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Evaluation of 10˚C as the optimal storage temperature for aspiration-injured donor lungs in a large animal transplant model

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KEYWORDS:
lung transplantation; organ preservation; mitochondrial health; ischemia reperfusion injury (IRI); inflammasome; necroptosis

BACKGROUND: Our recent work has challenged 4˚C as an optimal lung preservation temperature by showing storage at 10˚C to allow for the extension of preservation periods. Despite these findings, the impact of 10˚C storage has not been evaluated in the setting of injured donor lungs.
METHODS: Aspiration injury was created through bronchoscopic delivery of gastric juice (pH: 1.8). Injured donor lungs (n = 5/group) were then procured and blindly randomized to storage at 4˚C (on ice) or at 10˚C (in a thermoelectric cooler) for 12 hours. A third group included immediate transplantation. A left lung transplant was performed thereafter followed by 4 hours of graft evaluation.
RESULTS: After transplantation, lungs stored at 10˚C showed significantly better oxygenation when compared to 4˚C group (343 ± 43 mm Hg vs 128 ± 76 mm Hg, $p = 0.03$). Active metabolism occurred during the 12 hours storage period at 10˚C, producing cytoprotective metabolites within the graft. When compared to lungs undergoing immediate transplant, lungs preserved at 10˚C tended to have lower peak airway pressures ($p = 0.15$) and higher dynamic lung compliances ($p = 0.09$). Circulating cell-free mitochondrial DNA within the recipient plasma was significantly lower for lungs stored at 10˚C in comparison to those underwent immediate transplant ($p = 0.048$), alongside a tendency of lower levels of tissue apoptotic cell death ($p = 0.075$).
CONCLUSIONS: We demonstrate 10˚C as a potentially superior storage temperature for injured donor lungs in a pig model when compared to the current clinical standard (4˚C) and immediate transplantation. Continuing protective metabolism at 10˚C for donor lungs may result in better transplant outcomes.

[#]Both authors have equal contributions.

Data Availability: The data sets generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request.*Abbreviations:* EVLP, ex vivo lung perfusion; ccf mtDNA, circulating cell-free mitochondrial DNA; P/F, PaO_2/FiO_2; ALI, acute lung injury.

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For patients with end-stage lung disease, lung transplantation is a lifesaving therapy. One of the most important limitations faced within the field today involves the limited number of viable donor lungs available to meet the demand of a growing recipient list. In some regions, over 80% of donor lungs are discarded based on concerns of graft quality due to injuries sustained to the organ prior to and during organ retrieval.[1] Example of common injuries include contusions, ventilator-induced lung injury, injury due to aspiration of gastric contents, the presence of pulmonary emboli, and more. Although efforts have been made to improve the utilization of these organs, transplantation of lungs with these injuries may increase the risk of developing post-transplant primary graft dysfunction (PGD), an important cause for early mortality within lung transplant recipients.[2−7]

The current standard of care for the preservation of lungs prior to transplantation involves cold flushing the organ with a dedicated solution, and subsequently storing on ice at ∼4˚C.[8] Recently, we have identified 10˚C as a superior temperature for prolonged lung storage.[9] During 10˚C lung preservation, cytoprotective metabolites are formed and mitochondrial health is preserved. Based on these findings, we have launched of a multi-center human clinical trial (ClinicalTrials.gov Identifier: NCT04616365) investigating the use of this new preservation temperature for planned semi-elective human lung transplantation, which is currently ongoing.

Despite these advances, one important limitation of the studies performed to date is that they were conducted using healthy large animal donor lungs, hence, not reflecting the clinical reality of donor lung procurement. Thus, the influence of 10˚C lung preservation in the setting of injured donor lungs remains unknown. Here, we aimed to evaluate the effects of 10˚C lung preservation in comparison to the standard 4˚C for injured lungs. Lung injury was accomplished through gastric juice aspiration, followed by transplantation using a large animal model. An additional group of immediate transplantation (short ischemia) was included into the study design to evaluate the functional, metabolic, and biological influences of the 2 temperatures more appropriately.

Methods

Study design

To evaluate the potential physiological and biological benefit of 10˚C vs 4˚C over marginal donor lungs, we conducted retrieval and transplant experiments using adult male Yorkshire pigs. All animals received humane care and study protocol approvals were evaluated and delivered by the Animal Care Committee following the Canadian Council on Animal Care and Certificate of Good Animal Practices Guidelines. After each procurement, partially injured donor lungs (n = 5/group) were randomized into 2 storage temperatures for which the investigators were blinded. Storage would take place for 12 hours either at 10˚C in a thermoelectric cooler (accuracy of ± 0.5˚C) or at 4˚C in a walk-in cooler. A third group included a minimum cold (approx. 1 hour at 4˚C) storage prior to transplant. Large animal models of this kind require important infrastructures and manpower. Therefore, the sample size we used (n = 5/group) was considered sufficient to detect significant differences. After each respective storage condition, left lung transplants were performed and followed by 4 hours of graft evaluation. Both organ retrieval and transplant procedures followed previously described methods.[10]

