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EXPERIMENTAL EX-VIVO LUNG PERFUSION FOR RECONDITIONING OF LUNG GRAFTS

Wang Xingyu

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Thèse de doctorat ès sciences de la vie (PhD)

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de l'Université de Lausanne

par

Xingyu WANG

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Université des Sciences et Technologie de Huazhong
Wuhan, Chine en 2012

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EXPERIMENTAL EX-VIVO LUNG PERFUSION FOR RECONDITIONING OF LUNG GRAFTS

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1. **Wang X**, Wang Y, Parapanov R, Abdelnour E, Gronchi F, Perentes JY, Piquilloud L, Ris HB, Letovanec I, Liaudet L, Krueger T. Pharmacological reconditioning of marginal donor rat lungs using inhibitors of peroxynitrite and poly (ADP-ribose) polymerase during ex-vivo lung perfusion. *Transplantation* Apr 2016. DOI: 10.1097/TP.0000000000001183

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1. **X Wang**, R Parapanov, I Letovanec, et al. Ex vivo pharmacological inhibition of Poly (ADP-ribose) polymerase (PARP) reduces ischemia reperfusion injury and improves function of transplanted lung grafts. 102nd Swiss Surgery Congress. Lugano, Switzerland. 06/2016

2. S Cavin, **X Wang**, J Perentes, et al. Interstitial fluid pressure: a novel functional biomarker for the monitoring of drug uptake in normal and tumor tissue after photodynamic therapy. 24th Annual Meeting of European Society of Thoracic Surgery. Naples, Italy. 05/2016

3. **X Wang**, Parapanov R, Letovanec I, et al. Ex vivo pharmacological inhibition of Poly (ADP-ribose) polymerase (PARP) reduces ischemia reperfusion injury and

improves function of transplanted lung grafts. 24th Annual Meeting of European Society of Thoracic Surgery. Naples, Italy. 05/2016

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5. **X Wang,** C Marcucci, C Francioli, et al. Experimental ex-vivo lung perfusion with sevoflurane: effect on damaged donor lung grafts. 23rd Annual Meeting of European Society of Thoracic Surgery. Lisbon, Portugal. 06/2015

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8. **X. Wang**, JY. Perentes, Y. Wang, et al. Light dose plays a critical role for the distribution of chemotherapy and tumor mesothelioma xenografts pre-treated by photodynamic therapy. 22nd Annual Meeting of European Society of Thoracic Surgery. Copenhagen, Denmark. 06/2014

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Abstract

Lung transplantation is the only therapeutic option for patients suffering from end-stage lung disease aiming to increase quality and expectancy of life. However, due to the limited number of donor organs eligible for lung transplantation, only a limited number of patients can benefit from this therapeutic option.

The use of "marginal donor lungs" that do not fulfill the standard selection criteria for donor lungs was shown to be valuable option to increase the number of available organs; however, using these lungs may bear an increased risk for graft dysfunction after lung transplantation. Thus the accurate evaluation of these potentially damaged organs prior to transplantation becomes critical to avoid unwanted outcomes. As shown recently, ex-vivo lung perfusion (EVLP) is an appropriate tool to assess donor lungs before transplantation. After procurement, donor lungs are mounted in a specially designed circuit to perfuse and ventilate them at physiologic and protective conditions. Instead of cold ischemic preservation, resulting in a highly slowed down metabolism of the graft, EVLP preserves the organ at body temperature before transplant and allows assessing lung function in a well defined environment outside the body. Since lungs remain metabolically active the concept of normothermic lung preservation also bears the potential to serve for ex-vivo drug delivery to recondition donor lungs for transplantation.

The goal of this thesis is to assess EVLP as a platform to deliver therapeutic agents for repair of damaged lung grafts and to prepare them for transplantation.

In a first step we have established a novel experimental rodent EVLP model to assess and treat donor lung grafts mimicking the clinical setting. This model allows us to keep rodent lungs in physiologic conditions over 4 hours and to assess quantitatively and qualitatively donor lung damage related to typical graft injuries including bacterial contamination or warm ischemia (WI).

WI is an important risk factor for ischemia-reperfusion injury (IRI), known to result in primary graft dysfunction in clinical practice.

a) One of the key processes involved in IRI are the formation of reactive oxygen/nitrogen species (ROS/RNS) and the activation of poly (ADP-ribose) polymerase (PARP). Therefore we sought to investigate whether rat lungs obtained after extended warm ischemia could be reconditioned during EVLP using the inhibitors of ROS/RNS or PARP.

b) Another known effect of WI resulting in IRI is the up-regulation of the Nuclear factor-kappa B (NF-κB), a family of transcription factors, playing a critical role in the inflammatory response. We therefore studied the potential of ex-vivo inhibition of NF-κB pathway to reduce WI induced lung damage.

c) Tissue damage due to IRI is triggered by the release of various inflammatory cytokines. Sevoflurane, a volatile anesthetic, recognized to affect the release of cytokines

in-vitro, was therefore administrated using the EVLP platform and its effect on WI induced lung injury was determined.

We found that all three approaches of ex-vivo lung therapy improved the functional status of damaged lung grafts mounted in the EVLP circuit with significant attenuation of WI induced lung inflammation and tissue injury.

In a further step we have assessed if EVLP can reduce the bacterial load of streptococcus pneumoniae infected donor lungs, and if this affects lung function. We have found that in our experimental setting ex-vivo antibiotic treatment reduces the bacterial load without having a relevant effect on the impaired functional status of infected lungs.

Subsequently we have developed a new experimental model of EVLP followed by unilateral lung transplantation. We than assessed the effects of donor lung reconditioning by EVLP on lung function during blood recirculation in the recipient.

We found that transplantation of damaged lung grafts undergoing sham EVLP displayed severe dysfunction after transplantation. Pharmacological inhibition of PARP during ex-vivo perfusion of injured lungs resulted in significantly reduced IRI and excellent initial lung function after transplantation.

We conclude that EVLP bears the potential to be used as a platform to treat donor lungs before transplantation: (1) to repair preexisting donor lung injuries and (2) to prime the lung to attenuate deleterious effects of blood reperfusion after transplantation. Once translated to clinical practice this may become a pivotal strategy to expand the donor pool with donor grafts initially considered inappropriate to transplant.

Résumé

La transplantation pulmonaire est la seule option thérapeutique pour les patients souffrant d'une maladie pulmonaire chronique terminale qui permette d'augmenter la qualité et souvent l'espérance de vie. Néanmoins, en raison du manque d'organes disponibles pour la transplantation pulmonaire, seul un nombre limité de patients peut bénéficier de cette option thérapeutique. L'utilisation des poumons de donneurs dits "marginaux", qui ne remplissent pas les critères habituels pour une transplantation, a démontré être une option possible pour augmenter le nombre de poumons à disposition pour la transplantation. Toutefois, l'utilisation de ces poumons peut augmenter le risque de dysfonction du greffon après la transplantation. Une évaluation précise de ces poumons potentiellement endommagés avant la transplantation devient donc une étape importante dans le processus de transplantation. Comme montré récemment, la perfusion ex-vivo du poumon est un outil approprié pour évaluer des poumons de donneurs avant la transplantation. Suite au prélèvement, les poumons du donneur sont montés dans un circuit permettant de les perfuser et les ventiler dans des conditions physiologiques et protectrices. On parle de perfusion pulmonaire ex-vivo ou ex-vivo lung perfusion (EVLP). A la place d'une préservation au froid qui diminue le métabolisme du greffon, l'EVLP autorise une préservation de l'organe à 37° avant la transplantation, et ainsi permet d'évaluer la fonction pulmonaire dans un environnement bien défini à l'extérieur du corps humain. Comme les poumons sont métaboliquement actifs, l'EVLP permet aussi d'administrer des traitements médicamenteux ex-vivo, avec pour objectif d'améliorer les poumons avant transplantation (concept de reconditionnement pharmacologique).

Le but de cette thèse a été d'évaluer la perfusion ex-vivo du poumon comme plateforme pour l'administration de substances thérapeutiques aux poumons endommagés et ainsi de les préparer pour une transplantation pulmonaire. Dans un premier temps, un modèle expérimental d'EVLP du poumon de rat a été établi, modèle qui est proche de l'application clinique. Ce modèle permet de garder des poumons de rongeurs dans des conditions physiologiques pendant 4 heures et d'évaluer des poumons endommagés de façon quantitative et qualitative. Des lésions typiques des poumons donneurs ont été évaluées, comme notamment les lésions dues à l'ischémie chaude et à la contamination bactérienne.

L'ischémie chaude est un facteur important déclenchant des lésions d'ischémie-reperfusion, qui aboutit en clinique à la dysfonction primaire du greffon après transplantation. Un des processus impliqués dans l'ischémie-reperfusion est la formation de radicaux libres d'oxygène (reactive oxygen species, ROS) et d'azote (reactive nitrogen species, RNS), ainsi que l'activation de l'enzyme nucléaire Poly-ADP-ribose Polymerase (PARP). Nous avons investigué si les poumons obtenus après un temps d'ischémie chaude prolongé pouvaient être reconditionnés lors de l'EVLP en administrant des inhibiteurs pharmacologiques des ROS/RNS et de la PARP.

Un autre mécanisme de l'ischémie-reperfusion favorisant la dysfonction primaire du greffon est l'activation précoce d'une réponse inflammatoire non spécifique induite par la reperfusion. Cette réponse est orchestrée par un facteur de transcription appelé nuclear factor-kappa B (NF-κB). Nous avons donc investigué la possibilité de reconditionner le poumon au cours de l'EVLP par un inhibiteur du NF-κB).

De plus, nous avons également évalué la capacité d'un agent anesthésique volatil, le sevoflurane, administré dans le circuit d'EVLP, à réduire l'inflammation et les lésions pulmonaires induites par l'ischémie chaude, eu égard à certains effets anti-inflammatoires connus de cet agent.

Nous avons ainsi pu démontrer, dans notre modèle d'EVLP, que les trois approches décrites ci-dessus, permettaient d'améliorer l'intégrité et la fonction des poumons endommagés et que ces trois approches permettaient également une diminution de l'inflammation pulmonaire due à l'ischémie chaude.

L'étape suivante a été d'évaluer si la perfusion isolée du poumon permettait de diminuer la charge bactérienne dans des poumons contaminés par la bactérie *Streptococcus pneumoniae*, et si une telle réduction permettait d'améliorer la fonction pulmonaire. En administrant un traitement antibiotique ex-vivo dans le circuit d'EVLP, nous avons ainsi pu montrer que la charge bactérienne des poumons était réduite, toutefois, sans que cela n'ait d'effet relevant sur la fonction pulmonaire des poumons infectés.

Lors de la dernière étape, un modèle expérimental de perfusion ex-vivo du poumon suivi par une transplantation uni-pulmonaire chez le rongeur a été développé. Nous avons évalué des poumons donneurs reconditionnés par l'EVLP dans ce modèle de transplantation pulmonaire. En utilisant ce modèle, nous avons montré que des poumons endommagés par une ischémie chaude, perfusés dans un circuit d'EVLP sans ajout de substance thérapeutique montraient une dysfonction sévère après la transplantation. Par contre, l'application d'un inhibiteur pharmacologique de la PARP lors de l'EVLP de ces poumons endommagés a permis une réduction significative des lésions d'ischémie-reperfusion après transplantation, se traduisant par une fonction excellente des poumons reconditionnés, après la transplantation.

En résumé, la perfusion ex-vivo du poumon a le potentiel d'être utilisée comme plateforme de traitement des poumons de donneurs endommagés : 1) pour réparer des lésions préexistantes chez le donneur 2) pour préparer le poumon afin de diminuer des effets qui auront lieu lors de la reperfusion du poumon lors de la transplantation.

Une fois introduite en clinique, cette stratégie permettra d'élargir le pool des donneurs en utilisant des poumons initialement considérés comme non éligibles pour la transplantation.

List of Abbreviation

3-AB	3-aminobenzamide
3-NT	3- Nitrotyrosine
4-HNE	4-Hydroxynoneal
AU	Arbitrary Unit
BALF	Bronchoalveolar Lavage Fluid
BOS	Bronchiolitis Obliterans
CI	Cold Ischemia
CINC-1	Neutrophil Chemoattractant Factor 1
CMV	Cytomegalovirus
CO	Carbon Monoxide
DAMPs	Damage-Associated-Molecular-Patterns
DBD	Donation after Brain Death
DCDD	Donation after Circulatory Determination of Death
$DppO_2$	Differential Partial Pressure of Oxygen
ET	Expiratory Time
EVLP	Ex-vivo Lung Perfusion
FiO_2	Fraction of Inspired Oxygen
HSPs	Heat Shock Protein
IFN-α	Interferon Alpha
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IRI	Ischemia Reperfusion Injury
ISHLT	International Society for Heart and Lung Transplantation
IT	Inspiratory Time
LAP	Left Atrial Pressure
LDH	Lactate Dehydrogenase
LLP	Left Lung Parenchyma
LPD	Low Potassium Dextran
Ltx	Lung transplantation
MAP	Mean Arterial Pressure
MDA	Malondiaadehyde

MNs	Mononuclear Cells
MnTBAP	Mn(III)-tetrakis(4-benzoic acid) porphyrin chloride
M_{PAW}	Mean Airway Pressure
NAD^+	Conenzyme Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate-oxidase
NF-KB	Nuclear Factor kappa B
NO	Nitric Oxide
O_2^-	Superoxide Anion Radical
OI	Oxygenation Index
PaO_2	Partial Pressure of the Oxygen
PA	Pulmonary artery
PAP	Pulmonary Artery Pressure
PAR	Poly (ADP-ribose) Polymerase
PARP	Poly (ADP-ribose) Polymerase
PAWP	Peak Airway Pressure
PDTC	Pyrrolidine Dithiocarbamate
PEEP	Positive End Expiratory Pressure
PGD	Primary graft dysfunction
PIP	Peak Airway Pressure
PMNs	Polymorphonuclear Cells
PN	Peroxynitrite
PV	Pulmonary vein
QoL	Quality of life
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SEVO	Sevoflurane
SPC	Static Pulmonary Compliance
SVR	Systemic Vascular Resistance
TLRs	Toll like Receptors
TNF-α	Tumor necrosis
WI	Warm Ischemia

Table of contents

List of Figures

List of Tables

Chapter 1 General Introduction

1.1 Lung transplantation

Since the first successful lung transplantation (LTX) in men three decades ago (1), lung transplantation gained wide acceptance and is currently the only therapy of end-stage lung disease (e.g. cystic fibrosis, emphysema, pulmonary fibroses, etc) aiming to improve quality of life and survival. A major drawback is donor lung shortage which makes this treatment option only available to a very selected group of patients suffering from terminal respiratory insufficiency.

1.1.1 Brief history and current trend

In 1940s, Demikhov demonstrated the feasibility of the technique of Ltx using a canine model (2). Human Ltx was reported by Hardy in 1963. Although surgery was successful, the patient died on post-op day 18 due to kidney failure (3). In the following 10 years, multiple attempts of Ltx failed because of post-transplant graft rejection, as well as issues with healing of the bronchial anastomosis. An acceptable long-term survival after Ltx was only achieved following the development of posttransplant immunosupression (4). In 1983, Cooper from Toronto performed the first successful Ltx with long-term survival (5). Five years later the same group reported on the first successful bilateral Ltx using a sequential technique (6), which became the standard in clinical practice.

In 2014, the registry of the International Society of Heart and Lung Transplantation counted a cumulative number of over 50,000 Ltx worldwide since the introduction of this treatment modality in 1985, of which 96 % were primary Ltx and 4% were lung re-transplantations [Figure 1.1, (7)]. Patients undergoing Ltx during this timeframe had a median survival of 5.7 years, with unadjusted overall survival rates after single or double Ltx of 89% at 3 months, 80% at 1 year, 65% at 3 years, 54% at 5 years, and 31% at 10 years. Survival after lung transplantation is known to be higher when reported from single centers and more recent periods. Survival of all consecutive patients undergoing double or single lung transplant at the Lausanne university hospital from 2006 to 2014 (n=200, heart-lung and retransplantation excluded, Centre Universitaire Romand de Transplantation) have a 1-, 3-, 5- and 10-year survival of 91, 82, 75 and 65 %. Although a survival benefit has not yet been proven for all transplant indications, quality of life is significantly improved with 80% of survivors presenting no limitations of daily activities up to 10 years post Ltx (8). The major causes of death reported during the early post-transplant period are primary graft dysfunction (PGD), infection, cardiovascular and surgery related events. Chronic lung allograft dysfunction, infections and malignancy account for most deaths after the first post-transplant year (7). Figure 1.2 shows the current survival curves of primary adult lung transplant as reported by the international registry.

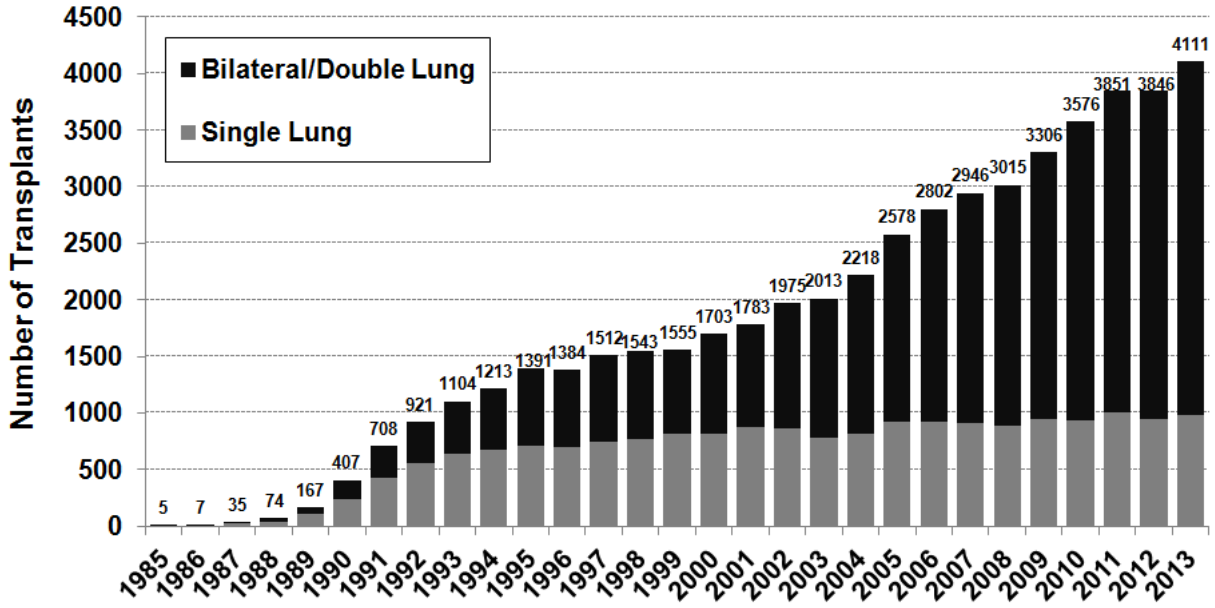

Figure 1.1 Number of adult lung transplantation reported by year and procedure type. [Cited from the 2015 official adult lung and heart-lung transplantation report (7)]

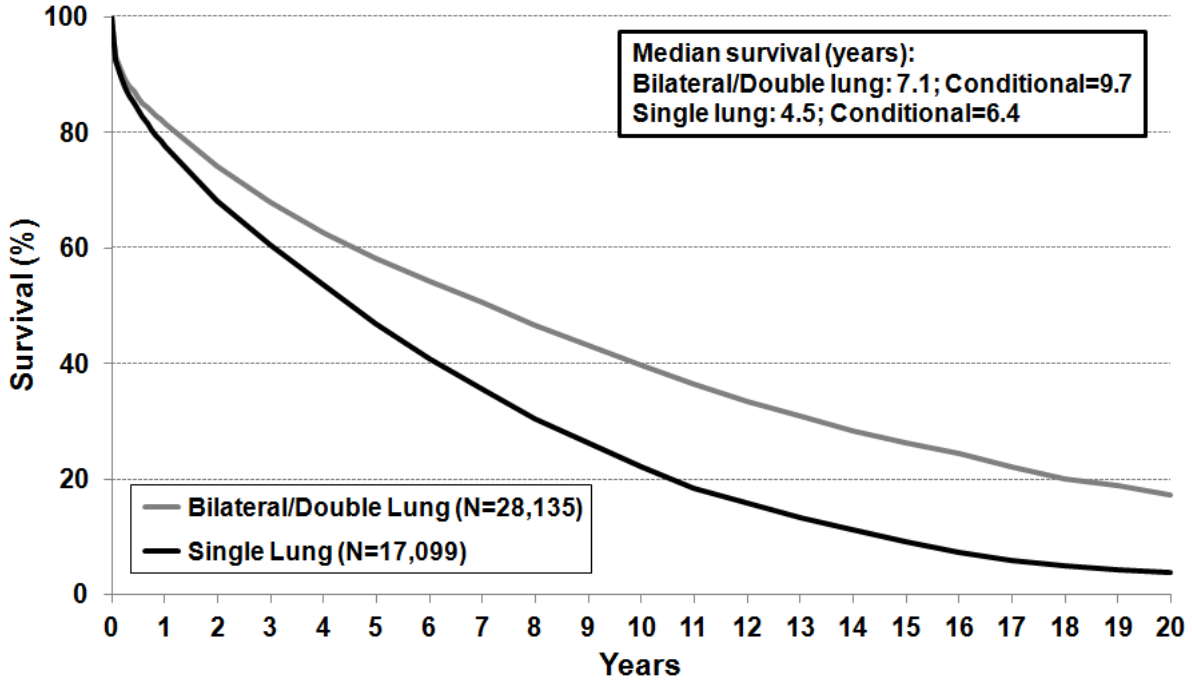

Figure 1.2 Primary adult lung transplant recipient Kaplan-Meier survival, stratified by procedure type. Cited from the 2015 official adult lung and heart-lung transplantation report (7)

1.1.2 Selection of donor lungs and organ shortage

Selection criteria for donor lungs were established during the development of Ltx. According to an expert consensus, the ideal donor must fulfill a number of criteria outlined by the international Society for Heart and Lung Transplantation (ISHLT), Table 1.1 (9). Furthermore, surgeons perform a physical examination of the potential donor lung at retrieval including macroscopic observation and palpation to exclude any detectable intrinsic lung disease and abnormalities. Such stringent criteria are considered to limit the risk of PGD, a type of acute lung injury and dysfunction which plays an essential role for post-transplant morbidity and mortality (10), and which increases the risk of chronic lung allograft dysfunction in transplanted patients (10).

The application of restrictive selection criteria contributes to a low utilization rate of donor lungs from multi-organ donors, with an estimated refusal rate around 80%. Consequently critical organ shortage is inevitable and translates into increased waiting times and waitlist mortality.

• Age<55years
• ABO compatibility
• Clear chest radiograph
• PaO_2/FiO_2 (1.0)>300, PEEP 5cmH2O
• Tobacco history<20 packs/year
• Absence of chest trauma
• No evidence of aspiration/sepsis
• No prior cardiopulmonary surgery
• Sputum gram stain-absence of organisms
• Absence of purulent secretions at bronchoscopy

Table 1.1 Current ISHLT ideal donor lung selection criteria (9)

1.1.3 Donation after neurological determination of death lungs

The principle source of donor lungs is the so called "neurological determination of death" donor (DNDD). In this type of donor, the complete brain function is irreversibly damaged, indicating that the legal definition of death is fulfilled. In potential donors the heartbeat and ventilation are maintained by intensive medical care, preserving for a limited period of time the viability of organs (11). Unfortunately, acute lung injury occurs to a certain degree in all potential donors as a consequence of brain death. Brain stem death leads to sympathetic stimulation with increased heart rate and cardiac index (12). The sudden increase of systemic vascular resistance leads to a drop in ventricular output and therefore an enhanced pulmonary capillary pressure, causing increased epithelial permeability (13, 14). The so-called neurogenic lung edema develops. In addition, an up-regulated systemic inflammatory response takes place (13, 14). The over-expression of pro-inflammatory cytokines such as Interleukin-1beta (IL-1β), Interleukin-6 (IL-6), Tumor necrosis factor alpha (TNF-α) induces cell adhesion molecules on the epithelial and endothelial surfaces and results in the recruitment of neutrophils and monocytes, and causes lung inflammation (15).

Next to these endogen mechanisms, excessive volume administration, lung trauma related to mechanical ventilation, and oxygen toxicity contribute to the lung injury (16, 17). In addition, neurological death multiorgan donors, who all need to be mechanically ventilated, are at very high risk to develop ventilator associated pneumonia (18).

1.1.4 Donation after circulatory determination of death lungs

To overcome the shortage of lung donors, alternative sources of lungs have been considered. Donors in whom the circulatory arrest precedes brain death are nowadays used to extend the donor pool. The procedure is called donation after circulatory determination of death (DCDD). Lungs remain viable after cardiac arrest for certain period of time (19). The current classification of DCDD is based on the timing of cardiac arrest prior to organ procurement, so called "Maastricht Categories" and is subdivided in 5 categories. Patients dead on arrival at hospital (Cat. I) and patients undergoing unsuccessful resuscitation (Cat. II) are considered as uncontrolled DCDD donors; patients undergoing cardiac arrest after withdrawal of therapy (Cat. III) and patients undergoing cardiac arrest following brain death (Cat. IV) are considered as controlled DCDD donors; the 5^{th} category of patients describes cardiac arrest in hospital patients (uncontrolled) (20).

Since the first clinical experience with DCDD in 1995 by Love et al (21), numerous transplant centers worldwide have adopted controlled DCDD (Cat.III) in their Ltx program,

and more than 300 donor lungs of this category have been transplanted between 2003 to 2013 (22). The use of DCDD lungs results in comparable post-transplant outcomes to the DBD lungs, with an overall survival at 1, 3, 6, 12 and 24 months after transplantation of 94%, 94%, 94%, 94% and 87% respectively from DCDD donors, and 92%, 88%, 84%, 78% and 69% (23).

Given the increased risk of unidentified lung damage in uncontrolled DCDD and the uncertainty about the effects of the agonal phase before cardiac arrest (24), this strategy is still underutilized (e.g. the utilization rate in the United States is 1.9% (25). Indeed, a longer agonal time with hypoxia and low systemic blood pressure before organ retrieval in DCDD donors, may be associated with an increased risk of PGD following Ltx (26).

1.1.5 Extended criteria donor lungs

As a consequence of the shortage of ideal donor organs and the high waitlist morbidity and mortality numerous centers use today "extended criteria" or "marginal" organs for transplant which fail to fulfill ISHLT standard selection criteria but may still be transplantable. This strategy demonstrated equivalent short-term outcomes (27-32).

However, standardization of extended criteria appears to be difficult, due to the variable definition of extended criteria lungs among different centers. The Toronto lung transplant group has defined different types of lung donor as shown in table 1.2 (10).

Selection Criteria	Standard Criteria (*Ideal Donors*)	Extended Criteria (*Extended Donors*)	Contraindications (*Marginal Donors*)
ABO compatibility	Identical	Compatible	Incompatible
Donor history			
Age, yr	< 55	> 55	
Smoking history, pack-years	< 20	> 20	
Chest trauma	No trauma	Localized trauma	Extensive lung trauma
Duration of mechanical ventilation, h	< 48	> 48	
History of asthma	No	Yes	
History of cancer	No (except low-grade skin cancer and carcinoma *in situ*)	Primary central nervous system tumors	History of cancer
Sputum gram stain	Negative	Positive	
Oxygenation, mm Hg*	> 300	< 300	
Chest X-ray	Clear	Localized abnormality	Diffuse infiltrates
Bronchoscopy	Clear	Secretions in main airways	Persistent pus/signs of aspiration

* Last blood gas performed in the operating room with an F_{IO_2} of 100% and positive end-expiratory pressure of 5 cm H_2O.

Table 1.2 Ideal, extended and marginal donor selection criteria suggested by the Toronto lung transplant group

A donor scoring system based on five items has been proposed to help clinical decision making, including age, smoking history, chest x-ray, secretions and the ratio of partial pressure of the oxygen on fraction of inspired oxygen (PaO_2/FiO_2) (33). Today, the cut off

values for most parameters describing donors and donor lungs are not well defined and rather based on expert opinion than on a higher level of evidence.

The use of donor lungs not fulfilling standard criteria may bear an increased risk of graft dysfunction, as shown in various studies (30, 32, 34). There is an agreement that new strategies need to be developed to better predict the post transplant function of potential lung graft, especially when organs do not fulfill standard criteria.

1.2 Normothermic ex-vivo lung perfusion

Recent alternative approaches to expand the donor pool such as the utilization of DCDD and extended criteria lung grafts increase the complexity of donor lung selection with potential fatal outcomes in recipients if graft damage is not recognized before transplant. Thus a detailed and careful evaluation of potential lung grafts becomes even more important. With the recent introduction of ex-vivo lung perfusion (EVLP) transplant teams have a potent platform at their disposal to assess potential donor lungs and to identify and repair damaged organs before transplant.

1.2.1 Standard static hypothermic preservation

It is current clinical practice that the decision whether to use or not a lung for transplantation has to be taken based on donor history, laboratory tests, radiological imaging, and physical examination of the graft in the donor, at least in centers where EVLP is not available. Once the lung is accepted, the organ is flushed with a preservation solution at 4°C, procured and preserved at 4°C until it is placed in the empty cavity of the recipient for implantation. The duration of cold ischemia (CI) is usually limited to 8 hours (26). The reason of cold preservation is to reduce the rate of metabolic activity, to reduce ischemic injury and thus maintaining the viability of the graft until blood reperfusion. During the procurement procedure, a number of strategies are applied to minimize graft damage, such as protective ventilation to avoid barotrauma, cold flushing with a low potassium dextran-glucose solution, administration of anticoagulants and vasodilators; inflation of the lung for cold ischemic storage. Figure 1.3 illustrates the graft temperature during ischemia, starting with the clamping of the donor's aorta and ending at the moment of re-ventilation and the blood reperfusion in the recipient.

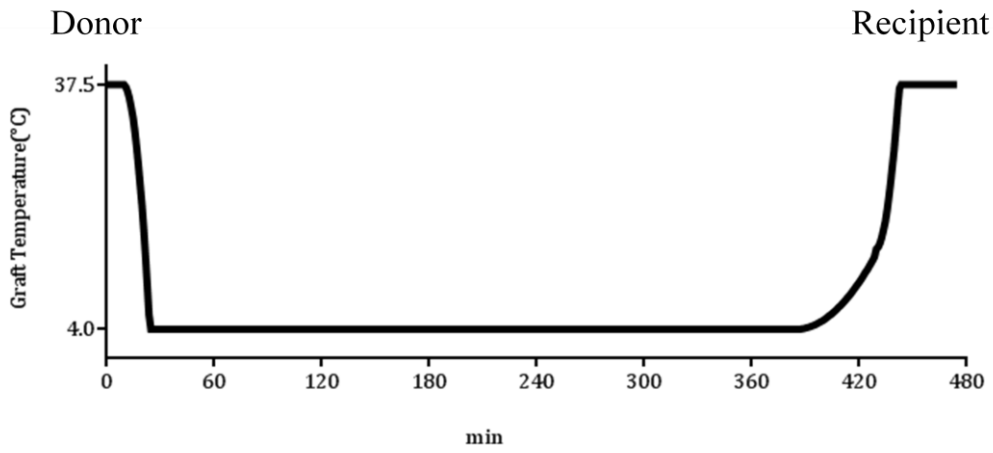

Figure 1.3 Current standard donor lung preservation

1.2.2 Ex-vivo preservation and assessment

With the standard procedure of cold preservation the graft evaluation is limited to the time before organ procurement, which can be largely influenced by the clinical experience of the retrieval team, the donor management, and systemic effects induced by donor brain death.

In contrast, EVLP gives the opportunity to assess organs once they have been harvested. The lung is procured at 4°C as described above, transported to the transplant center and mounted in the EVLP circuit where it undergoes normothermic preservation. Metabolic activity is restored at that time thus allowing for assessment of lung function.

Described by Steen and coworkers (35-37), normothermic EVLP was initially employed to assess DCDD lungs. Following this pioneer work, Shaf Keshavjee from Toronto has extensively investigated the use of EVLP in extended criteria donor lungs which resulted in a specific EVLP protocol of protective ex-vivo lung perfusion and ventilation (38).

Once the harvested lung is equipped with the PA and LA cannulae, and an endotracheal tube, it is connected to the primed perfusion circuit and its vascular system is de-aired. It is then connected to the ventilator in a semi-inflated state. The perfusion is performed with an extracellular solution complemented with Dextran and Albumin to achieve optimal rheological properties and a high colloid pressure (Steen Solution, XVIVO, Sweden). Antibiotics and steroids are added. The inflow pCO2 is corrected by controlling the CO2 supply through the gas-exchange membrane. Perfusion is initiated slowly, the graft is progressively warmed, the flow is increased gradually and when the circuit reaches a temperature of 32°C, ventilation is started. Respiratory rate is 7/min, tidal volume is 7ml/kg, positive end-expiratory pressure is 5cmH_2O, and the FiO_2 is 21%. When the lung temperature reaches 37°C, the perfusion flow is increased to a maximum of 40% of the cardiac output calculated according to the ideal bodyweight of the donor. The LA pressure

is set to 3-5mmHg, the PA pressure is monitored and depends on the vascular resistance of the lung and the preset flow. In general, clinical EVLP is performed for a period of 4 to 6 hours, the graft is cooled down, and preserved at 4°C until implantation (Figure 1.4).

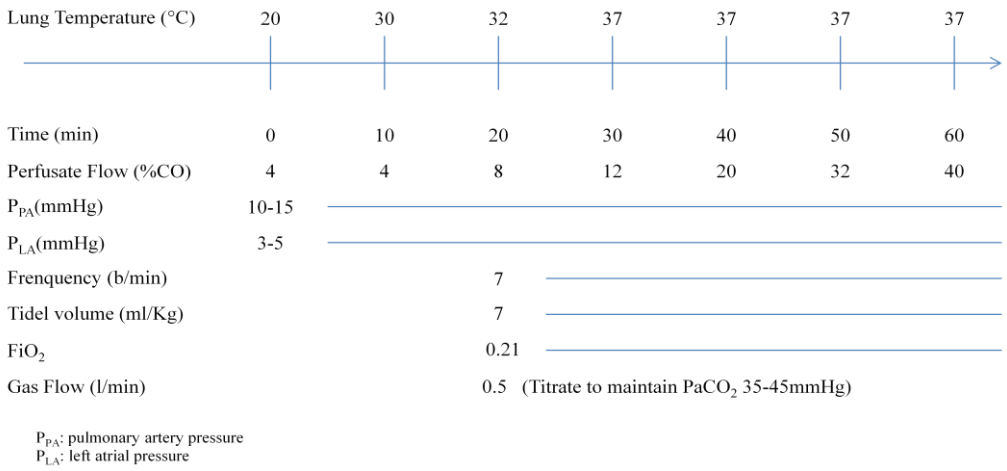

Figure 1.4 Toronto clinical EVLP protocol (38)

During the ex-vivo perfusion, several aerodynamic (lung compliance, airway pressure) and hemodynamic (pulmonary vascular resistance) measurements, and measurements of the grafts' oxygenation capacity (differential partial pressure of oxygen of affluent and effluent perfusion solution) are repeatedly performed. Therefore the donor graft can be repeatedly and reliably evaluated in order to determine its suitability for transplantation. The decision to accept or decline the EVLP lung for Ltx is primarily based on the trend of the parameters mentioned above over 4 to 6 hours of EVLP. Once the lung is accepted for transplantation the lung is cooled down again, stored again at 4°C and then implanted (Figure 1.5).

While ex-vivo evaluation was initially limited to a short time frame, the Toronto group has successfully *performed* prolonged normothermic EVLP up to12 hours, by using an acelluar perfusate and a centrifugal pump with reduced perfusion flow and maintained positive left atrium pressure (39).

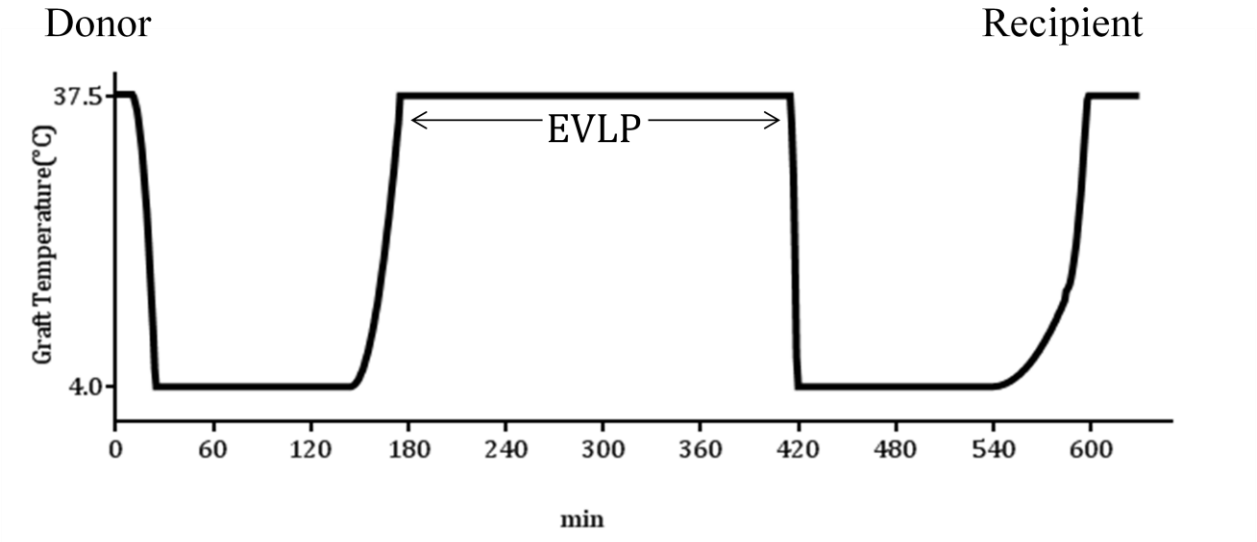

Figure 1.5 EVLP to preserve or assess donor lung grafts at body temperature

1.2.3 Ex-vivo reconditioning and therapeutic interventions during EVLP

Apart from being a potent tool for organ assessment and preservation, EVLP provides an opportunity to treat donor lungs since the metabolism of the lung is preserved. It is a well recognized effect that normothermic EVLP favors atelectatic lung areas to be recruited, thus allowing a better ventilation-to-perfusion ratio in the graft. Ex-vivo bronchoscopies and ventilatory recruitment maneuvers may be used to further improve the homogeneity of ventilation.

More importantly, EVLP may serve as a therapeutic platform to deliver therapeutic agents though airway or/and pulmonary vessels. Drugs can be ex-vivo administered at high doses since doses are only limited by direct tissue toxicities but not by systemic adverse effects.

Firstly, the ex-vivo treatment can be directed against preexisting donor lung injuries (e.g. edema, pulmonary embolism, pneumonia). Secondly, therapeutic interventions during EVLP might reduce the severity of the unavoidable adverse events induced by the blood reperfusion of the donor lung at the time of transplantation e.g. to prime the graft for transplant. (e.g. immunological events, ischemia-reperfusion injury).

The best established ex-vivo pharmacological intervention is the use of the acellular perfusate, the so called Steen® solution which has a high oncotic pressure, thus eliminating lung edema during EVLP. The therapeutic effects of various other drugs have been investigated in clinical or experimental settings, such as the use of anti-inflammatory

substances (40, 41), antioxidants (42), fibrinolytics (43), bronchodilators (44), and antibiotics (45).

1.3 Ischemia reperfusion induced pulmonary injury

After cold ischemic preservation, or cold ischemic preservation interrupted by normothermic preservation (EVLP), the lung is implanted into the recipients' chest cavity, and once the pulmonary vasculature is de-aired and vascular anastomoses are completed, blood circulation to the ischemic graft is progressively restored. The graft reperfusion by the recipients' blood, which is the final step of the surgical procedure, permits to reestablish organ function. However, the reperfusion also triggers a complex pathophysiological event, the so-called ischemia reperfusion injury (IRI). It is characterized by an increased alveolar-capillary permeability, enhanced pulmonary vascular resistance, interstitial edema, impaired oxygenation and pulmonary hypertension (46). It can be observed to various extents within the first 72 hours after transplantation in all patients. Severe IRI is an essential factor contributing to the occurrence of primary graft dysfunction, a significant cause of post-transplant morbidity and mortality and chronic lung allograft dysfunction (47). Recent studies have shown that up to 20% of transplants undergo PGD (48, 49). Therefore, substantial attention is drawn to strategies circumventing the damage elicited by IRI and the incidence of PGD.

1.3.1 Consequence of ischemic storage

Donor lungs from DNDD donors fulfilling the standard selection criteria for LTX are flushed with cold preservation solution and then stored at 4°C to decrease the metabolic rate. Although unavoidable to prevent irreversible ischemic damage, grafts exposed to cold preservation will still undergo a series of physiological changes that eventually deteriorate the organ function when re-perfused (47). When DCDD lungs are used, donor organs are inevitably exposed twice to a period of warm ischemia (WI): first of all, from the time of cardiac arrest in the donor until the start of cold organ perfusion (for legal and ethical reasons the patient cannot be touched during 5 to 10 minutes after cardiac arrest in most countries), and second, during transplantation, from removal of the organ from the cold storage until blood reperfusion in the recipient (50).

Additional WI amplifies the ischemic damage, due notably to the residual cellular metabolism while still lacking oxygen and nutrients (50). A number of key mechanisms are involved in the ischemia induced damage. Firstly, oxidative stress characterized by unstable free radical formation (reactive oxygen species [ROS]) promotes tissue injury by initiating lipid peroxidation and various oxidative damages in proteins and nucleic acids, leading to necrotic cell death (51). Secondly, the energy-dependent sodium pump (Na^+/K^+-

ATPase) is inactive during ischemia, resulting in influx of sodium and causing cell swelling (50). Thirdly, overloaded intracellular calcium and altered pH can derange cellular processes. Finally, during ischemia, the lack of mechanotransduction (52) in the arterioles and capillaries induces macrophages and endothelial cells mediated early inflammatory response, which play pivotal role in the subsequent reperfusion injury (47).

1.3.2 Consequence of reperfusion

Following hyperacute derangements triggered by ischemia of the graft, blood reperfusion itself paradoxically promotes further damage and dysfunction. Ischemia activated macrophages and endothelial cells induce the activation of platelet/thrombin and the formation of microvascular thrombosis (53), contributing to blood flow perturbations (47). Besides, when reperfusion occurs, macrophages and endothelial cells, associated with lymphocytes and epithelial cells release pro- and anti-inflammatory cytokines, chemokines (e.g. IL-1β, IL-6, TNF-α, IL-10, IL-8 and interferon alpha [IFN- α], etc) and adhesion molecules, modulating downstream leukocyte recruitment and infiltration, finally resulting in an inflammatory cascade (47, 50). Together with activated ROS and reactive nitrogen species (RNS) (54) signaling pathways, this inflammatory response results in reperfusion-induced vascular injury, translating into an increased pulmonary vascular resistance (PVR) and microvascular permeability (55). As a consequence, lung edema, poor gas exchange and ultimately graft dysfunction develops. Figure 1.6 briefly summarizes the mechanisms of IRI.

1.3.3 The role of peroxynitrite-poly (ADP-ribose) polymerase pathway in IRI

The oxidative stress leads to increased release ROS and RNS which generate a great amount of superoxide anion radical (O_2^-) and nitric oxide (NO), respectively (55). An important characteristic of O_2^- and NO is the capability to react with each other and to form a highly toxic new molecule, known as peroxynitrite (PN). PN interacts with lipids, DNA and proteins through a direct oxidative/nitrosative stress pathway or an indirect radical-mediated mechanism (56). This interaction triggers a cell response varying from some cell signaling modulation (57, 58) to overwhelming cellular oxidative injury (59). PN attacks nucleic acid and leads to DNA single-strand breaking, leading to the activation of a nuclear enzyme, called poly (ADP-ribose) polymerase (PARP) (60). Under normal physical conditions, the function of PARP is to facilitate DNA repair by binding to DNA strand breaks and transferring ADP-ribose units from the respiratory coenzyme nicotinamideadeninedinucleotide (NAD^+) to various nuclear proteins (60). However, in case of severe DNA damage, as a result of oxidative stress with a considerable formation of PN, PARP is over-activated and depletes NAD^+ store (60). As a pivotal factor of the

tricarboxylic acid cycle, the loss of NAD^+ decreases ATP levels, and finally results in cellular dysfunction and necrotic cell death (60). Another key pathophysiological function of PARP relies in its pro-inflammatory promotion. Activated PARP induces the over-expression of many inflammatory mediators, through up-regulation of various pro-inflammatory transcription factors, most importantly, the nuclear factor kappa B (NF-κB) pathway (61, 62), which is considered as a principle regulator of innate responses and inflammation during ischemia and reperfusion (63, 64).

