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RESEARCH ARTICLE | *Translational Physiology*

A method for translational rat ex vivo lung perfusion experimentation

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Ohsumi A, Kanou T, Ali A, Guan Z, Hwang DM, Waddell TK, Juvet S, Liu M, Keshavjee S, Cypel M. A method for translational rat ex vivo lung perfusion experimentation. *Am J Physiol Lung Cell Mol Physiol* 319: L61–L70, 2020. First published April 1, 2020; doi:10.1152/ajplung.00256.2019.—The application of ex vivo lung perfusion (EVLP) has significantly increased the successful clinical use of marginal donor lungs. While large animal EVLP models exist to test new strategies to improve organ repair, there is currently no rat EVLP model capable of maintaining long-term lung viability. Here, we describe a new rat EVLP model that addresses this need, while enabling the study of lung injury due to cold ischemic time (CIT). The technique involves perfusing and ventilating male Lewis rat donor lungs for 4 h before transplanting the left lung into a recipient rat and then evaluating lung function 2 h after reperfusion. To test injury within this model, lungs were divided into groups and exposed to different CITs (i.e., 20 min, 6 h, 12 h, 18 h and 24 h). Experiments involving the 24-h-CIT group were prematurely terminated due to the development of severe edema. For the other groups, no differences in the ratio of arterial oxygen partial pressure to fractional inspired oxygen (Pa_{O_2}/FI_{O_2}) were observed during EVLP; however, lung compliance decreased over time in the 18-h group ($P = 0.012$) and the Pa_{O_2}/FI_{O_2} of the blood from the left pulmonary vein 2 h after transplantation was lower compared with 20-min-CIT group ($P = 0.0062$). This new model maintained stable lung function during 4-h EVLP and after transplantation when exposed to up to 12 h of CIT.

ex vivo lung perfusion; ischemia-reperfusion injury; lung transplantation; organ preservation; rat

INTRODUCTION

Globally, the number of lung transplants performed annually has almost doubled within the last ten years (25). This significant increase may be in part due to the development and refinement of the normothermic ex vivo lung perfusion (EVLP) technique (5). The advent of this technology has allowed for the use of organs that would have otherwise been deemed unsuitable for transplantation (6). Indeed, EVLP provides surgeons the ability to functionally assess extended criteria lungs before making the critical decision of implanting them into a recipient. Although EVLP is effective for the assessment of lung quality, its true potential lies in the opportunity to provide therapeutic strategies to the graft to recondition and enhance the organ. Examples of interventions trialed to date within the EVLP system include gene therapy, mesenchymal stem cell therapy, and exogenous surfactant therapy (3, 11, 14). The first clinical trial demonstrating the safety and efficacy of EVLP was based on large animal (porcine) experimentation (5, 6). Large animal models are ideal for trialing novels therapies due to the compelling preclinical data that can be gathered; however, costs and logistical complexities can hinder their feasibility. In comparison, the use of small animal models allows for increased sample sizes and shortened experimental times at a fraction of the cost, which make them particularly well suited for screening new treatments.

To date, various attempts have been made to develop a rat EVLP model (1, 7–9, 13, 15–17, 19, 22, 23). The major limitation of these models for use translationally is the inability to achieve stable perfusion for extended periods of time, which is a key component of the EVLP technique (5). In 2014, Noda et al. (16) reported the first rat model of ex vivo lung perfusion followed by transplantation. The lungs were perfused for 4 h after just 1 h of cold ischemic time (CIT), and the data showed deterioration of lung function during EVLP. Soon after that, Nelson et al. (15) published a new rat EVLP technique; however, the lungs showed also increase in weight after just 1 h of perfusion, and the study did not provide any ventilation data. In 2016, a rat perfusion model developed by Bassani et al. (1) showed deterioration of lung function before lung recruitment, while their study did not provide any transplantation data.

To our knowledge, no previous studies have demonstrated stable donor function for at least 4 h in a rat EVLP model; as such, no good model exists that could be used to screen therapeutic interventions ex vivo. The aim of our study was to address this need by providing a new model for stable rat lung EVLP and to validate its appropriateness for viable lung transplantation after perfusion. We also investigated the effects of different lengths of CIT on donor lung quality to adapt our platform to model lung injury and therapeutic experimentation.

MATERIALS AND METHODS

Animal care. Male Lewis rats weighing 255 to 330 g (Charles River Laboratories Inc., Montreal, QC, Canada) were used. The animal use protocol was approved by the Animal Care Committee at University Health Network. All animals received humane care in compliance with the *Principles of Laboratory Animal Care* prepared by the National Society for Medical Research, the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication No. 86-23, revised 1996), and the *Guide to the Care and Use of Experimental Animals* published by the Canadian Council on Animal Care.

Donor lung procurement procedure. All donor rats were intraperitoneally injected with 50 mg/kg of ketamine (Vetalar, Bioniche Animal Health, Belleville, ON, Canada) and 5 mg/kg of xylazine (Rompun, Bayer Healthcare, Toronto, ON, Canada) for induction of

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anesthesia. After fully anesthetized, the animals were orotracheally intubated with a 14-gauge intravenous catheter, and the tracheal tube was connected to a ventilator (Harvard Model 683 Small Animal Ventilator, Harvard Apparatus Canada, Saint-Laurent, QC, Canada). Rats received volume-controlled ventilation at a rate of 60 breaths/min, with a tidal volume of 10 ml/kg, an inspired oxygen fraction (FI_{O_2}) of 0.5, and a positive end-expiratory pressure (PEEP) of 2 cmH_2O. Subsequently, a median laparotomy was performed and 400 USP units of sodium heparin were injected into the inferior vena cava (IVC). Thereafter, a median sternotomy was performed. To induce heart and lung block, the IVC and right atrium were incised along the apex of the left heart, and a 16-gauge intravenous catheter was inserted into the pulmonary trunk through an anterior incision in the right ventricular outflow tract. The lungs were then flushed with 20 ml of low potassium dextran glucose preservation solution (Perfadex, XVIVO Perfusion, Uppsala, Sweden) containing 10 μg/ml of prostaglandin E1. Immediately after flushing, the trachea was clamped at the end of inspiration to preserve the lungs in an inflated state. The heart and lung block was removed and placed in Perfadex at 4°C for storage. Graft lungs were stored for different times in each group after donor procurement: 20 min ($n = 4$); 6 h, 12 h ($n = 5$), 18 h ($n = 5$); and 24 h ($n = 2$).