Induced donor lung injury and procurement

Donor male Yorkshire pigs (34-41 kg) were sedated with ketamine (20 mg/kg intramuscular), midazolam (0.3 mg/kg intramuscular), and atropine (0.04 mg/kg intramuscular), and then anesthetized with inhaled isoflurane (1%-3%), followed by a continuous intravenous injection of propofol (3-4 mg/kg/h) and remifentanil (9-30 μg/kg/hr). Animals were placed supine; a tracheostomy was performed, and a subsequent pressure-control ventilation was initiated. Typical procurement settings were applied with an inspired oxygen fraction (FiO_2) of 0.5, a frequency of 15 breaths/min, a positive end-expiratory pressure (PEEP) of 5 cmH_2O, and a controlled pressure above PEEP of 15 cmH_2O. After a single recruitment maneuver, bilateral chemical lung injury was induced, using a bronchoscope to homogeneously instill 5 ml of gastric juice (pH 1.8) in each segment. All ventilator settings remained the same thereafter except for the inspired oxygen fraction that could be temporarily increased during the first hour to overcome bronchospastic reactions and to maintain saturation values above 85%. All animals were stabilized for 2 hours after the lung injury to help prevent hemodynamic instability during the donor operation. During this period, functional assessments were performed at baseline (pre-injury), 60 minutes after injury, 120 minutes after injury, and prior to lung flush. This included evaluation of lung dynamic compliance, peak airway pressures, tidal volumes and systemic P/F ratios. After the stabilization period, the procurement followed using a median sternotomy. After intravenous administration of 10,000 IU sodium heparin, the main pulmonary artery was cannulated, the superior and inferior vena cava were tied, the aorta was clamped, and the left atrial appendage was incised. A standard anterograde cold flush was performed by using 2 liters of Perfadex (XVIVO®). A ventilator inspiratory hold was achieved, the trachea was clamped, and the lungs were excised. Once on the back-table, an additional 1L retrograde flush was added. The lungs were immersed in preservation solution effluent during the static storage period.

Left lung transplant and post-transplant functional assessment

Each recipient was anesthetized and tracheostomized in a supine position. Ventilation was started using similar pressure-controlled settings of that used for donor procurement. Recipients with baseline P/F ratio <300 mm Hg were not considered suitable and

therefore excluded from the study. 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 to 0 synthetic, monofilament, nonabsorbable polypropylene suture interrupted in 2 places. The PA anastomosis was performed next with a continuous 5 to 0 PROLENE suture interrupted in 2 places. Lastly, the left atrial anastomosis was performed with a continuous 5 to 0 PROLENE suture interrupted in 2 places. After that, the transplanted lung was re-inflated to a volume of 10ml/kg of mean donor/recipient 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 FiO_2 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. Thereafter, the right bronchus was clamped, and the tidal volume was reduced to 5ml/kg. Peak airway pressure and dynamic compliance of the transplanted graft was then noted.

Sample collection protocol

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 and lower lobes at the beginning post-harvest and pre-transplant. During reperfusion, plasma was collected at 1 hour and 4 hours and snapped frozen and stored at -80˚C. After reperfusion, the peripheral−lateral portions of the left upper and lower lobes were sampled. All assessments were performed in a blinded fashion with experimental group allocation concealed.

Inflammatory cytokines

Tissue lysates were assayed using ELISA kits for porcine interleukin IL-1β (Cat#DY6226, IL-1β porcine ELISA Kit, R&D Systems), interleukin IL-6 (Cat #P6000B, IL-6 porcine ELISA Kit, R&D Systems), interleukin IL-8 (Cat#P8000, porcine IL-8/CXCL8 ELISA Kit, R&D Systems) and TNF-α (Cat #PTA00, porcine TNF-α ELISA Kit, R&D Systems) based on the manufacturer's instructions.

Metabolomic analysis

Tissue samples were assayed for untargeted measurements of metabolites (Metabolon Inc., Durham, NC). Samples were extracted and prepared using Metabolon's standard solvent extraction method just prior to profiling analysis using Gas Chromatography (GC)-Mass Spectrometry (MS) and Liquid Chromatography (LC)-MS/MS platforms. Data extraction, peak identification and compound identification were provided by Metabolon. Metabolomics data analysis of raw peak intensities was performed using MetaboAnalyst software.[11] Data was processed by imputing missing or 0 values with 1 of 5 minimum value, and metabolites with more than 50% missing values were deleted from further analysis. Subsequently data was normalized (quantile), $\log_2$ transformed and auto scaled. Principal Component Analysis (PCA), and statistical tests were performed on normalized data, as described in Statistical Analysis section.

Circulating cell-free mitochondrial DNA (ccf-mtDNA) measurements

ccf-mtDNA was extracted from pig plasma samples (QIAamp DNA Mini Kit, Cat#51304, Qiagen) following the manufacturer's protocol for DNA purification from blood or bodily fluids spin columns. 50μL of plasma was used for the collection of ccf-mtDNA and 100μL of Ultra Pure distilled water free from RNAse and DNAse was used to elute from the column. The estimated absolute value was quantified in copies/μL by using a standard curve, of known concentration, synthesized from an oligonucleotide of the PCR product (Integrated DNA technologies; IA) and serially diluted to concentrations ranging from 1.0×10^2 to 1.0×10^8 copies/μL. Mitochondrially encoded gene, ND4, was used to represent the mitochondrial genome and was amplified using MT-ND4 primers. qPCR was performed using Bio-Rad CFX96 (BioRad Laboratories Inc) with the following cycling conditions: initial denaturation at 95˚C for 10s, 60˚C for 20s, and fluorescence measurement. Finally, it is followed by a melt curve analysis- 65˚C to 95˚C, increasing at increments of 0.5˚C every 5 seconds then proceeding with fluorescent read.