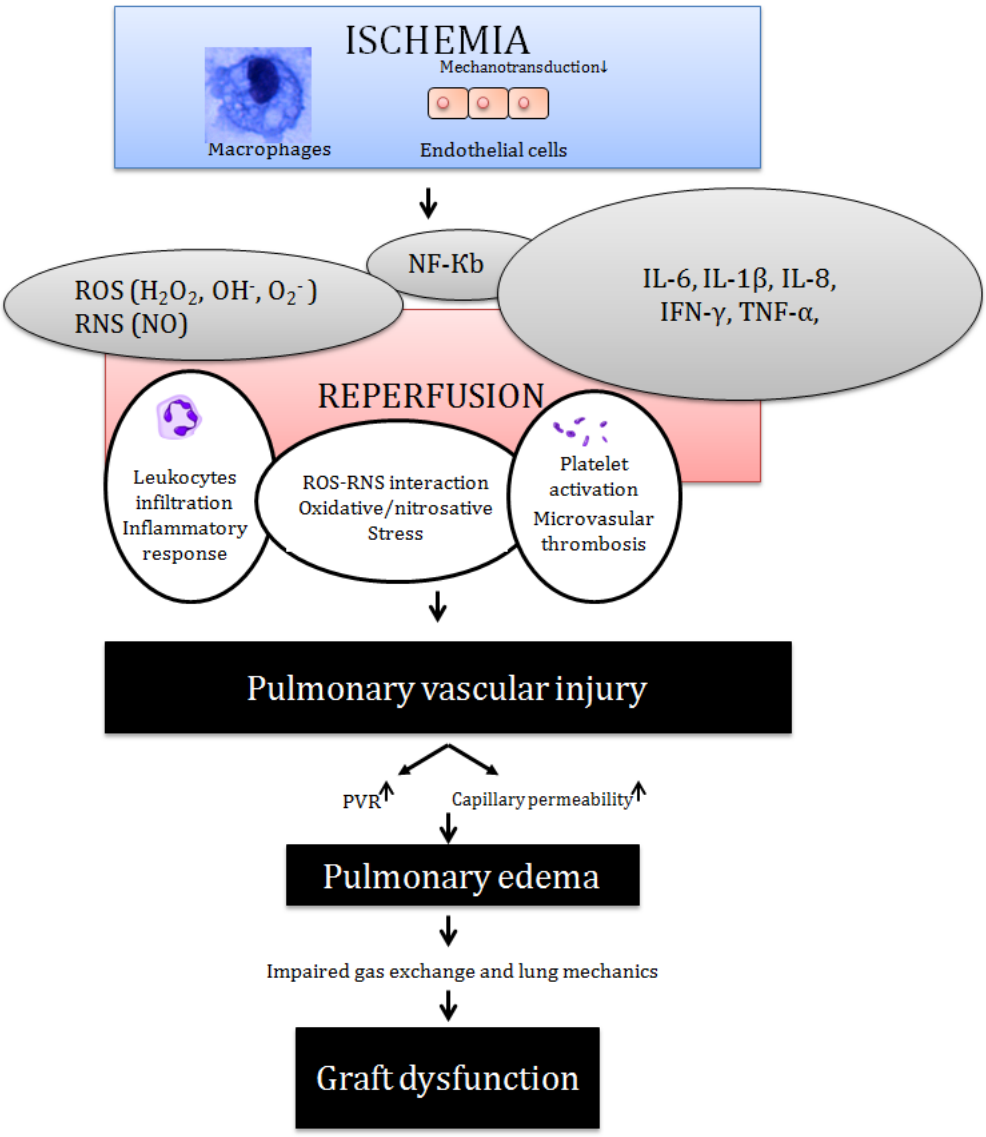

Figure 1.6 Principle mechanisms involved in ischemia reperfusion induced lung injury

Increased formation of PN occurs in the reperfused lung following Ltx, as shown by the detection of nitrotyrosine, a footprint of PN (53, 65). PN synthesis related cytotoxicity was shown to be related to unwanted outcomes after lung transplantation, such as obliterative bronchiolitis, a major form of chronic lung allograft dysfunction (66, 67).

In contrast, few evidence exists about the role of PARP in ischemia reperfusion injured lungs, although its implication in this context has been shown in other transplanted organs, such as the heart (68, 69) and the kidney (70), or in other experimental lung injury models, such as sepsis (71) and hemorrhagic shock (72).

1.3.4 Strategies to circumvent lung IRI

Numerous strategies to limit ischemia reperfusion induced lung damage have been proposed using various experimental settings. Most of the preclinical work has been performed in animal models of transient *in-situ* ischemia followed by lung reperfusion, or in models of transplantation with donor treatment before harvesting or during cold preservation.

Those therapeutic strategies can be briefly summarized as (1) anti-oxidant strategies, applying free radical scavenger [e.g. edaravone(73)] to protect mitochondria, inhibition of xanthine oxidase (74) or NADPH oxidase (75); (2) anti-inflammatory strategies, inhibition of pro-inflammatory transcription factors [NF-κB (76), activator-protein-1(77)], depletion of alveolar macrophage/neutrophil, inhibition of inflammatory-related mediators [cytokines (78), complement (79)]; (3) Intra-airway/venous delivery of vasodilators and modulation of cytoprotective pathways, such as NO (80), prostaglandins (81), carbon monoxide [CO (82)]; (4) others approaches such as therapeutic preconditioning (83, 84), gene therapy (85) and protective lung preservation and reperfusion (86-89).

1.4 Summary

Lung transplantation represents the most promising therapy for end stage lung disease. The number of organs considered as transplantable is significantly lower than the number of patients waiting for lung transplant. This leads to an increased waiting time, and waiting list mortality. The use of DCDD lungs or extended criteria lungs will at least in part alleviate this problem, but may conversely increase the risk of unwanted bad outcome after lung transplantation, if irreversibly damaged donor lungs are transplanted.

EVLP emerges as an opportunity to better evaluate and preserve potential lung grafts not fulfilling standard donor selection criteria. It may also serve as a platform to repair damaged grafts or to prime donor lungs for adverse effects occurring after lung transplantation with the goal to expand the pool of available organs. In this PhD thesis, I

investigate in an experimental setting the potential of EVLP-mediated drug delivery to recondition donor lungs injured by warm ischemia.

Chapter 2

General Hypotheses, Objectives and Thesis Structure

2.1 General hypotheses

1. Experimental ex-vivo lung perfusion accurately assesses the severity of donor lung injury.

2. Warm ischemia induced donor lung injury can be repaired by ex-vivo therapeutic intervention

2.2 General objectives

In this research project the candidate evaluates in an experimental setting the potential of EVLP to repair typical donor lung injuries. The objectives are:

- to develop a rodent model of damaged donor lungs and to assess these lungs in a downsized EVLP system,

- to apply different ex-vivo therapies during EVLP addressing donor lung injuries related to ischemia or bacterial contamination, with the aim to repair damaged lung grafts ex-vivo and to render these lungs eligible for transplantation,

- to establish a rodent unilateral lung transplant model, and

- to select a promising ex-vivo therapy and to assess in the transplant model if the benefit of ex-vivo reconditioning translates into an improved graft function after transplantation.

2.3 Thesis structure

Chapter 3: Development of a novel rodent model of EVLP for the functional assessment of variably damaged lung grafts.

Chapter 4: Ex-vivo inhibition of peroxynitrite and poly (ADP-ribose) polymerase in lungs injured by warm ischemia.

Chapter 5: Ex-vivo inhibition of NF-κB using pyrrolidine dithiocarbamate in lungs injured by warm ischemia.

Chapter 6: Ex-vivo conditioning of ischemic lungs by sevoflurane.

Chapter 7: Ex-vivo delivery of antibiotics to lungs colonized by *Streptococcus pneumonia.*

Chapter 8: Development of an acute unilateral rodent lung transplant model.

Chapter 9: Transplantation of injured donor lungs reconditioned by ex-vivo pharmacological inhibition of poly (ADP-ribose) polymerase

Chapter 10: General conclusions and future directions

Chapter 3

Establishment of a rat EVLP platform: Transfer of the Toronto protocol of human EVLP to a rodent model and ex-vivo assessment of ischemic lungs

Abstract

Background: Normothermic ex-vivo lung perfusion (EVLP) provides an opportunity to assess and repair injured donor lungs for subsequent transplantation. Here we describe a downsized experimental EVLP platform based on the Toronto technique of human EVLP. We assess the effect of different preservation conditions on ex-vivo lung function of rodent lungs.

Methods: Following cardiac arrest, lungs underwent either 3 hours cold storage (C3h), or 1 hour warm ischemia followed by 2 hours of cold storage (W1h), or 2 hours warm ischemia followed by 1 hour cold ischemia (W2h). All the lungs were then ex-vivo perfused for 3 hour, during which static pulmonary compliance (SPC), pulmonary vascular resistance (PVR), and differential partial pressure of oxygen ($DppO_2$) were measured. At the end of EVLP, privascular edema was histologically evaluated, lung weight gain was calculated, protein and lactate dehydrogenase (LDH), protein carbonyl and 3-nitrotyrosine (3-NT) were determined in bronchoalveolar lavage (BAL) or lung tissue.

Results: SPC improved in C3h, remained stable in W1h and decreased significantly in W2h. PVR increased in all groups although no significant difference was found. $DppO_2$ remained stable in C3h and W1h but significantly decreased in W2h. Perivascular edema, lung weight gain, protein in BAL and LDH remained stable in C3h, increased moderately in W1h but markedly in W2h. Protein carbonyl and 3-NT levels increased non-significantly in group of C3h while significantly in W1h and W2h.

Conclusion: This rat EVLP model allows for quantitative assessment that reflects the degree of damaged lung in accordance with the presence and the length of warm ischemia. It may be used to assess ex-vivo pharmacological treatments of damaged donor lungs.

Introduction

Cold static preservation (CSP) has been the only strategy to preserve donor lungs from procurement to implantation until the concept of normothermic ex-vivo lung perfusion (EVLP) has been introduced in clinical practice. While CSP aims to reduce the metabolism to prevent irreversible damage due to ischemia, EVLP maintains lung metabolism since the lung is ventilated and perfused outside the human body at normothermia (38). This allows to assess ventilatory mechanic and gas exchange capacity of donor lungs ex-vivo and to better select grafts before transplantation (90). Since the maintenance of lung tissue metabolism is the main principle of normothermic EVLP, this technology also gives an opportunity for ex-vivo therapy. Two principle strategies may evolve in the future: (1) Treating pre-existing donor organ injuries to render a damaged organ eligible for transplantation [e.g. oedema, embolism (85, 91)], and/or (2) Preparation of the organ for adverse events following lung transplantation, such as ischemia-reperfusion injury, or rejection (92).

The concept of EVLP has recently been introduced in clinical practice (36). Various approaches of ex-vivo lung perfusion and ventilation have been described (35, 38, 93, 94). Most cases from series in the literature describe the outcome of EVLP applying the Toronto technique, which can be considered as the best evaluated approach to date (93, 95-97). Today, EVLP mainly serves for organ preservation and assessment. The utilization of ex-vivo drug therapies is restricted to the use of a defined hyperoncotic perfusion solution to reduce oedema, and heparin, steroids as well as antibiotics.

We here describe in detail a downsized experimental model of the Toronto technique of EVLP for assessment of lungs variably damaged by ischemia. This model may serve as a platform for development of innovative ex-vivo therapies of donor lungs.

Material and Methods

Animal and housing

Eighteen male adult Sprague Dawley rats (mean weight 377g, Charles River, L'Arbresle, France) were used. All the animals were *ad libitum* fed with standard diet and drink, housed in laminar flow cages in a conventional animal facility, and treated in accordance with National Institute of Health Guidelines for the Care and Use of Laboratory Animals and the cantonal commission (Authorization Nr. VD2637)

Animal model

The rats were anesthetized by intraperitoneal injection of pentobarbital (50mg/kg, Streuli Pharma AG, Uznach, Switzerland). The rats were then placed on an animal surgical table (Hugo Sachs Electronik (HSE), Hugstetten, Germany)in a supine position. This was followed by tracheotomy and intubation. Mechanical ventilation was done by a standard rodent ventilator (model 683, Harvard Apperatus, Holliston, MA) using a room air supplemented with oxygen (FiO_2 0.21). Tidal volume was of $7ml \cdot kg^{-1}$ and respiratory rate was 75 min^{-1}. A midline sternotomy was performed and the chest cavity was exposed by a retractor (Medicon eG, Tuttlingen, Germany). Heparin (600IU, Drossapharm AG/SA, Basel, Switzerland) was injected into right ventricle. Following systemic heparinization, 2 manufactured metal cannulas (Hugo Sachs, Hugstetten, Germany) were introduced into the pulmonary artery (PA, ID=1.7mm, OD=2.0mm) and the left atrium through ventricular incisions (LA, ID=3.4mm, OD=4.0mm). Subsequent to the asystole, animals were randomly assigned to one of 3 experimental.

Study design

Different warm and cold ischemia conditions were tested

1) C_{3h} group, (N=6): Lungs were immediately flushed through the PA- and vented trough the LA cannula with 15ml of 4°C Perfadex® (Xvivo Perfusion, Goteborg, Sweden), heart and lung were *en bloc* extracted, lungs were kept inflated, and preserved at 4°C Perfadex® for 3 hours, followed by 3 hours of EVLP;

2) W_{1h} group, (N=6): Lungs were left *in situ*, exposed to room temperature for 1 hour, then flushed through PA- and vented trough the LA cannula with 15ml of 4°C Perfadex®, heart and lung were *en bloc* extracted, lungs were kept inflated, and preserved at 4°C Perfadex® for 2 hours, followed by 3 hours of EVLP;

3) W_{2h} group, (N=6): Lungs were left *in situ*, exposed to room temperature for 2 hours, flushed through the PA- and vented trough the LA cannula with 15ml of 4°C Perfadex®, heart and lung were *en bloc* extracted, lungs were kept inflated, and preserved at 4°C Perfadex® for 1 hour, followed by 3 hours of EVLP.

4) Baseline group, (N=6): lungs were procured immediately after cardiac arrest, without cold flush.

During cold Perfadex® flush, infusion pressure was set at 15mmHg) and all the lungs were ventilated with respiratory rate of $15 \cdot min^{-1}$ and tidal volume of $7ml \cdot kg^{-1}$. Study groups are illustrated as Figure 1.

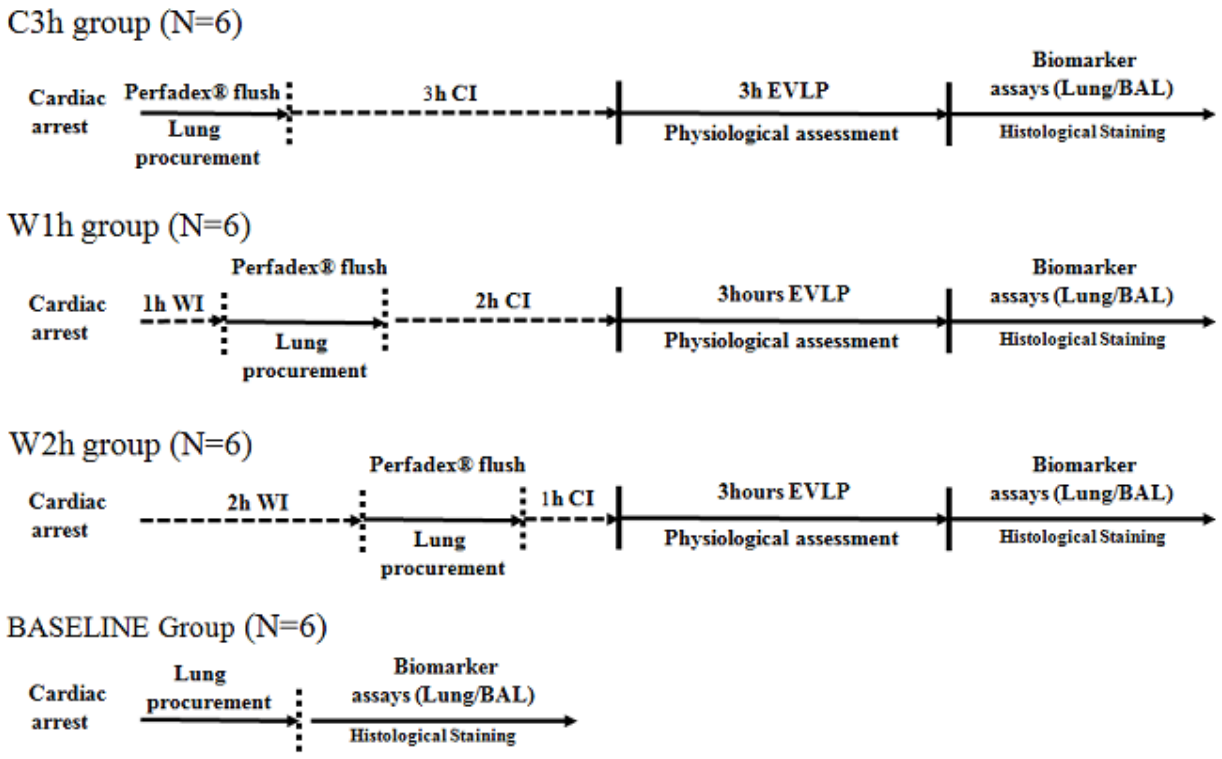

Figure 3.1 study design

Experimental Ex-vivo lung perfusion & ventilation system

A modified isolated lung perfusion system [Harvard IL-2 System, TYPE 829/2, Hugo Sachs Electronik (HSE), Hugstetten, Germany] was used in our study. The primary functional units of the perfusion system are a speed adjustable roller pump (ISM 827/230V, HSE,

Hugstetten, Germany), a gas-exchange membrane (Hemofilter D150, MEDICA S.P.A, Italy,) a heat exchanger, a jacketed lung chamber, a pressure equilibration vessel close to the LA cannula allowing to control the pressure of the venous outflow, a jacketed perfusate reservoir and a closed system of perfusion tubes. The temperature of the perfusion system was regulated and maintained by a water thermostatic circulator (Alpha immersion thermostat 6, LAUDA-Brinkmann, Delran, NJ, USA) and a heater-cooler unit (Sarns TCMI, 3M, Saint Paul, MN, USA) connected to the lung chamber, a heat exchanger connected to the tubing system, and the perfusate reservoir. The perfusate was deoxygenated by a gas mixture of 6% O_2, 10% CO_2 and 84% N_2 supplied through the gas-exchange membrane.

PA- and LA- pressures of the graft were measured by pressure transducers (P75 TYPE 379, HSE, Hugstetten, Germany) which were placed in the tubing system close to the PA and LA cannula. Two flow-through oxygen electrodes (HSE, Hugstetten, Germany) were employed to measure in real-time the partial pressure of oxygen (pO_2) of the perfusate before and after the lung, respectively. Signals from the pressure transducers and the oxygen electrodes were converted and monitored using a data collector (USB data acquisition hardware, HSE, Hugstetten, Germany). A perfusion control unit was integrated to allow for lung perfusion with constant flow or constant pressure (TYPE 704, HSE, Hugstetten, Germany). Ex-vivo lung ventilation was performed using a Scireq Flexivent ventilator (FX3, SCIREQ Inc, Montreal, Canada) connected to the tracheal tube of the graft. The ex-vivo lung perfusion and ventilation system is shown in Figure 2A and 2B.

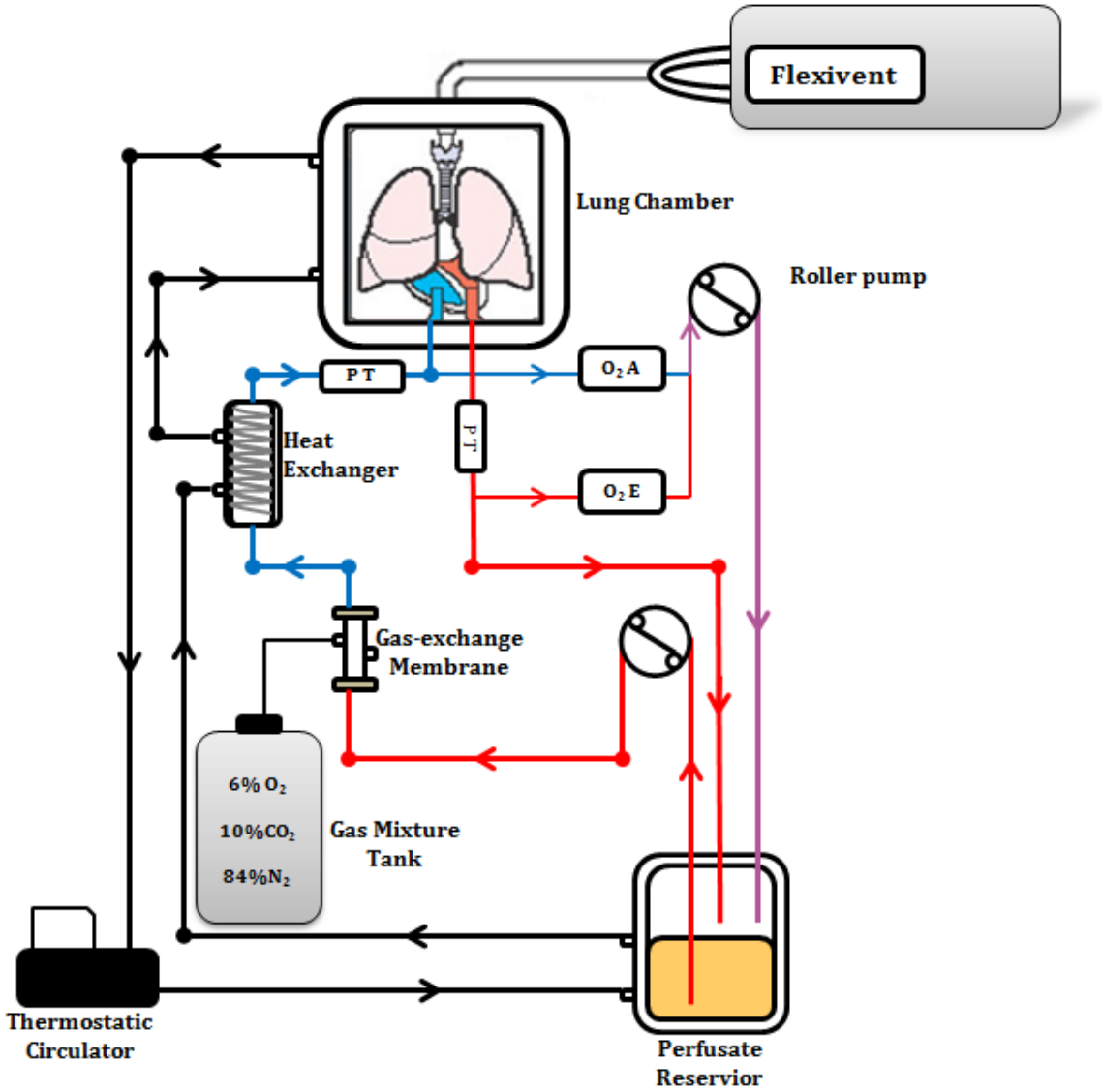

Figure 3.2.A Schematic diaphragm of Ex-vivo lung perfusion and ventilation system

Normothermic EVLP requires ventilation and perfusion of the organ: A closed circuit of lung and perfusion system is constructed, comprising an organ chamber to create a protective environment for the graft during perfusion, a pump to drive the perfusate, a reservoir and tubing containing the perfusion solution, a gas exchange membrane to supply the circuit with a gas mixture to remove oxygen and add carbon dioxide, a heat exchanger for temperature control. PA as well as the LA pressures are monitored during perfusion (PT: pressure transducer). The perfusate flows from the lung to a reservoir, then to the gas - and heat exchange system before entering the lung again. In order to ventilate the lung, a tube is inserted in the trachea and connected to a ventilator.

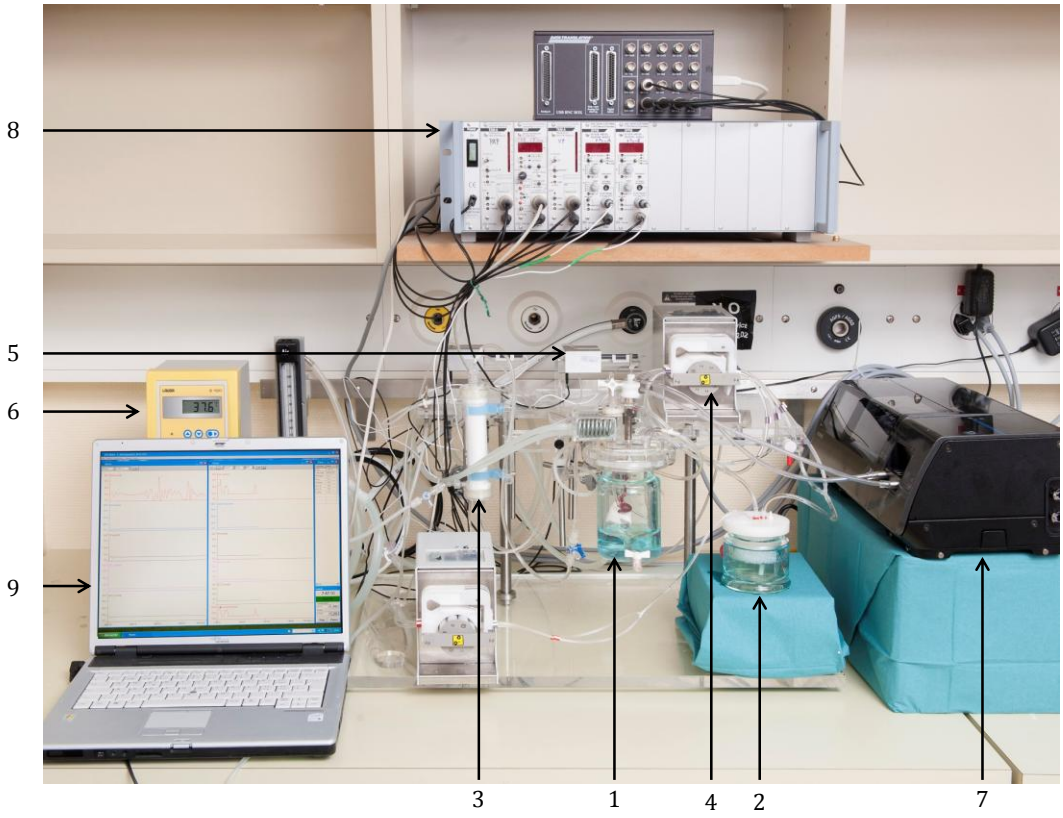

Figure 3.2.B Rodent EVLP platform

1: Lung chamber; 2: Perfusate reservoir; 3: Gas-exchange membrane; 4: Roller pump; 5: Flow-through oxygen electrode; 6: Thermostatic circulator; 7: Ventilator; 8: Data collection unit; 9: Data recording and analysis.

Preparation of EVLP circuit

The EVLP circuit was primed with 100ml of Steen® solution (XVIVO Perfusion AB, Goteborg, Sweden) at room temperature. The roller pump, pressure transducers, oxygen electrodes and ventilator were calibrated in compliance with the manufacturer instruction. The circuit was maintained at 10°C prior to the onset of lung perfusion.

Initiation, rewarming and normothermic phase of EVLP

Following cold storage, the heart-lung block and cannulas were weighted using a laboratory balance (PB-602C, METTLER TOLEDO, Greifensee, Switzerland) before

mounting it in the EVLP circuit. Perfusion was flow-controlled, with the initial flow set at 2ml·min^{-1} and the temperature at 10°C. The PA cannula was connected to the circuit, and an antegrade washout of the lung was performed to discharge clots and bubbles which may have remained in the pulmonary vascular bed. Then the LA canula was connected to the perfusion circuit and perfusion was started.

The perfusion flow and temperature were gently elevated to reach the target flow of 8 % of the estimated cardiac output and 37.5°C within 30min. Cardiac output was estimated according to following formula: 3.75*60*bodyweight(kg)$^{0.72}$ (98). The LA pressure was maintained at 4cmH_2O by adjusting the height of equilibration vessel. The perfusate pH was maintained stable between 7.35 and 7.45 by adding gas mixture or buffer (THAM-Kohler 3M, Kohler pharma GmbH, Alsbach-Hahnlein, Germany)

In initial experiments the perfusion mode was switched to a pressure-controlled mode with a constant PA pressure preset to 15mmHg. Since this pressure-controlled perfusion mode resulted in healthy lungs in a perfusate flow through the lung of 7-8 % of the animals' estimated cardiac output, the perfusion was always flow controlled in later experiments.

When normothermia was achieved, the mechanical ventilation was started (V_t=3ml·min^{-1}, frequency=7·min^{-1}, PEEP=3cmH_2O, FiO_2=0.21). After 10 minutes V_t was increased to 6ml·min^{-1} and the lung was recruited with the plateau pressure gradually increased from 15cmH_2O to 18cmH_2O. EVLP duration was 180min. The EVLP protocol is shown in Figure 3A.

Physiological assessment of Ex-vivo lung

Real-time PA and LA pressure were continuously recorded to calculate pulmonary vascular resistance (PVR): PVR (cmH_2O·ml^{-1}·min^{-1}) = (mean PA pressure-LA pressure)/Flow. PaO_2 of Steen® solution in influent and effluent of the grafts was recorded to calculate differential partial pressure of oxygen ($DppO_2$) to determine the graft oxygenation capacity. At selected time points during EVLP (at 30min, 90min, 150min 180min), static pulmonary compliance (SPC) was measured by the ventilator analyzing inspiratory pressure-volume curves during a defined perturbation. The time points of Ex-vivo assessment of these parameters during EVLP is illustrated in Figure 3B. After EVLP the heart-lung bloc including cannulas was weighed again to calculate the weight change. Then 2ml of sterile PBS (pH 7.4) was injected in the airway to perform bronchoalveolar lavage (BAL), and the samples were snap-frozen and kept at -80°C (U570 premium, Eppendorf, Hamburg, Germany). The lungs were stored at -80°C

A

Time (Minutes)	0	10	20	30	40	50	60	90	120	150	180
T(C°)	12	20	30	35	37.5						
Constant flow perfusion (Percentage of estimated cardiac output)	2	2	4	7	8	8	8	8	8	8	8
V_T(ml/kg)	no			**3**	6						
RR(breath/min)	no			7							
PEEP(cmH_2O)	no			3.0							
FiO_2	no			0.21							

B

Time (Minutes)	0	10	20	30	40	50	60	90	120	150	180
Physiological Assessment	pH										
	PO_2 (PA, mmHg)										
	PO_2 (LA, mmHg)										
	PVR ($cmH_2O \cdot ml^{-1} \cdot min^{-1}$)										
	---------						SPC($cmH_2O \cdot ml^{-1}$)				
	WG	---------									WG

Figure 3.3.A EVLP protocol. Ventilation and perfusion settings

(F-Ctrl: Flow controlled mode; P-Ctrl: Pressure controlled mode; V_T: Tidal volume; RR: Respiratory rate; PEEP: Positive end-expiratory pressure)

Figure 3.3.B Lung function assessments during EVLP

pO_2: Partial pressure of oxygen; PA: Pulmonary artery; LA: Left atrium; PVR: Pulmonary vascular resistance; WG: Weight gain.

Biomarker assays

Lung tissue and BAL samples were proceeded to perform biomarker assays as described below. A group of rats (Baseline group, N=6) were sacrificed and the lungs were procured without other intervention to determine the baseline value of these assays.

Rat lung protein extraction and quantification

Lung tissue was grinded to powder in liquid nitrogen and homogenized in lysis buffer (TrisHCL 10mM, NP40 0.5%, NaCl 0.15 M, Na3VO4 1 mM, NaF 10mM, PMSF 1 mM, EDTA 1 mM, aprotinin 10µg/ml, leupeptin 10µg/ml, and pepstatin 1µg/ml), sonicated and incubated 20min on ice. Then samples were centrifuged at 13000rpm for 10min and proteins were quantified by the BCA assay according to manufacturer's guide (Thermo Scientific Pierce, Rockford, IL, USA).

Protein and lactate dehydrogenase (LDH) level in BAL

Protein in BAL was determined using the Pierce BCA assay (Thermo Scientific, Rockford, USA), and expressed in $mg \cdot ml^{-1}$. LDH was measured using a commercial kit (Cytotoxicity Detection KitPLUS; Roche, Basel, Switzerland), and expressed in arbitrary units.

Protein carbonyl concentration in lung tissue

Protein carbonyl was quantified in lung tissue using an ELISA-based kit (OxiSelect Protein Carbonyl ELISA Kit; Cell BIolabs Inc., San Diego, CA, USA) with accordance to the user manual. The absorbance was measured at 450nm and the results was expressed as $nanomol \cdot mg\ protein^{-1}$.

3-nitrotyrosine (3-NT) concentration in BAL

3-NT was determined as a footprint of peroxynitrite generation using a commercial ELISA kit (Rat 3-nitrotyrosine ELISA kit; Amsbio, Abingdong U.K.), and expressed in $nanomol \cdot ml^{-1}$.

Histopathological analysis

Histopathological examination of the right lung was performed in all animals following 3 hours of EVLP. Lungs were fixed with OCT and 4% paraformaldehyde injected intrabronchially. Five micrometer sections were sliced from paraffin-embedded lung tissue, stained with hematoxylin-eosin (HE) and analyzed by two investigators independently in a blinded fashion, using an image analysis program (Slidepath, Leica Biosystems). On each slide, 20 pulmonary vessels (arteries and veins) were assessed, and the ratio of perivascular edema thickness to the inner diameter of the surrounded vessel was calculated to describe the degree of edema.

Statistical analysis

Data were analyzed using Graphpad prism 6 (Graphpad Software Inc., La Jolla, CA, USA) and are presented as means±SEM. Normality test was performed using *Kolmogorov-Smirnov* test. For physiological measurements (SPC, PVR and $DppO_2$), *2-way ANOVA* was performed, followed by *Dunnett's* test to analyze the effects of time and *Sidak's* test to evaluate the effect of different preservation conditions. For other comparisons, 1-way ANOVA followed by *Tukey's* correction was applied if data passed normality test, otherwise *Kruskal-Walllis* test and Dunn's correction was applied. P values of less than 0.05 were considered to indicate statistical significance.

Results

Macroscopic appearance of lungs undergoing 3 hrs of EVLP

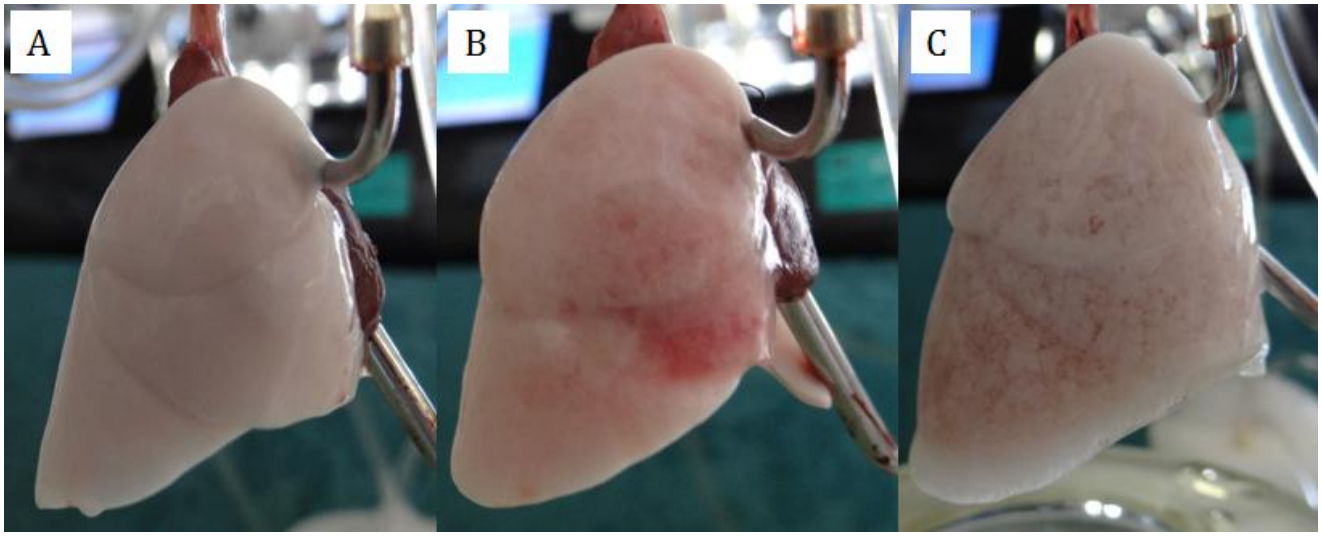

Figure 3.4 Appearance of lungs at the end of EVLP

Appearance of lungs undergoing either A: 3 hours of cold preservation (C3h), B: 1 hour of WI and 2 hrs of CI (W1h) and C: 2 hours of WI and 1hr CI (W2h) followed by 3 hrs of normothermic EVLP.

Ex-vivo assessment of lung function

Ex-vivo pulmonary mechanics as assessed by SPC slightly improved over 3 hours of EVLP in group C3h, were stable in group W1h throughout EVLP, but lower as compared to group C3h, and were lowest and deteriorated over time in group W2h (Fig. 3.5.A). The W2h group had significantly decreased SPC from 90 to 180 minutes of EVLP as compared to group C3h.

PVR decreased over time during the first 90 minutes and remained then stable in all three experimental groups. The highest PVR values were measured in group W2h, however no statistically significant difference was observed (Fig. 3.5.B).

The lungs' diffusion capacity as assessed by the difference of pO2 in the influent and effluent perfusate was lowest in the W2h group and this difference reached statistically

significance when compared to both other groups at 90 minutes and later. The oxygenation capacity was similar in C3h and W1h groups (Fig. 3.5.C).

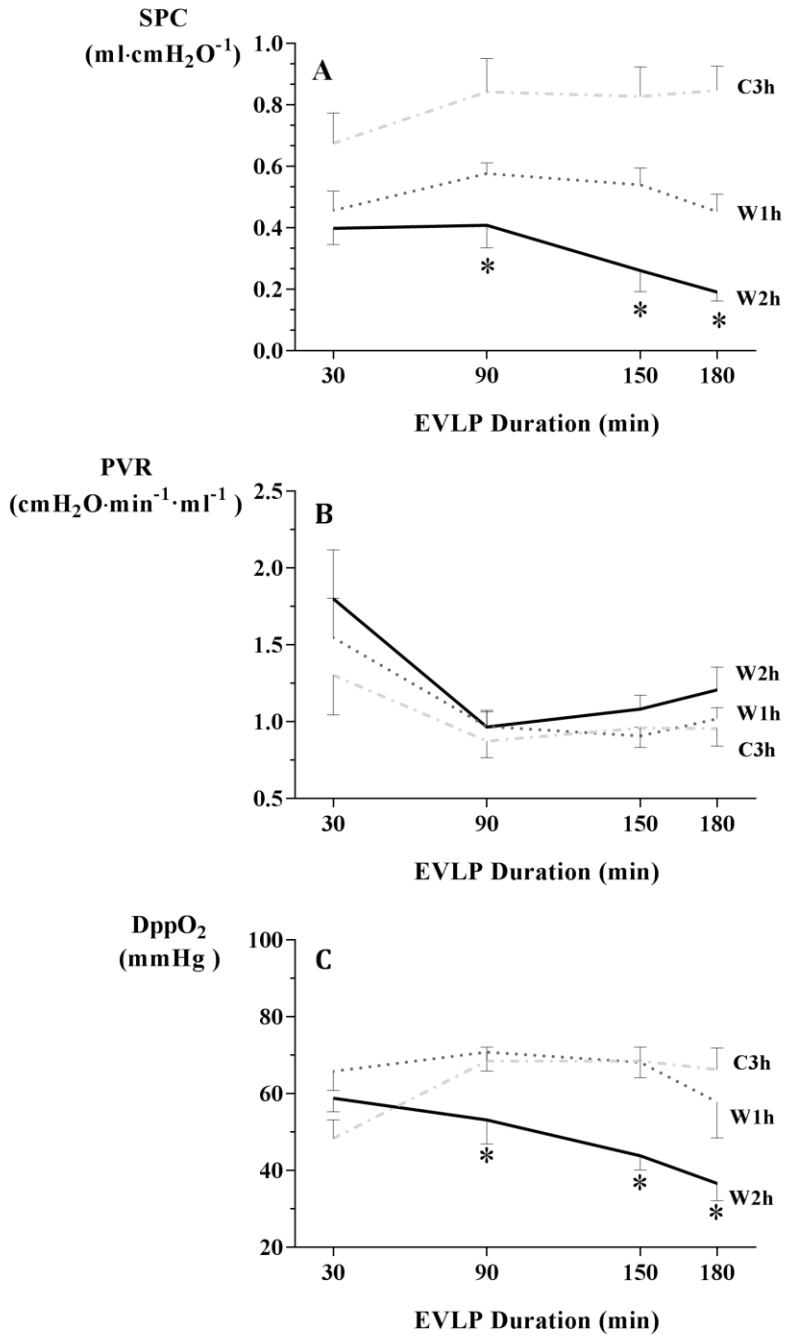

Figure 3.5 3-hour-EVLP: ex-vivo assessment of pulmonary compliance, pulmonary vascular resistance and oxygenation capacity *:$p<0.05$

Necrosis, oxidative and nitrosative stress in lung tissue

Significant higher LDH levels were found in tissue samples from lungs in W2h group (Figure 3.6.A) as compared to C3h and W1h groups ($p<0.05$). Significantly increased levels of protein carbonyl in lung tissue were found in all EVLP groups as compared to the baseline group, levels in W2h group were also significantly higher than in group C3h (Figure 3.6.B). 3-NT levels were highest in group W1h and W3h and significantly increased as compared to baseline (Figure 3.6.C)

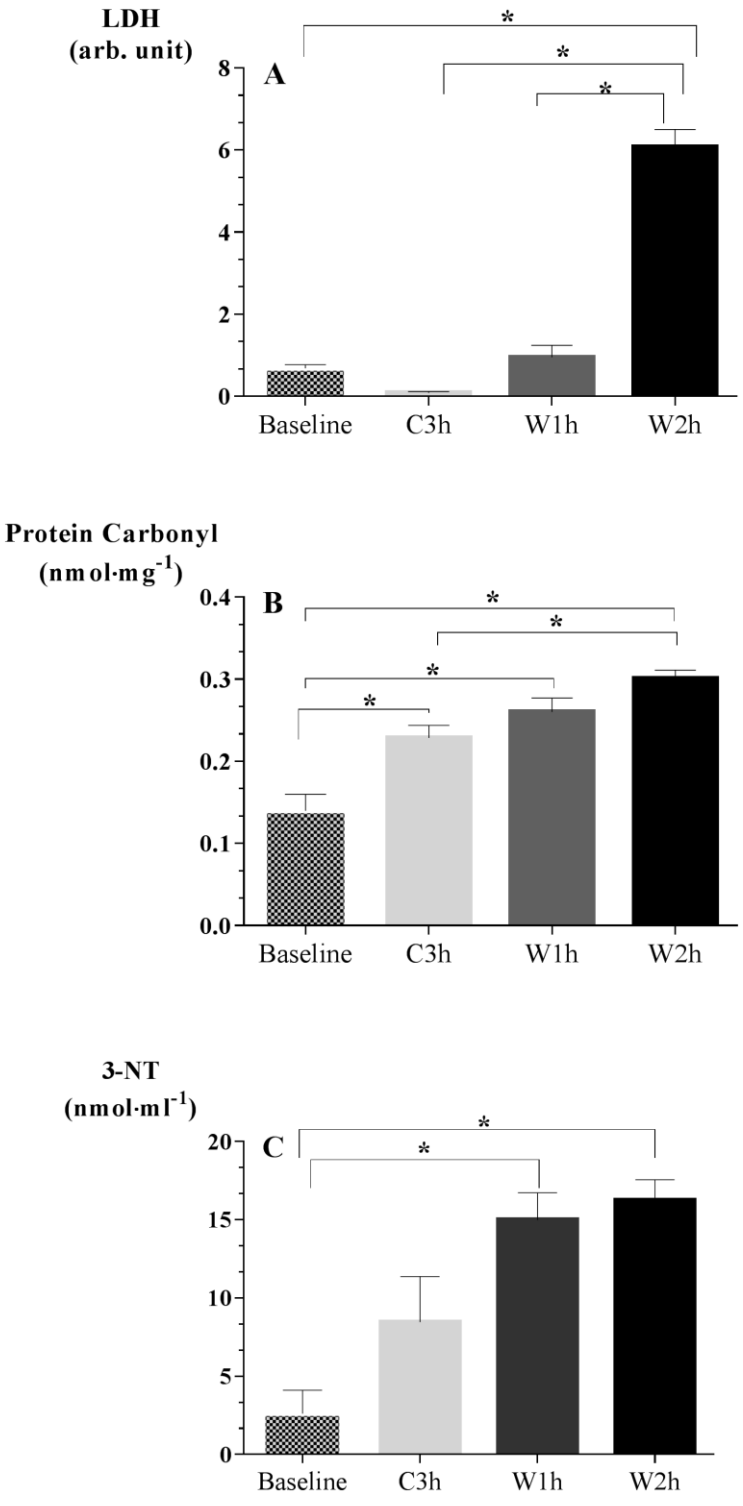

Figure 3.6 Lung tissue necrosis, oxidative and nitrosative stress during EVLP *:p<0.05

Lung edema devolvement during EVLP

Lung interstitial edema as assessed by lung weight gain during 3 hours of EVLP significantly increased in W2h group as compared to all other groups (Figure 3.7.A). In parallel, lungs in Baseline, C3h and W1h group displayed low protein content in the BAL, while lungs with extended warm ischemia had a significantly higher leakage of protein to the bronchoalveolar space. (Figure 3.7.B and Table 3.2)

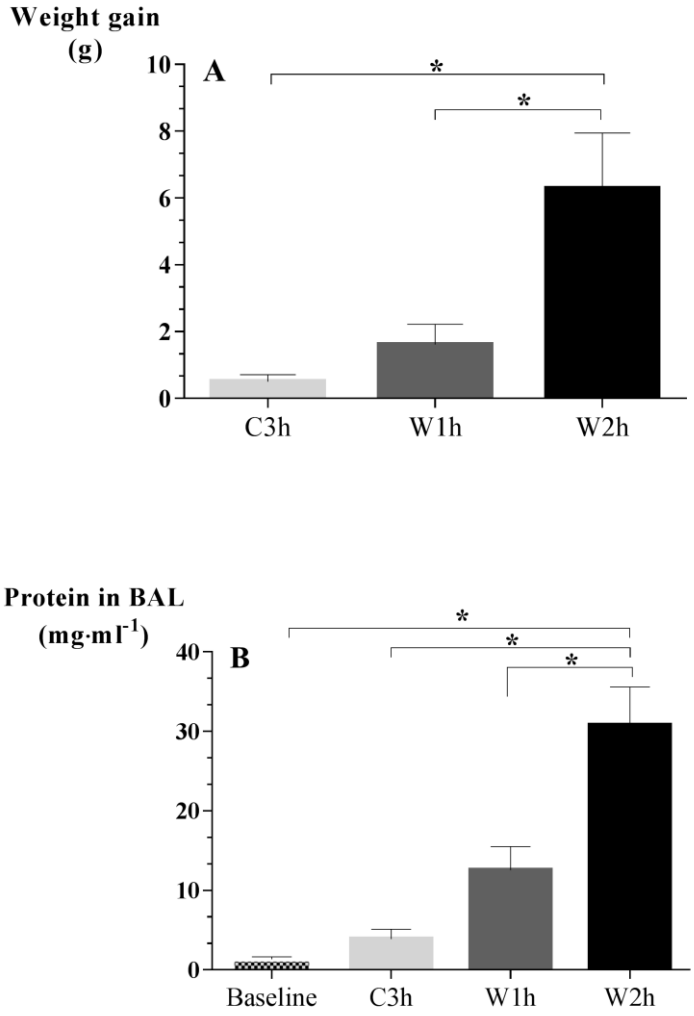

Figure 3.7 Protein content in BAL and edema development as assessed by weight gain during EVLP

Histology and histomorphometry of lung edema

A various degree of pulmonary perivascular edema was visible on histological lung tissue sections in all EVLP groups, but not in the baseline group, where normal parenchyma architecture was observed. Representative histological sections indicating how quantification of perivascular lung edema was performed are shown in Figure 3.8. The ratio of edema thickness to the diameter of the examined vessel was calculated. Lungs from group C3h presented mild perivascular edema, while lungs undergoing 1 hour (W1h) or 2 hours (W2h) of WI showed increased perivascular edema. Histomorphometric analysis of edema showed significant differences in-between experimental groups.

