



Successful 3-day lung preservation using a cyclic normothermic ex vivo lung perfusion strategy

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Summary

Background Cold static preservation (CSP) at higher temperatures (10°C) has been recently shown as an optimal strategy up to 24-36h of preservation. Here, we hypothesized that alternating 10°C static storage with cycles of normothermic *ex vivo* lung perfusion (EVLV) would provide conditions for cellular “recharge”, allowing for multi-day lung preservation.

Methods Donor lungs from male Yorkshire pigs were preserved using 10°C CSP with two cycles of 4h EVLP. After a total of 3 days of preservation, a left lung transplant was performed followed by 4h of graft evaluation. As controls, 2 lungs were preserved solely with continuous 10°C preservation for 3 days and transplanted.

Findings For animals receiving lungs preserved using a cyclic EVLP protocol, lung function and histological structures were stable and the recipient systemic partial pressure of oxygen/fraction of inspired oxygen (P/F Ratio) after excluding the contralateral lung was 422 ± 61 mmHg. In contrast, lungs preserved solely in continuous cold static storage at 10°C for 72h developed massive lung failure, resulting in recipient death. Metabolomic analysis revealed that EVLP plays a critical role in the re-vitalization of key central carbon energy metabolites (Glucose, Succinate, N-Acetyl Aspartate) and reducing the expression of the inflammasome activation marker *CASP1*.

Interpretation In conclusion, we demonstrate for the first time the feasibility of 3-day lung preservation leading to excellent early post-transplant outcomes. The thoughtful combination of cold storage (10°C) and intermittent EVLP can open new opportunities in organ transplantation.

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Introduction

For those with end-stage lung disease, lung transplantation is a lifesaving therapy. One of the obstacles faced in clinical transplantation is the amount of time in which

the graft can be preserved in a viable state prior to transplant. Using the current gold standard practice of cold static preservation within a cooler of ice, clinical preservation times are limited to approximately 6-8h.¹ Recently, we have shown the superiority of 10°C for the cold static storage of donor lungs in comparison to the conventional ice cooler approach. Through usage of 10°C cold static storage, donor lungs were found to have maintained mitochondrial health and demonstrated

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Research in context

Evidence before this study

The clinical standard of lung preservation involves storage on ice (4°C), which is currently limited to 6-8h. We recently have identified 10°C to be an optimal temperature for lung storage, allowing for preservation times of up to 24-36h. Despite this information, even by storing lungs at 10°C, cold static preservation is not unlimited. More recently, normothermic (37°C) *ex vivo* lung perfusion (EVLP) was developed as a platform for donor lung assessment. During EVLP, lungs are brought back to physiological temperature, re-oxygenated, and metabolism is restored. Pre-clinical studies have demonstrated that by interrupting cold storage with EVLP (cold-warm-cold), longer preservation times can be tolerated. Like conventional cold storage, EVLP is also limited to clinical times of around 4-6h.

Added value of this study

Here, we explored the concept of multi-day lung preservation by pairing 10°C lung preservation with short cycles of EVLP. For the first time, we demonstrate successful 3-days of lung preservation with exceptional immediate post-transplant graft function. Furthermore, we identify the importance of EVLP to re-vitalize metabolites lost during the cold storage period and reduce mitochondrial injury.

Implications of all the available evidence

Advancements towards multi-day lung preservation will allow for the implementation of organ banking approaches and open new opportunities in organ preservation and transplantation.

excellent graft function after a 36h storage period.² A clinical trial investigating the feasibility of 10°C lung preservation to allow for semi-elective lung transplantation is currently underway (ClinicalTrials.gov Identifier: NCT04616365). Despite these positive findings, continuous static storage cannot be prolonged unlimited, and it is still unknown whether donor lungs can tolerate storage at 10°C for periods beyond 24-36h. If preservation of lung grafts for a period of multiple days could be achieved, several important implications would ensue. Some of these implications include the overcoming of geographical hurdles faced in organ donation, the ability to explore the concept of organ banking, the ability to perform time dependent lung repair, and a revolution of organ allocation practises.

Within the setting of lung preservation and repair, the recently developed technology, normothermic *ex vivo* lung perfusion (EVLP), has important implications. Normothermic EVLP is a novel platform which simulates an *in vivo* environment prior to transplantation.

During EVLP, lungs are brought back to human body temperature, re-oxygenated, and metabolism is restored. Although conventionally used as a platform for organ assessment and reconditioning, EVLP has recently demonstrated itself as a viable option for the safe extension of preservation time. By interrupting cold storage with EVLP, longer preservation times (in the order of 24h) can be tolerated.^{3,4} Furthermore, analysis of our own clinical experience supports this notion in which lungs preserved for more than 12h (through a short period of EVLP usage) have similar outcomes to our conventional transplant patients where donor organs were preserved solely with cold static preservation at 4°C for shorter periods.⁵ However, currently clinicians have not extended EVLP for long periods, generally restricting application to periods of 4-6h clinically on the platform.⁶ Attempts at longer normothermic perfusion times can often lead to the development of organ edema and accumulation of inflammatory bioproducts, rendering the organ non-transplantable.⁷ Thus, even at experimental level, more than 24hs continuous perfusion has not been achieved to date in *ex vivo* models.

Therefore, to achieve multi-day preservation, we hypothesized that alternating an optimized cold static preservation method at 10°C with EVLP in repeated cycles (cold-warm-cold preservation) would provide the ideal conditions to preserve organ quality for a much longer period compared to each of these strategies alone. In this study, we aimed to investigate the feasibility and efficacy of this approach within a well-established pig lung transplant model of graft evaluation. Furthermore, we provided mechanistic insights by evaluating the dynamics of organ metabolism during cycles of cold and warm preservation.

Methods

Ethics

All animals received humane care and all protocols were evaluated and approved by the Animal Care Committee (Animal Use protocol Reference #5491.5.3), Toronto General Hospital Research Institute, UHN, following the Canadian Council on Animal Care Certificate of Good Animal Practices Guidelines (<https://www.ccac.ca/en/program-features/certificate-of-gap-good-animal-practice.html>).⁸

Study design

With the purpose of achieving multi-day lung preservation, we first began by performing a set of pilot experiments to evaluate whether continuous 10°C static preservation would be suitable to achieve lung viability after 72h. Lung viability was determined by performing functional assessments according to the Toronto EVLP protocol, encompassing measurements of lung airway

pressures, compliances, and gas exchange.^{6,9} A pre-determined perfusion termination criteria of excessive perfusate loss (>1000ml/hr) was put forth. Results of those experiments showed that continuous 10°C static preservation was not able to sustain graft function after the 72h period ($n=3$).

