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Normothermic Liquid Ventilation as a Method to Rehabilitate Lungs with Ischemia-Reperfusion Injury for Transplantation in an *Ex-Vivo* Rat Model

D i s s e r t a t i o n

zur Erlangung des Doktorgrades

Dr. med.

(doctor medicinae)

an der Medizinischen Fakultät
der Otto-von-Guericke-Universität Magdeburg

vorgelegt von Helena Linge

aus Hamburg

Magdeburg 24.03.2024

Bibliographische Beschreibung:

Linge, Helena:

Normothermic Liquid Ventilation as a Method to Rehabilitate Lungs with Ischemia-Reperfusion Injury for Transplantation in an *Ex-Vivo* Rat Model. - 2023. - 85 Bl., 15 Abb., 16 Tab.

Kurzreferat:

Lung transplantation remains the only curative treatment for patients with end-stage pulmonary disease. Lung ischemia-reperfusion injury is a major contributor to post-transplant allograft dysfunction and a major cause of donor organ non-utilization. The alveolar macrophage is a key inflammatory mediator in lung ischemia-reperfusion injury. *Ex-vivo* lung perfusion has been investigated to rehabilitate lungs before transplant but has failed to provide significant improvements after ischemic insult. Here a novel method for *ex-vivo* lung reconditioning after significant ischemia-reperfusion injury with improved, clinically relevant physiologic parameters using liquid ventilation is described. These physiologic improvements are the result of the mechanical clearance of alveolar macrophages and inflammatory cytokines which blunt the release of additional inflammatory cytokines halting the propagation of ischemia reperfusion injury. While the wide applicability of these findings to large animal or human transplantation is yet to be explored, these findings represent a method for lung reconditioning from significant ischemic injury that could widen the lung organ donation pool and make a positive clinical impact on patient outcomes.

Schlüsselwörter

Alveolar Macrophage, *Ex-Vivo* Lung Perfusion (EVLP), Ischemia-reperfusion Injury, Liquid Ventilation

Kurzreferart (deutsch):

Lungentransplantation bleibt weiterhin die einzige kurative Behandlungsmethode für Patienten mit terminaler Lungenerkrankung. Die Lunge erleidet während der Transplantation aufgrund von Ischämie und Reperfusion (Ischemia-reperfusion Injury, IRI) eine Schädigung, die zu einer Beeinträchtigung der Transplantatfunktion nach der Transplantation führt. Diese IRI ist weiterhin eine Hauptursache für das ungenutzte Potential von Spenderorganen. Alveoläre Makrophagen spielen eine wichtige Rolle als Entzündungsmediatoren bei der Lungen-IRI. Die *Ex-vivo* Lungenperfusion (EVLP) wurde etabliert, um Lungen vor der Transplantation zu rehabilitieren. Allerdings konnte EVLP noch keine signifikanten Verbesserungen nach IRI aufweisen.

In dieser Studie, wurde über eine neuartige Methode zur *Ex-vivo* Rekonditionierung von Lungen nach schwerer IRI berichtet. Dabei werden verbesserte physiologische Parameter erreicht, bei der die Atemwege mit Flüssigkeit gefüllt und gespült wurden. Die erzielten physiologischen Verbesserungen resultieren aus der mechanischen Entfernung von alveolaren Makrophagen und entzündlichen Zytokinen. Dadurch wird die Freisetzung weiterer entzündlichen Mediatoren minimiert und IRI gehemmt. Obwohl die breite Anwendbarkeit dieser Ergebnisse bei großen Tieren und die Übertragbarkeit auf die Lungentransplantation bei Menschen noch weiter erforscht werden müssen, stellt diese Methode eine Möglichkeit zur Regeneration der Lunge nach schweren ischämischen Verletzungen dar. Sie könnte dazu beitragen, den Spenderorgan-Pool zu erweitern und sich positiv auf die klinischen Ergebnisse der Patienten auszuwirken.

The research and data presented in this dissertation were produced in the laboratory of the Center for Organ Engineering in collaboration with Harvard Medical University and Massachusetts General Hospital, under the guidance of David Becerra, M.D., and principal investigator Prof. Harald Ott, M.D. This project was part of a research scholarship from the Biomedical Education Program. During the research visit, the studies conducted at Otto-von-Guericke University provided the foundational knowledge of the research topic and inspired the development of independent research ideas and skills.

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Index of Abbreviations

Abbreviation	Meaning
%	Percent
%v/v	Volume percent
°	Degree
°C	Degrees celsius
Δ	Change
AM	Alveolar macrophages
ARDS	Acute respiratory distress syndrome
ATP	Adenosintriphosphate
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin fraction
CA	California
CaCl	Calcium chloride
CCR2	Chemokine receptor 2
C_{dyn}	Dynamic compliance
cmH_2O	Centimeters of water
CO	Carbon monoxide
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
COVID	Coronavirus disease
CT	Connecticut
CXCL1	C-X-C motif chemokine ligand 1
CXCL2	C-X-C motif chemokine ligand 2
DAMPs	Damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DCD	Donor after cardiac death
DE	Germany
DMEM	Dulbecco's modified eagle medium
DPBS	Dulbecco's phosphate-buffered saline
DS	Donkey serum
DSO	German organ transplantation foundation
ECAD	E-cadherin
ECD	Extended criteria donors
ECM	Extracellular matrix
ECMO	Extracorporeal membrane oxygenation
et al.	et alii
EVLP	Ex-vivo lung perfusion
F4/80	Major macrophage marker
FBS	Fetal bovine serum
FDA	Food and drug administration

FiO_2	Fraction of inspired oxygen
FL	Florida
g	Gramm
GA	Georgia
Ga	Gage
GRO-α	Growth-regulated alpha protein
H	Hydrogen
h	Hour
H&E	Hematoxylin & eosin
H_2O	Water
HEPA	High-efficiency particulate air
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMGB1	High-mobility group box 1
ICU	Intensive care unit
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IL	Illinois
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 Beta
IL-2	Interleukin 2
IL-6	Interleukin-6
IN	Indiana
Inc.	Incorporated
iNKT	invariant natural killer T
IRI	Ischemia-reperfusion injury
ISHLT	International Society for Heart and Lung Transplantation
iSTAT	Intelligent system for temperature and treatments
KCl	Potassium chloride
kg	Kilogramm
L	Liter
L/S	Large-scale
LAP	left atrial pressure
LIRI	Lung ischemia reperfusion injury
LV	Liquid Ventilation
LW	Lung weight
MA	Massachusetts
MAPK	Mitogen-activated protein kinase pathway
MCP-1	Monocyte chemoattractant protein-1
MD	Maryland
MFI	Mean fluorescence intensity
mg	Milligram
MgCl Hex	Magnesium chloride hexahydrate
MGH	Massachusetts General Hospital

MI	Michigan
min	Minute
mL	Milliliter
mM	Millimolar
mmHg	Millimeters of mercury
MMPs	Matrix metallopeptidasen
MO	Missouri
n	Number
Na	Sodium
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NC	North Carolina
NE	Nebraska
NETs	Neutrophil extracellular traps
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NH	New Hampshire
NJ	New Jersey
NL	Netherlands
NOX	NADPH oxidase
ns	Not statistically significant
NY	New York
O_2	Oxygen
OCS	Organ Care System
OH	Ohio
p	Statistical significance
P/F	Ratio of arterial partial pressure of oxygen to the fraction of inspired oxygen
PA	Pennsylvania
PaO_2	Partial pressure of oxygen
PBS	Phosphate-buffered saline
PEEP	Positive end expiratory pressure
PGD	Primary graft dysfunction
pH	Potential of hydrogen
PLV	Partial liquid ventilation
pMAP	mean pulmonary arterial pressure
PMNs	Polymorphonuclear neutrophils
PRRs	Pattern recognition receptors
PVR	Pulmonary vascular resistance
ROS	Reactive oxygen species
SCS	Static cold storage
SEM	Standard error of the mean
SSC	Saline-sodium citrate

TBS	Tris buffer
TdT	Terminal deoxynucleotidyl transferase
TLR4	Toll-like receptor 4
TLV	Total liquid ventilation
TNF-α	Tumor necrosis factor alpha
TUNEL	terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling
TV	Tidal volume
TX	Texas
UK	United Kingdom
µl	Microliter
µm	Micrometer
USA	United States of America
VECAD	VE-cadherin
W/D	Wet-to-dry ratio
WA	Washington
WI	Wisconsin
XPS	XVIVO System
ZO-1	Zonula occludens 1

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1. INTRODUCTION

Lung transplantation remains the only curative treatment modality for patients with end-stage pulmonary diseases, such as COPD, cystic fibrosis, pulmonary fibrosis, or pulmonary arterial hypertension among other pathologies[1]. The shortage of donor lungs and the troublingly low rate of donor organ utilization increases waitlist mortality[1]. According to the data from the Global Observatory on Donation and Transplantation, as of 2020, approximately 39,000 patients were waiting for a lung transplant worldwide. The number of patients waiting for lung transplantation has been steadily increasing in recent years, and there is a growing need for effective strategies to increase the number of available donor lungs for transplantation. According to the data from the German Organ Transplantation Foundation (DSO), as of December 31, 2021, approximately 593 patients were waiting for a lung transplant in Germany. This number has been fluctuating in recent years, with a decrease in the number of patients waiting for lung transplantation in 2020 due to the COVID-19 pandemic. Only 15% of donor lungs are deemed to be acceptable for transplantation after organ harvest[2]. Certain parameters have to be met to allow a lung to be considered for transplantation, which secures the success and well-being of the patient but also limits the number of available donor lungs[3], [4].

Generally, the donor lung must be able to provide adequate oxygenation and carbon dioxide removal for the recipient. The specific parameters for blood gas exchange in a lung transplant vary depending on the individual case. The lung must also be able to effectively exchange gases during mechanical ventilation. The pulmonary vascular system must be functioning to support blood flow. Furthermore, the donor lung must be free from malignant tumors, or infections that could spread to the recipient. Significant damage may compromise the function of the lung. Lastly, the donor lung must be appropriately matched to the recipient based on factors such as blood type, size, and immunological compatibility. Regarding the ideal lung donor criteria, the age of the donor should be between 20 and 45 years old, have no history of smoking, have a clear chest X-ray, have under 5 days of ventilation, have a negative gram stain, have clear bronchoscopy and a ratio of PaO_2/FiO_2 over 350 mmHg as well as an ischemic time below 4 hours [4], [5]. Many transplant centers have embraced the use of extended criteria donors (ECD) in lung transplantation due to the realization that the traditional empiric criteria may be too strict. Although ECDs may not fully meet the standard criteria, certain centers still deem them suitable for transplantation[6]. Mulligan MJ *et al.* published a review utilizing the United Network of Organ Sharing database to investigate the impact of using extended criteria donors on one-year survival rates in high-risk lung recipients. The study revealed that the utilization of these donors resulted in a decrease in the one-year survival rate among these recipients. This emphasizes the importance of meticulous donor selection for lung transplantation in high-risk recipients and the importance of finding approaches to regenerate marginal lungs before transplantation[7].

1.1 The Idea behind *Ex-vivo* Lung Perfusion

Ex-vivo lung perfusion (EVLP) was developed to assess potential donor lungs after procurement to increase the pool of available lungs for transplantation[8], [9]. It soon became apparent that this platform could be used to treat and improve lung function before transplantation, transforming an organ that would have initially been rejected into a usable allograft[10]. The system makes it possible to evaluate the parameters needed for successful transplantation and also shorten ischemic periods during the evaluation time before actual *in vivo* transplantation. The potential benefit to patients is profound, increasing the donor organ pool and decreasing both waitlist time and waitlist mortality. EVLP has also become an emerging tool in lung transplant research to evaluate mechanisms of lung injury and recovery in an organ-specific environment[11]–[13].

1.1.1 Short History of EVLP

Normothermic *ex-vivo* organ perfusion was first described in 1935 when Carrel and Lindbergh successfully perfused the thyroid glands of animals for up to a week[14]. Human lung transplantation started in 1963, but the survival rate was low due to complications such as rejection, infections, and bronchial anastomotic issues[15]. However, advancements were made including the first successful heart/lung transplantation in 1981 as well as single and double lung transplantations[16]–[18]. Normothermic EVLP was explored in the 1980s but had limited success[19]. In the mid-1990s, Steen *et al.* developed a new method for *ex-vivo* lung assessment, allowing objective evaluation of non-heart-beating donor lung and transplantation in 2000[20]. Subsequent studies demonstrated successful lung transplantation after *ex-vivo* lung reconditioning using EVLP[21]–[23]. The Toronto protocol, initiated by Cypel *et al*., introduced extended EVLP reassessment of lung function for transplantation[24].

1.1.2 The Concept of EVLP

EVLP is a technique used to assess and treat donor lungs that are considered marginal for transplantation[25], [26]. During EVLP, donor lungs are removed from the body and perfused with a sterile, oxygenated solution, allowing for assessment of lung function and repair of any injury that may have occurred during the preservation period. This process also provides an opportunity to administer therapeutic interventions, such as antibiotics or anti-inflammatory agents, to improve lung function and viability[27]. EVLP has the potential to increase the number of viable donor lungs available for transplantation, ultimately improving outcomes for patients waiting for a lung transplant[26]. EVLP is employed for donor lungs that are considered high-risk and fulfill at least one of the following five conditions: (I) PaO_2/FIO_2 ratio lower than 300 mmHg; (II) evidence of pulmonary edema on the last chest X-ray; (III) poor lung/lobe compliance; (IV) high-risk history; (V) lung from marginal donor after cardiac death (DCD)[8], [28].

1.1.3 General EVLP Assembly

The basic assembly of an *ex-vivo* lung perfusion system usually consists of several components. First, the donor lungs are carefully removed from the donor and transferred to a sterile chamber. Next, the lungs are connected to a specialized ventilation system that supplies oxygen and eliminates carbon dioxide via an endotracheal tube.

Subsequently, the lungs are connected to a perfusion circuit, which facilitates the circulation of a sterile, oxygenated solution through cannulas connected to the pulmonary artery and left atrial cuff. The perfusion circuit commonly includes a pump, a heat exchanger, a reservoir, an oxygenator, an air filter, O_2 sensor to ensure the required temperature, pressure, and sterility of the solution[29].

To monitor the function of the lungs during EVLP, various sensors may be attached to the circuit to measure parameters such as oxygen and carbon dioxide levels, pulmonary artery pressure, and airway resistance[26], [29].

Furthermore, within the circuit, therapeutic interventions may be administered to improve lung function. Overall, the assembly of an EVLP system necessitates specialized equipment and expertise to guarantee the optimal perfusion and functionality of the donor's lungs[26].

1.1.4 Commercially available EVLP Systems

There are several EVLP systems available, each with unique features. The XVIVO Perfusion System (XPS™) (XVIVO, Gothenburg, Sweden) is a self-contained device designed for the Toronto protocol, featuring a perfusion and ventilation circuit and the acellular Steen Solution™ (XVIVO, Gothenburg, Sweden) as the perfusion solution[30]. Notably, the XPS™ distinguishes itself from other systems by enabling X-ray imaging while undergoing EVLP[31].

Vivoline Medical offers the LS1 system (Vivoline Medical AB, Lung, Sweden), which is a static system that requires the use of the Disposable Lung Set™. This system is designed to complement the XPS™system[32]. The Lung Assist (Organ Assist b.v., Groningen, Netherlands), developed by Organ Assist, uses a rotary pump and allows in situ and ex-situ assessment [31], [33]. The Organ Care System™ (TransMedics, Inc., Andover, Massachusetts, USA) is a portable device for transporting donor organs, reducing cold ischemic time. The OCS™Lung is currently involved in various international trials such as the INSPIRE and EXPAND trials [31]. Each of these EVLP systems has its advantages and limitations, and the choice of system may depend on the specific needs of the patient and transplant center[26].

1.1.5 Clinical Relevance - Clinical Trails

The purpose of clinical trials involving EVLP systems is to evaluate the safety and efficacy of these systems for lung transplantation. The trials aim to determine whether EVLP can improve outcomes such as post-transplant lung function, survival, and incidence of primary graft dysfunction (PGD) compared to standard lung preservation methods. The trials also assess the

feasibility and practicality of using EVLP in clinical settings. Overall, the goal is to advance the field of lung transplantation by improving organ preservation and increasing the number of viable donor lungs available for transplantation.

The INSPIRE trial (Trial ClinicalTrials.gov Identifier: NCT01630434) is a large international study that compares the use of normothermic storage for donor lungs using the Organ Care System™Lung (EVLP) to static cold storage (SCS) treatment. The trial included 320 patients who were randomly assigned to either treatment group. The main comparison criteria are the 30-day survival rate and the presence of PGD grade 3 within the first 72 hours after transplantation. Results showed that the EVLP group had a significantly lower incidence of PGD grade 3 after transplantation, indicating improved clinical outcomes compared to SCS. The trial also demonstrated the safety of using OCS™Lung for perfusion times of about 4 hours[31], [34].

The EXPAND (ClinicalTrials.gov Identifier: NCT01963780) trial is a phase 3 international, multicenter study that is prospective and non-randomized. It focuses solely on assessing the safety and efficacy of OCS™Lung while also considering the recruitment, preservation, and evaluation of expanded-criteria lungs for transplantation. The study involves 55 participants, and its evaluation criteria include observing the occurrence of grade 3 PGD within 72 hours and monitoring 30-day mortality rates.

The NOVEL Lung Trial (ClinicalTrials.gov Identifier: NCT01365429) is an ongoing, non-randomized, multicenter trial mandated by the FDA. The trial compares EVLP-treated lungs with a control group of lungs selected based on standard criteria after lung transplantation. The interim results published between May 2011 and May 2014 showed that 42 out of 76 EVLP-treated lungs (55%) were transplanted and compared to 42 control lungs. The EVLP was performed using the Toronto protocol and the XPS™. Early outcomes, such as PGD, duration of mechanical ventilation, ICU stay, hospital stay, 30-day mortality, and 1-year survival rate were comparable between the two groups[26], [35], [36].

The HELP trial (ClinicalTrials.gov Identifier: NCT01190059) is the first prospective clinical trial published in 2011. It involved 20 out of 23 high-risk donor lungs (87%) that underwent EVLP treatment and were subsequently transplanted. There were no significant differences in PGD, days on mechanical ventilation after transplantation, stay in the ICU, hospital stay, and 30-day mortality when compared to the control group of 116 patients who underwent normal transplantation criteria[8], [36], [37].

The DEVELOP-UK trial (Current Controlled Trials ISRCTN4492241) conducted in the UK between April 2012 and October 2015, aimed to compare non-randomized reconditioned extended-criteria lungs to a group of standard-criteria lungs. The trial involved the comparison of extended-criteria donor lungs (EVLP group, 102 patients) and standard-criteria donor lungs (control group, 306 patients) using the Lund protocol and Vivoline LS1 device. However, due to a poor

recruitment rate for the EVLP arm and unfavorable outcomes of the EVLP group, the Trial Steering Committee decided to stop further recruitment of patients for EVLP-treated lungs[38].

Out of 54 EVLP-treated lungs, 18 (34%) were transplanted in the study. The estimated survival rate at one year was lower in the EVLP group than in the control group, although this difference was not statistically significant. The EVLP-treated patients had a higher incidence of early graft injury and required more extracorporeal membrane oxygenation (ECMO) support. As a result, the estimated survival rate was lower in the EVLP group than in the control group[36], [37].

1.2 Current Issues with Lung Regeneration

1.2.1 Lung Ischemia-reperfusion Injury

Despite efforts to minimize lung ischemia during organ procurement and transport, lung ischemia-reperfusion injury (IRI) is still a major contributor to post-transplant allograft dysfunction[39]. Reperfusion injury is a common occurrence in clinical practice when dealing with tissue ischemia. The extent of the injury is directly proportional to the length of the ischemic period, prompt reperfusion may not be operational[40]. Even with short ischemic periods, reperfusion can still result in cellular stress. IRI denotes the functional and structural changes that occur during the restoration of blood flow after a period of ischemia. While restoring blood flow can reverse ischemia, it can also cause harmful effects such as cell necrosis, cell swelling, and non-uniform restoration of flow to all tissue[40], [41]. Dysfunctional alterations in the vascular, endothelial, and mitochondrial systems result in the reflow phenomenon, which causes reduced local perfusion, edema, and other related consequences. Hence, it is imperative to recognize IRI and explore novel treatment modalities to improve patient outcomes. The interplay between reactive oxygen species (ROS), inflammatory cytokines, and complement activation can lead to a cascade of events that exacerbate tissue injury and dysfunction during IRI[42].