ND4 Forward 5' GCA ACA CTA GTA CCC ACA CTA AT 3'

ND4 Reverse 5' TCC TGC TAG GGT GTA GAA TAG G 3'

Gene block: 5'—ATT CTA TAT CCT ATT CGA AGC AAC ACT AGT ACC CAC ACT AAT TAT CAT CAC ACG CTG AGG AAA CCA AAC AGA ACG ACT CAA TGC AGG ACT TTA TTT CCT ATT CTA CAC CCT AGC AGG ATC CCT ACC ACT GCT AGT AGC AC- 3'

Tissue analysis of acute lung injury scores and cell death markers

Post-harvest and post-transplant lung tissue samples were collected 4 hours after reperfusion from both left upper and lower lobes, which were embedded in paraffin after fixation in 10% buffered formalin for 48h, followed by 5μm sectioning as well as hematoxylin and eosin staining. These slides were histologically evaluated in terms of the presence of acute lung injury. Brightfield images were acquired using an Olympus VS120 slide scanning system. 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.[12] 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 4 parameters was taken for each animal.

To assess cellular apoptosis, tissue sections were stained with deoxynucleotide transferase-mediated deoxy uridine triphosphate nick-end labeling (TUNEL) (In Situ Cell Death Detection Kit, POD; Roche Diagnostics GmbH, Mannheim, Germany). Sections were counterstained with DAPI and mounted with Fluorescent Mounting Medium (IHC-World, cat # E19-18). All TUNEL-stained slides were scanned using a whole slide scanner for Fluorescence (Axio Scan.Z1, Carl Zeiss Microscopy GmbH). TUNEL-positive cells were then quantified using image analysis software (HALO Image Analysis Software, Pelkin Elmer) and expressed as a percentage of total cells.

Gene expression measurement

Mitochondrial gene expression was evaluated using a Custom RT2 PCR Array for Pigs (CLAS44858) which includes genes for biogenesis (*PPARG, PPARGc1A, PPARGC1B, and TFAM), fusion (MFN1, MFN2, and OPA1), fission (MFF and FIS1), and mitophagy (PARK2 and PINK1).*

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.

Statistical analysis

Experimental results are expressed as mean ± standard error of mean. Parametric one-way ANOVA testing followed by Bonferroni's multiple comparison test was performed to compare difference for 2 or 3 groups at fixed timepoints. For data involving several time-points, two-way analysis of variance for repeated measures was used followed by Bonferroni's multiple comparison test. Statistical tests were also performed on metabolomic data after data normalization, in which 2 group comparisons were compared using a *t*-test. Graph Pad Prism Version 9 (GraphPad Software) computer software was used to conduct all statistical analyses. Statistical significance was considered for a p-value < 0.05.

Results

Aspiration-induced lung injury results in the deterioration of donor lung function

To evaluate the impact of the different storage conditions on the preservation of injured lungs, a swine model of aspiration-induced injury was developed. After donor injury, the lungs were blindly randomized to storage at 10˚C or 4˚C for a period of 12 hours, and the left lung was subsequently transplanted. A third arm evaluating minimal storage was also included. The full study design is shown in Figure 1. Donor animal weight and oxygenation was similar amongst the groups (Table S1, $p = 0.71$). To perform the injury, homogenized gastric juice (pH:1.8) was instilled into the airways (5 ml/segment) using bronchoscope. After the acid was given, the donor animal was stabilized for a period of 2 hours, with hourly assessments being performed prior to lung extraction. The donor PaO_2 /FiO_2 (P/F ratio) prior to injury was 451 ± 43 mm Hg, which dropped to a final P/F ratio of 251 ± 77 mm Hg for all animal included in the study. Based on the International Society of Heart Lung Transplantation guidelines, a donor P/F ratio less than 300 mm Hg categorizes the lungs as extended criteria and confer higher risk for transplantation.[13] No differences were found when comparing systemic donor P/F ratios (Figure 2A, $p = 0.91$), dynamic lung compliance (Figure 2B, $p = 0.99$) and lung tidal volume (Figure 2C, $p = 0.95$) amongst the 3 study groups during the donor observation period. A representative image of the lung post-harvest is shown in Figure 2D. No differences in acute lung injury scoring of tissue histology were seen amongst the groups (Figure 2D, $p = 0.95$). A representative image of the lung post-harvest is shown in Figure S1.

10˚C lung preservation results in superior post-transplant graft function and protects against acute lung injury

After donor lung flush and retrieval, lungs were allocated to a dedicated storage condition and a left lung transplant was performed into a recipient animal. Baseline characteristics of recipient animals were similar amongst the 3 groups (Table S3). Following implantation, the recipient animal was monitored for 4 hours of graft reperfusion. This time point is critical for the assessment of ischemia-reperfusion injury related to the quality of preservation.[14] At the end of the reperfusion period, the right pulmonary artery and bronchus was clamped to independently evaluate the performance of the transplanted left lung. During reperfusion, lungs stored at 10˚C showed significantly better oxygenation when compared to 4˚C demonstrated through selective pulmonary vein gases (Figure 3A, 2H, $p = 0.027$; 3 hour, $p = 0.008$; 4h, $p = 0.004$) and exclusion of the contralateral native lung (343 ± 43 mm Hg vs 128 ± 76 mm Hg, Figure 3B, $p = 0.03$). When compared to all other storage conditions, lungs stored at 10˚C for 12 hours tended to have higher lung dynamic compliances (Figures 3C, 4˚C vs 10˚C, $p = 0.08$; Min CIT vs 10˚C, $p = 0.08$) and lower peak airway pressures (Figures 3D, 4˚C vs 10˚C, $p = 0.22$; Min CIT vs 10˚C, $p = 0.15$) after clamping the right bronchus. Histological analysis of post-transplant lung tissue revealed that lungs stored at 10˚C showed significantly lower signs of acute lung injury compared with those stored at 4˚C (Figure 3E, $p = 0.039$).