Ex vivo lung perfusion procedure. The procedure is summarized in Table 1. Before perfusion, the circuit was primed with 150 ml of Steen solution (XVIVO Perfusion AB, Göteborg, Sweden) supplemented with 1,000 USP units of sodium heparin and 50 mg of methylprednisolone (SOLU-MEDROL, Pfizer, Inc., New York, NY). After cold storage, the inflow cannula was inserted into the main pulmonary artery (PA) with the perfusion at 10% of maintenance flow. We set this point as the initiation of perfusion. The drainage cannula was placed from the apex of the heart into the left atrium (LA) through the mitral valve and then connected to the circuit. The trachea was also cannulated. Then, the heart-lung block was applied to the Isolated Perfused Lung System for Rat and Guinea Pig (IPL-2; Harvard Apparatus, Holliston, MA; Hugo Sachs Elektronic, Hugstetten, Germany). The flow rate of perfusion was gradually increased to 1 h to reach the target flow rate according to our clinical EVLP protocol (5). We used 20% of the cardiac output (CO) as the maintenance flow rate to perfuse both lungs with an estimated CO of 75 ml/ml for 250 g rats (12, 16). LA pressure was also monitored and increased to 2 cmH_2O at the time when total flow was achieved. Twenty minutes after the initiation of perfusion, lungs were ventilated with ambient air (VCM-P, Harvard Apparatus, Holliston, MA) at an inspiratory/expiratory pressure of 10/5 cmH_2O and a rate of 40 breaths/min. Subsequently, a deoxygenation sweep was turned on with 6% O_2, 8% CO_2, and balanced N_2 using a gas exchange membrane (D-150 Hemofilter, Medsulfone, Italy). During EVLP, PA pressure, peak airway pressure, and airway flow were monitored continuously, and dynamic lung compliance and pulmonary vascular resistance (PVR) were analyzed in real time. Physiological data were gathered every 30 min. At 5 min before these assessments, the lungs were automatically expanded to an inflation pressure of 20 cmH_2O. Perfusate samples were taken every hour and used to measure pH, P_{CO_2}, P_{O_2}, electrolytes, glucose, and lactate. After the last assessment, the lungs were ventilated with 50% oxygen for 5 min for lung storage using a second ventilator (Harvard Model 683 Small Animal Ventilator). The ventilation conditions used were as follows: rate of ventilation = 40 breaths/min; tidal volume = the same volume as the end of EVLP; and PEEP = 2 cmH_2O. Thereafter, perfusion was stopped, and the trachea was clamped to maintain the lungs in an inflated state. Subsequently, on ice, the donor lungs were prepared by placing a cuff into the left PA, LA, and left main bronchus (MB) using 16-, 14-, and 14-gauge cuffs, respectively. Finally, the donor lungs were stored at 4°C in Perfadex until implantation.

Lung transplant procedures. The procedure that we used for rat lung transplantation has been previously described in detail (18). All recipient rats were anesthetized with an intraperitoneal injection of 60 mg/kg ketamine and 6 mg/kg xylazine. Under continued parenteral anesthesia, orotracheal intubation was accomplished. The tracheal tube was connected to a ventilator (Harvard Model 683 Small Animal Ventilator), and rats were ventilated under the following conditions: tidal volume = 10 ml/kg; respiratory rate = 60 breaths/min; PEEP = 2 cmH_2O; and $FI_{O_2} = 1.0$. The right jugular vein was dissected and a catheter was inserted. Then the continuous administration of 15 $mg \cdot kg^{-1} \cdot h^{-1}$ ketamine and 1.5 $mg \cdot kg^{-1} \cdot h^{-1}$ xylazine was started for maintenance; in addition, saline was administered at 1.5 ml/h for volume replacement. The right internal carotid artery was dissected, and a catheter was inserted to enable arterial blood pressure to be monitored continuously. A left thoracotomy was performed through the fifth intercostal space. After the hilar structures (PA, LA, and MB) were dissected, each was clamped with a microsurgical aneurysm clamp. The donor lung cuffs were placed into the corresponding recipient structures through each ventral incision, and anastomoses were secured with 7-0 polypropylene ties. Lungs were then reinflated and ventilated with 100% oxygen; next, blood was reintroduced by first unclamping the LA, followed by unclamping of the PA. Recipient rats were ventilated and reperfused for 2 h. Blood samples, to be used for blood gas analysis, were taken from the arterial line and left pulmonary vein (PV) using a 27-gauge needle directed toward the transplanted lungs. Lungs were removed for further examination and all rats were euthanized by exsanguination.

Wet-to-dry weight ratio. Right lung tissue after 4-h EVLP and left lung tissue at 2 h after reperfusion were used to calculate the wet-to-dry weight (W/D) ratio. Tissues were excised from the upper one-third of the lung. Wet weight (mg) was measured first; then, the tissue was dried for 3 days at 80°C before dry weight (mg) was measured. The W/D ratio was calculated as wet weight/dry weight.

Cytokines. After each blood gas analysis, 2 ml of blood were taken and centrifuged at 6,000 rpm for 5 min. Plasma and the middle one-third of each lung was collected and stored at −80°C until use.

Table 1. *Rat EVLP perfusion strategy*

Technique	Details
Perfusion	Constant flow perfusion
Perfusion flow	20% of estimated cardiac output (estimated cardiac output: 75 ml/kg for 250-g rats)
Perfusate	150 ml of Steen solution with 1,000 USP units of sodium heparin and 50 mg of methylprednisolone
LA pressure	3 cmH_2O
Ventilation	Pressure control ventilation
Inspiratory/expiratory pressure	10/5 cmH_2O
Respiratory rate	40 breaths/min
FI_{O_2}	room air
Recruitment maneuver	Up to 20 cmH_2O 5 min before assessment, every 30 min
Deoxygenation	Using a gas exchange membrane
Mixed gas	6% O_2, 8% CO_2, and balanced N_2

EVLP, ex vivo lung perfusion; LA, left atrium.