Research indicates that ROS is the initial trigger in IRI, particularly during reperfusion[43]. ROS are highly reactive molecules that activate various signaling pathways. During reperfusion oxygen is reintroduced leading to an imbalance between ROS generation and clearance[43], [44]. ROS production elevates in various cell types including macrophages, endothelial cells, vascular smooth muscle cells, and alveolar type II cells[45]. Increased ROS levels contribute to apoptosis, intracellular calcium overload, and innate immune responses[46], [47]. Usually, mechanisms counterbalance ROS, but these protective processes are overwhelmed by reperfusion[48]. Subsequently, ROS initiates the activation and synthesis of inflammatory cytokines, sterile inflammation, and the emergence of ionic derangements downstream[49].

The response to IRI is rapid and complex and results in a major release of cytokines, immune activation, and damage-associated molecular patterns (DAMPs)[50]. As mentioned above, ROS plays a pivotal role in initiating these cascades. Proinflammatory molecules such as IL-1β, IL-2, TNF- and IFN-γ increase after reperfusion[51]. A group of inflammatory mediators, DAMPs,

including high-mobility group box 1 (HMGB1), fibronectin, heat shock proteins, and oxidized phospholipids activate pattern recognition receptors (PRRs) specifically, toll-like receptors (TLRs) are associated with IRI and sterile inflammation through NF-κB signaling[52]–[54]. The release of these cytokines and DAMPs induces tissue damage and triggers sterile immune responses through both innate and adaptive mechanisms[55].

Sterile inflammation arising from non-infectious injuries such as IRI involves immune resembling those observed during microbial invasion[56]. Both innate and adaptive immune mechanisms are activated in regions of sterile injury and cell death[56], [57]. The innate immune response is initiated first[58]. This leads to the infiltration of polymorphonuclear neutrophils (PMNs) and activation of invariant natural killer T (iNKT) cells and alveolar macrophages. Various cellular adhesion molecules facilitate the binding of PMNs to endothelial cells and chemokines produced by lung macrophages, epithelium, and endothelium propel the recruitment and adherence of immune cells in IRI[42], [50]. The migration of activated leukocytes into extravascular and alveolar spaces induces microvascular permeability by releasing ROS, proteases, and elastases and promoting the formation of gaps in the endothelium[59]. The adaptive immune system is also activated during IRI with T-Cells being activated by antigen-dependent and -independent pathways[60]. B-cells are also activated contributing to antibody-mediated tissue injury[60].

Other cellular mechanisms include metabolic changes, ionic imbalances, and mitochondrial dysfunction. Oxygen depletion causes a decrease in oxidative phosphorylation resulting in reduced ATP levels and a shift to anaerobic metabolism[61], [62]. The accumulation of ATP breakdown products and lactate leads to increased ROS production and cellular acidosis[45], [61]. Elevated levels of hydrogen ions during acidity lead to intracellular accumulation of sodium (Na+/H+), which attracts water resulting in cellular swelling[63]. Ischemia also induces an elevation in intracellular calcium and sodium due to the dysfunction of ATP-dependent cellular ion pumps[64]. Calcium overload contributes to vasoconstriction, induction of apoptosis, and loss of endothelial junctions resulting in higher vascular permeability[41], [65]. Ion imbalance also impacts mitochondria leading to swelling and apoptosis[66].

The inflammatory response triggers the activation of the complement system and coagulation cascade leading to elevated platelet aggregation and microvascular permeability[67]. It also results in the release of anaphylatoxins and the formation of membrane-destructive complexes further triggering endothelial vascular permeability[68], [69]. Additionally released circulating cytokines initiate platelet aggregation and coagulation[43]. Activated platelets play a role in pulmonary edema formation by releasing vasoactive mediators (ROS, Serotonin, thromboxane A2, and platelet-activating factor) and binding to the pulmonary endothelium leading to microthrombus formation, microvascular constriction, and leukocyte adhesion which contributes to neutrophil infiltration[69], [70].

IRI targets the pulmonary vascular endothelium leading to increased permeability of the alveolar-capillary barrier and the development of pulmonary edema, which reduces oxygenation[71], [72]. Additionally, IRI causes neutrophils to migrate into the extravascular space that releases cytokines, ROS, and neutrophil extracellular traps (NETs), which when accumulated, can mediate atelectrauma, epithelial injury, and damage endothelial barrier[73]. Leukotrine B4 and Matrix metallopeptidases (MMPs) further increase inflammation and lung pyroptosis [73], [74]. Histones secreted by NETs activate neutrophils via TLRs and promote epithelial cell death.[75], [76]. The damage to the basement membrane of the alveolar-capillary barrier induces the formation of hyaline membrane and fibrine and surfactant is inactivated. Neutrophil adherence to the endothelial lining triggers a proinflammatory response involving β2-integrin and calcium-dependent cytoskeletal rearrangement, which contributes to the formation of gaps between endothelial cells[42]. Edema is caused by altered hydrostatic pressures, osmotic forces, and increased permeability allowing fluid and proteins to extravasate[43], [76]. Protein-rich fluid floods the alveoli, creating edema and reducing gas exchange[76]. Intestinal flooding is facilitated by intravascular coagulation leading to platelet aggregation and microthrombi formation due to increased vascular permeability[67], [77]. Lastly, alveolar epithelium is negatively impacted, exhibiting increased permeability and a compromised ability to remove alveolar fluid, resulting in edema[78]. However, research is still lacking in investigating the harm inflicted on the alveolar and bronchial epithelium.

When looking into the role of alveolar macrophages (AM), research in this area indicates that activation of alveolar and interstitial macrophages plays an instrumental role in the initiation and progression of lung IRI[79], [80]. AMs are immune cells that reside in the lung alveoli and play an important role in the response to lung injury and inflammation. Different subsets of macrophages, such as M1 and M2, are involved in the initiation and resolution of inflammation. M1 macrophages promote inflammation and tissue damage, while M2 macrophages have anti-inflammatory and tissue repair functions[72]. During ischemia, macrophages are exposed to hypoxic and oxidative stress and can be activated by DAMPs leading to cytokine secretion which promotes neutrophil recruitment, endothelial activation, and mast cell degranulation[39], [80], [81]. The subsequent inflammatory response results in the physiologic hallmarks of IRI such as interstitial and alveolar edema, decreased lung compliance, and increased pulmonary vascular resistance followed by poor oxygenation and primary graft failure[82].

Naibu B *et al*. described that the depletion of AMs resulted in a 53% reduction in lung vascular permeability compared to the control group. This reduction in permeability was accompanied by a 50% reduction in tissue myeloperoxidase content and a decrease in leukocyte accumulation in the bronchoalveolar lavage fluid. Additionally, the depletion of AMs led to a significant decrease in the production of proinflammatory chemokines cytokines and nuclear transcription factors in the lung tissue[79].

During ischemia-reperfusion, AMs are activated partly through the binding of DAMPs to toll-like receptor 4 (TLR4)[83]. Activation of TLR4 leads to the activation of nuclear factor-κB (NF-κB) which is responsible for inducing inflammation and causing injury to the lungs[52]. The activated Inflammatory M1-like macrophage secretes neutrophil chemokines CXCL1 and CXCL2 and proinflammatory cytokines, which then recruit neutrophils and monocytes [39], [84].

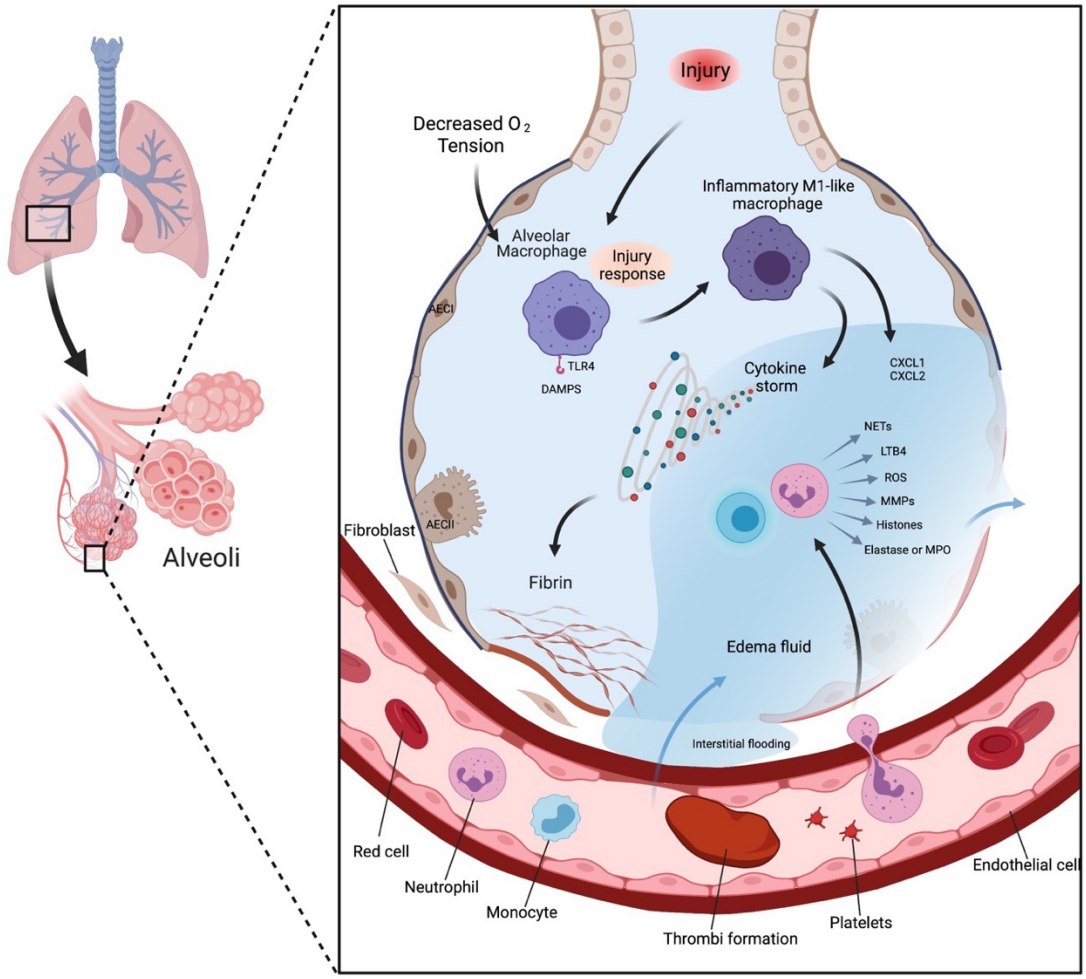

Figure 1. *Overview of ischemia-reperfusion injury in the lung. The process of IRI involves a robust inflammatory response and heightened oxidative stress. These responses are initiated during the initial ischemic phase and further exacerbated during reperfusion. Macrophages are subjected to hypoxic and oxidative stress, potentially leading to their activation by DAMPs. This activation triggers the secretion of cytokines, which in turn promotes the recruitment of neutrophils, endothelial activation, and mast cell degranulation. This cascade further leads to the migration of white blood cells and the activation of platelets. TNF-mediated expression of tissue factor encourages platelet aggregation, microthrombus formation, intraalveolar coagulation, and hyaline membrane formation. These processes collectively culminate in damage to the alveolar-epithelial barrier. The inflammatory response results in the physiologic hallmarks of IRI, including interstitial and alveolar edema, decreased lung compliance, and increased pulmonary vascular resistance resulting in compromised oxygenation and primary graft failure. IRI: Ischemia-reperfusion injury, DAMPs:*

Damage associated molecular patterns, TNF: Tumor necrosis factor. Figure created with BioRender.com.

1.2.2 Primary Graft Dysfunction

Primary graft dysfunction (PGD) is a serious complication that can occur after lung transplantation. It is a form of acute lung injury that can result from ischemia-reperfusion injury. PGD can manifest within the first 72 hours after transplantation[85]. The International Society of Heart and Lung Transplantation (ISHLT) established a standardized definition of PGD in 2005. The assessment involves evaluating the PaO_2/FiO_2 ratio and the presence of bilateral infiltrates on a chest radiograph (findings of PGD are nonspecific and may include perihilar ground glass opacities, reticular interstitial and parenchymal opacities, and perivascular thickening). These assessments are conducted at specific time points (6h, 24h, 48h, and 72h) after surgery using FiO_2 of 1.0 and PEEP of 5 cmH_2O. In 2016, an updated statement from ISHLT specified the starting time for the PGD clock as the removal of the pulmonary artery cross-clamp of the second lung[86]. PGD is characterized by acute respiratory distress syndrome (ARDS)-like symptoms such as hypoxemia, bilateral infiltrates on chest imaging, decreased lung compliance, and increased pulmonary vascular resistance[87]. The severity of PGD can range from mild to severe, and it can lead to prolonged mechanical ventilation, longer hospital stays, and increased mortality[88].

1.3 Liquid Ventilation

Acute respiratory distress syndrome (ARDS) can lead to respiratory failure and death. The mortality rate of ARDS remains high despite advances in critical care medicine. This highlights the need for alternative therapies that can improve the outcomes of patients with ARDS[89]. The idea of Liquid ventilation (LV) as a treatment method is advancing from a theoretical and basic research standpoint to a clinical implementation. Since 1962 this fringe concept has been subject to various studies Kylstra *et al*. investigated the effects of breathing saline oxygenated at 6 atmospheres on mice and their ability to sustain gas exchange and the effects of hypoxia to tissue[90]. LV was first introduced to minimize lung injury by reducing alveolar surface tension during mechanical ventilation to prevent injury related to repeated atelectasis and barotrauma[91], [92]. Published in 1989, LV has shown promise in the neonatal population for premature lung diseases by decreasing alveolar surface tension, promoting homogenous lung expansion, and increasing gas exchange at a tolerable pressure[93]. Partial liquid ventilation in adults, children, and neonates with respiratory failure using perfluorocarbon as an agent with a high capacity to carry O_2 and CO_2 showed safe use and improved lung function[94]. LV has been investigated in different animal models over the past five decades, with healthy models, models with lung injury, and premature lungs[92]. LV can be categorized into two types in the literature: partial liquid ventilation (PLV) and total liquid ventilation (TLV). While in PLV only a part of the

lung is filled with liquid, the rest with air, in TLV the entire lung Is filled with liquid, and no air is issued for ventilation[92], [95]. An issue with traditional TLV in the treatment of ARDS was that TLV was associated with lung injury and decreased lung compliance due to high pressures[96]. Sage *et al.* presented that TLV with lower tidal volume and lower pressure has the potential to reduce inflammation, lower lung injury, and improve oxygenation[96]. Patients with severe respiratory failure on extracorporeal life support have also been treated with LV using an oxygen carrier as a ventilant as a savage treatment with some improvement in gas exchange[94], [97]–[99]. LV has also been useful in introducing ultrafast hypothermia to prevent early inflammatory events. LV had the additional benefit of mechanically removing airway fluid and debris from the respiratory system[100]. Nakajima *et al.* introduced the concept of airway lavage to improve post-transplant lung function after gastric aspiration injury which reduced pro-inflammatory cytokines and lung inflammation[101].

When considering methods for lung rehabilitation from significant IRI, we revisited the concept of LV in an *ex-vivo* setting. The literature is sparse regarding the benefits and effects of LV as a respiratory treatment. Presently, there are no published data on the use of *ex-vivo* LV in the reconditioning of potential lungs for transplantation.

1.4 The Objective of the Project

When considering mechanisms of lung injury, multiple studies have identified activated alveolar macrophages as a key inflammatory mediator in lung ischemia-reperfusion injury[79], [80], [82], [102]–[104]. Depleting macrophages could prevent the development of macrophage-propagated lung injury[79]. Macrophage depletion has been well established in murine models using liposomal clodronate to investigate the role of macrophages in various pathologic processes[105]–[107]. Preliminary data showed that *in vitro*, 1%v/v and 2%v/v selectively deplete macrophages in a population of cells derived from a digested rat lung (analyzed on flow cytometry) in a dose-dependent fashion.

In this study, we describe a unique and novel method for *ex-vivo* lung reconditioning for use in transplantation. The primary aim is to establish an *ex-vivo* LV and perfusion circuit to maintain optimal conditions for lung regeneration. Secondly, a protocol was devised to assess the reconditioning of ischemic lungs using the developed LV circuit. A standardized EVLP circuit was constructed to provide an objective evaluation of the lung functions following reconditioning. We hypothesized that alveolar macrophage-mediated inflammation and ischemia-reperfusion injury can be reduced by treatment on an LV *ex-vivo* circuit. We believe LV clears harmful damage-associated molecular patterns (DAMPs) which facilitate macrophage activation, neutrophil migration into the interstitium, and endothelial dysfunction. Additionally, the mechanical

clearance of pro-inflammatory cytokines from the alveolus released due to cellular damage in the early ischemia-reperfusion phase may allow for cellular metabolic recovery during LV. Thirdly, as a part of the protocol, we implemented a therapeutic intervention aimed at attempting to isolate the macrophage contribution to the inflammatory response by depleting macrophages in the organ with liposomal clodronate.

2. MATERIAL AND METHODS

2.1 MATERIAL

2.1.1 Animal Use Protocol

All animal studies were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee Protocol #2019N000059 and conducted following the Guide for the Care and Use of Laboratory Animals. Lungs were explanted from outbred adult male Sprague-Dawley rats weighing 250-450 g from Charles River Laboratories, Wilmington, MA.