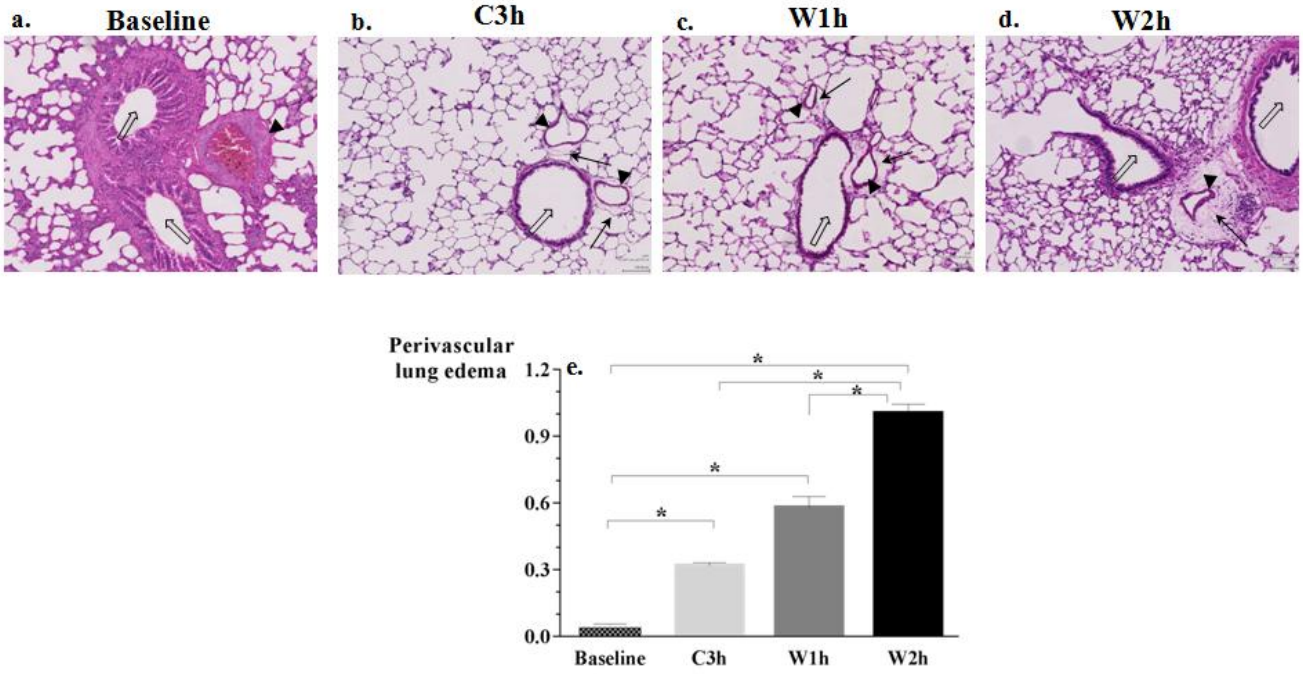

Figure 3.8 Lung edema after EVLP

a.: Baseline group; b.: C3h group; c.: W1h group; d.: W2h group. Black arrows show perivascular edema around arteries and veins (arrowheads). Open arrows indicate bronchial structures. Graph e. displays the quantification of perivascular lung edema for all experimental group (ratio of the perivascular edema thickness to the inner diameter of the examined vessels).

Parameters	Time of EVLP (min)	C3h	W1h	W2h
SPC $ml \cdot cmH_2O^{-1}$	30	0.674±0.994	0.456±0.062	0.398±0.053
	90	0.842±0.108	0.576±0.035	0.408±0.073*
	150	0.827±0.097	0.539±0.055	0.261±0.069*
	180	0.847±0.079	0.452±0.057	0.190±0.030*
PVR $cmH_2O \cdot min^{-1} \cdot ml^{-1}$	30	1.304±0.261	1.548±0.257	1.800±0.320
	90	0.874±0.108	0.968±0.095	0.965±0.109
	150	0.960±0.128	0.908±0.057	1.082±0.091
	180	0.956±0.113	1.020±0.074	1.207±0.148
$DppO_2$ mmHg	30	48±4.8	66±5.0	59±3.6
	90	69±3.6	71±4.9	53±6.3*
	150	69±3.7	68±4.0	44±3.7*
	180	66±5.7	58±9.4	37±4.5*

Table 3.1 Summary of EVLP physiological parameter Means±sem *: p<0.05 vs C3h

Parameters	Baseline	C3h	W1h	W2h
Weight Gain (g)		0.505±0.205	1.613±0.608	6.288±1.657*
Protein In BAL $(mg \cdot ml^{-1})$	1.340±0.309	3.873±1.237	12.560±2.963	30.780±4.813*
LDH (AU)	0.683±0.088	0.080±0.039	0.952±0.293	6.074±0.430*
Protein Carbonyl $(nmol \cdot mg^{-1})$	0.140±0.020	0.228±0.015	0.260±0.0170‡	0.301±0.010†
3-NT $(nmol \cdot ml^{-1})$	2.603±1.507	8.451±2.922	14.980±1.740‡	18.550±0.309‡

Table 3.2 Summary of lung weight gain and biomarker assays Means±sem *: p<0.05 vs all other groups; †:p<0.05 vs Baseline and C3h; ‡:p<0.05 vs Baseline

Discussion

Here we describe (a) how to transfer the principle concepts of the Toronto protocol of human EVLP to a rodent model, and (b) how experimental ischemic lung injury can be detected with a miniaturized ex-vivo lung perfusion circuit.

In this study we have assessed injured rat lungs in a downsized EVLP circuit. We have found that warm in-situ ischemia of lungs procured from healthy rats translates into worsened lung ventilatory mechanics and perturbed gas exchange during ex-vivo reperfusion and re-ventilation, as compared to lungs undergoing immediate cold ischemic preservation after cardiac arrest. We have observed a good correlation of the ex-vivo functional results and analysis of damage in BAL and tissue.

Ischemia is the most relevant trigger of graft injury in solid organ transplantation. In clinical lung transplantation cold ischemic times (CIT) up to 8 to 12 hours are considered acceptable in lungs originating from donation after neurological determination of death (DNDD) donors (99). Longer CITs may negatively affect outcome after lung transplantation (100). The broad interest in donation after circulatory determination of death (DCDD) donor lungs has stimulated the research on acceptable warm ischemic times (WIT) and pharmacological intervention to decrease the warm ischemic damage(92). Van Raemdonck et al. have shown in a large animal model that WIT of 1 hour leads to an acceptable lung function after reperfusion. Interestingly, the authors have not tested these injured lungs in a transplant model but in an ex-vivo lung perfusion model (101), demonstrating the significance of EVLP for the assessment of potential donor lungs where the risks of unidentified graft damage is high.

The rat model of EVLP

The goal of our study was to determine if a downsized experimental model of EVLP allows assessing ischemic injury to lungs, with particular interest in warm ischemic damage. We have chosen the rodent model of EVLP since it has several advantages as compared to large scale EVLP models, in particular when ex-vivo pharmacological treatments are tested. In general, researchers can develop high success rates performing rat models and their excellent reproducibility increases the chance to achieve in short periods of time higher sample sizes with statistically meaningful results. Rat models are considered as excellent for the testing of therapeutics against ischemia-reperfusion injury (102). In the context of EVLP the rat model is of particular interest since most effects observed during ex-vivo perfusion need to be translated to graft function after blood reperfusion; thus, a corresponding model of transplantation is mandatory. Various rat left lung transplant models are well described, validated and used in multiple studies (103-105). In addition, are considered as cost effective, which holds especially true for the required infrastructure (perfusion and ventilation system), the running costs for consumables and animals, as well as the required manpower. Less

than 0.1L of the expensive Steen solution is required per procedure, as compared to 3L needed for EVLP in a pig. If drug discovery research for ex-vivo lung repair is conducted, the low priming volume of the rat EVLP circuit becomes relevant in so far as less amount of the compound of interest is needed. However, potential advantages of the pig model as compared to rat-EVLP are the greater similarity of the pigs' immune system and physiology (106), thus making study results easier comparable to humans. Since there is a good size match of human and pig lungs, the results from animals studies regarding ventilatory and perfusion parameters could be directly transferred to clinical trials.

Only very few studies describe EVLP in rats. Most of them describe closed perfusion circuits and use of Steen solution for perfusion. For instance, the Pittsburgh group assessed a new EVLP dual perfusion technique including the bronchial artery circulation (107) and also hydrogen preconditioning of lungs (108) using an advanced rat model of prolonged normothermic EVLP. This model is very close to the one we describe in this paper (109); in contrast to our study, native, non-injured lungs were used. EVLP systems described in other studies show important differences regarding the ventilatory management, the achieved length of perfusion, and the ex-vivo assessment of lung function. Egan et al assessed rat DCDD lungs in a simplified ex-vivo circuit. The circuit did not allow assessing lung function ex-vivo, neither compliance nor oxygenation capacity (110). A study of the Kyoto Group has described the inhomogeneity of ex-vivo perfusion (111) and the use of plasmin in a partially thrombosed donor lungs (112) in an easy 1 hour ex-vivo perfusion model, using negative pressure chamber ventilation. The group from Ohio described a model of 1 hour EVLP in native non-damaged lungs, using volume controlled ventilation (113).

We have based our experimental model on the Toronto technique of human EVLP since this protocol has been introduced successfully in clinical practice and promising results of this protocol are published (38, 39). The Toronto protocol is characterized by normothermic perfusion, use of acellular Steen solution, constant flow perfusion of the lung at 40% of the donors' cardiac output, a closed circuit with a defined LA pressure, and protective ventilation; below we describe the principle aspects of the human procedure and how they were transferred to the model.

Components of the circuit: To perform EVLP of human lungs a perfusion circuit with following principle elements is needed: an organ chamber to create a protective environment for the graft during perfusion, a pump to drive the perfusate, a reservoir and tubing containing the perfusion solution, a gas exchange membrane to supply the circuit with a gas mixture to remove oxygen and add carbon dioxide, and a heat exchanger for temperature control. In order to ventilate the lung, it is connected to a standard ICU ventilator (114). In the experimental setting described in this paper we have used the well described IL-2 lung perfusion which has been established many years ago for lung physiology research [Harvard IL-2 System, TYPE 829/2, Hugo Sachs

Electronik (HSE), Hugstetten, Germany] (115). We have made essential changes to the hardware, as well as to the applied perfusion and ventilation procedure.

The first principle modification is the use of a rodent ventilator (FX3, SCIREQ Inc, Montreal, Canada) for positive pressure ventilation with a set tidal volume replacing the negative pressure chamber ventilation set-up in the original circuit. As described in the method section the pressure ventilation chamber was maintained but served as organ chamber to control the lungs' microenvironment (temperature, humidity, fluid loss). The use of the Flexivent ventilator allows for pressure and volume controlled ventilation, and more importantly, it simplifies the automatic repeated assessments of lung ventilatory mechanics including lung compliance and peak airway pressure, since this is a preset functionality of the device. This allows assessing organ function ex-vivo repeatedly, as done in the clinical protocol of EVLP.

The next important modification to mimic the clinical situation was the use of Steen solution instead of Krebs-Henseleit solution, which has been used in most studies using the circuit in the field of lung physiology. Acellular Steen solution is used in the clinical setting of EVLP. It is an extracellular solution complemented with Dextran and Albumin to achieve optimal rheological properties and a high colloid pressure (Steen Solution, XVIVO, Sweden). Confirming results from other investigators (109), we show that Steen solution allows preserving rat lungs at normothermic conditions during prolonged EVLP.

In human EVLP the inflow pCO2 is corrected by controlling the CO2 supply through the gas-exchange membrane in the human setting (114). The experimental circuit uses a dialysis hemofilter for gas exchange. The gas mixture supplied to the perfusion solution was composed according to the human EVLP protocol, containing 84% of Nitrogen, 6% oxygen and 10% CO2. The pH value of the perfusion solution is controlled every 30 minutes with a ph meter. As described in the Toronto protocol for human lungs, pH is maintained between 7.35-7.45. A buffer was systematically added to Steen solution to obtain the target pH value before connecting the lung to the circuit. Gas flow was adapted to attain a pCO2 of approximately 35mmHg as assessed by occasional blood gas analysis. pH values were found to be very stable and depended rather on CO2 supply and buffer in the perfusion solution, than on CO2 removal by the graft. The circuit was equipped with sensors for influent and effluent perfusate oxygen partial pressure measurements (flow-through oxygen electrodes, HSE, Hugstetten, Germany). This allows for automatic and continuous assessments of the oxygenation capacity given as the delta v-a PO_2 of the perfused lung, similar to the human setting.

Ex-vivo perfusion and ventilation of the lung: In the Toronto protocol the human lung undergoes cold static preservation until EVLP. To mount the human lung in the perfusion circuit the system is primed with the perfusion solution, a straight cannula is inserted in the pulmonary artery and a funnel shaped cannula is sutured to the left

atrium. As a result a closed circuit of lung and perfusion system is constructed. This allows control of the PA as well as the LA pressures during perfusion (114). In contrast, in the experimental setting PA and LA cannula for EVLP are already used for cold flush and kept in place during cold ischemic preservation. The cold flush at 4 °C is performed in situ in the donor animal using Perfadex which is perfused at a low pressure of 15mmHg through a cannula inserted in the pulmonary artery trunk. The lung vasculature is vented through a second cannula introduced in the left ventricle, passing through the mitral valve with its tip in the left atrium. Consequently, heart and lung are not separated for EVLP. Both cannulas are connected to the EVLP circuit and the lungs' vascular system is de-aired. As in the human setting the lung is intubated and connected to the ventilator in a semi-inflated state.

According to the Toronto protocol perfusion is initiated slowly, the graft is progressively warmed, the flow is increased gradually. The perfusate flows from the lung to a reservoir, then to the gas - and heat exchange system before entering the lung again. Ventilation is started when the circuit reaches a temperature of 32°C. Respiratory rate is 7/min, tidal volume is 7ml/kg, positive end-expiratory pressure is 5cmH2O, and the FiO2 is 21%. In the experimental setting respiratory rate was 7/min, TV was 6ml/kg, PEEP was 3, and FiO2 was 21%.

When the lung temperature reaches 37°C, the perfusion flow is increased to a maximum of 40% of the cardiac output calculated according to the ideal bodyweight of the donor. This is considered as a protective perfusion strategy. The LA pressure is set to 3-5mmHg, the PA pressure is monitored and depends on the vascular resistance of the lung and the preset flow. In general, clinical EVLP is performed for a period of 4 hours. Then the graft is cooled down, and preserved at 4°C until implantation (114). In the animal model the perfusion flow was set lower than in the clinical setting. Since preliminary studies had shown (1) that a perfusate flow from 20 to 40% leads to worsening ex-vivo lung function over time (data not shown), and (2) that a pressure controlled perfusion of healthy lungs with a PA pressure set to 15mmHg results in a perfusion rate around 10% of the cardiac output, we decided to set a constant perfusate flow at 8% of the estimated cardiac output. Thus, we performed EVLP in this model with a constant flow like in the clinical setting, but at a lower rate. This appeared to be a safe for prolonged normothermic EVLP in our experimentalmodel. Other investigators have used constant flow modes for rat EVLP ranging from 8% to 20% of the estimated cardiac output (108(Dong, 2013 #195, 111, 113)As in the clinical protocol the target LA pressure was 3 to 5 mmHg.

The clinical EVLP protocol requires methylprednisolon, heparin and antibiotics as adjuncts to the Steen solution. In contrast we do not apply these drugs in the experimental setting in order to avoid interference with the experimental hypothesis to be tested. In particular, methylprednisolon would have affected the inflammatory tissue

response. The administration of heparin was considered unnecessary since the lung was flushed and contained no blood when mounted in the circuit.

Assessment of the lung

In the clinical protocol various parameters are assessed during EVLP to determine transplant suitability including lung compliance and airway pressures, oxygenation, pulmonary artery and left atrial pressures, and pulmonary vascular resistance. Next to lung mechanics and hemodynamics, the lung's oxygenation capacity is determined by measuring the difference of oxygen partial pressure in the effluent (pulmonary veins or left atrium) and the inflow perfusion solution (pulmonary artery). These measurements need to be performed repeatedly with set pulmonary perfusion, flow and ventilatory parameters. In the clinical setting the decision if a lung can be accepted or needs to be declined is based on the trend of the above mentioned parameters. So far no single individual parameter has been identified to indicate the transplant suitability. According to the Toronto EVLP protocol a lung will be declined if after 4 hours of perfusion the ratio of partial pressure of oxygen in the left atrium to the inspired fraction of oxygen is below 400 mmHg, the PA pressure, the compliance or the peak airway pressure worsen during EVLP more than 15%; conversely, lungs are accepted with a partial pressure oxygen ratio above 400 mmHg, stable or decreasing PA and peak airway pressures, and stable or improving compliance (116).

Similar to the clinical setting ventilatory mechanics were assessed repeatedly. Each measurement of lung compliance was preceded by a lung recruitment maneuver with a peak airway pressure of 15cmH_2O. In contrast to the clinical setting the FiO_2 was not increased to 1.0 to measure the lungs' oxygenation capacity. This was necessary since the experimental set-up did not allow for rapid and valid changes of FiO_2 in our experimental setting.

Ex-vivo lung function and tissue damage of ischemic lungs

The principle goal of this EVLP model is to evaluate lung damage due to ischemia. We have shown that lungs undergoing 3 hours cold ischemic preservation have excellent ex-vivo lung function with improving compliance, lowest vascular resistance and a stable oxygenation capacity in our model. Analysis of ischemia induced tissue damage in these lungs showed a low degree of oxidative stress (protein-carbonyl), nitrosative stress (3-NT) and tissue necrosis (LDH). The edema development during EVLP was less pronounced as compared to all other groups.

In clinical practice, healthy human lungs undergoing three hours of CI would be considered as ideal donor lungs. As known from human studies those lungs would show stable ventilatory mechanics and diffusion capacities if mounted in a human EVLP system (117). Here we demonstrate that lungs preserved under standard ideal conditions have good organ function in this experimental setting. Besides, we

demonstrate a good correlation of excellent functional results and low ischemia induced tissue injury.

Lungs damaged by one or two hours of WI had worse lung function in the experimental ex-vivo system as compared to “ideal donor lungs”, including pulmonary mechanics and oxygenation capacity. This correlated very well to the extent of tissue damaged as determined in lung parenchyma and BAL in the same lungs at the end of EVLP. Protein-carbonyl, 3-NT, LDH were highest after 2 hours of WI, and were also increased when the lung was harvested one hour after cardiac arrest. Interestingly, lungs obtained after 1 hour of WI displayed still a quite stable lung function during ex-vivo perfusion during 2 hours; however their function tended to decrease during the third hour of EVLP, indicating some degree of ischemic damage. In contrast, lungs injured by 2 hours of WI showed a bad initial function as well a rapid decline of their function during EVLP, most probably related to irreversible ischemic tissue damage as documented by tissue analysis.

In the clinical setting, lungs obtained after prolonged times of warm ischemia are grafts obtained from uncontrolled or controlled donation after circulatory determination of death donors, with some of these lungs safely usable and other lungs irreversibly damaged. Our experimental setting demonstrates how EVLP may help to decide whether lungs after prolonged ischemic times are still eligible for transplant of not. According to the clinical protocol of EVLP, lungs with declining compliance, increasing vascular resistance and worsened oxygenation capacity are not eligible for transplant. In our experimental model lungs showing these functional characteristics of non-transplantable lungs showed the highest degree of oxidative/nitrosative stress and tissue necrosis.

In conclusion, this experimental model of rat EVLP allows for 3 hours of normothermic preservation of rat lungs. The model presents principle characteristics of the Toronto protocol of human EVLP, including a closed perfusion circuit, acellular perfusion, protective ventilation and perfusion settings. Moreover, it allows to asses reliably lung function ex-vivo. A good correlation of ex-vivo lung function and the degree of ischemic tissue damage was found. This model may be used to assess ex-vivo pharmacological treatments of damaged donor lungs. It will allow assessing the initial organ function, to perform pharmacological ex-vivo treatments aiming to improve graft function, and to reassess the effect of the applied treatment ex-vivo. In a final step an extension of the model with transplantation of pretreated lungs to a recipient is required to confirm the effects of ex-vivo therapies.

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Chapter 4

Pharmacological reconditioning of marginal donor rat lungs using inhibitors of peroxynitrite and poly (ADP-ribose) polymerase during ex-vivo lung perfusion

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Abstract

Background: Donor lungs obtained after prolonged warm ischemia (WI) may be unsuitable for transplantation due to the risk of reperfusion injury, but could be treated and reconditioned using ex-vivo lung perfusion (EVLP). Key processes of reperfusion injury include the formation of reactive oxygen/nitrogen species (ROS/RNS) and the activation of poly(ADP-ribose) polymerase (PARP). We explored whether rat lungs obtained after WI could be reconditioned during EVLP using the ROS/RNS scavenger Mn(III)-tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP) or the PARP inhibitor 3-aminobenzamide (3-AB).

Methods: Rat lungs obtained after 3h cold ischemia (CI group, control), or 1h WI plus 2h CI (WI group) were placed in an EVLP circuit for normothermic perfusion with Steen solution for 3h. Lungs retrieved after WI were treated or not with 3-AB (1mg/ml; WI-3-AB group) or MnTBAP (0.3mg/ml; WI-MnTBAP group), added to the perfusate. Measurements included physiological variables (lung compliance, vascular resistance and oxygenation capacity), lung weight gain, as well as levels of proteins, lactate dehydrogenase (LDH), protein carbonyl (marker of ROS), 3-nitrotyrosine (3-NT, marker of RNS), poly(ADP-ribose) (PAR, marker of PARP activation) and interleukin 6 (IL-6), in the bronchoalveolar lavage (118) or the lung tissue,and histopathological analysis.

Results: In comparison to CI group, the lungs from WI group displayed higher protein carbonyls, 3-NT, PAR, LDH and proteins in BAL, lung weight gain, perivascular edema, as well as reduced static compliance, but similar oxygenation. All these alterations were markedly attenuated by 3-AB and MnTBAP.

Conclusions: EVLP of lungs obtained after warm ischemia exhibit significant oxidative stress, PARP activation and tissue injury, which is suppressed by pharmacological inhibitors of ROS/RNS and PARP.

Introduction

Lung transplantation is limited by the shortage of suitable organs, and increasing the pool of eligible donor lungs is an urgent priority. This could be achieved by using lungs obtained after cardiac death (DCD lungs), or lungs displaying some damage and dysfunction ("marginal" donor lungs), although such options increase the risk of primary graft dysfunction (PGD) after transplantation. In this respect, evaluation of the donor lung using ex vivo lung perfusion (EVLP) represents a strategy to determine the suitability of the organ for transplantation. EVLP could also permit to treat the donor lung to improve its condition, thereby increasing the number of transplantable organs (28, 85, 119, 120).

Ischemia-reperfusion (IR) represents the critical process triggering PGD after transplantation, and its severity depends on the duration of graft ischemia (121). In the case of DCD lungs, the unavoidable warm ischemic time represents therefore an important risk factor for reperfusion injury and PGD (122). Whether such lungs can be reconditioned by targeted therapies during EVLP requires prompt investigation. A key mechanism of IR injury is the generation of oxidants and free radicals, initiating widespread cellular damage (56). These species encompass reactive oxygen species (ROS), primarily the superoxide radical, and reactive nitrogen species (RNS), primarily peroxynitrite (56). A major cytotoxic pathway triggered by ROS/ RNS is related to DNA injury and subsequent activation of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) (56). Activated PARP initiates a series of molecular events leading to cell death via regulated necrosis (necroptosis), and fostering inflammation via interactions with nuclear factor kappaB and additional signaling pathways (56). Pharmacological inhibition of PARP has been therefore proposed to reduce tissue injury and inflammation under conditions associated with ROS/RNS formation (123).

We therefore conducted the present study to address two main hypotheses. First, we postulated that EVLP of rat lungs obtained after prolonged warm ischemia is associated with significant oxidative stress, PARP activation and tissue injury; and second, that such lungs can be reconditioned by pharmacological treatment with inhibitors of PARP and peroxynitrite.

Material and Methods

Surgical preparation and experimental design

The animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals", and experiments were approved by our Local Ethical Committee (Authorization Nr 2637). Twenty-eight male adult Sprague-Dawley rats (10-14 weeks, mean weight 410 grams, Charles River, L'Arbresle, France) were used. Anesthesia was induced with inhaled isoflurane and maintained with pentobarbital sodium (50mg/kg i.p.). The animals were placed on heating plates to maintain temperature at 37.5°C, tracheotomized and mechanically ventilated (Respiratory rate -RR- = 75·min^{-1}, tidal volume -V_t- = 8 ml·kg^{-1}, fraction of inspired oxygen -FiO_2- = 0.21), using a rodent respirator (model 683, Harvard Apparatus, Holliston, MA). Following a median sternotomy, heparin (600 U) was injected into the right ventricle in order to prevent clotting and thrombus formation within the pulmonary circulation. Perfusion cannulae (Hugo Sachs, Hugstetten, Germany) were inserted into the pulmonary artery-PA- (ID = 1.7mm, OD = 2.0mm) and left atrium –LA- (ID = 3.4mm, OD = 4.0mm). Animals were then killed by pentobarbital overdose and bleeding, and they were allocated to one of 4 groups (as depicted in Figure 4.1):

Cold Ischemia (CI group, N=6): Lungs were first flushed through the PA cannula with 15ml of 4°C Perfadex® (Xvivo Perfusion, Göteborg, Sweden), at a perfusion pressure of 20 cm H_2O, while ventilated at a RR of 15/min and a V_t of 7 ml/kg (FiO_2=0.21). Lungs were then harvested and preserved in an inflated (FiO_2 = 0.21) status for 3 hours in 4°C Perfadex®, hence, the lungs were ventilated during Perfadex flushing, and were then kept inflated for 3 h, after which they were exposed to EVLP for 3 hours.

- Warm Ischemia (WI group, N=7): Lungs were first kept deflated *in situ* for 1 hour at room temperature, after which they were flushed through the PA cannula with 15ml of 4°C Perfadex®, at a perfusion pressure of 20 cm H_2O, while ventilated at a RR of 15/min and a V_t of 7 ml/kg (FiO_2=0.21). Lungs were then harvested and preserved in an inflated (FiO_2 = 0.5) status for 2 hours in 4°C Perfadex®, hence, the lungs were ventilated during Perfadex flushing, and were then kept inflated for 2 h, and then exposed to EVLP for 3 hours.

- Warm Ischemia treated with the PARP inhibitor 3-aminobenzamide (WI-3-AB group, N=6): Same as WI group, except that 3-AB (see dose below) was added to the perfusate during EVLP. The lungs were therefore continuously perfused with 3-AB during the 3 hours of EVLP.

- Warm Ischemia treated with the superoxide dismutase mimetics and peroxynitrite scavenger Mn(III)-tetrakis(4-benzoic acid) porphyrin chloride (WI-MnTBAP group, N=6): Same as WI group, except that MnTBAP (see dose below) was added to the perfusate during EVLP. The lungs were therefore continuously perfused with MnTBAP during the 3 hours of EVLP.

In addition to the 4 groups above, a group of n=6 rats was sacrificed by pentobarbital overdose, their lungs were immediately retrieved without any further intervention, and kept frozen at -70°C until analysis (Baseline group). This group served to determine the basal (physiological) levels of some biochemical markers, as detailed below.

It is important to mention here that we purposely let the lungs deflated during the 1 hour period of warm ischemia in order to further enhance the damaging effect of warm ischemia, whereas lungs in the CI group were not deflated. Therefore, lung injury following warm ischemia in our study cannot be ascribed solely to the warm ischemic time, but also, to some extent, to the deflation procedure. This protocol was designed to evaluate the effects of MnTBAP and 3-AB on significantly damaged lungs, whereas lungs from the cold ischemia group served as control, undamaged lungs.

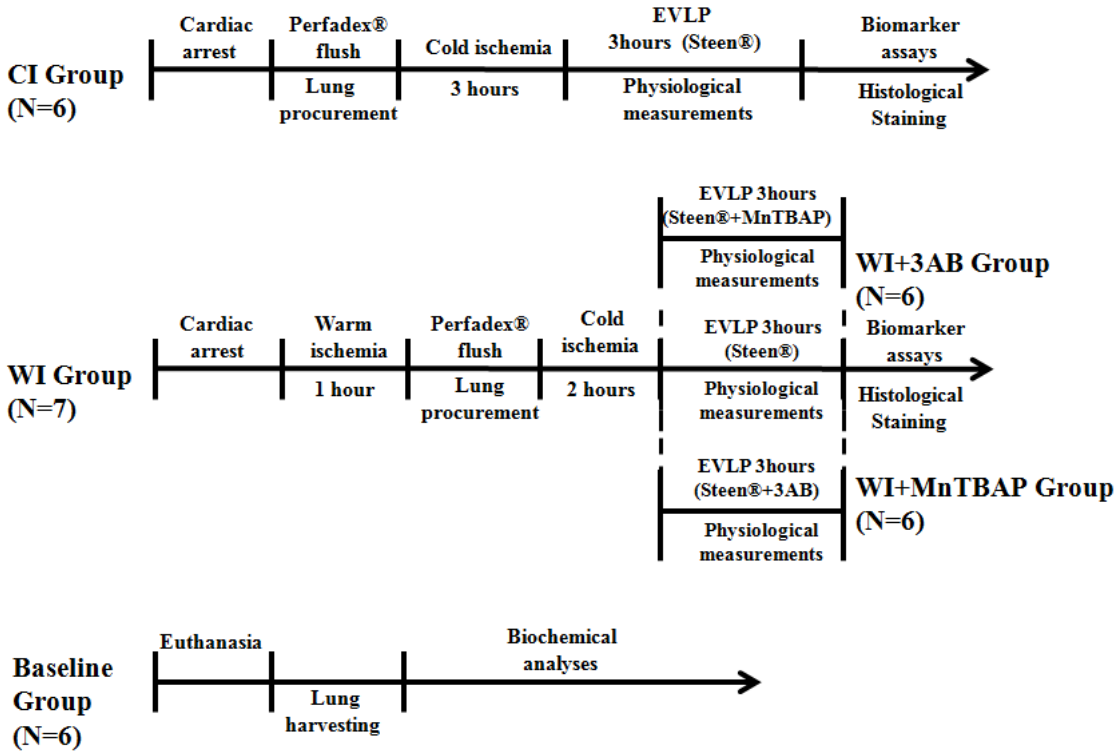

Figure 4.1 Experimental Design

Ex-vivo lung perfusion

The heart-lung block was weighted, then mounted in a customized rat EVLP system (Harvard IL-2 System, Hugo Sachs), as detailed in Figure 4.2. The circuit was perfused with Steen® solution (Xvivo Perfusion), equilibrated with a mixture of 6% O_2, 10% CO_2 and 84% N_2 through a gas-exchange membrane (Hemofilter D150; MEDICA S.P.A, Italy) and maintained at pH 7.4. Left atrial pressure was set at 4cm H_2O. Perfusion was started in a flow-controlled mode, at 2 ml·min^{-1} and at a temperature of 10°C, progressively increased to 7 ml·min^{-1} and 37.5°C (using a 3M TCMII heater, Saint Paul, MN, USA), over 30 minutes, after which ventilation was started (V_t = 6 ml·kg^{-1}, FiO_2 = 0.21, RR = 7· min^{-1})

using a Flexivent FX3 ventilator (SCIREQ Inc., Montréal, Canada). This ventilation protocol was used in order to reproduce the strategy used in clinical EVLP and detailed in the landmark article by Cypel et al (38). The perfusion was then switched to a pressure-controlled mode, with a preset constant PA pressure of 15cm H_2O for 150 minutes. In the treatment groups, Steen® solution was supplemented either with 3-AB (1mg·ml^{-1}, Sigma-Aldrich, Buchs, Switzerland) or MnTBAP (0.3 mg·ml^{-1}, Calbiochem, Sand Diego, CA), according to previous experimental studies (124, 125). At the end of EVLP, the heart-lung block was retrieved and weighted. The difference with the initial weight was calculated as an index of lung edema. A bronchoalveolar lavage (118) was performed with 2 ml PBS, pH 7.4, via the tracheal cannula. The left lungs were then flash-frozen in liquid nitrogen and kept at -80°C until processing, and the right lungs were fixed in 4% parafornaldehyde for further histological analysis.

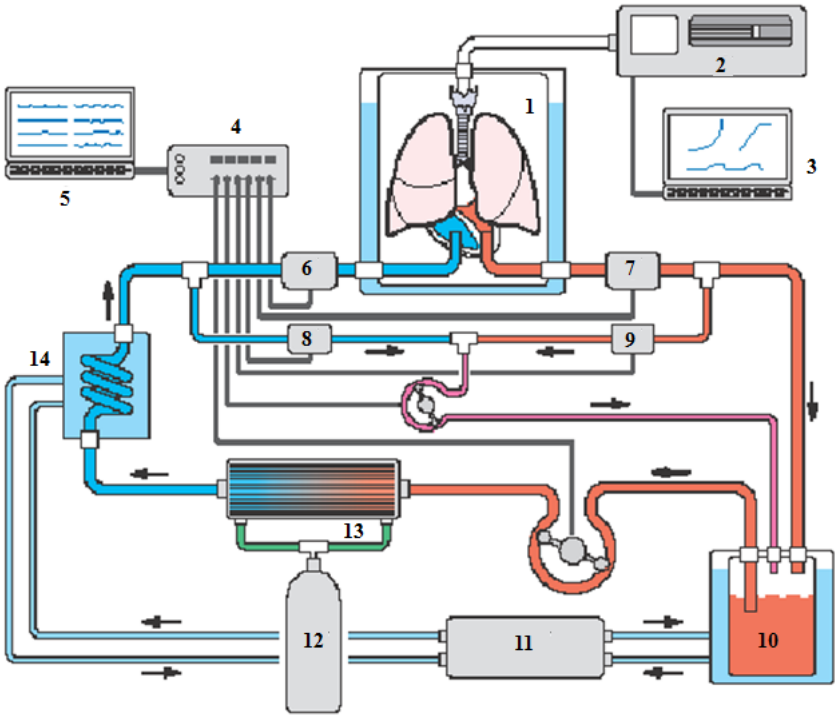

Figure 4.2 The ex vivo rat lung perfusion circuit

1: Jacketed artificial thorax; 2: Flexivent FX3; 3: Laptop (Flexivent data recording); 4: EVLP data converter; 5: Laptop (EVLP data recording and display); 6: Pressure transducer connected to the pulmonary artery canula; 7: Pressure transducer connected to the left atrial canula; 8: Oxygen electrode (affluent circuit); 9: Oxygen electrode (effluent circuit); 10: Perfusate reservoir; 11: Thermostatic circulator; 12 Gas mixture tank; 13: Gas exchange membrane; 14: Heat exchanger.

Measurements

Physiological variables

Pulmonary vascular resistance (PVR) was calculated as: PVR= (mean PAP-LAP) /Flow, expressed in cm $H_2O{\cdot}ml^{-1}{\cdot}min^{-1}$. At selected time-points, static pulmonary compliance (SPC) was determined during transient increase of inspiratory pressure to 15cm H_2O.

The partial pressure of O_2 was measured in the affluent and effluent of the EVLP circuit, using 2 O_2 electrodes (Hugo Sachs Elektronik, Hugstetten, Germany), to compute differential partial pressure of O_2 ($DppO_2$) as an index of O_2 transfer.

Protein, lactate deshydrogenase (LDH), and 3-nitrotyrosine (3-NT) content in BAL

Proteins were determined using the BCA assay (Thermo Scientific, Rockford, USA), and expressed in $mg \cdot ml^{-1}$. LDH activity, an index of tissue necrosis, was measured using a commercial kit (Cytotoxicity Detection Kit^{PLUS}; Roche, Basel, Switzerland), and expressed in arbitrary units. 3-NT was determined as a footprint of peroxynitrite generation using a commercial ELISA kit (Rat 3-Nitrotyrosine ELISA kit; Amsbio, Abingdon, U.K.), and expressed in $nanomol \cdot ml^{-1}$.

Protein carbonyl formation in lung tissue

Protein carbonyl formation was quantified in lung tissue as an index of oxidative stress. Frozen lung samples were homogenized by pulverization in liquid nitrogen, and protein carbonyl were measured using an ELISA-based assay (OxiSelect Protein Carbonyl ELISA Kit; Cell Biolabs Inc., San Diego, USA) and expressed in $nanomol \cdot mg\ protein^{-1}$.

Poly (ADP-ribose) and Interleukin-6 (IL-6) concentrations in lung tissue

PARP activation was evaluated by the measurement of its product, poly(ADP-ribose) (PAR) in lung homogenates using a commercial kit (Pharmacodynamic Assay II, Trevigen Inc., Gaithersburg, USA), and expressed in $picogram \cdot mg\ protein^{-1}$. IL-6 was determined in lung homogenates using a commercial rat ELISA kit (Rat IL-6 DuoSet; R&D Systems, Minneapolis, USA) and expressed in $nanogram \cdot mg\ protein^{-1}$. Control experiments for PAR and IL-6 were done using lungs not exposed to EVLP, retrieved from rats immediately after euthanasia (baseline group), in order to determine the physiological, basal levels of PAR and IL-6. Therefore, this group served as the control group (instead of the cold ischemia group) for PAR and IL-6 levels.

Histological analysis

Histopathological changes were evaluated in the right lung, obtained from each animal at the end of the EVLP protocol. Lungs fixed with OCT and 4% paraformaldehyde were embedded in paraffin, longitudinally sectioned at 5 µm and stained with hematoxylin and eosin. All slides were digitalized using Hamamatsu NanoZoomer HT Digital slide scanner (Hamatsu Photonics, K. K., Japan). They were uploaded to an image analysis programme (Slidepath, Leica Biosystems) for morphometric measurements. As an index of lung injury, we determined the severity of perivascular edema (since alveolar edema could not be evaluated due to the broncho-alveolar lavage), by computing the ratio of perivascular edema thickness to the inner diameter of the examined vessel. On average, a number of 20 symmetrically cross-sectionned vessels per lung (arteries and veins) were evaluated twice, by two independent investigators who were blinded with respect to the different experimental groups.

Analysis of data

All data are expressed as means±sem. For physiological measurements (compliance, resistance and $DppO_2$), the effects of time and treatments were analyzed by 2-way ANOVA, followed by Dunnett's test for the effect of time (with 30 minutes as a control), and Tukey's adjustments for the effects of treatments. For all other comparisons, one way ANOVA followed by Tukey's correction was used. $p<0.05$ was considered significant. Data analysis was performed by Graphpad prism 6 (GraphPad Software Inc., La Jolla, CA, USA).

Results

MnTBAP and 3-AB alleviate lung nitro-oxidative stress and tissue injury during EVLP

In comparison with the CI group, the WI group displayed significant increases of protein carbonyl adducts (p<0.01 WI vs CI; Fig. 4.3A), 3-NT content (p<0.01 WI vs CI; Fig. 4.3B) and LDH activity (p<0.01 WI vs CI; Fig. 4.3C). All these alterations were suppressed by treatment with MnTBAP (p≤0.01 WI-MnTBAP vs WI for all variables) and 3-AB (p<0.01 WI-3-AB vs WI for all variables)

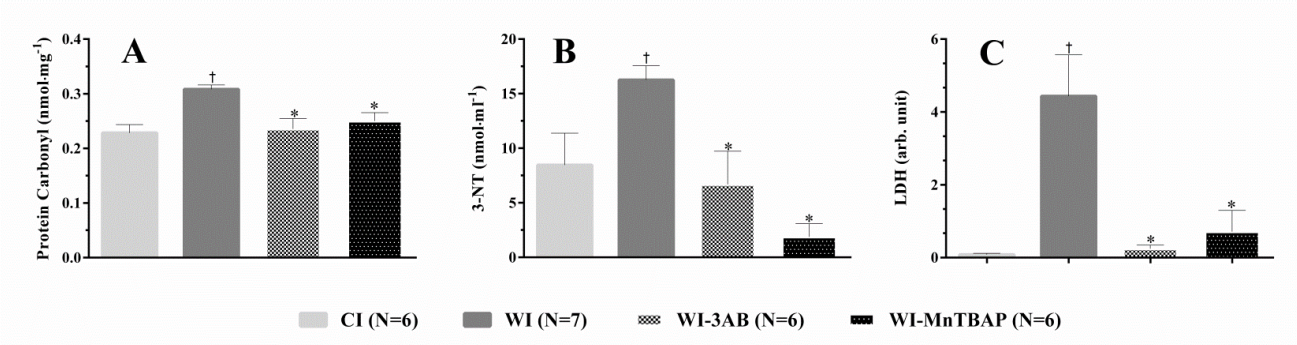

Figure 4.3 Lung oxidative/nitrosative stress and LDH release after EVLP

A: Protein carbonyl in lung tissue after EVLP. B: 3-nitrotyrosine (3-NT) in broncho-alveolar lavage fluid. C: LDH in broncho-alveolar lavage fluid Means ± sem. †: p<0.05 vs CI; *: p<0.05 vs WI

PARP activation and lung inflammation during EVLP are reduced by 3-AB and MnTBAP

A massive increase of PAR content (Fig. 4.4A) occurred in the WI group, as compared to baseline conditions (determined in lungs retrieved from normal rats) (p<0.01 WI vs baseline). This was suppressed both by 3-AB (p<0.01 WI-3-AB vs WI) and MnTBAP (p<0.01 WI-MnTBAP vs WI) (Fig. 4.4A). Furthermore, when compared to baseline conditions, WI lungs displayed a significant elevation of IL-6 (p<0.01 WI vs baseline), pointing to the activation of inflammatory cascades (Fig. 4.4B), whereas such increase was not observed in the WI-3AB and WI-MnTBAP groups (p>0.05 WI-3-AB vs baseline and WI-MnTBAP vs baseline). At the end of EVLP, lung IL-6 was however only significantly reduced in the WI-3-AB group in comparison to WI (p<0.05), whereas the difference was non-significant (p>0.05) between WI and WI-MnTBAP groups.

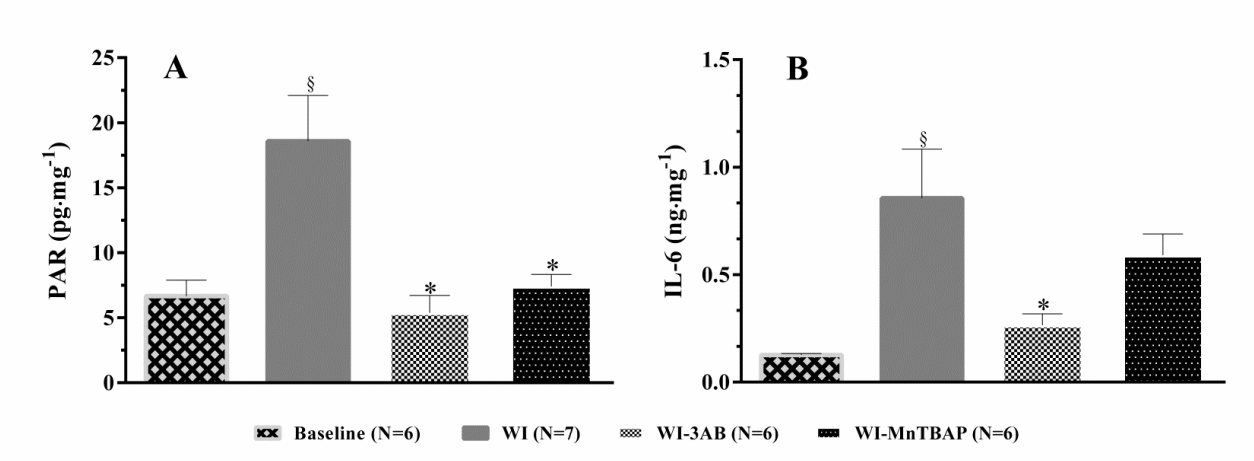

Figure 4.4. Activation of PARP and expression of IL-6 in lungs after EVLP

A: poly(ADP-ribose) content in lung tissue (a marker of PARP activation).

B: IL-6 in lung tissue.

Means ± sem. *: p<0.05 vs WI ; §: p<0.05 vs Baseline

MnTBAP and 3-AB mitigate the development of lung edema during EVLP

Protein content in BAL (Fig. 4.5A) increased massively in the WI group (p<0.01 vs CI), and this was suppressed by 3-AB (p<0.01 WI-3-AB vs WI) and MnTBAP (p<0.01 WI-MnTBAP vs WI). In parallel, a marked weight gain (Fig. 4.5B) occurred in the WI group (p<0.01 vs CI), which was strikingly reduced by3-AB (p<0.01 WI-3-AB vs WI) and MnTBAP (p<0.01 WI-MnTBAP vs WI).

