UNIVERSITÀ
DEGLI STUDI
DI PADOVA

University of Padova

Department of Cardiac, Thoracic, Vascular Sciences and Public Health

Ph.D. Course: Translational Specialistic Medicine "G.B. Morgagni"

Curriculum: Thoracic and Pulmonary Sciences

XXXIV CYCLE

DEVELOPMENT OF A MURINE MODEL OF EX-VIVO LUNG PERFUSION FOR TRANSPLANTATION RESEARCH

Coordinator: Prof. Annalisa Angelini

Supervisor: Prof. Marco Schiavon

Ph.D. Student: Dr. Marco Mammana

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ABSTRACT

Background. Ex vivo lung perfusion (EVLP) is an alternative to static cold storage (SCS) for lung preservation in clinical lung transplantation. Its advantages include the possibility of assessing, reconditioning, and preserving for longer time the lungs prior to transplantation. This technology was introduced in the early years of 2000, and, since then, different devices and protocols were introduced into widespread clinical practice. However, several aspects of EVLP are still controversial; moreover, the potential of EVLP for organ reconditioning and regeneration is still largely unexplored. For these purposes, several investigators have created animal models of EVLP. Rat models of EVLP constitute a potentially invaluable tool for transplantation research, since they allow a higher number of experiments to be performed than with a large animal model, and a wide array of experimental treatments to be investigated. The aim of this project is to create a reliable experimental rat ELVP protocol for transplantation research, and to assess the role of albumin as an additive of EVLP perfusion solution.

Methods. Rat heart-lung blocks were harvested from Sprague-Dawley rats after 1 hour of warm ischemia. Grafts were kept immersed for 1 hour in low-potassium dextran solution and then placed on EVLP for 3 hours and perfused with a low-potassium dextran solution, additioned or not with 70 g/L of albumin (EVLP and EVLP+albumin groups). Every 30 minutes perfused lungs were evaluated for gas exchange, dynamic lung compliance (C_{dyn}), and pulmonary vascular resistance (PVR). Control lungs, after harvest, were kept in low-potassium dextran solution at 4°C for 4 hours (SCS group). Lung injury was evaluated by wet/dry ratio, histology, immunohistochemistry and TUNEL assay.

Results. A significant decrease in PVR was observed from 30 to 60 minutes of EVLP, while other lung function parameters remained stable throughout the 3 hours. No significant differences were observed in lung function parameters or in pathologic assessment between EVLP and EVLP+albumin groups. Pathologic specimens of lungs

treated by EVLP, with or without albumin, were characterized by significantly higher edema score and apoptotic index ($p < 0.05$), as compared to lungs preserved by SCS.
Conclusions. This project describes the first institutional experience with a rat EVLP model which allows for safe preservation of rat lungs for 3 hours with stable lung function parameters. After an initial learning phase, consistent and reliable results were obtained. Albumin did not demonstrate to be an essential component of the perfusion solution; however, this result needs further testing for longer perfusion times and on different animal models. Pathologic findings suggest that, compared to SCS, EVLP may cause a higher extent of lung injury; which, if confirmed, should be acknowledged as an inherent limitation of this model.

RIASSUNTO

Introduzione. La perfusione ex vivo dei polmoni (EVLP) è un'alternativa al trasporto in cella frigorifera (static cold storage, SCS) per la preservazione degli organi ai fini di trapianto. I suoi vantaggi includono la possibilità di valutare, ricondizionare e preservare più a lungo i polmoni prima del trapianto. Questa tecnologia è stata introdotta nei primi anni 2000, e, da allora, diversi dispositivi e protocolli per l'EVLP sono entrati a far parte della pratica clinica internazionale. Tuttavia, alcuni aspetti legati all'EVLP sono ancora controversi; inoltre, le potenzialità dell'EVLP nel ricondizionare e rigenerare gli organi rimangono ancora in larga parte da esplorare.
Per dare risposta a questi quesiti, diversi investigatori hanno creato dei modelli animali di EVLP. Il modello murino di EVLP costituisce uno strumento estremamente utile per la ricerca in trapiantologia, poiché, rispetto all'animale di grande taglia, tramite esso è possibile realizzare un maggior numero di esperimenti, e testare un ampio ventaglio di approcci terapeutici. Lo scopo di questo progetto è creare una piattaforma sperimentale di EVLP nel ratto, e valutare il ruolo dell'albumina come componente di una soluzione di perfusione acellulare.
Metodi. Blocchi cuore-polmone di ratto sono stati prelevati da ratti Sprague-Dawley dopo un'ora di ischemia calda. Gli organi sono stati immersi per un'ora in soluzione di preservazione, a 4°C, e successivamente connessi alla piattaforma EVLP per 3 ore e perfusi con una soluzione acellulare, a basso contenuto di potassio e a base di destrano, a cui sono stati aggiunti oppure no 70 gr/L di albumina, a seconda del gruppo sperimentale (EVLP e EVLP+albumina). Ogni 30 minuti i polmoni sono stati valutati per l'efficienza degli scambi gassosi, la compliance polmonare dinamica (C_{dyn}), e le resistenze vascolari polmonari (PVR). Il grado di danno polmonare è stato valutato istologicamente attraverso il rapporto wet/dry, istologia, immunoistochimica, e test TUNEL.

Risultati. Tra i minuti 30 e 60 di EVLP è stata osservata una significativa riduzione delle PVR, mentre gli altri parametri di funzionalità polmonare sono rimasti stabili nell'arco delle 3 ore. Non sono state osservate differenze significative nei parametri di funzionalità polmonare o nella valutazione patologica tra i gruppi EVLP e EVLP+albumina. I campioni di tessuto dei polmoni sottoposti a EVLP, con o senza albumina, sono risultati caratterizzati da un grado significativamente maggiore di edema e da un maggior indice apoptotico ($p<0.05$), rispetto ai polmoni del gruppo SCS.

Conclusioni. Questo Progetto descrive la prima esperienza istituzionale con un modello di EVLP murino, il quale consente la preservazione di polmoni di ratto per 3 ore in uno stato fisiologicamente attivo, con parametri di funzionalità polmonare stabili. Dopo una fase iniziale di apprendimento, la sperimentazione ha dato luogo a risultati affidabili e riproducibili. L'albumina non si è rivelata una componente indispensabile per la corretta riuscita della perfusione ex-vivo; tuttavia, questo risultato necessita di ulteriori test per periodi più lunghi e su differenti modelli animali. I risultati anatomopatologici suggeriscono che, a confronto con la preservazione in cella frigorifera, l'EVLP potrebbe dare luogo ad un danno polmonare di maggior entità, che, se confermato, dovrebbe essere tenuta in considerazione come un limite intrinseco del modello murino.

1. BACKGROUND

Current hurdles in lung transplantation

Lung transplantation (LTx) is an effective, life-saving therapy for patients with end-stage lung disease such as chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), cystic fibrosis, and primary pulmonary hypertension. Unfortunately, the number of patients waiting for LTx greatly exceeds the number of donors available.

According to the latest reports from the Italian National Transplant Network, average waiting list time for lung transplantation is 2.59 years, and, in 2020, waiting list mortality was 7.5% (1). This is due on one hand to an overall low number of potential multi-organ donors (28.9 potential donors per million population in Italy), and, on the other hand, to the low rate of clinically acceptable lungs among available donors. The utilization rate for the lung is lower than for most other transplantable organs (Fig. 1); in fact, a number of events occurring in donor lungs, such as brain death, trauma, mechanical ventilation, infection, and aspiration, can exacerbate the ischemia-reperfusion-induced lung injury (IRI) following lung transplantation.

IRI manifests clinically as primary graft dysfunction (PGD) and occurs in up to 25% of lung transplants (2). PGD increases morbidity and mortality in the short-term and is a risk factor for the development or chronic lung allograft dysfunction, which occurs in up to half the recipients at 5 years (3). Chronic lung allograft dysfunction remains the Achilles' heel of lung transplantation, preventing it from reaching its full potential.

Different strategies have been adopted to expand the donor pool, such as the use of lungs from donors with extended criteria or non-heart beating donors (NHBD); however, concerns over quality of the organs, and the resulting increased risk of PGD, has led the majority of lung transplant programs to adopt a conservative

strategy in the selection of donor lungs, resulting in a wait list mortality as high as 30-40% (4, 5).

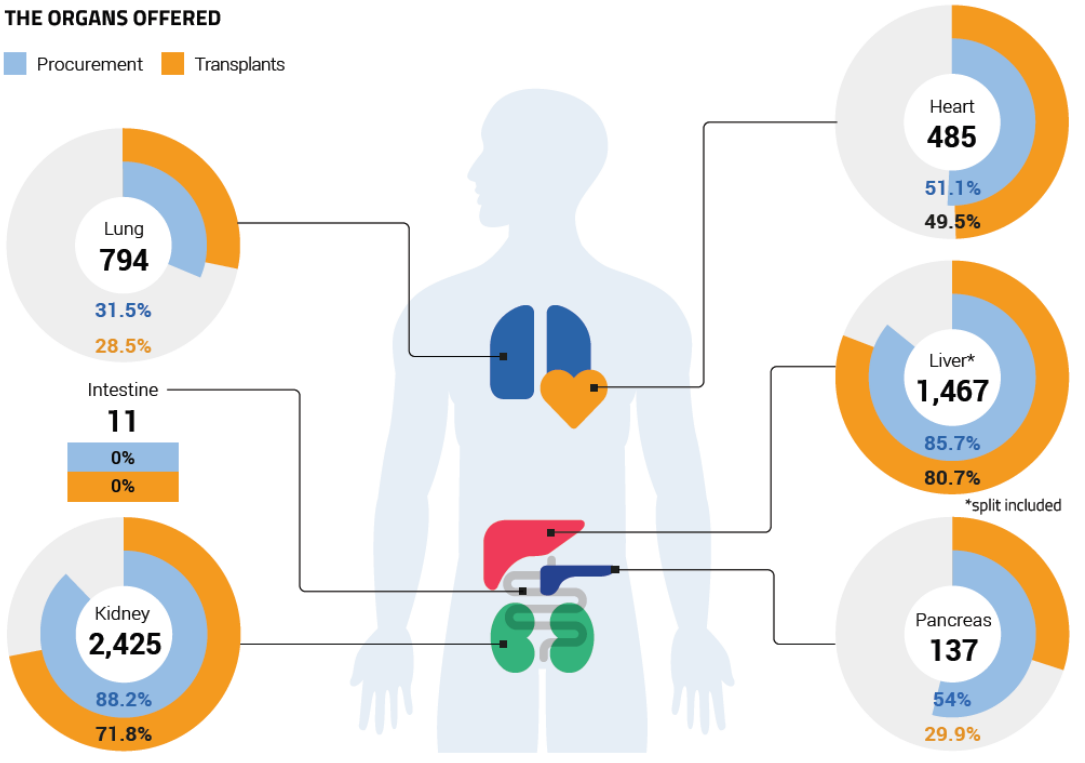

Figure 1. Relationship between the number of organs offered and the organs harvested. Note how these rates for lung are lower than for most other organs. From Centro Nazionale Trapianti, 2021. Report 2020, Annual activity of Italian National Transplant Network.

The current standard for organ preservation: static cold storage

The current primary clinical practice for donor lung preservation is static cold storage (SCS). During donor lung retrieval, a cold pulmonary flush is performed using a low-potassium dextran preservation solution that aids in topical cooling and lung ventilation. In this technique, the lungs are stored at 4°C (placed on the “ice box”)

and transported in a static inflated state. The induced hypothermia effectively reduces cell metabolism, oxygen requirement, and essential nutrient consumption.
Because of its simplicity and efficiency (for good quality organs and short transportation times) SCS has been pivotal to the developmental phase of transplantation, its increased application and its remarkable success. In contrast with many aspects of transplantation medicine, the clinical practice of organ preservation has remained unchanged for over 40 years, and still represents the standard at most transplant centers today. However, SCS has some intrinsic limitations, and since the early years of 2000, clinicians have started to "think outside of the (ice) box" (6).
Firstly, metabolism at 4°C is slowed down but is still present. Energy stores progressively decrease, acidosis develops, cells start to swell and reactive oxygen species are produced. All this leads to progressive and time-dependent attrition of the tissue that is exacerbated by ischemia reperfusion upon warm reoxygenation of the organ in the recipient.
Secondly, the type of organs that are procured and transplanted nowadays are completely different compared with 40 years ago. Shortage of organ donors has led clinicians to consider "extended criteria donors" and NHBD, which are more susceptible to cold ischemic and preservation injury. Transplant surgeon are permanently confronted with the dilemma of trying to offer transplants to the largest number of patients, whereas, at the same time, not taking excessive risks for individual patients. Most of the time, the final decision to transplant or not to transplant is taken empirically and based on experience, but not on reliable surrogates, simply because in the era of cold storage, these surrogates do not exist.

Rationale for ex-vivo lung perfusion

The rationale for ex-vivo lung perfusion (EVLP) is to keep the lungs in a physiologic status prior to transplantation. In contrast with cold static lung preservation whereby

cell metabolism is slowed down and requirement for oxygen and essential nutrients is reduced to prevent organ deterioration, normothermic EVLP under physiologic conditions allows pulmonary cells and tissues to remain metabolically active and viable for several hours. This period provides a window for prolonged lung preservation, assessment, and reconditioning of previously less than optimal performing pulmonary grafts by several mechanisms: dehydration of lung tissue by the high oncotic pressure in the perfusate, removal of harmful and toxic waste products (blood clots, neutrophils, inflammatory cytokines) with filters and membranes in the circuit, and recruitment of atelectatic areas resulting in better ventilation/perfusion matching. Finally, EVLP offers a platform to study avenues for preconditioning and protection of the pulmonary graft against subsequent inflammatory and immune insults following LTx.

