compared to the CSP group, final blood oxygenation was significantly higher in the D-EVLP group (D-EVLP: 508.7±90.4 vs. CSP: 159.4±70.1, $p<0.001$), while mean pulmonary artery pressure (D-EVLP: 22.4±5.9 vs. CSP: 30.2±2.8 mmHg, $p=0.03$) and mean airway pressure (D-EVLP: 6.8±0.8 vs. CSP: 11.8±1.0 mmHg, $p<0.001$) were significantly reduced. The D-EVLP group also demonstrated significantly higher blood oxygenation (D-EVLP: 508.7±90.4 vs. I-EVLP: 228.5±130.7, $p<0.01$), significantly lower mean airway pressure (D-EVLP: 6.8±0.8 vs. I-EVLP: 10.1±2.6 mmHg, $p=0.03$), and reduced mean pulmonary artery pressure (D-EVLP: 22.4±5.9 vs. I-EVLP: 29.2±11.0 mmHg, $p=0.26$) compared to the I-EVLP group. Blood oxygenation (I-EVLP: 228.5±130.7 vs. CSP: 159.4±70.1, $p=0.33$), pulmonary artery pressure (I-EVLP: 29.2±11.0 vs. CSP: 30.2±2.8 mmHg, $p=0.85$), and mean airway pressure (I-EVLP: 10.1±2.6 vs. CSP: 11.8±1.0 mmHg, $p=0.27$) did not significantly differ between the CSP and I-EVLP groups. There were no significant differences observed among all groups in age, weight, mean total anastomotic time, mean arterial pressure, or heart rate (data not shown).

All groups had similar pre-euthanasia donor blood oxygenation levels. A divergence was, however, present thereafter as a function of the preservation strategy utilized (Figure 2C). In the CSP group blood oxygenation dropped from 419.4±108.9 pre-euthanasia to 159.4±70.1 post-transplantation. Similarly, pre-euthanasia blood oxygenation in the I-EVLP group was high (406.1±120.0) and perfusate oxygenation levels steadily decreased throughout the EVLP period to a final blood oxygenation level of 228.5±130.7 after transplantation. Oxygenation in the D-EVLP group started high (358.2±151.1) and improved after the period of cold-static preservation and one-hour period of EVLP (450.3±95.0). Oxygenation levels in the D-EVLP group continued to improve through the end of EVLP (477.6±88.7) and reached a final blood oxygenation level of 508.7±90.4 after transplantation.

Proinflammatory Cytokine Expression

The effect of the various preservation strategies on the expression of proinflammatory cytokines in BAL fluid was assessed at the end of reperfusion (Figure 3). In parallel with the lung function results, the D-EVLP group had significantly decreased expression of IL-1β (D-EVLP: 259.5±87.3 vs. CSP: 851.1±262.8 pg/ml, p=0.001), IL-8 (D-EVLP: 112.1±74.8 vs. CSP: 531.5±331.4 pg/ml, p=0.03), and TNF-α (D-EVLP: 233.2±84.9 vs. CSP: 1050.2±353.8 pg/ml, p=0.001) compared to the CSP group. Additionally, the D-EVLP demonstrated significantly decreased IL-1β (D-EVLP: 259.5±87.3 vs. I-EVLP: 566.3±202.1 pg/ml, p=0.01), IL-8 (D-EVLP: 112.1±74.8 vs. I-EVLP: 248.4±96.3 pg/ml, p=0.04), and TNF-α (D-EVLP: 233.2±84.9 vs. I-EVLP: 568.1±218.2 pg/ml, p=0.01) in comparison to the I-EVLP group. As shown in Figure 3, the I-EVLP group had intermediate cytokine levels with significantly decreased expression of TNF-α (p=0.03) compared to CSP group. In addition, IL-1β (p=0.09) and IL-8 (p=0.10) were also decreased, but this did not reach statistical significance.