2.1.2 Chemicals

Table 1: Index of applied chemicals

Chemicals	Manufacturer
Bovine serum albumin fraction	Fisher Scientific, Waltham, MA, USA
Calcium chloride	Sigma-Aldrich, St. Louis, MO, USA
D-(+)-glucose	Sigma-Aldrich, St. Louis, MO, USA
DAPI fluromount-G	Invitrogen, Carlsbad, CA, USA
Deionized water	Center for Organ Regeneration, Boston, MA, USA
Dextran	Sigma-Aldrich, St. Louis, MO, USA
Donkey serum	Sigma, St. Louis, MO, USA
Dulbecco's modified eagle medium	Gibco, Life technologies, Carlsbad, CA, USA
Dulbecco's phosphate-buffered saline (DPBS)	Life Technologies Corporation, Grand Island, NY, USA
Eosin	Sigma-Aldrich, St. Louis, MO, USA
Ethanol	national diagnosics, Atlanta, GA, USA
Fetal bovine serum	Gibco, Life technologies, Carlsbad, CA, USA
Hematoxylin, Gill 3	Sigma-Aldrich, St. Louis, MO, USA
HEPES	Sigma-Aldrich, St. Louis, MO, USA
Histo-clear	national diagnosics, Atlanta, GA, USA
Magnesium chloride hexahydrate	Sigma-Aldrich, St. Louis, MO, USA
MAGPIX® Drive Fluid PLUS 4PK	Millipore, Billerica, MA, USA
Mounting medium	Histomount, ThermoFisher Scientific, MA, USA
Mucasol®	Schülke, Norderstedt, DE
Paraformaldehyde	national diagnosics, Atlanta, GA, USA
Perfadex®	XVIVO Perfusion Inc., Gothenburg, Sweden
Potassium chloride	Sigma-Aldrich, St. Louis, MO, USA
Sodium bicarbonate	Sigma-Aldrich, St. Louis, MO, USA
Sodium chloride	Carl Roth, Darmstadt, DE
SSC buffer, 20X	Promega, Walldorf, DE
Tris buffer	Sigma-Aldrich, St. Louis, MO, USA
Triton X-100	Sigma-Aldrich, St. Louis, MO, USA

2.1.3 Medication

Table 2: Index of applied medication

Medication	Manufacturer	Administration
5% Isoflurane	Piramal Pharma Limited, Telangana State, IN, USA	5 mL/min with 2 L/min O2
Oxygen	Center for Organ Regeneration, Boston, MA, USA	
Heparin 1000 units/1ml x10ml	Lambert Vet Supply, Fairbury, NE, USA	no dilution
Liposomal clodronate kit	Liposoma BV, Amsterdam, NL	5.25 mg in 30 mL Perfadex
Antibiotic-Antimycotic (100X), penicillin, streptomycin, and Gibco Amphotericin B	Gibco, Life Technologies, Carlsbad, CA, USA	1 % in Perfusion Solution
Surfactant	CUROSURF, Chiesi Pharmaceuticals, Cary, NC, USA	0.3 mL in 3 mL DPBS

2.1.4 Devices

Table 3: Index of utilized devices

Devices	Manufacturer
Accessory Kit (Induction chamber, Absorption filter, Tubing)	Harvard Apparatus, Inc., Holliston, MA, USA
Anesthetic vaporizer	Harvard Apparatus, Inc., Holliston, MA, USA
Animal clipper	Oster Animal Care, Boca Raton, FL, USA
Autoclave	Thermo Scientific, Waltham, MA, USA
Benchtop vortex mixer	Labnics, Fremont, CA, USA
Centrifuge	Fischerbrand, Pittsburgh, PA, USA
CG4+ Cartridges	Abbott, Chicago, IL, USA
GlucCell	Chemglass Life Science, Vineland, NJ, USA
Computer screen	Acer, New-Taipeh, Taiwan
Data acquisition hardware	Harvard Apparatus Inc., Holliston, MA, USA
Digital stirring hotplate	Thermo Scientific, Waltham, MA, USA
FMS Lab refrigerator (4 °C)	Thermo Fischer, Waltham, MA, USA
Freeze flask	Labconco, Kansas City, MO, USA
Fume hood, MSC-Advantage, Class A2	Thermo Scientific, Waltham, MA, USA
Glucose test strips	Chemglass Life Science, Vineland, NJ, USA
Handheld magnetic separator block	Millipore, Billerica, MA, USA
Histological tissue slide drying plate	Histo-Line Laboratories, Pamtigliate, Italy

In-line pressure transducers	Harvard Apparatus, Inc., Holliston, MA, USA
Incubator 37° C	Thermo Scientific, Waltham, MA, USA
iSTAT	Abbott, Chicago, IL, USA
Lab Upright freezer (- 20°C)	Thermo Fischer, Waltham, MA, USA
Laptop	Dell Technologies, Round Rock, TX, USA
Light microscope	Leica, Wetzlar, DE
Luminex MAGPIX	Millipore, Billerica, MA, USA
Lyophilizer	Labconco, Kansas City, MO, USA
Microplate shaker	VWR International, Radnor, PA, USA
Microtome	Leica Camera, Wetzlar, DE
Nikon Ti-PFS inverted microscope	Nikon, Tokyo, Japan
Oven	Thermo Scientific, Waltham, MA, USA
pH electrode	Thermo Scientific, Waltham, MA, USA
Precision scale	Kern & Sohn GmbH, Balingen, DE
Pressure cooker	Instant Brand, Downers Grove, IL, USA
Pump	Masterflex, Gelsenkirchen, DE
Pump	Harvard Apparatus Inc., Holliston, MA, USA
Suction system	Bench Integrated, Center for Organ Engineering, Boston, MA, USA
Ultra-low freezer (- 80°C) TSX700	Thermo Fischer, Waltham,MA, USA
Ultrasonic cleaner model B200	Branson Ultrasonics, Danbury, CT, USA
Ventilator	VentElite, Harvard Apparatus Inc., Holliston, MA, USA
Vetenary Fluovac	Harvard Apparatus, Inc., Holliston, MA, USA
Vetenary Flurosorber	Harvard Apparatus, Inc., Holliston, MA, USA
Water bath	Fisher Scientific, Waltham, MA, USA
Water purification system	Thermo Scientific, Waltham, MA, USA

2.1.5 Laboratory Material

Table 4: Index of utilized laboratory material

Laboratory Material	Manufacturer
Arterial cannula	David Becerra, Center for Organ Engineering, Boston, MA, USA
Catheter adapter	Harvard Apparatus, Holliston, MA, USA
Connection cap system, wide mouth, with screw cap, 4 GL 18 ports	DWK Life Science, Wertheim, DE
Connector Luer, female/male	B. Braun, Melsungen, DE
Electronic pipette controller	Falcon, Corning, Corning, NY, USA
Funnel	Fischerbrand, Pittsburgh, PA, USA
Glass beakers	Fischerbrand, Pittsburgh, PA, USA
Glass media bottles with caps: 1000mL, 500mL, 250 mL, 100mL	Fisherbrand, Waltham, MA, USA
Instech Luer stub 16 ga	Harvard Apparatus, Holliston, MA, USA
Magnetic stirring bar	Fischerbrand, Pittsburgh, PA, USA
Measuring cylinder	Fischerbrand, Pittsburgh, PA, USA
Mechanical transfer pipettes	Brand, Wertheim, DE
Membrane oxygenator	Harvard Apparatus Inc., Holliston, MA, USA
Membrane ventil, Safeflow	B.Braun, Melsungen, DE
Microscope slide storage box	Carl Roth, Karlsruhe, DE
Organ chamber	David Becerra, Center for Organ Engineering, Boston, MA, USA
Screw caps with boreholes	Carl Roth, Karlsruhe, DE
Silicone tubing (Platinum) L/S 16, 17, 18	Masterflex, Cole-Parmer Instruments Co., Verron Hills, IL, USA
Single use pressure sensor	PendoTECH, Princeton, NJ, USA
Slide holder	Carl Roth, Karlsruhe, DE
Stain tray	Simport, Quebec, Canada
Staining trough	Carl Roth, Karlsruhe, DE
Stirring rod	Thermo Fischer, Waltham, MA, USA
Stopcock	B.Braun, Melsungen, DE
Tube rack	Fischer Scientific, Waltham, MA, USA
Vial rack	Fischer Scientific, Waltham, MA, USA
Vial storage box	Fischer Scientific, Waltham, MA, USA
Whatman HEPA-Vent filter	Fischer Scientific, Waltham, MA, USA
Y-Luer adapters	Harvard Apparatus, Holliston, MA, USA

2.1.6 Consumable Material

Table 5: Index of utilized consumable material

Consumable Material	Manufacturer
Aluminum foil	Fischerbrand, Pittsburgh, PA, USA
Bed underlays	McKesson, Irving, TX, USA
Cannula	B. Braun, Melsungen, DE/ BD Medical Franklin Lakes, NJ, USA
Centrifuge tubes 15 mL, 50 mL	Falcon, Corning, Corning, NY, USA
Chemical absorbent pads	Fischerbrand, Pittsburgh, PA, USA
Closed top screw cap	Thermo Scientific, Waltham, MA, USA
Cover slips 0.15 mm	Fischerbrand, Pittsburgh, PA, USA
Disposable spatula	Cole-Parmer, Quebec, Canada
Disposable sterilization filters	Millipore, Billerica, MA, USA
Disposable transfer pipettes	Thermo Scientific, Waltham, MA, USA
Disposable scalpel	B. Braun, Melsungen, DE
Embedding cassettes	Epredia, Portsmouth, NH, USA
Gauze sponge	McKesson, Irving, TX, USA
Glass beakers	Fischerbrand, Pittsburgh, PA, USA
Glass slides	Fischerbrand, Pittsburgh, PA, USA
Hydrophobic barrier pen	Thermo Scientific, Waltham, MA, USA
Microscope slide with grids	Kova International Inc., Garden Grove, CA, USA
Microtome disposable blades	Leica, Wetzlar, DE
Nitrile exam gloves	McKesson, Irving, TX, USA
Petri dish 35 mm x 10 mm, 100 mm x 15 mm	Falcon, Corning, Corning, NY, USA
Pipette tips (0,5-10 µL, 10-100 µL, 100-1000 µL)	Thermo Fischer, Waltham, MA, USA
Plastic wrap	Total Home, CVS, Woonsocket, RI, USA
Screw clear glass vial 20 mL	Thermo Scientific, Waltham, MA, USA
Serological pipets (50 mL, 25 mL, 10 mL, 5 mL, 1 mL)	Falcon, Corning, Corning, NY, USA
Sharps disposal container	CardinalHealth, Dublin, OH, USA
Sterilization sleeves	McKesson, Irving, TX, USA
Syringes	BD Medical, Franklin Lakes, NJ, USA
Ultrasonic cleaner model B200	Branson Ultrasonics, Danbury, CT, USA
Weighing boat	Carl Roth, Karlsruhe, DE
Zip bag with biohazard symbol	Therapak, Claremont, CA, USA

2.1.7 Other Material

Table 6: List of utilized surgical instruments

Surgical Instruments
Adson tissue forceps straight
Derf needle holders straight, serrated
Eye scissors straight, sharp/sharp
Iris forceps straight, serrated
Micro forceps with round handle curved, smooth
Operating scissors straight, blunt/blunt
Scalpel handle
Scalpel blades, sterile
Sterilizing instrument tray
Tungsten carbide Metzenbaum dissecting scissors straight, blunt/blunt
Tungsten carbide operating scissors straight, sharp/sharp
Vannas spring scissors straight, sharp

(Harvard Apparatus Inc., Holliston, MA, USA)

Table 7: List of applied suture material

Suture Material	Manufacturer
2-0 Silk suture	Ethicon, Inc., Raritan, NJ, USA
Vessel Loops	Aspen Surgical, Caledonia, MI, USA

2.1.8 Mediums, Solutions, Buffers

Table 8: Index of utilized mediums, solutions, and buffers

	Composition	
Albumin-based electrolyte solution	5.03 g/L	NaCl
	0.34 g/L	KCl
	1.67 g/L	D-(+)-Glucose
	0.40 g/L	CaCl
	0.25 g/L	MgCl Hex
	2.53 g/L	$NaHCO_3$
	4.77 g/L	HEPES
	5.00 g/L	Dextran
	70.0 g/L	BSA
	20 mL/L	FBS
	10 mL/L	Antibiotic – Antimycotic (penicillin, streptomycin, Amphotericin B)
	1000 mL	Deionized Water

Antibody diluting solution	5% Donkey Serum in TBS
Blocking solution for immunohistochemistry	5% Donkey Serum in TBS
Ethanol 70%	700 mL Ethanol 95%/ Methanol 5% in 300 mL deionized water
Ethanol 95%	950 mL Ethanol 95%/ Methanol 5% in 50 mL deionized water
Paraformaldehyde 4%	10 g Paraformaldehyde in 1L PBS
Perfusate LV	Dulbecco's Modified Eagle Medium (1X) + 1% Antibiotic-Antimycotic + 2% FBS
Sodium citrate buffer	1:15 Tri-sodium citrate buffer pH 6.0 to deionized water
Tris buffer solution (TBS)	100 mL Tris Buffer in 900 mL deionized water
Wash solution for immunohistochemistry	0.025% Triton in TBS

2.1.8.1 Composition of Commercial Solutions

Table 9: Index of commercial solutions with composition

Perfadex®	XVIVO Perfusion Inc., Gothenburg, Sweden	
	50.0 g/L	Dextran 40
	8.0 0 g/L	Sodium Chloride
	1.00 g/L	Glucose Monohydrate
	0.40 g/L	Potassium Chloride
	0.098 g/L	Magnesium Sulphate 7 H_2O
	0.046 g/L	Disodium Phosphate 12 H_2O
	0.063 g/L	Monopotassium Phosphate
	g.s.	Water for Injection
Dulbecco's Modified Eagle Medium (1X)		Gibco, Life technologies, Carlsbad, CA, USA
	1 g/L	D-Glucose
		L-Glutamine
	25 mM	HEPES
	110 mg/L	Sodium Pyruvate
	1%	Antibiotic - Antimycotic
	2%	FBS

2.1.9 Antibodies

Table 10: Primary Antibodies

Antibody	Host	Dilution	Manufacturer
F4/80	Rabbit	1:1000	Thermo Fischer, Waltham, MA, USA
VECAD	Rabbit	1:1000	Invitrogen, Carlsbad, CA, USA
ZO-1	Rabbit	1:150	Invitrogen, Carlsbad, CA, USA
ECAD	Mouse	1:200	Novus Biologicals, Littleton, CO, USA

Table 11: Secondary Antibodies

Antibody	Dilution	Manufacturer
Donkey anti-Rabbit IgG (H+L) Alexa Flour 647	1:1000	Invitrogen, Carlsbad, CA, USA
Donkey anti-Mouse IgG (H+L) Alexa Flour 647	1:1000	Invitrogen, Carlsbad, CA, USA

2.1.10 Kits

Table 12: Index of utilized kits

Kit	Manufacturer
MILLIPLEX® Rat Cytokine/Chemokine Magnetic Bead Panel	Millipore, Billerica, MA, USA
DeadEnd™ Fluorometric TUNEL System	Promega Corporation, Madison, WI, USA

2.1.11 Software

Table 13: Index of applied software

Software	Manufacturer
GraphPad Prism	GraphPad Software, Boston, MA, USA
HART Regen	Harvard Apparatus Inc., Holliston, MA, USA
Image J	National Institutes of Health, Bethesda, Maryland, USA
Microsoft Excel	Microsoft Corporation, Redmond, WA, USA
Microsoft Word	Microsoft Corporation, Redmond, WA, USA
xPONENT	Luminex, Austin, TX, USA

2.2 METHODS

2.2.1 Experimental Groups and Study Design

Thirty lungs were sorted into two control groups and three experimental groups (n=6 per group). The "non-ischemic control" lungs were procured immediately after cardiac death, flushed with preservation solution Perfadex® (XVIVO Perfusion Inc., Gothenburg, Sweden), exposed to 4 hours of cold preservation stored on ice with lungs inflated, then placed on EVLP for 6 hours for assessment. The "ischemic control" lungs were exposed to 4 hours of *in vivo* warm ischemia after animal sacrifice, followed by lung procurement (described below), flush with preservation solution Perfadex®. Lungs in this group were stored on ice inflated for 4 hours of cold preservation. They were then assessed during 6 hours of EVLP. The "liquid ventilation" lungs were similar to the "ischemic control" lungs with the exception that the 4 hours of cold preservation was replaced with 4 hours of liquid ventilation (described below). The "EVLP-Clodronate" and "LV-Clodronate" groups were similar to the "ischemic control" and "liquid ventilation" groups, respectively, with the exception that liposomal clodronate was added to the flush preservation solution Perfadex® after lung procurement. Figure 2 provides an experimental flowchart for the treatment groups. After the according treatment, lungs underwent either W/D assessment or Bronchoalveolar Lavage (BAL) sampling and histological preparation (n=3 per group).

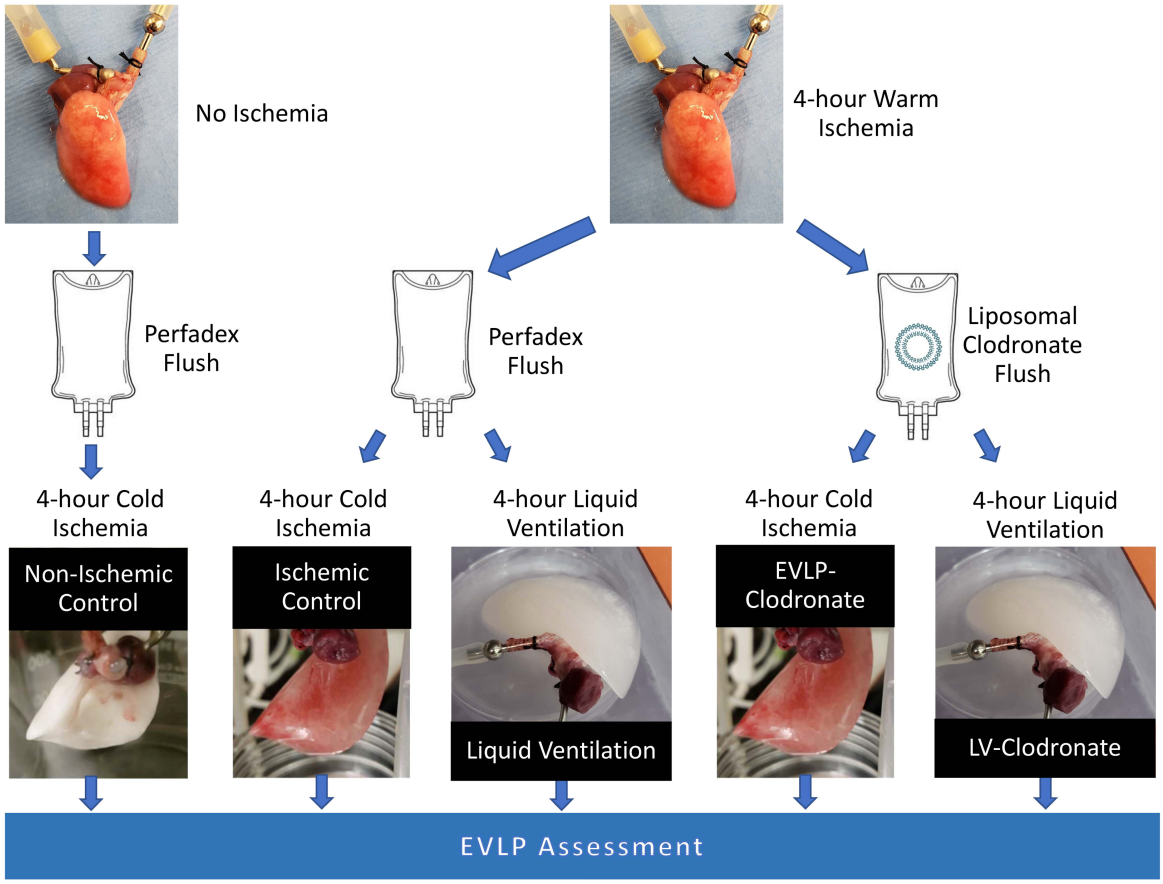

Figure 2. *Experimental flowchart. The experimental flowchart outlines the protocol for each treatment group. EVLP: Ex Vivo Lung Perfusion. LV: Liquid Ventilation.*

2.2.2 Lung Procurement, Treatment, and Assessment

2.2.2.1 Animal Procurement

All rats were pair-housed and given unrestricted access to chow and water before use. Animals were anesthetized with 5% isoflurane and a flow of 2 L/min Oxygen firstly in a constructed box. Before lung harvest, rats were weighed. Fur was removed by an electric shaver from the pelvic area to the neck, preparing for abdominal and thoracic access. To ensure further sufficient anesthesia, the animal was placed once more in the box before being fixed on its back for lung harvest. The skin was disinfected with 70 % Ethanol and the skin was removed from the abdominal and thoracic area. A laparotomy was performed: The abdominal cavity was opened above the liver and a T-incision was performed from under the ribcage to the pelvic area. The intestinal organs were moved aside, out of the cavity, and secured with a blunt instrument. With forceps, the abdominal Vena cava inferior and Aorta under renal vessels were bluntly exposed from surrounding tissue. 0.5 mL heparin was administered intravascularly via the inferior vena cava and left to circulate for 1 minute before the animal was sacrificed via exsanguination by severing the abdominal vessels according to approved protocols.

2.2.2.2 Lung Treatment

Following sacrifice and warm ischemia time (if applicable), a sternotomy was performed: To ensure a collapsed lung, the xiphoid process was elevated, and the diaphragm was removed from the ribcage. The ribs were cut bilaterally from the Sternum ending at both Claviculae. To allow for more operative room, bilateral ribcage, and Sternum were removed (Figure. 3A). To mobilize the lung, pulmonary ligaments were eradicated from the esophagus and the vena cava inferior was severed, without injuring the esophagus. Thymus and tissue lateral of the trachea were bluntly cleared with forceps (Figure 3B). The pulmonary truncus and ascending aorta were secured with a loose 2-0 silk suture. The trachea was cut rather distal from the lung and elevated ventrally from the esophagus. The heart apex and left atrial appendage were resected to allow for pulmonary venous outflow. A custom 18-gauge arterial cannula (Figure 2B) was inserted under continuous PBS flush in the pulmonary artery through the right ventricular outflow tract and secured with the pre-located suture. The heart and lungs were harvested *en-block*[108]–[110]. Similarly, a blunt cannula (Instech Luer Stub 16 Ga (Harvard Apparatus, Holliston, MA, USA)) was secured in the trachea with a 2-0 silk suture and a proximally located vessel loop (Figure 3C & 3D).