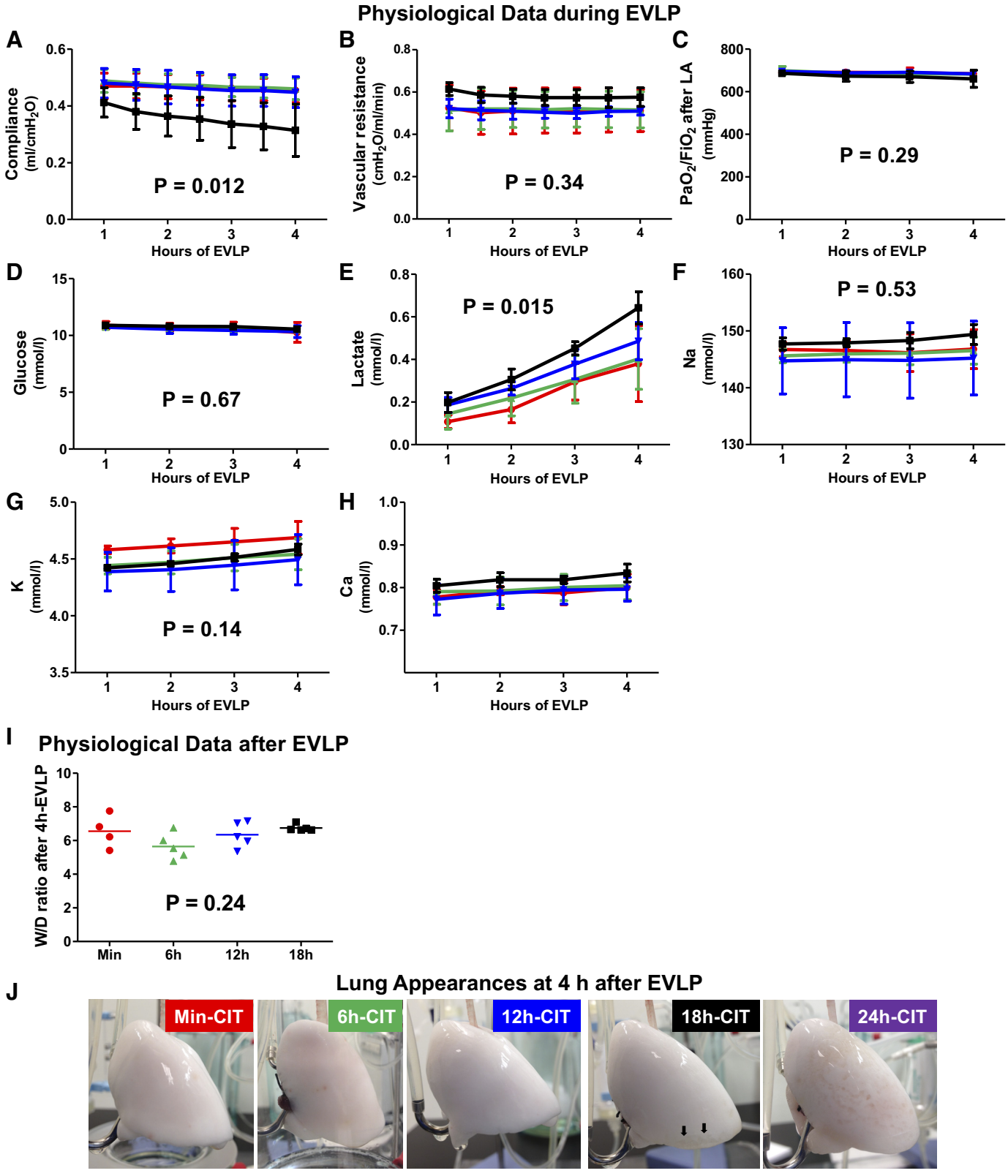

Fig. 1. *A–I*: lung function and blood gas analysis of donor lungs undergoing ex vivo lung perfusion (EVLP) after exposure to different cold ischemic times (CITs). Each CIT group is depicted in a different color: 20 min (20min-CIT) in red, 6 h (6h-CIT) in green, 12 h (12h-CIT) in blue, and 18 h (18h-CIT) in black. *J*: rat donor lung appearance after 4-h EVLP in each cold ischemic time (CIT) group. W/D, wet-to-dry weight.