In a new series of experiments, 72h lung preservation using an intermittent EVLP protocol ($n=4$) was put forth. Although it may be considered small, this sample size is typical of large animal experiments of this magnitude, and allows for the demonstration of a proof-of-concept. The protocol was designed to follow a 24h schedule, in which perfusion times were in accordance to what is currently practised in clinical settings today (4h).⁶ The full protocol is shown in [Figure 2B](#). Lung evaluation was performed after a single-left lung transplant within an established porcine model.¹⁰ In this model, venous gases from the implanted graft can be measured hourly to determine gas exchange properties. At the end of the reperfusion phase, the right pulmonary artery was clamped to exclude the native lung from the animal's circulatory system. After waiting for 5 minutes, a systemic blood gas was taken to directly measure the performance of the transplanted graft. As a negative control, two lungs which were preserved for 72h using 10°C static preservation alone were also transplanted. Tissue biopsy samples were taken during the preservation period for biological analyses. Data reporting was performed following the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.¹¹

Lung procurement procedure

Donor lung procurement and transplantation followed previously published methods.¹⁰ Donor Yorkshire pigs (28-35kg) were sedated with ketamine (20 mg/kg IM), midazolam (0.3 mg/kg IM), and atropine (0.04 mg/kg IM), and then anesthetized with inhaled isoflurane (1-3%), followed by a continuous intravenous injection of propofol (3-4 mg/kg/h) and remifentanyl (9-30 µg/kg/hr). The animals were placed in supine position, intubated and subsequently pressure-control ventilated at an inspired oxygen fraction (FiO₂) of 0.5, a frequency of 15 breaths/min, a positive end-expiratory pressure (PEEP) of 5 cmH₂O, and a controlled pressure above PEEP of 15 cmH₂O. After a median sternotomy, the main pulmonary artery was cannulated, the superior and inferior vena cava was tied, the aorta was clamped, and the left atrial appendage was incised. A 2L antegrade flush was performed in the donor at a height of 30 cm above the heart. A ventilator inspiratory hold was performed, the trachea was clamped, and the lungs were excised and placed on the back-table. Once on the back-table, an additional 1L retrograde flush was performed. The lungs were placed in an organ bag and kept at 4°C. Donor animals with a P/F ratio < 300 mmHg were excluded from the study.

Ex vivo lung perfusion (EVLP)

EVLP was performed according to the Toronto protocol, involving an acellular perfusate, a closed left atrium, protective flows and protective ventilation.¹² The lung bloc was placed in the XVIVO chamber (XVIVO, Denver, CO). The trachea was intubated and connected to the ventilator. The pulmonary artery (PA) was cannulated, and the LA and PA were directly connected to the perfusion circuit. The EVLP perfusate consisted of 1.5 L of an extracellular albumin (70 g/L) solution (STEENTM). The perfusate was driven by a centrifugal pump at a constant flow rate. The temperature of the perfusate was gradually increased to 37°C. When the temperature of the perfusate reached 32°C, volume-control ventilation (VCV) was initiated. The perfusate flow rate was gradually increased to the full flow rate of 40% estimated cardiac output (CO = 100 ml/kg). EVLP was performed for 4h, in which physiologic assessments were taken hourly. These included ventilator parameters (dynamic compliance, static compliance, peak airway pressure, plateau pressure) and perfusate blood gas analysis. Lungs were weighed prior to and after EVLP (Model CS 2000, OHAUS Corporation, USA). The net weight gain was calculated and used to as a measure of lung edema. After the first EVLP, the EVLP circuit was stored in a walk-in cooler at 4°C overnight and re-connected to the lung for the next EVLP cycle using snap-cannulas. An ultra-violet C device was added to the circuit and run continuously during the perfusion periods to prevent potential microbial contamination. Standard bacterial cultures were performed on EVLP perfusate samples to quantify changes in bacterial load during the preservation period.

Lung transplantation procedure

To begin the transplant procedure, a left thoracotomy was performed through the fifth intercostal space. The pulmonary hilum was dissected, and the left azygous vein was carefully elevated from the left atrium and ligated. The inferior pulmonary ligament was divided. Both the right and left main pulmonary arteries were carefully dissected. After administration of heparin, a left pneumonectomy was completed. The bronchial anastomosis was performed first with a continuous 4-0 synthetic, monofilament, nonabsorbable polypropylene suture interrupted in two places. The PA anastomosis was performed next with a continuous 5-0 PROLENE suture interrupted in two places. Lastly, the left atrial anastomosis was performed with a continuous 5-0 PROLENE suture interrupted in two places. After that, the transplanted lung was re-inflated to a volume of 10ml/kg of mean donor/recipient body weight. The lungs were de-aired through the left atrial anastomosis. Hourly ventilator assessments (peak airway pressure, plateau pressure, dynamic compliance, static) and blood gases at an FiO₂ of 100% from the left upper vein and lower vein were taken. The right pulmonary artery was clamped 4 hours after reperfusion to assess functions of

the transplanted lung only and a systemic arterial blood gas sample was taken.

Lung tissue collection

Tissue samples were taken at specific timepoints and separated to be either snap-frozen and stored at -80°C or formalin-fixed, paraffin-embedded, and sectioned for histological analysis. Lung biopsies were taken from the anterior portions of the right upper lobe at the beginning of preservation (oh), after EVLP1 (28h), after EVLP2 (52h). Before transplantation, the peripheral–lateral portions of the right upper and lower lobes were sampled, and the left lung was spared for transplantation. After reperfusion, tissue from the peripheral–lateral portions of the left upper and left lower lobes of the transplanted lung were sampled. For lungs in the negative control group stored using continuous 10°C storage alone, tissue biopsies were taken in a similar fashion and at identical corresponding timepoints.

Metabolomic analysis

Tissue samples (oh, 28h, 52h, and 72h) were taken and snap-frozen during 3 day preservation studies. Samples were stored at -80°C and sent to Metabolon (Metabolon Inc., Durham, NC) for analysis of a single-carbon metabolism panel (Pyruvic Acid, Lactic Acid, 2-Ketoglutaric Acid, Succinic Acid, Fumaric Acid, Malic Acid, N-Acetyl aspartic Acid) as well as glucose using LC-MS/MS.

Gene expression measurement

Gene expression for cell death was evaluated using a Custom RT2 PCR Array for Pigs (CLAS40678D) which includes genes for necroptosis (*TNFA*, *FASLG*, *CASP8*, *NFKB*), pyroptosis (*CASP1*, *GPX4*), apoptosis (*CASP3*, *CASP8*, *CASP9*, *TP53*, *NFKB*) and ferroptosis (*MPO*, *GPX4*, *TP53*). Gene expression for mitochondrial injury was performed in a similar fashion using a Custom RT2 PCR Array for Pigs (CLAS41706) including mitochondrial injury genes (*B2M*, *LOC100739238*, *LOC100624950*, *HSP70.2*, *ARRDC3*, *DNAJB1*, *EDN1*, *ASB1*, *SLC25A25*, *GADD45B*).

Following manufacturer's protocols, RNA was converted to cDNA (Cat #330404, RT2 First Strand Kit, Qiagen). cDNA was loaded into plates, in triplicate, mixed with RT2 SYBR Green qPCR Master Mix (Cat#330503, RT2 SYBR Green qPCR Master Mix, Qiagen). Ct values were analyzed using Qiagen RT2 Profiler PCR Data Analysis Software. Genes were normalized using the Geometric Mean of Housekeeping Genes *ACTA1* and *ACTG1*.