Gross and Histologic Evidence of Lung Injury

Overall, the D-EVLP group had improved histologic and gross appearance (Figure 4) in addition to decreased lung injury scores (Figure 5) compared to both the CSP and I-EVLP groups. Individual parameters of the lung injury severity score demonstrated less injury in the D-EVLP group compared to the CSP group with significantly fewer neutrophils per high-powered field (D-EVLP: 1.6±0.7 vs. CSP: 2.6±0.6, p=0.04) (Figure 5). The D-EVLP group also had decreased alveolar edema (D-EVLP: 0.1±0.2 vs. CSP: 0.5±0.6, p=0.13) and less interstitial infiltrate (D-EVLP: 0.9±0.6 vs. CSP: 1.7±1.0, p=0.12) compared to the CSP group, but these variables did not achieve statistical significance. Additionally, composite lung injury severity scores were less for the D-EVLP group compared to the CSP group (D-EVLP: 2.5±1.2 vs. CSP: 4.8±2.0, p=0.06). The D-EVLP group had values less than the I-EVLP group for each parameter yet achieved statistical significance only for the grade of interstitial infiltrate (D-EVLP: 0.9±0.5 vs. I-EVLP: 1.7±0.4, p=0.03). The I-EVLP group did not demonstrate a statistically significant reduction in any lung injury parameter in comparison to the CSP group.

Discussion

This study used a preclinical porcine lung transplantation model to demonstrate that EVLP can rehabilitate lungs from uncontrolled NHB donors (Maastricht category I) with extended warm ischemic times to an acceptable functional status for successful transplantation. Interestingly, the results of this study, regarding the timing and initiation of EVLP and inclusion of a CSP period, were contrary to our initial hypothesis. These results demonstrate that a combination of 4 hours of CSP followed by 4 hours of normothermic EVLP is significantly more protective than either 4 hours of CSP or 4 hours of immediate EVLP alone. This conclusion is supported by the finding that the D-EVLP group demonstrated significantly improved lung physiology, decreased proinflammatory cytokine expression, decreased neutrophil infiltration and conserved lung histology despite a doubling of the preservation period. Although the I-EVLP group did demonstrate decreased proinflammatory cytokine expression and a trend toward improved lung physiology compared to the CSP group, these differences were not significant, unlike those observed with the D-EVLP group. Therefore, while our hypothesis was incorrect, we are encouraged by the finding that excellent post-transplantation lung function can be achieved with uncontrolled NHB donor lungs with extended warm-ischemic times through the use of delayed normothermic EVLP. Additionally, we believe that this study provides the foundation for further research directed toward the goal of expanding the limited human donor pool through the use of uncontrolled NHB donation.

One aspect of this study that warrants further discussion is the use of ATL-1223, a selective A2AR agonist, in the perfusion circuit for both EVLP groups. Our laboratory has extensive experience with the use of selective A2AR agonists in animal models of transplantation[11–13]. It is well-established that specific A2AR activation decreases the release of TNF-α and other proinflammatory cytokines, down-regulates adhesion molecules (P-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1), and blocks neutrophil activation and infiltration[13,15,16]. We have recently demonstrated in a HB donor porcine transplant model that ATL-1223 attenuates ischemia-reperfusion injury after transplantation[9]. In addition, we have shown that administration of a selective A2AR agonist in the EVLP circuit effectively decreases inflammation and improves lung function in a porcine EVLP non-transplant model[10]. The present study was designed to evaluate the capability for rehabilitation of uncontrolled NHB donor lungs using EVLP. Given the known benefits of ATL-1223 treatment along with the unknown level of lung dysfunction incurred by either the mechanism of donor euthanasia or the subsequent 60-minute warm ischemic time, we included ATL-1223 in the EVLP circuit for both groups to enhance the probability of achieving or exceeding acceptable lung function outcomes. We recognize that one limitation of this study is that we are unable to make specific conclusions on the role of ATL-1223, just as we are unable to make any conclusions on the roles of other agents that are currently utilized in standard EVLP protocols. Both the I-EVLP and D-EVLP groups underwent identical exposure to ATL-1223 and our results demonstrate that D-EVLP is superior to I-EVLP in the setting of uncontrolled NHB donor lung transplantation.