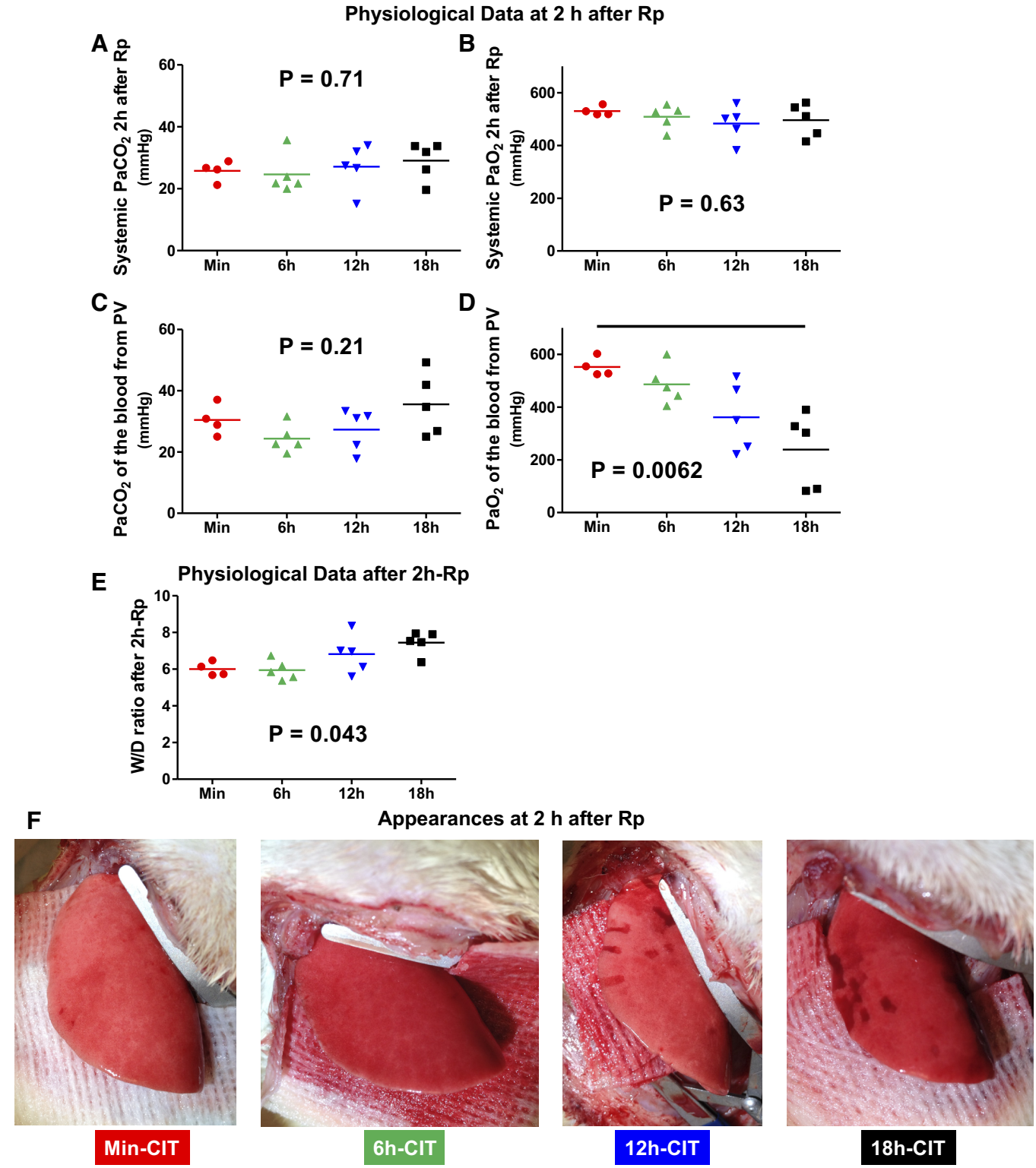

Fig. 2. *A–E*: arterial blood gas of donor lungs after 2-h reperfusion (Rp) for each cold ischemic time (CIT) group. *F*: rat donor lung appearance after 2-h reperfusion in each cold ischemic time (CIT) group. Arrows demonstrate areas of tissue edema. W/D, wet-to-dry weight.

Right lung tissue after 4 h EVLP and left lung tissue after 2-h reperfusion were homogenized, as described previously (10). Next, tissue was centrifuged and lysates were collected. Levels of interleukins were measured in these lysate samples, along with two additional samples: perfusate 4 h after EVLP and plasma 2 h after reperfusion. Interleukins that were measured were (IL)-1β, IL-2, IL-6, IL-10, and IL-17A and tumor necrosis factor-α (TNF-α); these were measured using a rat cytokine/chemokine magnetic bead panel (MILLIPLEX, EMD Millipore, Billerica, MA). Respective concentration levels in the tissue lysates were measured using a protein assay kit (Pierce BCA

Table 2. *Inflammatory mediators and soluble markers*

	Group				
	20-min-CIT (n = 4)	6-h-CIT (n = 5)	12-h-CIT (n = 5)	18-h-CIT (n = 5)	P Value
Interleukin-1β					
Perfusate after 4-h EVLP, pg/ml	4.85 ± 1.56	3.40 ± 2.36	3.55 ± 2.15	3.36 ± 1.67	0.56
Right lung tissue after 4-h EVLP, pg/mg protein	74.8 ± 61.6	110.3 ± 31.1	97.1 ± 61.6	165.2 ± 31.2	0.12
Left lung tissue after 2-h reperfusion, pg/mg protein	190.1 ± 216.1	295.8 ± 169.6	316.0 ± 285.0	454.5 ± 153.6	0.27
Interleukin-6					
Perfusate at after 4 h EVLP, pg/ml	297.1 ± 75.9	100.5 ± 89.2	247.0 ± 138.7	311.6 ± 237.6	0.11
Right lung tissue after 4 h EVLP, pg/mg protein	9.1 ± 18.1	7.3 ± 16.2	80.2 ± 179.3	137.1 ± 84.9	0.12
Left lung tissue after 2-h reperfusion, pg/mg protein	1,669.9 ± 1,441.9*	2,177.8 ± 1,204.4	2,960.3 ± 2,640.6	7,549.2 ± 3,086.8	0.026
M30, ng/ml					
Perfusate after 4-h EVLP	2.84 ± 0.61	3.00 ± 0.57	2.82 ± 0.39	2.91 ± 0.38	0.96
Plasma after 2-h reperfusion	5.04 ± 0.52	4.59 ± 0.44	5.75 ± 2.30	5.05 ± 1.40	0.57
M65, ng/ml					
Perfusate after 4-h EVLP	1.44 ± 0.62	1.19 ± 0.10	1.22 ± 0.33	1.61 ± 0.78	0.72
Plasma after 2-h reperfusion	1.43 ± 0.14	1.82 ± 0.68	2.10 ± 0.98	2.20 ± 0.44	0.20
HMGB1, ng/ml					
Perfusate after 2-h EVLP	13.06 ± 3.18	11.27 ± 2.73	9.75 ± 0.60	10.45 ± 2.61	0.30
Perfusate after 4-h EVLP	16.48 ± 3.70	12.84 ± 3.29	12.01 ± 1.13	13.32 ± 1.98	0.11
Plasma after 2-h reperfusion	4.52 ± 3.65	6.36 ± 6.33	48.81 ± 103.80	217.16 ± 287.81	0.17

Data are shown as means ± SD. EVLP, ex vivo lung perfusion; CIT, cold ischemic time. P values are for the differences between groups by Kruskal-Wallis test with Dunn's multiple comparison test. *Significant difference with the 18-h-CIT group.

Protein Assay Kit, Thermo Scientific, Rockford, IL) and were normalized to 1.0 mg of protein in the lung.

Cytokeratin 18 M30 neoepitope levels in perfusate and plasma. After the initiation of apoptosis, cytokeratin 18 is cleaved by caspases into three fragments, liberating a neoepitope that is specifically recognized by the M30 monoclonal antibody (20). An ELISA kit (Rat Cytokeratin 18-M30 ELISA Kit, MyBioSource, Inc., San Diego, CA) was used to measure M30-neoantigen levels in perfusate after 4-h EVLP and in plasma 2 h after reperfusion.

M65 levels in perfusate and plasma. M65 levels, which signify epithelial apoptosis and necrosis, were measured using an ELISA kit (Rat Cytokeratin 18-M65 ELISA Kit, MyBioSource, Inc., San Diego, CA) in perfusate after 4-h EVLP and in plasma after 2 h reperfusion.

HMGB1 levels in perfusate and plasma. The levels of high-mobility group box-1 (HMGB1) in perfusate after 4-h EVLP and in plasma after 2-h reperfusion were measured using an ELISA kit (HMGB1 ELISA; IBL International, GmbH, Hamburg, Germany).