Lung histology

Lung Tissue was collected at the beginning of the preservation period, after EVLP1, after EVLP2, and at the end of the reperfusion period. Lung tissue samples

were embedded in paraffin after fixation in 10% buffered formalin for 24h, followed by $5\ \mu\text{m}$ thick sectioning. Lung tissue samples were then stained to determine the degree of lung injury using standard Hematoxylin and Eosin (H&E) staining. A blinded external reviewer (M.K) was supplied with randomized digital scans. The degree of lung injury was determined using a grading system developed elsewhere.¹³ The main criteria used to score the lung injury were: white blood cell infiltration, fibrin exudates, alveolar hemorrhage and capillary congestion. The severity of each parameter was scored as 0, absent; 1, mild; 2, moderate; and 3, severe. The combined score of all four parameters was taken for each animal.

Indocyanine green (ICG) imaging

Near-infrared (NIR) fluorescent imaging with indocyanine green (ICG) has been used in various clinical intraoperative applications to evaluate tissue perfusion and can be used to non-invasively monitor and quantify lung microvascular perfusion and vascular permeability. To visualize tissue perfusion during EVLP, a single ICG (0.6 mg) dose was added to the EVLP perfusate and serial NIR imaging was performed with SPY Elite imaging system (Stryker, Kalamazoo, MI) during perfusion. Since the EVLP does not contain a liver (which is responsible for metabolizing ICG), image signal could be maintained without re-dosing the circuit.

Statistics

All results are expressed as mean \pm standard error of the mean. Mann-Whitney tests were performed to compare difference between groups. For data involving a time-component, two-way analysis of variance for repeated measures was used, followed by a Bonferroni correction for multiple comparisons. Graph Pad Prism Version 7 (GraphPad Software, La Jolla, CA) computer software was used to conduct all statistical analyses. Sample sizes used in this study were determined based on what is typical of large animal experimentation of this magnitude, using the minimum number of animal required to demonstrate the proof of concept. Due to the nature of the two experimental conditions (one with a daily EVLP intervention and the other without), blinding and randomization could not occur. Study animal were excluded if baseline arterial blood gas analysis revealed an arterial partial pressure of oxygen divided by the inspired oxygen concentration (P/F ratio) of less than 300 mmHg, as this would be indicative of poor baseline lung health.

Role of funders

Study funders did not have any role in the study design, data collection, data analysis, interpretation of writing of this manuscript.

Results

day 10°C static preservation alone results in non-viable lung function

With the goal of achieving multi-day preservation, we first attempted to evaluate whether continuous 10°C storage alone could allow for 72h of viable preservation. Pig lungs were procured from male Yorkshire pigs (28–35kg) using methods like that of current clinical standard, and then stored in a thermoelectric cooler (accuracy of $\pm 0.5^\circ\text{C}$) at 10°C for a period of 72h ($n=3$). After 72h of storage, lungs were placed on EVLP for functional analysis, using a well referenced method known as the Toronto Protocol.⁹ Using this method, over 700 lungs have been assessed at our transplant center, with strong capability of determining post-transplant lung function.^{14,15} When placed on the EVLP platform, lungs developed vascular failure (Figure 1a-c) and were terminated within 30min of perfusion based on a pre-determined termination criteria of excessive perfusate loss ($>1000\text{ml/hr}$). Indocyanine green (ICG) imaging of the lungs was performed in order to visualize the level of fluid accumulation in the lung.¹⁶ Post-imaging photos showed bright intensities within the lung tissue after 10min of perfusion, indicating the development of massive pulmonary edema.

To verify that these results were indeed indicative of poor lung quality, we further confirmed these findings using a syngeneic pig lung transplantation model ($n=3$). In a similar fashion to those experiments performed previously, pig lungs were retrieved and stored at 10°C for a period of 72h. Following the 72h period, the lungs were divided, and a single left lung transplant was performed. We aimed to monitor lung function for a period of 4h post-transplantation. This period of time marks the onset of major ischemia-reperfusion injury events and has been shown to be predictive of graft function at later timepoints.¹⁷ Similarly to our initial EVLP assessment findings, lungs stored at 10°C for 72h developed massive edema (Figure 1d) leading to death of the animals in less than 1h after reperfusion. Histology after reperfusion revealed severe interstitial edema, intra-alveolar edema, hemorrhage, cell infiltration and hyaline membrane formation (Figure 1e). These results reflect a severe injury phenotype, indicating that 10°C cold static preservation alone is not suitable for 3-day lung preservation.

3-day lung preservation at 10°C with intermittent 4h EVLP once a day allows for excellent post-transplant graft function

Considering that continuous static storage at 10°C is not viable approach to 3-day preservation, we then went on to evaluate whether the usage of EVLP during the cold storage period could allow for better post-transplant outcomes after an extensive preservation period. Porcine lungs were procured using identical methods as

described above, and then stored at 10°C with a cyclic once a day normothermic EVLP protocol ($n=4$). We specifically designed this protocol being logistically and translationally mindful, in which the length of perfusion followed that performed currently for the clinical assessment of donor lungs (4h).⁶ Moreover, we designed the protocol to allow for perfusion periods to be performed in the morning (9:00am–1:00pm) followed by 10°C cold storage for the rest of the day and into the night, allowing for minimization of operating room resources and overnight staff. After each EVLP cycle, the lungs were disconnected from the circuit using a quick-connector system and the perfusion circuit was stored in a 4°C walk-in fridge overnight for usage the next day. A perfusate ultra-violet C irradiation device was also added to the standard perfusion circuit to avoid circuit contamination during multi-day use (Figure 2a). Bacterial load in the perfusate during the two perfusion cycles remained low (Fig S1). The experimental protocol is shown in detail in Figure 2b. Briefly, pig lungs were retrieved and stored for 6h at 4°C to simulate transportation to a transplant center, followed by 18h of 10°C storage (Cold1). Lungs then underwent a first period of normothermic EVLP for 4h (EVLP1), followed by 20h of 10°C storage (Cold2), and an additional course of 4h of EVLP (EVLP2) the next day. Thereafter lungs were stored at 10°C for another 20h (Cold3), and then a single-left lung transplant was performed after the 72h period.

During the 2 cycles of EVLP, ex vivo lung function parameters remained stable (Figure 3a-f). The mean lung weight gain was 96 grams \pm 23 grams after the first cycle of EVLP, and 160 \pm 56 after the second cycle (Figure S2).

Furthermore, consistent trends in biochemical profiles of the EVLP perfusate (Figure 3g-i) were seen between the two cycles, indicating that the prolonged 10°C storage period between the EVLP assessments did not promote significant metabolic stress (increased glucose consumption and lactate production rates) on the organ. ICG imaging revealed stable perfusion patterns during both periods with no evidence of massive pulmonary edema formation (Figure 3j).