10˚C lung preservation results in cytoprotective metabolism

To understand the metabolic changes occurring during the preservation period, an untargeted global metabolomic tissue analysis from samples obtained during the experiment was performed. Principal component analysis (PCA) plot visualization showed that samples clustered together when comparing metabolic changes before and after the 12-hour preservation period at 4˚C suggesting almost no ongoing metabolism occurring at this temperature (Figure 4A). For lungs stored at 10˚C however, a distinct separation of samples into 2 clusters was seen, suggesting a continuation of lung metabolism (Figure 4B).

Analysis of post-preservation tissue revealed 56 metabolites upregulated in the 10˚C group vs in the 4˚C group and 59 significantly downregulated (Table 1, $p < 0.05$, $Log_2FC > 2$). Significantly upregulated metabolites included glutathione (Fold of change = 14.64; $p = 0.0079$) and ascorbate (Fold of change = 8.15; $p = 0.0079$), metabolites previously shown anti-oxidative protection in ischemia-reperfusion injury in the lungs.[15,16] The most down-regulated

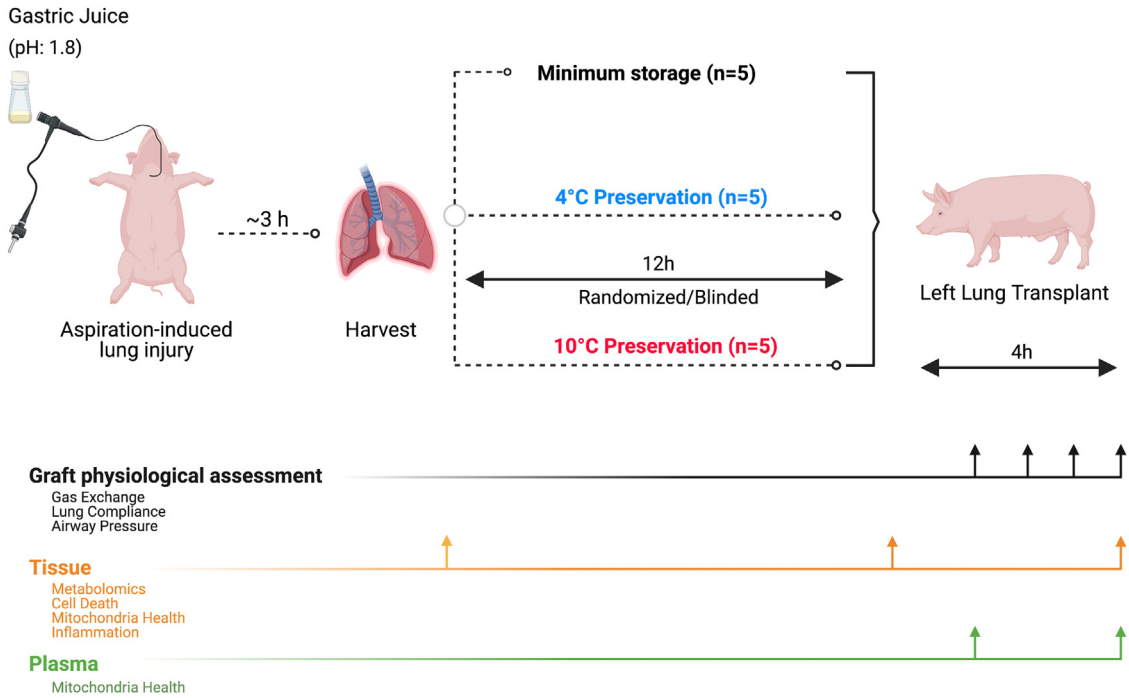

Figure 1 Graphical schema of experimental design. Partial aspiration injury was created through bronchoscopic delivery of gastric juice (pH: 1.8) using a reproducible method. Injured donor lungs (n = 5/group) were then procured and blindly randomized to storage at 4˚C (on ice) or at 10˚C (in a thermoelectric cooler) for 12 hours. A third group included minimal cold storage to control for preservation time. A left lung transplant was performed thereafter followed by 4 hours of graft evaluation.

metabolite is taurolithocholate (Fold of change = 0.037; p = 0.016), which has been shown to trigger mitochondrial depolarization.

10˚C lung preservation protects mitochondrial health

A well-described path of cellular stress involves mitochondrial impairment with further release of mitochondrial DNA (mtDNA) into the surrounding environment.[17] To assess mitochondrial stress, we measured the levels of circulating cell-free mtDNA (ccf-mtDNA) being released into the recipient plasma during graft reperfusion. Results of our analysis showed significantly less ccf-mtDNA in recipients who received lungs from the 10˚C group when compared to minimum storage (Figure 5C, p = 0.042).

As a consequence, mtDNA activates the intracellular inflammasome which turns back to release potent inflammatory cytokines such as IL-1β with further cellular damage (Figure 5A).[18,19] We measured the concentrations of IL-1β, Il-6 and IL-8 and TNF-α within the post-reperfusion lung tissue (Table S4). Results showed significantly lower concentrations of IL-1β in the post-reperfusion tissue for lungs stored at 10˚C vs those stored at 4˚C (Figure 5B, p = 0.019).