Conventional wisdom in organ transplantation holds that cold ischemic time is damaging to donor organs, and the United Network for Organ Sharing (UNOS) divides the United States into 11 geographic transplant regions with the goal of minimizing transportation times to limit donor organ preservation periods[17,18]. We found that a period of cold-static preservation prior to EVLP was beneficial in optimizing organ function and minimizing inflammation despite the prolonged preservation period this strategy employed. While our findings include an extended preservation period, the benefits of this timeframe are supported in the literature. In a 2011 human clinical EVLP trial, lungs from marginal and NHB Maastricht category III and IV donors underwent EVLP after a period of CSP. EVLP donor lungs yielded equivalent post-transplantation outcomes compared to standard non-marginal donor lungs despite an average of 10.9 hours of total preservation time (versus 6.2 hours for the non-marginal lungs)[8]. Additionally, in the first reported series investigating the use of EVLP in transplantation for standard HB donors, donor lungs underwent CSP followed by EVLP with a total preservation time averaging 17.4 hours, and excellent post-transplantation lung function was achieved in all patients[19].

The benefit of hypothermia is well-established in clinical protocols for organ preservation and protection. Hypothermia is the current clinical standard for neuroprotection during cardiac surgery and is an emerging treatment for patients following cardiac arrest. The benefit of hypothermia is proposed to involve mechanisms beyond the slowing of metabolism with colder temperatures[20,21]. Multiple mechanisms for hypothermic protection have been previously described and include: decreased free radical production[22], inhibition of apoptosis[23], suppression of the inflammatory response via inhibition of neutrophil infiltration[24], reduction of lipid peroxidation and leukotriene production[25], and attenuation of the nitric oxide response[26]. Applying this knowledge to the present study, we propose that establishment of lung hypothermia prior to the initiation of EVLP serves to effectively arrest the ongoing tissue damage and inflammatory response associated with the extended warm ischemic time. Subsequent acellular EVLP with the addition of anti-inflammatory compounds including methylprednisolone and ATL-1223 provides a therapeutic environment for tissue rehabilitation prior to transplantation. In contrast, transplantation directly after CSP results in an amplification of the ischemia-reperfusion inflammatory

cascade, resulting in subsequent donor organ injury. Similarly, we propose that initiation of EVLP immediately at the conclusion of the warm-ischemic period results in a sustained, hostile pro-inflammatory environment within the donor lung, ultimately leading to further organ damage. Certainly, further study of the cellular and molecular mechanisms behind the observed results will be required as our study is limited by the absence of mechanistic data both throughout the preservation process and following graft reperfusion. Despite this limitation, these data demonstrate that functional parameters during the EVLP period provide predictive information regarding the potential graft function and candidacy for transplantation. With this understanding, the results of this study demonstrate that the combination of CSP followed by normothermic EVLP effectively reduces the inflammatory response and tissue injury associated with NHB donor lung transplantation.

The findings of the present study support future studies to define the rehabilitative mechanisms and optimal timing of both CSP and EVLP. In the present study, both the donor organ harvest and subsequent storage procedure were consistent with current clinical HB donor protocols. In addition, 4 hours of CSP was chosen to approximate the average donor lung cold ischemic time. Therefore, with proper approvals and consent, transplant centers could adopt EVLP as a strategy to further study the assessment and rehabilitation of uncontrolled Maastricht category I and II NHB donor lungs. In addition to further study, our findings support the continued inclusion of CSP in clinical EVLP rehabilitation protocols. As we embark on the clinical adoption of EVLP, our findings provide promise for the inclusion of uncontrolled NHB donors in future human clinical trials for lung transplantation.

In conclusion, delayed *ex vivo* lung perfusion after a period of cold-static preservation is an effective strategy for the rehabilitation of uncontrolled NHB donor lungs for subsequent transplantation in a preclinical porcine transplant model. Clinical adoption of this lung preservation strategy could be easily applied to current organ procurement protocols. If clinically correlated in human NHB donor lungs, the findings of the present study will lead to improved human lung transplantation by allowing for safe transplantation of uncontrolled NHB donor lungs, thus significantly decreasing the donor organ shortage and saving lives.

Acknowledgments

NIH T32HL007849 and Roche Organ Transplant Research Foundation #58054922 (ILK)

We wish to thank Tony Herring, Cindy Dodson, and Sheila Hammond for their technical and surgical assistance.