Successful normothermic ex vivo organ experiences were first reported by Alexis Carrel and Charles Lindbergh in 1935, who demonstrated that organs could remain viable for several days (7). Isolated EVLP was first described in 1970 by Jirsch et al., detailing the preservation and evaluation of lungs in cases of distant procurement (8). Unfortunately, those first attempts of EVLP failed due to the inability to maintain the alveolar–capillary barrier within the lung, ultimately leading to the development of edema and increased pulmonary vascular resistance-related injuries in the lung during EVLP.

The concept of EVLP was reintroduced 30 years later by Professor Steen and colleagues in Lund, Sweden, as a technique to evaluate lungs from an uncontrolled DCD prior to transplantation (9). This unique case report demonstrated for the first time that lungs can be transplanted successfully after a period of warm ischemia, ex vivo perfusion and evaluation, and cold storage. Subsequent experimental work in Steen's laboratory (10), stimulated many research groups worldwide to further investigate the potential role of EVLP as a method to increase the number of suitable

pulmonary grafts, to reduce the incidence of primary and late graft dysfunction, and to improve outcome after LTx.

Clinical EVLP: technique

After cold pulmonary flush and retrieval, lungs are instrumented in the donor hospital or in the recipient hospital (after a period of cold ischemia during transport) for immediate or delayed normothermic perfusion, respectively. A perfusion cannula is inserted in the pulmonary artery and fixed. The left atrium (LA) can be left open for free drainage of the effluent, or a funnel-shaped cannula is sewn to the remnant of the muscular cuff depending on the preferred technique (see below). Finally, an endotracheal tube of an appropriate size is inserted in the trachea and fixed proximal to the bifurcation with the lungs still inflated.

The EVLP system consists of a perfusion circuit with tubing and a reservoir (Fig 2). The system is primed with the perfusate (2000 ml) and additives and warmed to 32°C. After mixing of the solutions, a sample of the perfusate can be drawn for biochemical analysis to correct pH, HCO3, and glucose levels as needed. The lungs are then placed in a specially designed organ chamber depending on the preferred equipment. A pump drives the perfusate from the reservoir through a gas exchange membrane, heat exchanger, and leukocyte filter before entering the lungs via the pulmonary artery. Pulmonary effluent from LA drains back to the reservoir and is recirculated.

Upon initiation of perfusion with careful monitoring of pulmonary artery pressure (PAP) maintained below 15– 20 mmHg, flow will gradually increase by increasing the pump speed over time according to the institution's protocol (30–60 min). Once the temperature of the outflowing perfusate has reached a preset temperature (32–34°C), protective lung ventilation is started (tidal volume 5–7 ml/ kg donor weight; respiratory rate 7–20 breaths per minute; positive end-expiratory pressure (PEEP) 5–

7 cm H2O; peak airway pressure <25 cm H2O; gas mixture preset for preservation or testing). Lung temperature will further increase to 37 °C reaching targeted flow. Alveolar recruitment maneuvers with airway pressure up to 25 cm H2O can then be performed to remove atelectatic areas if any.

The basic principle of EVLP is that lungs remain viable without additional injury reflected by edema formation. Three key elements for successful normothermic perfusion can be identified: (i) controlled gradual perfusion to avoid hemodynamic shear stress; (ii) perfusate with an extracellular, dextran 40-based solution with optimal colloid pressure; and (iii) controlled ventilation with low tidal volume and PEEP to protect against ventilator-induced lung injury (11).

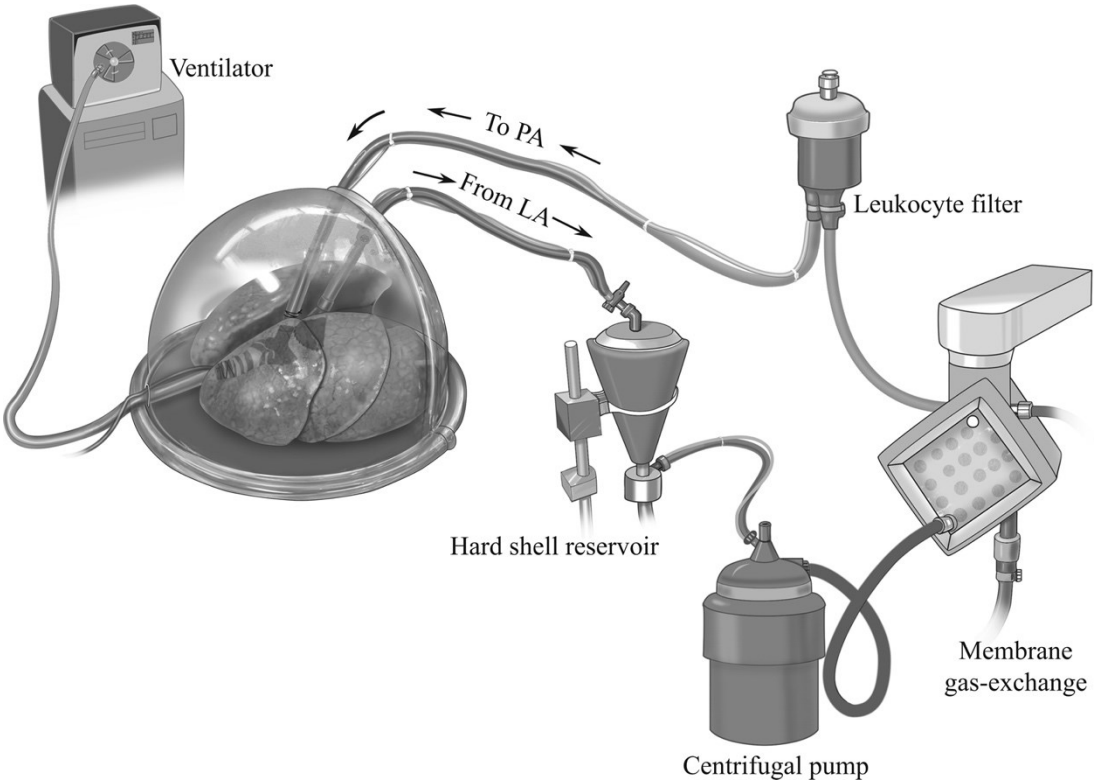

Figure 2. Overview of an Ex-Vivo Lung Perfusion platform. From Cypel M, Keshavjee S. Ex Vivo Lung Perfusion. Oper Tech Thorac Cardiovasc Surg 2014;19-433-442

Clinical EVLP: devices and protocols

Initially, many transplant teams started to utilize EVLP in the clinical setting using their own homemade circuit assembled with individual components available in the cardiac surgery department for extracorporeal support, including a centrifugal pump, heater/cooler, tubing, hard-shell reservoir, hollow-fiber oxygenator, leukocyte filter, in-line gas analyzer, saturation probes, and pressure transducers. Organs are placed in a specifically designed plastic chamber to fix the lungs in a stable position during ventilation and to provide a warm and humid environment (Fig 3).

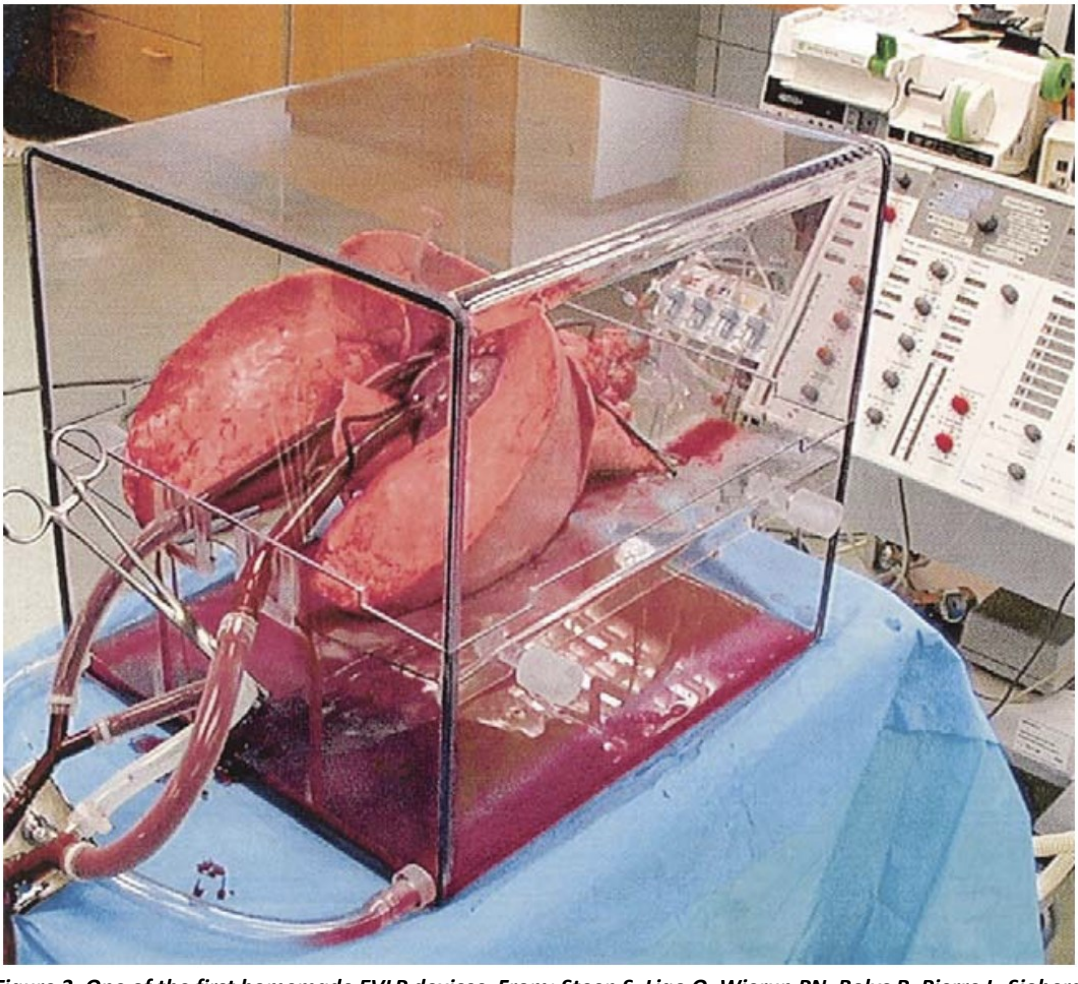

Figure 3. One of the first homemade EVLP devices. From: Steen S, Liao Q, Wierup PN, Bolys R, Pierre L, Sjoberg T. Transplantation of lungs from non-heart-beating donors after functional assessment ex vivo. Ann Thorac Surg 2003; 76: 244.

As the concept of EVLP gained further recognition and its use among transplant centers grew, it became clear that there was a need for standardization of technology and techniques, in order to simplify clinical practice and to facilitate comparison of outcomes between centers.

Several companies have started to market commercial devices for EVLP: OCS™ Lung (Transmedics); Vivoline® LS1 (Vivoline Medical, Lund, Sweden); Lung Assist® (Organ Assist, Groningen, the Netherlands); and XPS™ (XVIVO Perfusion AB). These are represented in Figure 4.

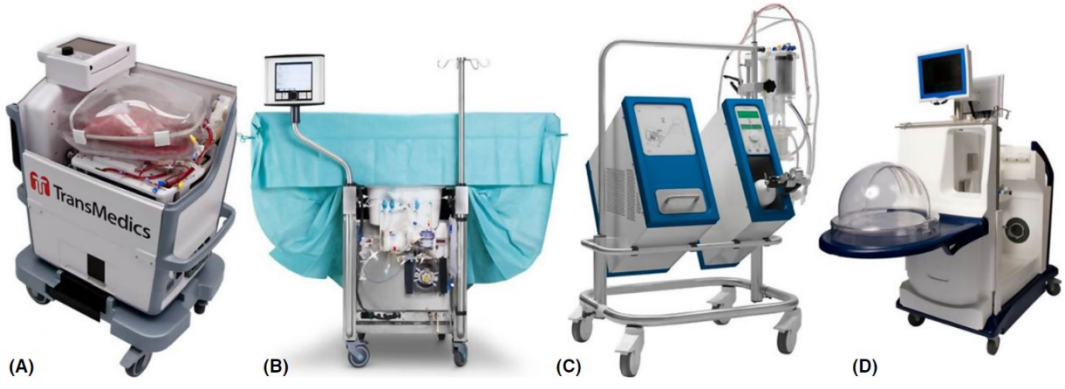

Figure 4. Commercial devices for ex vivo lung perfusion. (A) OCS™ Lung (Transmedics); (B) Vivoline® LS1 (Vivoline Medical); (C) Lung Assist® (Organ Assist); (D) XPS™ (XVIVO Perfusion AB).

These commercial devices present important differences between each other, and, consequently, they are used according to different protocols.

Three different protocols have been validated internationally in clinical trials. The Toronto protocol; the Lund protocol; and the Organ Care System™ (OCS) protocol.

All these protocols vary in composition of the perfusate, in perfusion and ventilation settings, and in the equipment used, these are summarized in Table 1.

In the following subparagraphs the main differences between protocols and their rationale will be described

Table 1. Ex vivo lung perfusion protocols (Adapted from Reeb J, et al. Clin Transplant 2016;30:183-94).