Mitochondrial biogenesis plays a critical role in maintaining functional mitochondria when cells experience metabolic or environmental stresses. Common pathways that play a role in the biogenesis process include mitochondrial fusion and fission. Mitochondrial fission is needed to create new mitochondria, but it also contributes to quality control by enabling the removal of damaged mitochondria and can facilitate apoptosis during high levels of cellular stress.[20] Activation of cell death pathways during lung preservation have also been shown to characterize post-transplant graft dysfunction.[14] To evaluate the role of mitochondrial biogenesis within the 2 preservation temperatures, gene expression analysis was performed on a panel of mitochondrial biogenesis markers on post-transplant lung tissue. Results of the analysis showed significantly lower fold regulations of *MFF* (Table 2, p = 0.019), a gene encoding for a mitochondrial fission factor protein, when storing lungs at 10˚C vs 4˚C. We then went on to evaluate the differences in cell death between the groups by performing terminal deoxynucleotidyl transferase dUTP (TUNEL) staining on post-transplant histological samples to quantify apoptosis. Results of our analysis showed a tendency of lower levels of cell death in grafts stored at 10˚C vs 4˚C (Figure 5D, p = 0.14) and 10˚C vs minimum storage (Figure 5D, p = 0.08).

Discussion

Here, we demonstrate 10˚C as a superior lung preservation temperature in comparison to the current standard of 4˚C by evaluating its efficacy for injured donor lungs within a large animal transplant model. We first began by establishing a reproducible model of lung injury through instillation of gastric juice into the segmental airways of donor pigs. The effectiveness of the injury was confirmed as donor animals had a mean pre-procurement P/F ratio of slightly under 300 mm Hg after receiving the injury. We then proceeded to allocate these injured lungs in a blinded and

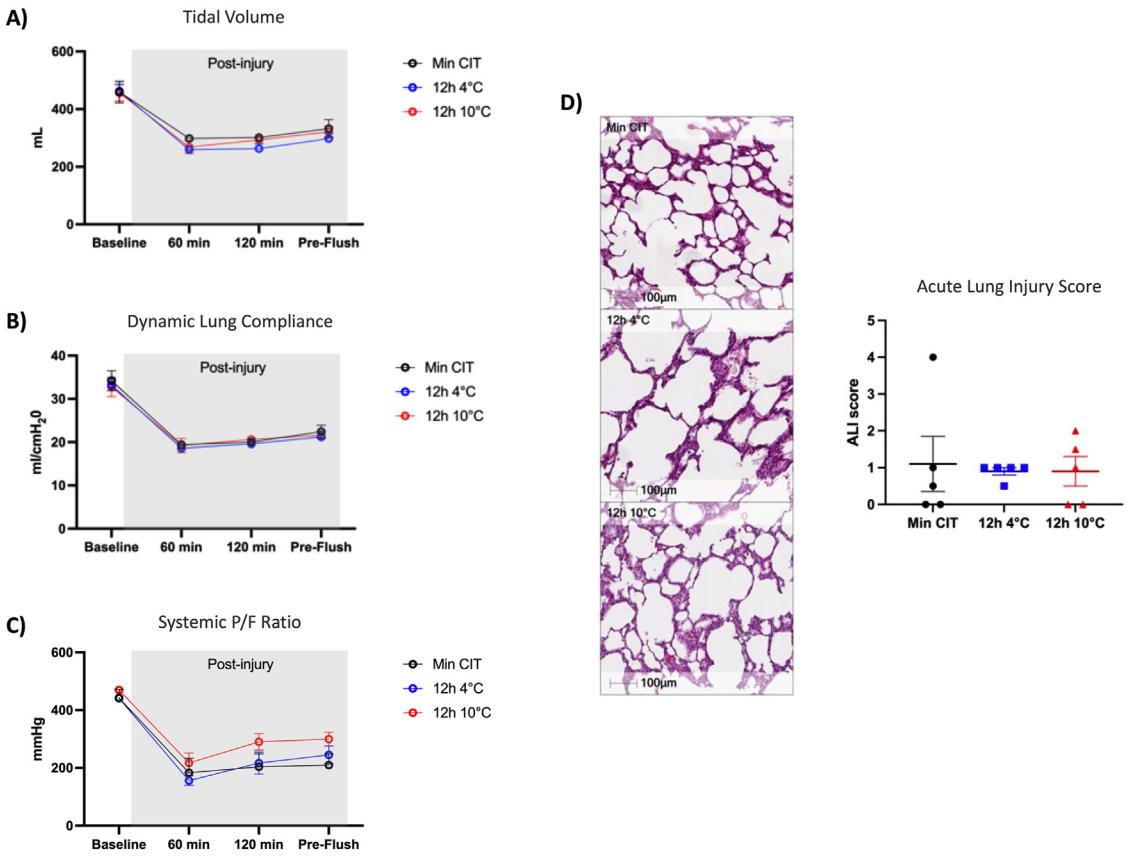

Figure 2 Evaluation of donor lung function before and after delivery of lung injury. (A) Tidal volume. (B) Dynamic Lung Compliance. (C) Systematic oxygenation. (D) Representative histology post lung retrieval alongside acute lung injury scores (scale bar = 100 μm). Experimental results are expressed as mean ± standard error of the mean. One-way ANOVA testing was performed followed by Bonferroni's multiple comparison testing for figures involving a single timepoint. Two-way ANOVA performed for all figures involving a time component followed by Bonferroni's multiple comparison testing. P/F ratio: ratio of oxygen partial pressure to fraction of inspired oxygen; CIT: Cold Ischemic Time; (* $p < 0.05$).