References

1. Kootstra G, Daemen JH, Oomen AP. Categories of non-heart-beating donors. Transplant Proc. 1995; 27:2893–4. [PubMed: 7482956]
2. de Antonio DG, Marcos R, Laporta R, et al. Results of clinical lung transplant from uncontrolled non-heart-beating donors. J Heart Lung Transplant. 2007; 26:529–34. [PubMed: 17449425]
3. Gomez-de-Antonio D, Campo-Canaveral JL, Crowley S, et al. Clinical lung transplantation from uncontrolled non-heart-beating donors revisited. J Heart Lung Transplant. 2012; 31:349–53. [PubMed: 22306439]
4. Wigfield CH, Love RB. Donation after cardiac death lung transplantation outcomes. Curr Opin Organ Transplant. 2011; 16:462–8. [PubMed: 21897244]
5. Steen S, Sjoberg T, Pierre L, et al. Transplantation of lungs from a non-heart-beating donor. Lancet. 2001; 357:825–9. [PubMed: 11265950]
6. Cypel M, Yeung JC, Hirayama S, et al. Technique for prolonged normothermic ex vivo lung perfusion. J Heart Lung Transplant. 2008; 27:1319–25. [PubMed: 19059112]

7. Cypel M, Rubacha M, Yeung J, et al. Normothermic ex vivo perfusion prevents lung injury compared to extended cold preservation for transplantation. Am J Transplant. 2009; 9:2262–9. [PubMed: 19663886]
8. Cypel M, Yeung JC, Liu M, et al. Normothermic ex vivo lung perfusion in clinical lung transplantation. N Engl J Med. 2011; 364:1431–40. [PubMed: 21488765]
9. LaPar DJ, Laubach VE, Emaminia A, et al. Pretreatment strategy with adenosine A2A receptor agonist attenuates reperfusion injury in a preclinical porcine lung transplantation model. J Thorac Cardiovasc Surg. 2011; 142:887–94. [PubMed: 21762933]
10. Emaminia A, Lapar DJ, Zhao Y, et al. Adenosine A(2)A agonist improves lung function during ex vivo lung perfusion. Ann Thorac Surg. 2011; 92:1840–6. [PubMed: 22051279]
11. Gazoni LM, Laubach VE, Mulloy DP, et al. Additive protection against lung ischemia-reperfusion injury by adenosine A2A receptor activation before procurement and during reperfusion. J Thorac Cardiovasc Surg. 2008; 135:156–65. [PubMed: 18179933]
12. Gazoni LM, Walters DM, Unger EB, et al. Activation of A1, A2A, or A3 adenosine receptors attenuates lung ischemia-reperfusion injury. J Thorac Cardiovasc Surg. 2010; 140:440–6. [PubMed: 20398911]
13. Sharma AK, Laubach VE, Ramos SI, et al. Adenosine A2A receptor activation on CD4+ T lymphocytes and neutrophils attenuates lung ischemia-reperfusion injury. J Thorac Cardiovasc Surg. 2010; 139:474–82. [PubMed: 19909990]
14. Koroskenyi K, Duro E, Pallai A, et al. Involvement of adenosine A2A receptors in engulfment-dependent apoptotic cell suppression of inflammation. J Immunol. 2011; 186:7144–55. [PubMed: 21593381]
15. Linden J. Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection. Annu Rev Pharmacol Toxicol. 2001; 41:775–87. [PubMed: 11264476]
16. Lisle TC, Gazoni LM, Fernandez LG, et al. Inflammatory lung injury after cardiopulmonary bypass is attenuated by adenosine A(2A) receptor activation. J Thorac Cardiovasc Surg. 2008; 136:1280–7. discussion 7–8. [PubMed: 19026816]
17. Snell GI, Rabinov M, Griffiths A, et al. Pulmonary allograft ischemic time: an important predictor of survival after lung transplantation. J Heart Lung Transplant. 1996; 15:160–8. [PubMed: 8672519]
18. Thabut G, Mal H, Cerrina J, et al. Graft ischemic time and outcome of lung transplantation: a multicenter analysis. Am J Respir Crit Care Med. 2005; 171:786–91. [PubMed: 15665320]
19. Ingemansson R, Eyjolfsson A, Mared L, et al. Clinical transplantation of initially rejected donor lungs after reconditioning ex vivo. Ann Thorac Surg. 2009; 87:255–60. [PubMed: 19101308]
20. Weng Y, Sun S. Therapeutic hypothermia after cardiac arrest in adults: mechanism of neuroprotection, phases of hypothermia, and methods of cooling. Crit Care Clin. 2012; 28:231–43. [PubMed: 22433485]
21. Lampe JW, Becker LB. State of the art in therapeutic hypothermia. Annu Rev Med. 2011; 62:79–93. [PubMed: 20854174]
22. Globus MY, Busto R, Lin B, et al. Detection of free radical activity during transient global ischemia and recirculation: effects of intraischemic brain temperature modulation. J Neurochem. 1995; 65:1250–6. [PubMed: 7643104]
23. Yang D, Guo S, Zhang T, et al. Hypothermia attenuates ischemia/reperfusion-induced endothelial cell apoptosis via alterations in apoptotic pathways and JNK signaling. FEBS Lett. 2009; 583:2500–6. [PubMed: 19596001]
24. Akriotis V, Biggar WD. The effects of hypothermia on neutrophil function in vitro. J Leukoc Biol. 1985; 37:51–61. [PubMed: 3917485]
25. Dempsey RJ, Combs DJ, Maley ME, et al. Moderate hypothermia reduces postischemic edema development and leukotriene production. Neurosurgery. 1987; 21:177–81. [PubMed: 2821445]
26. Fischer S, Renz D, Wiesnet M, et al. Hypothermia abolishes hypoxia-induced hyperpermeability in brain microvessel endothelial cells. Brain Res Mol Brain Res. 1999; 74:135–44. [PubMed: 10640684]