Parameter	Protocol		
	Toronto	Lund	OCS™
Perfusion			
Target flow (lead time)	40% Cardiac output (1 h)	100% Cardiac output (1 h)	2.0 -2.5 L/min (15-30')
Start flow	10% of the target flow	100 mL/min	200 mL/min
Flow characteristic	Continuous	Continuous	Pulsatile
PAP	Flow dictated ≤ 15 mmHg	≤ 20 mmHg	≤ 20 mmHg
LA	Closed	Open	Open
LA pressure	3-5 mmHg	0 mmHg	0 mmHg
Perfusate	Steen™ solution	Steen™ solution + RBC's hct 14%	OCS™ solution + RBC's hct 15-25%
Ventilation			
Start temperature	32°C	32°C	34°C
Tidal volume	7 mL/kg donor bw	5-7 mL/kg donor bw	6 mL/kg donor bw
Respiratory rate	7 bpm	20 bpm	10 bpm
PEEP	5 cmH_2O	5 cmH_2O	5-7 cmH_2O
FiO_2	21 %	50 %	12 %
Timing of EVLP			
Beginning of EVLP	Static device: recipient hospital or specialized center	Static device: recipient site	Mobile device: donor site
Perfusion time	4-6 h; until 12 h	2 h	Duration of transport

Perfusion settings

All perfusion protocols involve a ramp up phase, during which the flow is gradually increased until target flow is reached. The duration of this ramp up phase (lead time) is variable between protocols, and depends on the time needed for rewarming of the lungs. In fact, experimental studies suggest that excessive perfusion flow on a cold lung might cause significant hemodynamic stress (12). After target flow is reached, pulmonary artery pressure (PAP) is carefully monitored to avoid development of hydrostatic edema. Target flow is set at 100% of cardiac output in the Lund protocol, while it is only 40% in the Toronto protocol and 2–2.5 l/min in the OCS™ protocol (Table 1). The rationale for protocols using lower than physiologic (100%) flow is to protect the lungs from hydrostatic edema, especially in the dependent areas. It has been demonstrated that with lower perfusion flows it is possible to maintain lungs perfused for 12 hours without significant damage to the dependent areas of the lung (13).

Another important difference between perfusion protocols is whether the left atrium (LA) should be left open for drainage of pulmonary effluent (Lund and OCS™ protocols) or closed by suturing the atrial cuff to a specially designed plastic cannula to allow maintenance of a positive pressure between 3 and 5 mmHg by adjusting the height of the reservoir (Toronto protocol). Data deriving from experimental animal models suggest that maintaining a positive LA pressure prevents collapse of microvessels, decreases edema formation, and ultrafiltration coefficients, and ultimately allows for better lung function parameters and longer duration of EVLP (14,15).

Ventilation settings

A protective ventilation strategy, with tidal volumes ranging from 5 to 7 ml/kg of body weight is proposed by all three protocols. Based on experience on acute

respiratory distress syndrome (ARDS), protective ventilation improves lung injury histologically, decrease levels of proinflammatory cytokines and oxidative stress (16). Ventilation starts when the lung has rewarmed to 32-34°C, as recommended in all three protocols; however, the fraction of inspired oxygen (FiO_2) differs between protocols. According to the Toronto protocol, lungs are ventilated at room air (FiO_2=21%); a deoxygenator element in the circuit, which consists in a gas exchange membrane connected to a gas mixture of 8%CO_2, 6%O_2, and 86%N_2, is responsible for maintaining CO_2 and pH values levels between normality range. On the other hand, according to the OCS™ protocol, lungs are maintained most of the time in a preservation mode, wherein they are ventilated with a gas mixture of 12%O_2 and 5.5%CO_2, while no gas flow is delivered through the deoxygenator. When an assessment of gas exchange through blood gas analysis is required, (usually at donor hospital and at recipient hospital), the circuit is switched to the monitoring mode, where lungs are ventilated at room air, and deoxygenated with 6%CO_2.

Timing of EVLP

One of the main differences concerning available EVLP platforms is that between static and portable EVLP devices. A static EVLP device is intended for use at recipient hospital, which means that cold ischemia occurs during transportation time from donor to recipient hospital, and only then EVLP is initiated. On the other hand, a portable EVLP device (the OCS™ Lung) is composed of a mobile base which can easily be transported to donor hospital. It has all equipment on board including batteries for electrical supply, gas cylinders for preservation and monitoring as well as a ventilator for use during transport of organs from donor to recipient hospital. It offers a platform for normothermic lung preservation eliminating longer periods of cold ischemia, for continuous monitoring and assessment of graft function during storage, and for immediate and sustained recruitment and resuscitation.

However, in spite of the theoretical advantage of eliminating cold ischemia time, it is still to be determined which is the difference in the extent of ischemia-reperfusion injury between a static and a portable EVLP protocol. Different studies addressing this issue have provided conflicting evidence (17,18).

Perfusion solution

As shown in Table 1, all main EVLP protocols differ in the type of perfusion solution. In fact, it is still controversial whether adding red blood cells to the perfusion solution is needed to better mimic the physiologic and rheological conditions, and to reliably assess oxygenation capacity in a more physiologic manner. Red blood cells (either packed cells or full blood) are added in the Lund and OCS™ protocols (referred to as cellular perfusate) up to a hematocrit of ± 15–25%, while acellular perfusion is the preferred method in the Toronto protocol (Table 1). As no comparative clinical data between acellular versus cellular EVLP are available at the moment, available data come from experimental studies, which so far have produced conflicting evidence (19,20).

The only perfusion solution that is intended for use without red blood cells is the Steen Solution™ (XVIVO Perfusion, Goteborg, Sweden) as originally described by Stig Steen and coworkers from Lund University (10). This is an extracellular solution with dextran 40 to protect the endothelium from complement- and cell-mediated injury and to inhibit coagulation and platelet aggregation (low potassium dextran solution) with the addition of human albumin to maintain optimal colloid pressure (21). The OCS™ protocol is based on OCS™ solution (Transmedics) or Perfadex® (XVIVO Perfusion AB, Goteborg, Sweden), which are both low potassium dextran-based solutions, but without the addition of human albumin.

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

of human plasma. Furthermore, the oncotic pressure of a perfusate needed to maintain normal endovascular-interstitial fluid gradients may be derived from either physiological (albumin) or non-physiological sources (dextran). Table 2 reports the composition of most adopted commercially available lung perfusion solution in clinical lung transplantation, both for EVLP, static cold storage, or pneumoplegia. All products share a similar, extracellular-type, electrolyte composition, while they differ in the quantity of dextran, glucose and albumin (Table 2). Keeping these principles in mind, several investigators have started testing in-house EVLP perfusion solutions. The first aim is to reduce costs associated with commercially available products; in fact, cost and availability remain a significant hurdle to the wide adoption of EVLP technology. A recent report analyzing the cost of the DEVELOP-UK trial showed an estimated overall increase in cost of $47,000 per run for EVLP compared to conventional cold static preservation (22). The second aim is to find better perfusion solutions, which allow for longer preservation times and lung repair capabilities (23,24).

Table 2: Intended uses and composition of commercially available lung perfusion solutions

Perfusion solution	Steen ™	OCS Lung ™	Perfadex™
Intended use(s):			
Ex-vivo lung perfusion	•	• (+ RBC)	-
Pneumoplegia	-	•	•
Static cold storage	-	-	•
Components:			
NaCl	86 mmol/L	136.8 mmol/L	-
KCl	4.6 mmol/L	5.4 mmol/L	-
$CaCl_2.2H_2O$	1.5 mmol/L	-	-
$NaH_2PO_4.2H_2O$	1.2 mmol/L	-	-
$NaHCO_3$	15 mmol/L	-	-
$MgCl_2.6H_2O$	1.2 mmol/L	-	-
NaOH	1 mol/L	-	-
$MgSO_4.7H_2O$	-	0.8 mmol/L	-
$Na_2HPO_4.2H_2O$	-	0.3 mmol/L	-
KH_2PO_4	-	0.5 mmol/L	-
Na^+	-	-	138 mmol/L
K^+	-	-	6 mmol/L
Mg^{2+}	-	-	0.8 mmol/L
Cl^-	-	-	142 mmol/L
$PO4^{3-}$	-	-	0.8 mmol/L
$SO4^{2-}$	-	-	0.8 mmol/L
Human serum albumin	70 g/L	-	-
Glucose	11 mmol/L	10.1 mmol/L	5 mmol/L
Dextran 40	5 g/L	50 g/L	50 g/L
Sterile water	1000 ml	1000 ml	1000 ml

RBC, red blood cells

Clinical experience with EVLP

Since the report from Steen and coworkers (9), many centers have published case series and their institutional experience with EVLP, and several major trials have assessed the safety and efficacy of EVLP in donor lung evaluation and reconditioning (Table 3). The first successful clinical trial in Toronto was reported in the New England

Journal of Medicine in 2011, demonstrating no significant difference in the early post-transplant outcomes between 20 EVLP-treated lungs and 116 non-EVLP standard lungs: the incidence of PGD grade 2 or 3 at 72 h was 15% in the EVLP lungs vs. 30% in the non-EVLP standard lungs, and the 30-day mortality was 10% in the EVLP lungs vs. 5% in the non-EVLP standard lungs (25). A more recent study has focused on the long-term outcomes of 230 EVLP-treated allografts (24.6%) of 936 lung transplants performed in the Toronto Lung Transplant Program since 2008, showing that there was no significant difference in the CLAD-free survival (EVLP lungs: 70, 56, and 53% vs. non-EVLP standard lungs: 72, 56, and 36% at 3, 5, and 9 years after transplantation, respectively) or allograft survival (EVLP lungs: 73, 62, and 50% vs. non-EVLP standard lungs: 72, 58, and 44% at 3, 5, and 9 years after transplantation, respectively) (26). The INSPIRE trial was the first prospective randomized controlled study using the portable OCS Lung™ device for standard bilateral lung transplantation, performed at 21 academic lung transplant centers in USA, Europe (Belgium, France, Germany, Italy, Spain, and the UK), Australia and Canada (27). This study focused on the feasibility and safety of EVLP with the OCS™ device for 141 donor lungs meeting the current standard criteria, compared with 165 donor lungs preserved by standard cold storage. Although 30-day mortality was significantly higher in the OCS arm compared to the control arm (4.2% vs 0%, respectively, $p = 0.009$), incidence of PGD grade 3 within 72 h after transplantation was significantly lower in the OCS arm than in the control arm (17.7% vs 29.7%, respectively, $p= 0.015$). The EXPAND trial was a prospective single-arm study, performed at eight transplant institutions in the USA, Germany, and Belgium. This

Table 3: Summary of prospective clinical trials for ex-vivo lung perfusion (adapted from Prasad NK, et al. Transplantation 2021 1;105:979-985)

Trial	Number of patients	Location	System	Design	Key points
Standard criteria donors					
INSPIRE[27]	EVLP-151 Control-169	USA, Europe, Canada	OCS™ Lung	Randomized	PGD3 at 72 hours (EVLP vs control) – 17.7% vs 29.7% (p=0.015); 30d mortality – 4.3% vs 0% (p=0.009)
Extended criteria donors					
HELP[25]	EVLP-23, Control-116	Canada	XVIVO™	Nonrandomized	PGD3 at 72 hours (EVLP vs control) -15% vs 30% (p=0.11); median hospital stay (days) – 23 vs 27 (p=0.39); 30d mortality – 10% vs 5% (p=0.33)
Vienna[29]	EVLP-39, Control-41	Austria	XVIVO™	Randomized	PGD3 at 72 hours 0% in both groups; median hospital stay (days) – 20 vs 20.5 (p=0.8), 30d mortality- 0% vs 4.2% (p=0.6)
NOVEL[30]	EVLP-110, Control-116	USA	XVIVO™	Nonrandomized	PGD3 at 72 hrs (EVLP vs. Control)-8.9% vs 9.5% (p=0.12); median hospital stay (days)- 23.9 vs 28.5; 1yr mortality –6.8% vs. 3.5% (p=0.84)
EXPAND[28]	EVLP-79	USA, Europe	OCS™ Lung	Observational	Incidence of PGD3 at 72hours- 6.4%. 30d mortality- 1%, 1-year mortality – 7%.
DEVELOP-UK[22]	EVLP-53	UK	Vivoline®	Observational	Only 18 (34%) lungs were transplanted, longer ICU stay and ECMO support requirement (38.8%) than standard controls, trial terminated early

multicenter international study showed the safety and efficacy of portable EVLP preservation for 79 transplanted lungs from extended criteria or DCD donors, demonstrating favorable results, with the incidence of PGD 2 or 3 being 16% at 72 h post transplantation and the 30-day mortality being 1% (28).

Overall, the results of such trials suggest that EVLP is a safe technology, which allows to assess marginal donors, and in particular DCD donors; and which, consequently, allows to increase the number of transplantable organs. Recent studies have evaluated that the addition of the EVLP technology have contributed to an increase of transplantable lungs of 15% to 20% (31).

EVLP as an experimental platform

For its inherent characteristic of maintaining lungs isolated, in a physiologically functioning state, outside of human body, EVLP is an invaluable tool for experimental research. In fact, while performing EVLP, multiple factors can be assessed in real-time. These include pulmonary arterial flow, pulmonary arterial pressure and pulmonary resistance, as well as dissolved oxygen concentration in the perfusate before and after passing through the pulmonary circulation, which measures the gas exchange function of the lung. The wet/dry ratio of a lung can also be assessed, giving an accurate depiction of how edematous the lung has become. Table 4 lists all parameters than can be measured, as well as parameters that can be varied for the purpose of the experiment, during EVLP. Thanks to these properties, isolated lungs can be used as a model of acute lung injury or ventilation induced lung injury. The advantage of EVLP over in vivo models is that the effect of the variation of a specific ventilator or perfusion setting can be assessed without the confounding effects of interactions with other organs.