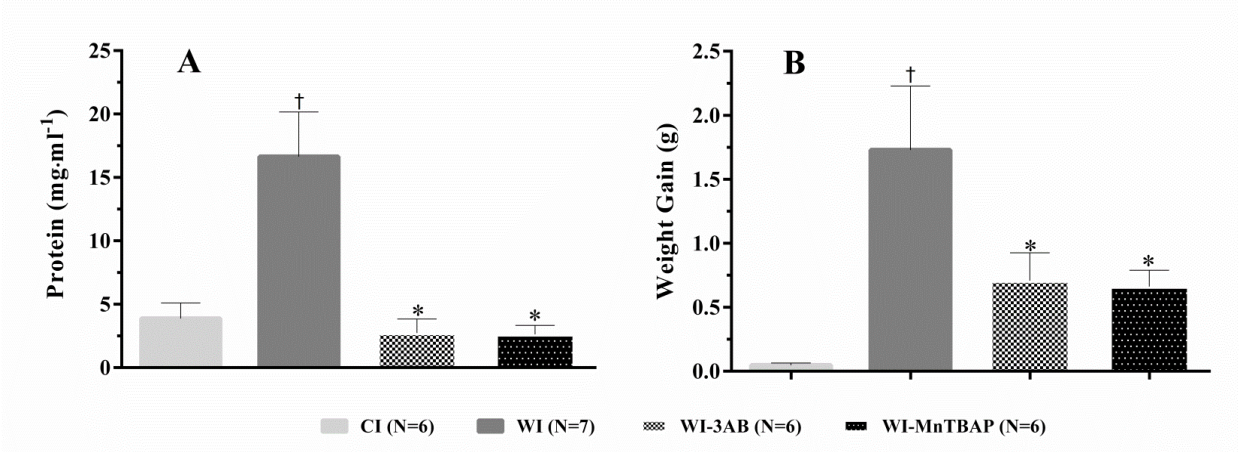

Figure 4.5. Protein rich pulmonary edema and lung weight gain after EVLP

A: Protein concentration in broncho-alveolar lavage fluid. B: Lung weight gain during EVLP.

Means ± sem. †: $p<0.05$ vs CI; *: $p<0.05$ vs WI

Contrasted effects of 3-AB and MnTBAP on pulmonary compliance, PVR and $DppO_2$ during EVLP

Whereas pulmonary static compliance remained stable in the CI group (Fig. 4.6 A and B), a progressive reduction occurred in the WI group ($p<0.05$ WI vs CI at 180 minutes). In contrast, WI-3AB (Fig. 4.6A) and WI-MnTBAP (Fig. 4.6B) lungs maintained stable compliance ($p>0.05$ WI-MnTBAP vs CI and WI-3-AB vs CI at all time points), so that the values were significantly higher than in the WI group from time 90 minutes ($p<0.05$ WI-MnTBAP vs WI and $p<0.05$ WI-3-AB vs WI at time 90 min and above). PVR did not vary significantly in CI, WI and WI-3-AB groups (Fig. 4.6C). In contrast, PVR was significantly higher in the WI-MnTBAP group ($p<0.01$ WI-MnTBAP vs CI and $p<0.01$ WI-MnTBAP vs WI at all time points; Fig. 4.6D). Finally, $DppO_2$ did not exhibit any significant changes over time in any of the groups (Fig. 4.7A and 4.7B).

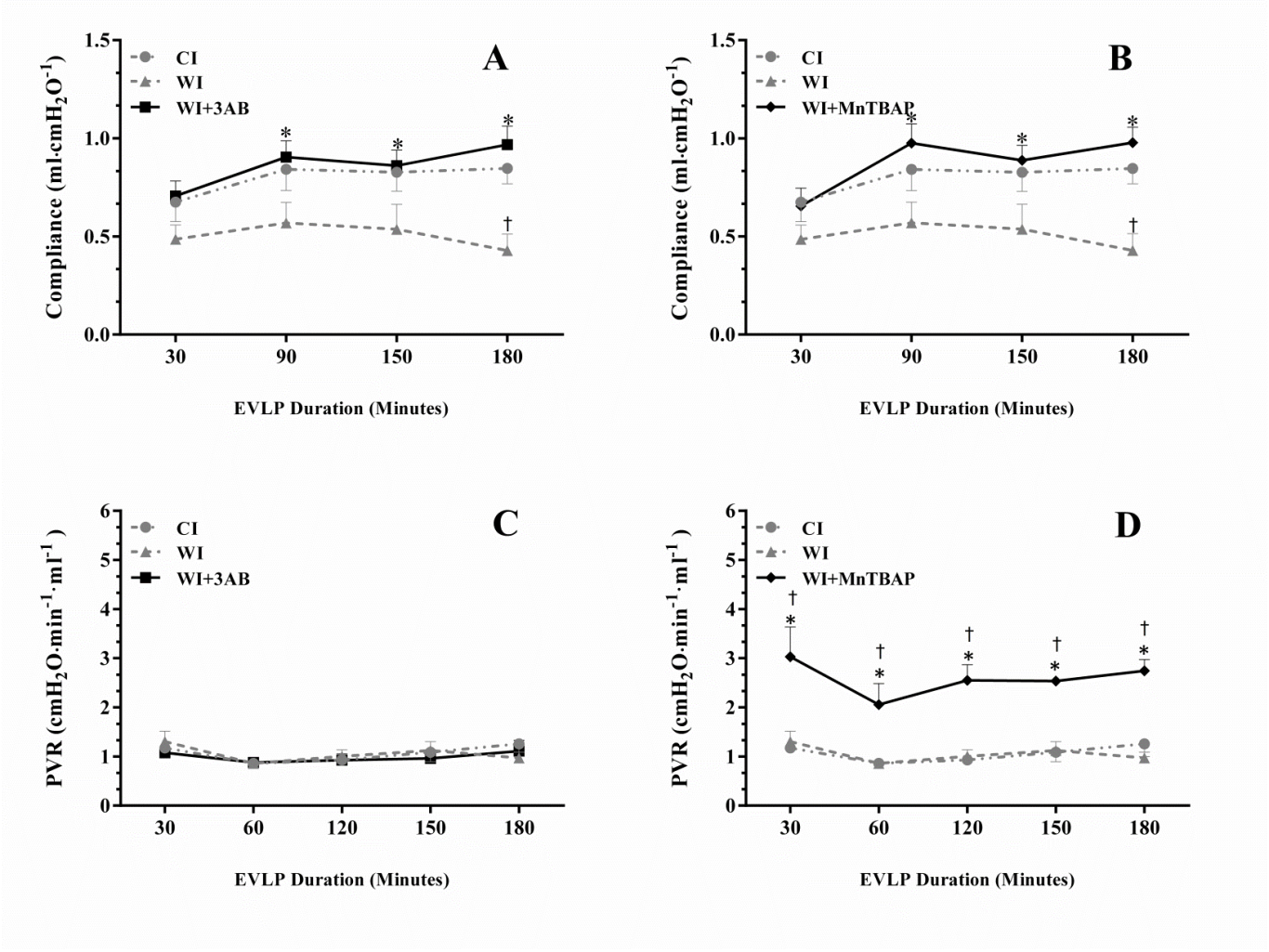

Figure 4.6. Time-course of pulmonary compliance and vascular resistance during EVLP

A-B: Pulmonary static compliance at 30, 90, 150 and 180 minutes of EVLP. Values for the CI and WI groups are the same in both graphs. The effects of 3-AB are shown in A, and the effects of MnTBAP are shown in B. C-D: Pulmonary vascular resistances (PVR) at 30, 60, 120, 150 and 180 minutes of EVLP. Values for the CI and WI groups are the same in both graphs. The effects of 3-AB are shown in A, and the effects of MnTBAP are shown in B

Means ± sem. †: $p<0.05$ vs CI; *: $p<0.05$ vs WI

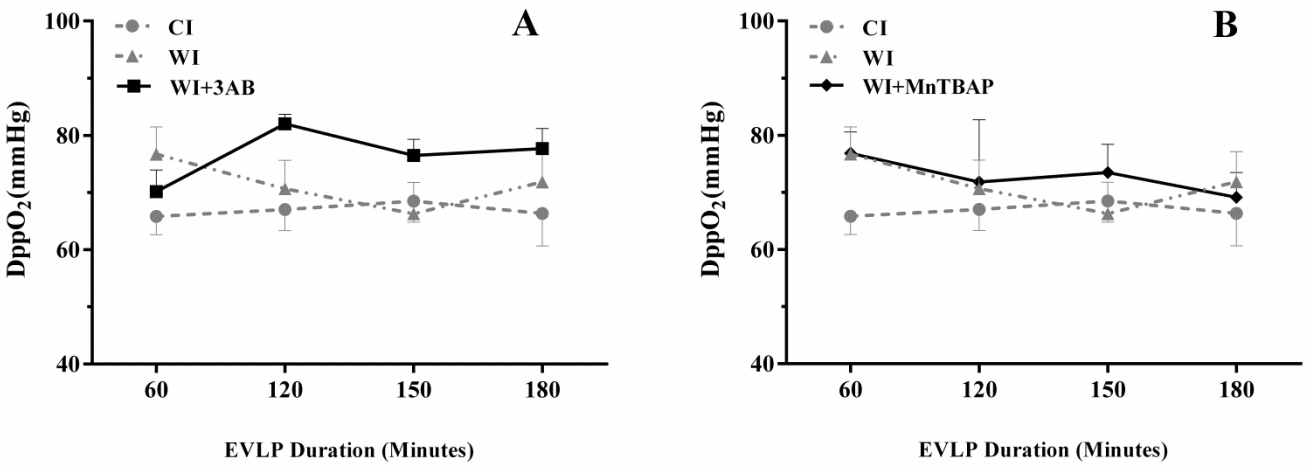

Figure 4.7. Time course of the differential partial pressure of oxygen between affluent and effluent arms of the EVLP circuit

Differential partial pressure of oxygen ($DppO_2$) calculated at 60, 120, 150 and 180 minutes of EVLP. Values for the CI and WI groups are the same in both graphs. The effects of 3-AB are shown in A, and the effects of MnTBAP are shown in B.

Histopathological changes

The major histopathological observation was the presence of perivascular edema (Fig.4.8A-H). We could not detect significant alveolar edema, due to the fact that the lungs were subjected to BAL before being fixed for histological evaluation. Perivascular edema was quatitatively assessed by calculating the ratio of edema thickness to the diameter of the examined vessels (Fig. 4.8I). When compared to the CI group, the severity of perivascular edema was significantly greater in the WI group ($p<0.05$ WI vs CI). In contrast, perivascular edema was not increased in the WI-MnTBAP and WI-3-AB groups when compared to the CI group ($p>0.05$ WI-MnTBAP vs CI and $p>0.05$ WI-3-AB vs CI), but was significantly reduced when compared to the WI group ($p<0.05$ WI-MnTBAP vs WI and $p<0.05$ WI-3-AB vs WI). A further histopathological observation was made in the WI-MnT BAP group, in which we found several deposits of dark brown material predominantly in the alveolar wall, most likely representing microaggregates of this porphyrin-based chemical in alveolar capillaries (Fig. 4.8G-H).

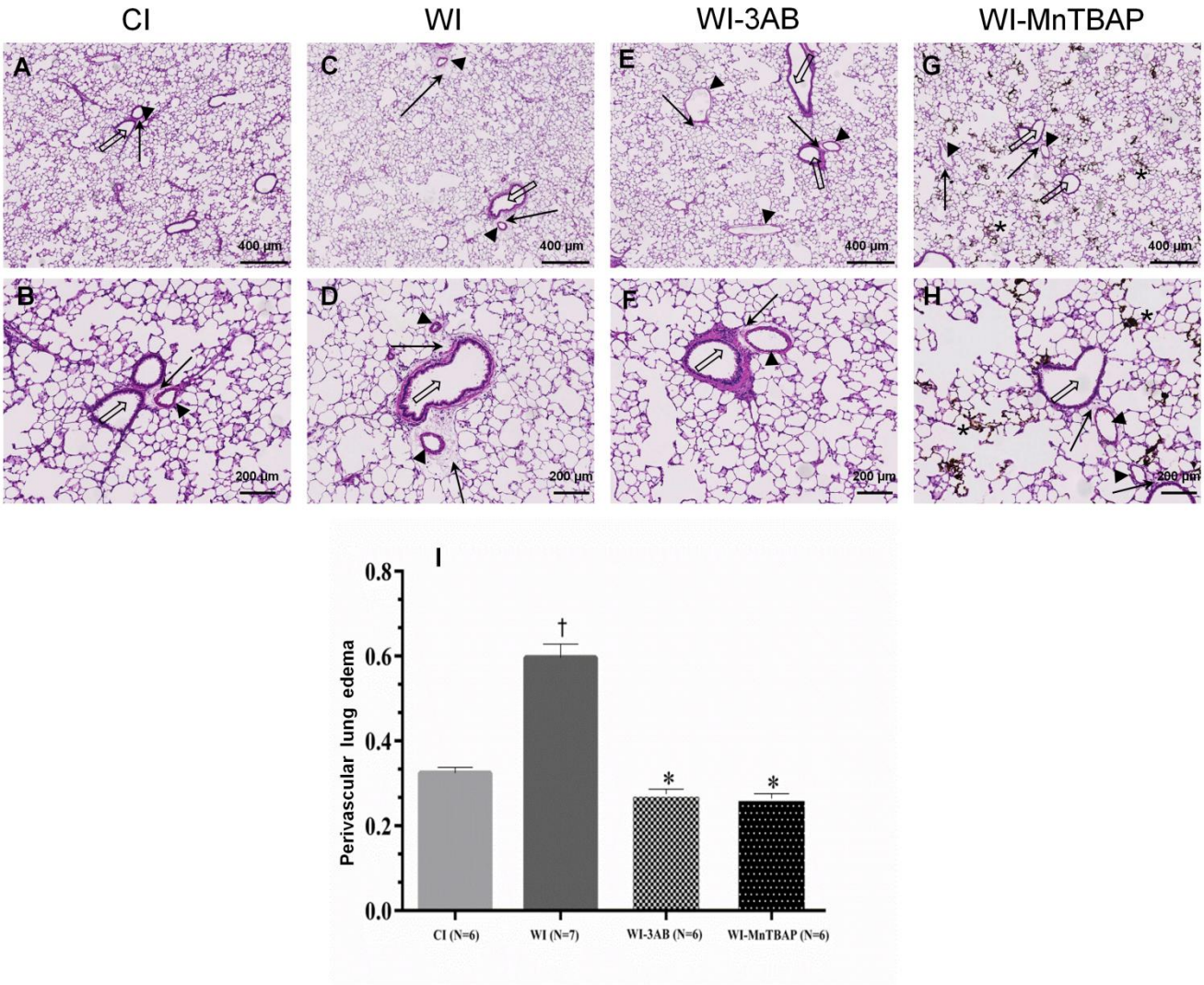

Figure 4.8. Histopathological findings following EVLP

Representative histopathological alterations (HE staining) following EVLP at a magnification of 4x (upper pictures) and 10x (lower pictures) in the CI group (A-B), the WI group (C-D), the WI-3AB group (E-F) and the WI-MnTBAP group (G-H). Black arrows show perivascular edema around arteries and veins (arrowheads). Open arrows indicate bronchial structures. Dark brown aggregates (stars) are found in the alveolar wall of lungs in the WI+MnTBAP group. The graph (I) displays the quantification of perivascular lung edema in each group (ratio of perivascular edema thickness to inner diameter of the examined vessels). Means ± s.e.m.. † $p < 0.05$ vs CI. *$p < 0.05$ vs WI

Discussion

Initially developed to assess the physiological status of the donor lung, EVLP has been extended to a technique for reconditioning the donor lung, and a platform to deliver drugs to treat the organ before transplantation (36, 126). In animal models, drugs including the adenosine A2 receptor antagonist ATL-123 (127), plasmin (128) and nitroglycerin (129) were recently shown to improve the status of DCD lungs after extended warm ischemia. Pre-clinical experiences using marginal human lungs treated during EVLP by adenoviral-mediated gene transfer of IL-10 (85), or by mesenchymal stem cells (130), also reported benefits in term of reduced inflammation and edema of the perfused lungs.

Our findings extend this emerging concept, by demonstrating a striking benefit of 2 drugs acting as inhibitors of redox-based cytotoxic pathways, administered during EVLP of lungs retrieved after prolonged warm ischemia. These lungs displayed greater formation of oxidized and nitrated proteins than cold-preserved lungs during EVLP, pointing to a more severe reoxygenation injury, in line with the established role of ROS and RNS in the pathophysiology of ischemia/reperfusion (131). It has been generally assumed that infiltrating polymorphonuclear neutrophils make up a large source of ROS/RNS during reperfusion/reoxygenation, as indicated by the reduced oxidative stress and lung injury in studies using leukocyte-depleted reperfusion solutions or antagonists of adhesion molecules (46, 132). In our study, the contribution of infiltrating neutrophils to ROS/RNS formation cannot be regarded, owing to the acellular perfusate used during EVLP, and other sources of reactive oxidants must be considered. There are indeed many possible cellular sources of ROS/RNS in the reperfused/reoxygneated lung independent from neutrophils, including endothelial cells, epithelial cells, and resident monocytes. Mechanisms for oxidant production in these cells include the activation of enzymes such as xanthine oxidase, NADPH oxidase or uncoupled NO synthase, as well as electron leakage from the mitochondrial respiratory chain (46, 56)

The enhanced oxidative stress in lungs after warm ischemia was suppressed by MnTBAP, which is in agreement with its activity as a superoxide dismutase mimetics and peroxynitrite decomposition catalyst (124). Furthermore, MnTBAP has also been associated with the upregulation of type I heme-oxygenase, an enzyme with cytoprotective and antioxidant properties (133). Such reduction of oxidative stress by MnTBAP is entirely consistent with previous data obtained with this compound in an unrelated model of lung oxidative stress (134). 3-AB produced comparable effects, which may suggest some direct anti-oxidant capacities of this compound, or simply reflect the reduced generation of reactive oxidants that follows the inhibition of PARP activity (135).

The heightened oxidative/nitroxidative stress in warm ischemic lungs was associated with copious generation of ADP-ribose polymers, which could be suppressed by MnTBAP and 3-AB, implying that a ROS/RNS-dependent PARP activation pathway took place in these lungs. PARP activation represents a crucial cytotoxic mechanism in

conditions associated with redox stress, as the latter is a major inducer of DNA damage, the obligate trigger of PARP activation. PARP uses NAD^+ as a donor of ADP-ribose units, and attaches them to multiple cellular proteins, modulating their biological activities (123). Such "PARylation" is critical for DNA repair, which is the basis for the development of PARP inhibitors as anticancer drugs (136). Besides its DNA repair functions, PARP promotes a series of molecular processes leading to necrotic cell death, including energetic collapse, parthanatos (a mode of cell death implicating some specific mitochondrial-to-nuclear crosstalk), and PARylation of cell death/survival kinases (123). Furthermore, activated PARP exerts pro-inflammatory actions, by directly affecting immune cells and by regulating numerous inflammatory mediators (63). Inhibiting PARP activity may therefore represent a potent therapeutic strategy to limit cell death, inflammation, and tissue injury in conditions associated with ROS/RNS generation and PARP overactivation, such as ischemia-reperfusion (137).

In our study, EVLP of warm ischemic lungs was associated with significant cell death, as evidenced by a massive release of LDH. In keeping with the biological roles of PARP, we found that 3-AB virtually abolished the increase in LDH, evidencing the instrumental role of PARP in the development of lung injury during EVLP of warm ischemic lungs. A comparable reduction of LDH was obtained with MnTBAP, pointing to the superoxide radical and peroxynitrite as crucial cytotoxic effectors in this setting, in agreement with previous investigations (138). Besides their PARP-activating role, these species exert additional cytotoxicity through lipid peroxidation and biomembrane injury, as well as protein oxidative damage leading to the dysfunction of ionic pumps and mitochondrial respiration, to cite only a few (56).

A further experimental finding was the increase of IL-6 in warm ischemic lungs following EVLP, a prototypical inflammatory cytokine elaborated during the sterile immune response triggered by ischemia-reperfusion (139). IL-6 is induced following the activation of various pro-inflammatory signaling pathways, primarily the p38 MAP kinase and nuclear factor kappa B (140). It is noteworthy that both ROS/RNS and PARP largely contribute to activate p38 and NF-κB (63), which likely explains the lack of IL-6 increase in lungs treated with 3-AB and MnTBAP, the former providing the most prominent effect, consistent with previously reported anti-inflammatory effects of 3-AB in unrelated models of lung inflammatory diseases (141-143). Such attenuated inflammatory response is particularly relevant, in view of its well established role in the pathophysiology of ischemia-reperfusion associated with lung transplantation (144). An important aspect of inflammation after reperfusion is related to endothelial activation in response to oxidative stress, promoting leukocyte adhesion and infiltration, thereby fostering the inflammatory response. Due to the use of an acellular perfusate during EVLP, we could not address the issue of leukocyte/endothelial interactions in our model. This will be the matter of future investigation in which we will evaluate leukocyte recruitment following transplantation of the ex-vivo reperfused lung.

A key feature of reperfusion/reoxygenation injury of the lung is the development of pulmonary edema, primarily related to increased endothelial permeability (145). Such

high-permeability edema was obvious in warm-ischemic lungs, which displayed massive weight gain, considerable increase in BAL fluid's proteins, and a substantially reduced compliance. In addition, histopathological analysis revealed a marked increase of perivascular edema in warm ischemic lungs, further confirming the alterations of vascular permeability under such conditions. The abrogations of protein leakage afforded by MnTBAP and 3-AB, as well as the significant attenuation of lung perivascular edema, imply, therefore, a considerable protection against endothelial injury, which may be attributed to the concomitant cytoprotective and anti-inflammatory actions of these two drugs. Indeed, the cytotoxicity of ROS/ RNS and of inflammatory mediators is the primary mechanism disrupting pulmonary endothelial integrity during lung reperfusion (145).

A major consequence of pulmonary edema is hypoxemia, consecutive to intrapulmonary shunt. Therefore, the fact that we did not notice any significant variations of oxygen transfer may appear puzzling. It is possible that our protocol of EVLP, using a FiO_2 of 0.21 throughout the procedure may have limited the interpretation of the oxygen transfer capacity. Indeed, in current clinical EVLP protocols, oxygen capacity is generally evaluated using a FiO_2 of 1 (38). However, the most likely explanation for the lack of alterations in oxygen transfer was the fact that we used an acellular (i.e. no hemoglobin) perfusate fluid in our EVLP protocol. It is here worth to mention that the relationship between PO_2 and the actual content of O_2 in such an acellular medium is linear, in contrast to the sigmoid shape of the PO_2/O_2 content relationship in a perfusate containing red cells and hemoglobin. Under conditions of low VA/Q (from simple VA/Q mismatch to true shunt), as expected in the conditions of our study due to the formation of pulmonary edema, the actual "venous admixture" from the affluent arm of the circuit will therefore have only a very limited influence on the O_2 content, hence the PO_2, of the effluent. This has very well been demonstrated in a recent paper by Yeung et al (146), who clearly showed in a porcine model of EVLP, that the consequences of VA/Q mismatch and true shunt on effluent PO_2 were only observed under conditions of a cellular perfusate, whereas there was no observable effect in conditions of acellular perfusate. Therefore, these characteristics imply that the interpretation of PO_2 values as an indicator of lung damage can be largely misleading when using an acellular perfusate. (146).

We did not identify differences among cold ischemic and warm ischemic lungs in term of PVR. It has been previously shown that an increase in PVR during EVLP is mainly related to vascular obstruction by thrombus formation in the absence of heparinization (128). We took care to avoid such process by injecting heparin within the right ventricle before pulmonary cannulation, which probably explains the lack of PVR increase in our model, a hypothesis which will be evaluated in the future in experiments without heparinization. Whereas 3-AB did not influence vascular reactivity, MnTBAP induced an unexpected increase of PVR, which may be explained by two possible mechanisms. The first one is microvascular clogging by drug microthrombi. Such mechanism appears likely in view of the histopathological findings of dark brown deposits in the alveolar

wall, suggesting drug microaggregates in alveolar capillaries. The second may be related to the pharmacological activity of the drug as a peroxynitrite decomposition catalyst. Although highly cytotoxic, peroxynitrite also possesses potent vasodilating properties, especially in the pulmonary circulation (147). We therefore suspect that the loss of such peroxynitrite-dependent vasodilation may have contributed to the increased PVR in lungs treated with MnTBAP. Obviously, in spite of the beneficial effects of MnTBAP noted in our study, such increase in PVR represents an adverse effect that would limit the translational potential of this compound. Additional studies addressing this issue are therefore needed to clarify the mechanisms of PVR increase by MnTBAP.

In summary, our study indicates that lungs retrieved from rats after cardiac death and prolonged warm ischemia exhibit severe reperfusion/reoxygenation injury upon perfusion in an EVLP circuit, characterized by oxidative/nitroxidative stress, PARP activation, cell death, inflammation, and high permeability pulmonary edema. These changes are markedly attenuated by the PARP inhibitor 3-aminobenzamide and the superoxide dismutase mimetics and peroxynitrite scavenger MnTBAP, administered within the ex vivo perfusion circuit. Although we may not directly translate our findings to the clinical situation, due notably to differences in the EVLP protocol and the lack of in vivo data on transplantation of the reconditioned lungs in our study, our findings support the concept that EVLP may serve as an useful therapeutic station for the reconditioning of marginal lungs. In this respect, drugs interfering with oxidant- and PARP-mediated cytotoxicity could represent promising tools for such reconditioning.

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Chapter 5

Pyrrolidine Dithiocarbamate Administered During Experimental Ex-Vivo Lung Perfusion Alleviates Lung Damage after Extended Warm Ischemic Time

This chapter of the thesis was performed in collaboration with Cyril Francioli. Both investigators contributed equally to this work.

Abstract

Purpose: The use of ex-vivo lung perfusion (EVLP) is of particular interest in grafts where the risk of unidentified lung damage is high, such as donation after circulatory death (DCD). From withdrawal of life support or cardiac arrest to cold preservation DCD lungs are at risk to undergo hypotension and warm ischemia. In this situation the up-regulation of the Nuclear factor-kappa B (NF-κB), a family of transcription factors, plays a critical role in the inflammatory response. We therefore studied the potential of pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF-κB pathway and antioxidant, to reduce the tissue damage of rat lungs harvested after circulatory death and an extended warm ischemic time.

Methods: Two groups of 6 Sprague-Dawley rats each were used. Following cardiac arrest, the lungs were exposed to 1 hour of warm ischemia and 2 hours of cold (4°C) ischemia. Normothermic EVLP during 4 hours was performed using a customized circuit primed either with Steen solution® only or supplemented with PDTC (2.5mg/ml). Differential partial pressures of oxygen in the perfusate ($DppO_2$), vascular resistance (PVR), static pulmonary compliance (SPC), peak airway pressure (PAWP) and lung weight gain were measured. At the end of EVLP, protein content level, lactate dehydrogenase (LDH), protein Carbonyl, IL-6, CINC-1 and TNF-α level were determined in bronchoalveolar lavage or in lung tissue.

Results: 4 hours of EVLP resulted in a significant decline of $DppO_2$, SPC, PVR, PAWP in untreated lungs, a clear increase of graft weight gain and significantly increased levels of LDH, protein, protein carbonyl, IL-6, CINC-1 and TNF-α in BAL or in lung tissue, indicating alveolar damage and inflammatory process. EVLP with PDTC significantly improved SPC, PVR, PAWP, weight gain and attenuated the increase of protein content, LDH, protein carbonyl, IL-6, CINC-1 and TNF-α in BAL or in lung tissue.

Conclusion: Pharmacological intervention during EVLP aiming to inhibit the NF-κB pathway markedly improves the functional status of DCD lungs procured after prolonged warm ischemic times. These results also correlate with lesser amount of inflammatory cytokines.

Introduction

Lung transplantation is the only definitive treatment available for end-stage lung diseases. A critical issue when considering such therapy is the significant shortage of donor lungs available for transplantation, due both to small number of organ donors, and to the high proportion of potential donor lungs ultimately deemed unsuitable for transplantation (148). A recent breakthrough in the field of lung transplantation has been the development of ex-vivo lung perfusion (EVLP), a technique which allows a precise functional assessment of potential lung grafts. EVLP was initially developed to assess the function and the potential for transplantation of lungs from donation after circulatory death (DCD) (37). EVLP also permits the ex-vivo administration of drugs to the graft, in order to improve its status and render it suitable for transplantation (concept of pharmacological reconditioning). Such reconditioning might be particularly useful for DCD lungs, as these lungs may be at higher risk for ischemia-reperfusion-mediated damage and dysfunction due to the unavoidable period of warm ischemia which occurs in this setting.

In line with this concept, we recently provided evidence, in an experimental model of DCD lungs, that such lungs could be significantly reconditioned during EVLP using drugs interfering with redox-dependent processes associated with ischemia-reperfusion.

Nuclear factor-kappa B (NF-κB) is a family of transcription factors whose activation plays a critical role in the inflammatory response to various tissue injuries (149). It regulates a wide range of gene implicated in inflammation, from proinflammatory cytokines and chemokines to cell adhesion molecules (118).

Pyrrolidine dithiocarbamate (PDTC) is a low molecular weight thiol compound which acts as an inhibitor of the NF-κB transcription factor family (150)and also has antioxidant properties; scavenging superoxide and hydroxyl radicals (151). It has already been shown to attenuate ischemia reperfusion injury when added to the organ preservation solution in a porcine lung transplant model (76).

We therefore hypothesized that treating damaged lungs with PDTC through EVLP would reduce lung tissue damage and improve lung function. We tested this hypothesis with on a model of rat lungs harvested after circulatory death and exposed to an extended warm ischemic time.

Materials and Methods

Animals

Twelve male Sprague-Dawley rats weighing 300 to 350g (Charles River Laboratory, L'Arbresle, France) were used in this study. All the animal experiments were performed in accordance with the Animal Welfare Act and the National Institutes of Health "Guidelines for the Care and Use of Laboratory Animals" were approved by our Local Ethical Committee (Service Vétérinaire Cantonal de l'Etat de Vaud, Authorization Nr. 2637)

DCD lung graft model

Animals were anesthetized using Isoflurane and pentobarbital sodium i.p. (50mg·kg^{-1}). Tracheotomy was performed and rats were mechanically ventilated with room air, tidal volume of 7ml·kg^{-1} and respiratory rate of 75·min^{-1}. Median sternotomy was performed and lung compliance was measured using a flexiVent ventilator (FX3, SCIREQ Inc, Montréal, Canada). One minute after injection of 600 IU of heparin in the right ventricule, animal was sacrified by exsanguination. Pulmonary artery and left atrium were canulated using specially designed canulas (Hugo Sachs Elektronik, Hugstetten, Germany). The chest was left open for one hour at room temperature. The lungs were then flushed with 15ml of cold low-potassium dextran solution (Perfadex®, Xvivo Perfusion, Göteborg, Sweden) through pulmonary artery at a pressure of 20cm H_2O. Heart lung bloc was inflated with a FiO_2 of 0.5, volume of 5ml·kg^{-1}, excised and kept for another 2 hours at 4°C.

Ex-vivo lung perfusion

The heart lung bloc was weighted, and then mounted to a commercial rodent's isolated organ perfusion platform (Harvard IL-2 System, Hugo Sachs Elektronik, Hugstetten, Germany). The EVLP circuit was primed with Steen® solution (Xvivo Perfusion, Göteborg, Sweden). For control group (CTRL, N=6), Steen® solution was used alone. THAM solution was used to adjust pH. For PDTC treatment group (PDTC, N=6), Steen® solution was supplemented with 2.5g·l^{-1} of pyrrolidine dithiocarbamate ammonium (Sigma-Aldrich, St. Louis, USA). A constant perfusion flow was applied, starting at 2% of cardiac output and at a temperature of 10°C. The flow and temperature were progressively increased over 40 minutes, to reach 7.5% of cardiac output and 37°C respectively. The left atrium pressure was set at 4cmH_2O. Ventilation was initiated after 30 minutes with room air, tidal volume was progressively increased to reach 6 ml·kg^{-1} after 40 minutes of EVLP. The perfusate was deoxygenated using a hemofilter (Hemofilter D150, MEDICA S.P.A, Italy) connected to the EVLP circuit and supplied with a 6%CO_2, 8%O_2, 86%N_2 gas mixture. The EVLP was carried out for a total of 4 hours.

Pulmonary artery pressure (PAP) and left atrium pressure (LAP) were continuously recorded using pressure transducer (Hugo Sachs Elektronik, Hugstetten, Germany).

Pulmonary vascular resistance (PVR) was calculated using the formula: PVR = (mean PAP-LAP)/Flow. Starting after 60 minutes of EVLP and every 30 minutes thereafter, lung compliance (19) and peak airway pressure (PAWP) were recorded using a flexiVent ventilator (FX3, SCIREQ Inc, Montréal, Canada). Oxygenation capacity (ΔppO2) of the lungs was determined as the oxygen partial pressure difference between outflow and inflow perfusate, determined using oxygen partial pressure electrode (Hugo Sachs Elektronik, Hugstetten, Germany).

At the end of EVLP the heart lung bloc was weighted. A bronchoalveolar lavage (118) was performed instilling 2ml of PBS inside trachea and 0.8 to 1.2ml of volume was recovered and stored at -80°C. The left lung parenchyma (LLP) was sampled and stored at -80°C.

Figure 5.1 summarizes the experimental design.

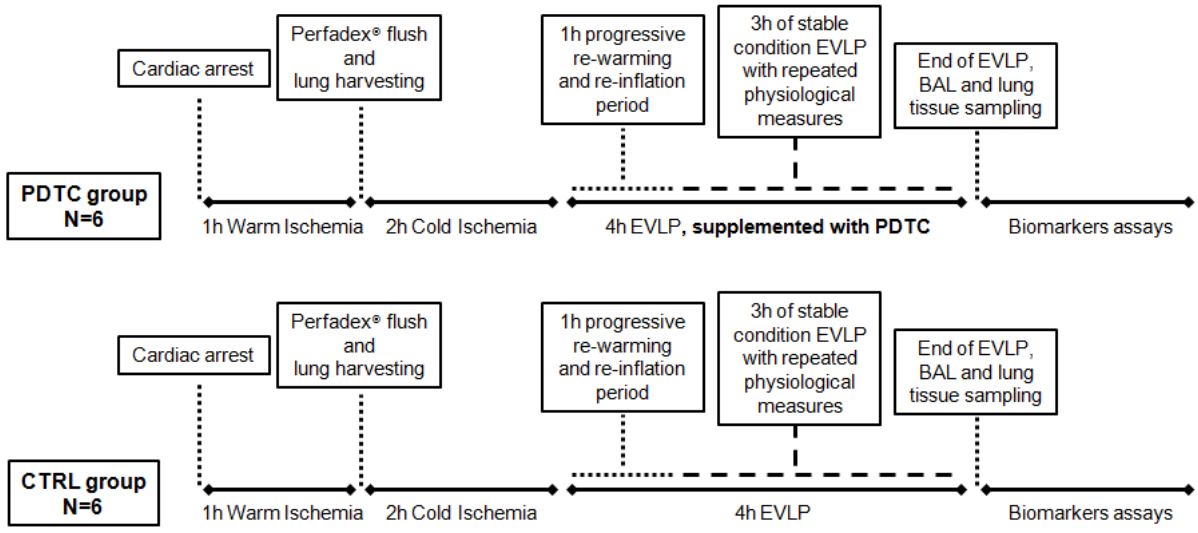

Figure 5.1 Study design

Baseline group

A group of rats (BASE, n=3) were sacrificed prior to any intervention and lungs were retrieved to assess a baseline level for the different biomarker assays.

Tumor necrosis factor alpha (TNF-α) production BAL

The productivity of TNF-α in the lung tissue and BAL was evaluated by a commercial ELISA kit (Rat TNF-alpha DuoSet) according to the manufacture manual. The results are normalized by the quantity of protein extracted for lung tissue and are expressed in nanogram·mg^{-1} for lung tissue and nanogram·ml^{-1} for BAL.

Cytokine-induced neutrophil chemoattractant Factor 1 (CINC-1) concentration in BAL

CINC-1 in the lung tissue and BAL was determined by a Duoset ELISA kit (Rat CXCL1/CINC-1; R&D System, Minneapolis, MN, USA). The results are normalized by the quantity of protein extracted for lung Tissue and are expressed in nanogram·mg^{-1} for lung tissue and nanogram·ml^{-1} for BAL.

Interleukin-6 (IL-6) concentration in BAL

A commercial rat ELISA kit (Rat IL-6 Duoset; R&D Systems, Minneapolis, MN, USA) was used to determine the IL-6 level in lung tissue and BAL. For lung tissue, the concentration of IL-6 was expressed in nanogram·mg^{-1} on a basis of calculating the linear portion of the standard curve created. For BAL, the results are expressed in nanogram·ml^{-1}.

Protein carbonyl concentration in lung tissue

Protein carbonyl was quantified in lung tissue using an ELISA based assay (OxiSelect Protein Carbonyl ELISA Kit; Cell Biolabs Inc., San Diego, CA, USA) with accordance to the user manual. The absorbance was measured at 450 nm thanks to an ELISA plate reader and the results was expressed in nanomol·mg^{-1}.

Protein concentration and Lactate dehydrogenase (LDH) activity in BAL

Total protein level in BAL was determined using the Pierce BCA assay (Thermo Scientific, Rockford, USA). The concentration was shown in mg·ml^{-1}. LDH was measured as an index of formazan from tetrazolium salt at 492nm (Cytotoxicity Detection Kit^{PLUS}; Roche Molecular Biochemicals, Basel, Switzerland) and was expressed in optical density (O.D.) units.

Statistical analysis

All the results in the study are presented as the mean plus or minus the standard error of the mean. Statistical analysis was performed using Graphpad prism 6 (GraphPad Software Inc., La Jolla, CA, USA). For the repeated physiological measurements during EVLP, data was analyzed by *2-way ANOVA,* significant difference were determined using the Bonferroni multiple comparison test. To determine the effect of time in physiological data the value at 60min was taken as control. For the weight gain comparison between control and treatment group, unpaired 2-tailed *t-test* was used. For the other comparisons, *one-way ANOVA* was used and *Tukey's* correction was used for significant difference determination. $p<0.05$ was considered statistically significant.

Results

EVLP physiological variables

In CTRL group the measured SPC declined during EVLP, especially during the last hour (LC=0.74 ± 0.05ml·cmH_2O^{-1} at 60min *versus* 0.61 ± 0.06ml·cmH_2O^{-1} at 210min (p=0.001) and 0.46 ± 0.08ml·cmH_2O^{-1} at 240min (p=<0.0001). In PDTC group the LC stayed stable during the whole EVLP procedure (LC=0.80 ± 0.06ml·cmH_2O^{-1} at 60min *versus* 0.83 ± 0.07ml·cmH_2O^{-1} at 240min). The CTRL group LC is significantly lower compared with PDTC at the end of the EVLP procedure (p=0.001). (Figure 5.2A)

The PAWP measured was stable in the PDTC group during the whole experiment (PAWP=6.47 ± 0.26cmH_2O at 60min *versus* 5.98 ± 0.22cmH_2O at 240min), while in CTRL group we see a progressive increase, starting at 210min (non significant) and increasing significantly in the last 30min (PAWP=6.94 ± 0.38cmH_2O at 60min *versus* 10.23 ± 1.97cmH_2O at 240min (p=<0.0015)). The difference between the two groups is significant at 240min (p=<0.0001)). (Figure 5.2B)

The PVR show a similar pattern as PAWP. It is stable in the PDTC group throughout experiment (PVR=0.44 ± 0.02cmH_2O·min·ml^{-1} at 60min *versus* 0.46 ± 0.02cmH2O·min·ml^{-1} at 240min). In the CTRL group the PVR is rising at 210min and 240min (PVR=0.46 ± 0.02cmH2O·min·ml^{-1} at 60min *versus* 0.77 ± 0.12cmH2O·min·ml^{-1} at 210min (p=0.0002) and 1.03 ± 0.16cmH2O·min·ml^{-1} at 240min (p=<0.0001). The PVR in CTRL group is significantly higher than in the PDTC group at 210min (p=0.0015) and 240min (p=<0.0001). (Figure 5.2C)

The PDTC group shows a stable $DppO_2$ during the experiment (40.4 ± 5.5mmHg at 60min *versus* 40 ± 7.4mmHg at 240min). The CTRL group shows a progressive decrease which is not statistically significant (40.4 ± 5.5mmHg at 60min *versus* 23.4 ± 6.2mmHg at 240min) as well as the difference between the two groups. (Figure 5.2D)

We measured an increase in weight gain of 0.47 ± 0.05g in the PDTC group, compared with 2.19 ± 0.53g in the CTRL group. The difference is statistically significant (p=0.009). (Figure 5.2E)

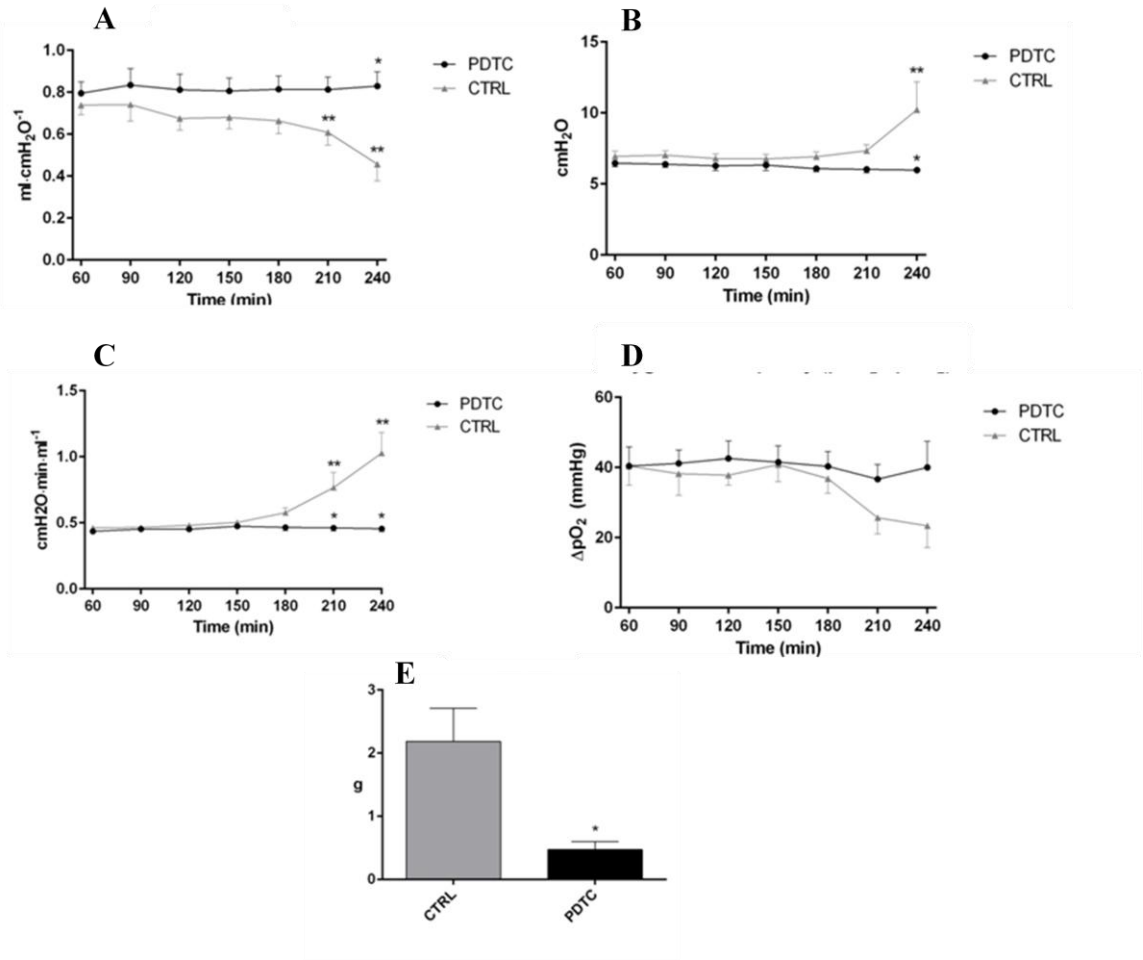

Figure 5.2 EVLP physiology

EVLP lungs reconditioned by PDTC benefited from an improved pulmonary physiological function than that of non-treated lungs, peculiarly in the last hour of perfusion, demonstrated as A: static pulmonary compliance (SPC); B: Peak airway pressure (PAWP); C: Pulmonary vascular resistance (PVR); D: Differential partial pressure of oxygen ($DppO_2$); E: Lung weight gain.

