TRANSPLANTATION

Static lung storage at 10°C maintains mitochondrial health and preserves donor organ function

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Cold static preservation on ice (~4°C) remains the clinical standard of donor organ preservation. However, mitochondrial injury develops during prolonged storage, which limits the extent of time that organs can maintain viability. We explored the feasibility of prolonged donor lung storage at 10°C using a large animal model and investigated mechanisms related to mitochondrial protection. Functional assessments performed during ex vivo lung perfusion demonstrated that porcine lungs stored for 36 hours at 10°C had lower airway pressures, higher lung compliances, and better oxygenation capabilities, indicative of better pulmonary physiology, as compared to lungs stored conventionally at 4°C. Mitochondrial protective metabolites including itaconate, glutamine, and *N*-acetylglutamine were present in greater intensities in lungs stored at 10°C than at 4°C. Analysis of mitochondrial injury markers further confirmed that 10°C storage resulted in greater protection of mitochondrial health. We applied this strategy clinically to prolong preservation of human donor lungs beyond the currently accepted clinical preservation limit of about 6 to 8 hours. Five patients received donor lung transplants after a median preservation time of 10.4 hours (9.92 to 14.8 hours) for the first implanted lung and 12.1 hours (10.9 to 16.5 hours) for the second. All have survived the first 30 days after transplantation. There was no grade 3 primary graft dysfunction at 72 hours after transplantation, and median post-transplant mechanical ventilation time was 1.73 days (0.24 to 6.71 days). Preservation at 10°C could become the standard of care for prolonged pulmonary preservation, providing benefits to both patients and health care teams.

INTRODUCTION

The ability to preserve donor organs before the time of transplantation has made this lifesaving procedure a clinical reality for those facing end-stage organ disease. In current practice, organ preservation is typically performed by flushing the organ with a cold specialized preservation solution followed by subsequent hypothermic storage on ice (~4°C) (*1*–*3*). This method continues to be used and applied across different organ systems due to its simplicity and low cost. Using this method for the preservation of donor lungs, maximum accepted preservation times have been limited to about 6 to 8 hours. In a large registry report from the International Society of Heart & Lung Transplantation (ISHLT), median preservation times of lungs used for adult lung transplantation were 5.5 hours (interquartile range, 2.3 hours) (*4*). Although the goal of hypothermic storage is to sustain cellular viability through reduction of cellular metabolism, reduction in organ temperature has been shown to progressively favor mitochondrial dysfunction (*5*–*9*). Therefore, the ideal temperature for donor organ preservation should be a balance between avoidance of mitochondrial dysfunction and prevention of cellular exhaustion.

Maintenance of appropriate cellular ionic gradients has also been demonstrated to play a critical role in the setting of cold static lung preservation (*10*). Specifically, the Na^+/K^+ adenosine triphosphatase (ATPase) has been described to play an important role in preventing lung injury (*11*), and inhibition of this enzyme has been shown to impair mitochondrial energetics (*12*).

Preliminary studies published more than 30 years ago have suggested that 10°C is the optimal lung storage temperature (*13*–*15*). Despite these reports, translation of these findings to clinical practice never occurred due to concerns about the margin of safety of this approach as opposed to a reliable and simple ice cooler and due to limited information regarding the underlying mechanisms by which 10°C was functionally superior 4°C storage. Thus, the safety and suitability of prolonged organ storage at 10°C has not been explored.

There remains a great need to extend the current window of preservation beyond what is currently practiced for lungs (*16*). Extending lung preservation times reliably would overcome geographic limits faced in transplantation, allow for optimized immunological matching among donor and recipients, and provide the opportunity to make lung transplantation a scheduled rather than urgent procedure, with advantages for patients, health care teams, and health systems. In this study, we evaluated the safety of static storage of porcine lungs at 10°C for prolonged pulmonary storage and provide mechanistic insights into the metabolic and biological impact of two different storage temperatures (4° and 10°C). We also report proof-of-concept clinical use of 10°C for prolonging organ preservation, leading to semi-elective lung transplantation in humans.