TUNEL staining and cell count. Apoptotic cells were stained using the terminal deoxynucleotidyl transferase-mediated deoxyuridinetriphosphate nick-end labeling (TUNEL) technique (In Situ Cell Death Detection Kit, POD, Roche Diagnostics, GmbH, Mannheim, Germany). After TUNEL labeling, cell nuclei were labeled with DAPI, examined under the Nikon A1R Resonance Scanning Confocal (Nikon Instruments, Inc., Melville, NY) and imaged in a blinded fashion. Ten fields were randomly chosen and imaged per section at a ×200 magnification. Apoptosis was morphometrically quantified using ImageJ 1.48 and is expressed as a mean of the ratio of TUNEL-positive cells to total cells.

VE cadherin staining. VE-cadherin was chosen as a representative endothelial intercellular junction marker and stained using an anti-VE cadherin antibody (VE-cadherin/CD144 Antibody Thermo Fisher Scientific, Inc.). After addition of a primary antibody and fluorescence tagging with a secondary antibody, each slide was imaged using a confocal fluorescence microscope (Olympus BX50).

Zonula occludens-1 staining. For zonula occludens-1 (ZO-1) immunohistochemistry, formalin-fixed paraffin-embedded lung tissue sections were stained as previously described (3).

Statistical analysis. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). Values are presented as the means ± SD. Physiological data were analyzed by repeated measures ANOVA with Bonferroni posttests and Kruskal-Wallis test with Dunn's multiple comparison tests. For inflammatory mediators, soluble markers, and histological score analyses, Kruskal-Wallis test with Dunn's multiple comparison tests were used. $P < 0.05$ was considered to be statistically significant.

RESULTS

Baseline characteristics. Animal weight and warm ischemic time (implantation time) were similar across all groups (Supplemental Table S1; see https://doi.org/10.6084/m9.figshare.12501200.v1).

Lung function during EVLP. All donor lungs except for the 24-h-CIT group, where massive edema developed very quickly, were able to be perfused for 4 h. Compliance in the 18-h-CIT group gradually decreased and was significantly lower than those of the other groups (P = 0.012; Fig. 1*A*). While vascular resistance tended to be higher in the 18-h-CIT group, there were no significant differences among groups (P = 0.34; Fig. 1*B*). No significant differences existed among groups for donor lung oxygenation (P = 0.29; Fig. 1*C*) or glucose levels (P = 0.67; Fig. 1*D*). Lactate levels were significantly higher in the 18-h-CIT group (P = 0.015; Fig. 1*E*). W/D ratio showed no significant differences (Fig. 1*I*) among groups up to 18 h of CIT; however, the 24-h-CIT group showed very high W/D levels (9.16 and 7.98 after just 31 and 38 min of EVLP). These findings indicate that, in our model, 18-h CIT seems to be the limit of lung viability under EVLP.

Lung function and pulmonary edema after transplantation. All the donor lungs except the 24-h-CIT group were transplanted successfully. Systemic blood gas analysis showed no difference among the groups (P = 0.71 and 0.63, respectively; Fig. 2, *A* and *B*). However, when oxygenation capacity of the transplanted graft was assessed, the 18-h-CIT group had a significantly lower Pa_{O_2} in comparison to the 20-min-CIT group (P = 0.0062; Fig. 2*D*). W/D was also higher after 18-h CIT compared with 20-min CIT (P = 0.043; Fig. 2*E*). Of note, the decrease in PV oxygenation was gradual and directly related to the increase in CIT (Fig. 2*D*).

Inflammatory markers. IL-6 levels in the lung after reperfusion were significantly higher in the 18-h-CIT group (P =