At the end of the 3 day preservation period using the intermittent EVLP protocol, lung function was evaluated by performing a left single lung transplant into a recipient animal, followed by 4h of graft reperfusion. Hourly functional assessments were performed to monitor post-transplant lung function, and the right pulmonary artery was clamped at the end of the reperfusion period to measure transplanted graft oxygenation independent of the contralateral native lung. Histological lung structures were maintained during the entire preservation period and at the end of the perfusion period (Figure 4a), which was supported by blinded analysis of the degree of lung injury performed through a standardized scoring system (Figure S3). Histological sections of

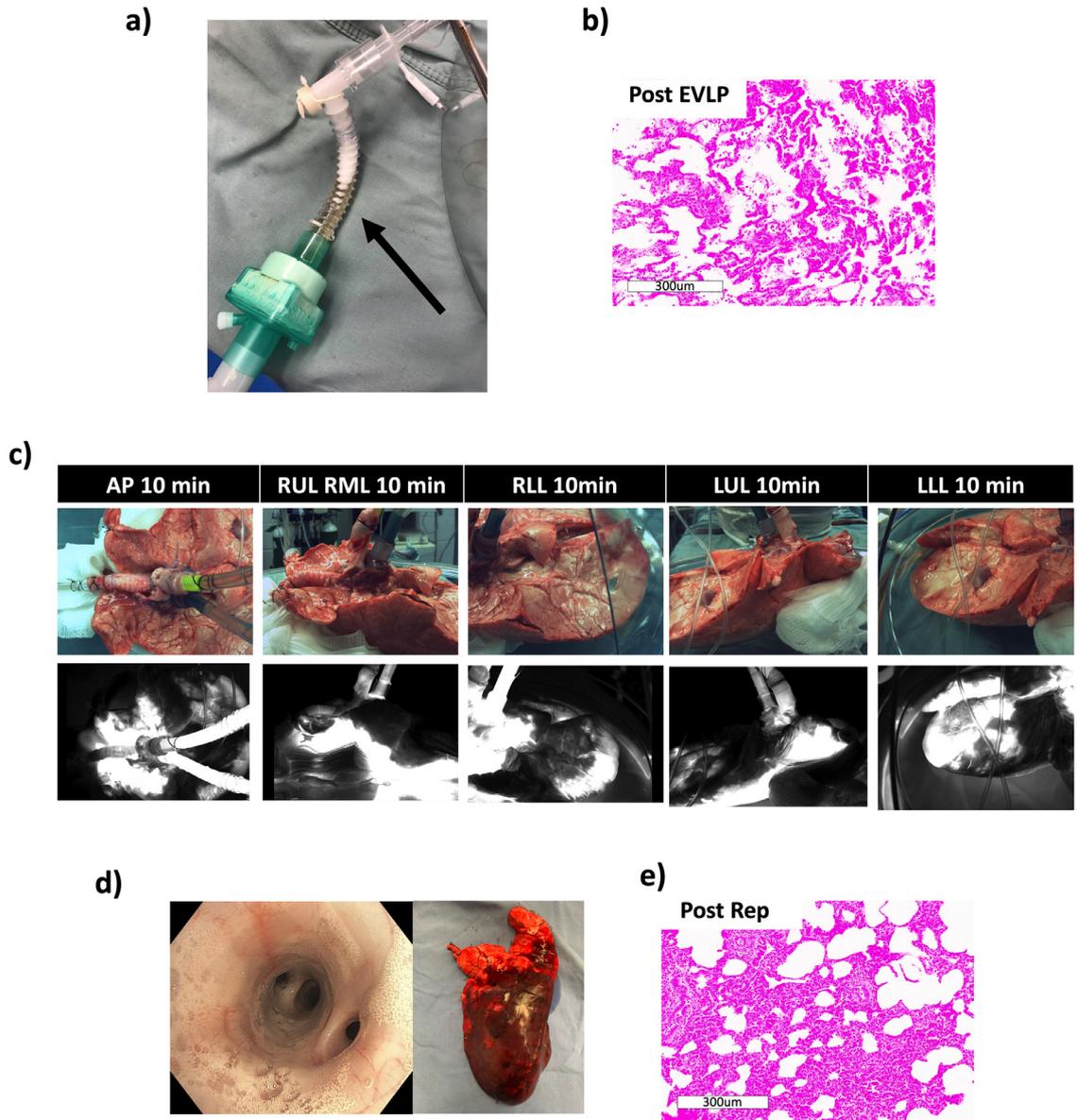


Figure 1. Physiological assessment of lungs stored for 72h using cold static preservation alone. *Ex vivo lung perfusion (EVLP) as primary mode of assessment.* a) Ventilator tubing attached to lung airway. Arrow shows perfusion solution filling the tube reflecting severe pulmonary edema. b) Representative histology after EVLP examination. (scale bar = 300 µm) c) Indocyanine green imaging (ICG) after the first 10 mins of perfusion. Bright intensities indicate development of pulmonary edema.

Post Transplant assessment. d) Post-reperfusion fiberoptic bronchoscopy images alongside explanted lung images of control lungs e) Post reperfusion representative histology of control lungs. EVLP: Ex vivo lung perfusion, Rep: Reperfusion AP: Anterior-Posterior, RUL: Right Upper Lobe, RML: Right Middle Lobe, RLL: Right Lower Lobe, LUL: Left Upper Lobe, LLL: Left Lower Lobe.

small airway structures can be found in Figure S4. Lungs preserved for 3 days using 10°C static preservation with the intermittent daily 4h EVLP had excellent post-transplant graft function and no pulmonary edema was observed in the bronchoscopic assessment after transplant. Lung function was stable during the 4h of reperfusion based on sampling the transplanted upper

and lower pulmonary veins (Figure 4b-c), and the systemic PaO₂/FiO₂ after excluding the contralateral lung was 422 ± 61 mmHg (Figure 4D). As a reference, PaO₂/FiO₂ above 300mmHg are typically considered excellent.¹⁸ Figure 4E shows representative images of lung gross appearance during the preservation period and post-transplantation.