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RESULTS

Lung preservation at 10°C results in superior prolonged graft preservation

To evaluate 10°C preservation as an optimal lung storage temperature, we performed experiments using a large animal pig model in which lung procurement was procedurally similar to that of current clinical methods (*17*). After explant, donor lungs (n = 5 per group) were randomized to storage at either 10°C in a thermoelectric cooler (accuracy of ±0.5°C) or 4°C in a walk-in cooler. After 36 hours of cold static preservation, the lungs were subjected to 12 hours of assessment in the normothermic ex vivo lung perfusion (EVLP) platform to evaluate functional and biological differences between the groups (Fig. 1A). A well-established protocol for EVLP was followed, referenced as the Toronto protocol (*18*). This method has been used to evaluate more than 700 human donor lungs, showing an excellent correlation with post-transplant outcomes (*19–21*). No differences in animal baseline characteristics such as donor oxygenation, donor weight, and donor lung compliance were found between the two experimental groups (table S1). After 36 hours of cold preservation, lungs stored at 10°C had a markedly better quality of preservation compared to those stored at 4°C, as demonstrated by the following functional parameters: significantly lower airway pressures (P < 0.0001; Fig. 1, B and C), higher lung compliances (P < 0.0001; Fig. 1, D and E), and better oxygenation function (P < 0.0001; Fig. 1F). In addition, lungs stored at 10°C developed less edema as reflected by a significantly lower lung weight gain during the perfusion period (30 ± 34.1 g versus 201 ± 33.2 g, P = 0.0159; Fig. 1G). A comparison of the lung gross appearance after the perfusion period within each experimental condition is shown in fig. S1. Lung function in the 10°C group after 36 hours of ischemia faired similarly to lungs subjected to minimal ischemia (~2 hours) in previous studies by our group (*18*, *22*).

Lung storage at 10°C leads to the accumulation of cytoprotective metabolites

Temperature is known to play a key role in the alteration of cellular energetics and metabolism (*23*). To have a high-fidelity view of the metabolome, we performed a global untargeted metabolomic analysis of tissue samples collected during the experiments described above. Samples were obtained before and after the 36-hour cold preservation period. Of the metabolites identified (table S2), four metabolites were found in significantly different abundance between the two groups after 36 hours of cold storage. Among these four metabolites, we identified significantly higher intensities of mitochondrial-related metabolites including itaconate, glutamine, and *N*-acetylglutamine (Fig. 2A). These metabolites have been previously described to promote the innate anti-oxidative system within mitochondria (Fig. 2B) (*24*). On the basis of this information, we further hypothesized that 10°C storage may sustain mitochondrial health during ischemia.

Lung storage at 10°C maintains mitochondrial and cellular health

Mitochondrial dysfunction states are characterized by changes in inflammatory, metabolic, and oxidative function (Fig. 3A). When under marked cellular stress, mitochondrial DNA (mtDNA) is released into both the intracellular and extracellular environment (*25*). Therefore, we measured the degree of mtDNA oxidation within the lung tissue, the amount of circulating cell-free mtDNA (ccf-mtDNA) during the perfusion period through quantitative polymerase chain reaction (qPCR) analysis (*26*), and the degree of oxidative protection among lungs stored at the two temperatures via immunohistochemical staining for 8-hydroxy-2-deoxyguanosine (8-OHdG), a known marker of DNA/RNA oxidation. Although we did not identify a significant difference in oxidative damage at 10°C versus 4°C during the cold storage period (P = 0.0952; fig. S2A) nor significant differences in the degree of mtDNA oxidation (P = 0.056; fig. S2B) in lung tissue at the end of the normothermic evaluation, we observed significantly more mtDNA release within the EVLP perfusate in the 4°C group as compared to 10°C ($P \leq 0.0001$; Fig. 3B). To supplement our analysis, inflammatory cell oxidation states were indirectly measured through quantification of tissue myeloperoxidase (MPO). No differences in MPO concentrations before or after cold storage, or after EVLP, were seen between the two groups (fig. S3), suggesting that passenger leukocytes present in the organ during preservation likely do not account for the observed difference in mitochondrial damage.