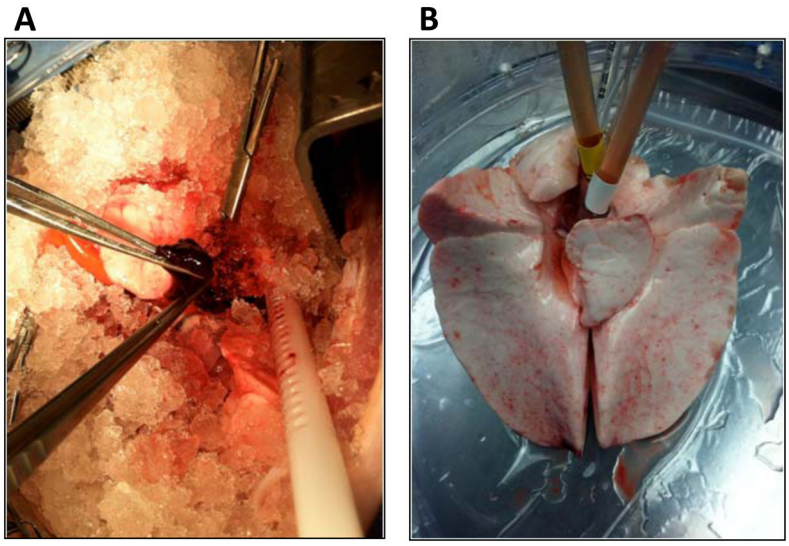

Figure 1.
Non-heart-beating donor lung harvest and *ex vivo* lung perfusion (EVLP) procedures. A. Fresh clot being removed from the donor left atrium and pulmonary veins during the antegrade lung flush. B. Donor lungs pictured during EVLP with the pulmonary artery cannula, left atrial cannula, and endotracheal tube secured in place.