Table 4: Dependent and independent variables with ex-vivo lung perfusion (Adapted from Nelson K, et al. World J Exp Med 2014;4:7-15.

Dependent variables (i.e. what can be measured with EVLP)	Independent variables (i.e. what can be varied in an EVLP)
Tracheal pressure	Tracheal pressure
End expiratory pressure	End expiratory pressure
End inspiratory pressure	End inspiratory pressure
Tidal volume	Tidal volume
Compliance	Respiratory rate
Respiratory rate	Pulmonary artery flow rate
Pulmonary artery flow rate	Pulmonary artery pressure
Pulmonary artery pressure	Left atrial outflow pressure
Left atrial outflow pressure	Perfusate composition
Pulmonary vascular resistance	Ischemic time
Lung weight	Temperature of perfusate
Wet to dry ratio	Temperature of organ
Pre-organ pO_2	Inspired gas concentration and components
Post-organ pO_2	
Perfusate pH	
Perfusate pCO_2	
Perfusate for molecular analysis	
Tissue for mRNA, protein, or histologic analysis	

Additionally, several EVLP-based therapeutic approaches have been studied, whereby injuries identified during lung graft assessment on EVLP can inform the application of personalized therapies during the EVLP time window. These therapies include successful administration of drugs, such as antibiotics, fibrinolytic and anti-inflammatory drugs, gene therapy, cell-based therapy, and application of therapeutic devices such as dialysis and an UVC irradiator. Given that the lung graft is isolated on EVLP, each of these strategies can be administered at high concentration via the vasculature of the airway without the risk of systemic toxicity.

Finally, EVLP is not only a tool for lung transplant research, but its use can be extended to other settings such as medical oncology (delivery of experimental chemotherapeutic agents), lung bioengineering (whole organ decellularization and delivery of stem cells), and thoracic surgery (autotransplantation techniques for advanced lung cancer). An in-depth review of experimental studies based on EVLP is

beyond the scope of this manuscript; however, there are several valuable papers covering this subject (32,33).

Animal models of EVLP

The "living" lungs maintained with perfusion and ventilation on EVLP provide valuable opportunities for science, allowing many types of research projects. Although most of the landmark work has been done on lungs from humans or from large animals (such as pigs), EVLP on lungs from rodents is also possible. By recognizing the limitations of each specific model, EVLP using small animal, large animal and human discarded lungs is a powerful system to advance science following a translational, bench to bedside approach.

Large animal models

Porcine EVLP has a direct translation to the human clinical setting. Its main advantage is that this model offers a very appropriate size comparison to humans. This not only makes it easier to translate information obtained on this model for clinical purposes, but it also offers the opportunity to use the same devices and settings for EVLP as in man. Additionally, the pig has a greater similarity to humans in gene sequence and physiology compared to mice and rats, which makes it a superior model.

The main limitation of the porcine model is the considerable costs needed for purchase and care of these animals. Moreover, the amount of perfusate used on an isolated pig animal is much higher than with small animal, making each experiment much more expensive.

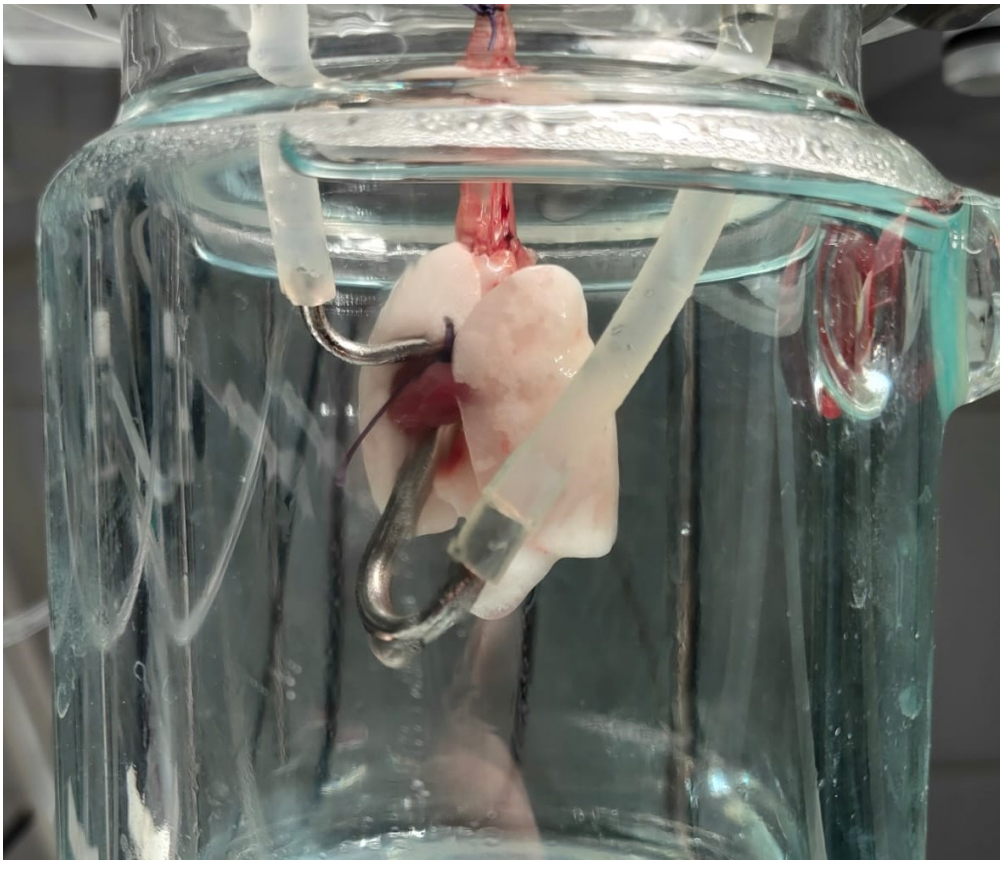

Figure 5. Small animal (rat) model of EVLP offer a convenient approach to study lung physiology and pathology.

Small animal models

Small animal models than have been employed in EVLP include rat, mouse, guinea pig and rabbit. These systems offer several distinct advantages compared to their larger counterparts. Overall, their cost for purchase and care is much lower. Because of this, one can complete more perfusion experiments than on a larger model, which aids in achieving statistical significance.

Of the different small animals used for EVLP experiments, rat, guinea pig and rabbit models have a larger thoracic cavity than mice, making surgical procedures easier. Initial cannulation can be done with or without the aid of a surgical microscope; moreover, a rat left lung transplantation technique has been developed and used in multiple studies (34, 35). One limitation, however, with the use of these three

animals is the relative scarcity of species-specific commercially available antibodies and molecular reagents.

Murine models of EVLP offer considerable advantages over rats because of the greater number of species-specific antibodies and gene probes available for experiments. Additionally, knock out (KO) lines of the mouse model are available. The greatest challenge in solely relying on the murine model of EVLP, however, is the technical difficulties involved during surgery. Mice have a smaller thoracic cavity and smaller organs than rabbits, rats or guinea pigs. Another drawback of this mouse model is the increased difficulty of training personnel on more technical mouse surgery procedures, which can create bottlenecks in experimental plans and ultimately slow down data acquisition.

Overall, small animal models of EVLP are very convenient. When considering their use, however, several key differences need to be taken into account. Mice and rats have much shorter perfusion times than human or pig lungs. In several studies on the rat EVLP model, perfusion time was limited to 60-75 minutes (36,37). While some studies have attempted to extend EVLP preservation time, deterioration in lung function parameters was frequently observed. Only recently, the research group from Toronto General Hospital have published a rat EVLP protocol wherein lungs remained stable for a maximum perfusion time of 4 hours (38). Table 5 provides a review of the Literature in regard of EVLP experiments and perfusion times on the rat model.

Table 5. Overview of perfusion times and perfusate types used in rat EVLP studies (one study for each research group is reported)

Author, year	City, Country	Perfusate type	EVLP runtime	Aim of the study
Inokawa, 2006[36]	Chapel Hill, USA	Earle's solution ± porcine RBC (Hct 12-15%)	75 min	Description of a rat EVLP protocol for non-heart beating donors
Menezes, 2012[37]	Sao Paulo, Brazil	Saline-diluted blood (Hct 15-20%)	60 min	Comparison between lung preservation solutions
Motoyama, 2013[39]	Kyoto, Japan	Steen™ solution	60 min	Administration of plasmin during EVLP for organ reconditioning
Noda, 2014[40]	Pittsburgh, USA	Steen™ solution	2 - 4 hours	Setting up of a prolonged rat EVLP protocol
Bassani, 2016[41]	Milan, Italy	Homemade (Perfadex™ + additives)	3 hours	Description of a rat EVLP protocol
Wang, 2018[42]	Lausanne, Switzerland	Steen™ solution	3 hours	Administration of pharmacological therapies during EVLP for organ reconditioning
Arni, 2021[43]	Zurich, Switzerland	Steen™ solution	4 hours	Subnormothermic perfusion temperature during EVLP
Ohsumi, 2022[38]	Toronto, Kanada	Steen™ solution	4 hours	Description of a rat EVLP model

RBC, red blood cells; Hct, haematocrit

Modeling of lung injury in animals

An important issue concerning animal models is the definition of lung injury. In humans, the definition of acute lung injury (ALI) is based on clinical parameters, which include acute onset, radiological evidence of bilateral pulmonary infiltrates, a ratio of the partial pressure of arterial oxygen to the fraction of inspired oxygen (PaO_2/FiO_2) of less than 300, and no clinical evidence for elevated pulmonary arterial pressure (44). Moreover, in the transplant setting, a specific type of ALI is often considered, that is ischemia/reperfusion injury (IRI). IRI is the inflammatory process that occurs in the transplanted lung, after a prolonged period of ischemia, following restoration of blood flow (45). Clinically, this injury results in a failure to recover lung function after transplantation (primary graft dysfunction, or PGD), which can be fatal. PGD, again, is defined upon clinical parameters, which include the PaO_2/FiO_2 ratio and the presence or absence of pulmonary infiltrates on chest radiography (Table 6). Moreover, PGD may occur at time 0 (ideally measured by blood gas analysis performed 6 hours after lung reperfusion) up to 72 hours after transplantation (46).

Table 6. Definition and grading of Primary Graft Dysfunction in humans

Grade	PaO_2/FiO_2	Radiographic infiltrates consistent with pulmonary edema
0	> 300	Absent
1	> 300	Present
2	200 – 300	Present
3	< 200	Present

However, these criteria cannot be directly translated to experimental animals. Although arterial blood gases, chest radiographs and even computed tomography can be performed in small experimental animals, the equipment required for these

measurements is available only in a few laboratories. Furthermore, such measurements may be incompatible with the design of many experimental systems. An alternative approach would be to define ALI in animals based on histopathological criteria similar to those seen in humans with ALI. In humans, the pathological correlate of ALI is diffuse alveolar damage (DAD), characterized by inflammatory infiltrates, thickened alveolar septae, and deposition of hyaline membranes (47). However, none of the available animal models reproduce all of the pathologic features of DAD in humans. Therefore, the criteria used to define lung injury in humans cannot be directly translated to most animal models.

To solve this issue, in 2011, a panel of expert of the American Thoracic Society has elaborated a set of features aimed at standardizing the definition of ALI in animal models, as well as setting a minimum of requirements for its assessment (48).

The Committee concluded that the main features of experimental ALI include: histological evidence of tissue injury, alteration of the alveolar capillary barrier, presence of an inflammatory response, and evidence of physiological dysfunction. In order to determine if ALI has occurred, at least three of these four features should be present. Moreover, for each of these features, a set of measurements classified as “very relevant” and “somewhat relevant” were identified, and it was recommended that at least one “very relevant” measurement for each of the four main domains be performed (48). These guidelines were recently revised in 2022, in view of the advances in imaging, genetic tool, “omics” technologies and cellular biology; however, the concept of ALI as a “multidimensional entity” characterized by the same four domains was retained (49). Table 7 lists all measurements recommended for each of the four main domains.

These guidelines are focused at the general definition of ALI; however, they still hold true in more specific settings, such as ventilator-induced lung injury, endotoxin-induced lung injury, or ischemia-reperfusion injury.

Table 7. The four domains of experimental ALI and recommended ("very relevant") measurements for their assessment according to the American Thoracic Society Guidelines[49]. It is recommended that at least 3 of the 4 main domains be assessed, by means of at least one of the "very relevant" measurements specific to that domain. "Somewhat relevant" measurements are not shown.

Histological evidence of tissue injury	Alterations of the alveolar-capillary barrier	Presence of an inflammatory response	Evidence of physiological dysfunction
Filling of the alveolar space with proteinaceous alveolar fluid and debris	Elevated BAL albumin, IgM, or other large circulating protein	Increase in chemokines or cytokines in BAL or lung tissue	Arterial blood gas analysis of oxygenation
A validated histologic injury score	Increased lung wet/dry ratio or extravascular lung water	Increase in neutrophil numbers in BAL or lung tissue	Lung compliance and/or elastance Alveolar fluid clearance
Evidence of alveolar epithelial injury (cell death, epithelial denudation, or ATII proliferation)	Elevated BAL total protein	Increase in inflammatory monocyte and macrophage subpopulations in BAL or lung tissue	Noninvasive measurement of oxygenation
Neutrophil infiltration of the alveolar space	Evan's blue dye accumulation in lung homogenate	Increase in neutrophil activity measured by elastase or myeloperoxidase in supernatant of BAL or lung tissue	Respiratory rate, difficulty breathing and minute ventilation
Thickening of alveolar septae and/or interstitial edema	Pulmonary vascular permeability index and/or filtration coefficient	Endothelial cell adhesion molecule expression or mediator release	
Hyaline membranes or presence of fibrin or derivates in the airspaces	Transport of large-molecular-weight substance		

A standardized definition of ALI increases the flexibility and applicability of the definition to multiple models while increasing the likelihood of translating preclinical findings to critically ill patients.