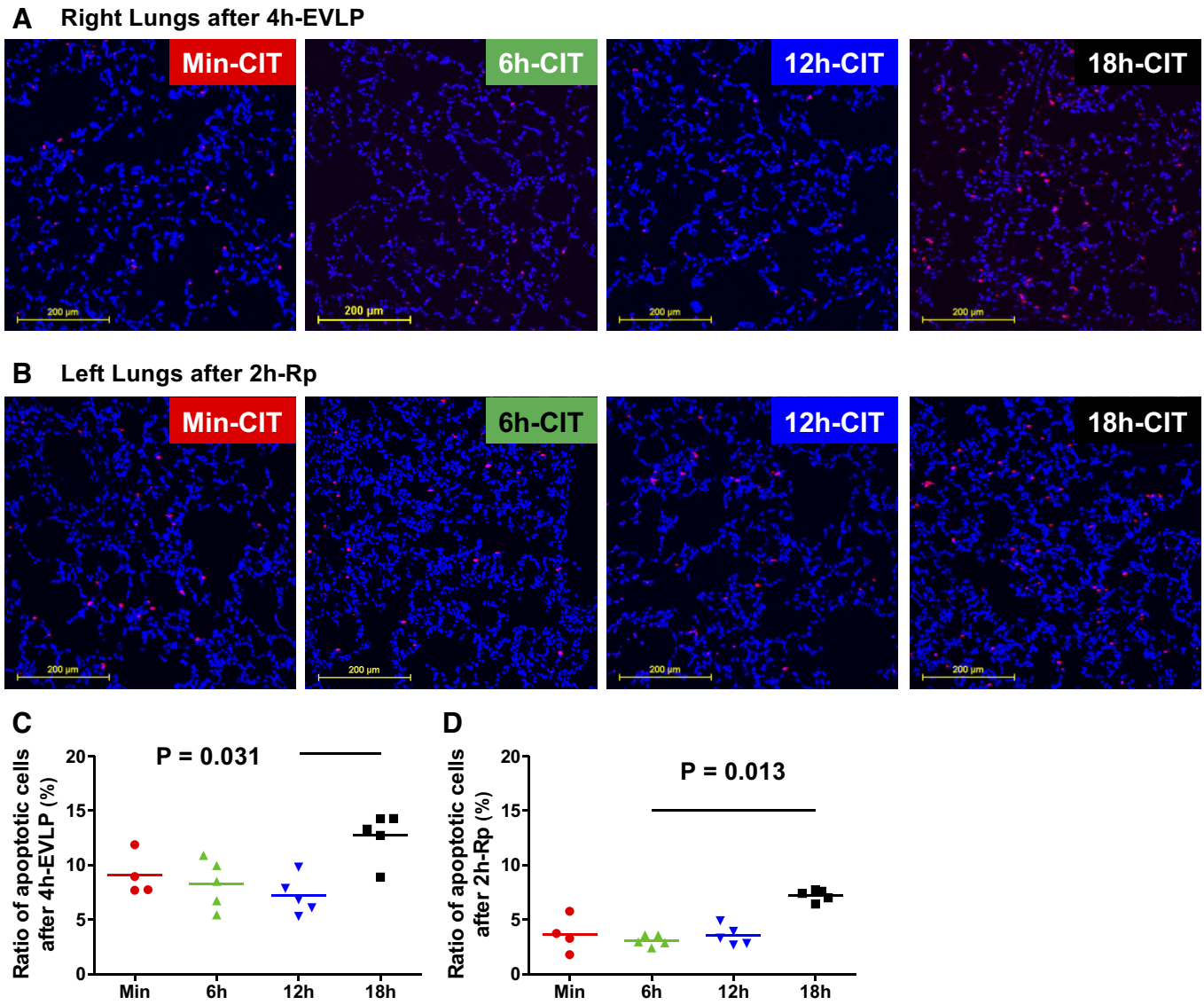

Fig. 3. *A* and *B*: TUNEL staining of the right lungs after 4-h ex vivo lung perfusion (EVLP) (*A*) and left transplanted lungs after 2-h reperfusion (Rp) (*B*). The lungs were exposed to different cold ischemic times (CITs): 20 min (20min-CIT), 6 h (6h-CIT), 12 h (12h-CIT), and 18 h (18h-CIT). *C* and *D*: the percentage of apoptotic cells (%) measured using TUNEL staining from right lungs after 4 h of EVLP under different CITs (*C*) and from left transplanted lungs after 2 h of reperfusion (*D*).

0.026; Table 2). IL1-β tended to be higher in the 18-h-CIT group; however, this was not statistically significant (Table 2). No significant differences were observed in the levels of other cytokines.

Cell death markers (M30, TUNEL, M65, and HMGB-1). More apoptotic cells were found in the 18-h-CIT group after both EVLP and reperfusion (post-EVLP: $P = 0.031$, Fig. 3, *A* and *C*; postreperfusion: $P = 0.013$, Fig. 3, *B* and *D*). This suggests that apoptosis occurs when CIT is longer than 12 h. No significant differences were found between groups for M30, M65, HMBG-1 at 2-h reperfusion and 4-h EVLP in plasma and perfusate, respectively (Table 2).

Tight junction of endothelial and epithelial cells. The expression of VE Cadherin after 4 h of EVLP was stable after up to 18 h of CIT; however, the 24-h-CIT group reduced expression after just 31 and 38 min of EVLP (Fig. 4, *A–E*). ZO-1 staining was preserved up to 18 h of CIT; while the 24-h-CIT group showed loss of epithelial tight junctions (Fig. 5, *A–E*). Taken together, these observations indicate that tight junctions of endothelial and epithelial cells are lost after 24 h of CIT leading to marked development of lung edema at commencement of EVLP.

DISCUSSION

EVLP is now an integral part of lung transplant programs around the world. To date, the main utility of EVLP has been in "testing" the quality of questionable donor organs from brain death and cardiac death donors. However, EVLP has even greater potential for use in the active treatment and repair of injured organs. Translational research relating to EVLP is predominantly carried out using an established swine model (5). This model, as for other large animal models, is associated with high costs and complex logistics. While many attempts have been made to

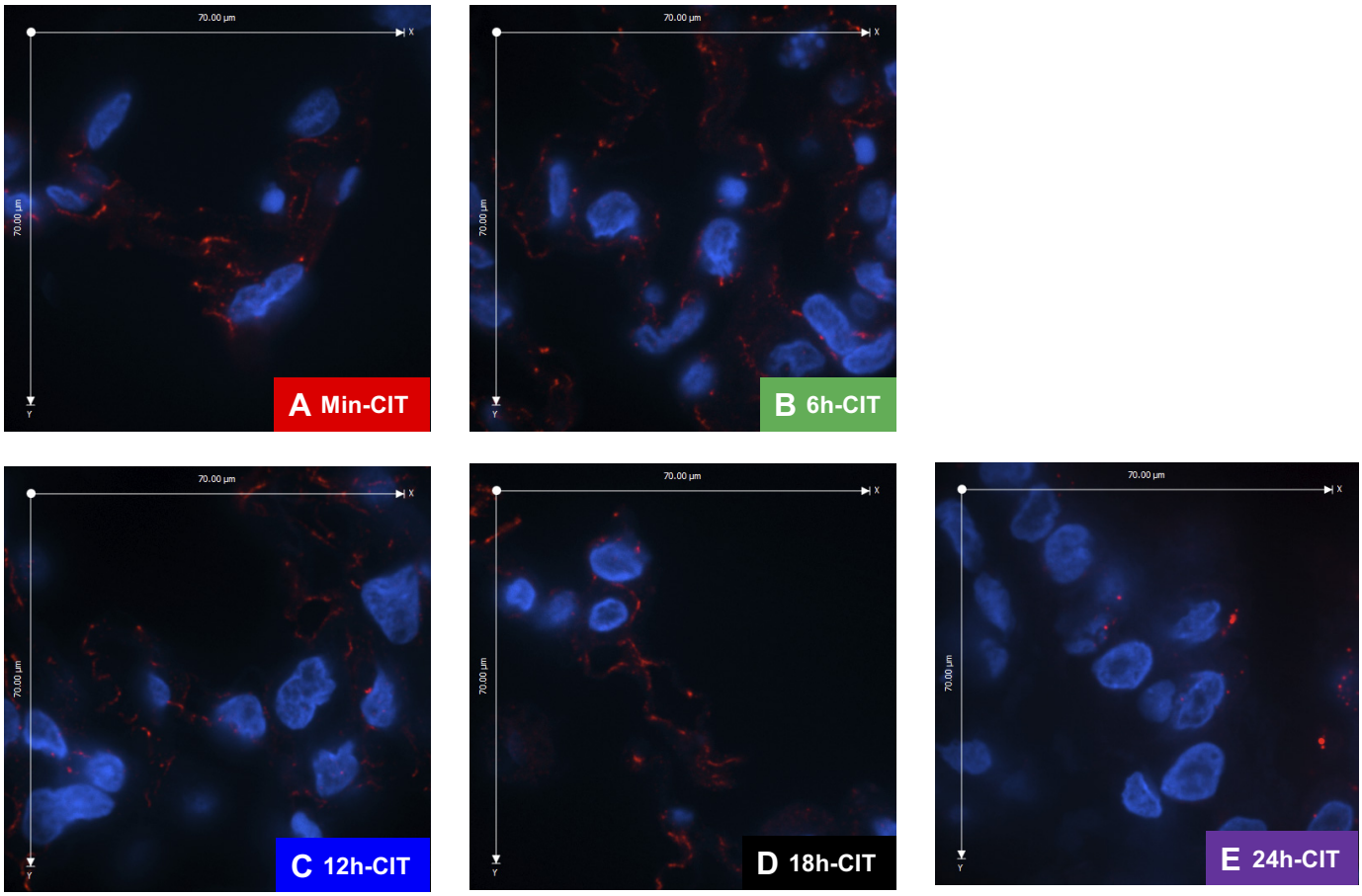

Fig. 4. VE-cadherin staining as a marker of endothelial cell integrity. Blue represents cells stained by DAPI, while red represents cells stained by VE-cadherin. Images were taken after the termination of EVLP in donor rat lungs exposed to different cold ischemic times (CITs): *A*: 20 min (20min-CIT); *B*: 6 h (6h-CIT); *C*: 12 h (12h-CIT); *D*: 18 h (18h-CIT); and *E*: 24 h (24h-CIT). Note that the 24h-CIT group was imaged after only 30 min (i.e., due to premature termination of EVLP).