*p<0.05 ; **p<0.01

Bioassay

Alveolar integrity and oxidative stress

The protein content in BAL was measured as a marker of alveolar cell integrity. The amount of protein is significantly increased in CTRL and PDTC compared to BASE (p=0.0004 and p=0.022). The difference between the CTRL and PDTC group is also significant (10.02 ± 1.12mg·ml^{-1} *versus* 6.1 ± 0.97mg·ml^{-1}). (Figure 5.3A)

The LDH content in BAL, marker of cell necrosis, is markedly increased in CTRL compared with BASE (6.45 ± 0.79OD492nm versus 0.21 ± 0.03OD492nm (p=0.002)). PDTC also shows a moderate increase of LDH level (3.72 ± 0.97OD492nm), which is not significant compared to BASE and CTRL (p=0.065 and p=0.081). (Figure 5.3B)

Protein Carbonyl level in lung tissue served as indicator of oxidative stress[i]. We measured an increase in protein carbonyl amount more pronounced in CTRL (0.38 ± 0.03nmol·ml^{-1}) than in PDTC (0.31 ± 0.05nmol·ml^{-1}) compared to BASE (0.17 ± 0.02nmol·ml^{-1}). This increase is significant for CTRL (p=0.03) but not for PDTC (p=0.16). (Figure 5.3C)

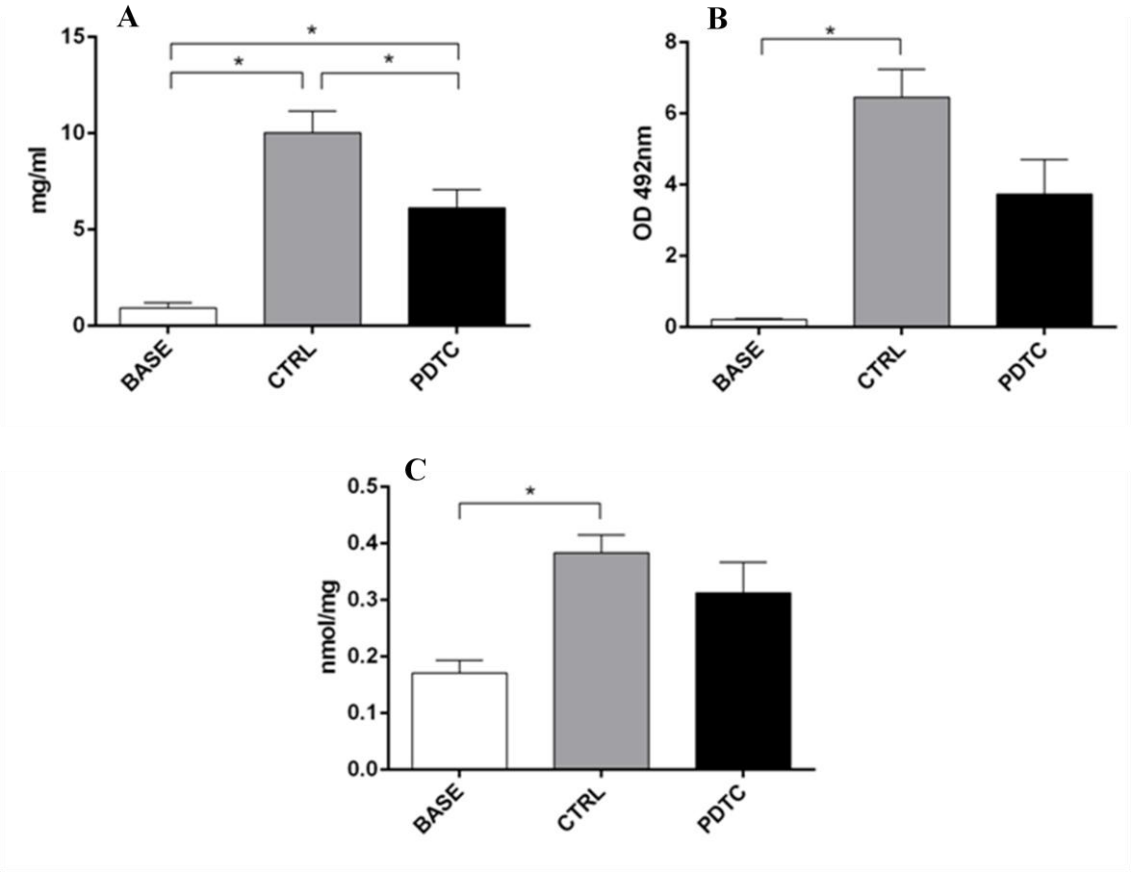

Figure 5.3 Alveolar integrity and oxidative stress

EVLP lungs reconditioned by PDTC showed a significantly less pronounced protein content (A) in BALF in comparison to control group, and displayed a trend of decreased LDH (B) and protein carbonyl (C).

*p<0.05

Inflammatory cytokines

IL-6 level determined in BAL was strongly increased in CTRL (2.97 ± 0.48ng·ml^{-1}) compared with BASE (0.05 ± 0.02ng·ml^{-1}, p=0.0005). PDTC level of IL-6 (1.15 ± 0.13ng·ml^{-1}) is non-significantly elevated compared to BASE and significantly reduced compared to CTRL (p=0.005) (Figure 5.4A).

CINC-1 is the rat homologue to human interleukin-8. Its level in BAL is significantly increased in CTRL (11.38 ± 1.25ng·ml^{-1}) and PDTC (7.4 ± 1.23ng·ml^{-1}) compared to BASE (0.14 ± 0.06ng·ml^{-1}) (p=0.0003 and p=0.008 respectively). This increase is less

pronounced in PDTC than in CTRL, although the difference between the two groups is not statistically significant (p=0.069) (Figure 5.4B).

TNF-α measured in BAL shows a very important increase in CTRL (1.71 ± 0.43ng·ml^{-1}) compared with BASE (0.11 ± 0.02ng·ml^{-1}, p=0.016) and PDTC (0.34 ± 0.07ng·ml^{-1}, p=0.012). The increase in PDTC is moderate and non-significant compared to BASE (Figure 5.4C).

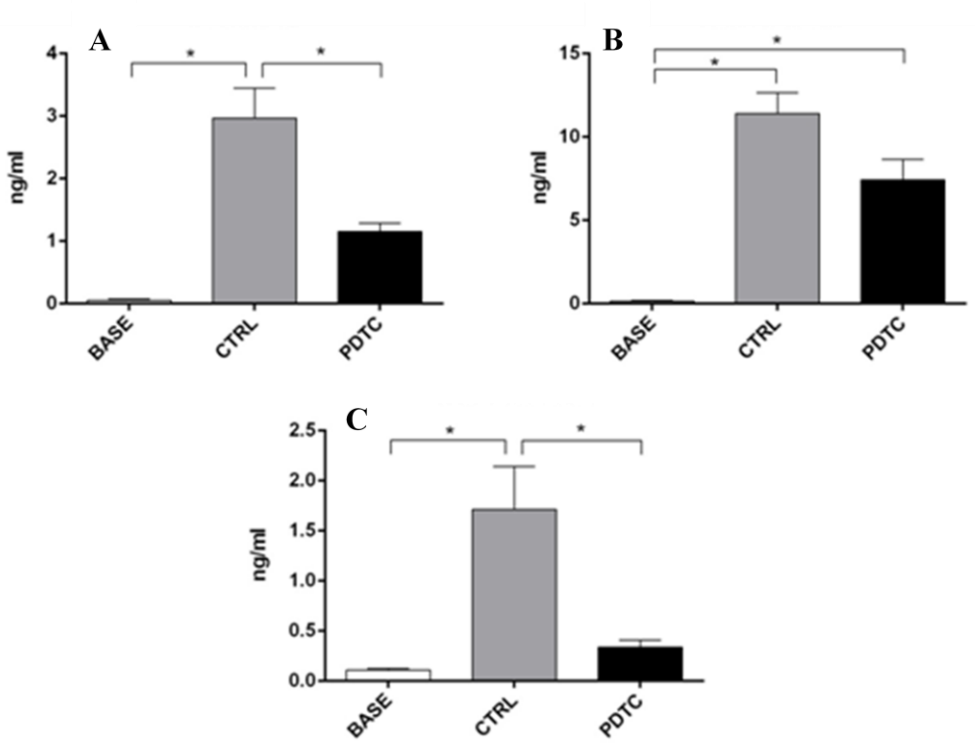

Figure 5.4 Inflammatory cytokines

Lung in control group was associated with high increased IL-6(A), CINC-1(B) and TNF-α(C) in BALF as compared to the Baseline group. These alterations have been significantly down-regulated by ex-vivo PDTC treatment.

Discussion

In this study, we describe the use of EVLP as a therapeutic platform to pharmacologically recondition injured lungs. We demonstrate the potential of pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF-κB pathway and antioxidant, to reduce the tissue damage of rat lungs harvested after circulatory death and an extended warm ischemic time.

Four hours of EVLP resulted in a significant decline of $DppO_2$, SPC, PVR, PAWP in untreated lungs, a clear increase of graft weight gain and significantly increased levels of LDH, protein, protein carbonyl, IL-6, CINC-1 and TNF-α in BAL or in lung tissue, indicating alveolar damage and inflammatory process. EVLP with PDTC significantly improved SPC, PVR, PAWP, weight gain and attenuated the increase of protein content, LDH, protein carbonyl, IL-6, CINC-1 and TNF-α in BAL or in lung tissue.

Nuclear factor-kappa B (NF-κB) plays an important role in the inflammatory response to ischemia reperfusion injury. The underlying mechanism is quite complex and has been described recently (REF): It is a family of dimeric proteins from the Rel family, including NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precuror p100), p65 (RelA), RelB and c-Rel (152), the commonest dimer being a heterodimer formed from a p50 subunit and a p65 subunit. NF-κB is normally held inactive in the cytoplasm, bound to an inhibitory protein termed IκB (the most common being IκBα) (153, 154). NF-κB activation follows the dissociation of the NF-κB dimer from IκB, due to stimulus-induced phosphorylation of IκBα and its polyubiquitination, leading to its degradation by the 26S proteasome (153, 154). NF-κB can then enter the nucleus to activate transcription of multiple NF-κB-dependent genes. The phosphorylation of IκB is triggered by IκB kinase (120), made up of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKY. The so-called canonical pathway of NF-κB activation, which prevails during innate immune reponses and inflammation, is related to the activation of IKK by various upstream signals which primarily include the inflammatory cytokines TNFα and IL-1 (153, 154), as well as ligands of innate immune cell surface from the toll-like receptors. Activation of NF-κB by TNFα is mediated by the TNF receptor (TNFR), an adaptor molecule (TRADD) and several signaling proteins including TNF receptor associated factors (TRAFs) and receptor interacting protein 1 (RIP-1), forming a complex activating IKK (155, 156). TLRs and IL-1 (via the IL-1 receptor), activate NF-κB through a common cascade involving the adaptor molecule MyD88 (Myeloid differentiation primary response gene 88), and several downstream proteins such as IRAK (IL-1R associated kinase) and TRAF6 to activate IKK (157-160). Besides these well-described mechanisms of NF-κB activation, it is important to stress the fact that NF-κB can also be activated by reactive oxidants, including PN, through complex, and yet incompletely understood mechanisms (161). As such, NF-κB is generally regarded as a "redox-sensitive" transcription factors, which is one important mechanism connecting oxidative stress and inflammation, as outlined in our recent review on the topics (63). Once activated, NF-κB activates the transcription of a myriad of genes primarily

involved in inflammation and, therefore, NF-κB is considered as the master regulator of innate immune responses and inflammation (63). In addition to NF-κB, some additional transcription factors play also important roles in the development of inflammation, such as activator-protein-1 (AP-1) and the interferon-regulatory factors (IRFs), to cite only a few. Evidence has been obtained that activation of NF-κB represents a key process leading to tissue injury and inflammation during ischemia and reperfusion (64) and notably after transplantation (76).

In conclusion, acute inflammation is a central pathophysiological process during ischemia-reperfusion. The activation of the transcription factor NF-κB is instrumental in the initiation of such inflammation. In this respect, we have demonstrated here, that inhibition of NF-κB activation using pyrrolidine dithiocarbamate, administered during EVLP of damaged lungs, resulted in significant decrease of inflammatory injury and improvement of functional status, suggesting that such approach could be useful to recondition low quality donor grafts for subsequent transplantation, which will be assessed in the future.

Acknowledgments

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Chapter 6

Experimental Ex-vivo Lung Perfusion with Sevoflurane: Effects on Damaged Donor Lung Grafts

This chapter has been submitted to Anesthesiology

Abstract

Background: Damaged donor lungs obtained after prolonged warm ischemia (WI) can be pharmacologically reconditioned during ex-vivo lung perfusion (EVLP). Here, we explored the ability of sevoflurane, administered within an EVLP circuit, to reduce damage and dysfunction of rat lungs obtained after circulatory death (DCD) and prolonged WI.

Methods: Fifteen rats were distributed in 3 groups. In the Baseline (N=3) group, heart-lung blocks were harvested immediately after euthanasia. In the Control (CONT, N=6) and Sevoflurane (SEVO, N=6) groups, the heart-lung blocks were harvested after 1h WI at 20º, stored for 2h at 4°C, and then perfused in an EVLP circuit for 3h with Steen® solution only or adding 2% sevoflurane during the first 30 min of EVLP). Physiological variables (compliance, vascular resistance, oxygenation capacity, peak airway pressure) were evaluated during EVLP. At the end of EVLP, lung weight gain was assessed, the levels of, protein and lactate dehydrogenase (LDH), protein carbonyls (markers of oxidative stress) and inflammatory cytokines (TNF-α, IL-6, CINC-1) were measured in bronchoalveolar lavage fluid (BALF) or lung tissue, and histopathological changes were assessed.

Results: When compared to CONT lungs, lungs from the SEVO group disclosed significantly reduced release of protein, LDH, protein carbonyls and cytokines, as well as a significant reduction of weight gain and perivascular edema, together with an improved static compliance. In contrast, Oxygenation, airway pressure and vascular resistance were unchanged by sevoflurane.

Conclusions: Sevoflurane administered during EVLP significantly attenuates pulmonary damage and dysfunction of rat lungs obtained after circulatory death and prolonged warm ischemia.

Introduction

The shortage of donor lungs due to a low acceptance rate remains a challenge in lung transplantation (LTX) (10, 162). To increase the number of eligible organs, one could harvest the lungs after circulatory death (DCD)(163), or use *ex-vivo* lung perfusion (EVLP) to evaluate and recondition damaged lungs previously denied for transplantation(38, 164) or combine both techniques. Due to an unavoidable and variable period of warm ischemia, DCD lungs may be at increased risk of primary graft dysfunction (PGD)(28). PGD is the main cause of short term morbidity and mortality following transplantation and may be associated with chronic allograft dysfunction (165). The leading contributors to PGD are the time of warm ischemia (WI) and the subsequent cellular ischemia reperfusion/reoxygenation (IR) injury (166). Theoretically, IR injury of donor lungs at risk of PGD could be reduced or alleviated by pharmacological reconditioning performed during EVLP (167).

Different therapeutic strategies have shown the efficacy in experimental IR injury of various organs. Evidence has notably accumulated that volatile anesthetics, such as sevoflurane, is a potent protective agent against IR, when administered either before or after ischemia, giving rise to the concept of anesthetic pre- and postconditioning, which has been essentially validated in the heart (168). With specific respect to the lungs, preconditioning with inhaled sevoflurane is associated with reduced IR injury in animal models of *ex vivo* isolated rat lungs (169) and auto-transplanted pig lungs *in vivo* (170). Furthermore, sevoflurane post-conditioning exhibits anti-inflammation property on lungs (171, 172), but with scarce explorations on the IR injury, particularly in the context of transplantation. The aim of the current experimental study was therefore to assess the therapeutic potential of postconditioning with sevoflurane added in the solution of an EVLP system on the development of IR injury in damaged rat lungs harvested after an extended period of WI.

Materials and Methods

Animals

All the animal experiments were performed in accordance with the Animal Welfare Act and the National Institutes of Health "Guidelines for the Care and Use of Laboratory Animals" and were approved by our Local Ethics Committee (Service Vétérinaire Cantonal de l'Etat de Vaud, Authorization Nr. 2637). Fifteen male adult (9-11 weeks) Sprague-Dawley rats weighing 300-350 g (Charles River Laboratory, L'Arbresle, France) were subdivided in 3 groups : Baseline (BASELINE group, N=3), Control (CONT group, N=6) and Sevoflurane (SEVO group, N=6).

Surgical preparation and lung harvesting

The procedures used in this study were comparable, with some modifications, to those presented in detail in our recent publication (92)**.** Animals were anesthetized with intra-peritoneal sodium pentobarbital (50mg/kg) and placed on a heating pad to maintain the core temperature at 37°C .The trachea was cannulated and mechanical ventilation was initiated using a rodent respirator (Model 683, Harvard Apparatus, Holliston, MA, USA). Ventilation was delivered in volume controlled mode with a respiratory rate at 75 breath·min^{-1}, a tidal volume of 7ml·kg^{-1} and an inspired fraction of oxygen of 0.21. Following median sternotomy, heparin (600 IU) was administered into the right ventricle, and both the pulmonary artery (PA) and the left atrium (LA) were cannulated using two specially designed metal catheters (Hugo Sachs Elektronik, Hugstetten, Germany) and secured with 3-0 silk sutures. The inner and outer diameters were 1.7 mm and 2.0 mm respectively for the PA cannula and 3.4 mm and 4.0 mm for the LA cannula.

The animals were then sacrificed by exsanguination through a left ventricular puncture and subdivided in 3 groups. In the BASELINE group, immediately after euthanasia, the heart-lung blocks were harvested and a bronchoalveolar lavage (118) was performed with 2 ml phosphate-buffered saline, pH 7.4, via the tracheal cannula. The left lungs were then flash-frozen in liquid nitrogen and kept at -80°C until processing, and the right lung were fixed in 4% paraformaldehyde for further histological analysis.This BASELINE group representsed the standard donor lung harvesting procedure and was used to determine the physiological levels of various biochemical markers and the baseline histology as detailed below. On the basis of preceding study (92), due to stable data reproducibility, we scarified only 3 animals for this purpose.

In order to mimic the DCD explantation procedure, lungs obtained after euthanasia in the CONT group and SEVO groups, were first kept deflated in situ during 1 hour of warm ischemia at room temperature. Then the lungs were perfused through the PA cannula with 15ml of 4°C Perfadex®, at a perfusion pressure of 20 cm H_2O, while ventilated at a

RR of 15/min and a V_t of 7 ml/kg and stored for 2 hours of cold ischemia in 4°C Perfadex® in an inflated status (FiO_2=0.50). The study design is illustrated in Figure 6.1.

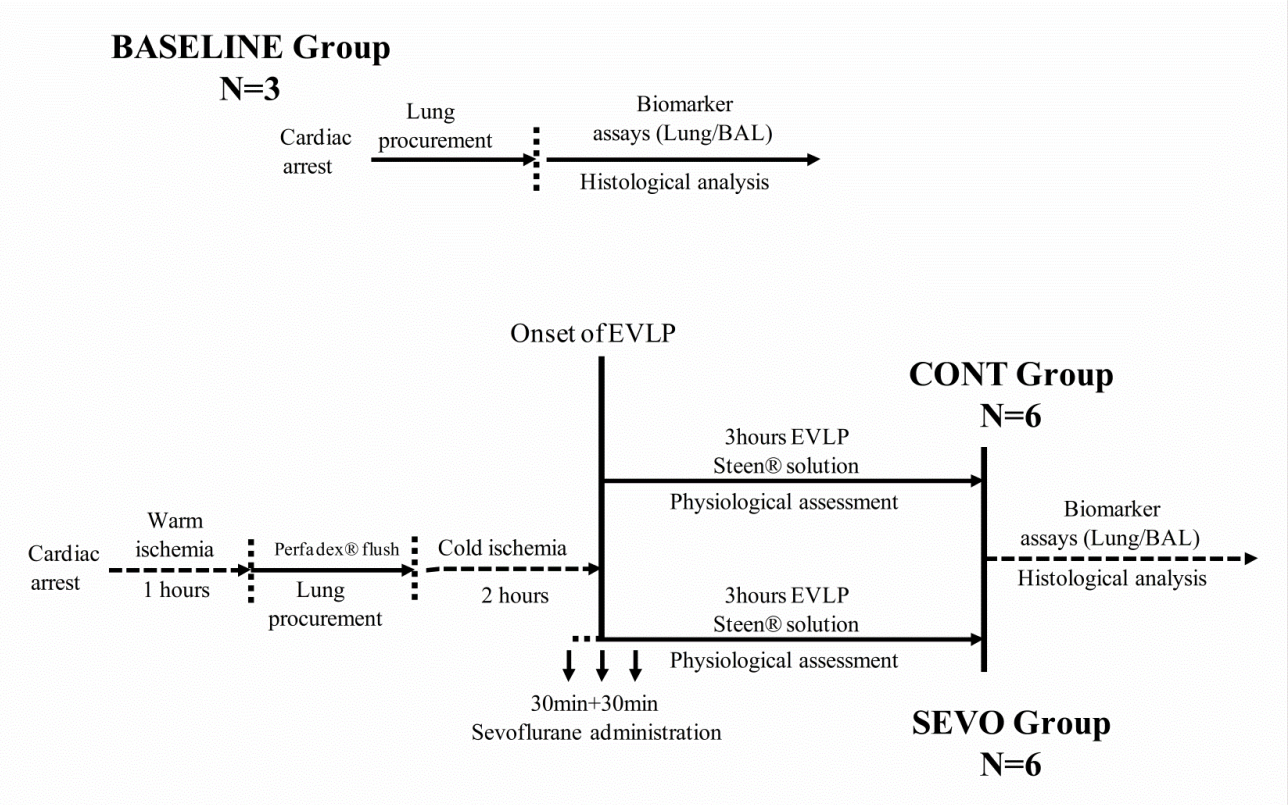


Figure 6.1 Experimental design

Baseline group (BASELINE, N=3): Rats were euthanized and the lungs were harvested directly without any further intervention, for the determination of baseline physiological variables in some of experiments.

Control group (CONT, N=6): subsequent to the cardiac arrest, the rat lungs underwent 1 hour warm ischemia in the room temperature were flushed with 4°C Perfadex® , harvested and stored in 4°C Perfadex® for 2 hours in an inflated status (FiO2=0.50), followed by 3 hours EVLP with only Steen® solution primed as the perfusate.

Sevoflurane group (SEVO, N=6): same as the CONT group, but with 2% sevoflurane continuously added to the circuit 30min before and after the initiation of EVLP.

Ex-vivo lung perfusion

After 2 hours of cold preservation, the heart-lung block of CONT and SEVO groups was weighed (PB-602C, METTLER TOLEDO, Greifensee, Switzerland) prior to be mounted on an isolated rat lung perfusion system (Harvard IL-2 System, Hugo Sachs Elektronik, Hugstetten, Germany). This EVLP system consisted of a perfusion circuit with tubing and a reservoir primed with Steen® solution (Xvivo Perfusion, Göteborg, Sweden). A pump drove the perfusate from the reservoir through a heat exchanger and a gas exchanger before entering the PA. Then the pulmonary effluent from LA drained back to the reservoir and was recirculated. Details of the circuit are provided in Figure 6.2.

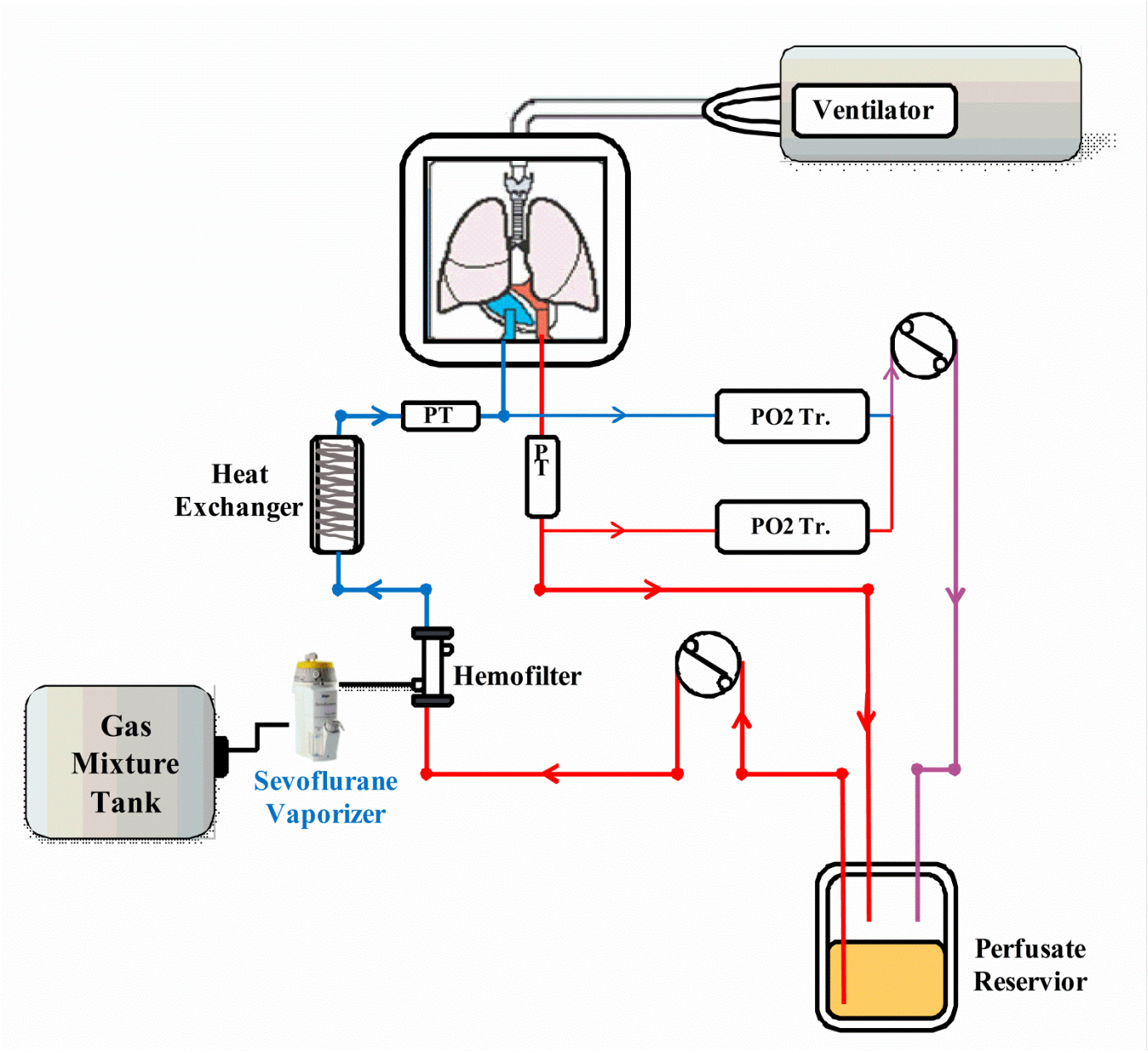


Figure 6.2. EVLP system

Oxygenated Steen® solution in Perfusate Reservoir was pumped through a Gas Mixture (6% O_2, 10% CO_2, 84% N_2) equilibrated Hemofilter to be deoxygenated and warmed through a Heat Exchanger prior to be perfused into pulmonary artery. The lung graft was mechanically ventilated with room air to artificially oxygenate Steen® solution which was circulated from the left ventricle then discharged to the Perfusate Reservoir. Only in the SEVO group, a customized flow-adaptable Sevoflurane Vaporizer was installed in line to the Hemofilter to supply 2% sevoflurane to the Steen® solution. Two transducers to continuously monitor PaO2 were respectively coped to the arterial and venous arms of the lung grafts.

PT: pressure transducer; PO2 Tr : PaO2 transducer

To evaluate the lung oxygenation capacity (ΔPO_2 = perfusate LA PO_2 - perfusate PA PO_2 [mm Hg]) during the EVLP, the perfusate was deoxygenated by using a 1 l/min gas mixture containing 6% O_2, 10% CO_2 and 84% N_2 (CHUV, Lausanne, Switzerland) delivered over a gas-exchange membrane (Hemofilter D150, MEDICA S.P.A, Italy) connected to the affluent (PA) arm of the heart-lung block (Figure 2). Thirty minutes prior to the EVLP procedure, the perfusate was cooled down to 10°C by an external heater-cooler unit (Sarns TCMII, 3M, Saint Paul, MN, USA). During this period, in CONT group, only Steen® solution was added in the EVLP circuit. In SEVO group, a flow adaptable sevoflurane vaporizer (Vapor2000 Sevoflurane, Drägerwerk AG, Lübeck,

Germany) was connected in line with the affluent of gas-exchange membrane, and 2% sevoflurane (169-171) at a flow of 1L/min was continuously added in the EVLP circuit during cooling down period (30min before the onset of EVLP) and 30 minutes during the initiation of EVLP, thus 1 hour of sevoflurane administration in total (Figure 1). EVLP was initiated at a flow rate of 2 ml·min^{-1} at 10°C, and was step-wise increased to the target flow defined as 7.5 ml·min^{-1}, which corresponds to 7.5% of theoretical cardiac output and (98) rewarmed to 37°C (Alpha immersion thermostat 6, LAUDA-Brinkmann, Delran, NJ, USA) during the next 30min. The left atrial pressure (LAP) was set at 4 cmH_2O by adjusting the height of an outflow vessel. The pH of the perfusate was maintained in the range of 7.35-7.45 by titrating THAM solution (Tham-Köhler 3M, köhler Pharma GmbH, Alsbach-Hähnlein, Germany) to the circuit. After the first 30 minutes of EVLP, mechanical ventilation of the lungs was initiated using a tidal volume of 3 ml·kg^{-1}, a respiratory rate of 7 min^{-1}, and a FiO_2 of 0.21 (flexiVent FX3 ventilator, SCIREQ Inc, Montréal, Canada). After 40 min of EVLP, the perfusate reached 37°C and the tidal volume was increased to 6 ml·kg^{-1}. After 180 minutes of EVLP, the heart-lung block was withdrawn from the circuit and weighed. 2 ml of sterile PBS, pH 7.4 was instilled into the trachea to perform and sample a BAL. The left lung was harvested from the block and stored at -80°C, and the right lung was fixed in 4% paraformaldehyde for further histological analysis. The EVLP protocol illustrated as Table 6.1 in this study was based on the strategy described for clinical EVLP by Cypel et al (38).

Sevorane® (%, CONT/SEVO)	0/2	0/2	0/2	0/2	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Time (Minutes)	0	10	20	30	40	50	60	90	120	150	180
T (C°)	10	20	30	35	37	37	37	37	37	37	37
Flow (%CO)	2	2	4	7	7.5	7.5	7.5	7.5	7.5	7.5	7.5
V_T (ml·kg^{-1})	no	no	no	3	6	6	6	6	6	6	6
RR (breath·min^{-1})	no	no	no	7	7	7	7	7	7	7	7

Table 6.1 Rodent EVLP protocol

CO: Cardiac output; T: Temperature; V_T: Tidal volume; RR: Respiratory rate

Measurements

Physiological variables

PA and LA pressures were continuously recorded and used to calculate pulmonary vascular resistance (PVR) according to the standard formula: PVR= (mean PAP -LAP pressure)/Flow. Before measuring the other physiological variables, a recruitment maneuver with an inspiratory pressure of 15 cm·H_2O^{-1} during 20 seconds was performed 60 minutes after the onset of EVLP and every thirty minutes thereafter. Static pulmonary compliance (SPC) was calculated by computing the change in lung volume elicited by an automated step-wise increase of inspiratory pressure up to 15 cmH_2O. Peak airway pressure (PAWP) measured by the ventilator from the pressure-time curve was documented. Finally, to compute the difference of partial pressure of O_2 ($DppO_2$), as an indicator of oxygenation capacity, partial pressure of O_2 in the Steen® solution was measured by 2 oxygen electrodes (Hugo Sachs Elektronik, Hugstetten, Germany) in the affluent and effluent arms of the circuit.

Rat lung protein extraction and quantification

The lung tissue was grinded in liquid nitrogen to a powder using a mortar and a pestle, then homogenized in lysis buffer (TrisHCl 10 mM, NP40 0.5%, NaCl 0.15 M, Na_3VO_4 1 mM, NaF 10 mM, PMSF 1 mM, EDTA 1 mM, aprotinin 10 µg/ml, leupeptin 10 µg/ml, and pepstatin 1 µg/ml), sonicated and incubated for 20 minutes on ice. Samples were centrifuged at 13000 RPM for 10 minutes and the clean supernatants were stored at -80°C until further processing. Protein content was measured with the BCA assay (Thermo Scientific Pierce, Rockford, IL, USA) and expressed in $mg \cdot ml^{-1}$.

Measurements of Cytokines

The concentrations of the cytokines: tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), as well as of the chemokine cytokine-induced neutrophil chemoattractant factor 1 (CINC-1) were measured in lung tissue extracts using specific ELISA kits **(**R&D System, Minneapolis, MN, USA) following the manufacturer's instructions. Concentration of cytokines were expressed in $nanogram \cdot mg^{-1}$ of proteins.

Protein carbonyl concentration in lung tissue

Protein carbonyls (a marker of oxidative modifications in proteins) were quantified in lung tissue using an ELISA based assay (OxiSelect Protein Carbonyl ELISA Kit; Cell Biolabs Inc., San Diego, CA, USA) according to the user manual and was expressed in $nanomol \cdot mg^{-1}$ of proteins.

Protein concentration and lactate dehydrogenase (LDH) levels in BAL

Total protein concentration in BAL was determined using the Pierce BCA assay (Thermo Scientific, Rockford, USA) and expressed in $mg \cdot ml^{-1}$. LDH in BAL was measured using a commercial kit (Cytotoxicity Detection Kit^{PLUS}; Roche Molecular Biochemicals, Basel, Switzerland) and was expressed in arbitrary units (A.U.).

Histological evaluation

Right lungs obtained either following the euthanasia (BASELINE group) or at the end of EVLP (CONT and SEVO groups) were for histopathological assessment. Lung tissue was formalin fixed (4% buffered formalin) and paraffin embedded. 5 µm thick slides were taken and stained with hematoxylin and eosin. All slides were digitalized using Hamamatsu NanoZoomer HT Digital slide scanner (Hamatsu Photonics, K K, Japan), and visualized by uploading to an image analysis program (Slidepath, Leica Biosystems) for morphometric studies. The pulmonary perivascular edema was indexed as the severity of tissue injury, by quantifying the ratio of perivascular edema thickness to the inner diameter of the encircled vessel. 20 symmetrically cross-sectioned vessels (arteries and veins), in average per slide, were independently evaluated by 2 investigators blinded to the experimental grouping of the specimens.

Statistical analysis

All the results in the study are presented as Means±SEM. Data analysis was performed by Graphpad prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Kolmogorov-Smirnov test was used for testing normality of the distribution. Then for the repeated

physiological measurements during EVLP (SPC, PAWP, $DppO_2$, PVR), data were analyzed by 2-way ANOVA, followed by Dunnett's test to determine the effect of time (data at 60 minutes during EVLP was taken as the control) and Sidak's test to evaluate the effect of sevoflurane treatment at selected time points. For the weight gain, comparison between CONT and SEVO groups was done using unpaired t-test was used. For the other comparisons (Protein Carbonyl, LDH, CINC-1, TNF, IL-6, protein in BAL and perivascular edema), one-way ANOVA followed by Tukey's correction was used. $p<0.05$ was considered statistically significant.

Results

Physiological variables during EVLP

SPC was greater in the SEVO group when compared to the CONT group throughout the experiment, the differences being statistically significant at 90 and 120 minutes (respectively 0.70±0.09ml·cmH_2O^{-1} vs 0.41±0.03 ml·cmH_2O^{-1}, and 0.70±0.08ml·cmH_2O^{-1}vs 0.44±0.04 ml·cmH_2O^{-1}) (Figure 6.3A). With respect to PAWP, we found that both experimental groups displayed a drop between 60 to 90 minutes of EVLP, followed by stabilization up to the end of the observation period. The values tended to be lower in the SEVO group but the differences did not reach statistical significance (Figure 6.3B). Both SPC and PAWP didn't show significant effect with respect to the effect of time in each group. No significant changes between time points or between groups were detected with respect to both PVR (Figure 6.3C) and $DppO_2$ (Figure 6.3D). The results for the physiologic parameters are summarized in Table 6.2.

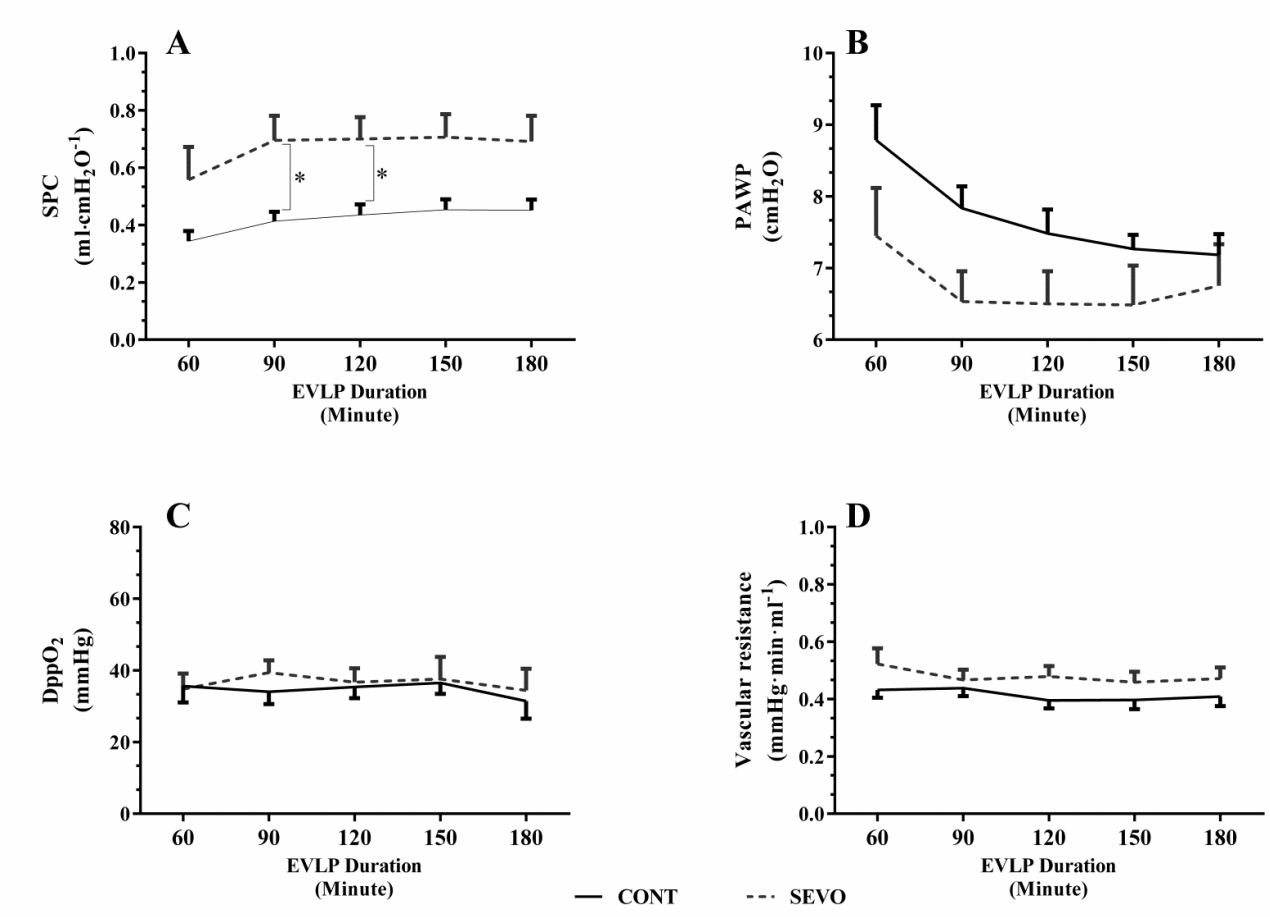


Figure 6.3 Pulmonary physiology during EVLP

A: Static pulmonary compliance (SPC) was stable in the CONT group throughout EVLP; lungs treated with sevoflurane showed enhanced compliance compared to the CONT group, B: Peak airway pressure (PAWP) in both experimental groups demonstrated a drop after the first measurement. In the CONT group PAWP

continuously declined while they remained stable in the SEVO group. Differential partial Oxygen pressure ($DppO_{2)}$ (C) and pulmonary vascular resistance (PVR) (D) were similar in both of experimental groups. All the physiological variables didn't demonstrate significant effect of time over groups.

Parameters	Groups	60min	90min	120min	150min	180min
SPC (ml·cmH2O^{-1})	CONT	0.34±0.04	0.41±0.03	0.44±0.04	0.45±0.04	0.45±0.04
	SEVO	0.56±0.11	0.70±0.09	0.70±0.08	0.71±0.08	0.69±0.09
PAWP (cmH_2O)	CONT	8.78±0.49	7.83±0.31	7.48±0.33	7.27±0.20	7.18±0.29
	SEVO	7.45±0.67	6.53±0.42	6.50±0.45	6.48±0.55	6.75±0.58
$DppO_2$ (mmHg)	CONT	35.6±4.57	34.00±3.44	35.33±3.17	36.50±3.05	31.40±4.93
	SEVO	34.80±4.33	39.99±3.47	36.67±3.93	37.60±6.20	34.40±6.05
PVR (mmHg·min·ml^{-1})	CONT	0.43±0.03	0.44±0.03	0.40±0.03	0.40±0.03	0.41±0.03
	SEVO	0.52±0.05	0.47±0.04	0.48±0.04	0.46±0.04	0.47±0.04

Table 6.2 Summary of the pulmonary physiologic parameters

SPC: Static pulmonary compliance; PAWP: Peak airway pressure; $DppO_2$: Differential partial pressure of oxygen; PVR: Pulmonary vascular resistance.

No statistical difference with respect to the effect of time for physiological parameters

Results was expressed as Mean±SEM

Biochemical variables

Biochemical data obtained in a group of normal lungs (BASELINE group) were used here as a baseline control, identifying the physiological values for the different variables. All the data are presented in detail in Table 6.3.

Lung protein carbonyl accumulation and LDH release in BAL

Protein carbonyls accumulation in lung tissue extracts was used as an index of oxidative modifications in proteins, hence of oxidative stress development during EVLP. As indicated in Figure 6.4A, protein carbonyls significantly increased following EVLP in the

CONT group in comparison to the BASELINE group (1.17±0.18 nmol·mg^{-1}vs 0.31±0.009 nmol·mg^{-1}; p=0.0004), pointing to the development of significant oxidative stress during EVLP. In contrast, protein carbonyls did not increase during EVLP in the SEVO group, and were therefore significantly reduced in comparison to the CONT group (0.55±0.05 nmol·mg^{-1}, p=0.0060 vs CONT, p= NS vs BASELINE).

LDH was measured in BAL as an index of cell death (Figure 6.4B). Following EVLP, a massive increase of LDH was noted in the CONT group in comparison to BASELINE (8.82±1.46 AU vs 0.46±0.17 AU, p=0.0002), implying significant cellular injury. The increase of LDH was significantly and markedly less pronounced in the SEVO group (3.80±1.23 AU, p=0.0153 vs CONT).

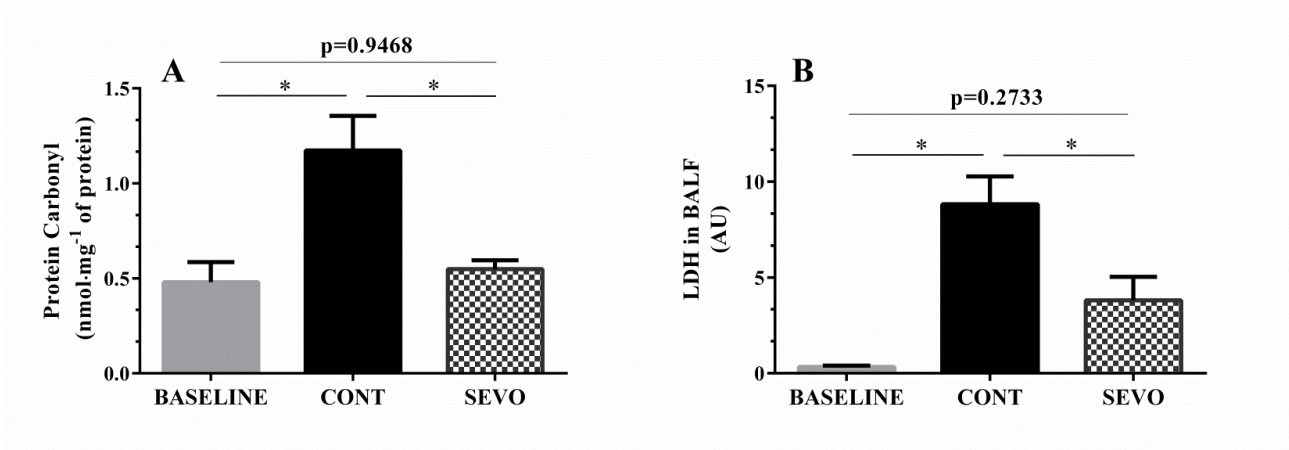


Figure 6.4. Evaluation of oxidative stress and tissue necrosis during EVLP

A: As an indicator of oxidative stress, protein carbonyl of lung tissue in CONT group was notably provoked in comparison with that in BASELINE group; but significantly reduced in SEVO group, B: LDH in BAL, a cell necrosis marker was markedly expressed in CONT group than that in BASELINE group, but less pronounced in the lungs treated with sevoflurane. *: p<0.05.

Lung tissue concentrations of inflammatory cytokines

We measured the tissue levels of TNF-α and IL-6 as prototypical biomarkers of acute innate inflammation in the pulmonary parenchyma, and we also evaluated the amount of CINC-1, a CXC chemokine analogous to human IL-8 playing an important role in attracting neutrophils at sites of inflammation, notably in the lung. As illustrated in Figure 6.5, tissue levels of TNF-α, IL-6 and CINC-1 all significantly increased (in comparison to BASELINE) following EVLP in the CONT group. In contrast, the increase was much less pronounced in the SEVO group, which disclosed significantly lower amounts of TNF-α and CINC-1 than the CONT group, and a trend towards reduced IL-6 levels (p= 0.11). Detailed results are presented in Table 6.3.