2. AIM OF THE RESEARCH

In light of the increasing use of EVLP, both in the clinical setting and the experimental setting; and of its still unexplored potentials in the field of organ reconditioning, the aims of this research were the following:

- To build an adapted EVLP circuit for the small animal model (rat)
- To set up a standardized and reliable experimental protocol which reproduces the use of EVLP for preservation, assessment and reconditioning of lungs from non-heart beating donors (NHBD)
- To assess the role of albumin as an additive of EVLP perfusion solution by comparing functional and pathologic outcomes of lungs undergoing EVLP with a standard low-potassium dextran solution, with or without the addition of albumin.
- To compare the extent of lung injury between lungs preserved by SCS or by EVLP, following a period of warm ischemia

3. MATERIALS AND METHODS

Animals and Study design

Outbred Sprague-Dawley rats weighing 250-350 gr from the internal breeding of the Interdepartmental Center of Experimental Surgery of Padua University were used. Animals were maintained in a specific pathogen free environment and fed with standard diet and water ad libitum.

Following terminal anesthesia, exsanguination and 1 hour of warm ischemia, rat heart-lung blocks were harvested and assigned to 3 experimental groups, each of them composed of 7 heart-lung blocks:

- In the Control group, heart-lung blocks were immersed in low-potassium dextran solution (Perfadex™) and stored at 4°C for 4 hours;
- In the EVLP group, heart-lung blocks were stored in Perfadex™ for 1 hour (cold ischemia time), and then connected to EVLP for 3 hours;
- In the EVLP+albumin group, heart-lung blocks underwent the same procedure as in the EVLP group, but the perfusion solution was additioned with 70 g/L of albumin

The research protocol was written in compliance with international standards for animal experimentation, and received approval by regulatory bodies (auth. n. 49/2020-PR). Moreover, the project received funding from a grant issued by the Veneto Region, as a part of the “Living, innovative, fully engineered, long-lasting and advanced bioreplacement (L.I.F.E.L.A.B.)” research program.

Rat ex vivo lung perfusion system

Based on a thorough search of the Literature, we found a commercially available lung perfusion system which was suited for the small animal model, and constituted the

core of the experimental EVLP platform (IL-2 Isolated Perfused Rat or Guinea Pig Lung System; Harvard apparatus, Holliston, MA, USA). The same device was used successfully by several research groups (37-43).

The system consisted of the following components:

- Roller pump (REGLO Analog 4-Channel roller pump, ISMATEC, Wertheim, Germany) with flow regulation between 0,003 and 35 ml/min;
- Tubing of 4 mm diameter (Tygon®) for the perfusion circuit;
- Rodent ventilator (VentElite, Harvard Apparatus, Holliston, Massachusetts, United States), which delivers tidal volume between 50 µl and 5 ml, allows for PEEP regulation, and enables both volume-controlled and pressure-controlled ventilation;
- Jacketed glass reservoir, with 2 L capacity, for the perfusion solution;
- Jacketed organ chamber;
- Circulating thermostatic water bath;
- Hollow-fiber oxygenator (D150; Medica S.p.A., Modena, Italy)

Water temperature was regulated by the thermostatic water bath and water was circulated through the outside of reservoir, respiratory circuit and organ chamber to keep the grafts, inhaled gas and perfusate at a constant temperature. The oxygenator was used to deoxygenate the perfusate using a gas mixture (8% CO2, 6% O2, 86% N2). The physiological parameters of the perfused lung were measured by a connected pressure transducer (P75, Harvard Apparatus) for PA pressure, a differential low-pressure transducer for respiratory flow (DLP2.5 Type 381; Harvard Apparatus) and a differential pressure transducer for airway pressure (MPX Type 399/2; Harvard Apparatus). The real-time signals from these transducers were recorded and analyzed using a data acquisition system (PULMODYN; Harvard Apparatus) (Figure 6).

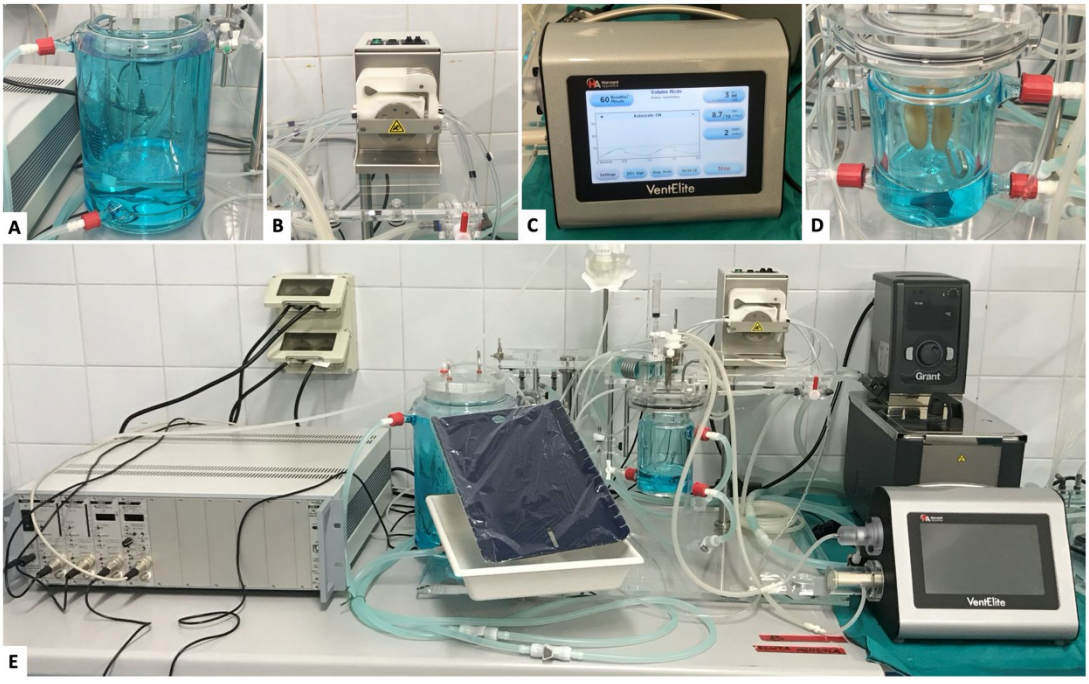

Figure 6. Components of the EVLP platform: jacketed reservoir (A), roller pump (B), rodent ventilator (C), organ chamber (D), platform overview (E).

Surgical procedures

Rats were anesthetized by intraperitoneal injection of Midazolam 15 mg/kg, Clonidine 100 mcg/kg, Tramadol 10 mg/kg, and, following 5 minutes, a second injection of Propofol 60 mg/kg. A median laparotomy was performed and 500 UI heparin were injected into inferior vena cava. Then, rats were tracheostomized and intubated with a 16G intravenous catheter fixed with silk ligature. Mechanical ventilation was initiated at room air, with a positive end expiratory pressure (PEEP) of 3 cmH_20, tidal volume of 6 ml/Kg, and respiratory rate of 60 breaths per minute. Following median sternotomy, the cardiac apex was cut down and the animal sacrificed by exsanguination. The animal was kept ventilated at room temperature for one hour (warm ischemia time). During this time, two specifically designed metal cannulae (Hugo Sachs Elektronic, Hugstetten, Germany) were inserted into the pulmonary artery (PA cannula) through an incision into the outflow tract of the right

ventricle, and into the left atrium (LA cannula), through the previous incision into the cardiac apex. The cannulae were secured with previously placed 2-0 silk ligatures. Cannulation was performed by the aid of an operating microscope. At the end of warm ischemia time, the lungs were flushed through the PA cannula with 20 ml of Perfadex™ at a temperature of 4°C, delivered from a height of 20 cm. Then, the trachea was closed at end-inspiration with a metal clip and the heart and lung block was excised. The organs were kept immersed in Perfadex™, at 4°C, for 1 hour (cold ischemia time.

Connection and initiation of EVLP

Fifteen minutes before the end of cold ischemia time, the EVLP circuit was primed with an in-house low-potassium dextran solution. For this purpose, we used a similar composition of an already published perfusion solution (50), which included: 1.5 L of Perfadex™, 2.5 gr of glucose, 1 gr cefazolin, 500 mg methylprednisolone, 50 mEq sodium bicarbonate, 0.18 gr calcium, and 30 UI of insulin. Depending on the experimental group (EVLP or EVLP+albumin), the solution was additioned or not with 70 g/L of bovine serum albumin (BSA lyophilized powder; Sigma-Aldrich, St. Louis, Missouri, USA). The solution was made fresh for each experiment; its pH was checked with a pHmeter (HI98190; Hanna Instruments, Woonsocket, Rhode Island, USA) and corrected with sodium bicarbonate. Approximatively 150 ml of perfusate were needed for priming of the circuit.

At the end of cold ischemia time, a retrograde flush (from LA cannula to PA) was performed with 15-20 ml of Perfadex™; and the PA cannula was connected with the inflow line of the EVLP circuit, making sure that the cannula was completely filled with fluid. Then, perfusion was started at low flow (2 ml/min) and the LA cannula connected with the outflow line; this was set as time 0 of EVLP.

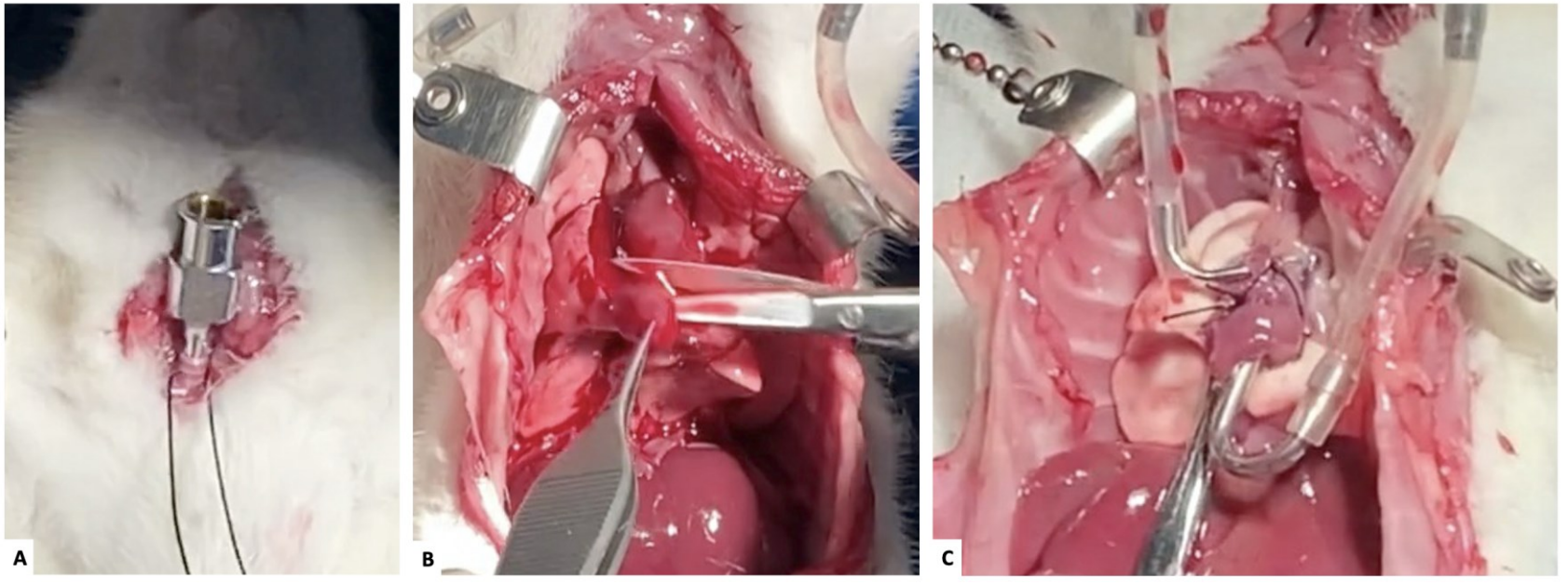

Figure 7. Surgical steps: tracheostomy and tracheal intubation (A), exsanguination by cutting the cardiac apex (B), cannulation (C).

Over the first 40 minutes, perfusion flow was gradually increased from 2 ml/min to a target flow of 7.5 ml/min. During the same time, the circuit was slowly rewarmed to reach the temperature of 37.5°C in 40 minutes. Gas exchange and ventilation were started after 20 minutes of perfusion, when temperature was above 32°C. Initial ventilation parameters were tidal volume: 3 ml/Kg, PEEP: 3 cmH_2O, respiratory rate: 60 breaths per minute. Tidal volume was gradually increased to 7 ml/Kg (approximatively 2 ml per breath for a rat weighing 300 gr) over the next 20 minutes. Every 30 minutes, starting from 1 hour of perfusion, a recruitment maneuver was performed by increasing PEEP to 5 cmH_2O and tidal volume up to a peak airway pressure (Pawp) of 25 cmH_2O. Then, a perfusate sample was taken after transit to the lungs and a blood gas analyzer (iSTAT; Abbot, Chicago, Illinois, USA) was used to measure the concentration of dissolved O_2, dissolved CO_2, electrolytes and glucose in the perfusate (CG4+ iSTAT Cartridge, Abbot). Additionally, the PA pressure, airway pressure and respiratory flow in the lung were monitored continuously through integrated transducers. Dynamic lung compliance (C_{dyn}) was determined by analysing pressure–volume curves. Pulmonary vascular resistance (PVR) was calculated from PA pressure and perfusion flow.