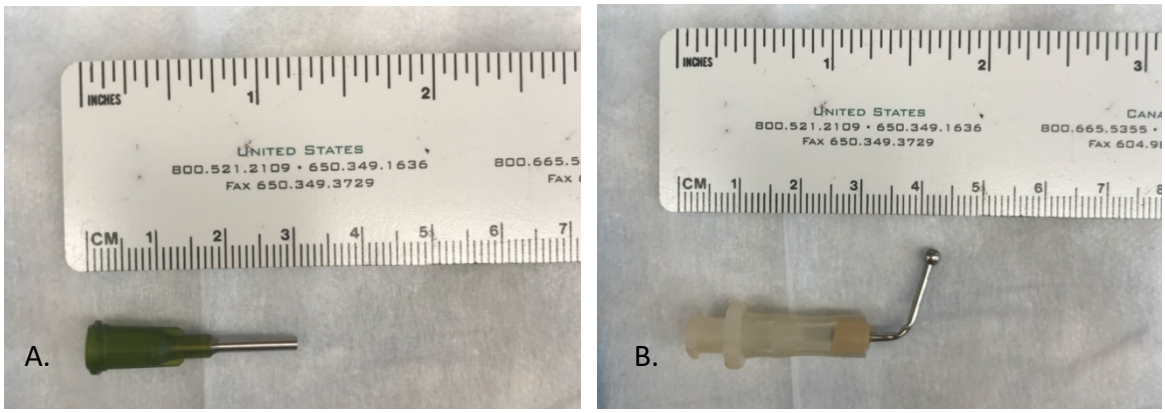

Figure 3*. Custom cannulas A. Tracheal cannula. B. The custom arterial cannula*.

A right pneumonectomy was performed after the right pulmonary hilum was securely ligated primarily with a 2-0 silk suture and finally with a vessel loop. The left lung airway was either recruited with approximately 3.0 mL air or 3.0 mL PBS (DPBS, (without Calcium Chloride and Magnesium Chloride), depending on if the lung was in an EVLP alone group or a liquid ventilation group, respectively. An anterograde vascular flush was performed with 30 mL of cold low-potassium dextran solution (PERFADEX®). If the lung was in a clodronate group, 5.25 mg liposomal clodronate as a large multilamellar liposome suspension buffered at pH 7.4 was added to the flush solution. The lungs underwent either cold storage in a sealed container or liquid ventilation for four hours prior to EVLP assessment, depending on group assignment.

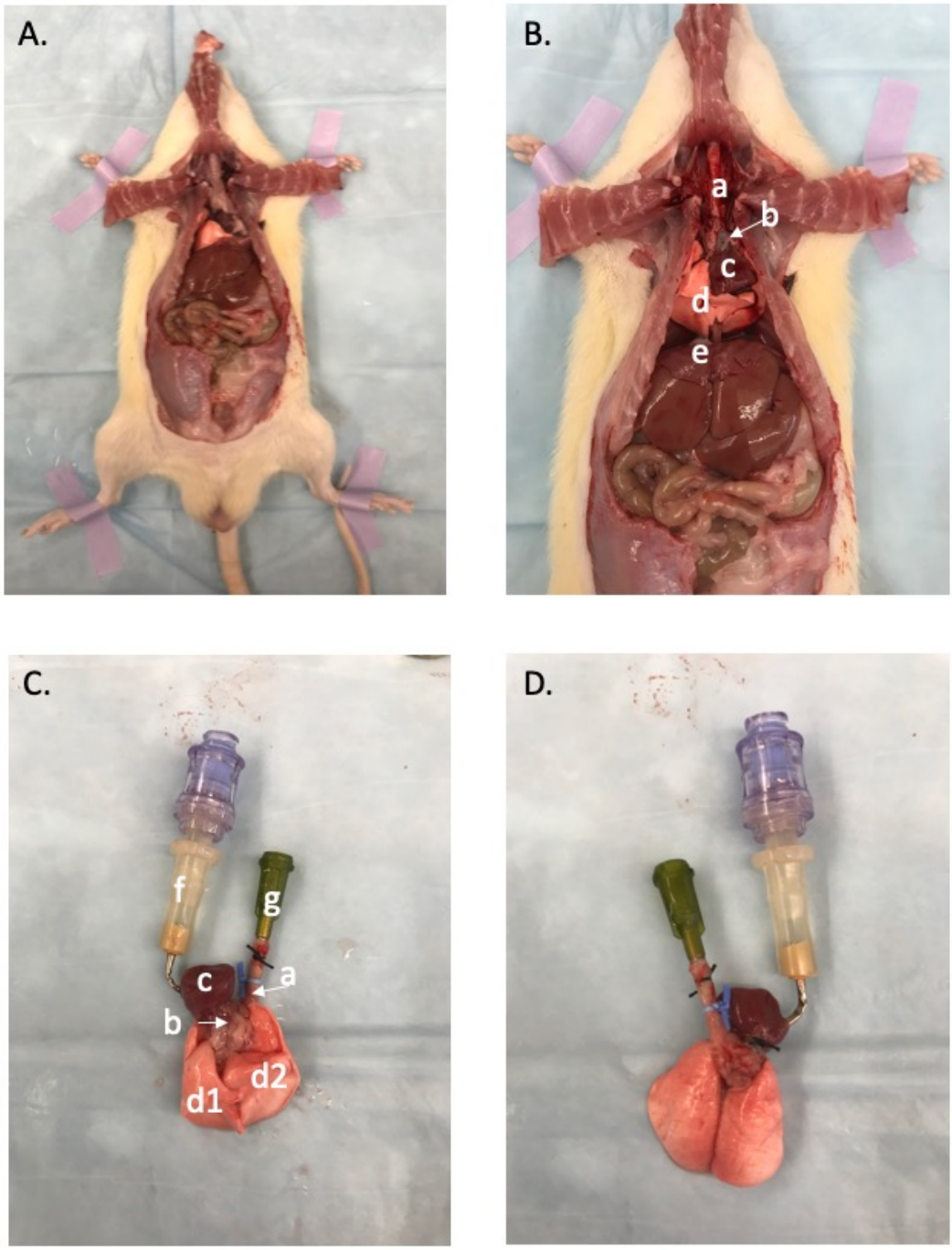

Figure 4. *Organ explantation. After sacrifice the thoracic cavity was opened and ribcage bilaterally opened for easier operative access (A). The situ after clearing Thymus and lateral tissue from the trachea (B; a: Trachea, b: Pulmonary arterial trunk, c: Heart, d: collapsed lung, e: severed diaphragm). Before harvesting the Lung and Heart en-block, the custom arterial cannula was inserted in the pulmonary trunk as described above and secured with a membrane valve, to ensure air-free vascular system. After harvest the tracheal cannula was inserted (C Organs from ventral; d1: right lung, d2: left lung, f: arterial cannula, g: tracheal cannula). D Organs presented from dorsal.*

2.2.3 *Ex-vivo* Lung Perfusion Protocol

A custom EVLP system was designed to allow constant arterial perfusion flowrate with passive venous outflow. A reservoir containing 100 mL of an albumin-based electrolyte solution (Table 8) was connected to an organ chamber via silicone tubing. Positive displacement roller pumps controlled the arterial inflow of perfusion solution and return of venous effluent from the lung (Figure 5). Before the pulmonary artery, silicone tubing with a larger lumen was installed and functioned as an air bubble trap to allow air to rise whilst fluid without air would access the lung. The EVLP system was housed in an incubator at 37°C for normothermic perfusion[110].

The lung was connected to the organ chamber by the previously described arterial and tracheal cannulas and was recruited with room air at 20 mL/kg before the initiation of perfusion. Perfusion flowrate was set initially to 20% of 6 mL/min/g of the predicted lung weight. The lung weight was calculated using the following formula: LW (g) = (0.0053 × body weight (g)) − 0.48) x 3. The flowrate was gradually increased over the first hour to the target, weight-based perfusion flowrate[111].

The utilized albumin-based electrolyte perfusion solution was composed starting with 1100 mL of deionized water stirring on a heating plate at 40° C. The electrolytes were added and dissolved. Dextran and BSA were added and let to dissolve completely before FBS and antibiotics were integrated. A total volume of 1500 mL was reached with deionized water. The solution was filter sterilized in three separate 500 mL Vessels and employed during EVLP.

Mechanical volume-controlled ventilation (VentElite, Harvard Apparatus Inc., Holliston, MA) commenced after 20 minutes of perfusion using a tidal volume (TV) of 6 mL/kg, a positive end-expiratory pressure (PEEP) of 5 cmH_2O, and a respiratory rate of 10 breaths per minute. Calculations were adjusted for single lung experiments, following the formula TV = $\frac{(6\ ml/kg)}{2}$ [112]. To measure pressure in both perfusate and ventilation tubing, pressure sensors were connected to both prior to entering the organ chamber. Pressure sensors were connected to the data acquisition hardware and the software collected the data. The software also allowed control and adjustment over the pumps.

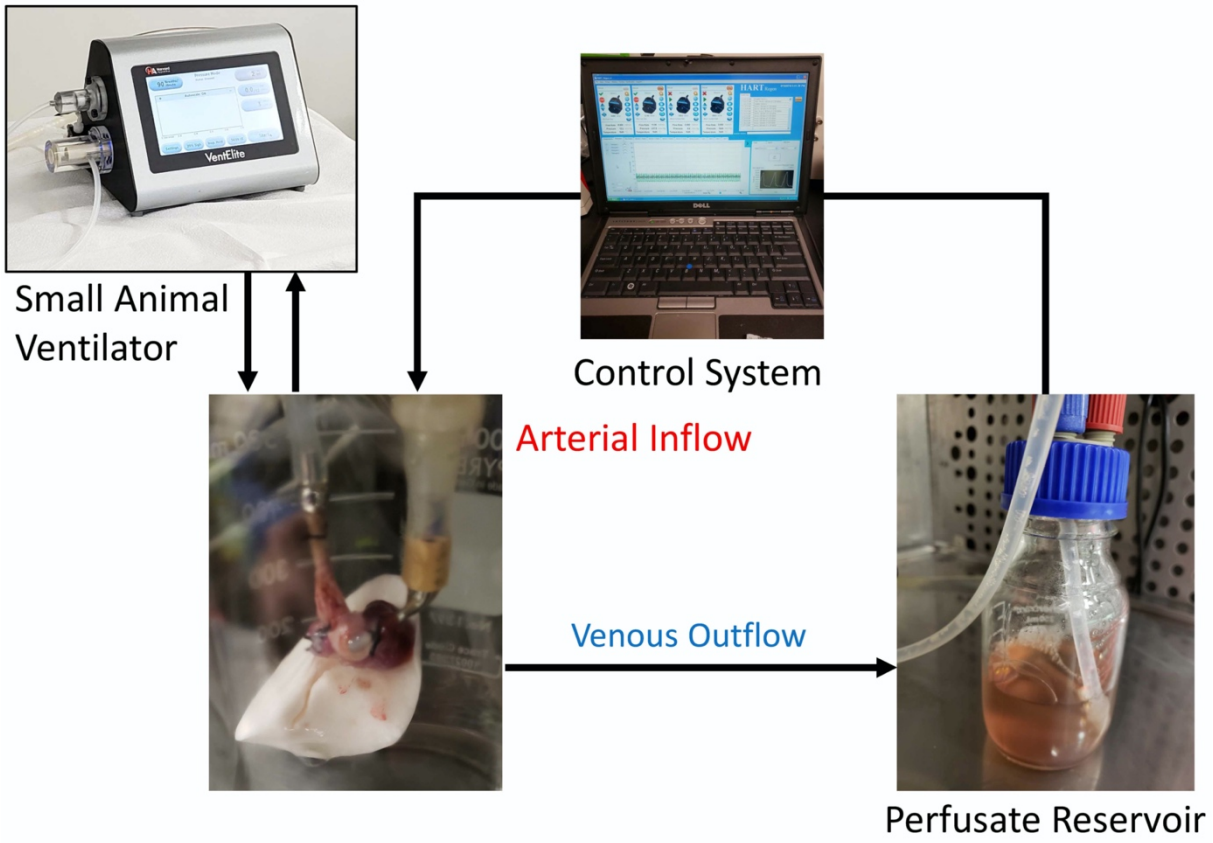

Figure 5. *Representative system diagram for EVLP. The EVLP circuit features a reservoir that delivers perfusate to the pulmonary arterial cannula through silicone tubing and a roller pump for lung perfusion. The venous outflow is passive, relying on gravity to return to the reservoir via another roller pump. Respiration is controlled by a digital small animal ventilator, which can adjust the respiratory rate, tidal volume, and PEEP. Additionally, a digital control system is in place to monitor and record arterial and airway pressures, as well as to regulate arterial flow rates. EVLP: Ex Vivo Lung Perfusion. PEEP: Positive end-expiratory pressure.*

2.2.4 Liquid Ventilation Protocol

A custom liquid ventilation system was designed which consisted of two separate closed circuits: one for vascular perfusion and one for liquid ventilation (Figure 6). The vascular perfusion circuit provided a constant arterial flowrate with passive venous outflow similar to the previously described EVLP setup (2.2.3 *Ex-vivo* Lung Perfusion Protocol) with the addition of a membrane oxygenator (Harvard Apparatus Inc., Holliston, MA). 1-2 L of oxygen was administered through the membrane oxygenator in a countercurrent flow to the perfusate to supply the lung. The lung was connected to the organ chamber by arterial and tracheal cannulas and recruited with the liquid ventilation solution before arterial perfusion (Figure 6B). The arterial perfusion flowrate was gradually increased over the first hour to a target, weight-based perfusion flowrate, as described above.

The LV circuit enabled a pressure-regulated mechanical LV with inflow and outflow Y-Luer adapter to minimize dead space ventilation. The inflow positive displacement pump was calibrated to a target positive inspiratory pressure of 6 mmHg and the outflow pump was set to a target end-expiratory pressure of 3 mmHg. The liquid ventilant flowrate was dependent on lung compliance and size and with flow loop gain adjusted to achieve 1-2 respirations per minute. To minimize lung injury, lower liquid volume and pressure were used based on lung compliance and tidal volume[96]. Pressure sensors measured vascular and airway pressure. The perfusate was a DMEM-based electrolyte solution (Table 9) with added 1% Antibiotic and 2% fetal bovine serum, and PBS was used as the liquid ventilant. LV was performed in a 37° C incubator allowing for normothermic conditions.

After four hours of LV, the lung was allowed to passively drain the liquid ventilant, 0.3 mL surfactant diluted in 3 mL PBS was instilled, and the lung was transferred to an EVLP circuit for assessment as previously described.

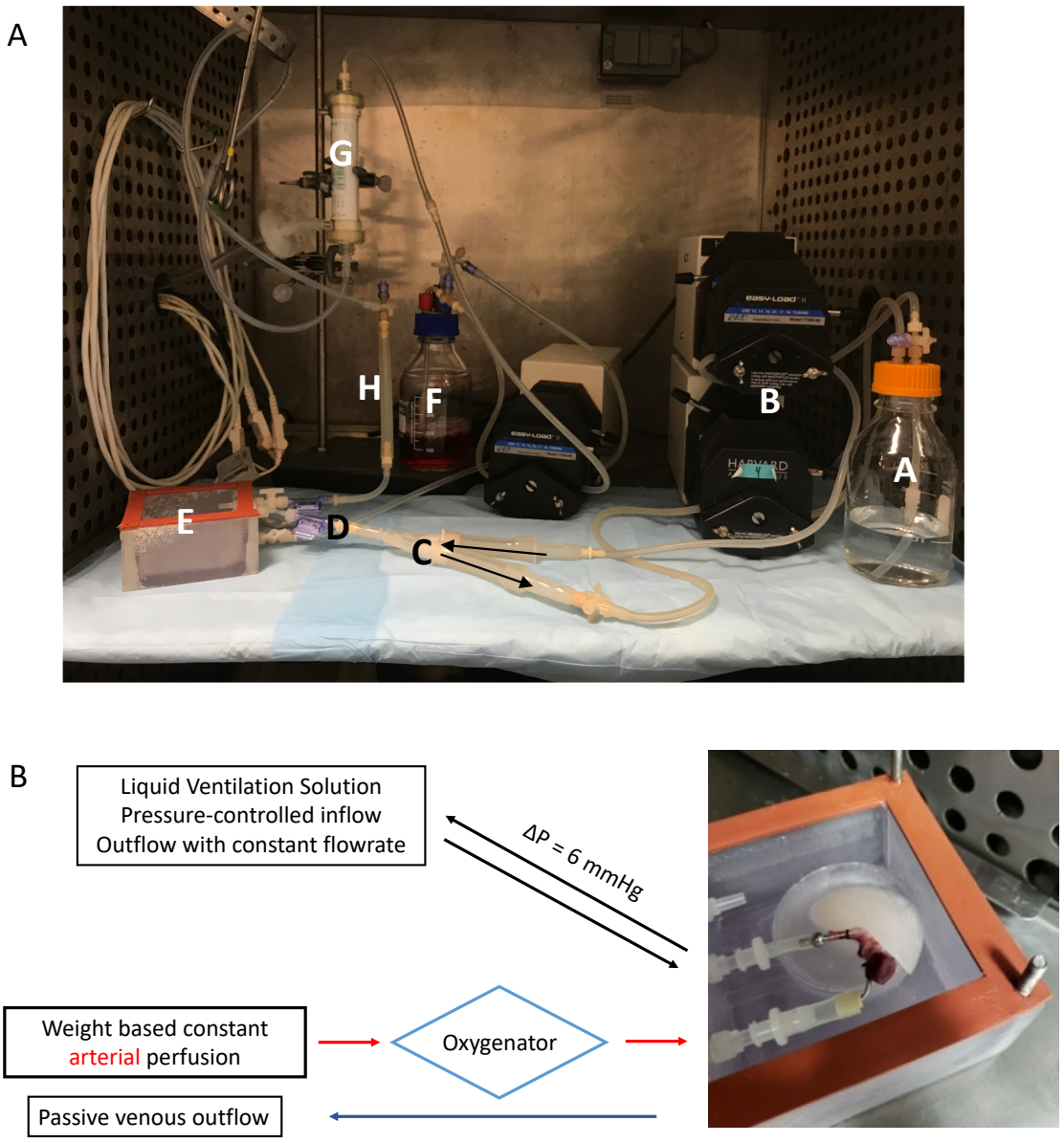

Figure 6. *Representative system diagrams for the Liquid Ventilation system. A Custom liquid ventilation circuit with a reservoir (A) delivers the liquid ventilant via silicone tubing and roller pumps (B) through silicone buffer tubing with an air-liquid interface for pressure damping (C) and a pressure sensor (D) to the custom murine liquid ventilation organ chamber (E). A digital system with predetermined high and low-pressure setpoints for liquid ventilation controls respirations with adjustable gain to modulate respiratory rate. A separate perfusate reservoir (F) supplies organ perfusion via silicone tubing, roller pumps, an oxygenator (G), an air trap to avoid arterial air embolization (H), and pressure monitors (D) to the organ chamber (E). The system is set up in an incubator at 37° C. B. Depicts the circuit described above systematically.*

2.2.5 Physiologic Assessment During EVLP

Every two hours during the EVLP assessment period, respiratory data were collected to calculate the dynamic compliance (C_{dyn}) of the experimental lung (*the lung volume change per unit pressure change)*. At the same time interval, arterial pressure data were collected to calculate the pulmonary vascular resistance (PVR).

After the EVLP period, C_{dyn} was calculated by TV/(peak pressure – PEEP), and PVR was calculated with (pMAP-LAP)/arterial flow rate, where pMAP = mean pulmonary arterial pressure and LAP = left atrial pressure.