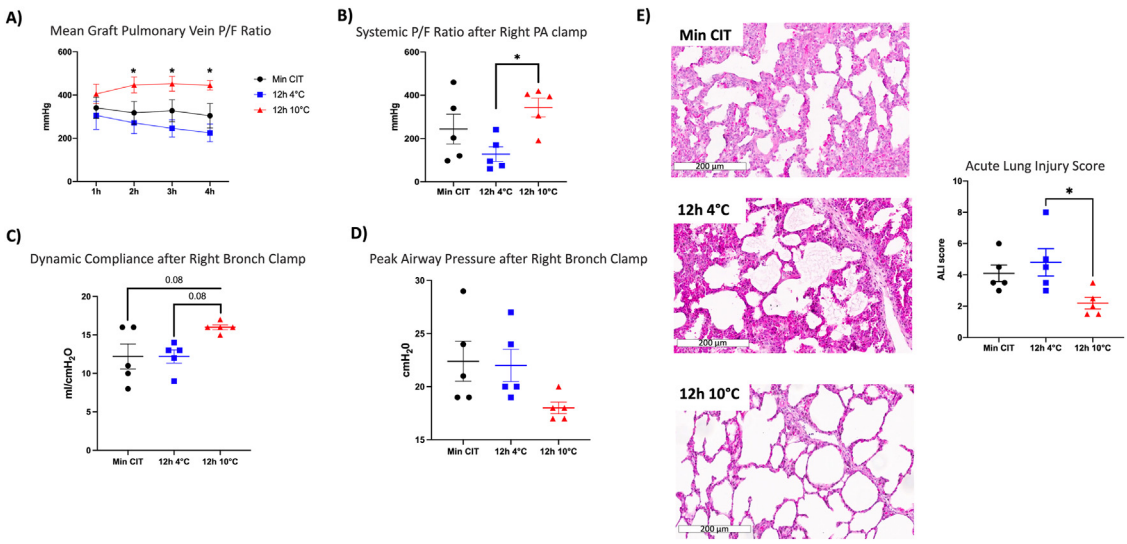

Figure 3 Post-transplant lung function and histological evaluation. (A) Mean graft (upper and lower) pulmonary vein oxygenation. (B) Systemic oxygenation after right pulmonary artery clamping at the end of the reperfusion period. (C) Dynamic lung compliance after right bronchus occlusion at the end of the reperfusion period. (D) Peak airway pressure after right bronchus occlusion at the end of the reperfusion period. (E) Representative histology post reperfusion alongside acute lung injury scores (scale bar = 200 μm). Experimental results are expressed as mean ± standard error of the mean. One-way ANOVA testing was performed followed by Bonferroni's multiple comparison testing for figures involving a single timepoint. Two-way ANOVA performed for all figures involving a time component followed by Bonferroni's multiple comparison testing. P/F ratio: ratio of oxygen partial pressure to fraction of inspired oxygen; ALI: Acute Lung Injury; Bronch: Bronchus; CIT: Cold Ischemic Time; PA: Pulmonary Artery (* $p < 0.05$).

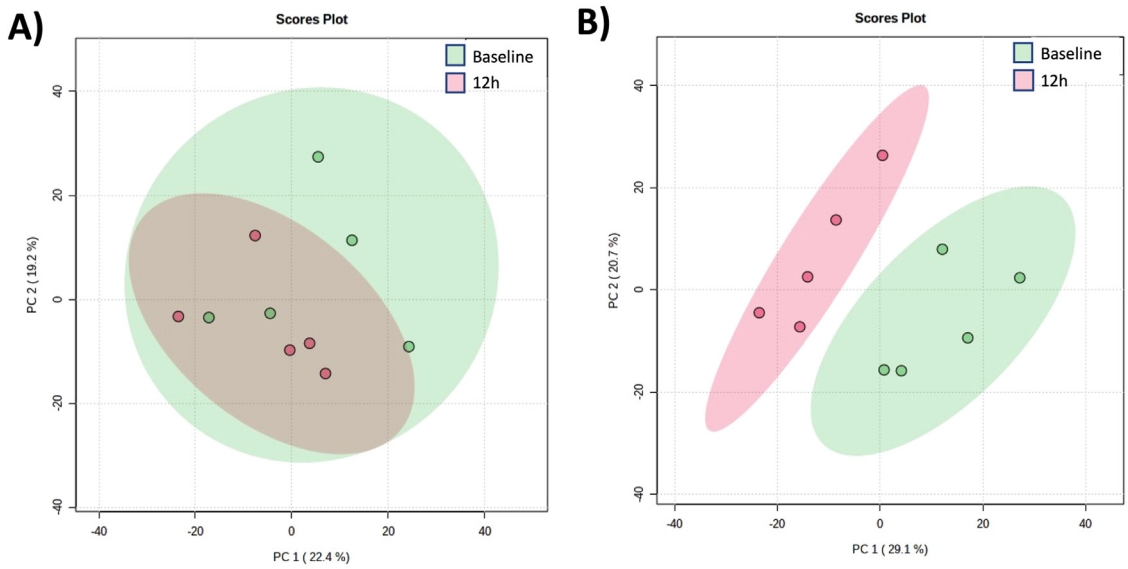

Figure 4 Principal component analysis of global metabolome. (A) Post-harvest (baseline) and post 12 hour preservation at 4°C. (B) Post-harvest (baseline) and post 12 hour preservation changes at 10°C. PC: Principal component; CIT: Cold Ischemic Time.

randomized fashion to different storage conditions and evaluated them through transplantation. A period of 12 hours of storage was selected to evaluate a timing that may be acceptable within current clinical practice, as well as to induce a moderate degree of reperfusion injury to compare the differences amongst the 2 storage temperatures. During 10°C storage, metabolomic analysis showed that the lungs were actively metabolizing cytoprotective molecules during the 12-hour period, while lungs stored at 4°C had minimal changes in metabolic changes occurring. Results of our study also showed that *injured* lungs stored at 10°C for a 12-hour period had superior physiologic performance post-transplantation in comparison to lungs stored using the current standard methods which is consistent with previously published literature examining 10°C lung preservation for the storage of healthy lungs.[9,21–23] Post-transplant lung tissue also showed reduced histological damage and had lower concentrations of the potent inflammatory mediator IL-1β. Further biological analysis showed protection of mitochondrial health to be a key mechanism in explaining the experimental results.