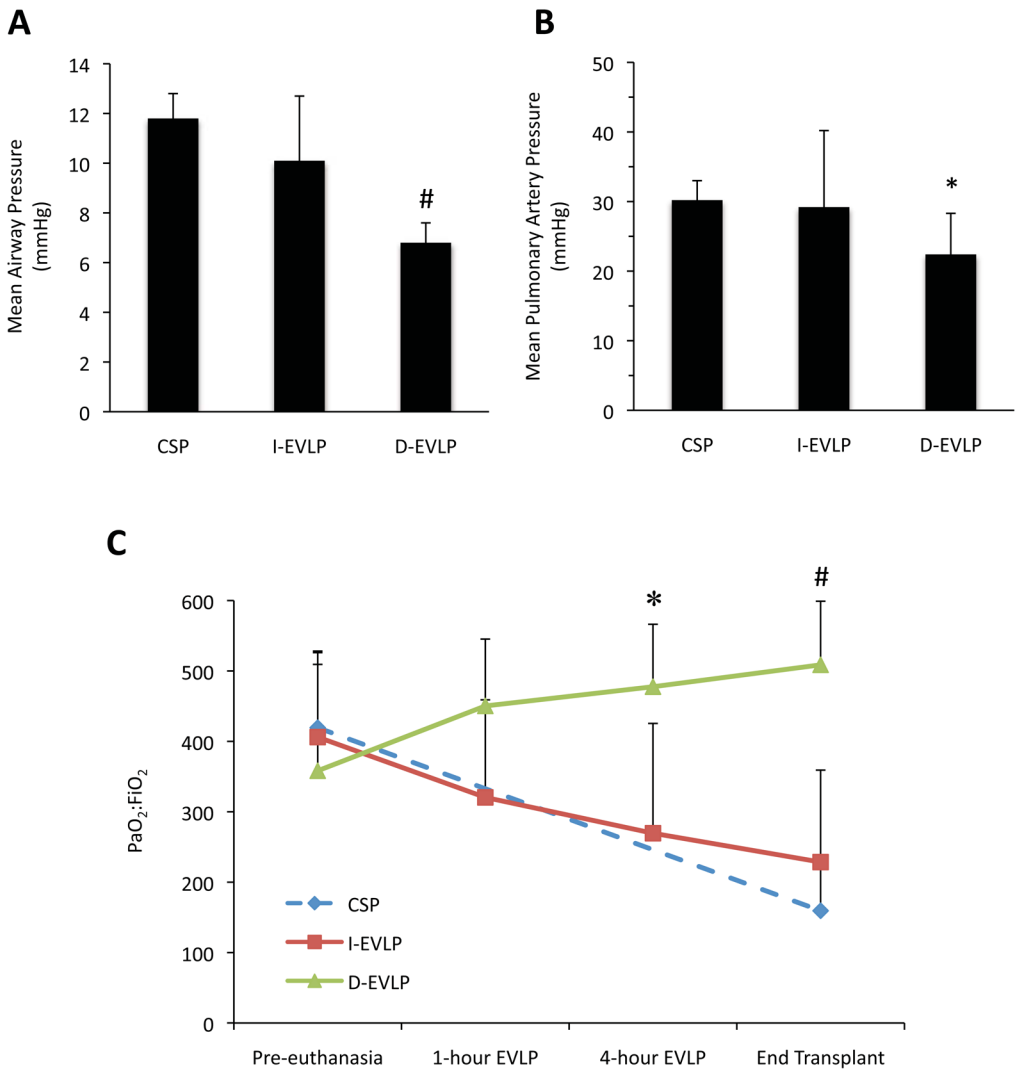

Figure 2.

Lung physiology after 4 hours of reperfusion. A. Mean airway pressure (mmHg). B. Mean pulmonary artery (PA) pressure (mmHg). C. Oxygenation as represented by the ratio of partial pressure of oxygen to fraction of inspired oxygen (PO_2:FiO_2) measured at four time points: donor arterial blood oxygenation prior to euthanasia, EVLP-perfusate oxygenation at one hour, EVLP-perfusate oxygenation at four hours, and recipient arterial blood oxygenation at the end of transplantation. Groups: cold-static preservation (CSP), immediate *ex vivo* lung perfusion (I-EVLP), and delayed *ex vivo* lung perfusion (D-EVLP). A., B.: *$p < 0.05$ vs. CSP, #$p < 0.05$ vs. CSP and I-EVLP. C.: *$p < 0.05$ vs. I-EVLP, #$p < 0.05$ vs. CSP and I-EVLP.