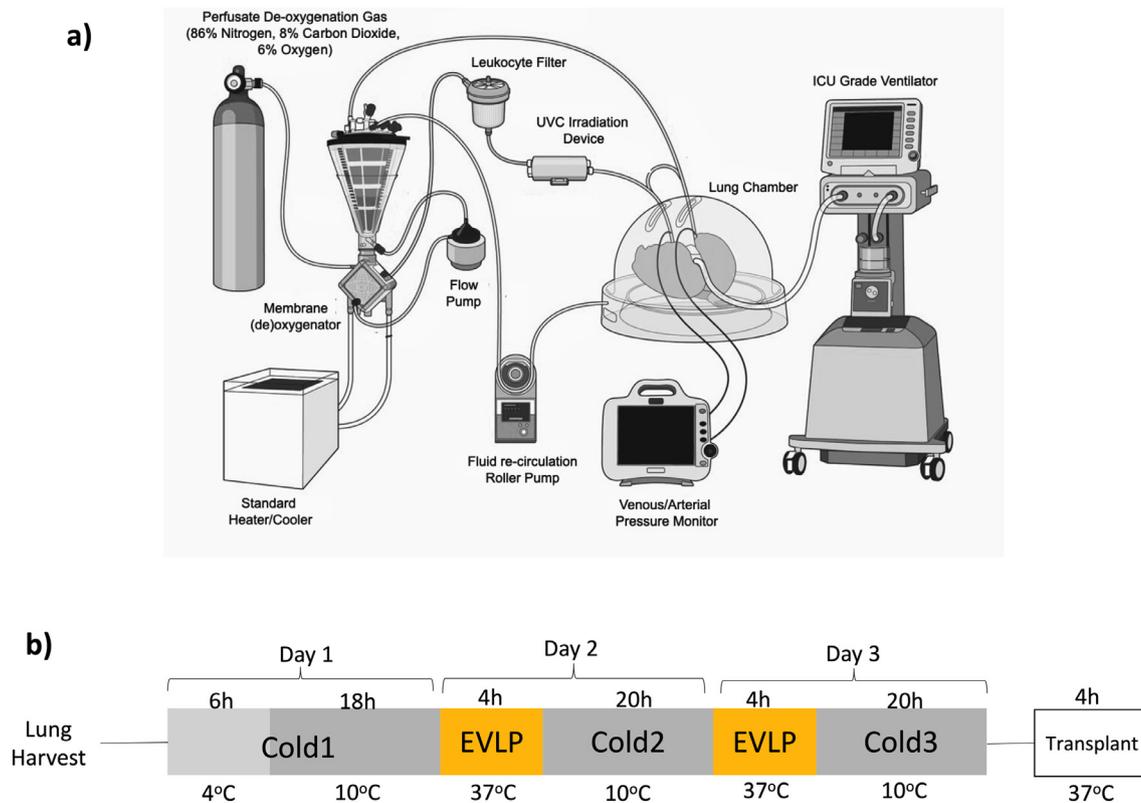


Figure 2. Extended 10°C cold storage with intermittent normothermic ex vivo lung perfusion (EVLP) protocol. a) Toronto EVLP circuit. b) Pig lungs were retrieved and stored for 6h at 4°C to simulate transportation to the transplant center, followed by 18h of 10°C storage (Cold1). Lungs then underwent a period of normothermic EVLP for 4h (EVLP1), followed by 20h of 10°C storage (Cold2), and an additional course of 4h of EVLP (EVLP2). Lungs were then stored at 10°C for 20h (Cold3), and then a single-left lung transplant was performed. The recipient animal was monitored post-transplant for 4h.

EVLP leads to restoration of central carbon metabolism during preservation: a key mechanism for multi-day preservation

We then sought to further investigate the potential mechanisms by which EVLP allowed significant extension of preservation times. Based on the properties of EVLP, we hypothesized that intermittent EVLP usage may provide better maintenance of key tissue metabolites in comparison to cold static storage alone. To characterize the metabolic restoration features of EVLP, we performed a targeted metabolomic analysis on lung tissue samples collected during 3-day preservation experiments (Metabolon, Durham, NC). The full biopsy schedule is shown in Figure 5a. Since cells may potentially utilize varying energy sources during preservation, we selected a panel of key metabolites involved in central carbon metabolism to gain a holistic overview of the most important energy-relevant pathways. Results showed significantly greater maintenance of tissue glucose (Figure 5b, $p = <0.0001$; oh, $p = >0.99$; 28h, $p = 0.039$; 52h, $p = 0.0003$; 72h, $p = 0.090$; Two-way ANOVA followed by a Bonferroni correction for multiple comparisons), succinate (Figure 5b, $p = <0.0001$;

oh, $p = >0.99$; 28h, $p = 0.040$; 52h, $p = 0.0008$; 72h, $p = 0.0023$; Two-way ANOVA followed by a Bonferroni correction for multiple comparisons), N-acetyl Aspartate (Figure 5b, $p = 0.0019$; oh, $p = 0.25$; 28h, $p = >0.99$; 52h, $p = 0.098$; 72h, $p = 0.48$; Two-way ANOVA followed by a Bonferroni correction for multiple comparisons), and 2-Ketoglutarate (Figure 5b, $p = 0.0004$; oh, $p = 0.18$; 28h, $p = 0.055$; 52h, $p = 0.027$; 72h, $p = >0.99$; Two-way ANOVA followed by a Bonferroni correction for multiple comparisons) in lungs subjected to an intermittent EVLP protocol versus lungs which were stored continuously in a cold static manner. Furthermore, we calculated the changes in tissue levels of lactate/pyruvate ratio to evaluate potential aerobic to anaerobic metabolic shifts during the preservation period. High levels of lactate/pyruvate ratio have been previously shown as a potential marker of poor graft quality.¹⁹ Results showed maintenance of lactate/pyruvate ratios in lungs undergoing the intermittent EVLP protocol, while this ratio became significantly elevated during continuous cold storage (Figure 5b, $p = 0.0405$; oh, $p = >0.99$; 28h, $p = >0.99$; 52h, $p = >0.99$; 72h, $p = >0.99$; Two-way ANOVA followed by a Bonferroni correction for multiple comparisons).