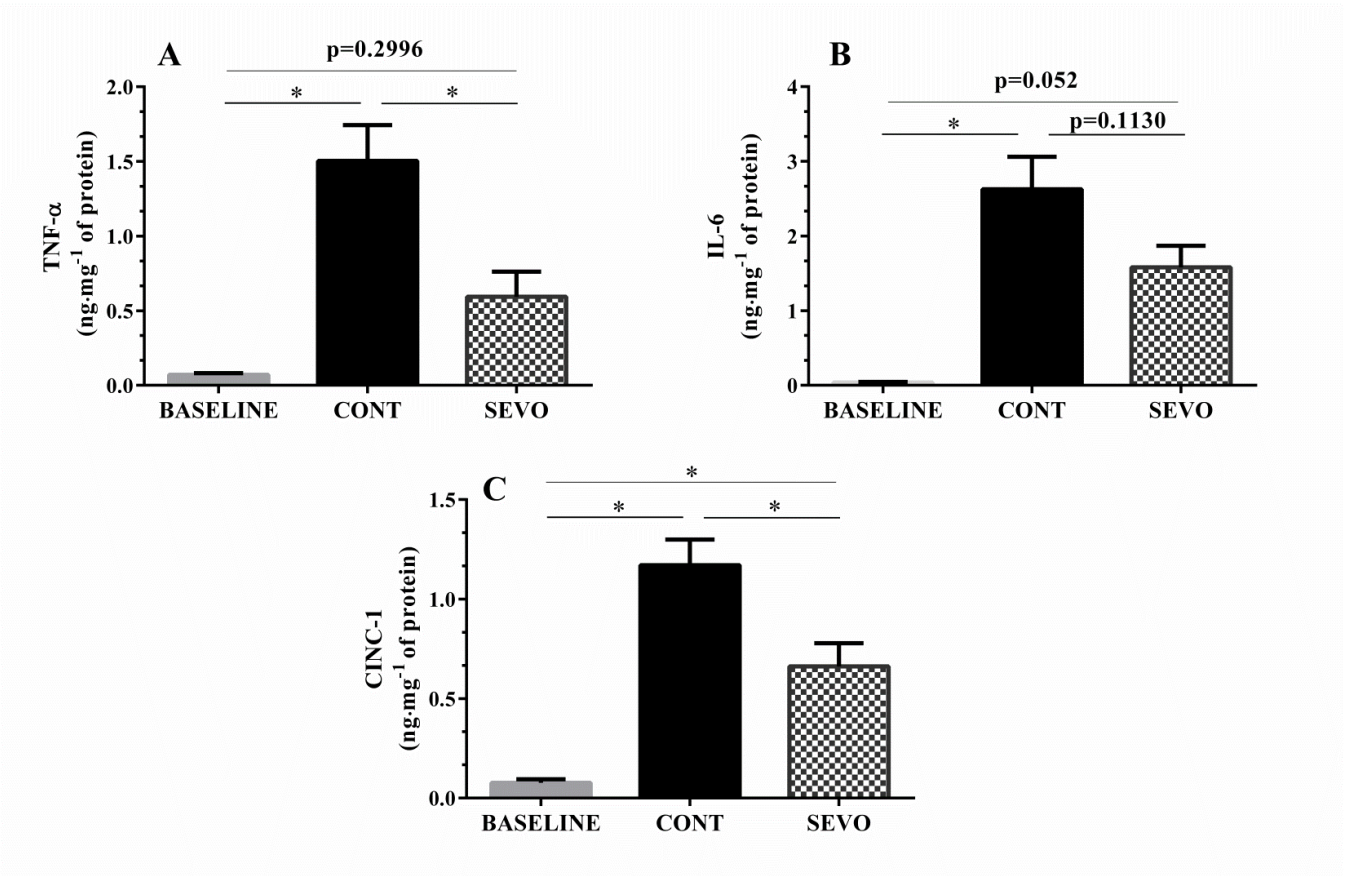


Figure 6.5 Evaluation of inflammatory mediators in lung graft tissue after EVLP

A: CINC-1 expression was significantly increased in the CONT group compared to the BASELINE group; but was suppressed in SEVO group, B: TNF- α expression in lungs of the CONT group was significantly increases compared to the BASELINE group; but attenuated in lungs treated by sevoflurane, C: IL-6 was up-regulated in CONT group than that in BASELINE group; but suppressed in SEVO group. *: $p<0.05$,

				P value		
	BASELINE	**CONT**	**SEVO**	Baseline vs CONT	Baseline vs SEVO	CONT vs SEVO
Protein Carbonyls (nmol·mg^{-1} of protein)	0.31±0.09	1.17±0.18	0.55±0.05	0.0193	0.9468	0.0106
LDH (AU)	0.46±0.08	8.82±1.46	3.80±1.23	0.0049	0.2733	0.0350
TNF (ng·mg^{-1} of protein)	0.07±0.01	1.50±0.24	0.59±0.17	0.0027	0.2996	0.0185
IL-6 (ng·mg^{-1} of protein)	0.04±0.01	2.63±0.44	1.58±0.29	0.0027	0.0520	0.1130
CINC-1 (ng·mg^{-1} of protein)	0.08±0.02	1.17±0.13	0.66±0.12	0.0003	0.0196	0.0277
Weight Gain (g)	NA	0.72±0.09	0.52±0.06	NA	NA	0.0443
Proteins in BALF (mg·ml^{-1})	0.76±0.15	4.41±0.90	3.61±0.30	0.0178	0.0713	0.6801

Table 6.3 Summary of the biomolecular test and lung weight gain

Results was expressed as Mean±SEM

Lung weight gain and protein levels in BAL fluid

Weight gain was determined as an index of lung edema following EVLP, and the amount of proteins recovered in the BAL was used to evaluate an increased permeability of the alveolo-capillary membrane permeability edema. As illustrated in Figure 6.6A, weight gain was significantly lower in the SEVO group compared to CONT group (0.52±0.06g vs 0.72±0.09g; p=0.044), implying less pulmonary edema formation in the SEVO group. With respect to proteins in BAL fluid, both CONT and SEVO groups displayed significantly greater values than the baseline group. The increased protein content in BAL fluid tended to be less pronounced in the SEVO vs CONT group (Figure 6.6B), although the difference was not significant (p=0.6801). Detailed results are summarized in Table 3.

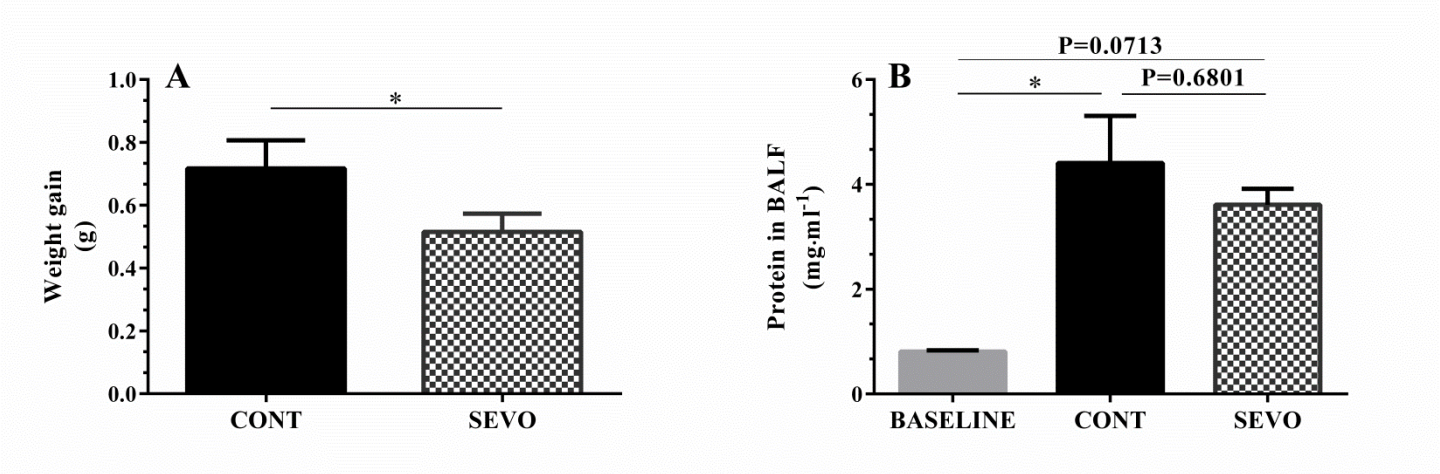


Figure 6.6 Evaluation of pulmonary edema during EVLP

A: Protein content in BAL, indexing the integrity of alveolar epithelium. Lung suffered from 1 hour warm ischemia in CONT group led to a substantial accumulation of proteins in BAL than that in BASELINE group, while mitigated in the SEVO group, as there was no significant increase of protein when compared to BASELINE group, B: Lung weight gain was significantly reduced with the presence of sevoflurane treatment compared to the control. *: $p<0.05$.

Post-EVLP histopathological findings

Perivascular edema was present in CONT and SEVO groups (Figure 6.7C-6.7F), but not in lungs of the BASELINE group in which normal pulmonary macrostructure was visible (Figure 6.7A-6.7B). Since the lungs were subjected to the BAL before histological fixation, we were unable to detect significant alveolar edema. Perivascular edema was determined by computing the ratio of edema thickness to the diameter of the vessels surrounded (Figure 6.7G). Following EVLP, the severity of perivascular edema was markedly increased in the CONT group in comparison with BASELINE group (0.58±0.04 vs 0.05±0.02; p=0.0001). Additionally, the edema was significantly lower when treated with sevoflurane (0.47±0.03, p=0.0355 vs CONT) compared to CONT group.

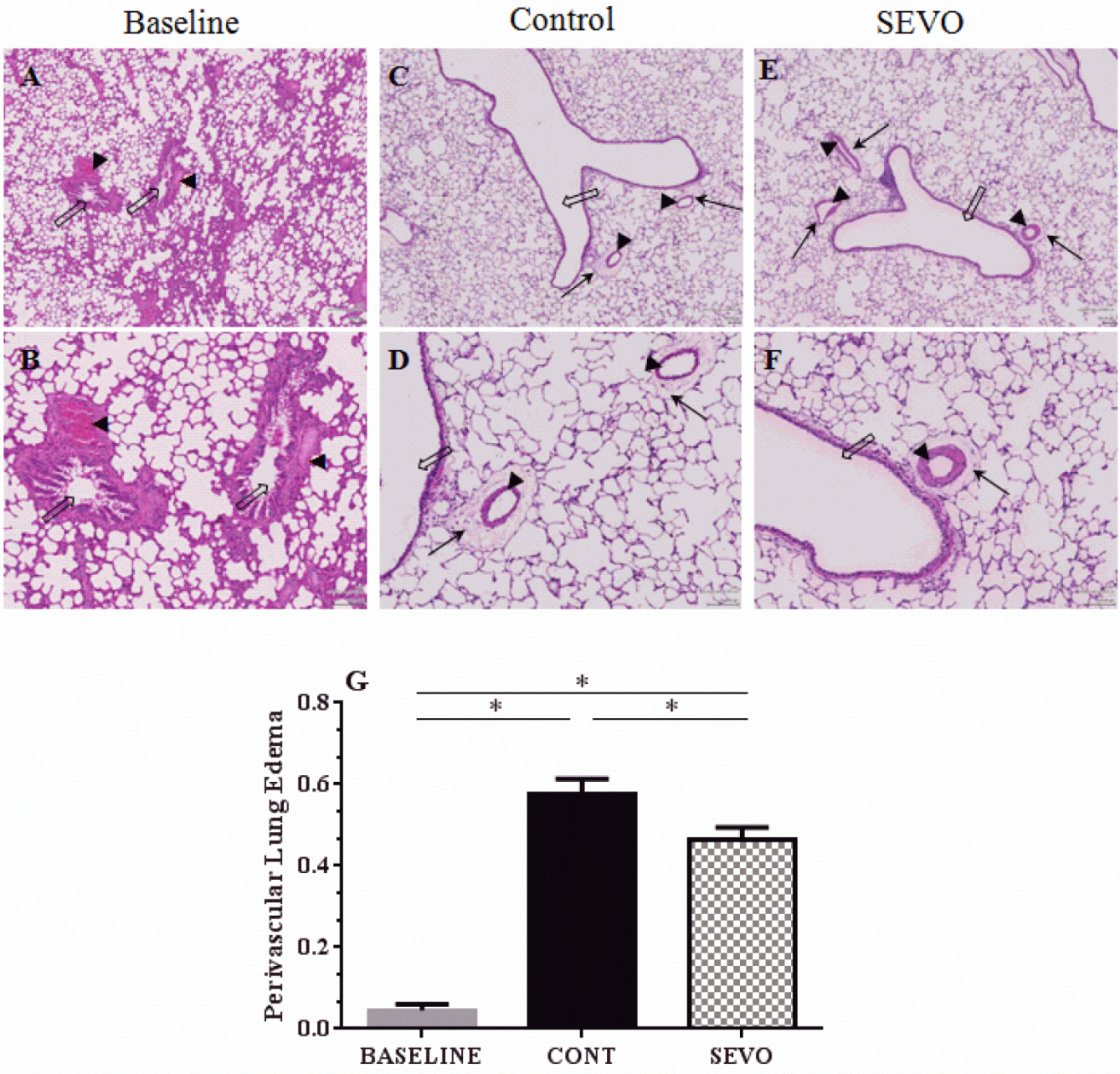


Figure 6.7 Histopathological changes

Representative histopathological alterations (HE staining) illustrated in a magnification of 4x (upper pictures) and 10x (lower pictures) in the Baseline group (A-B), the Control group (C-D), and SEVO group (E-F). Black arrows show perivascular edema around arteries or veins (arrowheads). Open arrows indicate bronchial structures. The graph (G) displays the quantification of perivascular lung edema in each group (ratio of perivascular edema thickness to inner diameter of the examined vessels).

*: $p<0.05$.

Discussion

In this animal study we evaluated the effect of sevoflurane, administered during EVLP, on IR injured in lungs harvested after 1 hour of warm ischemia.

EVLP, first developed as a tool for the evaluation and preservation of marginal donor lungs, has in recent years been studied as a modality to treat typical injuries of donor lungs such as brain death induced neurogenic edema, atelectasis, pulmonary embolism, (85, 91, 173) etc. It opens a platform for the administration of various agents such as anti-inflammatory agents (40), antioxidants (42), bronchodilators (44), and fibrinolytics (43) to minimize the damages caused by WI. Our findings strength this emerging concept, by showing the cytoprotective effect of sevoflurane post-conditioning during EVLP after a prolonged period of WI.

Sevoflurane had been reported to have protective effects against IR injury in solid organs such as the heart (174) liver (175) and brain (176), with a decrease in reactive oxygen species (ROS), an inhibition of apoptotic cascades and reduced neutrophil/platelet adhesion to the endothelial wall. Although more than a dozen experimental studies investigated the potential of sevoflurane in healthy and in IR injured lung tissue, the explicit pharmacological mechanism remains controversial. In an *ex-vivo* isolated rat model (169) and in an *in vivo* porcine model (170), preconditioning with sevoflurane attenuated TNF-α and NO metabolism. However, when evaluating pulmonary inflammation of healthy lung tissue after sevoflurane compared to thiopentone anesthesia, Takala et al. found contradictory results in a porcine model. Although sevoflurane was associated with a decreased gene expression of TNF-α and interleukin-1β (177), inflammatory mediator as leukotriene C4, NO_2 and NO_3 were highly increased in BAL samples (178).

In our DCD rodent model, sevoflurane was administered intravascularly early during EVLP to target the cellular signaling cascades involved in IR and limit the sequelae. A major consequence of IR injury is the development of pulmonary edema. EVLP lungs in SEVO group displayed a reduction in lung weight gain and perivascular edema, improved lung compliance and PAWP when compared to the CONT group. Giving the consideration that an increased endothelial permeability is one of the principal mechanisms in the development of pulmonary edema (179), these findings imply that the functional integrity of the pulmonary endothelium was better preserved. The stabilization of the endothelial glycocalyx by sevoflurane could contribute to this beneficial outcome as described in an IR model of isolated guinea pig hearts (180). In addition, the substantial mitigation of perivascular edema disclosed by histopathological observation further confirmed the cytoprotective effect of sevoflurane. However, this result was not supported by the protein content in the BAL, which lower but not statistically significant when comparing SEVO group to CONT. The small number of animals per group may explain this finding.

With regard to the oxygenation capacity, no clear difference in $DppO_2$ was identified between 2 experimental groups regardless the slightly higher mean values in the SEVO

group. Nevertheless, the use of $DppO_2$ as a parameter for the evaluation of lung function during acellular EVLP is controversial. Yeung JC et al. demonstrated a reduced effect of shunt on EVLP PO_2 when compared to addition of red blood cells to the perfusate. This result is attributable to the linearization of the relationship between oxygen content and PO_2, which occurs with acellular perfusate in opposition to the blood (146). Therefore, the interpretation of PO_2 values as an indicator of lung damage can be largely misleading when using an acellular perfusate.

Thirty minutes after sevoflurane administration, PVR was calculated by plotting PA to LA pressure gradients against circuit flow. PVR was not statistically different in both experimental groups throughout EVLP.

Despite few physiological arguments in favor to sevoflurane group during EVLP, we measured an important reduction of oxidative stress in warm ischemic lungs, as indicated by the lack of increase in protein carbonyl. The mechanisms involved in this protection are probably the same as described for the heart including the inhibition of: extracellular signal-regulated kinase ½, 70-kDA ribosomal protein s6 kinase and endothelial nitric oxide synthase (181), mitochondrial ATP-dependent potassium channel (182), GSK-3b (183), and mitochondrial permeability transition pore (mPTP) (184).

As a result of the reduction in oxidative stress, the inflammatory cascade activation after warm ischemia resulted in a decreased expression of inflammatory markers TNF -α, CINC-1 and IL-6 in SEVO group when compared to CONT group. Others mechanism like intrinsic anti-inflammatory properties of sevoflurane may contribute to this beneficial effect. Produced mainly by macrophages, TNF-α is involved in systemic inflammation, to stimulate the acute phase reaction (185) through the nuclear factor kappa B (NF-κb) pathway (186). Lungs treated with sevoflurane in our EVLP model showed decreased expression of TNF-α, suggesting that the protective effect may be mediated by reducing TNF-α release, which is consistent with the literature aforementioned (169). CINC-1 is the counterpart of the human growth-regulated gene product, a member of the interleukin-8 family (187), which plays a critical role in inflammation with neutrophil infiltration. The significant reduction of CINC-1 in lung tissue treated with sevoflurane in our setting indicates an attenuation of neutrophil recruitment in the inflammatory process. Taken together, the attenuated TNF-α expression and the reduction of CINC-1 confirms the finding, by Takaishi et al., that TNF-α blockade can reduce neutrophil chemotaxis and attenuate the lung injury process (188). Last but not least, acting as both a pro-inflammatory cytokine and an anti-inflammatory myokine, IL-6 is an important mediator stimulating the auto-immune process elicited by IR (139). Although no significantly, SEVO group has shown the lack of striking increase expression of IL-6 as in CONT group, which was in agreement of inflammatory response with TNF- α and CINC-1 expression.

To evaluate the extend of IR injury in our model, we measured LDH as a non-specific marker of cell necrosis in BAL. Sevoflurane administered in the early phase of

reperfusion result in a significant reduction of LDH release when compared to CONT group. This cytoprotective effect is in line with intrinsic necrosis pathway that involves the opening of the mPTP, the swelling of the internal mitochondrial membrane and the releasing of mitochondrial proteases into the cytoplasm. The following rupture of cellular membrane will release cytoplasmic proteins, including LDH.

There are several limitations in this study. The primary shortcoming may stand in the compromised translational probability to the clinical application, due conspicuously to the lack of in vivo evidence of lung transplantation following EVLP reconditioning. Besides, we measured static compliance by delivering positive pressure, which may induce volotrauma, particularly when compliance is good. This mechanism can explain the decline of the SPC at the end of EVLP in SEVO group.

In conclusion, our study indicates that 2% sevoflurane, administered in the perfusate at the beginning of the EVLP with an anesthetic vaporizer connected directly in the circuit, is associated with reduced oxidative stress, attenuated inflammatory response and cell necrosis as well as improved pulmonary physiological parameters. Sevoflurane may have protective properties against IR induced lung injury following warm ischemia in DCD lung grafts.

Acknowledgments

This study was performed in collaboration with Carlo Marcucci, Cyril Francioli, Roumen Parapanov, Christian Kern, Jean Perentes, Lise Piquilloud, Hans-Beat Ris, Lucas Liaudet , Thorsten Krueger, Fabrizio Gronchi.

Chapter 7

Experimental ex-vivo lung perfusion of donor lungs inoculated with streptococcus pneumonia

This chapter of the thesis was performed in collaboration with Etienne Abdelnour. Both investigators contributed equally to this work.

Abstract

Background: Normothermic EVLP has the potential to treat frequent donor lung injuries, such as pneumonia. Here we assess the ex-vivo antibiotic treatment on infected lung grafts inoculated with Streptococcus pneumoniae.

Methods: Three groups of 6 male Sprague-Dawley rats were used. Donor lungs were native or intratracheally inoculated four hours before harvesting with *Streptococcus pneumoniae* with a bacterial load of 1.0E+09 germs/500µl PBS. Subsequent to 4 hours of mechanical ventilation, pulmonary artery and left atrium were cannulated, heart-lung blocks were flushed and preserved at 4°C over 90 minutes in an inflated state (FiO_2 0.5). Normothermic EVLP was performed over 4 hours with Steen solution® only or supplemented with antibiotics (Meropenem: 2mg/ml perfusate; Vancomycin: 1mg/ml perfusate). Functional graft status during EVLP was assessed by measurements of differential oxygen partial pressures in Steen solution ($DppO_2$), pulmonary vascular resistance, lung compliance and weight gain. Bacterial load was assessed by repeated measurement in Steen solution, bronchoalveolar lavage and in lung parenchyma.

Results: Analysis of the perfusate sampled from EVLP with infected lungs displayed growth of *Streptococcus pneumoniae*: bacterial load resulted in growth from the 2nd hour until the end of EVLP in all animals. In lungs treated during EVLP with antibiotics no bacterial growth was detected. Analysis of BAL and lung parenchyma showed presence of Streptococcus pneumonia in the bacterial load group and was markedly reduced after antibiotic treatment. No bacterial growth was found in control animals. Bacterial load resulted in marked edema and significant deterioration of compliance, vascular resistance and $DppO_2$ during EVLP. Control lungs showed stable $DppO_2$, vascular resistance and lung compliance during 4 hrs of EVLP, without significant changes over time. Antibiotic treatment during EVLP of infected lungs did not affect ex-vivo lung function.

Conclusion: This experimental model of EVLP allows for ex vivo assessment and preservation of native and infected rat lungs during 4 hours. Ex-vivo antibiotic treatment reduces bacterial load in infected lung grafts, but does not result in a significant improvement of lung physiological function.

Introduction

Lung transplantation is an effective therapy in selected patients diagnosed with end-stage lung disease. The shortage of donor lungs suitable for lungs transplantation remains a critical issue. It is in part attributed to the impaired quality of donor lungs.

It is well known that donor lungs, as compared to other solid organs (heart, liver, kidney) are contaminated by microorganisms and present a high risk of established infection at the moment of organ procurement, above all due to the prolonged endotracheal intubation and mechanical ventilation (18). Clinical studies have shown that more than half of donor lungs are present bacterial infection, leading to an increased risk of donor-host transmitted lung infection and poor graft function after transplantation (189, 190). Thus, the treatment of donor lungs with broad-spectrum antibiotics against bacterial infection prior to transplant may represent an advantage for the recipient.

EVLP represents a novel opportunity modality to assess lung function and recondition damaged donor organs before transplantation. In this experimental study we sought to investigate the effect of ex-vivo antibiotic therapy on bacterial contamination in rat lungs. Bacterial load in BAL, lung parenchyma and perfusate as well as the ex-vivo lung function after treatment of *Streptococcus Pneumoniae* contaminated lungs were assessed.

Materials and Methods

All the animal experiments were performed in accordance with the Animal Welfare Act and the National Institutes of Health "Guidelines for the Care and Use of Laboratory Animals" and were approved by the cantonal authorities (Service Vétérinaire Cantonal de l'Etat de Vaud, Authorization Nr. 2637). Eighteen male adult Sprague-Dawley rats (10-14 weeks, mean weight 410 gr, Charles River, L'Arbresle, France) were used to harvest heart lung blocks. Following experimental groups were used:

a) Control group (CTRL, n=6): donor lungs were not inoculated with bacteria, no antibiotic treatment was performed

b) Infected group (SP, n=6): donor lungs were inoculated with Streptococcus Pneumoniae (10^9 *Streptococci Pneumoniae*) but left untreated

c) Infected and treated group (SP+ATB, n=6): lungs were inoculated with Streptococcus Pneumoniae (10^9 *Streptococci Pneumoniae*) and treated with antibiotics during EVLP (Meropenem: 2mg/ml perfusate; Vancomycin: 1mg/ml perfusate).

Harvest of heart-lung blocks: The animal was anaesthetized (induction with Isoflurane 5%, Pentobarbital injection i.p. (50mg/kg)), placed on a heating plate to maintain temperature at 37.5°C and tracheotomised. Either 0.5ml of PBS (control group) or 0.5ml of PBS containing 10^9 *Streptococci Pneumoniae* were injected into the trachea, half of the volume in left and half of the volume in right lateral decubitus position, followed by intratracheal injection of 1ml of air. Then a tracheal cannula was inserted and a mechanical ventilation was started (FiO_2 0.50, Isoflurane of 0.8%, Tidal Volume (V_T) of 7ml/kg and respiratory rate (RR) of 75/min), using a rodent respirator (model 683, Harvard Apparatus, Holliston, MA). Following 4 hours of ventilation, the animal underwent sternotomy, heparinization (600I.U.) and exsanguination. Perfusion cannulas (Hugo Sachs, Hugstetten, Germany) were inserted into the pulmonary artery (PA) (ID = 1.7mm, OD = 2.0mm) and left atrium (LA) (ID = 3.4mm, OD = 4.0mm). Lungs were flushed through the PA cannula with 15ml of 4°C Perfadex® (Xvivo Perfusion, Göteborg, Sweden) until outflow perfusate became clear. During the Perfadex flush, ventilation was maintained with FiO_2 of 0.50, RR of 20/min and V_T of was 5ml/kg. The lungs were semi-inflated with a 0.5 FiO_2 and preserved for 90 at 4°C (Figure 1).

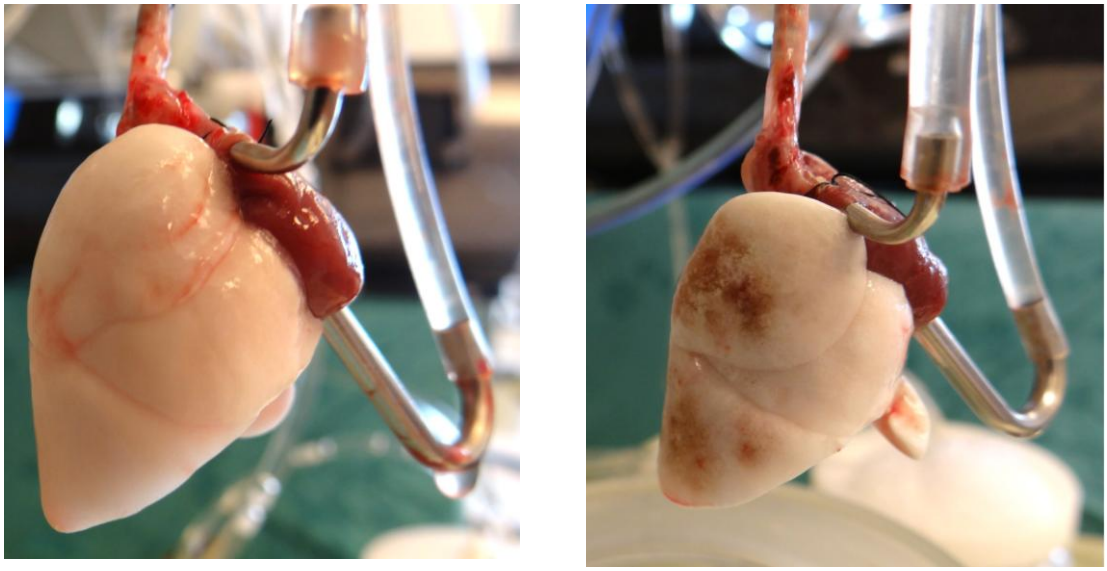


Figure 5.1 Heart-lung blocks harvested following PBS/bacterial inoculation

Left side: Control lung, inoculated with 0.5ml PBS;

Right side: Infected lung, inoculated with 10^9 *Streptococci Pneumoniae*

Rodent EVLP

Normothermic EVLP was preceded by cold ischemic preservation. The heart-lung block was weighted, mounted in the customized rodent EVLP system (Harvard IL-2 System, Hugo Sachs). The circuit was perfused either with Steen® solution (Xvivo Perfusion, Goteborg, Sweden) only (CTRL group and SP group), or supplemented with 2mg/ml of Meropenem and 1mg/ml of Vancomycin (SP+ATB group) A mixture of 6% O_2, 10% CO_2 and 84% N_2 was applied through a gas-exchange membrane (Hemofilter D150; MEDICA S.P.A, Italy) and pH was maintained at 7.4. Left atrial pressure was set at 4cm H_2O. Perfusion was started in a flow-controlled mode, at 2 $ml \cdot min^{-1}$ and at a temperature of 10°C, progressively increased to 7 $ml \cdot min^{-1}$ and 37.5°C within 30 minutes (using a 3M TCMII heater, Saint Paul, MN, USA), after which ventilation was started (V_T of 6 ml/kg, FiO_2 of 0.21 and RR of 7/min) using a Flexivent FX3 ventilator (SCIREQ Inc., Montréal, Canada). The perfusion was then switched to a pressure-controlled mode, with a preset constant PA pressure of 15cm H_2O for 150 minutes.

Assessment of bacterial load

Samples of Steen solution (10 ml) were taken at the onset, the 2nd hour and the end of EVLP. At the end of EVLP, the heart-lung block was retrieved and weighted to determine the weight gain during EVLP. A bronchoalveolar lavage (BAL) was performed at the end of the procedure with 1 ml of sterile PBS, pH 7.4, via the tracheal cannula. The left lung was harvested for assessment of bacterial load. All samples (Steen solution, BAL and fresh left lung tissue) were cultured for quantification of *Streptococci Pneumoniae*. Study design is illustrated as Figure 5.2.

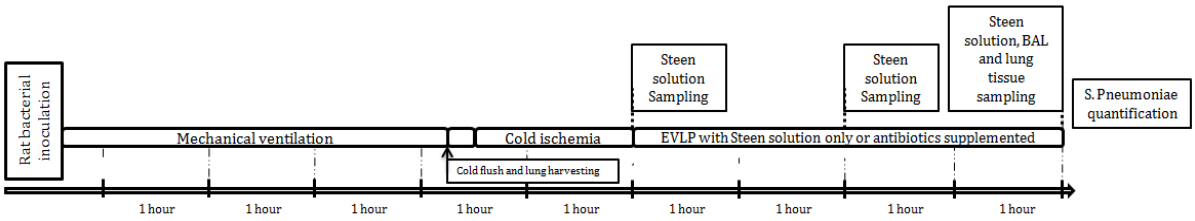


Figure 5.2 Study design

Functional evaluation during EVLP

During 4 hours of EVLP, partial pressure of oxygen was measured in the influent and effluent arms of the EVLP circuit, using two O_2 electrodes (Hugo Sachs Elektronik, Hugstetten, Germany), and the differential partial pressure of O_2 ($DppO_2$) was calculated. Pulmonary vascular resistance (PVR) was calculated as: PVR= (mean PAP-LAP) /Flow. At selected time-points, static pulmonary compliance (SPC) was measured using the Flexivent ventilator.

Data processing and statistical analysis

Results of physiological measurements during EVLP are expressed as means ± SEM. For SPC, PVR and DppO2, the effects of time and treatments were analyzed by 2-way ANOVA, followed by Dunnett's test for the effect of time (taking 30 minutes as a control), and Tukey's adjustments for the effects of treatments. For lung weight gain, one-way ANOVA followed by Tukey's correction was used. Bacterial quantification of Steen solution, BAL and lung tissue is expressed as absolute concentration of bacterial load in a given sample, categorized according to the related clinical significance and analyzed by fisher's exact test. $p<0.05$ was considered significant. Data analysis was performed using Graphpad prism 6 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Lung function assessment during EVLP and edema development

Ex-vivo antibiotic treatment did not affect the pulmonary mechanics of infected lungs as determined by repeated compliance measurements during EVLP. All infected lungs were associated with poor SPC as compared to the non-infected ones. In the control group, although stable initially, SPC tended to deteriorate after the first hour of EVLP. (Figure 5.3, A)

Graft $DppO_2$ (Figure 5.3.B) during EVLP was calculated to assess oxygenation capacity. Control lungs showed a stable DppO2 while infected lungs had a slightly worse DppO2, regardless the presence of ex-vivo antibiotic treatment. Differences were not statistically significant.

PVR (Figure 5.3.C) increased over 4 hours of EVLP in lungs inoculated with *Streptococcus Pneumoniae* and remained stable in lungs inoculated with PBS only.

Lungs loaded with *Streptococcus Pneumoniae* treated with or without antibiotics had a higher weight gain during EVLP (Figure 5.3.D) than the control lungs, although this was not statistically significant.

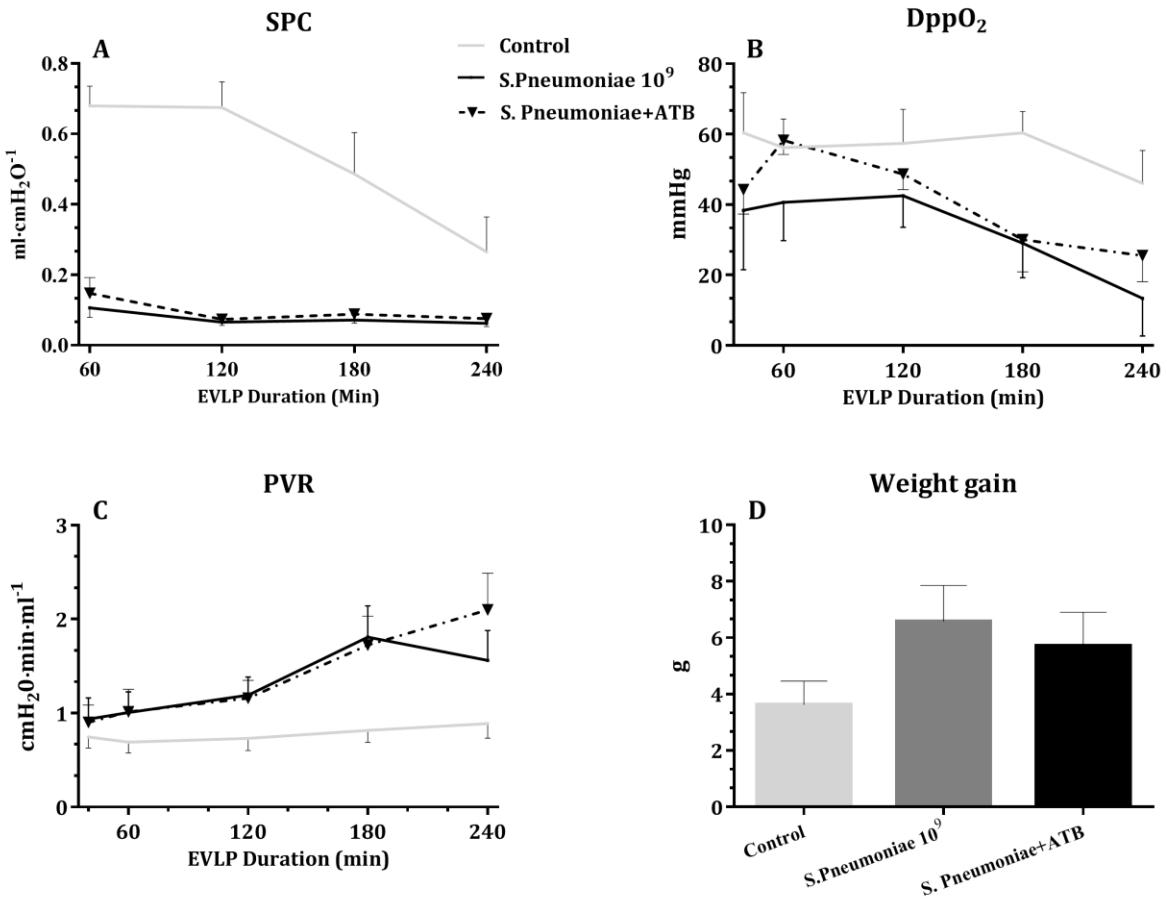
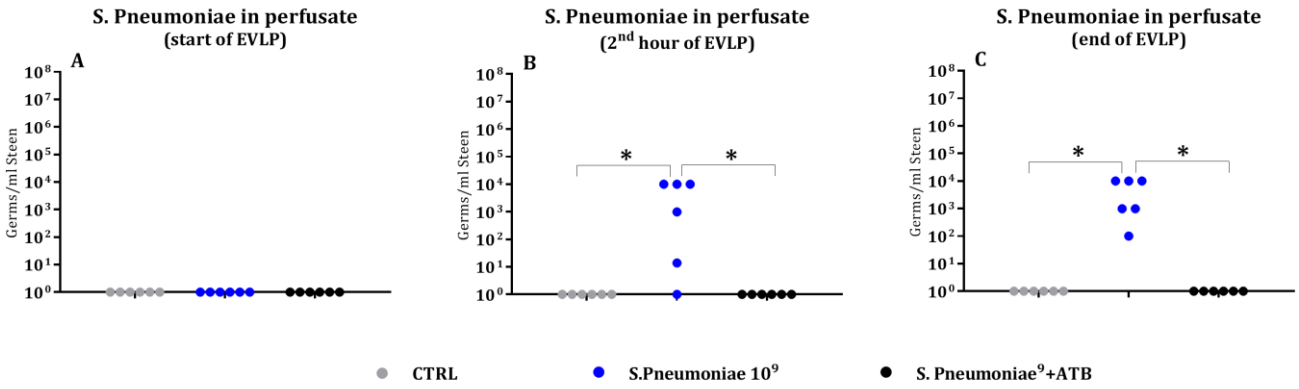


Figure 5.3 Lung function and weight gain during EVLP

A: Static pulmonary compliance; B: Differential partial pressure of O_2; C: Pulmonary vascular resistance; D: Weight gain

Analysis of bacterial load

The analysis of bacterial load in the perfusate at various time points and for various conditions is given in Figure 5.4. When control lungs were perfused, Steen solution was free of bacterial contamination throughout 4 hours of EVLP. When contaminated lungs



were perfused, the antibiotic treatment resulted in an undetectable level of bacteria in the perfusate, while perfusion of contaminated lungs without antibiotic treatment resulted in a high load of *Streptococcus Pneumoniae* in Steen solution. After 2 hours of EVLP 5 of 6 perfusion circuits were contaminated and after 4 hours all perfusion circuits were contaminated, when contaminated lungs were perfused without antibiotics.

Figure 5.4 Analyses of bacterial load in Steen solution during EVLP

*: $p<0.05$

The bacterial load in BAL after EVLP was highest in infected lungs kept untreated and increased in infected and treated lungs. *Streptococcus Pneumoniae* was undetectable in the BAL of three lungs initially contaminated and treated during 4 hours ex-vivo by antibiotics. *Streptococcus Pneumoniae* was not detectable in all control lungs.

No *Streptococcus Pneumoniae* was detected in tissue of control lungs, while both, treated and untreated contaminated lungs presented *Streptococcus Pneumoniae* tissue contamination, however, the number of lungs with a high bacterial loads was reduced in the antibiotic group, with one lung having no *Streptococcus Pneumoniae* contamination (Fig 5.5)

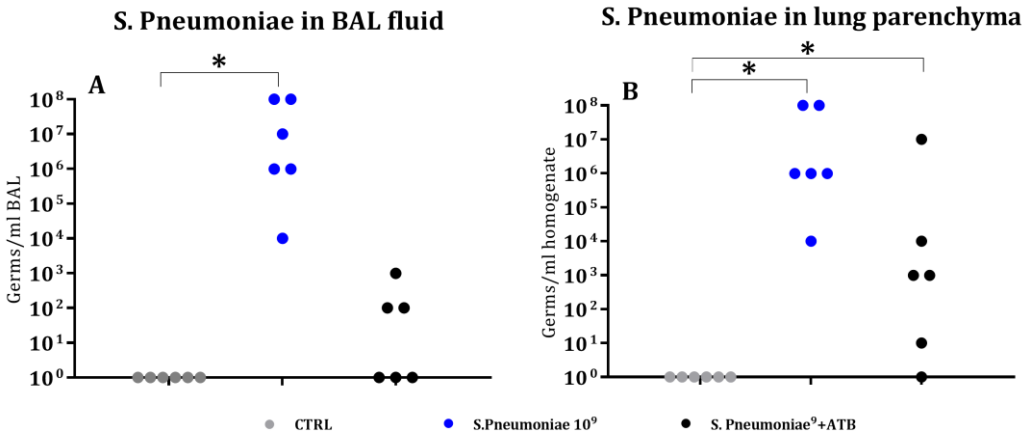


Figure 5.5 Analyses of bacterial load in BAL and lung parenchyma at the end of EVLP

*: $p<0.05$

Discussion

In this study we demonstrated the first time in an experimental setting that antibiotic therapy administered during EVLP reduces the bacterial burden of lungs contaminated with *Streptococcus Pneumoniae.* We also show that these bacteria translocate from the lungs' bronchial system to the perfusate of the EVLP system. This translocation leads to contamination of the perfusate and the perfusion circuit, with an increasing bacterial load throughout EVLP if no antibiotic treatment is added to the perfusate. The Steen solution and the normothermic perfusion mode present ideal conditions for bacterial growth. Adding antibiotics into the Steen solution avoids bacterial growth in the perfusate and results in a decrease of the grafts' bacterial load after 4 hours of EVLP (BAL and tissue). Contamination of lungs by *Streptococcus Pneumoniae* leads to impaired lung function in the ex-vivo setting, but the antibiotic treatment showed no beneficial effect on lung function.

The use of antibiotics administered during EVLP for infected donor lungs has been described in only two human studies, and was not addressed in experimental animal studies so far.

Andreasson et al. described the effect of EVLP with a perfusate containing high dose broad spectrum antibiotics on 18 human donor lungs considered unusable for transplantation. 13 of these lungs had positive cultures, with bacterial loads significantly decreasing after EVLP. Six out of 18 lungs were transplanted with favorable outcome. The authors conclude, that EVLP allows to effectively reducing the bacterial burden of donor lungs (191). No information is given about the evolution of lung function during EVLP; however some lungs were transplanted successfully. Our experimental study shows similar results in terms of reduction of the bacterial load; however, all contaminated lungs in our study displayed poor initial function in the EVLP system and a worsening lung function throughout EVLP and could most probably not have been transplanted with success.

The Toronto group has published an experimental human study with donor lungs rejected for transplantation because of concerns about infection. Half of the lungs underwent 12 hours of EVLP antibiotic therapy, half had EVLP without antibiotics (45). The authors observed a decrease of bacterial counts in BAL in most lungs undergoing the antibiotic treatment. Long term EVLP over 12 hours also resulted in improved compliance, pulmonary vascular resistance and lungs' oxygenation capacity. Endotoxin levels in the perfusate were found to be lowered by antibiotic treatment. Lungs were not transplanted.

Our results from the animal model are similar in terms of reduction of the bacterial charge, but are different in terms of the effect on lung function. This may be explained by the different bacterial charge as well as the different pathogenicity of the bacteria in the

clinical and animal setting. Besides, EVLP was performed during only 4 hours in our setting while in the above cited paper human lungs were perfused during 12 hours.

In conclusion this experimental model shows a beneficial effect of antibiotic treatment on bacterial load of lungs during EVLP. EVLP appears to be an ideal platform for the administration of antibiotics to infected lungs. The model may be used for the further development of this treatment strategy. An interesting topic in this context is the development of rapid real time detection systems to precisely assess the microbiological burden of potential donor lungs before transplant.

Acknowledgments

This study was performed in collaboration with Etienne Abdelnour, Cyril Francioli, Roumen Parapanov, Fabrizio Gronchi, Gay Prod'Hom, Christian Durussel, Jean Perentes, Michel Gonzalez, Hans-Beat Ris, Gilbert Groub, Lucas Liaudet , Thorsten Krueger.

Chapter 8
Development of an acute unilateral rodent lung transplant model

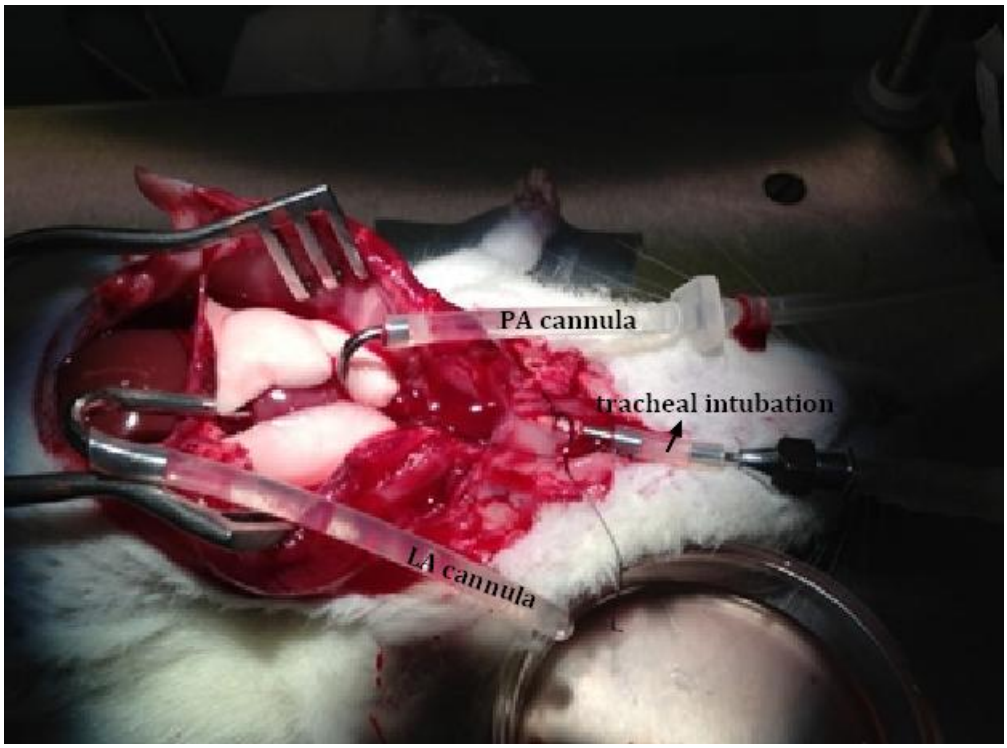
In this step, the candidate has established an acute unilateral rat lung transplant model based on the technique described by de Perrot et al (103). The principle idea of this acute model is to perform single lung transplantation followed by independent ventilation of the left-sided transplanted graft and the right-sided native lung with two different ventilators. This allows assessing accurately the graft function.

The model is an extension of the EVLP model described above and serves to correlate results obtained in the ex-vivo circuit to the in-vivo organ function. While the EVLP circuit affords graft reoxygenation and reperfusion with an acellular perfusate under well defined conditions, the transplant model permits to assess the effect of reoxygenation and blood reperfusion in-vivo.

The combination of both models will be an excellent tool to assess ischemia-reperfusion injury and potential therapeutic strategies in this field.