A well-described consequence of intracellular mtDNA release is the activation of the intracellular inflammasome and, in turn, the release of highly proinflammatory cytokines such as interleukin-1β (IL-1β) and IL-8 (*27*, *28*). To assess the potential downstream effects of mtDNA release on cytokine production, we measured the concentrations of IL-1β and IL-8 within the EVLP perfusate using standard enzyme-linked immunosorbent assay (ELISA) techniques. Results of our analysis showed significantly lower concentrations of IL-1β ($P \leq 0.0001$; Fig. 3C) and IL-8 ($P \leq 0.0001$; Fig. 3D) in the EVLP perfusate for lungs stored at 10°C versus those stored at 4°C.

Because mitochondria play an essential role in cellular respiration, we hypothesized that if mitochondrial dysfunction occurred during the preservation period, cellular metabolic dysfunction may be present. To gain insight into potential metabolic differences between the groups, we measured glucose and lactate concentration within the EVLP perfusate. Results showed that lung perfusate glucose consumption ($P \leq 0.0001$; Fig. 3E) and lung lactate production ($P \leq 0.0001$; Fig. 3F) were significantly higher at 4°C in comparison to 10°C. In addition, perfusate pH was lower at 4°C in comparison to 10°C (P < 0.0001; Fig. 3G). These results support the notion that 10°C storage results in favorable metabolic profiles.

Activation of cell death pathways during lung preservation has been shown to have implications in worsening post-transplant graft function (*29*). To evaluate differences in cell death markers between the groups, we measured concentrations of lactate dehydrogenase (LDH), a marker of cell necrosis (*30*), within the EVLP perfusate and performed terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining on post-EVLP histological samples to quantify apoptosis. LDH activity was significantly higher when lungs were stored at 4°C versus 10°C (P = 0.0065; Fig. 3H), whereas no differences in TUNEL$^+$ cells were observed (P = 0.6905; fig. S4). We then went on to evaluate differences in gene expression associated with cell death between the two groups. Using an RT^2 PCR array encompassing relevant genes for several cell death pathways (necroptosis: *TNFa*, *FASLG*, *CASP8*, and *NFKB*; pyroptosis: *CASP1* and *GPX4*; apoptosis: *CASP3*, *CASP8*, *CASP9*, *TP53*, and *NFKB*; and ferroptosis: *MPO*, *GPX4*, and *TP53*), we found lower expressions of *FASLG* (P = 0.0013; Table 1) in biopsied tissue after the 36-hour cold storage period and lower expressions of *CASP9* after EVLP (P = 0.0066; Table 1) for lungs stored at 10°C versus 4°C. *FASLG* and *CASP9* have been implicated in necroptosis and apoptosis, respectively (*31*, *32*).

Lung storage at 10°C better preserves Na$^+$/K$^+$ ATPase activity

Inhibition of the Na$^+$/K$^+$ ATPase has been shown to impair mitochondrial energetics and lead to reduced homeostatic control of