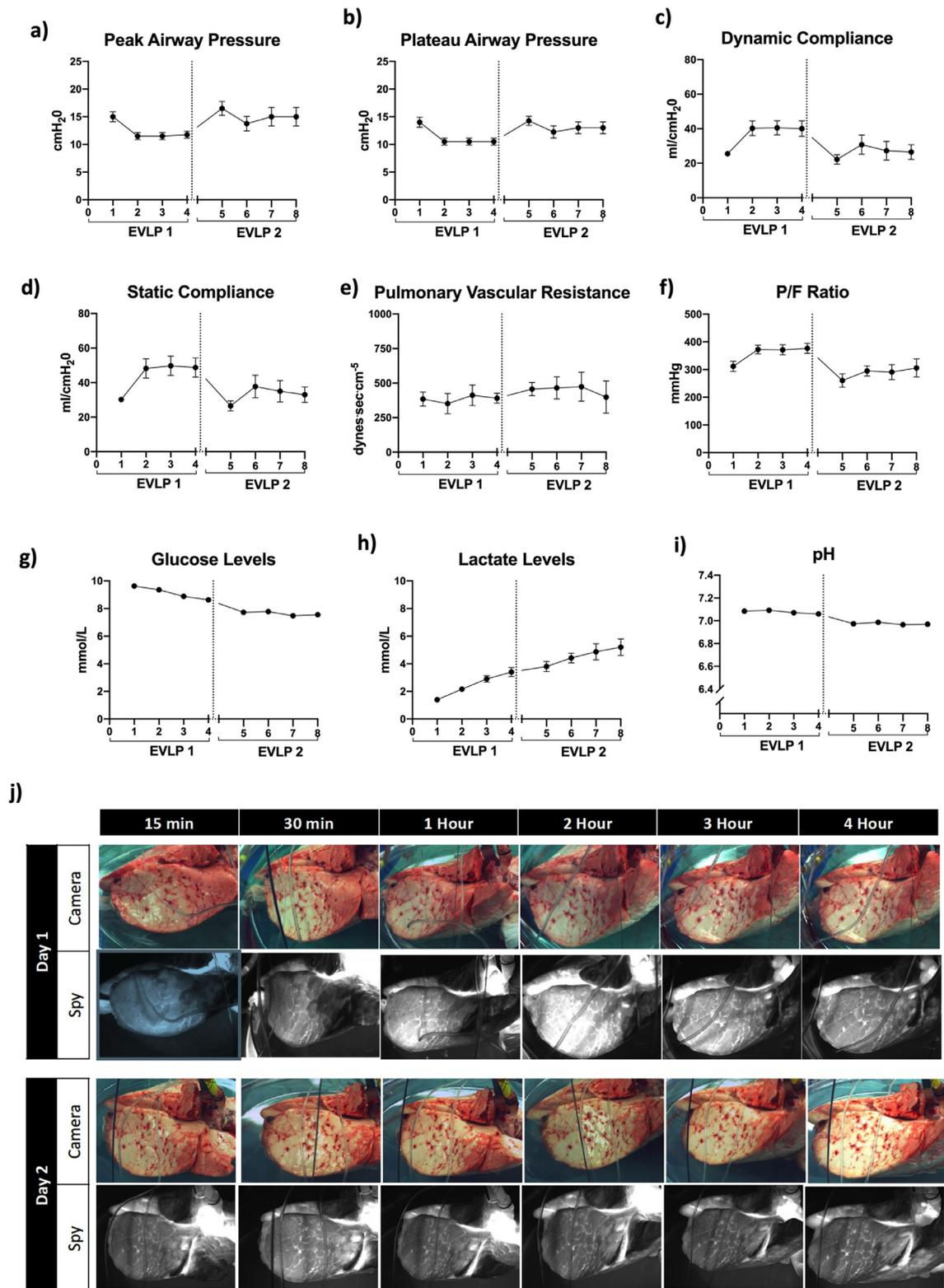


Figure 3. Lung assessment results during Intermittent ex vivo lung perfusion (EVL) periods. a-f) Physiologic results (data expressed as mean ± SEM) g-i) Perfusate biochemistry (data expressed as mean ± SEM) j) Serial Indocyanine green (ICG) images. Day 1 represented EVLP1 and Day 2 represents EVLP2. P/F ratio: ratio of oxygen partial pressure to fraction of inspired oxygen.

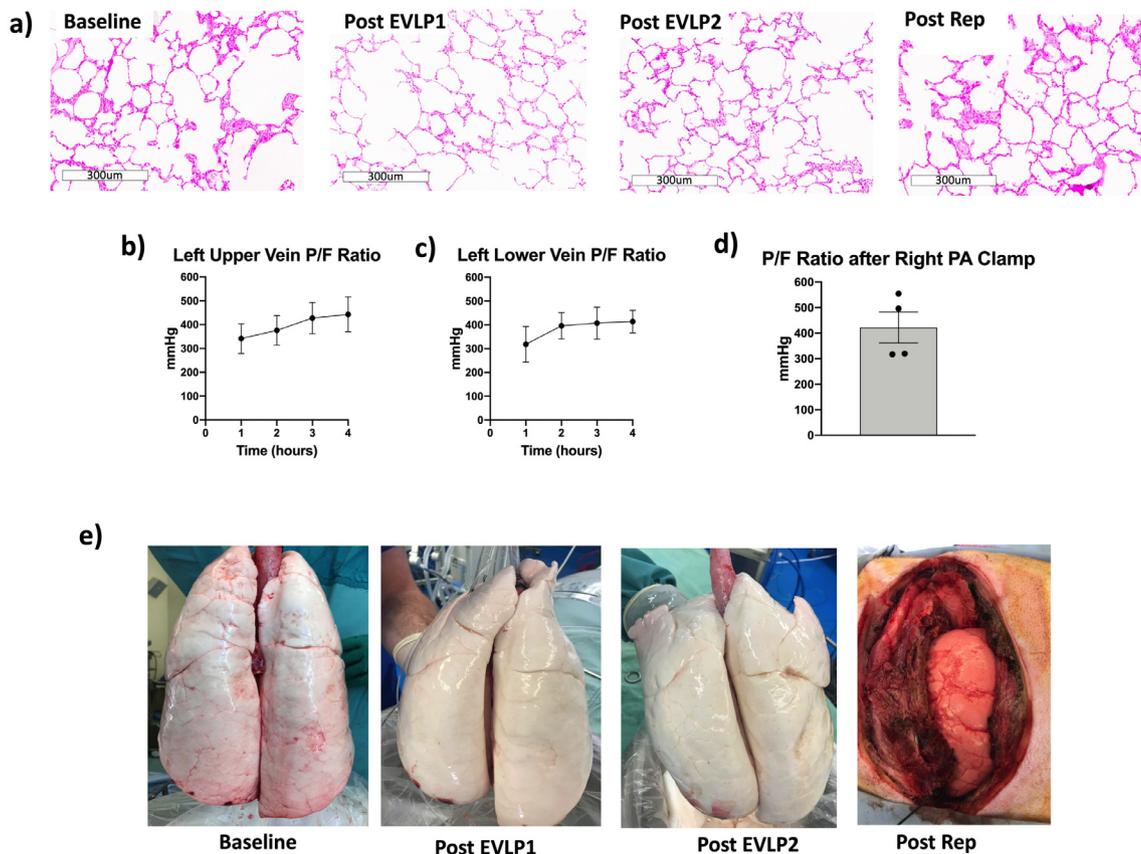


Figure 4. Lung function after transplantation. a) Representative histology before preservation, after EVLP1, after EVLP2, and post reperfusion during the intermittent EVLP protocol. (scale bar = 300 μm) b-c) Recipient P/F ratio taken during 4h of reperfusion (data expressed as mean ± SEM). d) P/F Ratio after contralateral (native lung) pulmonary artery clamping in the intermittent EVLP group (Data expressed as mean ± SEM). e) Representative gross lung appearance of lungs before preservation, after EVLP1, after EVLP2, and post reperfusion during the intermittent EVLP protocol. P/F ratio: ratio of oxygen partial pressure to fraction of inspired oxygen, EVLP: Ex vivo lung perfusion, PA: Pulmonary artery.

Cyclic EVLP results in downregulation of genes related to cell death and mitochondrial injury

Activation of cell death pathways during lung preservation has been shown to have implications in worsening post-transplant graft function.²⁰ To evaluate differences in cell death markers between the groups, we performed a gene expression analysis using a RT² PCR array encompassing relevant genes for several cell death pathways (Necroptosis [a programmed form of necrosis via inflammatory cell death]: *TNFA*, *FASLG*, *CASP8*, *NFKB*, Pyroptosis [highly inflammatory form of lytic programmed cell death]: *CASP1*, *GPX4*, Apoptosis [most common form of programmed cell death]: *CASP3*, *CASP8*, *CASP9*, *TP53*, *NFKB*, Ferroptosis [programmed cell death pathway accompanied by iron accumulation and lipid peroxidation]: *MPO*, *GPX4*, *TP53*).