Table 1 Short List of Significantly Changed Metabolites Detected After 12 hour Preservation in Porcine Lung Tissue Stored at 4°C vs 10°C

Metabolite	*p*-value	Fold change	$\log_2$(FC)
thymol sulfate	0.032	18.84	4.2358
glutathione, reduced (GSH)	0.0079	14.639	3.8718
Five-methyluridine (ribothymidine)	0.0079	11.563	3.5315
ethyl beta-glucopyranoside	0.012	10.816	3.4351
ascorbate (vitamin C)	0.0079	8.1466	3.0262
X-24582	0.032	7.8631	2.9751
Pyridoxamine	0.0079	7.2539	2.8588
adenosine 2′-monophosphate (2′-AMP)	0.016	6.8908	2.7847
Thymine	0.0079	5.4062	2.4346
bilirubin degradation product, C17H18N2O4 (3)**	0.032	0.19827	-2.3345
isobutyrylglycine	0.036	0.19581	-2.3525
caproate (6:0)	0.0079	0.19396	-2.3662
3-hydroxy-2-ethylpropionate	0.016	0.17663	-2.5012
X-21353	0.012	0.1646	-2.6029
4-hydroxyphenylacetylglycine	0.012	0.15536	-2.6863
Ectoine	0.0079	0.12049	-3.053
glycocholenate sulfate*	0.0079	0.068178	-3.8745
glycolithocholate sulfate*	0.0079	0.051852	-4.2694
taurolithocholate	0.016	0.037429	-4.7397

In orange are highlighted upregulated metabolites at 4°C, in blue are highlighted downregulated metabolites at 4°C.
FC, Fold Change.

Interestingly, our data also suggests that intentionally delaying transplantation by storing lungs at 10°C may have a therapeutic role in achieving favorable post-transplant outcomes. This finding contradicts the current conventional understanding. It is usually accepted that lengthening cold storage time results in organ deterioration.[24] In our study, we found that lungs stored at 10°C had less circulating cell-free mtDNA within the recipient plasma post-reperfusion and lower levels of tissue apoptosis compared to lungs which experienced minimal preservation. Furthermore, these lungs tended to have better function compared to minimal ischemia. Previous studies have cited a potential benefit of delaying graft reperfusion by keeping lungs at cold storage for a short period of time vs immediate reperfusion using ex vivo lung perfusion models.[25,26] Despite these findings, no literature has examined the direct biological consequences of continuing metabolism at 10°C in recovering injured organs with post-transplant assessment. In this study, we found that lungs stored at 10°C produced significant upregulation of reduced glutathione and ascorbic acid during the storage period. These metabolites have been