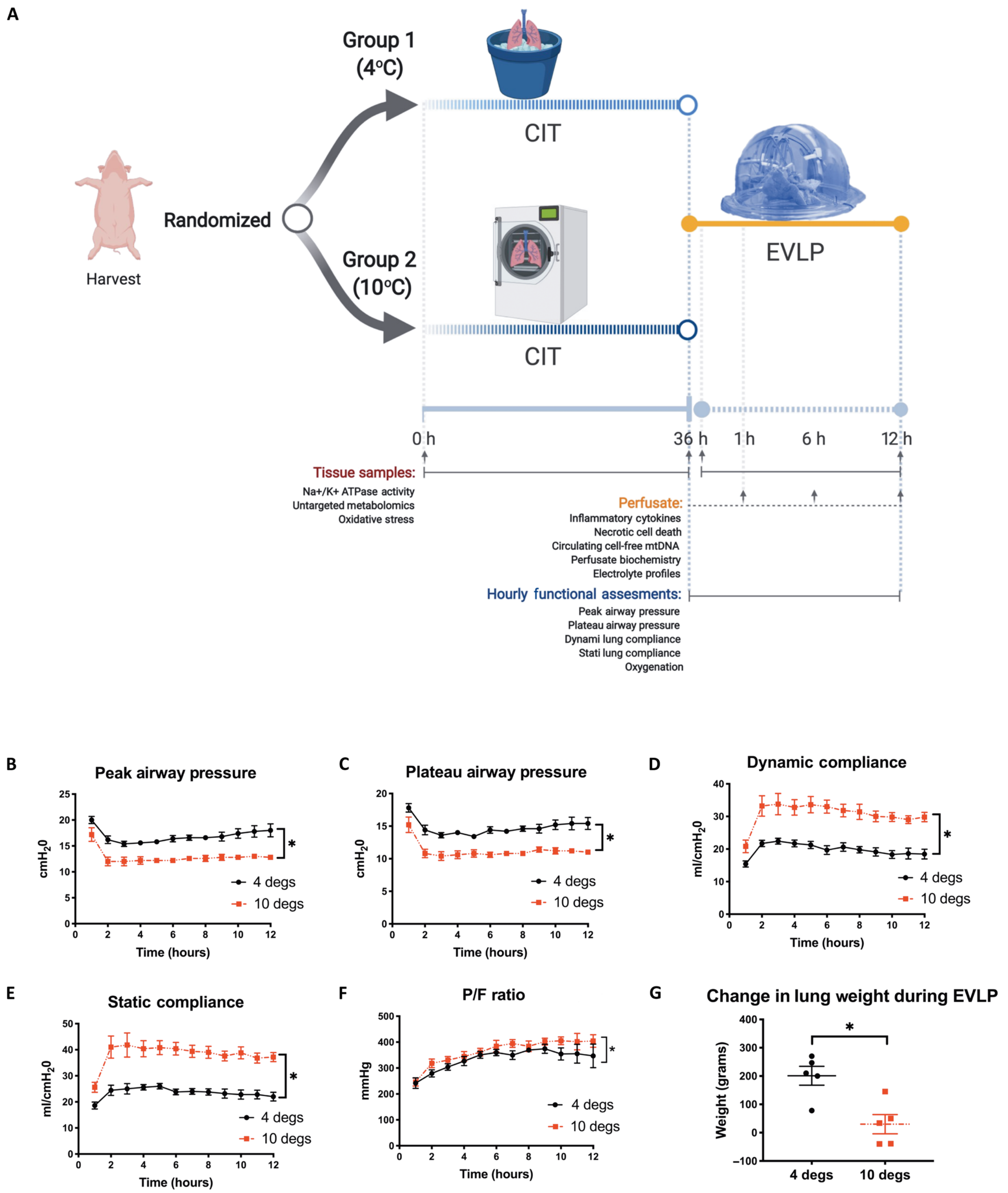

Fig. 1. Functional evaluation of porcine lungs after extended static storage at 10°C versus 4°C. (**A**) Schematic of study design. Donor pig lungs were retrieved and randomized to be stored at either 4°C or 10°C for 36 hours ($n = 5$ per group), followed by 12 hours of normothermic EVLP evaluation. Scheme created with BioRender.com (**B** to **G**) Results of physiologic evaluation during EVLP showing (B) peak and (C) plateau airway pressures, (D) dynamic and (E) static compliances, (F) P/F ratio (ratio of oxygen partial pressure to fraction of inspired oxygen), and (G) change in lung weight. *$P < 0.05$, two-way ANOVA performed for all figures involving a time component and Mann-Whitney test for comparison of two groups. Results are expressed as means ± SEM. degs, degrees; CIT, cold ischemic time.