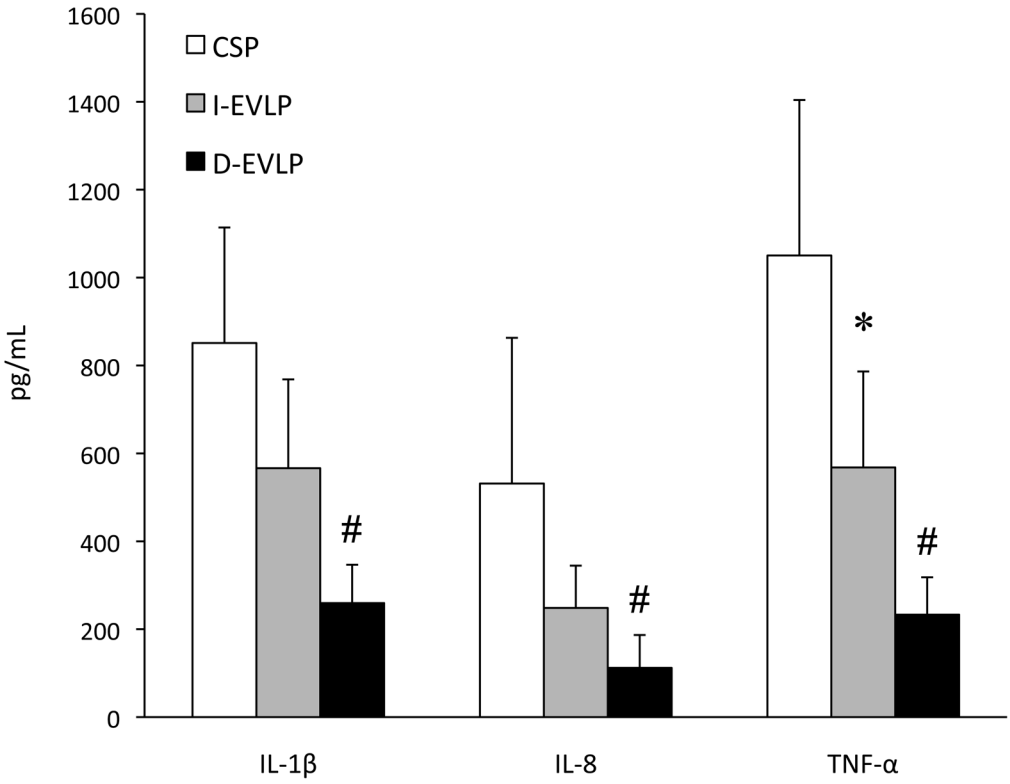

Figure 3.
Mean proinflammatory cytokine levels (pg/mL) in bronchoalveolar lavage fluid at the end of 4 hours of reperfusion. Groups: cold-static preservation (CSP), immediate *ex vivo* lung perfusion (I-EVLP), and delayed *ex vivo* lung perfusion (D-EVLP). *$p < 0.05$ vs. CSP. #$p < 0.05$ vs. CSP and I-EVLP.