At the end of 3 hours of EVLP, the cardiopulmonary block was detached from the circuit. The left lung was fixed in formalin and sent for pathology. The right superior lobe was used for determination of wet to dry ratio (the weight of the specimen was measured fresh, and after 24 hours at 60°C). The same sampling was applied to control lungs, after 4 hours of cold ischemia.

Pathologic analyses

Left lung samples were formalin-fixed and paraffin-embedded. Sections representative of the entire lung were obtained by cutting the specimen along the

sagittal axis. Histological evaluation was performed as previously described in human samples (51).

Different morphological parameters (edema, inflammation, fibrosis, hyperinflation, necrosis) were quantified and graded with the following scoring system: score 1 = mild, <30% of analyzed tissue; score 2 = moderate, 30%-50% of analyzed tissue; and score 3 = severe, >50% of analyzed tissue.

Additionally, the presence of intralveolar and interstitial neutrophils, hyaline membranes, proteinaceous material, and alveolar septa thickening were also evaluated and scored (score 0-2), as suggested by the latest ATS guidelines for assessment of lung injury on experimental animals (49).

Serial sections from the same paraffin-embedded lung specimens were used for terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) assay. Molecular analysis for apoptosis detection by the TUNEL technique was carried out. At least 300 cells were counted in 3 high power fields. Apoptotic index (AI) was expressed as number of TUNEL-positive cells/total cell number ×100. Expression of inflammatory cytokines IL-6, IL-8, IL-10, TNF-α and iNOS was performed, by using the following antibodies: IL-6 (Clone 1.2-2B11-2G10, Abcam, Cambridge, UK), IL-8 (Clone ab106350, Abcam, Cambridge, UK), IL-10 (Clone GT5111, Abcam, Cambridge, UK), TNF-α (Clone TNFA/1172, Abcam, Cambridge, UK), iNOS (Clone SP126, Abcam, Cambridge, UK). A semi-quantitative evaluation was carried out, based on a scale from 0 to 3. Score 1, 2, and 3 were assigned in case of single immunoreactive cells or single immunoreactive foci (i.e., focal to oligofocal), scattered foci (i.e., multifocal), and numerous to coalescing foci of immunoreactive cells, respectively. For all assessments, the pathologist was blinded to the experimental group of the specimens.

Statistical analysis

For estimation of sample size, the apoptotic index assessed by TUNEL assay in the three experimental groups was considered the primary outcome variable. Considering a one-way analysis of variance model, a sample size of 6 animals per group allowed to detect a difference of 5% in the apoptotic index at a 5% significance level, with 80% power, assuming a standard deviation of 1.5%. Sample size was increased to 7 animals per group (15%) to account for non-normal distributions and the use of non-parametric tests.

Numerical variables are presented as median and interquartile range, categorical variables are expressed as count and percentage. The comparison between pathologic variables was performed by Kruskal-Wallis test and Fisher's exact test as appropriate. Differences in lung function parameters between the two experimental groups were assessed using the Wilcoxon signed rank test with Benjamini-Hochberg correction for multiple tests. A p-value of <0.05 was considered statistically significant. All data were analyzed using the software R (R Foundation for Statistical Computing, Vienna, Austria).

4. RESULTS

Preliminary steps and learning curve for EVLP

The project received approval by the Animal Welfare Body of Padua University, after minor revisions, in July 2019; and by the Italian Ministry of Health in January 2020, without further modifications. Acquisition of all necessary equipment for the EVLP platform was completed in May 2020; however, due to delays attributable to Covid19 pandemic, the devices were installed at the Interdepartmental Centre of Experimental Surgery only in August 2020. The final setup and training for use were completed in October 2020. Due to precautions taken during Covid19 pandemic, there were restrictions in the number of animals available for experiments until the end of 2020.

As this was our first Institutional experience with the rat EVLP platform, the surgical procedures and the EVLP protocol were both associated with a learning curve. Therefore, we divided our experience into an early phase (from October 2020 to June 2021) and a later phase (from June 2021 to January 2022).

The early phase was necessary in order to set up and refine a reproducible protocol. In fact, no experiment conducted during this phase was considered for the purpose of final analyses. Overall, causes of failures during the early phase included: issues related to the surgical procedures (lung lacerations during harvest; n=2, tearing of the left atrium; n=4, malposition of the venous cannula; n=5), or issues related to the EVLP protocol, which resulted in overt lung edema during perfusion (Figure 8A).

EVLP-related causes of failure included mistakes in preparation of the perfusion solution (n=3), too high perfusion flow (n=2), air embolism during cannulation (n=2) or thermal shock due to a too quick rewarming phase (n=3). After having addressed all these issues, we no longer observed EVLP-related failures.

During the later phase, the overall failure rate was 10%, and was only due to surgical issues during cannulation of the left atrium (rupture of the atrium; n=1, malposition of the venous cannula, n=1).

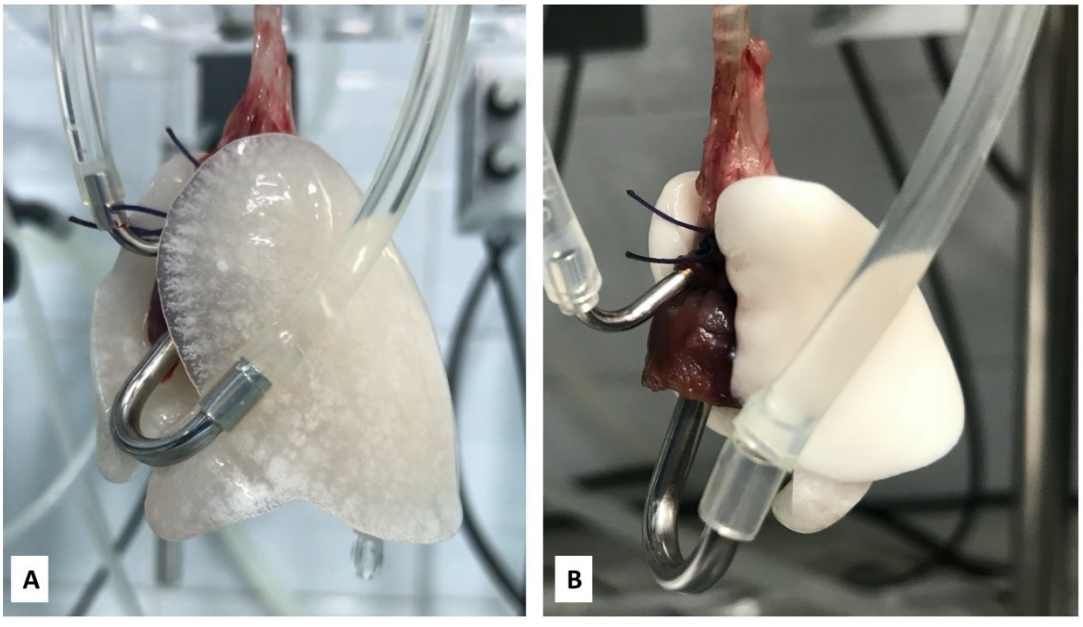

Figure 8. Macroscopic aspect lungs during EVLP. Edematous lungs (A) appear transparent, of increased volume, and fluid is clearly visible dripping from parenchyma or foaming. Normal lungs (B) have a white, matt surface, with normal volume and respiratory excursions

Lung function parameters during EVLP

The 3-hour EVLP protocol was completed for 14 grafts, 7 from the EVLP group and 7 from the EVLP+albumin group. Pulmonary gas exchange function remained stable in both groups. In particular, median pO2 values at 30 minutes were 153 mmHg (IQR 150, 158) in the EVLP group and 151 mmHg (IQR 147, 157) in the EVLP+albumin group; while median pO2 values at 180 minutes where 140 mmHg (IQR 137, 145) in the EVLP group and 137 mmHg (IQR 135, 145) in the EVLP+albumin group, ($p>0.05$) (Fig 9A). Pulmonary vascular resistances (PVR) decreased significantly in both groups between 30 and 60 minutes of perfusion [-26.3% (95%CI -35.3%, -22.2%), $p = 0.031$ in the EVLP group; -49.9% (95%CI -54.2%, -41.2%), $p = 0.016$ in the EVLP+albumin group], after which they remained stable over the 3 hour (Fig 9B). PVR values at 30 minutes were 2.66 mmHg/ml/min (IQR 2.05, 3.73) in the EVLP group and 3.63 mmHg/ml/min (IQR 3.32, 3.88) in the EVLP+albumin group; while PVR values at 180 minutes were 2.09 mmHg/ml/min (IQR 1.86,2.75) in the EVLP group and 2.06 mmHg/ml/min (IQR 1.85, 2.28) in the EVLP+albumin group.

Although a non-significant trend towards an increase in dynamic lung compliance (C_{dyn}) was observed over the first 90 minutes, its values remained stable in both groups over the 180 minutes. In particular, median C_{dyn} at 30 minutes was 0.09 ml/cmH2O (IQR 0.07, 0.17) in the EVLP group and 0.11 ml/cmH2O (IQR 0.07, 0.17) in the EVLP+albumin group; while Cdyn at 180 minutes was 0.14 ml/cmH2O (IQR 0.13, 0.20) in the EVLP group and 0.25 ml/cmH2O (IQR 0.21, 0.31) in the EVLP+albumin group.

Overall, no significant difference was observed in pO2, PVR or C_{dyn} between the two experimental groups ($p>0.05$).

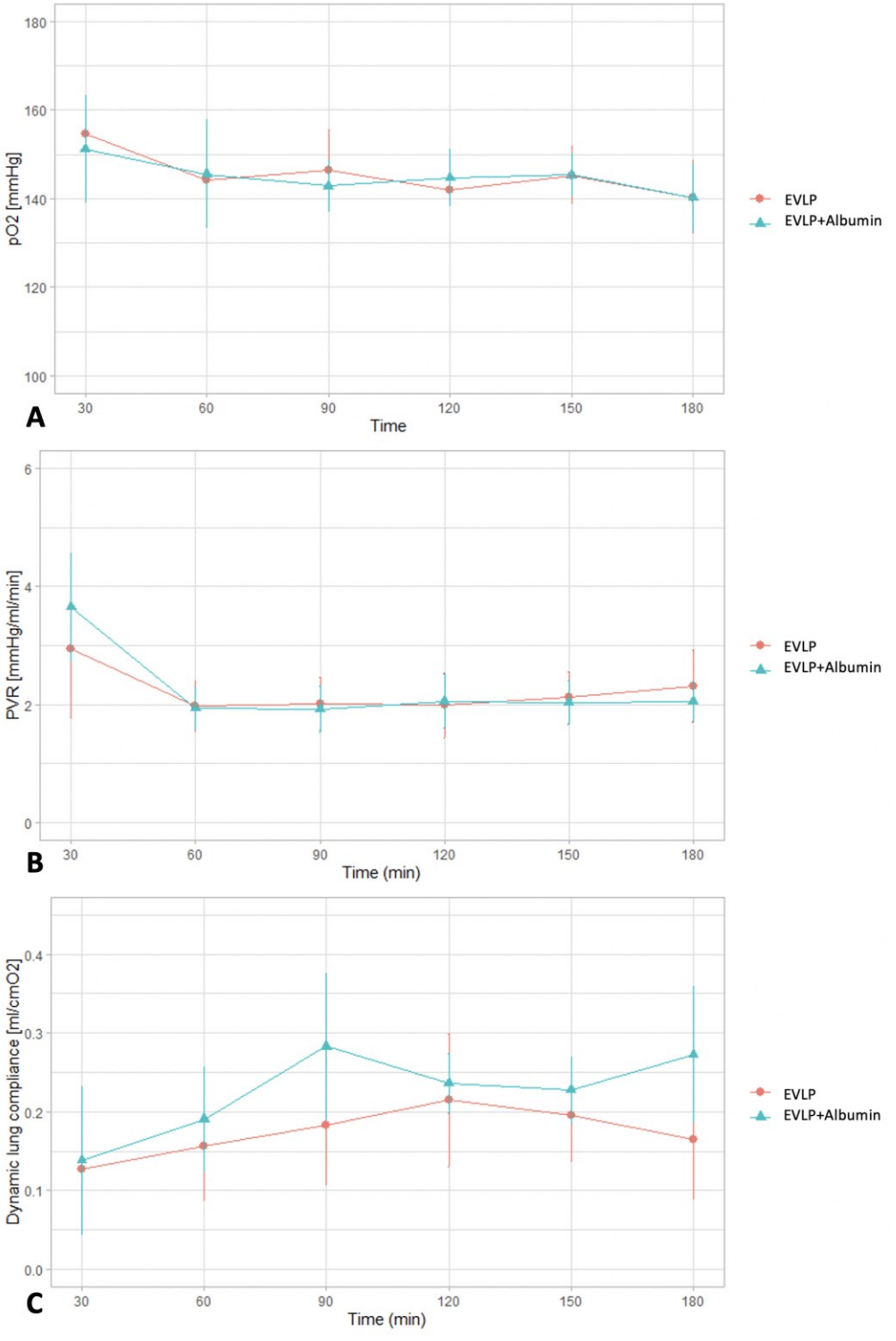

Figure 9. Trend of lung function parameters during 3 hours of EVLP. Reported parameters include pO_2 (A), pulmonary vascular resistance (PVR, B), and dynamic lung compliance (C).