2.2.6 Metabolic Assessment During EVLP

To monitor the function and condition of the experimental lung during EVLP, the perfusion solution was analyzed. Perfusate solution lactate concentration, pH, and oxygen content were measured using an i-STAT point-of-care analyzer (Abbott, Chicago, IL) with CG4+ cartridges (Abbott, Chicago, IL). Glucose levels were analyzed with a GlucCell® monitoring system and glucose test strips (Chemglass Life Science, Vineland, NJ). Perfusate samples were taken every two hours during the EVLP assessment period and immediately processed. For storage, the samples were then frozen at – 20 °C. The P/F ratio (Ratio of partial pressure of oxygen in the perfusate to the fraction of inspired oxygen) was determined throughout the 6-hour EVLP assessment period. The partial pressure of oxygen was analyzed using the i-STAT point-of-care analyzer. The fraction of inspired oxygen was 21% oxygen (FIO_2 of 0.21).

2.2.7 Wet-to-Dry Ratio

Fluid clearance and pulmonary edema were assessed using wet-to-dry ratio (W/D ratio). The parenchymal tissue of the left lung was weighed immediately after EVLP to determine the wet weight. The tissue was then lyophilized for 12 hours and weighed to determine the dry tissue weight. The W/D ratio was then calculated for three lungs from each group.

2.2.8 Bronchoalveolar Lavage Total Cell Count

Three lungs in each group underwent BAL sampling and cytokine analysis. A BAL sample was collected after EVLP, by instilling 2.0 mL PBS via the trachea and aspirating 1.0 mL for cell count and cytokine analysis. The suspension was analyzed using a manual hemocytometer, where 10 µl was installed per well. BAL cell counts were determined using a light microscope. The determination of the total cell count was a relevant aspect in evaluating the potential for inflammatory cell infiltration within the respiratory system. It should be noted that the focus was primarily on assessing the mechanical clearance of cells from the airway in response to LV or

apoptosis via clodronate, and as such, differentiation of the cell types was not a primary objective. To characterize the inflammatory response, the residual BAL fluid was stored at -80°C for cytokine analysis.

2.2.9 Bronchoalveolar Lavage Cytokine Analysis

At the conclusion of EVLP, Cytokines were analyzed to interpret the state of inflammation of the lung. Levels of IL-1α, IL-6, Gro-α, TNF-α, MCP-1, and IFN-γ were measured using a magnetic bead-based Luminex multiplex cytokine kit as per instruction of the manufacturer. This immunoassay allows simultaneous detection of multiple cytokines in a single sample. Briefly, BAL fluid was incubated overnight at 4°C with magnetic beads specifically coated with antibodies for the above-listed cytokines. A biotinylated detection antibody was then added and incubated for 1 hour at room temperature as well as Streptavidin-Phycoerythrin for 30 minutes. After this, samples were washed and analyzed using Luminex MAGPIX (Millipore, Billerica, MA, USA) as well as xPONENT software (Luminex, Austin, TX, USA).

2.2.10 Histology and Immunohistochemistry

2.2.10.1 Histology

After the EVLP assessment period, three lungs in each group were perfusion-fixed in a 4% paraformaldehyde solution for 24 hours. The paraformaldehyde solution was actively pumped through the respiratory system via the tracheal cannula for 24 hours at room temperature. After fixation, lungs were removed from the perfusion system sterilely and cut into tissue blocks with a disposable scalpel. These were sorted into labeled embedding cassettes and stored in plastic containers with 70% Ethanol until paraffin treatment. The samples were submitted to the MGH Center for Skeletal Research Core (NIH P30 AR075042) for paraffin processing and embedding of our tissue sections with a tissue processor.

The paraffin-embedded tissue was sectioned at 5 μm using a microtome creating a ribbon of sections. With forceps, the ribbon via the last section was placed in a water bath at 40°C. Smaller groups of sections were created by gently teasing apart the ribbon with clean forceps. To mount the sections on glass slides for staining, slides were positioned underneath the sections at a 45° angle in the water. The slides were carefully lifted from the water after ensuring that the sections were attached to the slide. Slides were left to dry for 2 hours before being transferred to a heating plate and dried at 37° C for 12 hours.

As most staining solutions are water-based, paraffin must be removed prior to staining. Firstly, the slides were kept on the heating plate to soften paraffin and then placed in a 60° C oven for 30

minutes to melt the paraffin before continuing with the deparaffinizing process through Histo-clear and the rehydrating process through graded ethanol to distilled water according to following protocol.

Table 14: Protocol of Deparaffinization and Rehydration

Solution	Time
Histo-clear	5 minutes
Histo-clear	5 minutes
100% Ethanol	3 minutes
100% Ethanol	3 minutes
95% Ethanol	3 minutes
95% Ethanol	3 minutes
Deionized Water	2 minutes

2.2.10.1.1 Hematoxylin – Eosin – Stain

Hematoxylin and eosin (H&E) staining is a technique used to visualize an overview with a brightfield microscope of the biological tissue structure.

The process involves two dyes: hematoxylin, a basic natural dye that stains acidic structures such as the nuclei of cells. Eosin is a synthetic acidic dye that stains basic structures such as cytoplasm and extracellular matrix[113].

Table 15: Hematoxylin and Eosin Staining Protocol

Process	Solution	Time
Nuclei staining	Hematoxylin dye	8 minutes
1. Wash	Deionized Water	5 minutes
2. Blueing	Deionized Water	3 minutes
Cytoplasm & ECM staining	Eosin dye	15 x immersions (dips)
Dehydration	95% Ethanol	wash
	100% Ethanol	3 minutes
	Histo clear	3 minutes

After staining, one drop of mounting medium was added to cover the section of tissue. A coverslip was applied at a 45° angle and let sink on slide slowly to ensure a bubble-free appliance. The mounting medium was left to harden at room temperature for 12 hours.

2.2.10.2 Immunohistochemistry

Tissue sections were deparaffinized, rehydrated, and mounted on glass slides for immunofluorescent staining as previously stated. Antigen retrieval was performed with a sodium

citrate solution at high temperature and pressure for 10 minutes. The residue of sodium citrate was removed by a continuous flow of water for 10 minutes.

2.2.10.2.1 Immunofluorescent Staining

Water residues were tapped off the slides carefully. For the following tissue washing, the slides were positioned in a staining box. The tissue sections were circled with a hydrophobic pen to confine the processing. A Tris buffer solution (TBS) with 0.025% Triton was added to the tissue samples for 5 minutes to wash the cell surfaces. This process was completed twice. To minimize off-target bindings, antigens need to be blocked. This was conducted with 5% donkey serum (DS) for 2 hours at room temperature. All fluid residue was removed by suction with a benchtop-incorporated vacuum system. A solution containing the primary antibody, TBS, and 0.5% DS was added to the tissue and incubated overnight at 4°C. Subsequently, the sections were washed with TBS with 0.025% Triton twice for 4 minutes. To reduce light pollution of secondary antibodies, the following steps were conducted in a darkened environment. The secondary antibodies and bovine serum were added to PBS. The tissue was incubated with secondary antibody solution for 1 hour at room temperature and then washed with TBS 3 times for 5 minutes. Dry slides were mounted with DAPI Fluoromount-G and covered with 0.15 mm coverslips under bubble-free conditions. The mounting medium was left to harden for 24 hours and stored light-protected. Images were captured using a Nikon Ti-PFS inverted microscope (Nikon, Tokyo, Japan).

2.2.10.2.2 F4/80, VECAD, ZO-1, ECAD

An analysis using immunohistochemistry was conducted to comparatively assess the relative expression of the cell junction proteins ECAD, VECAD, and ZO-1, along with the activated tissue macrophage marker F4/80. F4/80 is a protein expressed on the surface of activated alveolar macrophages and targeted to quantify the remaining cells after experimental treatment. The dilution 1:1000 was used for the primary antibody F4/80.

Endothelial cells lining the interior walls of blood vessels produce vascular endothelial l (VE)-cadherin, a protein that lines their surface. VE-cadherin plays an important role in the formation and maintenance of the endothelial barrier. The dilution 1:1000 was utilized for the primary antibody against VECAD.

ZO-1 is an antibody that specifically targets the zonula occludens protein 1, also known as tight junction protein 1. Tight junctions are critical for maintaining the reliability of endothelial and epithelial barriers formed by specialized cell-cell adhesion. The dilution 1:150 was used for the primary antibody targeting ZO-1.

E-cadherin is expressed on the surface of epithelial cells. E-cadherin belongs to the cadherin family of cell adhesion molecules that mediate homotypic cell-cell interactions to maintain tissue structure and function. The dilution 1:200 was applied when targeting ECAD.
An immunohistochemical stain uses a secondary antibody to amplify the signal generated by a primary antibody, which identifies and binds to a target antigen. Secondary antibodies are selected based on the species of animal from which the primary antibody was raised, as well as the detection method to be used.

2.2.10.2.3 TUNEL

For staining of apoptotic cells, a TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling) assay was used (Promega Corporation, Madison, WI). Only cells with distinct nuclear staining were analyzed. After treatment of deparaffinization and rehydration, apoptosis detection was applied using the following protocol.

Table 16: TUNEL Staining Protocol following Manufacturer Instructions

Process	Solution	Time
Fixation	4% formaldehyde in PBS	15 minutes
Wash	PBS	2 x 5 minutes
Proteinase K permeabilizes tissues and cells to the staining reagents in subsequent steps	Proteinase K solution diluted 1:500 in PBS	8-10 minutes at room temperature
Wash	PBS	5 minutes
Repeat fixation	4% formaldehyde in PBS	5 minutes
Wash	PBS	5 minutes
Equilibration	Equilibration buffer	10 minutes at room temperature
Label	TdT reaction mix	60 minutes at 37° C in humidified chamber
Stop Reaction	20x SSC	2 x 15 minutes
Wash	PBS	3 x 5 minutes
Mount and counterstain	DAPI Fluoromount-G	

Localized green fluorescence of apoptotic tissue was detected by Images captured using a Nikon Ti-PFS inverted microscope (Nikon, Tokyo, Japan). Only cells with distinct nuclear staining were analyzed. DAPI-stained nuclei were detected as blue.

2.2.10.3 Image Analysis

All fluorescent images for a given protein were captured with consistent exposure time and instrument gain. Images were analyzed using ImageJ software. Cell counts were obtained by isolating the DAPI color channel, subtracting the background signal, converting the image to a binary image, defining cell borders, and counting discrete nuclei.

Table 17: Protocol of TUNEL and DAPI positive nuclei count

Open image with ImageJ
Image -> Color -> split channels
Select channel (blue or red)
Process -> subtract background (keep default settings)
Image -> Adjust -> Threshold
Process -> Binary -> Make Binary
Process -> Binary -> Convert to Mask
Process -> Binary -> Fill Holes
Process -> Binary -> Watershed
Analyze -> Analyze Particles -> ^2 = 30-infinity
To visualize the counted objects: Pull down menu -> Outlines

Mean fluorescence intensity was obtained by isolating the appropriate secondary antibody fluorescent channel, analyzing each image from a particular protein staining with consistent brightness and contrast, then calculating the mean fluorescence. Quantitative data was generated as a mean fluorescence per cell for each slide. Three images were analyzed for each experiment, totaling nine data points per experimental group.

2.2.11 Statistical Analysis

Data are presented as the mean ± standard error of the mean (SEM). Continuous variables were compared using an analysis of variance test, followed by multiple subsequent t-tests with unequal variance if applicable. Statistical significance was defined as $p<0.05$.

3. RESULTS

The focus of the study is on developing a new method for *ex-vivo* lung reconditioning after significant ischemia-reperfusion injury using liquid ventilation. The study aims to improve physiologic parameters and decrease inflammatory cytokines and alveolar macrophages, which are major contributors to post-transplant allograft dysfunction. The method involves mechanically clearing alveolar macrophages and inflammatory cytokines, which can halt the propagation of ischemia-reperfusion injury.

3.1 Physiologic Performance During EVLP Assessment

3.1.1 Dynamic Compliance and Pulmonary Vascular Resistance

At the endpoint of the 6-hour EVLP assessment, all the ischemic lung groups showed a significant decrease in C_{dyn} in comparison to the non-ischemic control group ($p<0.05$ for all, Figure 7A). When comparing the ischemic groups, the liquid ventilation group showed a trend toward higher dynamic compliance than the ischemic control group, although this did not reach significance ($p=0.17$, Figure 7A). PVR in all ischemic lung groups was significantly higher than the non-ischemic control group following 6 hours of EVLP assessment ($p<0.05$ for all, Figure 7B). There was a tendency towards decreased overall PVR in both the liquid ventilation group and the LV-Clodronate group, in comparison to the ischemic control group when comparing the ischemic groups. However, this trend did not attain statistical significance ($p=0.11$ and $p=0.14$, respectively, Figure 7B).

An important observation in the temporal trends of the physiological parameters was that both liquid ventilation groups were able to significantly increase C_{dyn} over the course of the EVLP assessment ($p<0.05$ for both, Figure 7A). This indicated a sustained physiological improvement over the 6-hour EVLP assessment period, which was not evident in the other experimental groups or non-ischemic control lungs. Another trend that emerged during EVLP was a reduction in PVR observed in the LV group, although this did not reach significance.

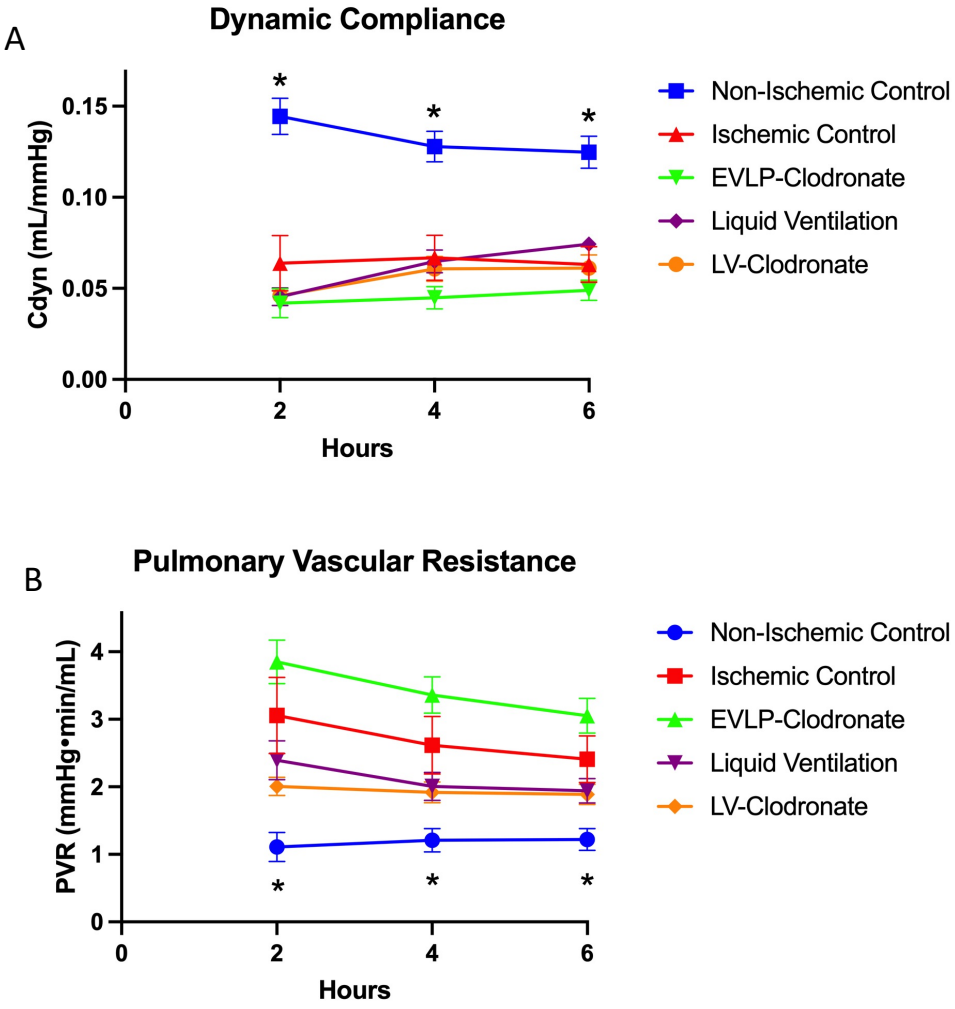

Figure 7. *Physiologic trends during EVLP assessment period. A. Dynamic compliance (C_{dyn}) demonstrated a significantly higher value in the non-ischemic control group as compared to the ischemic lung groups ($p<0.05$). The liquid ventilation group presented a tendency towards higher C_{dyn} at the 6-hour mark than the ischemic control group ($p=0.17$). Additionally, both liquid ventilation groups showed an increase in C_{dyn} during EVLP assessment period. B. Pulmonary vascular resistance (PVR) indicates a significant decrease in the non-ischemic control group compared to the ischemic lung groups over time ($p<0.05$). At the 6-hour mark, both liquid ventilation group and the LV-Clodronate group showed a tendency towards lower PVR compared to the non-ischemic control ($p=0.11$ and $p=0.14$, respectively). EVLP: Ex Vivo Lung Perfusion. LV: Liquid ventilation. * represents statistical significance.*

3.1.2 Wet-to-Dry Ratio

The W/D ratio was calculated as an indicator of pulmonary edema and barrier function. A trend toward a lower W/D ratio was revealed in both liquid ventilation groups compared with the other

ischemic groups, although this did not reach significance (p=0.16 and p=0.33 respectively, Figure 8A). Among all the groups, the non-ischemic lungs displayed pulmonary edema, as evidenced by the lowest observed W/D ratio.

3.1.3 Partial Pressure of Oxygen in the Perfusate to the Fraction of Inspired Oxygen Ratio

Throughout the 6-hour EVLP assessment period, the change in the ratio of partial pressure of oxygen in the perfusate to the fraction of inspired oxygen (P/F ratio) was analyzed. There was a significant improvement in the P/F ratio for the non-ischemic control group and the liquid ventilation groups, while the ischemic EVLP alone groups exhibited a reduction in the P/F. The change in the P/F ratio for the non-ischemic control group was significantly higher than the ischemic EVLP groups ($p<0.05$). However, it is worth noting that the difference between the non-ischemic control group and the liquid ventilation groups was not statistically significant, demonstrating improved oxygenation in the liquid ventilation groups (Figure 8B).

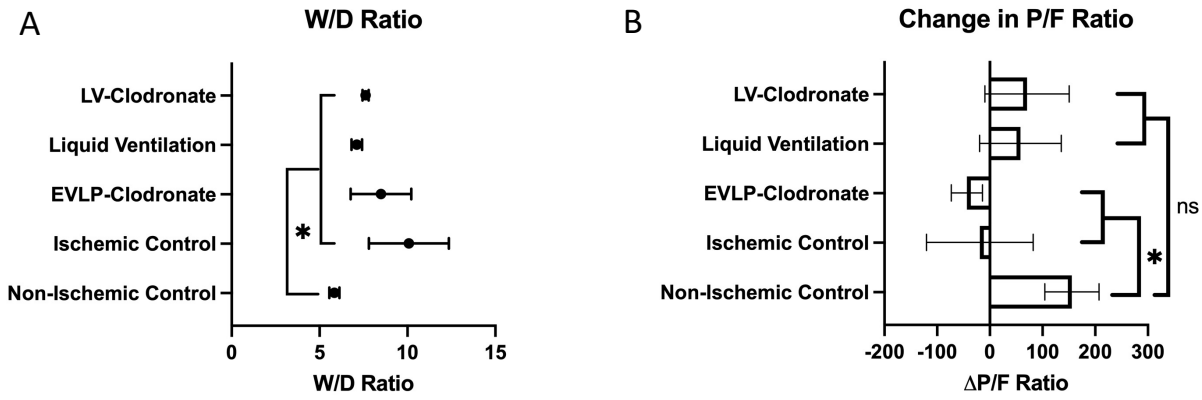

Figure 8. *The evaluation of pulmonary edema was conducted using wet-to-dry (W/D) ratio after EVLP, while oxygenation was assessed using the ratio of partial pressure of oxygen in the perfusate to the fraction of inspired oxygen (P/F ratio) during EVLP assessment period. A. The W/D ratio was significantly lower in the non-ischemic group compared to the ischemic lung groups (p<0.05). Both the liquid ventilation group and LV-Clodronate group showed a trend towards a lower W/D ratio compared to the ischemic control group (p=0.16 and p=0.33, respectively). B. The P/F ratio was significantly higher in the non-ischemic control group compared to the ischemic control group and EVLP-Clodronate group (p<0.05), but notably, there was no significant difference compared to either of the liquid ventilation groups. EVLP: Ex Vivo Lung Perfusion. LV: Liquid ventilation. Δ: change. ns: not statistically significant. * presents statistical significance*.