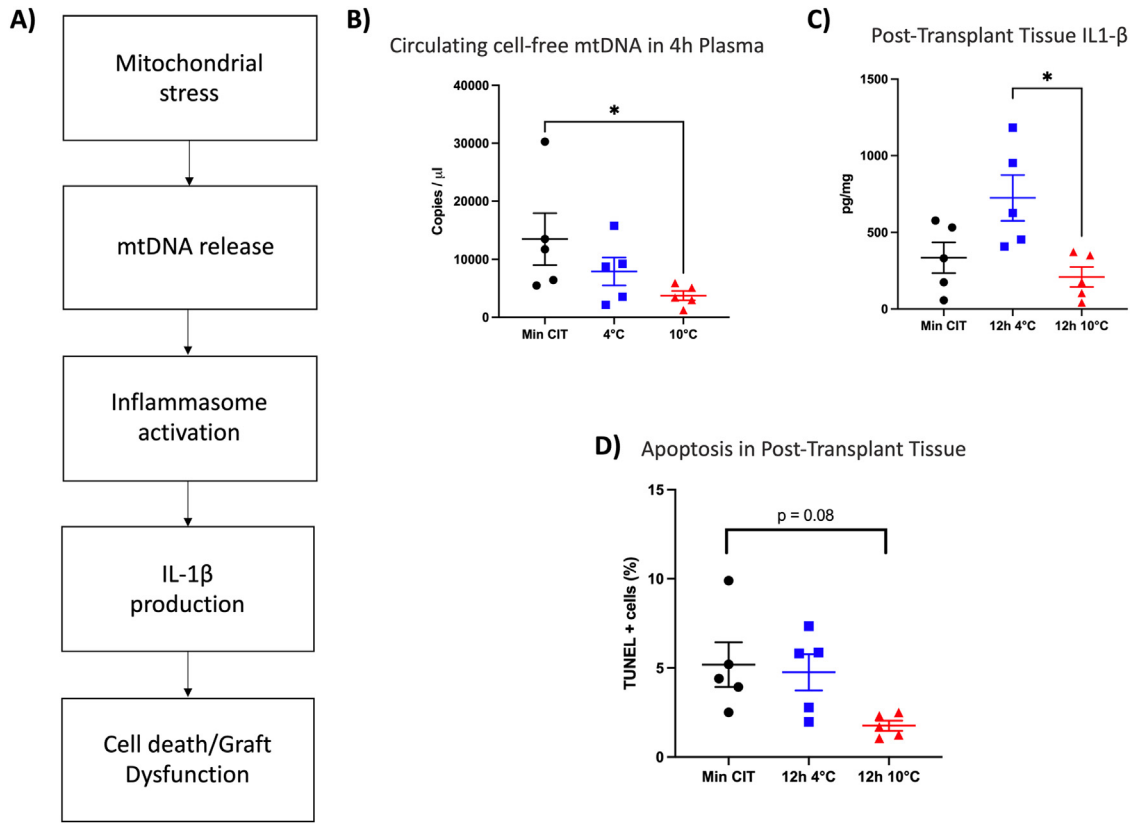

Figure 5 Mitochondrial health assessment. (A) Schema of graft dysfunction related to mitochondrial stress. (B) Circulating cell-free mitochondrial DNA measured in recipient plasma at the end of the reperfusion period. (C) Post-transplant tissue concentration of inflammatory cytokine IL-1β. (D) Lung tissue apoptotic cell death quantification. One-way ANOVA testing was performed followed by Bonferroni's multiple comparison testing for figures involving a single timepoint. IL-1β: Interleukin-1β; mtDNA: mitochondrial DNA (* $p < 0.05$).

shown to have implications in protecting lung grafts from ischemia-reperfusion injury through the upregulation of anti-oxidative systems.[15,16] Although these findings may lend in explaining our experimental findings, further in-depth analyses should be performed.

Our study has several limitations. First, lungs allocated to the 4˚C were placed on ice, where there is an inherent temperature difference in temperature distribution between the anterior and posterior aspects of the organ. On the hand, lungs preserved at 10˚C were placed in a temperature-controlled incubator where temperature is more uniform. Although this may be considered a limitation in our experimental design, we felt it was important to include the standard of care (lungs on ice) to have more translationally relevant findings. Next, mitochondrial health measurements were not performed using live tissue to properly extract mitochondria to run direct experiments such as mitochondrial electron transport chain function or mitochondrial membrane potential. However, the measurements chosen are well established in the field of mitochondrial research and represent a view of mitochondrial stress and gene expression.[27] More importantly, these are translational markers that can easily be accessed in patients/donor/recipient anywhere around the globe. Moreover, we performed direct transplantation of what would be classified as "extended criteria" lungs. In clinical transplant practise, these lungs would typically undergo normothermic ex vivo lung perfusion (EVLP) evaluation before being deemed suitable for transplantation.[28] Despite being a more clinically relevant model, direct transplantation of the lung grafts allowed us to have better evaluate the effects of the storage conditions on post-transplant lung function. Indeed, studies have reported a therapeutic role of EVLP, leading to superior post-transplant lung function.[29] Future studies should be performed to evaluate the utility of EVLP in recovering injured organs after 10˚C storage. Another limitation of our study relates to the choice of lung injury model which was selected for our investigation and the

Table 2 Fold Regulation of Mitochondrial Injury Gene Expression Markers in Post-Transplant Lung Tissue After Preservation at 10˚C Compared to 4˚C

Symbol	Fold regulation	*p* value
PPARG	-1.25	0.137639
PPARGC1A	3.10	0.136760
PPARGC1B	-1.19	0.522307
TFAM	-1.21	0.215283
MFN1	-1.10	0.563679
MFN2	2.14	0.145801
OPA1	-1.26	0.205977
MFF	-1.76	0.019014
FIS1	-1.21	0.872895
PARK2	1.79	0.177249
PINK1	1.03	0.752881

generalization of these findings to other types of organ injury. Aspiration was preferred over other mechanisms of lung injury based on its relatively high prevalence in potential lung donors. Aspiration was also preferred over warm ischemia only since the latter do not apply to lung procurement in brain dead donors. Finally, previous experimental studies have reported the creation of artificial aspiration lung injury large animal models for translational research, allowing us to build a reproducible model.[30,31] Lastly, this study was performed using porcine lungs where metabolic and biologic mechanisms may differ from that of humans. However, these results set the precedent for future experiments and mechanistic exploration.

In conclusion, our results support 10˚C as a potentially superior storage temperature compared to the current clinical gold standard (4˚C) for preservation of injured door lungs in a pig model. In a blinded and randomized large animal transplant experiment, we confirmed and further described the underlying mechanisms of lung protection related to improved mitochondrial preservation. In this study, we also demonstrate a potential therapeutic role of static lung storage at 10˚C in initiating reparative processes in injured organs prior to transplantation. By becoming a new standard of care, 10˚C as a cold storage temperature may improve the quality of injured lungs beyond that achieved by standard procurement strategies, which may increase the number of lungs available for transplantation or reduce the incidence of post-transplant primary graft dysfunction.

Author contributions

EAB, AA, CB, EB, AW, OH, YZ, and MCh participated in data acquisition and model development; MK performed blinded histological slide analyses.

EAB, AA, ACA, SK and MC were involved in the conception and experimental design of the study.

EAB, AA, SK, ACA and MC participated in writing and preparation of the manuscript

MC was the study supervisor.

Disclosure statement

MC, TW, SK are shareholders of Traferox Technologies Inc, Traferox devices were not used in any part of the study. MC, TW and SK are consultants for Lung Bioengineering Inc. None of the other authors have conflict of interest to disclose.

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Supplementary materials

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