establish a model of rat EVLP, current strategies fail to maintain viable perfusion for extended periods of time and cannot tolerate minimal amounts of CIT (1, 15, 16, 19, 22, 23).

A commercially available rat EVLP system has been developed by Harvard Apparatus. As far as we know, the first report using this device was published from a group in Japan; however, they used it to screen for ischemia-reperfusion injury and not for physiologic maintenance (9). Many groups have attempted to optimize this system for rat lung perfusion by changing different ventilator parameters and perfusion settings (1, 7, 8, 15, 16, 19, 22, 23). While the first publication reporting prolonged rat EVLP followed by transplantation was published in 2014 (16), lung deterioration was observed during perfusion despite a minimal ischemic injury. Recent work, from Cypel et al. (4) showed stable physiologic lung maintenance during EVLP after short or long CIT, which highlights the impact that perfusion and ventilatory techniques have on preserving lung quality during EVLP in a porcine model.

Using the described technique here, we were able to demonstrate stable rat lung function during 4 h of EVLP after up to 12 h of cold ischemia. Indeed, there were no differences in EVLP graft function among lungs that experienced 20-min, 6-h, and 12-h CIT. This was demonstrated by assessing lung compliance, Pa_{O_2}/FI_{O_2}, and vascular resistance. These findings were further validated by performing a single orthotopic lung transplant. Although results trended lower, no significant difference was found in Pa_{O_2} at the left PVs 2 h after reperfusion among 20-min-, 6-h-, and 12-h-CIT groups demonstrating that the EVLP platform and method described here does not appear to inflict significant lung injury in short ischemic times. For the 18-h-CIT group, the lung showed mild deterioration during EVLP and poor performance after reperfusion. Interestingly oxygenation capacity of the lungs was excellent during EVLP in all groups, including injured lungs after 18-h CIT. As we have previously demonstrated, P/F ratios during EVLP are a late marker of lung injury due to the use of an acellular solution. The underlying mechanism is well described by Yeung et al. (24), in which a reduced effect of shunt on EVLP Po_2 was found to be attributable to the linearization of the relationship between oxygen content and Po_2.

When 24-h CIT was trialed, the lungs showed rapid deterioration during EVLP and could no longer be ventilated. This suggests that 18-h CIT may be the limit for ischemic injury in our model, and thus, may serve as the best condition for trialing therapeutic interventions during EVLP aiming at reversing donor organ injury.

In previous studies using rat EVLP models, diluted blood was used as the perfusate and the authors only assessed ischemia-reperfusion injury without lung transplantation (9, 13, 17). Dong et al. (7, 8) reported the effect of nitric oxide and