3.2 Metabolic Parameters During EVLP Assessment

3.2.1 pH Assessment

The pH of the perfusion solution decreased in all study groups during the EVLP assessment period. There was no significant difference in the extent of pH reduction over the EVLP period between any experimental group. Notably, the perfusate pH was significantly higher in both liquid ventilation groups compared to the other experimental groups at multiple time points, which could indicate improved metabolic homeostasis in these lungs ($p<0.05$, Figure 9A).

3.2.2 Lactate Assessment

Lactate concentration in the perfusate increased significantly during EVLP in all groups ($p<0.05$ for all, Figure 9B). No significant association was noted between the lung's physiologic performance and the perfusate lactate concentration at the end of the EVLP assessment period.

3.2.3 Glucose Assessment

The overall glucose consumption during EVLP assessment period was comparable across all groups (Figure 9C). It is worth mentioning that the non-ischemic control group showed a consistent trend of lower glucose consumption than the other groups, particularly at the 2-hour and 4-hour time intervals ($p<0.05$, Figure 9C).

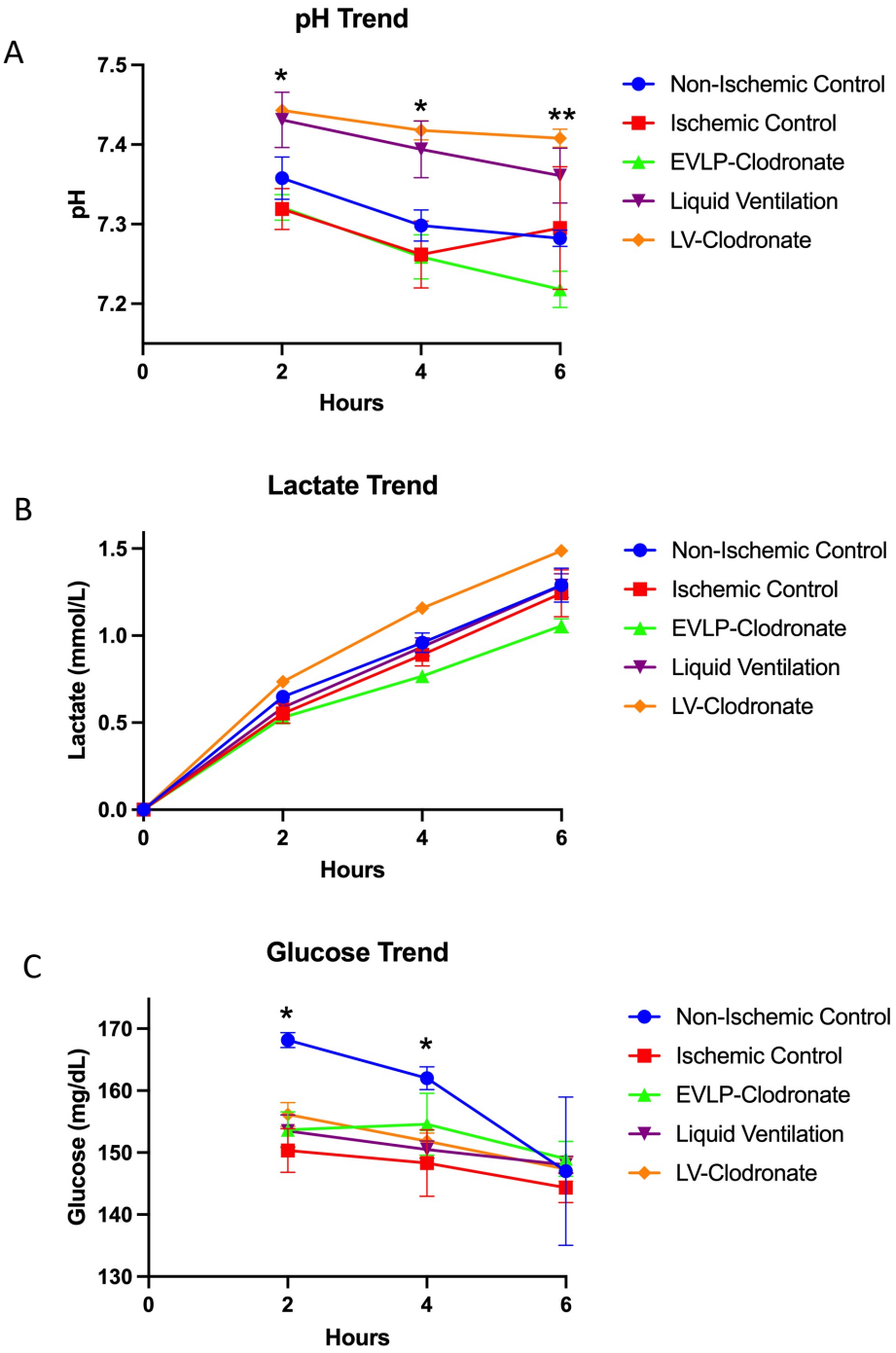

Figure 9. *Pulmonary metabolic trends during perfusate assessment. A. The pH trend indicated a reduction over time in all experimental groups, without any significant distinction in the extent of pH decline between any group. However, it is worth noting that both the liquid ventilation group and LV-Clodronate group had considerably higher pH levels at the 2- and 4-hour marks ($p<0.05$ for both, * indicates statistical significance). Additionally, the LV-Clodronate group had a higher pH value than all other groups at the 6-hour mark ($p<0.05$, ** indicates statistical significance). B. During the EVLP assessment period, the perfusate lactate concentration increased in all experimental groups. However, no correlation between lactate concentration and the physiologic performance of the lungs was observed. C. The glucose consumption trend indicated that the non-ischemic control group*

*consumed less glucose than the ischemic lung groups at the 2- and 4-hour marks. Glucose trend during the EVLP assessment period showed the non-ischemic control group consumed less glucose than the ischemic lung groups at the 2- and 4-hour marks. Otherwise, glucose consumption was similar in the groups. EVLP: Ex Vivo Lung Perfusion. LV: Liquid ventilation. * indicates statistical significance.*

3.3 BAL Total Cell Count

Upon direct analysis of the total cell count in the BAL fluid following the experimental procedure, it was observed that the ischemic lung groups that underwent either clodronate administration or LV, or a combination of both, exhibited a lower overall cell count trend in comparison to the ischemic control group. Furthermore, the non-ischemic control group demonstrated the lowest cell count trend. However, statistical analysis did not reveal any significant differences between the groups (Figure 10).

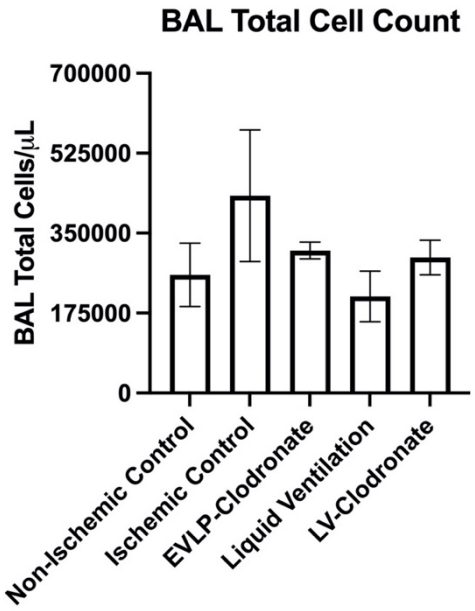

Figure 10. *BAL Total Cell Count. Total cells were counted directly after the EVLP period in the BAL fluid. Overall, the experimental lung groups trended towards a decrease in BAL total cells compared to the ischemic control group. The liquid ventilation group showed lowest total cell count compared to all other groups. No statistical significance was reached when comparing the groups.*

3.4 Cytokine Markers of Ischemia Mediated Inflammation

A quantitative analysis of IL-6, INF-γ, IL-1α, GRO-α, TNF-α, and MCP-1 was performed on BAL samples to determine their levels as markers of inflammation resulting from ischemia-reperfusion injury in the experimental groups. Compared to the non-ischemic control, all ischemic lung groups had a significantly higher concentration of both IL-6 and TNF-α in their BAL samples ($p<0.05$, Figure 11A-B). A tendency towards decreased TNF-α levels was observed in the LV group when comparing to the other ischemic lung groups, although statistical significance was not achieved (Figure 11B).

When analyzing INF-γ concentrations in BAL samples, both liquid ventilation groups and the EVLP-Clodronate group had a significantly lower INF-γ concentration than the ischemic control group ($p<0.05$, Figure 11C). The INF-γ concentration was significantly lower in the non-ischemic control group compared to the ischemic lung groups, which was expected ($p<0.05$, Figure 11C). In addition, a significant decrease in the concentration of IL-1α was noted in the BAL suspension from lungs treated with LV, in comparison to all other experimental groups ($p<0.05$, Figure 11D).

In the experimental groups where clodronate was administered for macrophage depletion, there was a noticeable increase in MCP-1 concentration in the BAL samples compared to other groups. This was probably caused by the injured and dying macrophages in these groups acting as DAMP signals ($p<0.05$, Figure 11E). As expected, the non-ischemic control group had a significantly lower BAL MCP-1 concentration than the ischemic lung groups (Figure 11E).

When assessing GRO-α, the LV group showed an increase in cytokine levels compared to the other groups, although it did not reach statistical significance compared to both non-ischemic control and ischemic control group ($p<0.05$, Figure 11F). The groups with administered clodronate presented a trend towards lower GRO-α levels than the ischemic control group. However, there was no significant difference in GRO-α concentrations between the groups overall ($p<0.05$, Figure 11F).

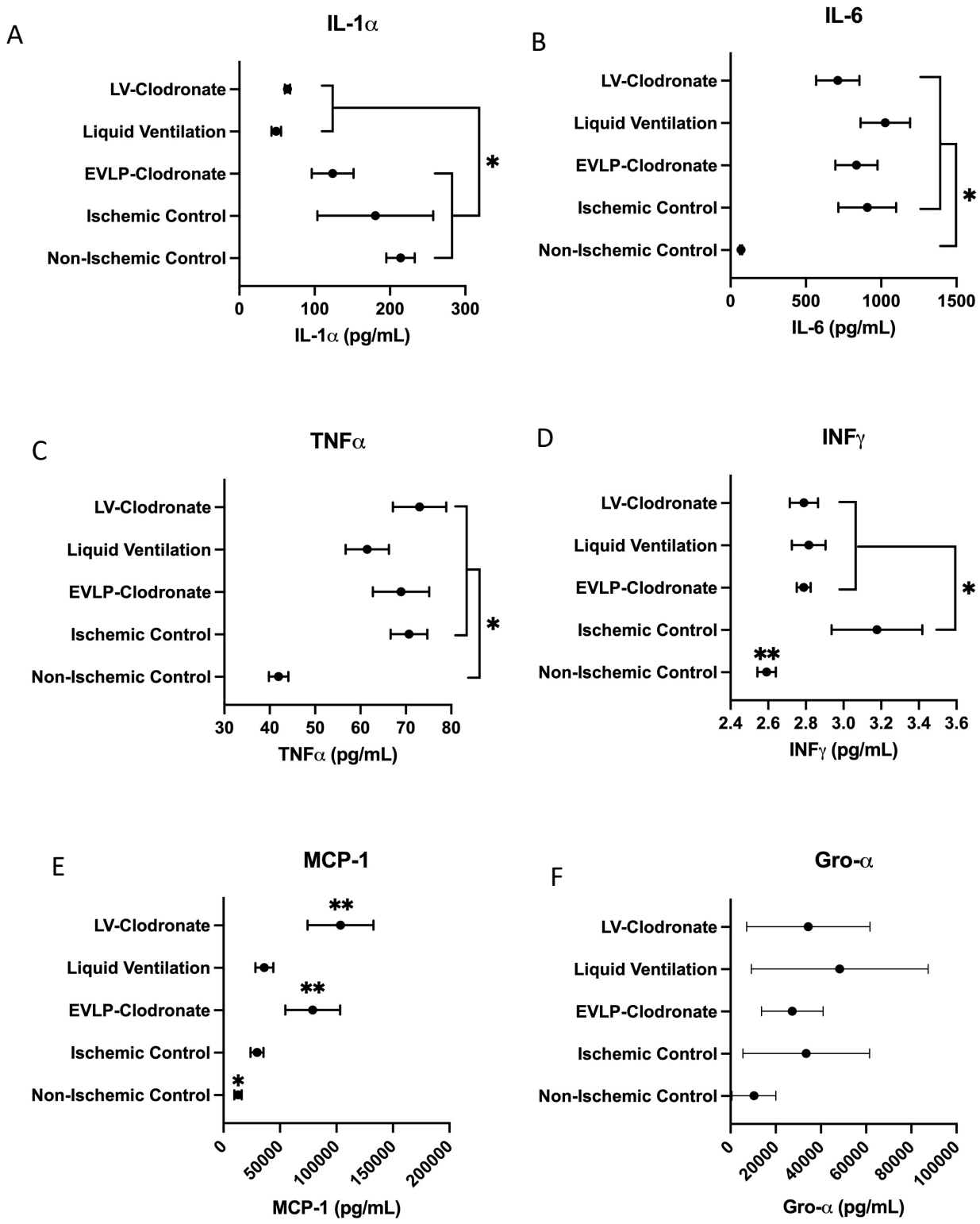

Figure 11. *BAL fluid cytokine concentrations. A. The comparison of IL-1α levels revealed a significant reduction in both liquid ventilation groups compared to all other experimental groups, including the non-ischemic control group ($p<0.05$). B. The non-ischemic control group demonstrated a significant lower concentration of IL-6 compared to all other groups with ischemic lungs ($p<0.05$). C. The comparison of TNF- α levels showed a significant decrease in concentration in the non-ischemic control group as compared to all the other ischemic lung groups ($p<0.05$). D. When comparing INF-*

*γ concentrations, the non-ischemic control group had significantly lower levels compared to all other ischemic lung groups (p<0.005, ** indicates statistical significance). It is worth noting that the liquid ventilation group, LV-Clodronate group and EVLP-Clodronate group exhibited significantly lower concentrations of INF-γ in the BAL samples compared to the ischemic control group(p<0.05). E. The measured levels of MCP-1 demonstrated a significantly lower concentration in the non-ischemic control group than all other ischemic lung groups (p<0.05). It is noteworthy that the Clodronate groups exhibited significantly higher levels of BAL MCP-1 compared to all other experimental groups (p<0.05, ** indicates statistical significance). F. The liquid ventilation group showed an increase in GRO-α levels compared to the other groups, although it did not reach statistical significance compared to the control groups. The Clodronate groups showed a trend towards lower GRO- α levels than the ischemic control group. However, there was no significant difference in GRO- α concentrations between the groups overall. EVLP: Ex Vivo Lung Perfusion. LV: Liquid ventilation. * indicates statistical significance.*

3.5 Immunohistochemistry and TUNEL Apoptosis Assay

3.5.1 Cell Junction Protein Analysis

To compare the relative expression of cell junction proteins VECAD, ECAD, ZO-1 and F4/80, a semi-quantitative analysis was conducted. F4/80 is an activated tissue macrophage marker F4/80. The measured MFI of ECAD, VECAD, and ZO-1 was not significantly different between all experimental groups (Figure 12A-C).

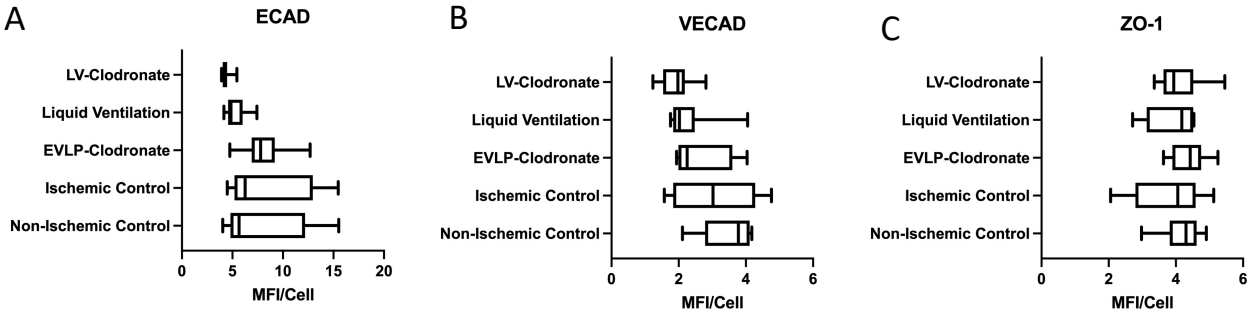

Figure 12*. Analysis of cell-cell junction proteins after the EVLP assessment period using a semi-quantitative approach. A. No variation in the protein content of ECAD was observed among the groups. B. Analysis of VECAD protein content revealed no significant differences between the experimental groups. C. There was no difference in ZO-1 protein content among the different groups, as determined by comparison. EVLP: Ex Vivo Lung Perfusion. LV: Liquid ventilation. ECAD: Epithelial cadherin. VECAD: Vascular endothelial cadherin. ZO-1: Zonula occludens-1.*

3.5.2 Activated Alveolar Macrophage Marker F4/80

The group that underwent LV alone had the lowest MFI for the macrophage marker F4/80, which was an unexpected finding when assessing the relative tissue quantity ($p<0.05$, Figure 13F). It is important to note that there was no significant difference observed in F4/80 expression between the groups treated with clodronate and the control groups.

3.5.3 TUNEL Apoptosis Assay

Tissue sections from each experimental group were subjected to a TUNEL assay, revealing a significantly lower cellular apoptotic rate in the non-ischemic control group as compared to the ischemic groups ($p<0.05$, Figure 14F). Although not statistically significant, there was a tendency towards increased cellular apoptosis in the clodronate group.