Results of our analysis showed significantly lower fold-change expressions of *CASP1* biopsied tissue after EVLP1 (Table 1, $P = 0.044$; Student's t-test) and after EVLP2 (Table 1, $P = 0.017$; Student's t-test) in

comparison to lungs stored using continuous cold static storage alone. Caspase-1 activation is mediated through the assembly of the cellular inflammasome, which are innate immune system receptors/sensors induced by pathological debris such as reactive oxygen species generated through mitochondrial dysfunction.²¹ Active caspase-1 is essential for the cleavage of pro-IL-1 β into IL-1 β , a potent inflammatory mediator involved in the manifestation of lung injury.²² To supplement our findings, we performed a similar gene expression analysis looking at a panel of markers relevant to mitochondrial injury (*B2M*, *LOC100739238*, *LOC100624950*, *HSP70.2*, *ARRDC3*, *DNAJB1*, *EDN1*, *ASB1*, *SLC25A25*, *GADD45B*). For lungs subjected to an intermittent EVLP protocol, heat shock protein markers (*HSP70.2*, *DNAJB1*) were expressed in higher fold changes after EVLP2 (Table 2, $P = 0.0023$ & $P = 0.01$, respectively; Student's t-test). However, prior to transplantation, significantly lower fold changes of mitochondrial injury markers such as *B2M* (Table 2, $P = 0.021$; Student's t-test),

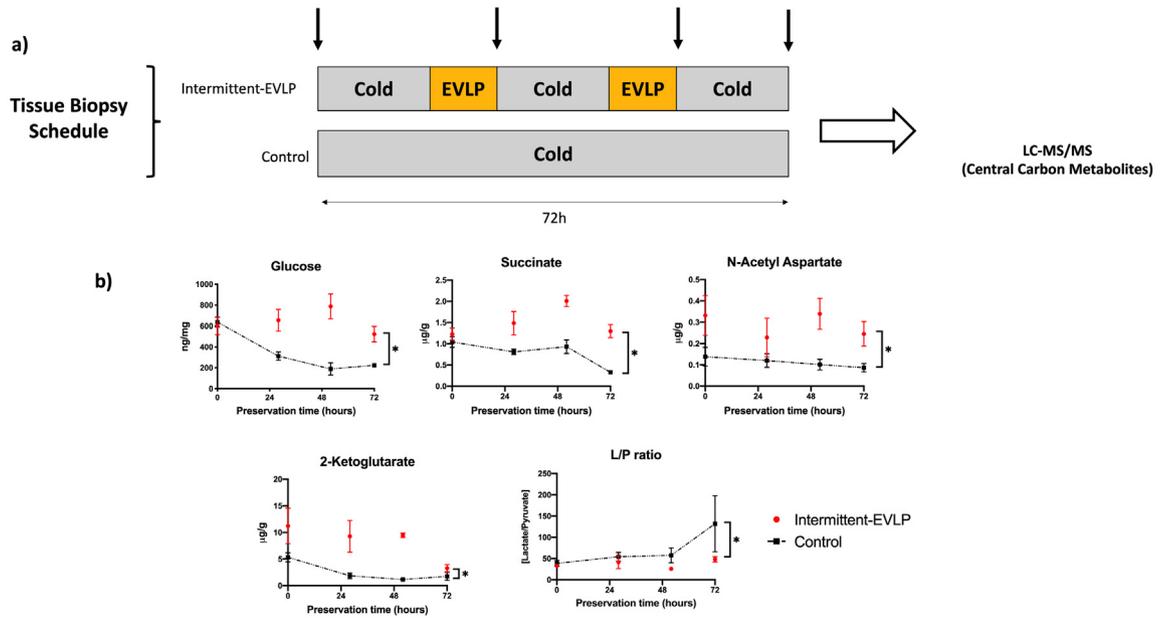


Figure 5. Metabolic restoration during normothermic ex vivo lung perfusion. a) Tissue biopsy schedule of 72h lung preservation studies. Tissue were collected at 0h, 28h, 52h, and 72h for lung undergoing the intermittent EVLP protocol ($n=4$) or lungs continuously stored at 10°C ($n=3$). Tissue was then subjected to targeted quantitative analyses using Liquid Chromatography with tandem mass spectrometry (LC-MS-MS). b) Quantitative measurement of central carbon metabolites (2-ketoglutarate, Succinate, N-Acetyl aspartate, glucose, lactate, pyruvate) during 3 day L/P preservation with and without intermittent EVLP. The ratio of lactate/pyruvate concentration was quantified and expressed as L/P ratio. (Two-way ANOVA followed by a Bonferroni correction for multiple comparisons. Data expressed as mean \pm SEM). L/P: Lactate/pyruvate, (* $p < 0.05$).

Symbol	0h		28h		52h		72h	
	Fold Change	p-value						
<i>FASLG</i>	-1.32	0.188750	-1.49	0.206571	1.04	0.516682	2.71	0.438171
<i>TP53</i>	-1.51	0.113879	-1.57	0.077564	2.79	0.440091	3.87	0.431132
<i>MPO</i>	-1.29	0.635392	-3.21	0.061336	-7.22	0.497035	1.94	0.442265
<i>CASP1</i>	-1.92	0.065584	-2.83	0.043734	-7.81	0.017392	-5.51	0.093603
<i>CASP3</i>	-1.26	0.366313	-1.13	0.609319	1.54	0.445331	2.85	0.064606
<i>CASP8</i>	-1.27	0.470621	-1.29	0.217024	-1.24	0.903804	3.01	0.080676
<i>CASP9</i>	1.54	0.558820	-1.03	0.777492	-1.19	0.650869	5.36	0.431136
<i>GPX4</i>	-1.29	0.330353	-1.38	0.167735	1.37	0.990061	-1.45	0.503817
<i>NFKB1</i>	-1.24	0.411644	-1.01	0.880403	1.46	0.256821	2.18	0.288193
<i>BID</i>	-1.23	0.541254	-1.29	0.206321	2.40	0.423408	2.18	0.114011

Table 1: Cell death gene expression in porcine lung tissue stored using an intermittent EVLP protocol ($n=4$) versus continuous static storage ($n=3$). Gene expression normalized using housekeeping genes *ACTG1*, *ACTA1*. p-values were calculated based on a Student's t-test of the replicate $2^{-\Delta\Delta CT}$ values for each gene in each experimental group.

LOC100624950 (Table 2, $P = 0.011$; Student's t-test) and *ARRDC3* (Table 2, $P = 0.012$; Student's t-test) were found in lungs subjected to an intermittent EVLP protocol versus control.

Discussion

In this study, we demonstrate a successful strategy to preserve donor lungs for 3 days with confirmation of

post-transplant function in a previously established translational model of graft transplantation. This could be achieved by modifying the current gold standard method of cold static preservation (from 4°C to 10°C) and interjecting two EVLP recharge periods for 4h each. We recently described the benefit of 10°C storage to be related to the protection of mitochondrial health and the preservation of cell membrane function. Despite these protective features, we hypothesized that

Symbol	0h		28h		52h		72h	
	Fold Change	p-value						
<i>B2M</i>	-1.37	0.117082	-1.40	0.351214	1.22	0.594492	-1.93	0.021091
<i>LOC100739238</i>	-1.26	0.304310	-1.39	0.231836	-1.67	0.153466	1.01	0.801472
<i>LOC100624950</i>	-1.19	0.415775	-1.57	0.215403	-3.88	0.197826	-2.17	0.010807
<i>HSP70.2</i>	-1.45	0.217479	1.52	0.279726	3.56	0.002363	1.89	0.108741
<i>ARRDC3</i>	-1.27	0.303136	-1.70	0.123053	-1.43	0.115733	-1.90	0.013758
<i>DNAJB1</i>	-1.08	0.665399	1.35	0.345922	1.83	0.010248	1.53	0.112598
<i>EDN1</i>	-1.38	0.232877	-1.43	0.358893	1.47	0.521563	-1.31	0.695647
<i>ASB1</i>	-1.28	0.259991	-1.51	0.178147	-2.29	0.278303	1.36	0.340678
<i>SLC25A25</i>	-1.38	0.234028	1.41	0.474820	1.18	0.710349	1.37	0.261271
<i>GADD45B</i>	-1.00	0.940080	3.27	0.040981	5.23	0.011042	1.00	0.817894

Table 2: Mitochondrial injury gene expression in porcine lung tissue stored using an intermittent EVLP protocol (n=4) versus continuous static storage (n=3). Gene expression normalized using housekeeping genes *ACTG1*, *ACTA1*. P-values were calculated based on a Student's t-test of the replicate $2^{-\Delta\Delta CT}$ values for each gene in each experimental group.

continuous cold storage alone would not be suitable to sustain the lungs for a period of multiple days. The findings of our study supported this hypothesis where lungs stored continuously at 10°C alone showed immediate failure post-transplantation.