Histologic assessment

Table 8 shows the result of pathologic assessment of lung injury according to the experimental group. Among standard histologic parameters, significantly higher edema score was observed in the two EVLP group compared to control group (p=0.023), while there were no differences in the extent of inflammation, fibrosis and hyperinflation. No signs histologic signs of necrosis were observed in any of the experimental groups; furthermore, W/D ratios were comparable among the 3 groups. Among histologic parameters suggested by ATS guidelines (49), marginally significant differences were observed in the scores of intraalveolar neutrophils, which were higher in the EVLP+albumin group (p=0.049); and interstitial neutrophils, which were higher in the Control group (p=0.043). No differences were observed in the presence of hyaline membranes or proteinaceous materials.

Table 8. Results of histology and wet/dry ratio

Variable	Controls N = 7[1]	EVLP N = 7[1]	EVLP+albumin N = 7[1]	p-value[2]
Inflammation				0.12
0	0 (0%)	1 (14%)	0 (0%)	
1	0 (0%)	4 (57%)	3 (43%)	
2	5 (100%)	2 (29)	3 (43%)	
3	0 (0%)	0 (0%)	1 (14%)	
Edema				**0.023**
0	5 (71%)	1 (14%)	2 (29%)	
1	2 (29%)	3 (43%)	0 (0%)	
2	0 (0%)	3 (43%)	5 (71%)	
Fibrosis	6 (86%)	7 (100%)	7 (100%)	>0.9
	1 (14%)	0 (0%)	0 (0%)	
Hyperinflation				0.2
0	2 (29%)	0 (0%)	0 (0%)	
1	3 (43%)	5 (71%)	4 (57%)	
2	0 (0%)	1 (14%)	3 (43%)	
3	2 (29%)	1 (14%)	0 (0%)	
W/D ratio	4.78 (3.93, 5.96)	5.50 (5.20, 6.36)	5.27 (4.58, 5.66)	0.5

Variable	Controls N = 7[1]	EVLP N = 7[1]	EVLP+albumin N = 7[1]	p-value[2]
Intraalveolar neutrophils				**0.049**
0	3 (43%)	2 (29%)	1 (14%)	
1	1 (14%)	4 (57%)	0 (0%)	
2	3 (43%)	1 (14%)	6 (86%)	
Interstitial neutrophils				**0.043**
0	0 (0%)	1 (14%)	0 (0%)	
1	0 (0%)	4 (57%)	3 (43%)	
2	7 (100%)	2 (29%)	4 (57%)	
Hyaline membranes				>0.9
0	6 (86%)	7 (100%)	7 (100%)	
2	1 (14%)	0 (0%)	0 (0%)	
Proteinaceous material				0.053
0	4 (57%)	3 (43%)	1 (14%)	
1	0 (0%)	4 (57%)	3 (43%)	
2	3 (43%)	0 (0%)	3 (43%)	

[1]n (%); Median (IQR)

[2]Fisher's exact test; Kruskal-Wallis rank sum test

Immunohistochemistry and TUNEL assay

A marginally significant difference (p=0.046) was observed in the percentage of apoptotic cells observed by TUNEL assay, which was lower in the Control group (median =1.55%, IQR=0.45%-2.9%) compared to the EVLP and EVLP+albumin groups (median=5.60%, IQR=4%-8%, and median=4.7%, IQR=3%-5%, respectively. The semi-quantitative scoring of inflammatory cytokines including IL-6, IL-8, IL-10, TNF-α, and iNOS, was comparable among groups, with the majority of specimens showing a mild to moderate (grades 1 to 2) expression of inflammatory cytokines (Table 9 and Figure 10).

Table 9. Results of TUNEL assay and immunohistochemistry

Variable	Controls N = 7[1]	EVLP N = 7[1]	EVLP+albumin N = 7[1]	p-value[2]
TUNEL[3]	1.55 (0.45, 2.90)	5.60 (4.00, 8.00)	4.70 (3.00, 5.00)	**0.046**
IL-6				0.2
1	5 (71%)	3 (43%)	3 (43%)	
2	2 (29%)	4 (57%)	1 (14%)	
3	0 (0%)	0 (0%)	3 (43%)	
IL-8				>0.9
1	6 (86%)	5 (71%)	5 (71%)	
2	1 (14%)	2 (29%)	1 (14%)	
3	0 (0%)	0 (0%)	1 (14%)	
IL-10				0.6
1	4 (57%)	2 (29%)	1 (14%)	
2	2 (29%)	4 (57%)	4 (57%)	
3	1 (14%)	1 (14%)	2 (29%)	
TNF-α				>0.9
1	6 (86%)	5 (83%)	5 (71%)	
2	1 (14%)	1 (17%)	1 (14%)	
3	0 (0%)	0 (0%)	1 (14%)	
iNOS				0.083
1	6 (86%)	2 (29%)	5 (71%)	
2	1 (14%)	5 (71%)	1 (14%)	
3	0 (0%)	0 (0%)	1 (14%)	

[1]n (%); Median (IQR)

[2]Fisher's exact test; Kruskal-Wallis rank sum test

[3]Results are expressed as % of apoptotic cells/total cell number

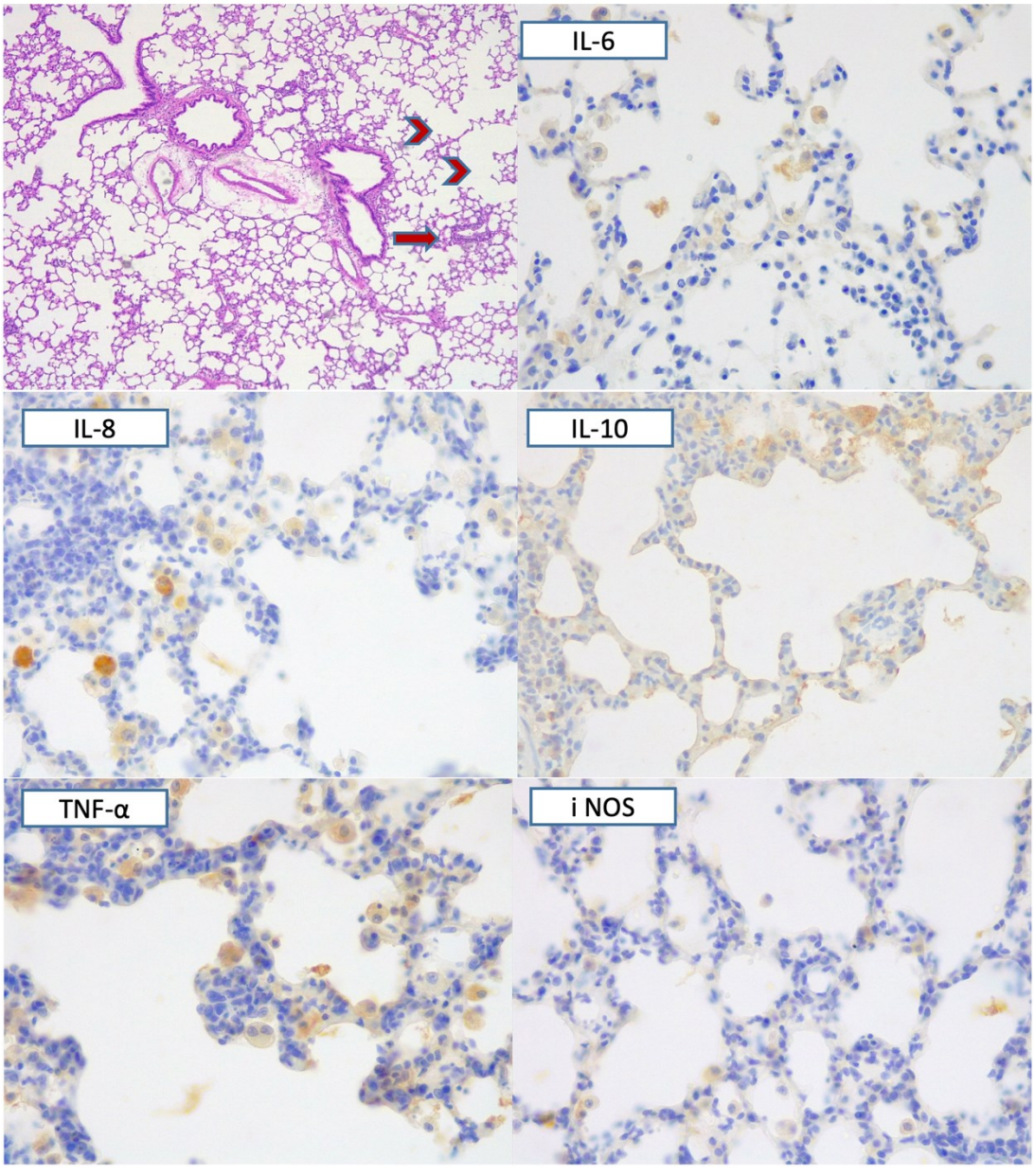

Figure 10. Pathologic specimen from the EVLP+albumin group. At histologic examination, the lung parenchyma shows mild inflammation (arrow) and moderate emphysema (arrow head). Immunohistochemical staining for IL-6, IL-8, IL-10, TNF-α and i NOS is detected in a quite low number of cells.

5. DISCUSSION

Development and setting up of the rat EVLP model

EVLP is a key tool for translational science in the lung. In fact, initially, it was in large part thanks to the experiments performed on a porcine model by Professor Steen that this technology could be refined, gaining the interest of the scientific community (10). At present, EVLP is used for clinical lung transplantation by many lung transplant centers worldwide; however, several aspects of such technique still remain controversial. Among them, probably the main one concerns the optimal composition of the perfusion solution (19,20); but areas for improvement include all aspects of EVLP, including the modality of ventilation, the composition of inhaled gas mixture, or the overall duration of perfusion (52). Moreover, several therapeutic approaches for organ reconditioning or regeneration are currently being tested, and this is where animal models of EVLP become fundamental (32).

Typically, a translational pathway requires that a potential novel therapy be tested on the small animal model first, and then translated to large animals and humans. However, the majority of research with EVLP has been performed on large animals (Figure 10). While the pig model offers better clinical resemblance to humans, it significantly increases costs of experimentation, which can be a limitation to the number of potential novel therapies to be tested. The EVLP rat model, on the other hand, may overcome such issues, but it is associated by a greater technical complexity associated with the downscaling of EVLP devices used in man. Moreover, rat lungs are particularly susceptible to several kind of injuries, and perfusion times are significantly shorter than with porcine EVLP (53), which are probably the main reasons for its relative underuse compared to the large animal counterpart (Figure 11).

Table 3 EVLP as a platform for specific treatments (13-16,37,48-67)

EVLP-based treatment	Experimental			Preclinical human EVLP	Clinical EVLP
	Small animal EVLP	Large animal EVLP	Human Lung EVLP		
Fibrinolysis for PE		✔(48)			✔(15)
Surfactant		✔(14)			
α1 antitrypsin		✔(49)		✔(50)	
adenosine A2B receptor		✔(51)			
β_2-adrenergic receptor agonists		✔(53,54)	✔(52)		
High dose antibiotics				✔(13)	✔(55)
Gene therapy		✔(56)		✔(16)	
Cell based therapy					
Mesenchymal stromal cells	✔(61)	✔(58,59)	✔(57)	✔(60)	
Regulatory T cells	✔(62)				
UVC irradiation		✔(37)		✔(37)	✔(63)
Hemofiltration/hemodialysis		✔(65)			✔(64)
Cytokine filtration		✔(66)			
Prone EVLP		✔(67)			

The numbers in the table indicate references. EVLP, ex vivo lung perfusion; PE, pulmonary embolism; UVC, ultraviolet C.

Figure 11. Novel EVLP-based therapies distributed according to the animal model (or human) where they have been tested. From Watanabe T, et al. J Thorac Dis 2021;13:6602-6617 (ref n. 33). Full references of the studies reported in the table can be found in the article.

According to our experience, it took approximatively 20 experiments in order to refine a standardized protocol. As far as the surgical procedure is concerned, the most critical part is cannulation of the left atrium. In fact, the cannula is inserted through the cardiac apex and the atrioventricular valve, which may be particularly narrow. Forcing through the valve may result in tearing of atrium. Moreover, the distal end of the cannula could easily be misplaced, causing the rapid onset of edema during EVLP. While other authors have chosen not to cannulate the atrium, letting the perfusate free to drain from the pulmonary veins (41), a closed circuit has the advantage that venous pressure can be regulated. In fact, data deriving from experimental studies suggest that maintaining a positive LA pressure prevents collapse of microvessels, decreases edema formation, and ultrafiltration coefficients, and ultimately allows for better lung function parameters and longer duration of EVLP (14,15). A closed circuit with a LA pressure between 3 and 5 mmHg is also an

important principle of the Toronto EVLP protocol for humans (13). In our experimental setting, LA pressure could easily be adjusted by regulating the height of the outflow part of the circuit, equipped with a venous equilibrator vessel.

Edema during EVLP was the main cause of experiment failure during the earlier part of our experience (Figure 8A). Beside malposition of the venous cannula, edema could occur due to several other causes, including: mistakes in preparation of the perfusion solution, too high perfusion flow, air embolism during cannulation or thermal shock due to a too quick rewarming phase. After having addressed all these issues, we no longer observed this event.

Overall, our experience was similar to that of other investigators, who reported that the model was associated with a tedious learning curve, and that more than 30 perfusions necessary to achieve reproducible results (54).