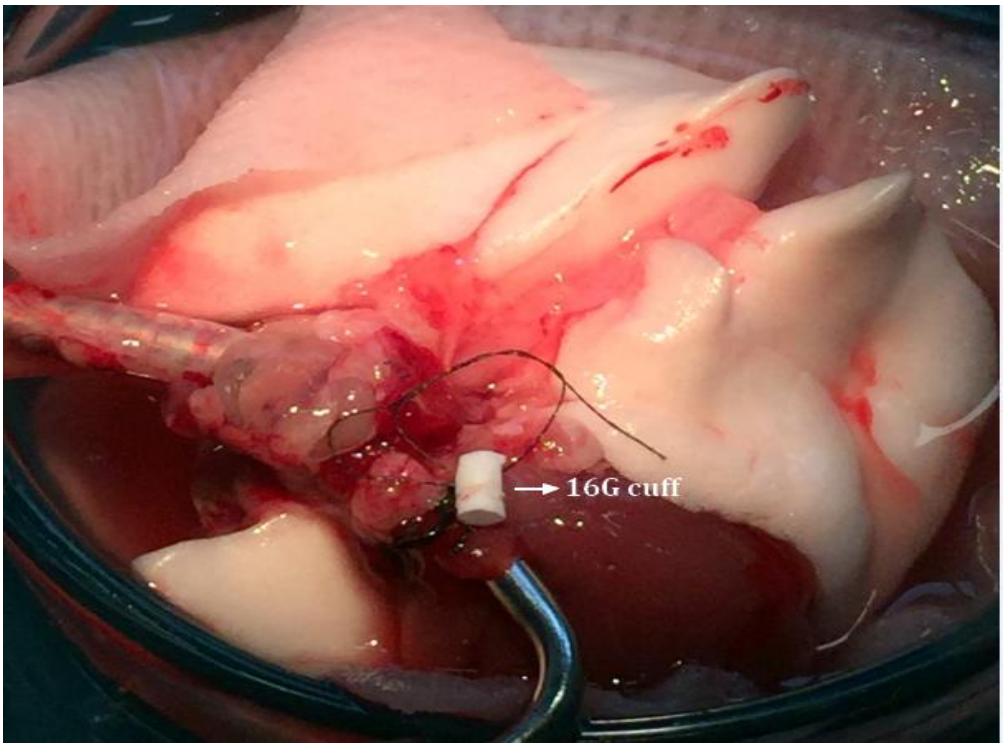
The following chapter describes key steps of the model as established today:

1)



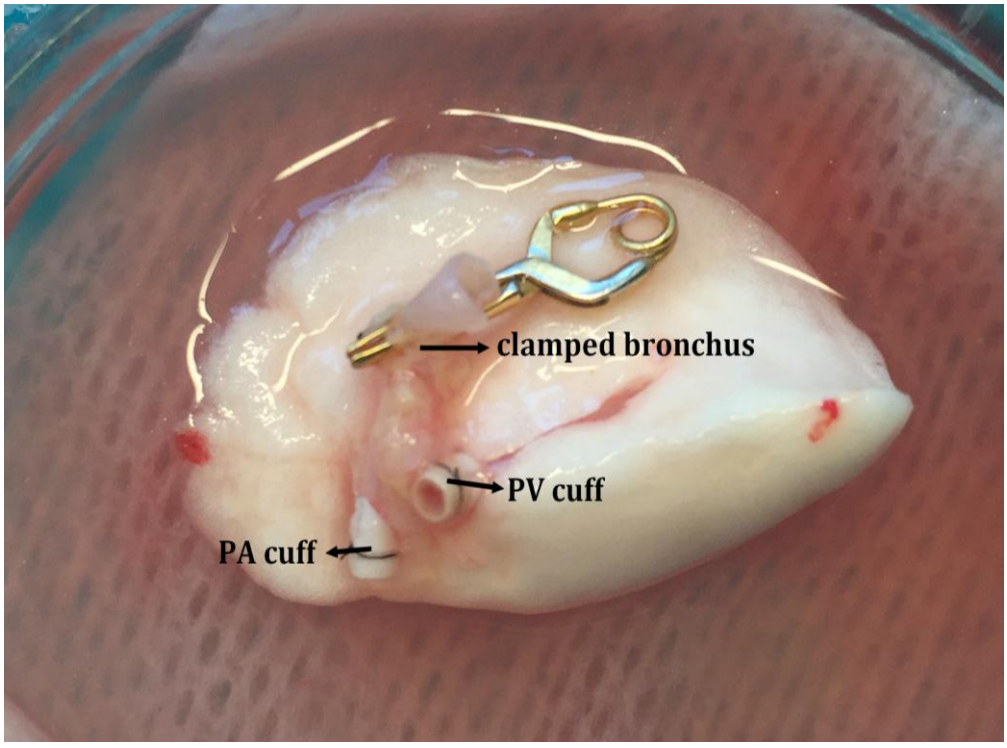
Following cardiac arrest, pulmonary artery (PA), left atrium (with introduction of the cannula in the left ventricle) and trachea are cannulated. The heart- lung block is flushed at 20cmH_2O with 15ml of 4°C Perfadex (Xvivoperfusion, Sweden), during which the lung is mechanically ventilated (tidal volume 7ml/kg, frequency 20/min, PEEP 5cmH_2O, FiO_2 0.21)

2)



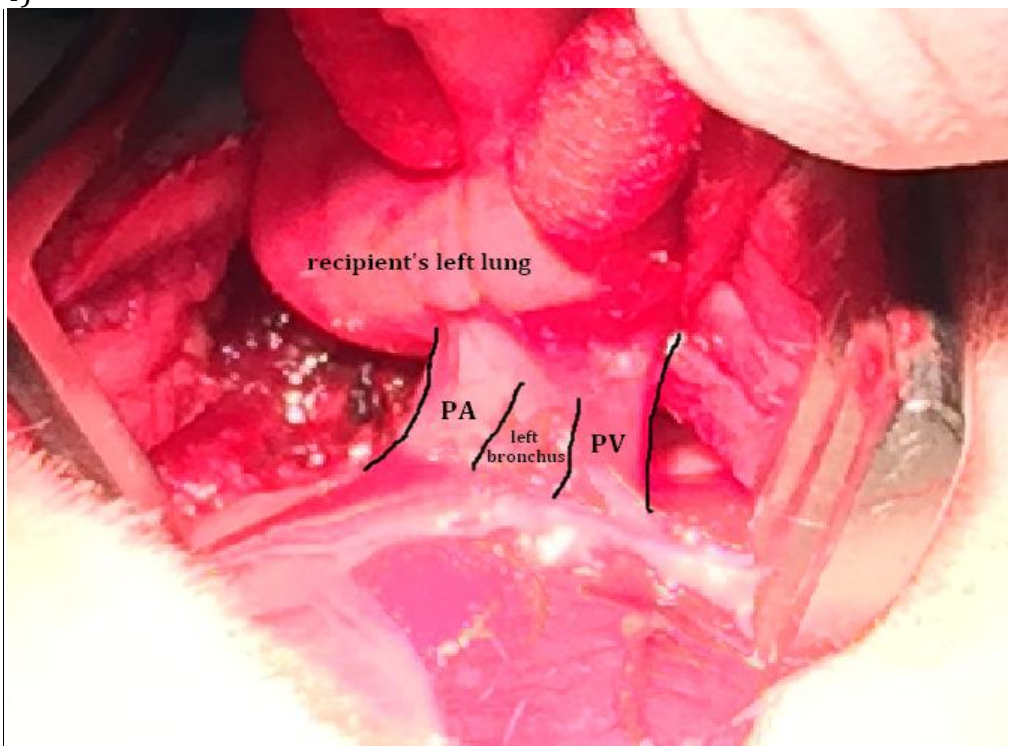
Following cold flush the heart-lung block is harvested and stored in 4°C Perfadex in an inflated status.

3)



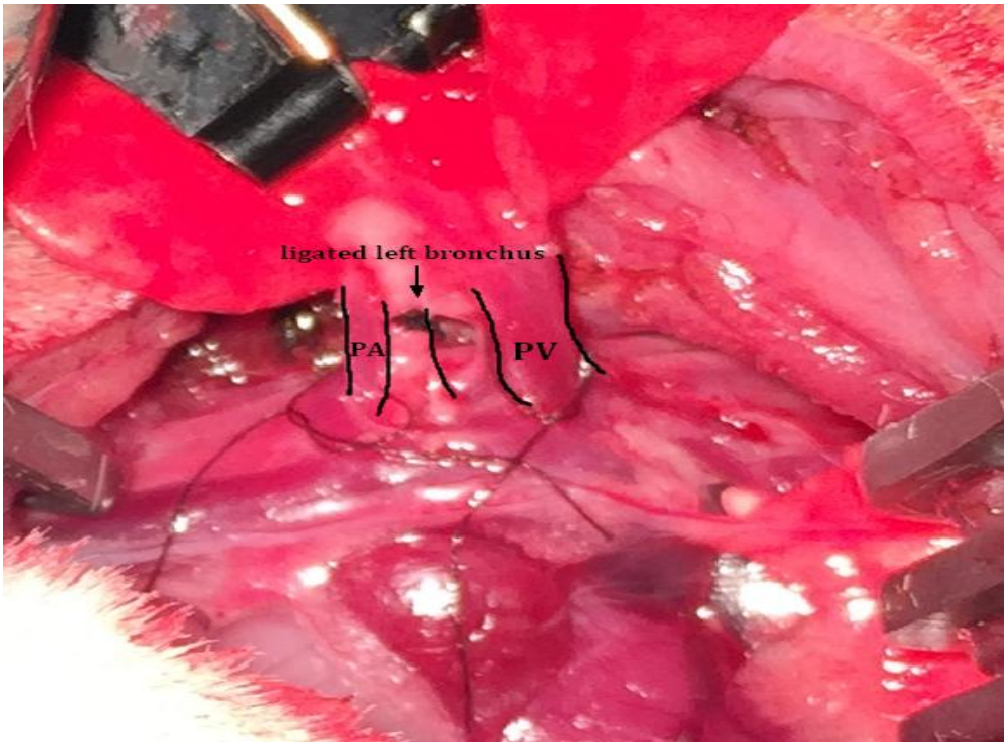
The left lung is isolated and the pulmonary hilus is carefully dissected. 16-Gauge cuffs are placed into the PA and PV. The left main bronchus is clamped, keeping the graft inflated.

4)



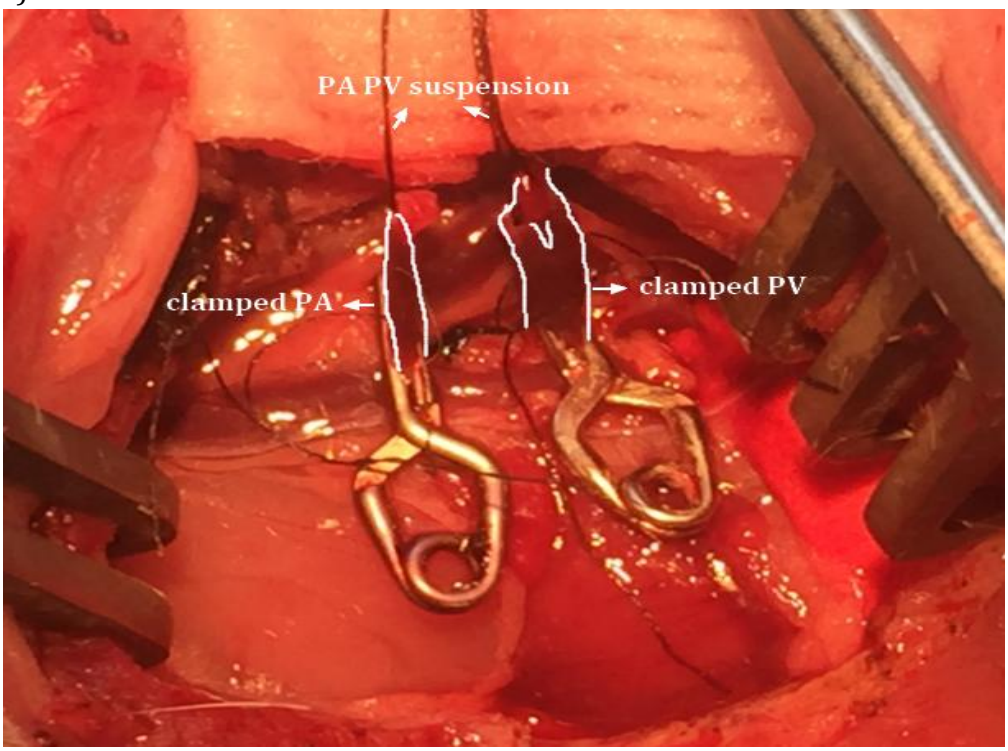
The recipient rat is anesthetized and mechanically ventilated (tidal volume 7ml/kg, frequency 90/min, PEEP 5cmH_20, FiO_2 0.50). The left hilus is exposed following a thoracotomy.

5)



Anterior view: The left PA, PV and the main bronchus of the recipient are carefully dissected. Then the left main bronchus is ligated following lung deflation. The recipients' right lung is then ventilated with a reduced tidal volume (5ml/kg).

6)



The recipient PA is proximally clamped by a microclip, followed by the PV clamping. The left native lung is resected. The recipient PA and PV are suspended distally by 7-0 silk suture to facilitate the following anastomosis.

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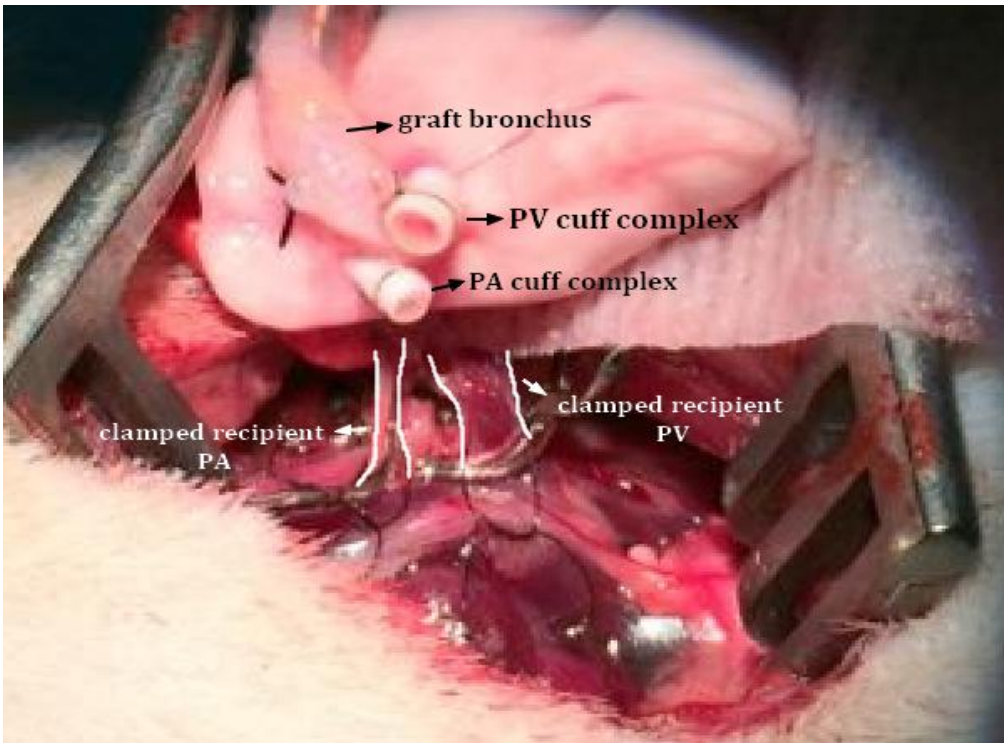

The graft is placed into the recipient chest cavity, taking care that the PA and PV are not twisted.

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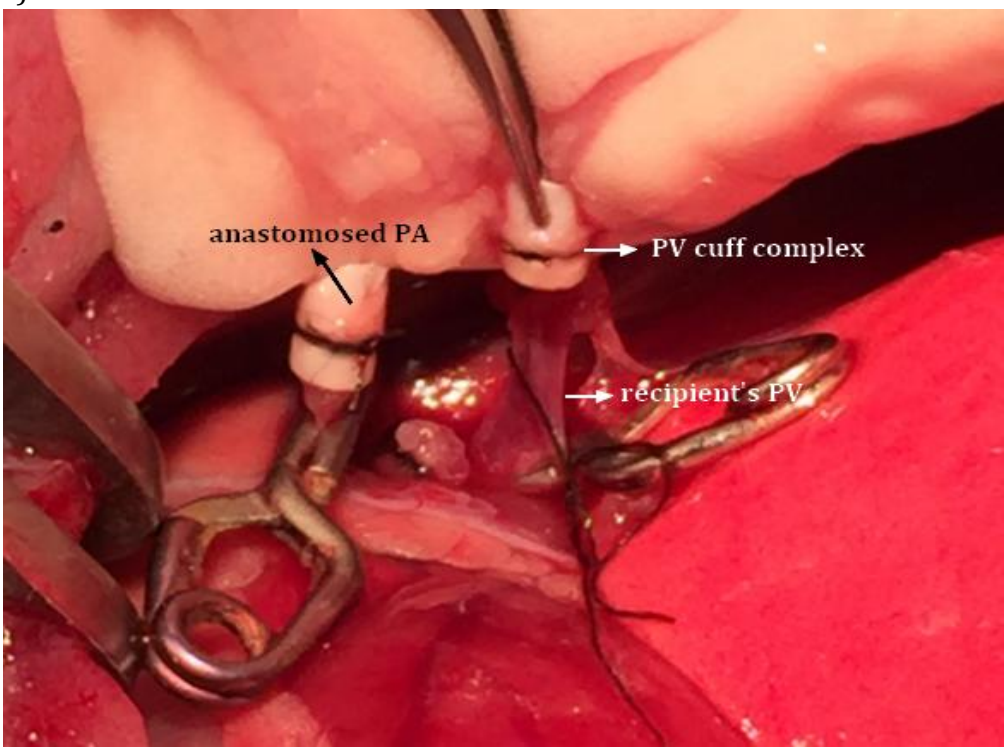

Transverse incisions are made anteriorly in recipient PA and PV. The grafts' PA and PV cuffs are introduced into the corresponding vessels of the recipient, and secured by 7-0 silk sutures.

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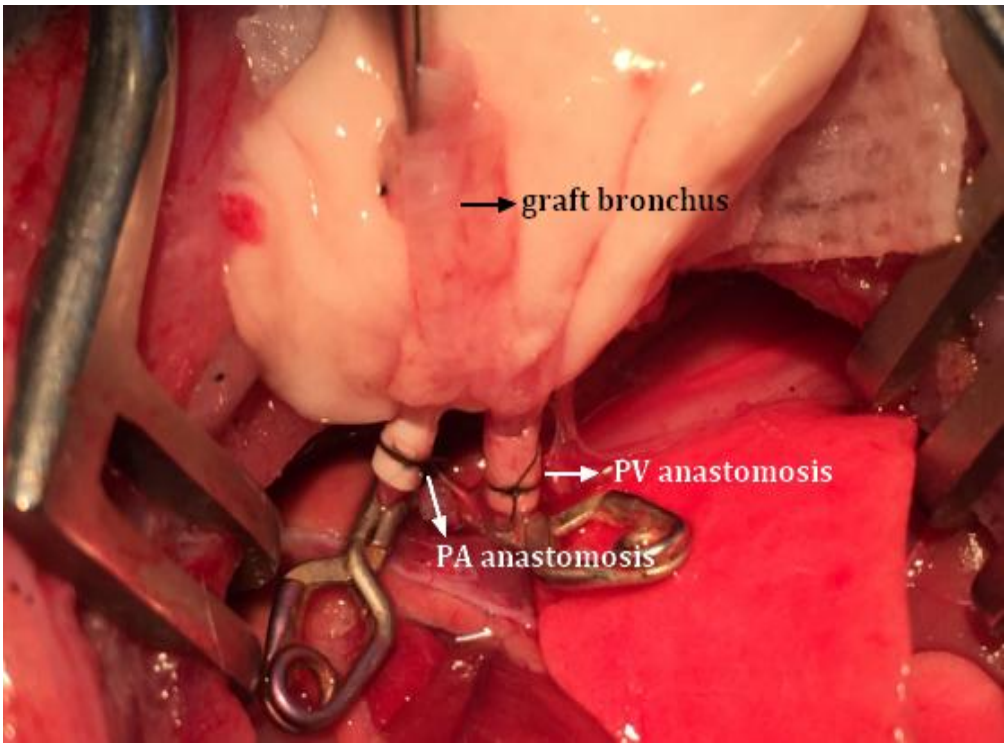

Here, both anastomoses are completed. The left main bronchus of the graft is intubated and connected to a second ventilator.

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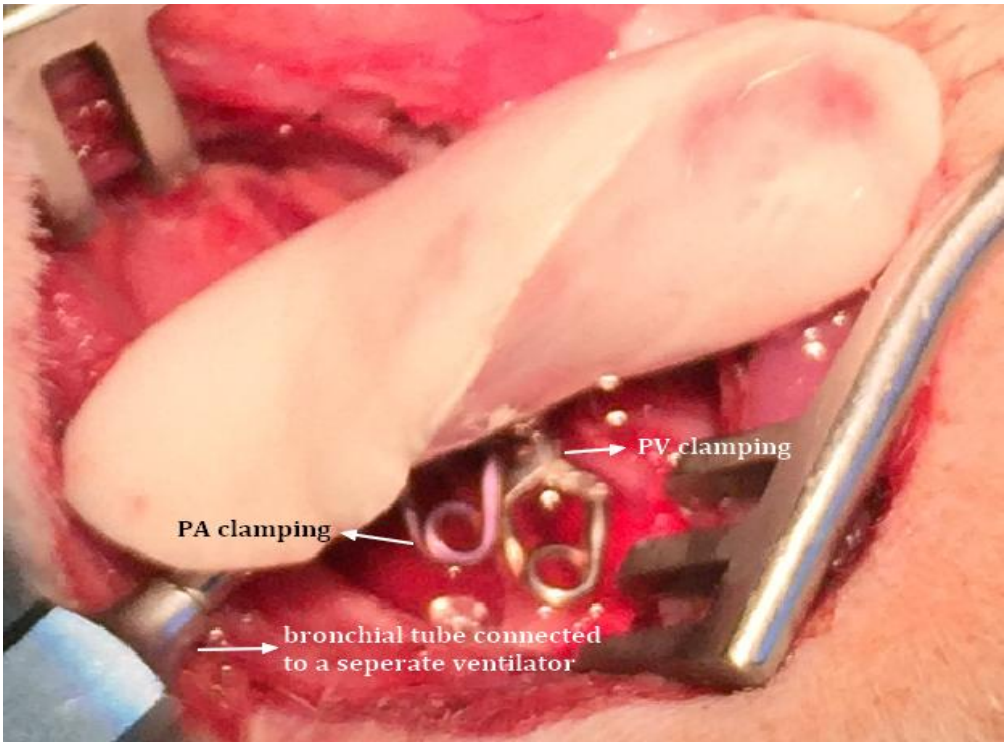
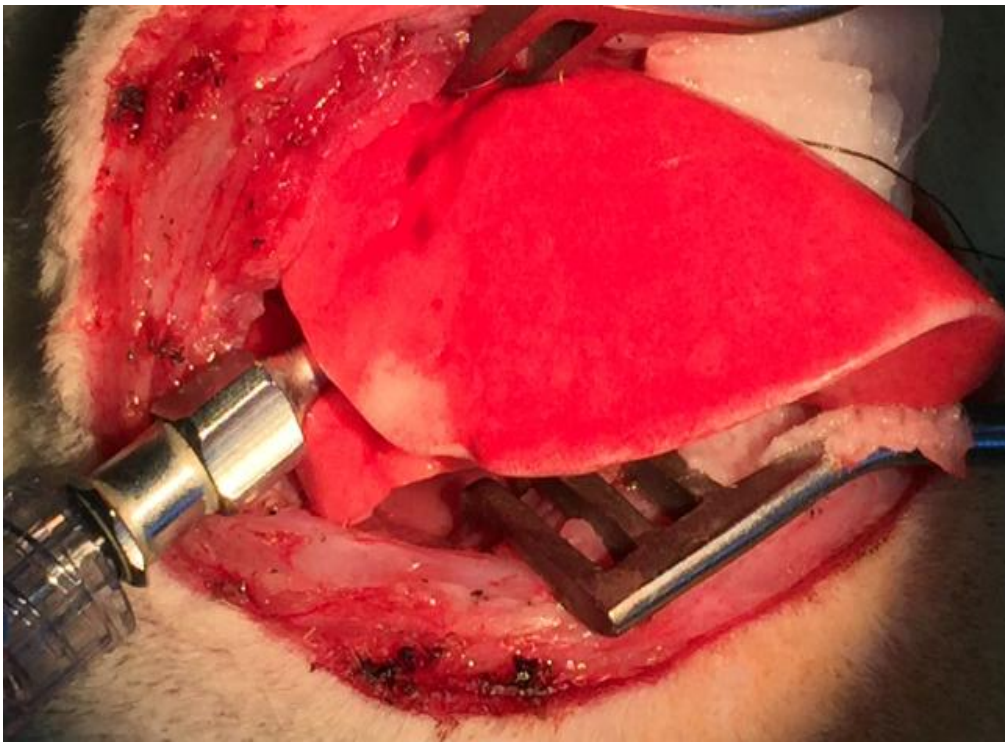


Following the anastomoses of PA and PV, the graft is mechanically ventilated using a separate ventilator (tidal volume 4ml/kg, frequency 90/min, PEEP 5cmH_20, FiO_2 0.50) for 3 min prior to the blood reperfusion

11)



The PV is declamped, followed by the declamping of PA. The graft is reperfused and separately ventilated for 2 hours, during which the in-vivo graft function (oxygenation, lung compliance, peak airway pressure) is assessed.

Chapter 9

Ex-vivo pharmacological inhibition of poly (ADP-ribose) polymerase (PARP) reduces ischemia reperfusion injury and improves function of transplanted lung grafts

Abstract

Introduction: Ischemia-reperfusion injury (IRI) is a key mechanism of graft damage following lung transplantation, which could be targeted by therapies applied during ex-vivo lung perfusion (EVLP). Production of oxidants and activation of the enzyme poly (ADP-ribose) polymerase (PARP) are key processes involved in IRI. Previously, the pharmacological inhibition of PARP during EVLP was shown to alleviate reperfusion injury and improve graft function *ex-vivo*. Here, we hypothesized that inhibition of PARP with 3-aminobenzamide (3-AB) during EVLP improves lung graft function and reduces IRI in a rodent lung transplantation model.

Methods: Male Sprague-Dawley rats (n=24) underwent left single lung transplantation (Ltx). Donor lungs were procured and allocated to 3 groups: lungs were flushed with cold Perfadex following cardiac arrest and stored for 4 hours at 4°C before transplantation (CI group), or lungs underwent 1hr warm ischemia following cardiac arrest, cold Perfadex flush, and were stored for 1 hour at 4°C, followed by 3 hours of normothermic EVLP either with Steen solution (WI group) or Steen solution supplemented with 3-AB (WI-3-AB group) and then transplanted. Graft physiological function (static pulmonary compliance, peak airway pressure, pulmonary vascular resistance, oxygenation and weight gain) were assessed during EVLP and the following 2 hours reperfusion after transplantation. Bronchoalveolar lavage fluid (BALF) harvested after EVLP or Ltx was analysed to determine protein content, lactate dehydrogenase (LDH), malondialdehyde (135), inflammatory cytokines (IL-6, TNF-α, CINC-1). Total cell count and cell differentiation in BALF was assessed.

Results: Warm ischemia lungs exerted significantly enhanced IRI after Ltx, in line with poor physiological function and elevated oxidative stress, lung inflammation and edema during EVLP. IRI after Ltx was markedly alleviated by inhibition of PARP administrating 3-AB during EVLP.

Conclusion: Pharmacological intervention during EVLP inhibiting PARP protects damaged donor lungs from ischemia-reperfusion injury after transplantation and improves post-transplant graft function in this experimental setting.

Introduction

We have previously shown in an ex-vivo model that ischemia-reperfusion injury can be alleviated by pharmacological inhibition of PN and PARP during EVLP (92). Here we apply this strategy in a lung transplant model with donor lungs undergoing prolonged warm ischemia.

Materials and methods

Animals

Adult male Sprague-Dorley rats weighing 300-350g were used as donors and recipients. All the animal experiments were performed in accordance with the Animal Welfare Act and the National Institutes of Health "Guidelines for the Care and Use of Laboratory Animals" and were approved by the cantonal authorities (authorization 2637).

Donor lung procurement and preservation

General anesthesia was induced by intraperitoneal administration of Ketamine (80mg·kg^{-1}, SINTETICA S.A., Mendriso, Switzerland) +Xylazine (8mg·kg^{-1}, Provet, S.A., Lyssach, Switzerland). The animal was placed on a heating pad maintaining the core temperature at 37°C, the trachea was intibated and the animal was ventilated using a rodent respiratory (Model 683; Harvard Apparatus, Holliston, MA, USA). Mechanical ventilation was adjusted with a fraction of inspired oxygen (FiO2) of 0.21, a respiratory rate (RR) of 80 breaths·min^{-1}, a tidal volume (V_T) of 7ml·kg^{-1} and positive end-expiratory pressure (PEEP) of 3cmH_2O. Following a median sternotomy, systemic anticoagulation was achieved by injecting heparin (600U) to the right ventricle. Pulmonary artery (PA) and left ventricle (LV) were cannulated and flushed as described previously (92). Then the animals were randomly divided into 4 groups (see also Figure 9.1):

a) Cold ischemic lungs (CI group, N=8): the lung was flushed with 4°C Perfadex (XVIVO Perfusion AB, Goteborg, Sweden) through PA at a perfusion pressure of 20 cm H2O, being ventilated (FiO_2=0.21, RR=15/min, V_T =7ml·kg^{-1} and PEEP=3cmH_2O), procured and stored in 4°C Perfadex for 4 hours in an inflated status prior to left lung transplantation (L-Ltx).

b) Warm ischemic lungs undergoing EVLP (WI_E group, N=7): the lung was kept *in situ* for 1 hour at room temperature, followed by 4°C Perfadex flush through PA, ventilation (FiO_2=0.50, RR=15·min^{-1}, V_T =7ml·kg^{-1} and PEEP=3cmH_2O). The lung was procured and stored in 4°C Perfadex for 1 hour in an semi-inflated status. Then the heart-lung blocks underwent normothemic EVLP up to 3 hours, after which the L-Ltx was performed.

c) Warm ischemic lungs undergoing EVLP the PARP inhibitor 3-aminobenzamide (WI_E-3-AB group, N=9): same procedure as described for WI_E group, except that WI_E-3-AB (1mg·ml^{-1}, Sigma-Aldrich, Buchs, Switzerland) was added to the perfusate.

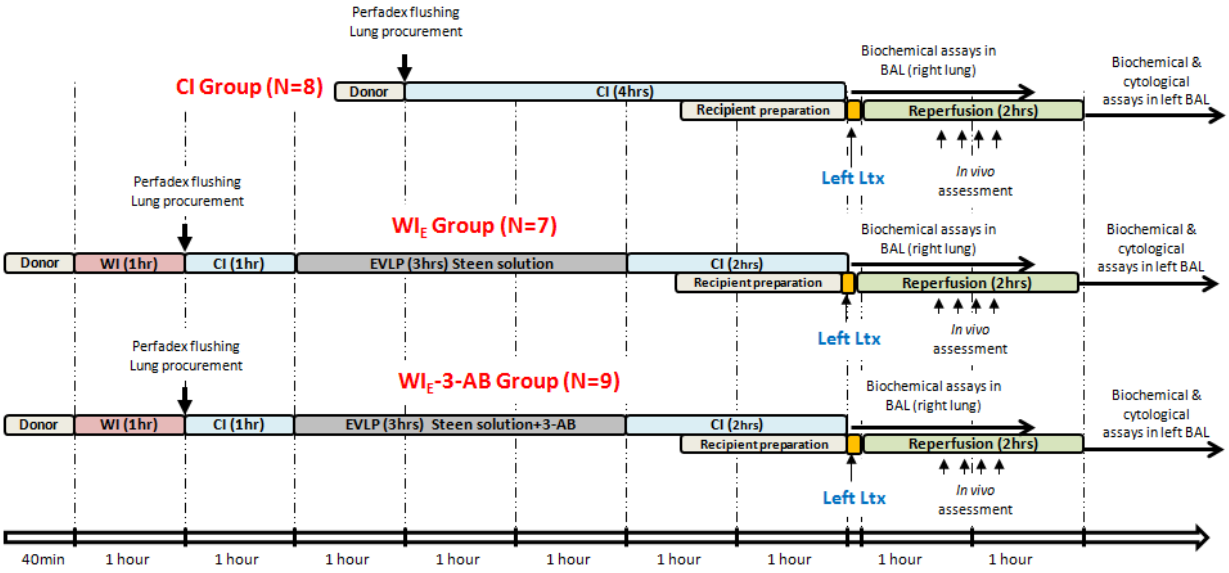


Figure 9.1 Study design

Rat Normothermic EVLP

EVLP was preceded by warm and cold ischemia as described above. Animals in groups WI_E and WI_E-3-AB underwent 3 hours of EVLP using a customized rat EVLP system (Harvard IL-2 System; Hugo Sachs Elektronik, Hugstetten, Germany) as described in detail previously (92). Briefly, the heart-lung blocks were weighted and then mounted in the circuit for protective ventilation and constant flow normothermic perfusion with Steen® solution (Xvivo Perfusion, Göteborg, Sweden). In the WI group, the circuit was primed with Steen® solution only, while 3-AB (1mg·ml^{-1}, Sigma-Aldrich, Buchs, Switzerland) was added to the Steen solution in WI_E-3-AB group. The perfusion was initialized at flow rate of 2ml·min^{-1} at 10°C, and was step-wise increased to the target flow which corresponds to 7.5% of theoretical rat cardiac output (98) at 37.5°C. Mechanical ventilation was started once normothermia was reached with a FiO_2 of 0.21, RR of 15/min, V_T of 6ml·kg^{-1} and PEEP of 3cmH_2O (flexiVent FX3 ventilator; SCIREQ Inc, Montréal, Canada). The circuit was deoxygenated using a mixture of 6% O_2, 10% CO_2 and 84% N_2 supplied through a hemofilter (D150, MEDICA S.P.A, Italy). During 3 hours of EVLP, partial pressure of O_2 of affluent (P_aO_2) and effluent (P_eO_2) arms, pulmonary artery pressure (PAP) and left ventricular pressure (LVP) were continuously recorded to calculate differential partial pressure of O_2 ($DppO_2$= P_eO_2- P_aO2) and pulmonary vascular resistance (PVR=[PAP-LAP]/Flow). Static pulmonary compliance (SPC), peak airway pressure (PAWP) were repeatedly measured and monitored. At the end of EVLP, heart-lung blocks were weighed again, immediately cooled in 4°C Perfadex and stored in an inflated status (FiO_2=0.21) for a second cold preservation of 2 hours.

Orthotopic left lung transplantation

Rat left lung transplantation with independent ventilation of the graft and the native lung was performed according to a technique described before (103). During the cold ischemic preservation, the left bronchus was isolated from the trachea of retrieved heart-lung blocks and clamped to keep the lung inflated, the left lung was separated from the heart lung block, the left PA and left PV were carefully mobilized, and a homemade 16-gauge cuff were placed in each vessel (Abbocath Catheters, Hospira, Inc., Lake Forest, IL, USA).

General anesthesia of the recipient was the same as in donor rats, but Buprenorphone (0.3mg·kg^{-1}, i.p., Temgesic®, Reckitt Benckiser AG, 8304 Wallisellen, Switzerland) was added. Then the animals were placed on a heating surface maintaining the body temperature at 37.5°C, tracheostomized and ventilated using a respirator (model 683, Harvard Apparatus, Holliston, MA) with FiO_2 of 0.50, RR of 90 breaths·min^{-1}, V_T of 7ml·kg^{-1} and PEEP of 3cmH_2O. The right external jugular vein was cannulated with a microtip polyethylene catheter (ID=0.28mm), and 0.9% NaCl of 10ml·kg^{-1}·h^{-1} was given to the recipient animal. A Micro-Renathane® tubing (ID=0.37mm) was introduced into the left carotid artery to monitor arterial blood pressure during the surgery. A rat foot sensor (FootClip, STARR life science Corp, Oakmont, PA, USA) was attached to obtain the arterial oxygen saturation (SpO_2). Through the left posterolateral thoracotomy, the left pulmonary inferior ligament was mobilized, and the left hilar structures were dissected with left PA, left PV and left main bronchus freely exposed. The left main bronchus was ligated and divided, and the V_T of the respiratory was reduced to 5ml·kg^{-1}, since ventilation was limited to the recipients' right lung only. The left PA and PV were then proximally clamped and the left native lung was removed. The recipients' PA and PV were suspended, anteriorly incised and circumferentially encircled with pre-looped 7-0 silk sutures (Teleflex, Coventry, CT, USA). The donor lung was weighed and then the pre-cuffed PA and PV were gently introduced into the corresponding PA and PV of the recipient, with the cuffs secured by sutures. The graft was connected to a second ventilator (Flexivent FX3; Scireq Inc, Montreal, Canada; FiO_2=0.50, RR=90 breaths·min^{-1}, V_T =4ml·kg^{-1} and PEEP=5cmH_2O). Thus, the recipients' native right lung and left transplanted lung were separately ventilated. Following a recruitment maneuver, graft reperfusion was started by declamping the PV and then the PA. Care was taken to prevent twist of the graft bronchus. The animal was kept under general anesthesia for two hours of reperfusion, after which the transplanted left lung was harvested to determine the graft weight gain during reperfusion, as well as for further analysis.

In-vivo recipient and graft functional assessment during blood reperfusion

During 2 hours of blood reperfusion, mean arterial pressure (MAP, F-LMP1-00-00, Datex Engstam, Helsinki, Finland) and SpO_2 were continuously monitored. At selected time-points, PAWP and SPC were measured using the Flexivent ventilator. The gas exchange of the graft was assessed by blood gas analyses in blood taken from the grafts' PV. The the oxygenation index (OI) was calculated using the equation: $OI=FiO2 \cdot M_{PAW} \cdot PO_2^{-1}$

(192), where PO_2 was the partial pressure of O_2 of the blood sample and M_{PAW} was the Mean Airway Pressure of the ventilated graft (M_{PAW}=Peak inspiratory pressure (PIP)· inspiratory time (IT)+PEEP·expiratory time (ET)) (193). PIP, IT and ET were automatically recorded by the ventilator.

Biochemical analyses of bronchoalveolar lavage at the end of EVLP

At the end of EVLP, BAL was performed in all the right lungs from the donor rats by instilling 6ml of PBS (pH=7.4) through the right main bronchus. After recovery, the BAL fluid (BALF) was centrifuged at 5,000 rpm for 10minutes at 4 °C and the cell-free supernatant was kept. The proteins content, as an index of the integrity of the alveolar-capillary membrane, was determined using BCA assay (Thermo Scientific, Rockford, USA) and expressed in $mg \cdot ml^{-1}$; lactate dehydrogenase (LDH) activity, an general index of tissue necrosis, were measured using a kit (Cytotoxicity Detection Kit PLUS; Roche, Basel, Switzerland) and expressed in arbitrary unit (AU) $\cdot ml^{-1}$; Malondialdehyde (135), a byproducts of lipid peroxidation during oxidative stress was measured using a rat ELISA kit (MyBioSource, San Diego, CA, USA); inflammatory cytokines as interlukin-6 (IL-6), tumor necrosis factor alpha (TNF-α) and chemokine cytokine-induced neutrophil chemoattractant factor 1 (CINC-1) were assessed using ELISA kit (R&D system, Minneapolis, MN, USA) and expressed in $nanogram \cdot ml^{-1}$.

Biochemical and cytological analyses of BAL at 2 hours after transplantation

Two hours following transplantation, BAL was performed in the transplanted left lung by instilling 4ml of PBS (pH=7.4). Analysis (Protein content, LDH, MDA, IL-6, TNF-α and CINC-1 determined in BALF) in the cell-free supernatant of the BALF were done as described above. The sedimented cells in the BALF were resuspended in a volume of 0.5ml, a total cell count was performed in 0.1ml of this volume using a hemocytometer, while the remained cell suspension was cytospinned (800 rpm for 5 min, Cytospin 2, SHANDON southern products Ltd, UK), stained (Diff-quik, Medion, Diagnotic AG, Dudingen, Switzerland) to assess differential cell count under a light microscope. Cells were categorized as mononuclear cells (MNs) or polymorphonuclear cells (PMNs). The calculated cell concentration was corrected to the recovered BALF volume and finally expressed as actual counts.

Statistics

Data analysis was performed by Graphpad prism 6 (GraphPad Software Inc., La Jolla, CA, USA). All the results in this study are presented as Means±SEM. For the repeated measurements (SPC, PAWP, $DppO_2$, PVR, MAP and SpO_2) during EVLP or reperfusion time, 2-way ANOVA was applied, followed by Dunnett's test for the effect of time and Tukey's test for the effect of treatment. One-way ANOVA plus Tukey's correction was used for all other comparisons. $P<0.05$ was considered statistically significant.

Results

The effect of 3-AB on SPC, PAWP, $DppO_2$ and PVR of warm ischemic lung during EVLP

SPC in WI_E group remained stable throughout EVLP and was improved in lungs undergoing EVLP with administration of 3-AB (Figure 9.2A). This effect was significant at time 90 min (p=0.0036) and 120 min (p=0.0812). PAWP during EVLP (Figure 9.2B) was lower in lungs treated with 3-AB as compared to lungs undergoing sham EVLP (P<0.05 at all time-points). In both groups (WI_E and WI_E-3-AB) a PAWP drop after 30 min of EVLP was observed, corresponding to the the onset of ex-vivo ventilation and preceding recruitment maneuvers. $DppO_2$ (Figure 9.2C) and PVR (Figure 9.2D) were stable during EVLP and did not reveal differences in-between both groups.

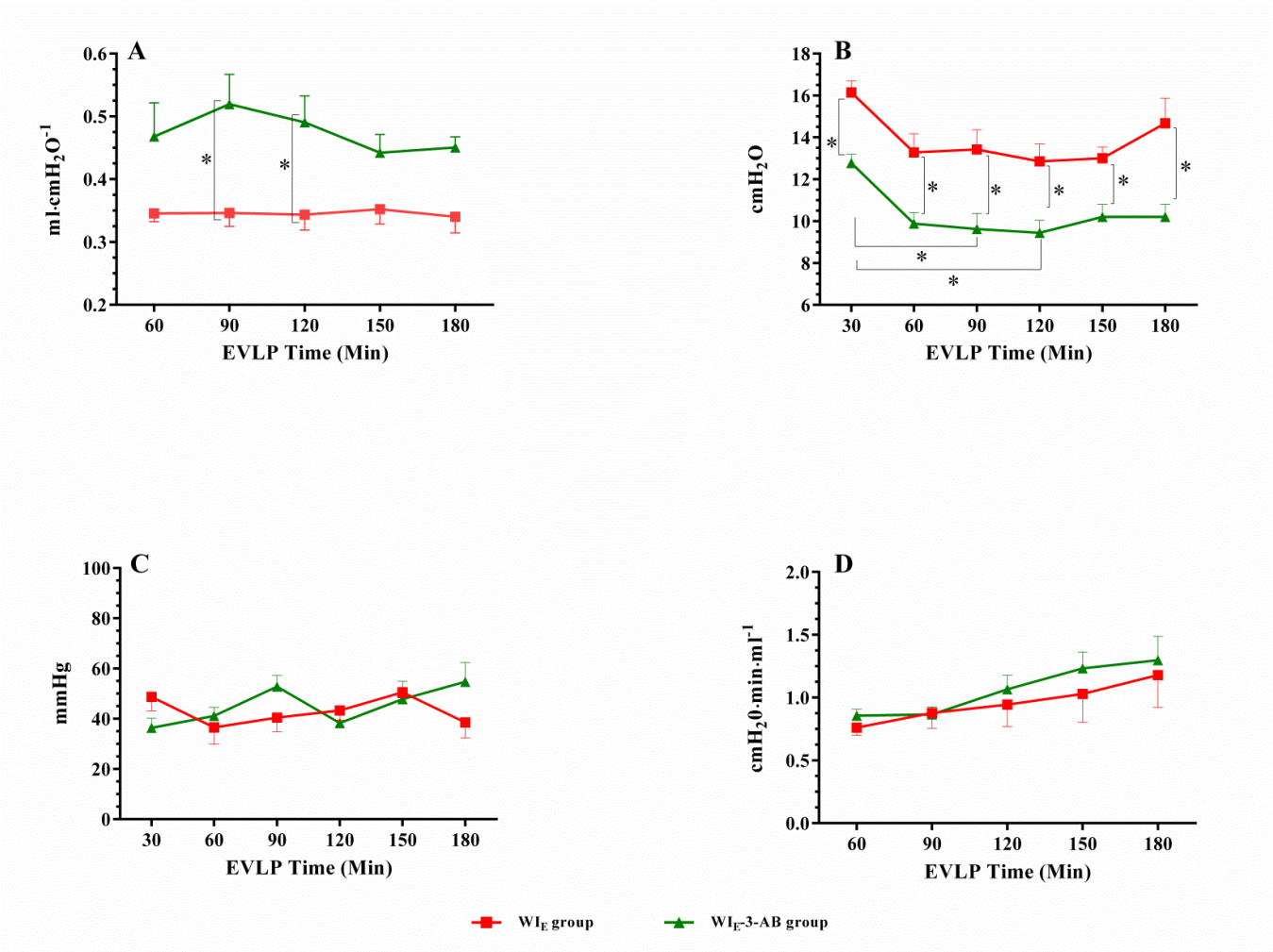


Figure 9.2 EVLP lung physiology for WI_E and WI_E-3-AB groups

A: Static pulmonary compliance (SPC) during EVLP; B: Peak airway pressure (PAWP) during EVLP; C: Differential partial pressure of the oxygen ($DppO_2$) and D: Pulmonary vascular resistance (PVR). Means±sem. *: $p<0.05$.