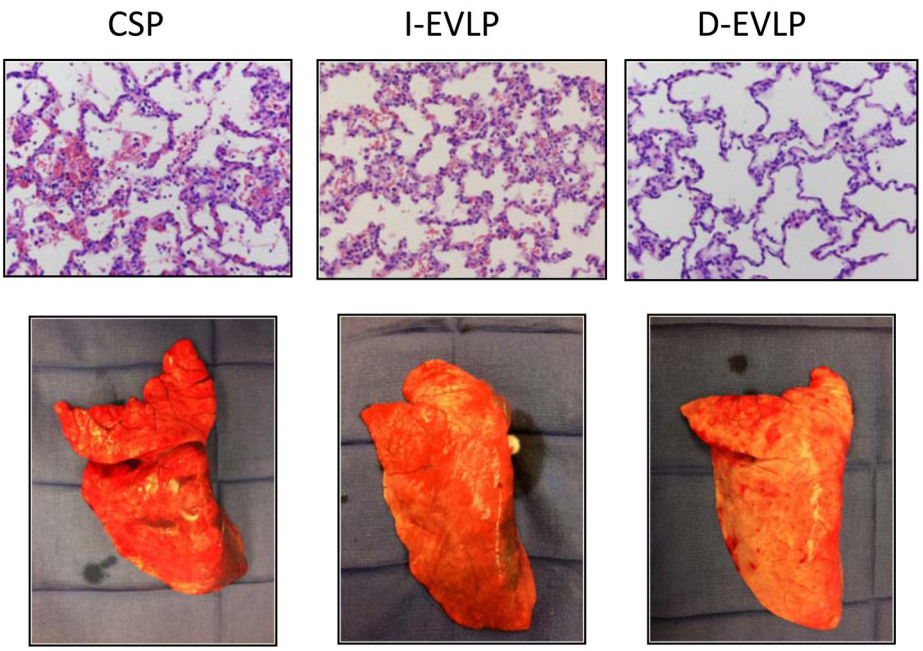

Figure 4.
Representative histologic (*top*, hematoxylin-eosin sections, 20X) and gross (*bottom*) appearance of lungs after 4 hours of reperfusion. Groups: cold-static preservation (CSP), immediate *ex vivo* lung perfusion (I-EVLP), and delayed *ex vivo* lung perfusion (D-EVLP).

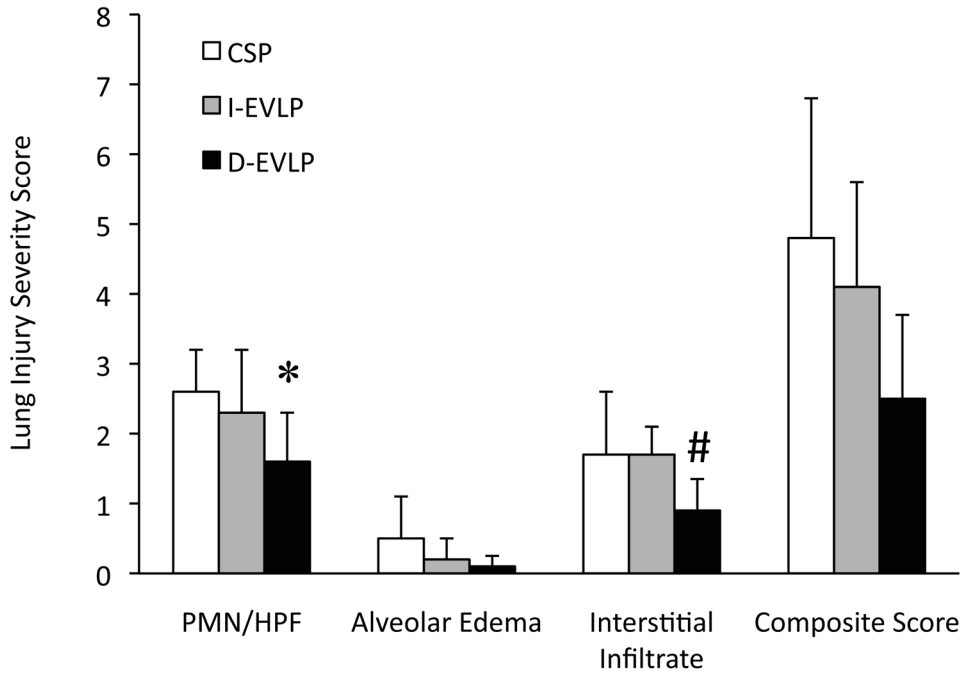

Figure 5.

Mean lung injury severity scores by histology. A score on a scale of 0–3 was assigned for each section: neutrophils per high powered field (score: 0=<5, 1=6–10, 2=11–20, 3=>20), alveolar edema (score: 0=<5%, 1=6–25%, 2=26=50%, 3=>50%), interstitial infiltration (score: 0=none, 1=minimal, 2=moderate, 3=severe), and a composite score obtained by summation of these three criteria (0–9). Groups: cold-static preservation (CSP), immediate *ex vivo* lung perfusion (I-EVLP), and delayed *ex vivo* lung perfusion (D-EVLP). *$p < 0.05$ vs. CSP. #$p < 0.05$ vs. I-EVLP.