cellular calcium and pH through inhibition of Na^+/Ca^{2+} exchangers (*12*, *33*). Na^+/K^+ ATPase activity was measured within lung tissue before and after the cold preservation period. Although we did not observe significant differences in pump activity after preservation between the groups ($P = 0.0952$; fig. S5A), perfusate analysis showed significantly lower concentrations of perfusate calcium during

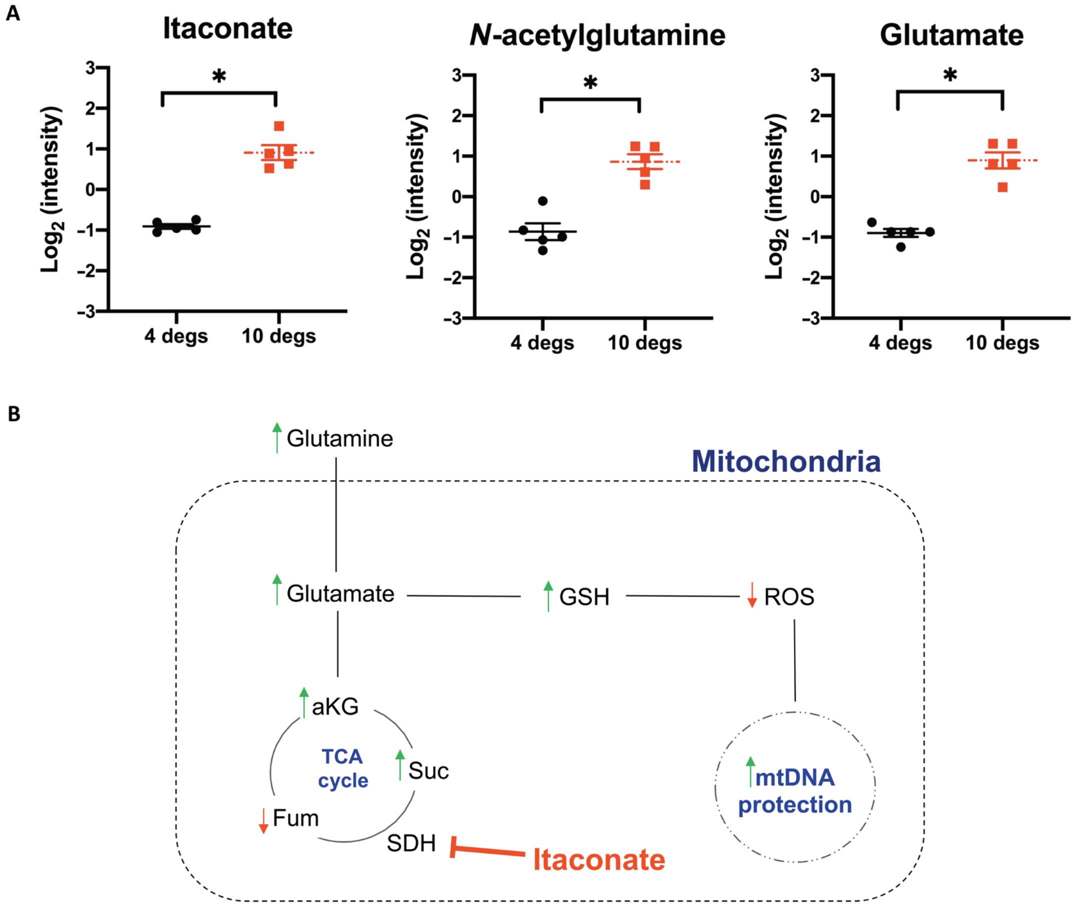

Fig. 2. Metabolic differences after prolonged 4°C and 10°C lung storage. (**A**) Differentially expressed metabolites within porcine lung tissue after 36 hours of cold preservation for lungs stored at 4°C versus 10°C. *$P < 0.05$, *t* test; values expressed as means ± SEM. (**B**) Graphical image showing the potential mechanism of metabolite accumulation. Itaconate inhibits succinate dehydrogenase, leading to generation of metabolites involved in mitochondrial oxidative protection such as glutamine. Fum, fumurate; Suc, succinate; SDH, succinate dehydrogenase; aKG, α-ketoglutarate; GSH, glutathione; ROS, reactive oxygen species; TCA, tricarboxylic acid cycle.

perfusion ($P \leq 0.0001$; fig. S5B), when lungs were stored at 10°C versus 4°C.