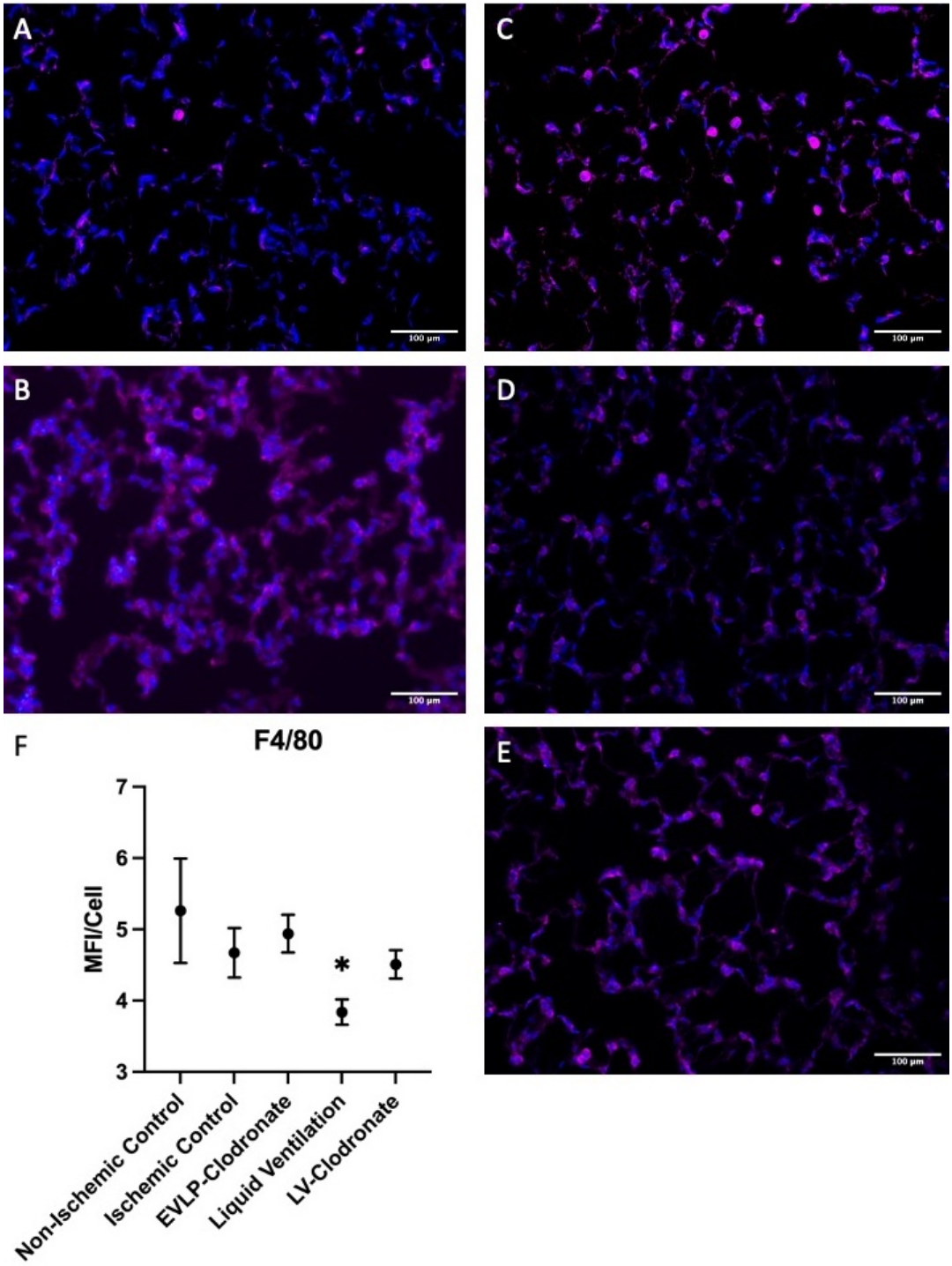

Figure 13. *A semi-quantitative analysis of the macrophage cell surface protein F4/80 was performed after EVLP. A.-E Fluorescent images with x20 magnification and 100 µm scale. DAPI blue: positive nuclei. F4/80 red: activated macrophages and immune cells (non-lymphocytes). A. Non-ischemic Control group B. Ischemic-Control group C. EVLP-Clodronate group D. Liquid Ventilation group E. LV-Clodronate group F. The comparison of F4/80 revealed a significant reduction in the liquid ventilation group as compared to all other experimental groups ($p<0.05$). EVLP: Ex Vivo Lung Perfusion. LV: Liquid ventilation. * indicates statistical significance*

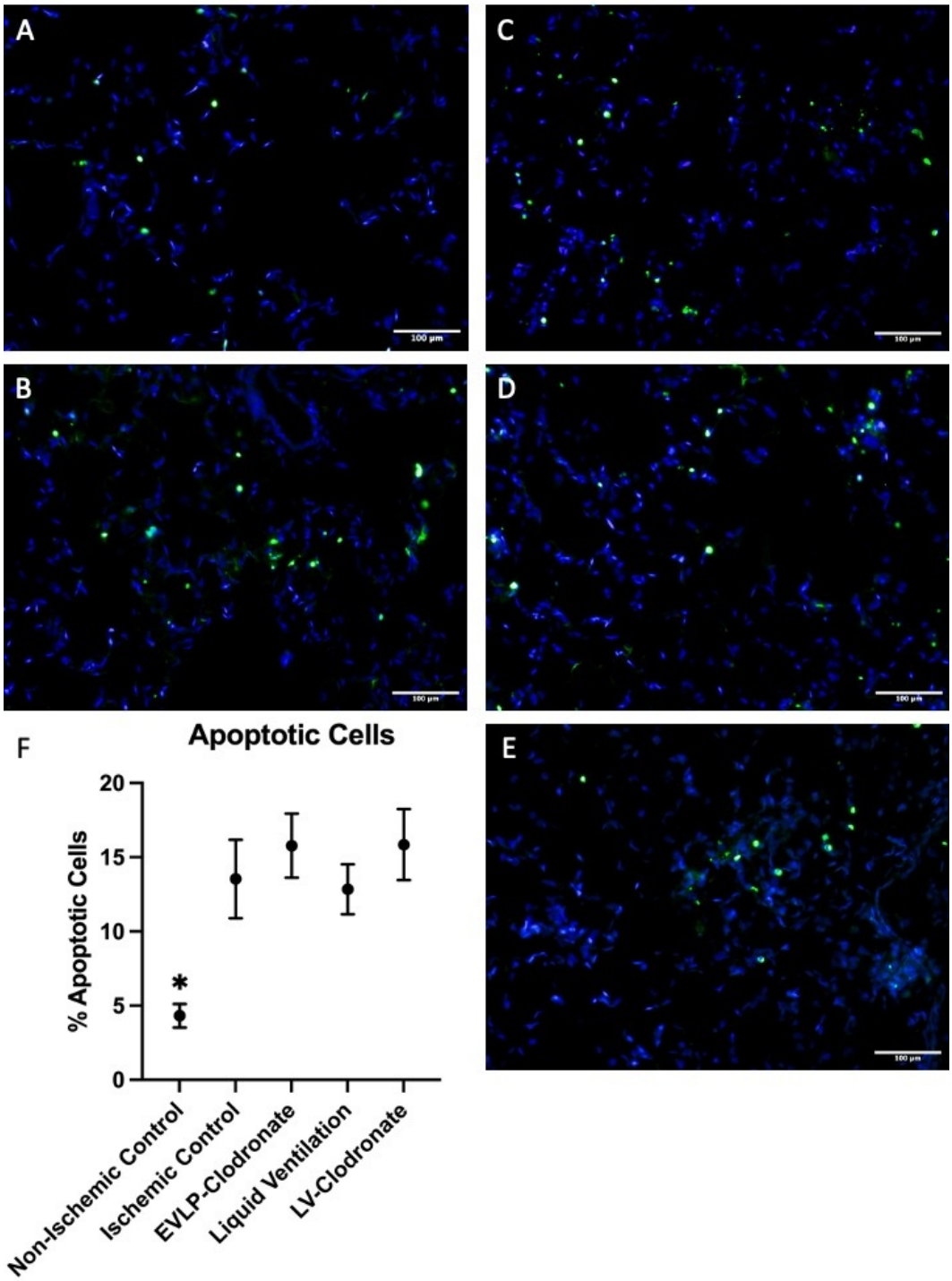

Figure 14. *The level of apoptosis in the lung samples was assessed with a TUNEL assay after EVLP. A.-E. Fluorescent images with x20 magnification and 100 μm scale. DAPI blue: positive nuclei. TUNEL green: apoptotic signal. A. Non-Ischemic Control B. Ischemic Control C. EVLP-Clodronate D. Liquid Ventilation E. LV-Clodronate The comparison of the percentage of total cells undergoing apoptosis revealed a significantly lower rate in the non-ischemic control group compared to all other groups ($p<0.05$). However, no difference was observed among the ischemic groups. EVLP: Ex Vivo Lung Perfusion. LV: Liquid ventilation. * indicates statistical significance.*

4. DISCUSSION

Liquid ventilation has long been a fringe concept and treatment modality in both the medical and scientific community. With the advent of *ex-vivo* perfusion strategies for lung preservation and recovery, new and innovative approaches to treating lungs that would otherwise be discarded are needed. EVLP has yet to recover lungs with severe warm ischemic times and therefore ischemia-reperfusion injury to functions, that allow use in transplantation. Using total liquid ventilation for the reconditioning of lungs with ischemia-reperfusion injury has not been previously documented. This is the first proof-of-concept experiment demonstrating the potential to treat lungs that have experienced extreme (4-hour warm ischemia) injury with LV.

Preliminary data suggested that LV aids in recovering an ischemic lung. Mechanistically, this was likely due to the additional time allowing for the epithelial and endothelial cell compartments to recover metabolically and re-establish barrier function, compared to conventional cold storage. This is critical for lung function recovery. Additionally, LV provides clearance of harmful cytokines and DAMPs during the period of maximal cellular injury, which are considered major contributors and propagators of IRI after a significant ischemic period. These include interleukins, cytokines, and small molecules released by injured cells that further signal neighboring cells to change their phenotypic profile and cause organ dysfunction. In pulmonary ischemia-reperfusion injury, these DAMPs collect in the alveolar space and lead to further tissue injury and organ dysfunction.

The macrophage is the main regulator of IRI releasing a plethora of cytokines that activate neutrophils to release granules and free radicals which cause direct cellular damage and organ dysfunction. These cytokines also activate the endothelium and epithelium via DAMP pathways to alter cellular function and reduce barrier function.

Suggested was that post-mortem depletion of macrophages can alter the inflammatory milieu and allow for an environment of reconditioning before transplantation. The data did not demonstrate complete recovery of significantly injured lungs to baseline function. However, improved pulmonary performance over a comparable lung treated with EVLP alone, which is the current standard approach for clinical lung assessment and recovery before transplantation was demonstrated. While the experimental groups did not fully return to baseline function, there was a notable improvement in dynamic compliance and PVR observed in the ischemic groups that underwent liquid ventilation compared to those subjected to EVLP alone.

Significantly, both LV groups exhibited a substantial enhancement in C_{dyn} and showed a promising trend toward reduction in PVR over the 6-hour EVLP assessment period, suggesting sustained physiological improvement. LV led to lungs exhibiting P/F ratios similar to healthy, non-ischemic lungs, while those treated with EVLP alone had significantly lower P/F ratios.

The preservation technique best suitable during and after warm ischemia remains undecided. *Ex-vivo* lung perfusion has become an important tool in assessing marginal lungs.

4.1 Development of the Ischemic Rat Lung Model

A reproducible ischemic model with IRI was established. Ischemic time refers to the duration between procurement and implantation into the recipient without undergoing perfusion. Ischemia can be categorized into cold or warm ischemia. Warm ischemia occurs when the tissue remains unaltered after cardiac death. The organ experiences cold ischemia during a controlled preservation period to lower metabolism and decrease cell death by regulating temperature. Ideally, it is recommended to restrict total ischemic time to 4–6 hours to ensure the optimal performance of the lung allograft. However, certain studies indicate that an extended duration of up to 9–10 hours might still be considered acceptable[114]. In this study, an established ischemic model with 4 hours of in situ warm ischemia, followed by an overall 10 hours of experimental procedures was established, making it a meaningful and comparable model.

As a standard practice, a translational pathway involves initially testing a potential novel therapy on a small animal model before progressing to trials on larger animals and eventually humans. A rodent EVLP model offers established benefits, as demonstrated by several research groups[110], [111], [115]–[117]. Recently, Ohsumi *et al.* introduced a reliable rat EVLP protocol, showing that rat lungs exposed to cold ischemic times of up to 12 hours maintained stable function during 4 hours of EVLP and after transplantation[118]. In addition to its cost-effectiveness and simplified handling, this model allows for easier execution of multiple cases and experimental groups compared to large animal models. This is especially advantageous for the preliminary screening of new treatments before moving on to large animal studies [119].

The disadvantages of using a rat model are that rat lungs are delicate and prone to developing pulmonary edema quickly, making them less resilient to suboptimal perfusion conditions. This could pose a challenge for testing therapies requiring extended perfusion. Furthermore, some rat strains are hypersensitive to dextran, a component in the standard perfusion solution. This may lead to an allergic reaction, potentially confounding research results. This should be considered in further investigation, though we were able to establish control groups with no prominent allergic reactions.

Rat physiology and biochemistry differ from humans, and gene editing in rats is complex. Thus, transitioning findings from rats to humans remains challenging. Further validation in larger animal models may be necessary for therapeutic development. Although the pig model provides a closer clinical representation of humans, it substantially raises experimental costs, potentially limiting the number of novel therapies that can be tested[119].

4.2 Liquid Ventilation Circuit

A custom LV system was developed, consisting of separate closed circuits for vascular perfusion and liquid ventilation. Experimental LV prototypes are described and can be used to ventilate

rabbits and larger animals. Nadeau *et al*. introduced such a ventilator to induce fast hypothermia over the high density of PFC liquid ventilation in a lamb model to predict the temperature in every phase[120]. Rambaud *et al*. described hypothermic TLV in rabbits with ARDS using a liquid ventilator to continuously control liquid pressures and volumes to reduce lung inflammation and hemodynamic failure[121]. These described LV systems are used *in vivo* and rely on the intact circulatory system of the animal. The LV system we present has a unique setup for extracorporeal lung ventilation and perfusion. The separate closed circuits for vascular perfusion and LV allow for independent regulation of perfusate and liquid ventilant parameters, optimizing conditions for lung reconditioning. Similar to the system described here, the liquid ventilator used by both groups also uses a Y-adapter to enable pressure-regulated mechanical LV with inflow and outflow to minimize dead space ventilation. Pressure-controlled ventilation also allows simplified clinical use and can be adapted more easily to patients in a clinical setting[120].

Perfluorocarbons are widely used when focusing on LV, due to high solvability for gases[122]. Choosing an aqueous fluid with electrolytes ensures a non-toxic and isotonic environment, in contrast to using an oxygenated perfluorocarbon. This choice was made for improved compatibility. The addition of supplements like glucose, specific amino acids, and extra electrolytes to the PBS ventilant led to issues such as septal edema and fluid transgressing from the vascular circuit to the respiratory circuit. To address this, osmolarity measurements were conducted on the ventilate both with and without the additional supplements. As a result, exclusively PBS was used as the ventilant.

The choice of perfusion solution composition, which includes electrolytes, antibiotics, and FBS, is aimed at creating an optimal environment for lung tissue while maintaining appropriate osmotic intravascular pressure to minimize the risk of lung edema. It is crucial to emphasize that the primary objective was to mitigate inflammation processes and support epithelial hemostasis through continuous airway lavage, rather than inducing hypothermia or employing LV for lung ventilation. The use of a membrane (de)oxygenator ensures effective oxygenation of the perfusate[120], [121].

The concept of using an airway lavage to inhibit the inflammation process has been studied by different groups. The findings of Avoine *et al*. support the idea of inhibiting inflammation, coupled with improved gas exchange. The group utilized continuous total liquid ventilation to effectively wash out meconium[123]. Nakajima *et al.* introduced the concept of airway lavage to improve post-transplant lung function after gastric aspiration injury which reduced pro-inflammatory cytokines and lung inflammation[124]. By capitalizing on the washout effect to reduce injury-inducing substances, the results indicate a reduction in inflammation mediators. Expanding on these findings, lungs subjected to significant ischemia displayed a trend of diminished inflammation and enhanced physiological parameters as a result of regeneration through continuous LV.

Utilizing pressure sensors and regulation in both circuits helps minimize the risk of lung injury and enables customized ventilation tailored to lung compliance and size[96]. However, it is important to acknowledge the potential risk of barotrauma, which was addressed by employing low ventilation pressures and volumes. The application of reduced pressures and end-expiratory volumes, maintained below anticipated functional residual capacity, was observed to result in improved respiratory recovery and the preservation of lung structure[122]. This approach may be better tolerated in a long-term experimental setting.

The perfusate and ventilant were not modified throughout the 4-hour LV period. Although our previous studies evaluated the metabolic parameters and confirmed acceptable parameter levels for 4 hours. It should be noted that the potential for the accumulation of harmful cellular byproducts and subsequent metabolic instability remains.

Following the reconditioning period of LV, the surfactant is administered through a syringe over the trachea. However, it should be noted that certain regions of the lung may not receive sufficient surfactant distribution, leading to inadequate ventilation in those areas and potentially resulting in poorer oxygenation during EVLP assessment.

4.3. Development of the EVLP System and Protocol

EVLP platforms offer a means to assess lung function and determine the suitability of marginal donor lungs for transplantation. In addition, EVLP offers the potential to reduce ischemic time, implement interventions, and recondition lungs. We developed a custom rodent EVLP system to assess experimental single-left lungs. Custom-designed cannulas (see Figure 3), incorporated pressure sensors and a specifically designed organ chamber to allow easy access and more importantly protective upright positioning of the lung hindering any complications from compression (Figure 15). This also facilitated an unhindered pulmonary venous outflow.

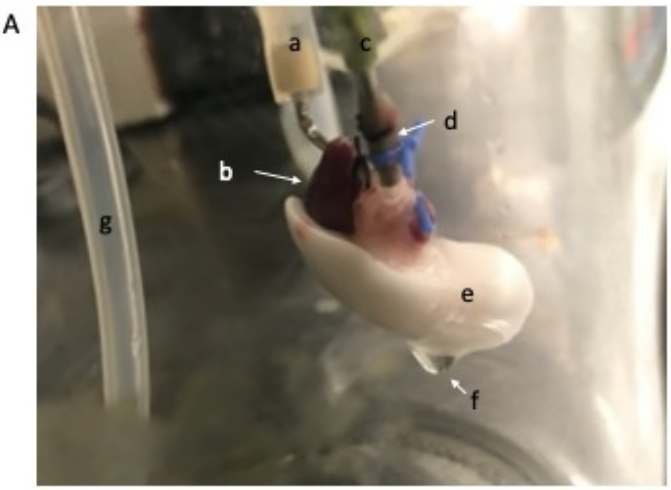

Figure 15. A. *Upright positioning of the lung in the organ chamber. a. arterial cannula b. heart c. tracheal cannula d. trachea e. left lung f. passive venous outflow droplet g. perfusion solution collecting tube.*

The system allowed constant perfusion flowrate, passive venous outflow, and mechanical volume-controlled ventilation, providing stable and controlled circulation, hydrostatic pressure, and oxygenation. To avoid high hydrostatic pressures and edema the atrium was not cannulated, letting the perfusate free to drain from the pulmonary veins. The target perfusion flowrate was set at 100% of cardiac output, initially starting at 20% to let the lung slowly initiate the rewarming phase. Protocols suggest that a lower flowrate at 20% or 40% of cardiac output may further reduce hydrostatic pressure and pulmonary edema[114]. This could be beneficial during EVLP in the model described here and should be further investigated. Ventilation was initiated after the lung reached physiological temperature[111].

The optimal composition of perfusion solution is still controversial, yet there does not seem to be a preferred solution, especially for extended preservation periods[125]. The greatest difference between perfusates is extracellular or cellular solutions. Steen solution is the only perfusion solution clinically approved for use without additional blood products[126]. As initially outlined by Stig Steen and colleagues at Lund University, this involves employing an extracellular solution containing dextran, which shields the endothelium from complement- and cell-triggered damage, while also suppressing coagulation and platelet aggregation (referred to as low potassium dextran solution). Additionally, human albumin is introduced to uphold optimal colloidal pressure[127]. Upon comparison of cellular and acellular perfusates, there appear to be no notable differences in respiratory parameters or graft functionality in lungs with ischemic injury[128]. Using acellular perfusion is more straightforward due to the reduced lifespan of red blood cells in the extracorporeal circuit, which may lead to hemolysis over time[129]. As a result, a blood cellular solution restricts perfusion times to under two hours, which would not be productive in our protocol.

The inclusion of albumin in the Steen solution plays a crucial role in maintaining elevated oncotic pressure, thereby delaying the onset of pulmonary edema. Through the experience with preliminary experiments, we chose an acellular perfusion solution. The perfusate was an electrolyte-based albumin solution (table 8), to ensure physiological tissue metabolism and osmotic pressure stability. The W/D ratio in the non-ischemic group did not show a significant increase compared to lung tissue without any experimental protocol ($p=0.07$), suggesting limited edema buildup. This implies that the custom perfusate formulation did not induce significant edema.

Potential factors such as air embolism during cannulation or rapid rewarming leading to thermal shock could be limitations that exacerbate pulmonary dysfunction. While these concerns were taken into account and addressed, they cannot be completely dismissed.

4.4 Assessment during EVLP

In addition to comparing the efficacy of LV against the current standards of static cold storage and EVLP regeneration, we also evaluated the degree of IRI among the experimental groups.

To comprehensively assess lung injury in this protocol, a multidimensional approach was employed. The dysfunction of the lungs during EVLP was evaluated by tracking trends in lung function parameters and observing metabolic changes in the perfusate. Following the EVLP evaluation phase, we measured alterations in the alveolar-capillary barrier using the W/D ratio, an indirect indicator of extravascular lung water accumulation. Additionally, we examined the presence of an inflammatory response by measuring inflammatory cytokines in the BAL and evaluating the activation of the alveolar marker F4/80, along with detecting cell apoptosis in lung tissue.