Trends of physiologic parameters during EVLP

Our results suggest that, over the 3 hours of perfusion, no significant deteriorations could be observed in oxygenation capacity, PVR or C_{dyn} (Figure 9). The initial decrease observed in PVR in both experimental groups undergoing EVLP is consistent with what is commonly observed in our clinical practice, and usually, it parallels the stepwise increase in perfusion flow during the earlier phase of the protocol. This trend is consistet with that observed by other investigators with the rat EVLP model (41,55). A trend towards an increase in C_{dyn} was observed over the first 90 minutes (Figure 9C), although that was not statistically significant. By analyzing the individual curves, we could observe that increases in C_{dyn} could be observed after each recruitment maneuver, and that the first recruitment maneuvers (i.e., those performed at 60 and 90 minutes) were more beneficial than the subsequent ones (Figure 12). This effect can be explained by the reversal of atelectatic areas of the lung, which may be present after a prolonged period of absence of ventilation. This

finding highlights the role of recruitment maneuvers as a fundamental component of an EVLP protocol.

Overall, the trends of physiologic parameters herein reported compare favorably with those reported by other investigators with a similar model. In particular, Noda et al. (40) in 2014 described a model of rat EVLP followed by transplantation. Lungs were perfused for 4 h after 1 h of cold ischemia time; however, during EVLP, they observed a progressive decrease in C_{dyn} and oxygenation capacity, paralleled by an increase in PVR. Similarly, Nelson et al. (55) observed an increase in lung weight with stable vascular resistance during 1 hour of EVLP, while other functional parameters were not reported. On the other hand, in more recent reports, stable physiologic parameters could be observed over comparable EVLP perfusion times by the Lausanne and Toronto groups (38,42).

The role of albumin as an additive of EVLP perfusion solutions

Albumin is a key component of Steen solution, which is the only perfusion solution approved for clinical use without the addition of blood products (56). The rationale for its use is to provide a high oncotic pressure to the perfusion solution, which helps to delay the onset of lung edema (57). In addition, other investigators have suggested that Steen solution may exert its beneficial effects thanks to cytoprotective and antioxidant properties (58). On the other hand, the OCS™ lung perfusion solution, relies solely on high-molecular weight dextran, rather than albumin, in order to maintain a normal endovascular-interstitial fluid gradient (Table 2). Therefore, with the intention of reducing costs associated with EVLP, it is important to determine what the role of albumin, and if is by omitting it, significant differences in physiologic parameters, and/or lung injury scores may be observed. In fact, cost and availability remain a significant hurdle to the wide adoption of EVLP technology. A recent report analyzing the cost of the DEVELOP-UK trial showed an estimated overall increase in

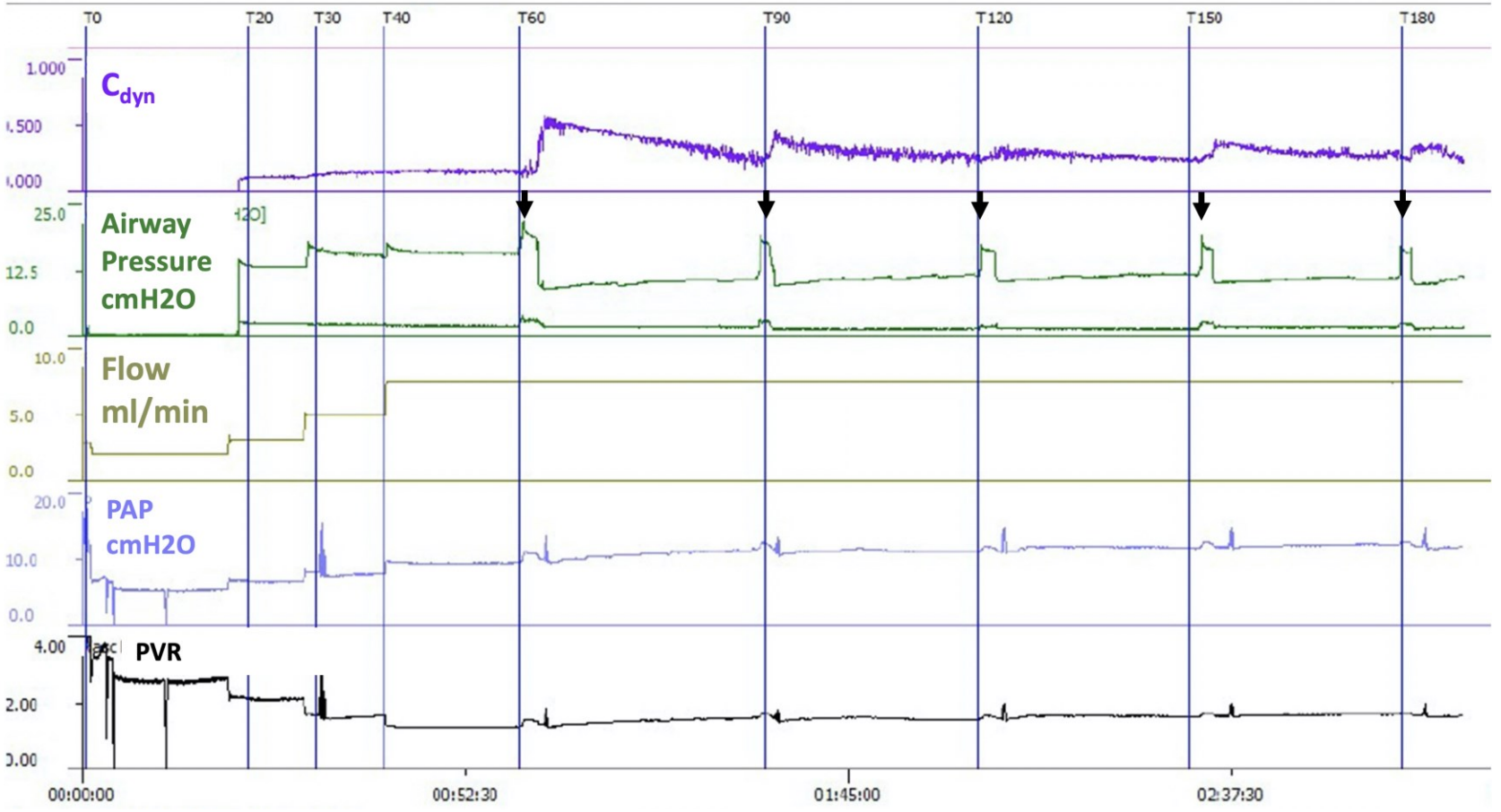

Figure 12. Rough curves relative to the main physiologic parameters during and individual EVLP experiment. Black arrows indicate recruitment maneuvers, which are reflected by temporary increase in airway pressure. A beneficial effect can be observed by an increase in dynamic lung compliance (C_{dyn}) displayed in the corresponding curve. PVR, pulmonary vascular resistances.

cost of $47,000 per run for EVLP compared to conventional cold static preservation (22); which has prompted other investigators to test in-house perfusion solutions (23,24). According to our results, no significant differences could be observed both in the trend of lung function parameters (Figure 9), and in lung injury scores (Table 8). This finding, however, should be interpreted with caution, since perfusion time in this experiment was only 3 hours, which, in most cases, is less than the average duration of clinical EVLP. We don't know the effect of the omission of albumin from the perfusion solution for longer EVLP times. Therefore, further investigation is necessary with longer perfusion times and/or different animal models, in order to confirm such results, which could help making EVLP more affordable and cost/effective in clinical practice.

Pathologic evaluation of lung injury

Evaluation of lung injury in animal models is a critical issue, owing to differences between the clinical and the experimental setting, and to a lack of standardization in measurement of outcome parameters. In this particular case, the assessment of lung injury was carried using a multidimensional evaluation. Histologic evidence of lung injury was assessed by the use of two different scoring systems. A conventional scoring system, borrowed from our Institutional clinical research protocols (59), and a specific scoring system for animal models, compliant with ATS guidelines (49). Alterations of the alveolar-capillary barrier were assessed by measuring the wet/dry ratio, which is an indirect measure of the extent of extravascular lung water. Presence of an inflammatory response was assessed by measurement of inflammatory cytokines and iNOS in lung tissue. Finally, for the EVLP groups, physiological dysfunction could be easily measured with the trend of lung function parameters. Therefore, all four domains recommended by ATS guidelines were investigated.

Beside the comparison of the extent of lung injury between the two EVLP groups, which resulted comparable in all four domains, we assessed the validity of this EVLP model by comparing it with the current standard of care, namely static cold storage (SCS). While most of measured parameters resulted comparable, significantly higher lung edema scores and apoptotic indexes were evident in the EVLP groups compared with the control group. These results are not conclusive, but seem to suggest that EVLP results in a higher degree of lung injury and edema than SCS, although in the absence of necrosis on histology, or gross alterations in physiologic parameters and W/D ratio. The apparent contrast between the result of the W/D radio and of the edema score may be a consequence of the different lung regions that were sampled for the two analyses. In fact, the right superior lobe was used for analysis of W/D ratio, while sagittal sections of the entire left lung were used for assessment of the edema score. Since the lungs were kept in upright position for the duration of EVLP, gravity may have played an important role in the development of edema preferentially in the lower lung areas, leaving relatively unaffected the right upper lobe.

Overall, these findings are in contrast with studies performed with human EVLP, where even lower apoptotic indexes and overall lung injury scores could be observed in lungs preserved by EVLP compared with lungs preserved by SCS (59). On the other hand, the results of TUNEL assay performed on rat lungs preserved by EVLP were comparable with prior studies using the same animal model, in similar experimental conditions (38).

The interpretation of such results should take into account several factors. First, EVLP represents a non-physiological condition, wherein the lung is kept at normothermia, and comes in contact with exogenous substances and materials. The rationale for this is to assess the organs, and potentially, reverse an initial injury by the administration of specific therapies. However, when the initial injury is not evident, the potential for organ recovery cannot be adequately assessed. In this specific case,

lungs were left in situ for 1 hour, at room temperature and ventilated, prior to assigning them to either the EVLPs or the SCS group. However, it is known from experimental studies and clinical practice that the lungs resist better than other parenchymatous organs to warm ischemia. When lungs are ventilated, their retrieval is considered safe up to 3 hours from cardiac death, and experimental studies have demonstrated that safe warm ischemic time in such conditions may extend up to 5 hours (60).

Second, compared to human, the rat animal model is more susceptible to several types of injury, and very few groups have extended EVLP duration beyond 3 hours, with the maximum reported time being 4 hours (38,43). This is significantly different from experiments performed on the porcine EVLP model, where EVLP duration could be extended up to 12 (13) or even 24 hours (20), and can be considered a limitation of this model.

The rat EVLP model is increasingly being used in scientific literature; however, in many studies, the comparison with a control, non-EVLP group is lacking (37,38,41,55), or when this is present, it is constituted by fresh lung tissue, sent for analysis after lung harvesting, under the same experimental conditions as the EVLP groups, without any further intervention (36,42). Therefore, to the best of our knowledge an assessment of the degree of lung injury in EVLP lungs compared to lungs undergoing SCS has never been reported. Our results suggest that, as opposed to the clinical setting, where EVLP is a safe preservation strategy, which may lead to even reduced rates of lung injury compared with static cold storage (59), the same may not apply to the rat model. However, these findings are not conclusive, and need further testing.

In any case, the utility of this model should not be underestimated. In fact, the EVLP platform constitutes an invaluable tool for evaluation of experimental treatments, as well as for studying physiology of isolated organs in different experimental conditions, which cannot easily be replicated otherwise (53). Authors approaching

this model, however, should acknowledge that, to some extent, a certain degree of lung injury may occur, and results obtained from the comparison with non-EVLP groups may not be easily be interpretable or translatable to larger animal models. Moreover, it is possible that with further research, refinement of materials and techniques of rat EVLP will lead to an improvement of the characteristics of this model.

To conclude, this study describes the setting up of a reliable rat EVLP platform for transplantation research. The addition of albumin to EVLP perfusion solution did not lead to an evident benefit in lung function or lung injury parameters, but this result should be interpreted with caution, and tested on larger animal models, for longer perfusion times. Compared to SCS, EVLP rat lungs experienced a higher rate of edema and higher apoptotic index, although in the absence of overt physiological dysfunction. These findings are not conclusive, but hint towards a higher degree of lung injury in EVLP lungs compared to SCS lungs; which, if confirmed by further experiments, should be acknowledged as an inherent limitation of rat EVLP models.

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ACKNOWLEDGEMENTS

This project was funded by a grant issued by Veneto Region (DRG n. 1017 of 17/07/2018), and is part of the "Living, innovative, fully engineered long-lasting and advanced bioreplacement (L.I.F.E.L.A.B.)" program.
The L.I.F.E.L.A.B. program is directed by Prof. Gino Gerosa, as Scientific Coordinator, and by a scientific board composed of researchers and physicians from Padua University and Hospital. CORIS provided administrative support for the project.

I would like to thank L.I.F.E.L.A.B. scientific board and CORIS for their support in the realization of this project.
Moreover, my gratitude goes to all people who supervised or actively took part in the research, and, in particular:

- To Prof. Federico Rea and Prof. Marco Schiavon for giving me the opportunity to start this project and for the guidance provided during its realization;
- To Prof. Fiorella Calabrese and Dott. Federica Pezzuto, for their support in the project and for carrying out the pathologic assessments;
- To Dott. Giulio Faggi for its expertise with the anesthesia protocol and with ventilation settings;
- To Dott. Arben Dedja for helping me to learn the surgical procedures described in this manuscript;
- To all residents and researchers from the Thoracic Surgery Unit who assisted me during the realization of the experiments.