Administration of 3-AB diminishes lung edema development, tissue injury and oxidative stress during EVLP

Donor lung weight gain during EVLP, as a marker of lung edema (Figure 9.3A), was more pronounced in WI_E group than in WI_E-3-AB group (p=0.0428 WI_E vs WI_E-3-AB). In comparison to the lungs undergoing cold static preservation only, donor lungs exposed to warm ischemia (WI_E group) showed significantly increased tissue necrosis and oxidative stress as demonstrated by the protein content (Figure 9.3B), LDH (Figure 9.3C) and MDA (Figure 9.3D) in BALF (p=0.0415, p=0.0002 and p=0.0261 WI_E vs CI). When 3-AB was added during EVLP, the protein content and MDA in BALF were less pronounced (p=0.6841, p=0.08, respectively; WI_E-3-AB vs WI_E), and the LDH level in BALF significantly reduced (p=0.0002 WI_E-3-AB vs WI_E).

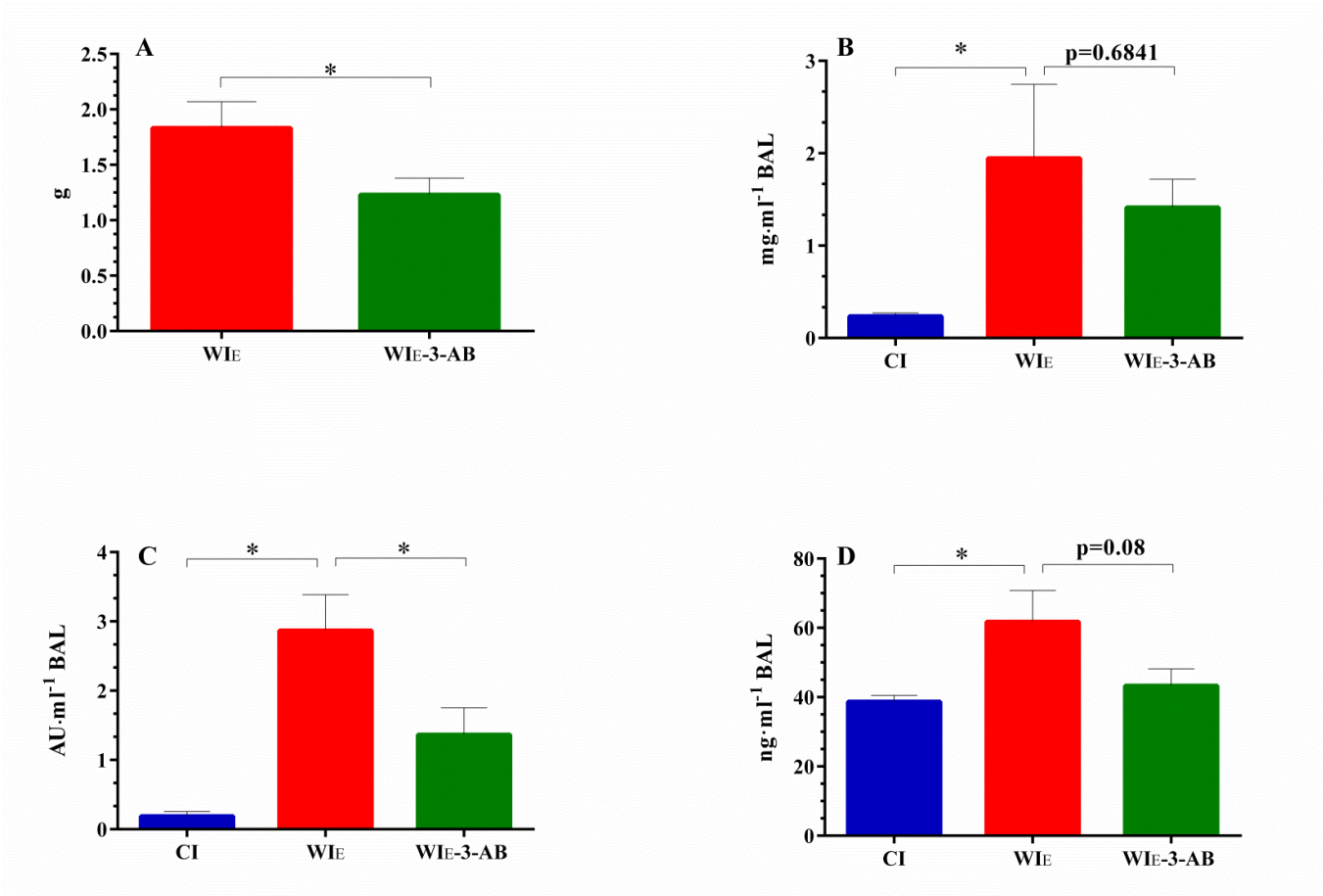


Figure 9.3 lung edema, tissue injury and oxidative stress at the end of EVLP

A: Lung weight gain during EVLP. B: Protein content in broncho-alveolar lavage fluid (BALF). C: Lactate dehydrogenase (LDH) in BALF. D: Malondialdehyde in BALF. Means±sem. *: $p<0.05$.

Lung inflammatory response was attenuated by 3-AB at the end of EVLP

The levels of IL-6 and TNF-α in the BALF as markers of acute innate inflammation were evaluated at the end of EVLP. In addition, the concentration of CINC-1, a member of the CXC family of chemokines, attracting neutrophils and corresponding to the human IL-8 was measured in BALF. Levels of IL-6 (Figure 9.4A), TNF-α (Figure 9.4B) and CINC-1 (Figure 9.4C) in BALF from lungs undergoing warm ischemic damage were elevated as compared to lungs undergoing cold ischemic preservation only (p=0.0003, p=0.0017 and p<0.0001 WI_E vs CI). Conversely, the levels of IL-6 and CINC-1 were significantly attenuated by 3-AB administration during EVLP (p=0.0001 and p=0053 WI_E vs WI_E-3-AB). TNF-α was reduced, without a significant difference (p=0.1255 WI_E vs. WI_E-3-AB).

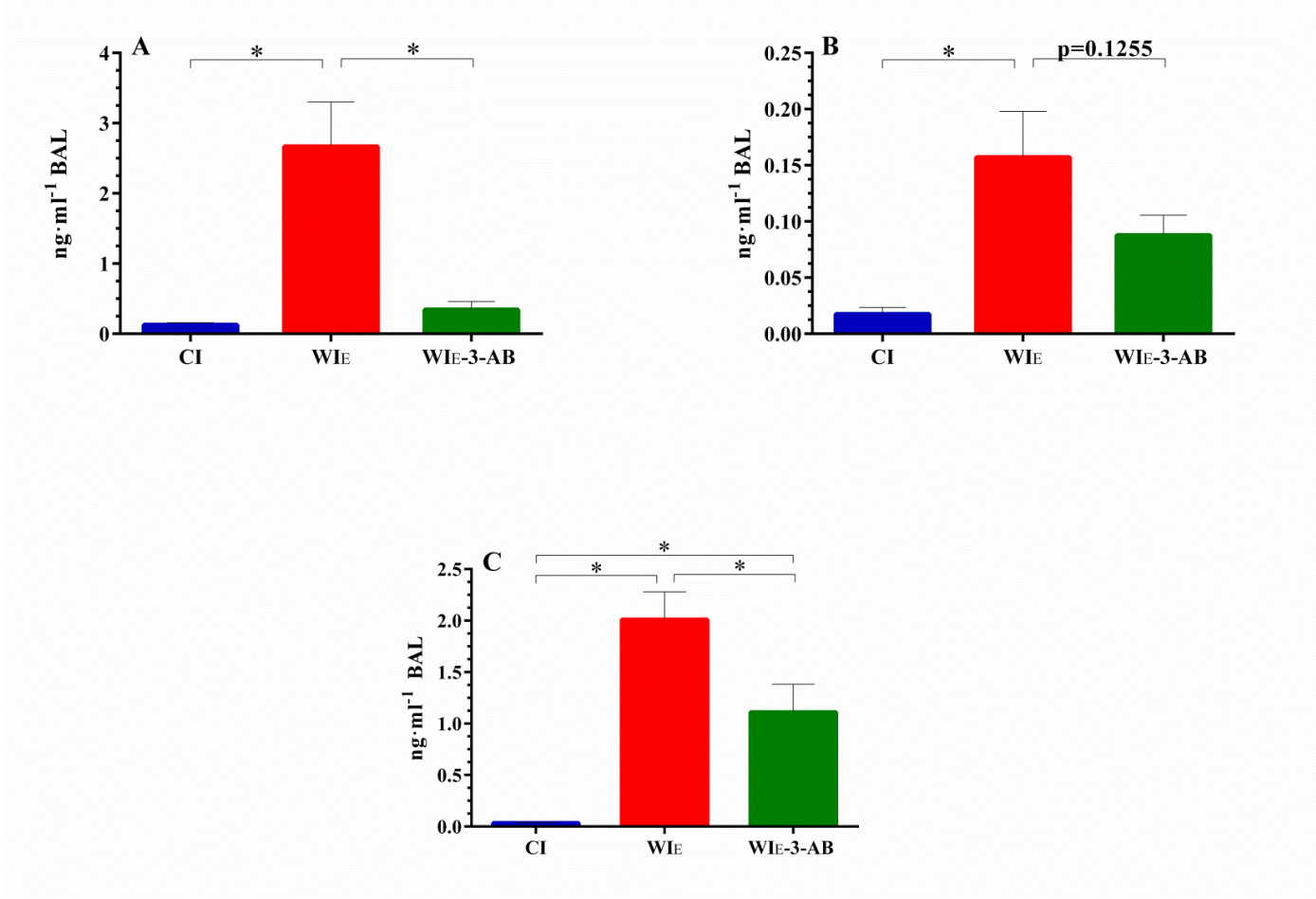


Figure 9.4 Lung inflammatory response assessed at the end of EVLP

A: Interleukin-6 (IL-6) in BALF. B: Tumor necrotic factor alpha (TNF-α) in BALF. C: Chemoattractant for neutrophils 1 (CINC-1) in BALF. Means±sem. *: $p<0.05$.

The effect of EVLP with 3-AB on graft function after transplantation

During two hours of blood reperfusion after transplantation, injured grafts which underwent inhibition of PARP with 3-AB during EVLP (WI_E-3-AB group) exerted

comparable SPC (Figure 9.5A) and PAWP (Figure 9.5B) to those grafts preserved at ideal conditions at 4°C ($p>0.05$ WI_E-3-AB vs CI group). The lung function of these grafts was significantly improved as compared to those in WI_E group ($p<0.05$ WI_E-3-AB vs WI_E). Furthermore, normal and similar MAP (Figure 9.5C) and SpO_2 (Figure 9.5D) were observed for all the transplanted grafts in different groups.

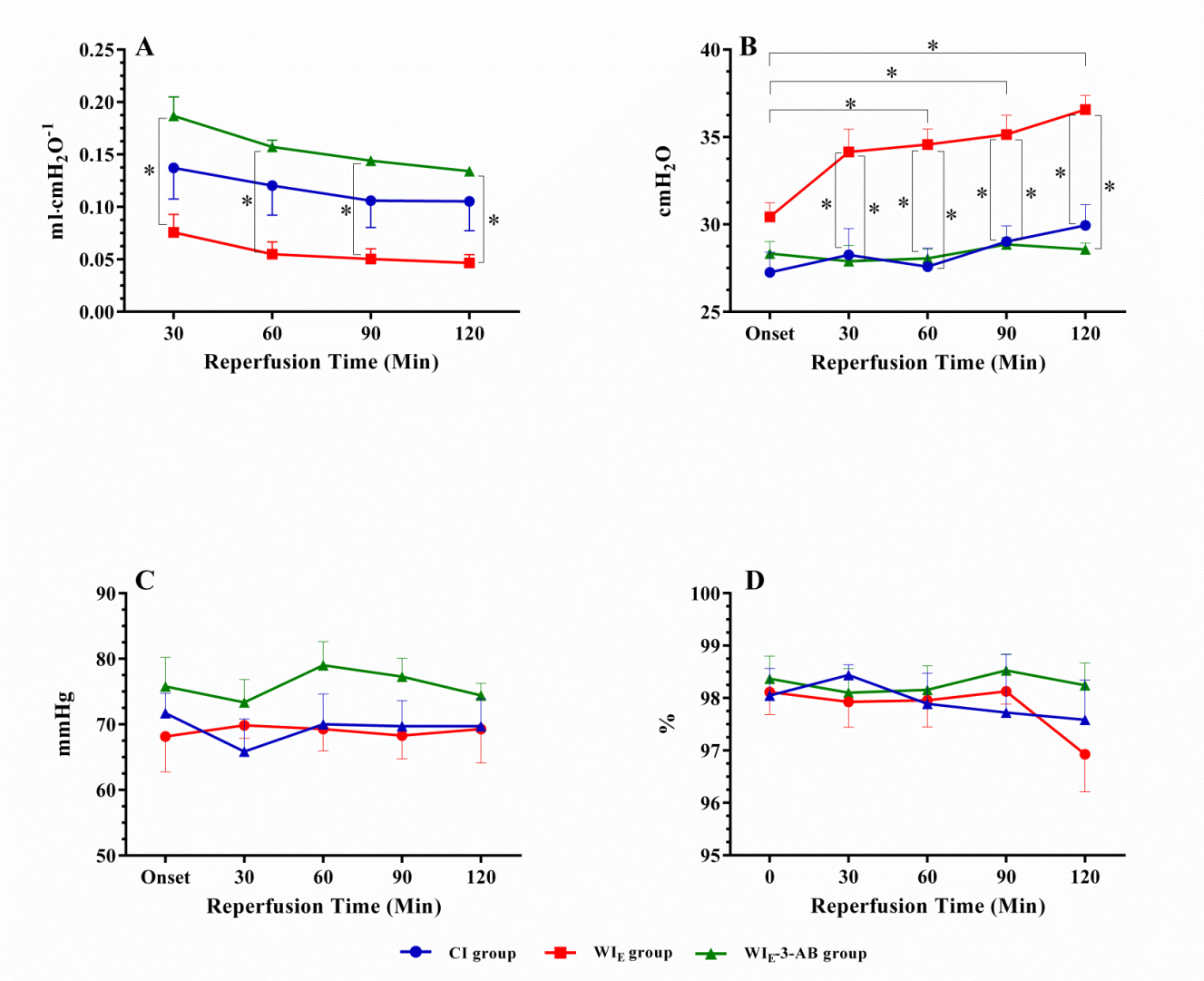


Figure 9.5 Graft function after transplantation

WI_E-3-AB group showed improved pulmonary compliance (A) and decreased peak airway pressure (B) as compared to the WI_E group. Mean arterial pressure (MAP) and oxygen saturation (SpO_2) were stable and comparable for all the experimental groups. Means±sem. *: $p<0.05$.

Improved graft gas-exchange at 2 hours after transplantation

Lungs from the WI_E-3-AB had a significantly lower OI than those from the the WI_E group ($p=0.0267$ WI_E-3-AB vs WI_E). (Figure 9.6).

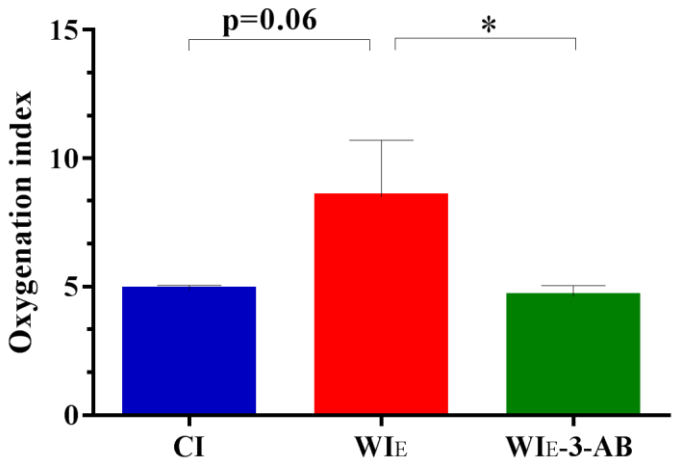


Figure 9.6 Graft gas-exchange function at 2 hours after transplantation

Means±sem. *: $p<0.05$.

EVLP with 3-AB diminished lung edema development, tissue necrosis and oxidative stress after transplantation

A considerable increase of graft weight (Figure 9.7A) occurred after 2 hours of blood reperfusion in WI_E group, as compared to the CI group (p=0.0054). This effect was alleviated by 3-AB treatment during EVLP (p=0.0001 WI_E vs WI_E-3-AB). Likewise, when compared to CI group, WI_E grafts displayed a significant elevation of protein content (Figure 9.7B), LDH (Figure 9.7C) and MDA (Figure 9.7D) in BALF harvested after transplantation, indicating an increased alveolar-capillary permeability, cell necrosis as well as lipid peroxidation after transplantation ($p<0.05$ WI_E vs CI) These phenomena were significantly reduced in animals receiving injured grafts after EVLP reconditioning with 3_AB (WI_E-3-AB group; p=0.0009, p=0.0325 and p=0.0419; WI_E-3-AB vs WI_E).

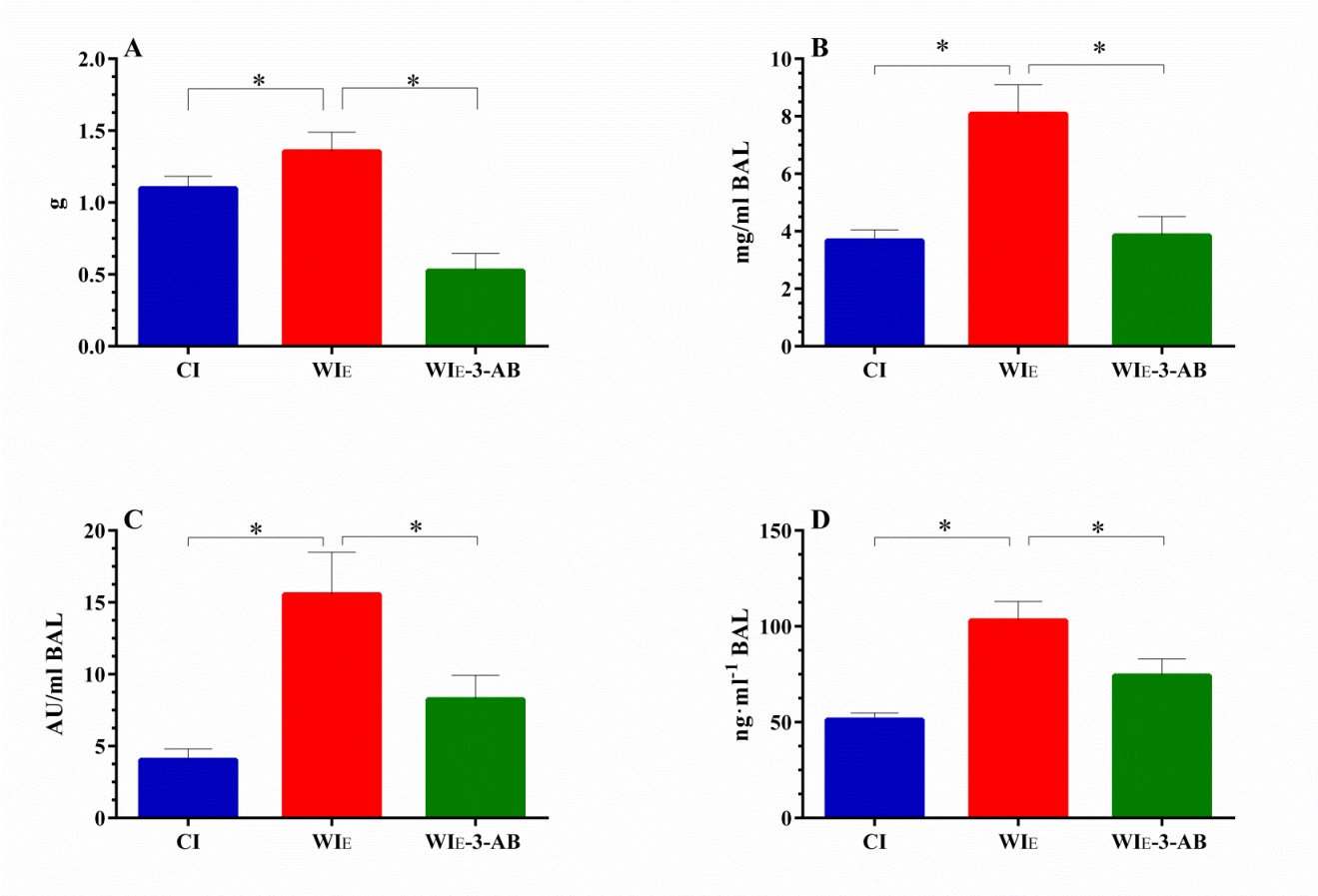


Figure 9.7 Graft edema, tissue necrosis and oxidative stress after lung transplantation

A: Graft weight gain at 2 hours after transplantation. B: Protein content in BALF. C: LDH in BALF. D: MDA in BALF. Means±sem. *: $p<0.05$.

Graft inflammatory response was down-regulated by 3-AB after transplantation

Following 2 hours of blood reperfusion, IL-6 (Figure 9.8A), TNF-α (Figure 9.8B) and CINC-1 (Figure 9.8C) in BALF were all substantially up-regulated as compared to the status before transplantation (Figure 9.4), indicating the inflammatory response. Furthermore, transplantation of lungs from the WI_E group was associated with significant activation of IL-6, TNF-α and CINC-1 in comparison to both other groups. The expression of inflammatory cytokines in lungs undergoing 3-AB ex-vivo treatment was higher than in CI lungs ($p<0.05$), but was less as compared to those lungs injured by warm ischemia undergoing sham EVLP ($p<0.05$ for IL-6 and CINC-1, $p=0.0584$ for TNF-α WI_E vs WI_E-3-AB).

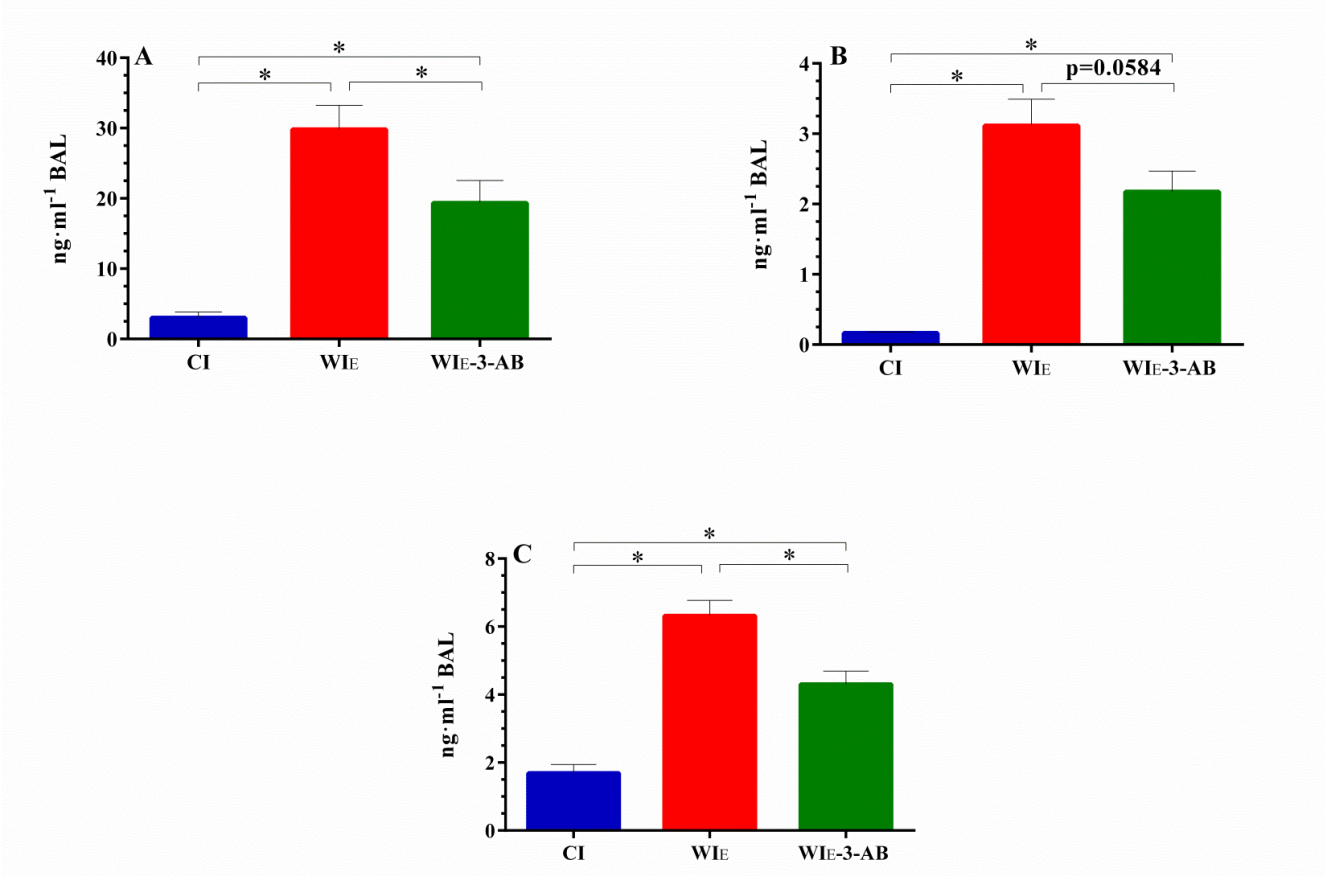


Figure 9.8 Graft inflammatory response after transplantation

A: IL-6 in BALF. B: TNF-α in BALF. C: CINC-1 in BALF. Means±sem. *: $p<0.05$.

Inflammatory cell infiltration after transplantation

Two hours following transplantation, an increased total cell counts in the BALF was noted in WI_E group (p=0.0108 WI_E vs CI), whereas a significantly lower cell count was found in BALF of lungs undergoing 3-AB treatment (p=0.0382 WI_E vs WI_E-3-AB) (Figure 8.9A). Cell differentiation in BAL (Figure 9.9 B-E) showed that cells recruited in WI_E (Figure 9.9C) and WI_E-3-AB (Figure 9.9D) groups were predominately MNs. In contrast, MNs and PMNs were equally presented in CI group (Figure 9.9B). Furthermore, both cell types were present at high levels in the BALF of grafts of WI_E group but not of WI_E-3-AB group (Figure 9.9E).

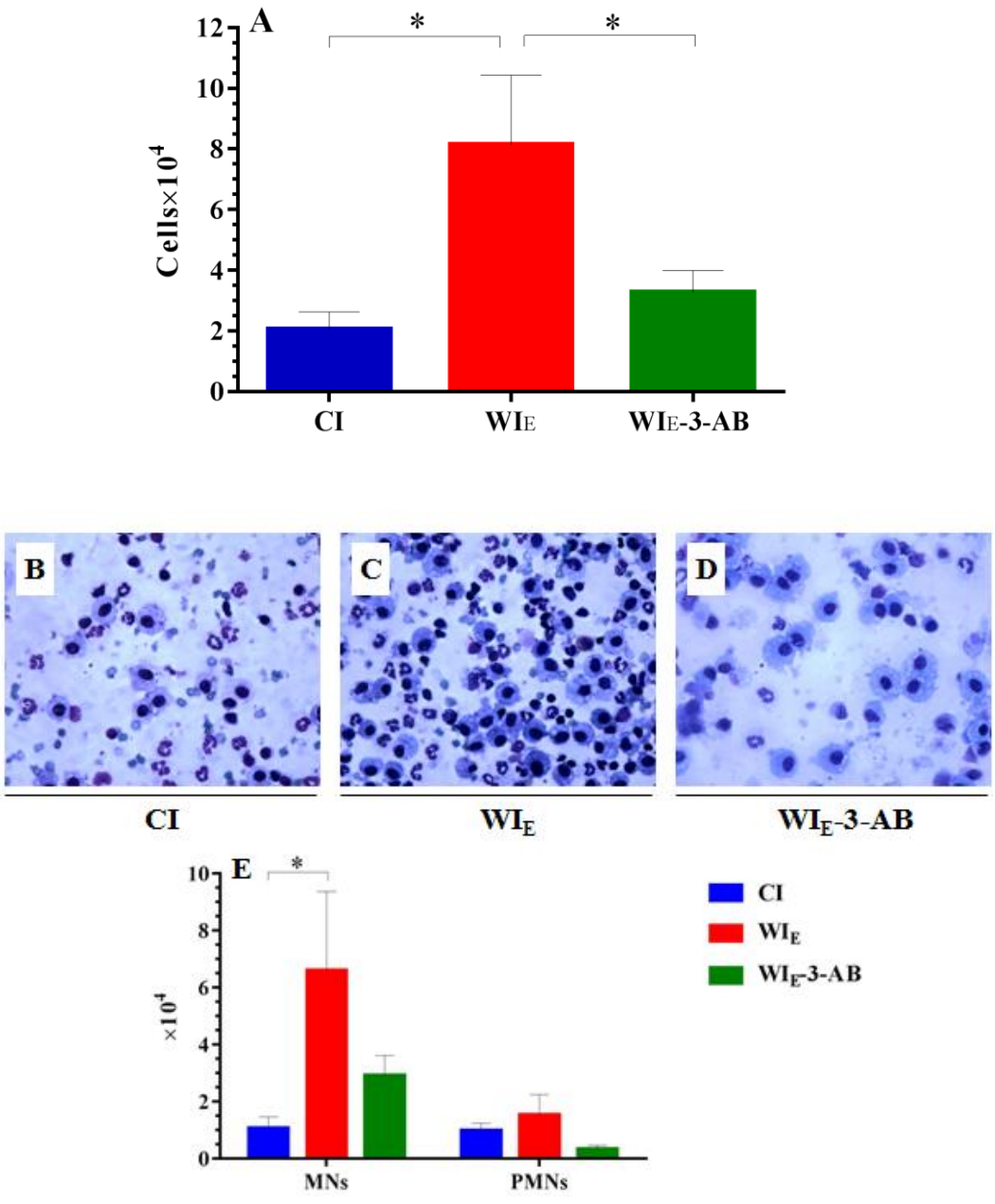


Figure 9.9 Cytological assessment after transplantation

A: Total cell counts in the BALF at 2 hours after transplantation. Microscopic view of cells in BALF at 2 hours after transplantation in CI group (B), WI_E group (C) and WI_E-3-AB group (D). Graph E shows the cell counts of MNs and PMNs in all groups.

Means±sem. *: $p<0.05$.

MNs: mononuclear cells; PMNs: polymorphonuclear cells

Magnification for B, C and D: 400 X

Discussion

In this study, we demonstrated by using a rat model that ex-vivo inhibition of PN on damaged donor lungs obtained from extended warm ischemia led to a significant improvement of pre-transplant graft functional/biochemical outcomes, and remarkable benefit of alleviated IRI following transplantation. Focusing on the redox-based cytotoxicity during lung graft reperfusion/reoxygenation, our ex-vivo mediated pharmacological therapy further advances the fact that EVLP as a treatment platform to recondition injured donor lungs to be candidatable for the transplantation (38, 119, 120).

The application of damaged DCDDs, conceptually aiming to expand the lung donor pool is potentially at highly increased risk of PGD, particularly in the cases of uncontrolled DCDDs (uDCDDs) due to the warm ischemic damage ascribed to the uncertain period of agonal phase (28). It is noteworthy to underline that the donor lungs exposed to warm ischemia were intendedly allowed to become atelectatic before cold Perfadex flushing, in order to further detriment the graft. This procedure deprived lungs of oxygen in the alveoli and airways. Therefore, lung injury following WI was attributed not only to the warm ischemic time, but also partially the deflation procedure. In contrast, CI lungs were kept inflated during either the cold perfadex flushing or storage thereafter to assort as control, undamaged lung grafts, representing an ideal preservation.

In this study, donor lungs in all experimental groups were preserved bilaterally to undergo the same conditions before transplant, then the left lungs were isolated for the following unilateral Ltx while the right lungs, which virtually contained identical pre-transplant consequences of ischemia with (WI_E or WI_E-3-AB group) or without (CI group) EVLP as the left lungs were subjected to the biochemical assays. Given the interest to rule out the effect of EVLP before transplantation, separate handling of the bilateral lungs gives an opportunity to investigate the causal role of pharmacological EVLP to repair donor lung injuries and the preparation for post transplantation events. Furthermore, independent ventilation with precise control of transplanted graft following reperfusion allowed us to directly obtain samples or measurements reflecting the functional status of the grafts and the degree of lung injury in vivo (103).

Donor lungs retrieved after prolonged WI elicited significant formation of MDA during EVLP, a marker of lipid peroxidation (194), pointing to the oxidative degradation of lipids with the process of lipid electrons loss and cell membrane damage mediated by the free radicals (195). MDA, as well as 4-hydroxynonenal (4-HNE) of reactive aldehydes, are two end products of in vivo lipid peroxidation (194) initiated by the PN and finally the activation of nuclear enzyme PARP for the induced DNA damage (56). WI lungs with copious MDA formation implied the triggering of “PN/PARP pathway”, which was entirely in line with massively increased 3-nitrotyrosine (3-NT) and poly (ADP ribose) (PAR), a footprint of PN generation and a marker of PARP activity respectively, as shown in our previous study (92). It is particularly noteworthy that due to the acellualr EVLP, there are possible other reactive oxidants instead of well recognized

infiltrating neutrophils as the main source of ROS/RNS contributing to the IRI. Indeed, the phase of our EVLP is more appropriately to be regarded as “anoxia-reoxygenation”, of which the endothelium and epithelium in the perfused lungs are the origins of oxidant production related injury (46). In addition, WI_E lungs were associated with considerable MDA production following Ltx and 2 hours blood reperfusion, as compared to either CI lungs or per se before Ltx (Figure 8.3D), further reflecting the pathogenic role of PARP in oxidant injury in the lung grafts after transplantation.

Ex-vivo delivery of 3-AB largely suppressed the enhanced oxidative stress of WI lungs with reduced MDA following transplantation, as compared to the non-treated lungs and was identical to the CI lungs, suggesting potent protective effect of such ex-vivo treatment. In addition, WI lungs treated with 3-AB before Ltx was associated with non increased MDA, consistent with mitigated PAR and 3-NT in the previous observation (92), pointing to the reduced generation of reactive oxidants owing to the PARP inhibition, probably as well as its direct anti-oxidant property (135).

Lung edema development is of the prominent consequences presented in IRI. Lungs underwent WI revealed significant edema formation during EVLP, as evidenced by the markedly enhanced lung weight gain and protein content in BALF, and was further pronounced when reperfused, implicating increased dysfunction of epithelial integrity (145). This alteration of protein-enriched edema, however, was significantly abrogated by 3-AB, either during EVLP or post Ltx, shedding light on its protective effect of alveolar-capillary membrane. In fact, biomembrane toxicity of ROS/RNS particularly lipid proxidation during reperfusion is the key factor inducing disruption of pulmonary endothelium and increased permeability (145, 195).

In this study, we demonstrated clearly that cell necrosis was drastically promoted during EVLP lung of WI, shown as its general marker LDH. Taken together with the elevated PAR (substrate of PARP) (92), this result was consistent with the view of oxidant-dependent PARP activation pathway incurred in those lungs, which led to subsequent nicotinamide adenine dinucleotide (NAD) and ATP depletion then ultimately resulted in necrotic cell death (56). In contrast, 3-AB abolished the increased LDH release during both EVLP and blood reperfusion period, indicating the cytoprotective property of this compound mediated by the PARP inhibition. Similar results were reported in other type of cells as myocytes (196), renal (197) epithelial cells, thymocytes (198) etc, to cite only a few.

Another major finding of the study was the inflammatory response. WI_E lungs exhibited heightened pro-inflammatory cytokine IL-6 following EVLP, pointing to a redox-dependent modulation of inflammatory signaling in the ischemic lung. Notably, such up-regulation further responded up to 10-fold at 2 hours after transplantation, reflecting the critical role of cellular restoration featured reperfusion in the development of inflammation. Mainly secreted by macrophages, IL-6 is induced following the activation of signaling pathway, namely, p38 MAP kinase (MAPK) and nuclear factor kappa B (NF-κB) (140), the activation of both are also attributed to the initiation of TNF-α (199), an

important signaling protein highly involved in systemic inflammation. Furthermore, prevailing in innate immune response and inflammation, pro-inflammatory cytokines mediated NF-κb activation yields the expression of chemokines, which acts as a chemoattractant for the neutrophil recruitment, such as IL-8 and its counterpart CINC-1 in rats (200). WI lungs at the end of EVLP or after subsequent blood reperfusion exerted considerably over-expressed TNF-α and CINC-1, reinforcing the fact of activated inflammatory cascade. Anti-inflammatory effect of 3-AB on damaged WI lung was associated with markedly attenuated pro-inflammatory cytokines and chemokine as discussed above, implying the central role of PARP in mediating inflammatory response. It must be underscored that a principle function of activated PARP relies in its pro-inflammatory promotion of many inflammatory mediators, cytokines, chemokines and adhesion molecules (201). Therefore inhibition of PARP and PN promotes the down-regulation of pro-inflammatory signal pathway, which produces a self amplifying cycle and noxious cellular spilling to neighboring tissue for inflammatory cell injury (56).

A straightforward consequence of lung edema and activated inflammatory response is the exacerbation of pulmonary physiological function, as observed in this study. WI injured lungs were associated with poor compliance and elevated PAWP over the period of EVLP, and displayed a persistent physiological deterioration after transplantation. This observation was in agreement of the development of interstitial and alveolar edema that resulted from the dysfunction of endothelial barrier, in the context of PGD featured IRI (202). As we expected, improved lung compliance and decreased airway pressure were shown in the WI_E lungs treated by 3-AB, reflecting the protective property of endothelial hyperpermeability owing to the PARP inhibition. Surprisingly, beneficial effect of 3-AB treatment in WI lungs was limited with regard to the unimproved PVR and $DppO_2$ during EVLP. It has been shown that PN possesses potent pulmonary vasodilating activity (147); therefore we speculate that the lack of such PN-dependent vesodilation due to the possible direct antioxidant capacity of 3-AB (92, 203), may make up to the unchanged PVR. Based on our previous observation (92) and disclosed by Yeung et al (146), alteration of $DppO_2$ as an index of oxygenation capacity on the EVLP lungs is probably misleading, due to the application of acellular perfusate. Nevertheless, after transplantation when graft circulation reestablished, WI_E-3-AB lung was characterized with improved respiratory function at the end of reperfusion, as shown with significantly lower OI. It is worthy to mention that we use OI, defined as the reciprocal of PaO_2/FiO_2 ratio times MAP, to better represent the severity of oxygenation dysfunction because it takes airway pressure into account (204). Therefore, lower OI refers to a better *in vivo* lung oxygenation capacity in our experimental Ltx setting.

Injured WI lungs presented an elevated cell migration after 2 hrs reperfusion in the BALF, deriving from cytokines-mediated inflammatory cascade. Ex-vivo 3-AB treatment prevented the increase of cell migration, underscoring its preponderant property of down-regulated inflammation in IRI (47). Interestingly, we found that accumulated leukocytes in BALF were predominated by MNs instead of PMNs, although CINC-1(neutrophil attractant) was overexpressed in all the transplanted lungs when

reperfused (Fig 7C). Vigorous experimental evidences have been assembled to show a biphasic pattern of IRI in Ltx, of which donor macrophages mainly mediate the early phase of reperfusion injury, whereas in the delayed phase, recipient neutrophils are mostly involved (205, 206). Since we particularly sought to assess acute consequences of 2 hours reperfusion of transplanted donor grafts with or without *ex-vivo* 3-AB reconditioning, our observation was in agreement of the exhibition of bimodal pattern in IRI. Indeed, study with specific antibodies against PMNs confirmed that neutrophil-mediated inflammatory events occurred primarily after 4 hours of reperfusion (207), conversely, first few hours of reperfusion is independent from neutrophils, during which macrophages exert more important roles (208-210).

In summary, our study demonstrated that EVLP of DCDD lungs underwent extended warm ischemia displayed notable ischemia reperfusion injury following transplantation, as evidenced by increased oxidative stress, lung inflammation, pulmonary edema, cell death and deteriorated lung function. Ex vivo delivery of PARP inhibitor 3-AB markedly alleviated these alterations, ensuring the adequacy of damaged DCDDs lungs for the subsequent transplantation. Our results elucidate the central role of intervening PARP-mediated IRI after lung transplantation, and further highlight the efficacy of EVLP as a therapeutic mean to recondition marginal lungs to eventually expand the donor pool.

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Chapter 10

General conclusions and future directions

Today lung transplantation is the only curative treatment for various end-stage lung diseases, but the proportion of donor lungs considered usable for transplantation remains low, leading to high waitlist mortality. It is therefore of paramount importance to increase the availability of donor lungs.

One of the principle reasons for the low acceptance rate of potential donor lungs is the concern about graft dysfunction after transplantation resulting from ischemia reperfusion injury.

The donor lung needs precise evaluation prior to transplantation. Since introduction of LTX in clinical practice the donor lung evaluation consisted of bronchoscopic and radiological examination and lung functional tests, etc, allowing identifying usable organs. With the increasing number of transplants performed using extended criteria donor lungs or lungs from DDCD donors, this may approach may become too imprecise and fail to detect usable lungs for transplant.

Improved graft evaluation is mandatory when using extended criteria donor lungs. Ex-vivo lung perfusion provides a more reliable approach to assess potential donor lungs and to identify injured organs before transplant. Moreover, EVLP can serve as a therapeutic platform to treat injured lungs previously deemed to be not transplantable.

In this thesis we have established a model of EVLP to accurately assess rodent lungs and to investigate how different graft ischemic preservation conditions affect ex-vivo lung function. The lungs underwent a period of cold or warm ischemia before EVLP to mimic clinical conditions of ideal or marginal donor lungs. We have demonstrated that this EVLP model allows for quantitative assessment of lung injury related to warm ischemic times. Ischemic damage was associated with worsened pulmonary compliance, vascular resistance and edema. Lung injury as determined by ex-vivo lung function paralleled the biochemical and histopathological assessment of oxidative/nitrosative stress, cell necrosis and perivascular edema.

We have assessed various ex-vivo treatment strategies. We sought to investigate whether damaged rat lungs after warm ischemia could be reconditioned during EVLP by pharmacological inhibition of (a) ROS/RNS formation, or (b) PARP activity, or (c) NF-κB activation, aiming to reduce ischemia reperfusion induced lung injury. We have also determined the therapeutic properties of volatile sevoflurane for ex-vivo post-conditioning on damaged lungs. As expected, lungs exposed to extended warm ischemia developed severe ischemia reperfusion injury, characterized by poor pulmonary function, oxidative stress, PARP activation, tissue injury, and upregulation of inflammation either during EVLP or after lung transplantation. These alterations were significantly reduced by all tested therapeutic strategies delivered during EVLP, demonstrating, how EVLP could be used as a treatment platform for ex-vivo therapies, either to repair donor lung injuries or to prepare for post-transplant events.

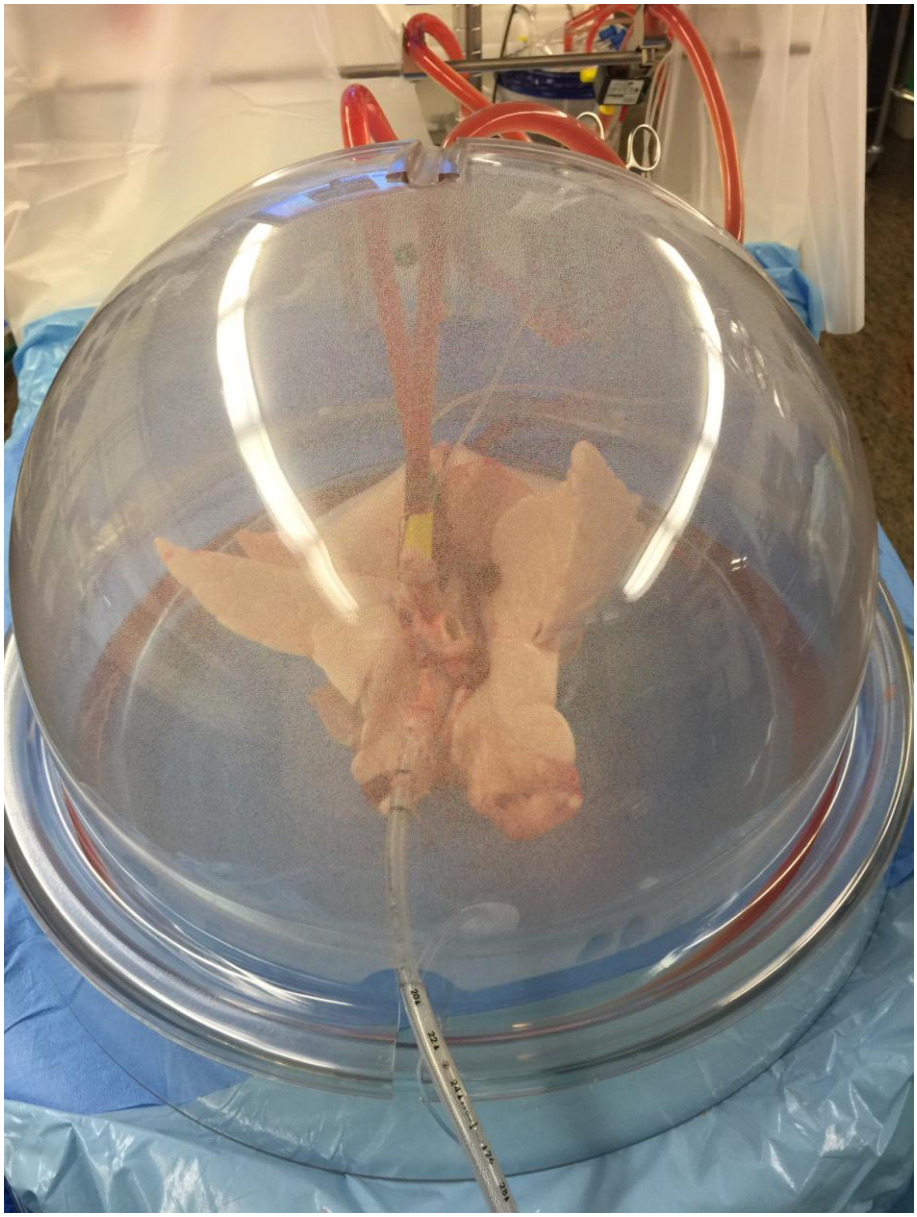

Figure 10.1 EVLP in University Hospital of Lausanne

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