The adoption of EVLP as an organ assessment tool in clinical transplantation has continued to become widespread. Its utility as a platform for precision organ diagnosis, treatment, and repair continues to be investigated with several promising applications. An important extension of this work is the usage of EVLP to provide extended preservation times. Although advocated through observational data, little is known about the mechanism by which EVLP can allow for extended preservation periods. One central concept of EVLP is that preservation of the donor organ during the period of EVLP occurs at normothermia, hence with full metabolic activity to potentially mitigate cellular damages. Cellular inflammasome activation has been shown to lead to Caspase-1-dependent mitochondrial damage, resulting in mitochondrial reactive oxygen species production, dissipation of the mitochondrial membrane potential and mitochondrial permeabilization.²³ The results of this study support these findings as the gene expression of *CASP1* was found to be in significantly lower fold changes after each EVLP cycle. In further support of this notion, prior to transplantation, significantly lower fold changes of mitochondrial injury markers such as *B2M*, *LOC100624950* and *ARRDC3* were found in the graft.

Maintaining mitochondrial health is directly associated with central carbon metabolism.²⁴ Central carbon metabolism uses a complex series of enzymatic steps to convert sugars into metabolic precursors, which ultimately carry out cellular function and generate the entire biomass of the cell.²⁵ Example of these pathways include the tricarboxylic acid cycle, glutamine metabolism and the pentose phosphate pathway. When

performing a targeted analysis to investigate the influence of normothermic EVLP on these metabolites, we found glucose, succinate, and N-acetyl Aspartate to be better maintained using the intermittent EVLP protocol in comparison to prolonged cold storage alone. This is in accordance to previous work which has been performed that showed that addressing metabolism through EVLP perfusate modification may improve donor lung quality.^{26–29} Taken together with our findings, we believe that strategies that further promote metabolic recovery during the intermittent EVLP periods may prove to enhance our currently proposed protocol, which may lead to an extension of preservation periods significantly beyond 3 days.

To our knowledge, this is the first study to demonstrate multi-day extracorporeal lung preservation in which graft function has been confirmed through transplantation. Hozain and colleagues demonstrated 4-days of extracorporeal lung preservation by means of cross-circulation (lungs connected to an alive swine host for all 4 days).³⁰ Although the results of this study were encouraging, the intention of this method described by the authors was to allow for extracorporeal lung repair, and would most likely not be used for the purposes of preservation due to significant logistical, ethical, and feasibility constraints.³⁰ On the other hand, our proposed intermittent EVLP strategy overcomes these barriers by providing a very practical approach using novel technology. Our study does have limitations. Firstly, lungs used in this study did not have common baseline donor-related injuries (such as aspiration, infection, ventilator injuries, contusions, or emboli). However, we felt that controlling these variables allowed us to directly study the effects of our proposed strategy on quality of preservation without the influences of these confounders. Furthermore, by using a large animal model (rather than human lungs rejected for transplantation), we were able to confirm the quality of the preservation

through recipient transplantation- an important consideration after an extensive preservation period. Secondly, our primary endpoint for graft success was the evaluation of lung function during a 4h reperfusion period. We did not feel the need to keep the animal alive for longer than this as the ischemia-reperfusion injury associated with prolonged graft storage has been described to occur within the acute phases (first four hours) of reperfusion post-transplantation.³¹ Therefore, positive findings at this timepoint would most likely suggest positive outcomes during the rest of the post-transplant course. Extending to this point, keeping the animal alive for multiple days post-transplantation may result in the influence of several recipient factors to interfere with our graft evaluation such as the early onset of acute rejection and other immunological factors. In addition to this, our biological analysis regarding the mechanism of benefit of EVLP was limited to the scope of mitochondrial injury markers and central carbon metabolism. Because animals which received lungs in the control group did not survive to the first hour of reperfusion, post-transplant biological evaluation could not be performed at a direct timepoint evaluation. However, tissue biopsies taken during the 72h period provided insight in the mechanisms which created these post-transplant differences. Further studies should be performed to further define the role of EVLP as a recharge mechanism during prolonged ischemic preservation. Moreover, in this study, cyclic EVLP using the current standard of care for lung preservation (4°C) was not evaluated. Because our protocol requires an extensive period of cold static preservation (20 h), we felt that prolonged preservation at this temperature would result in too much injury, impeding the ability to achieve 3 day lung preservation. Therefore, only 10°C static lung preservation was evaluated. Lastly, our sample size may be considered small. However, this is typical of large animal experimentation which is associated with complexity, logistical hurdles, and costs. Moreover, the consistency of results obtained during the transplantation phase gave us a lot of confidence in the ability of a cyclic EVLP protocol to allow for extended preservation.

In conclusion, we were able to successfully achieve 3 days of lung preservation by combining 10°C preservation and intermittent 4h normothermic EVLP. Extension of preservation periods will allow for improved transplant logistics, the opportunity to explore organ banking concepts, allow for better immunological matching amongst donor and recipients and the opportunity to perform time-dependent therapeutics. Further metabolic rehabilitation through enhancement of this protocol may allow for stronger lung recovery during these periods. The results of this study have the potential to greatly extend preservation times and allow for advanced lung repair and regeneration which will revolutionize the landscape of current lung transplantation practices.

Contributors

AA, AN, EB, EBr, AM, VM, AW, MK, RVPR, and YZ participated in data acquisition and model development. AA, JY, LB, EF, TW, ML, ACA, SK and MC were involved in the conception and experimental design of the study. AA, ACA, SK and MC participated in writing, revision and preparation of the manuscript. AA, AN, EB, EBr, AM, VM, AW, RVPR, YZ, JY, LB, EF, TW, ML, ACA, SK, and MC critically revised and approved the manuscript. AA, MC, EBr, ACA have verified the underlying data. MC was the study supervisor. All authors have read and approved the final version of this manuscript.

Data sharing statement

The data sets generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Declaration of interests

MC, TW and SK are shareholders of Traferox Technologies Inc and consultants for Lung Bioengineering. MC, AA, TW, SK have an international patent pending pertaining to donor organ preservation using both static cold storage and ex vivo organ perfusion has been filed (17/714,593).

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:[10.1016/j.ebiom.2022.104210](https://doi.org/10.1016/j.ebiom.2022.104210).

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