The physiologic improvement of the LV groups during the EVLP assessment period gave additional support to the idea that these lungs are being rehabilitated throughout the experiment. In clinical lung transplantation, elevated dynamic compliance in a lung allograft can lead to failure to wean from mechanical ventilation[130]. Lung compliance decreases due to lung edema and the absence of surfactant protein[131]. The dysfunction of surfactant protein, caused by dilution and reduced production, leads to increased alveolar wall tension and can result in alveolar collapse. Interstitial edema interferes with lung parenchyma elasticity, leading to decreased compliance. Additionally, there is a tendency for small airways to collapse due to diminished support from the surrounding tissue and the congestion of central lymphatics, resulting in further obstruction to the aeration of lung parenchyma.

The shift from interstitial to alveolar edema is characterized by changes in ventilation parameters, including decreased compliance. Alveolar flooding limits lung volume and further decreases compliance. The lower regions of the lung become consolidated due to collapsed alveoli under increased lung weight, decreasing the aerated volume[132]. Mechanical ventilation during and after lung transplantation contributes to PGD by causing volume trauma in higher regions and atelectrauma in less ventilated regions[131], [132]. Hence, it is important to assess dynamic compliance. In successful post-transplantation management, the assessment of C_{dyn} during EVLP can predict early outcomes after transplantation. While the experimental groups did not demonstrate a significant correction in C_{dyn} at the 6-hour EVLP endpoint compared to the ischemic control group, there was a noticeable trend towards improved C_{dyn} in the LV group. It is also noteworthy that the LV group showed a significant improvement in C_{dyn} over the 6-hour EVLP period. This suggests a sustained physiological enhancement throughout the assessment period. This was not observed in the other experimental groups or non-ischemic control lungs. Additionally, this aligns with the lower W/D ratio, indicating reduced edema in both LV groups.

Increased PVR is a common occurrence during and after lung transplantation leading to elevated pulmonary vascular pressures. Several factors contribute to this increase in PVR. Hypoxic vasoconstriction restricts blood flow to consolidated lung areas, while engorged central lymphatics exert pressure on arterioles within the broncho-vascular axis[132]. Overdistended areas generate tension on the alveolar wall, reducing capillary flow[132], [133]. Elevated PVR can lead to right heart strain and failure in at-risk patient populations[134]. These points underscore the importance of the improvements in these parameters experimentally. Another trend that emerged during EVLP was a reduction in PVR observed in the LV group, although this did not reach significance. At the 6-hour endpoint, the experimental groups trended toward lower PVR than the ischemic control group. These physiological findings indicate, that LV with perfusion flushes the inflammation mediating debris and allows for the regeneration of IRI. It is unclear that a longer EVLP period may allow for a continued sustaining of these physiological parameters and must be investigated.

Additionally, the P/F ratio has been a longstanding evaluation tool for clinical lung transplantation. Lung edema due to IRI leads to impaired gas exchange and hypoxemia in post-transplant PGD due to ventilation-perfusion inequality. The severity of hypoxemia is assessed using P/F ratio[88].

The experimental results demonstrated that lungs rehabilitated with LV exhibited P/F ratios similar to those of healthy, non-ischemic lungs. In contrast, lungs treated solely with EVLP showed significantly lower P/F ratios compared to healthy lungs. This emphasizes the significance of the physiological findings in the LV groups. Moreover, the experiments showed that lungs rehabilitated with LV achieved P/F ratios akin to non-ischemic lungs, demonstrating the lung tissue's capability for oxygenation. As all lung groups experienced ischemic periods and cell death, there was a consequent rise in lactate levels during the EVLP period across all experimental groups. This signifies heightened metabolic activity and cellular stress. The elevated lactate levels account for lower pH of the perfusion solution. Nevertheless, there was no notable difference in pH reduction between the groups, implying that not only the experimental groups were adversely affected to a greater extent. The elevated pH levels noted in the LV groups, in contrast to the other groups at various time points, suggest enhanced metabolic stability in those lungs. This observation implies that LV could potentially have positive effects on sustaining pH balance and perhaps preserving lung function. However, the absence of a correlation between lung performance and lactate concentration indicates that lactate levels alone may not be a reliable indicator of lung function during EVLP assessment in this study protocol.

The similar glucose consumption across all groups during EVLP assessment indicates that the lungs in the various experimental groups had comparable metabolic demands. The consistently lower glucose consumption in the non-ischemic control group indicates a potential difference in metabolic activity compared to the other groups, which may be attributed to the absence of

ischemic insult. During the entire 6-hour duration of EVLP assessment, there was no alteration made to the perfusate. A potential approach to ensure optimal perfusion and metabolic homeostasis could involve the addition of glucose based on measured glucose levels and/or modifying the perfusate composition based on lactate and pH levels.

To explore sustained improvements in physiological parameters, it would be valuable to consider conducting longer periods of EVLP. This avenue could be explored in future experiments.

4.5 Pathological Assessment after EVLP

Lung edema, characterized by the accumulation of fluid in the extravascular spaces of lung tissue, plays a crucial role in the pathophysiology of PGD. It leads to reduced lung compliance, increased PVR, and hypoxemia[132]. The W/D ratio was utilized as a measure to assess pulmonary edema and barrier function[135]. The results demonstrated a trend towards a lower W/D ratio in both LV groups compared to the other ischemic groups, although statistical significance was not achieved. These findings suggest a potential benefit of LV in reducing pulmonary edema.

The total cell count in BAL fluid can provide information about the presence and quantity of different types of cells. This can give insights into the presence of inflammation, infection, or allergic reactions in the lungs. While differentiating between cell types was not the primary objective, the focus was on assessing the mechanical clearance of cells during LV and the impact of clodronate-induced apoptosis. After analyzing the BAL fluid, it was observed that the ischemic lung groups treated with clodronate, LV, and the group with a combination of both showed a lower cell count compared to the ischemic control group. The results indicate that LV had a mechanical flushing effect on the respiratory tract, while clodronate demonstrated an apoptotic effect on macrophages. However, to further confirm these findings, future studies could involve differentiating the BAL cells. Additionally, characterizing the presence of activated AMs could be achieved by sorting for the F4/80 marker during immunohistochemistry.

4.5.1 Cytokine Analysis and Inflammatory Markers

Even more interesting than the physiologic improvements in lung function were the mechanistic insights gained from our cytokine analysis. The following cytokines were analyzed: IL-6, INF-γ, IL-1α, GRO- α, TNF- α, and MCP-1.

Interleukin (IL-1α) is an inflammatory cytokine that is released by endothelial cells, macrophages, and lymphocytes in times of oxidative or metabolic stress which initiates and propagates inflammatory responses[136]. IL-1α is a proinflammatory cytokine that plays a significant role in ischemia-reperfusion injury (IRI) in the lungs. During the ischemic phase, IL-1α is released from damaged cells and activates the innate immune system. Upon reperfusion, IL-1α is further upregulated, leading to the recruitment of neutrophils and macrophages to the lungs, resulting in an inflammatory response. IL-1α also contributes to endothelial and epithelial cell injury, which

can lead to capillary leak, pulmonary edema, and impaired gas exchange. The inhibition of IL-1α has been shown to reduce IRI and improve outcomes in lung transplantation models. Therefore, targeting IL-1α signaling pathways may be a potential therapeutic strategy for the treatment of IRI in lung transplantation. This cytokine has been identified as an important mediator of pulmonary ischemia-reperfusion injury[137]. INF-γ is another prominent cytokine that is produced by macrophages and acts as a primer of alveolar macrophages during inflammatory states[49]. Interferon-gamma (IFN-γ) is a pro-inflammatory cytokine that plays a role in ischemia-reperfusion injury (IRI) in various organs, including the lung. In the context of lung IRI, IFN-γ is produced by both resident lung cells and infiltrating immune cells, such as T cells and natural killer cells.

IFN-γ promotes the activation of alveolar macrophages and induces the production of additional pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β), which contribute to tissue damage and dysfunction in the lungs.

IFN-γ also activates endothelial cells, leading to increased expression of adhesion molecules and recruitment of leukocytes to the lung tissue, further exacerbating the inflammatory response.

Overall, IFN-γ plays a significant role in the pathogenesis of lung IRI, and targeting its signaling pathways has been suggested as a potential therapeutic strategy for preventing or ameliorating this condition[138]. The observed decrease in INF-γ in the liquid ventilation groups compared with the ischemic control group further contributes to the decreased inflammatory response seen in these groups. The significant decrease in both IL-1α and INF-γ concentrations in the liquid ventilation lungs allows us to postulate that the LV process may clear harmful cytokines from the alveolus which may halt the propagation of the inflammatory cascade. This anti-inflammatory property of the LV process makes it a unique treatment possibility for *ex-vivo* lung rehabilitation. In their study, Wang *et al*. affirmed that EVLP elevated the levels of IL-6 and IL-10 in injured lungs and they utilized 3-aminobenzmide as a treatment to reduce these cytokines. Evaluated levels of IL-6 were consistently observed in all of our experimental groups compared to the non-ischemic control group. This could be attributed to the extended perfusion, including the LV phase. Further investigation might warrant consideration of a treatment option to mitigate the exacerbated damage induced by IL-6[117].

It was further attempted to explore whether macrophage activation was responsible for the physiologic and inflammatory changes observed in the lungs. The clodronate groups were intended to deplete macrophages after lung harvest to mitigate the inflammatory process during EVLP assessment. This paradoxically resulted in increased concentrations of Monocyte chemoattractant protein-1 (MCP-1), an inflammatory cytokine associated with antigen-presenting cell recruitment, in the BAL samples. MCP-1 is a chemokine that plays a critical role in recruiting monocytes and other immune cells to sites of inflammation[139]. In the context of lung IRI, MCP-1 is upregulated in both animal models and human lung transplant recipients[39].

Studies have demonstrated that blocking MCP-1 or its receptor (CCR2) can reduce lung injury in animal models of IRI, suggesting that MCP-1 plays a key role in the pathogenesis of IRI. MCP-1 can activate various inflammatory pathways, such as NF-κB, MAPK, and ROS, leading to increased oxidative stress, apoptosis, and cellular damage[140]. This finding may be related to macrophages undergoing apoptosis and cell lysis leading to DAMPs and inflammatory cytokine release.

When investigating the Gro-α levels, the LV group displayed higher levels compared to the other groups. The groups that received clodronate treatment showed a trend towards lower GRO-α levels compared to the ischemic control group. Note, no statistical significance was reached between any of the groups. GRO-α also known as CXCL1 expressed in alveolar type ll epithelial cells, which is elevated due to an increase of iNKT cells and TNF-α from macrophages. CXCL1 then recruits neutrophils to the lung, causing inflammation[141], [142]. In studies, EVLP showed a decrease in neutrophil count, however, triggered pro-inflammatory chemokines like CXCL1[141], [142]. The trend towards lower levels in the clodronate group can be explained by the depletion of AM and the resulting interruption of the signaling cascade. The depletion of AM may contribute to the altered GRO-α levels.

A key mechanistic finding that further supports the physiologic improvements in the LV group is that this group had the lowest detection of the activated macrophage marker F4/80. This adhesion protein is present on macrophages and certain other immune cell, excluding lymphocytes[143]. A possible explanation for this result is that LV may mechanically clear AMs thus decreasing the cell type most responsible for propagating ischemia-reperfusion injury in the lung. This may provide additional insight into the reduction of IL-1α, a potent inflammatory mediator released by macrophages that propagate IRI. This reduction is likely due to the mechanical reduction of AMs. In contrast, this depletion of AM and subsequent reduction in cytokine levels was not noted in the clodronate groups, possibly because there was not sufficient time for macrophage apoptosis following clodronate administration.

However, there is some conflicting evidence as to whether depletion of AMs is protective or injurious[144].

As described before AMs differ depending on their distinct subpopulations and localization in the lung. M2 macrophages have anti-inflammatory and tissue repair functions and play a critical role in maintaining lung homeostasis[72], [145], [146].

Consequently, the depletion of macrophages can result in compromised pathogen and debris clearance, elevating the susceptibility to infections and injury. Moreover, the depletion of AMs can disrupt the cytokine balance, resulting in dysregulated immune responses and inflammation[144]. AMs also play a vital role in lung tissue repair and remodeling and their depletion can impede these processes, leading to persistent tissue damage and fibrosis[147].

These factors highlight the significance of investigating long-term consequences on lung health following AM depletion and developing targeted strategies to address specific AM populations.
These findings provide a broad overview of the inflammation process in this model. To gain a deeper understanding of IRI following LV regeneration and its advantages, it is crucial to assess cytokines and active AMs after varying durations of EVLP. This data can then be compared to baseline levels in a healthy lung after 6 hours of EVLP.

4.6 Future Considerations

There are several overall considerations to our conclusions that deserve mention. The most important is whether the physical and immunologic principles reported here will translate to large animal or human scale models. The mechanical LV process will certainly have different challenges with a two or three-lobe human lung compared to a single-lobe rat lung. There are also many noted differences between the innate immune system of a rat and a human, which have the potential to alter the magnitude of our anti-inflammatory findings associated with LV. Additionally, logistical concerns will arise if this technology were to be applied in the clinical realm, specifically defining which lungs should be trialed on LV rather than traditional EVLP. While the translation of these data to the clinical realm will certainly require significant additional research and development, the limitations of this study should not undermine the potential conceptual advances in the field of lung transplantation. This model and regeneration method should be verified by transplanting the experimental left lung. It is imperative to define specific parameters to develop a protocol for performing an orthotopic left lung transplant using lungs that have undergone experimental interventions. An orthotopic left lung transplant should be done via left thoracotomy on syngeneic Lewis rats. Three transplants should be performed per group. Transplanted rats are to be analyzed with survival to a maximum of 28 days (elective sacrifice and harvest), histology and cytokine analysis, and computer tomography controls after 7 days after transplant[148], [149].

4.7 Conclusion

LV is considered an unconventional concept and treatment method within the scientific community. However, with the development of EVLP techniques for lung preservation and rehabilitation, there is a growing need for novel approaches to salvage lungs that would otherwise be deemed unsuitable for transplantation. In this study, a proof-of-concept experiment that showcases the potential of LV as a treatment strategy for severely injured lungs that have undergone prolonged warm ischemia (4 hours) was presented. Although our data did not demonstrate complete restoration of lung function to baseline levels, we did observe improved

pulmonary performance compared to lungs treated solely with EVLP, which currently serves as the standard approach for clinical lung assessment and recovery before transplantation.

5. SUMMARY

Patients with end-stage pulmonary disease currently have lung transplantation as the only curative treatment option. The number of patients awaiting lung transplantation has been steadily rising, highlighting the urgent requirement for effective approaches to expand the pool of viable donor lungs for transplantation. EVLP was developed to evaluate potential donor lungs post-procurement to enhance the number of available lungs for transplantation. However, it became evident that this method could also serve as a means to treat and enhance lung function before transplantation, effectively converting initially rejected organs into usable allografts. Despite attempts to reduce lung ischemia during organ procurement and transportation, IRI remains a significant factor contributing to post-transplant allograft dysfunction. Studies have identified activated AMs as a crucial inflammatory mediator in lung IRI. To prevent the progression of macrophage-mediated lung injury, depleting macrophages has shown promise. In murine models, macrophage depletion has been extensively studied using liposomal clodronate to investigate the role of macrophages in various pathological processes.

The objective of this study was to establish a reliable rat EVLP model and a novel method for *ex-vivo* lung reconditioning following extensive IRI using LV in preparation for transplantation. We hypothesized that utilizing a LV *ex-vivo* circuit would reduce alveolar macrophage-mediated inflammation and IRI and may facilitate cellular metabolic recovery during LV. A novel circuit was created for lung perfusion and LV. Additionally, a protocol for depleting AMs using liposomal clodronate was established, along with an EVLP circuit for lung assessment. The method results in substantial enhancements in physiological parameters that are crucial for clinical outcomes. The observed improvement in physiological performance can be attributed to the elimination of AMs and inflammatory cytokines. This not only reduces the release of additional inflammatory cytokines but also effectively halts the progression of IRI. Although the extensive applicability of these findings to transplantation in large animals or humans has not been investigated, the results present a promising approach for reconditioning lungs that have suffered significant ischemic injury. Implementing this method has the potential to expand the pool of available lung organs for donation and positively impact clinical outcomes for patients.

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Acknowledgments

First and foremost, I would like to express my gratitude to my doctoral advisor, Professor Thorsten Walles. Without your continuous support, this dissertation would not have been possible. You ignited my interest in medical research and reliably accompanied me through every stage of the work. I am particularly thankful for your academic mentorship in Magdeburg and the opportunity to participate in the Biomedical Education Program, which brought me to Boston. My research year in Boston not only bolstered my confidence in biomedical research but also provided extraordinary experiences I would not want to miss anymore.

My heartfelt thanks extend to Professor Harald, MD, for granting me the opportunity to join his laboratory team in Boston allowing me to conduct experiments in the Laboratory for Tissue Engineering and Regenerative Medicine. His exceptional expertise and guidance in transplant research offered me profound insights into a previously unfamiliar world, reinforcing my passion for transplantation research and regenerative medicine. I am forever grateful for this opportunity.

A special acknowledgment goes to Doctor David Becerra and the entire Ott Lab team for their unwavering guidance and supervision. Whether in planning the first experiments, dealing with failed attempts, or preparing nervously for presentations, you were always a reliable and humorous pillar of support. I fondly recall the pleasant and motivating atmosphere in the lab and the team's all-encompassing willingness to help.

During my time in the experimental thoracic surgical laboratory under Professor Thorsten Walles's guidance, I extend my thanks to Dr. Cornelia Wiese-Rischke and Marion Möckel. Their availability for expert guidance, and teaching of methods and processes, has been instrumental in building the foundation I now possess. Thank you for your support throughout.

I am grateful to Christoph Maeß for his professional support in setting up the Magdeburg lung perfusion system.

Special thanks to IMTR Rottmersleben, ZEMM Würzburg, and the dedicated support staff for the opportunity for organ retrieval and support in the process.

Furthermore, heartfelt thanks to the Experimentelle Fabrik Magdeburg for providing access to their Angiography system and to Professor Pech of the Department of Radiology at the University

Hospital Magdeburg for enabling lung scans with staff expertise and the computer tomography system.

In conclusion, I express my deepest gratitude to my parents, Carolin and Michael, and my two sisters, Teresa and Matilda. Your unwavering support has been my constant throughout this journey, encouraging even in challenging situations. Without you, this project would not have been possible. You are my greatest motivation.

Declaration of Honesty

Ich erkläre, dass ich die der Medizinischen Fakultät der Otto-von-Guericke-Universität zur Promotion eingereichte Dissertation mit dem Titel

Normothermic Liquid Ventilation as a Method to Rehabilitate Lungs with Ischemia-Reperfusion Injury for Transplantation in an *Ex-Vivo* Rat Model

in der Universitätsklinik für Herz- und Thoraxchirurgie mit Unterstützung durch Herrn Prof. Dr. med Thorsten Walles und in The Laboratory for Organ Engineering and Regeneration unter der Leitung von Herrn Prof. Harald Ott, M.D

ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe.

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Ich habe diese Dissertation bisher an keiner in- oder ausländischen Hochschule zur Promotion eingereicht. Ich übertrage der Medizinischen Fakultät das Recht, weitere Kopien meiner Dissertation herzustellen und zu vertreiben.

Magdeburg, den 29.10.2024

Presentation of Educational Background

Der Lebenslauf ist in der Version aus Datenschutzgründen nicht enthalten.

Publications and Conference Participation

November 2022	Becerra D, Linge H, Jeffs S, Roberts S, O J, Ott HC. Liquid Ventilation Reconditions Isolated Rat Lungs Following Ischemia-Reperfusion Injury. Tissue Eng Part A. 2022 Nov;28(21-22):918-928. doi: 10.1089/ten.
August 2021	Normothermic Liquid Ventilation Attenuates Inflammation from Ischemia-Reperfusion Injury in an Ex Vivo Rat Model, Vortrag 20th Congress of the European Society for Organ Transplantation, Milan, Italy
Oktober 2020	Optimierung der Organperfusion in einem präklinischen *ex-vivo* Lungenperfusionsmodell, Poster 29. Jahrestagung der Deutschen Gesellschaft für Thoraxchirurgie, Erfurt. DOI:10.13140/RG.2.2.32299.67369