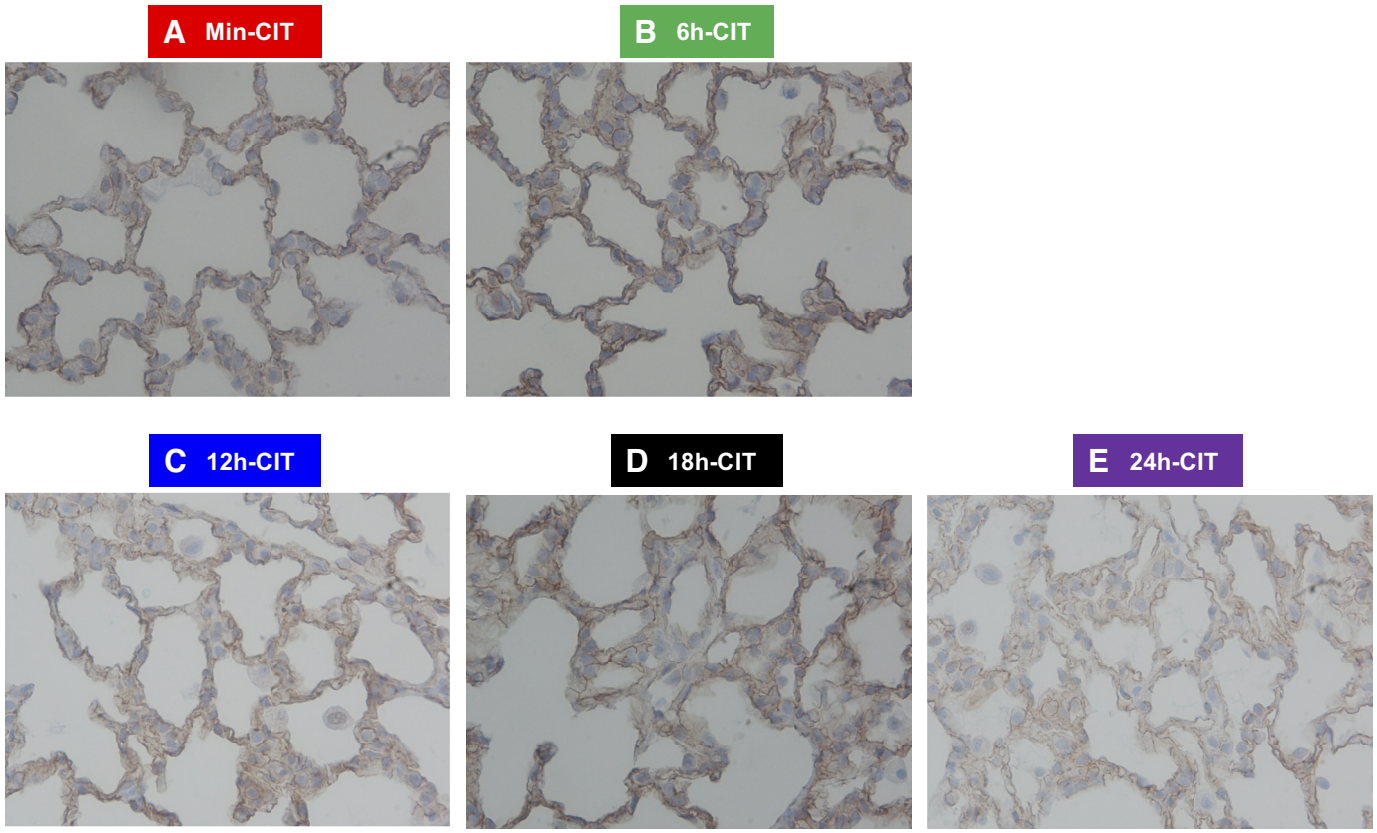

Fig. 5. Zonula occludens-1 (ZO-1) staining as a marker of tight junction integrity. Images were taken after the termination of ex vivo lung perfusion (EVLP) in donor rat lungs exposed to different cold ischemic times (CITs): *A*: 20 min (20min-CIT); *B*: 6 h (6h-CIT); *C*: 12 h (12h-CIT); *D*: 18 h (18h-CIT); and *E*: 24 h (24h-CIT). Note that the 24h-CIT group was imaged after only 30 min (ie, due to premature termination of EVLP).

carbon monoxide using Steen solution as the perfusate, in which they used their own custom EVLP system. Later, another group published some reports using the commercial EVLP system from Harvard Apparatus using Steen solution as the perfusate (19, 22). In 2014, Noda et al. (16) reported a model for rat EVLP followed by transplantation. Lungs were perfused for 4 h after 1 h of CIT; however, during EVLP they observed a marked decrease in oxygenation, pulmonary compliance, and glucose level, accompanied by a gradual increase in vascular resistance demonstrating inability to maintaining function of normal lungs in the system. This model has several similarities to the one described here; however, we believe the high ventilatory settings in that study likely explains the inability to achieve stable 4 h of EVLP. They described 15 cmH_20 of airway pressure, which normally leads to a tidal volume of 10 ml/kg or more in a normal rat lung ex vivo. In ex vivo studies, higher tidal volumes can lead to lung injury not only due to alveolar overdistension but also to impacting capillary flow. Thus high lung ventilatory volumes translate into increased airway and alveolar pressures that are transmitted to the alveolar perivascular space. The net effect is increased alveolar pressure squeezing the capillaries and cyclic increase PVR. With PVR increasing cyclically with each inspiration, it leads to a repetitive vascular collapse (at low flows such as EVLP) and reopening phenomenon that causes endothelial damage and subsequent lung edema as previously demonstrated (2, 21). Similarly, Nelson et al. (15) published a new EVLP technique but observed an increase in lung weight with stable vascular resistance during 1 h of EVLP and did not report any other functional data. Wang et al. (23) also reported the use of the same EVLP apparatus with Steen solution as the perfusate without transplantation; the lungs were maintained in cold ischemia for 3 h followed by perfusion for 3 h, during which compliance and vascular resistance remained stable. Very recently, Bassani et al. (1) reported another rat EVLP approach. A comparison of our rat EVLP technique with these approaches is shown in Table 3.

One of the limitations of this study as a translational model is that the lungs were only perfused for 4 h. Although this is similar to clinical EVLP protocols, stable swine lung perfusion has been maintained for at least 12 h (5). Future studies should explore the maximal perfusion time using this method, while maintaining stable lung physiology. In addition, human lungs may face multifactorial donor injuries, whereas this study only evaluated cold ischemic injury. Finally, perhaps a larger number of animals per group could have demonstrated some differences between groups not apparent in our study. Despite these limitations, the new rat EVLP protocol that we present here overcomes many of the current challenges encountered by existing methods and has the potential to advance preclinical research into enhancing donor lung function and repair.

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Table 3. *Comparison of perfusion and ventilation settings*

	Institution					
	Kyoto (9, 13, 17)	Sao Paulo (19, 22)	Pittsburgh (16)	Ohio State (15)	Lausanne (23)	Toronto (This study)
Perfusion settings						
Perfusate	Homologous blood	Homologous blood	Steen solution	Steen solution	Steen solution	Steen solution
Constant pressure or flow?	Constant flow	Constant flow?	Constant flow		Constant flow	Constant flow
Flow rate or PA pressure	10 ml/min	5–7 ml/min	20% of CO		PAP = 10 mmHg	20% of CO
LA pressure			3 mmHg			3 cmH_2O
Duration	60–80 min	60 min	2 or 4 h		3 h	4 h
Time to total flow	8 min		60 min	15 min		60 min
Drugs in perfusate			Heparin, mPSL, CEZ			Heparin, mPSL, CEZ
Ventilation settings						
Positive or negative ventilation	Negative	Positive	Positive	Positive	Positive	Positive
Ventilation mode		VC (flexiVent)	PC	VC	VC (flexiVent)	PC
Tidal volume		10 ml/kg		4 ml/kg	6 ml/kg	
Inspiratory pressure	−8/−4 cmH_2O		14–15 cmH_2O			10 cmH_2O
PEEP		1–3 cmH_2O	5 cmH_2O	2 cmH_2O		5 cmH_2O
FI_{O_2}	0.21		0.21		0.21	0.21
For assessment			1.0			
Respiratory rate	60	60 or 70	30		7	40
I:E ratio	50%	60%	1:3			50%
Deoxygenation gas						
O_2			6%			6%
CO_2	8%	10%	8%			8%
N_2	92%	90%	86%			86%

PAP, pulmonary artery pressure; CO, cardiac output; mPSL, methylprednisolone; CEZ, cefazolin; VC, volume control; PC, pressure control; PA, pulmonary artery; LA, left atrium; PEEP, positive end-expiratory pressure; I:E, inspiratory/expiratory.

Perfadex solution and Steen solution were provided by XVIVO Perfusion.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.O., M.L., S.K., and M.C. conceived and designed research; A.O. and Z.G. performed experiments; A.O., T.K., Z.G., D.M.H., and M.C. analyzed data; A.O., S.J., M.L., S.K., and M.C. interpreted results of experiments; A.O. prepared figures; A.O., T.K.W., S.J., M.L., S.K., and M.C. drafted manuscript; A.O., A.A., S.J., M.L., S.K., and M.C. edited and revised manuscript; A.O., S.J., M.L., S.K., and M.C. approved final version of manuscript.

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