As a means to demonstrate the importance of Na^+/K^+ ATPase activity during 10°C lung preservation, we recovered porcine lungs ($n = 4$) and cold flushed them with ouabain (100 mg/liter; a known Na^+/K^+ ATPase inhibitor) supplemented in the standard low-potassium dextran flush solution. Lungs were subsequently stored at 10°C for 36 hours and evaluated on EVLP for 12 hours (Fig. 4A). Donor animal baseline characteristics showed no differences before lung retrieval for lungs, which would go on to be stored at 10°C alone versus those stored at 10°C with ouabain (table S3). Measurement of tissue Na^+/K^+ ATPase activity confirmed the successful inhibition of pump activity with ouabain delivery ($P \leq 0.0001$; fig. S6). Results showed significantly higher airway pressures ($P \leq 0.0001$; Fig. 4, B and C), lower lung compliances ($P \leq 0.0001$; Fig. 4, D and E), worse oxygenation function ($P \leq 0.0001$; Fig. 4, F and I), and higher lung weight gain during perfusion ($P = 0.0159$; Fig. 4G) in the lungs stored at 10°C with ouabain when compared to 10°C alone. Furthermore, analysis of perfusate pH ($P \leq 0.0001$; Fig. 4H) and calcium concentrations ($P \leq 0.0001$; Fig. 4I) showed similar trends with high concentrations of perfusate calcium and low pH levels in the lungs that received ouabain versus 10°C preservation alone. Analysis of changes in ccf-mtDNA in the EVLP perfusate during perfusion showed significantly more mtDNA release for lungs stored with ouabain in comparison to those stored without ($P \leq 0.0001$; Fig. 4J). These results demonstrate the importance of the preserved Na^+/K^+ ATPase function during the 10°C cold preservation period, which may have implications for the maintenance of overall mitochondrial health.

Proof-of-concept 10°C prolonged lung storage and transplantation in humans

Under a clinical protocol with research ethics board approval, we investigated the suitability of extended human lung preservation using 10°C (fig. S7). In this specific pilot study, the goal of prolongation of preservation was to move night transplants to occur during the morning. Recent data suggest that outcomes of organ transplantation and other surgical procedures are improved when performed electively during day time with a rested team (*34*, *35*). Lungs meeting criteria for transplantation based on standard assessments were retrieved and then transported in usual fashion (in an ice cooler at 4°C with a median transport time of 2.97 hours) to our hospital. Upon arrival at the transplant hospital, lungs were immediately stored at 10°C within a temperature-controlled incubator. Five patients received bilateral lung transplants, in which each lung was transplanted sequentially. The mean recipient age for patients transplanted was 70 years old, and four of the five recipients had a diagnosis of pulmonary fibrosis. Three of the five donors involved donation after neurologic determination of death (NDD), whereas the other two were donation after circulatory determination of death (DCD). The median total preservation time for the first implanted lung was 10.4 hours (9.92 to 14.8 hours) and 12.1 hours (10.9 to 16.5 hours) for the second lung. No patients had primary graft dysfunction (PGD) grade 3 (ratio of arterial oxygen partial pressure to fractional inspired oxygen < 200 mmHg) at 72 hours nor required post-operative extracorporeal membrane oxygenation (ECMO) or post-transplant nitric oxide (NO) usage. The median time on the ventilator after lung transplant was 2 days (range, 0 to 7 days), and the median time to hospital discharge was 17 days (range, 14 to 26 days). For comparison, the approximate median hospital length of stay after standard lung transplantation at our center

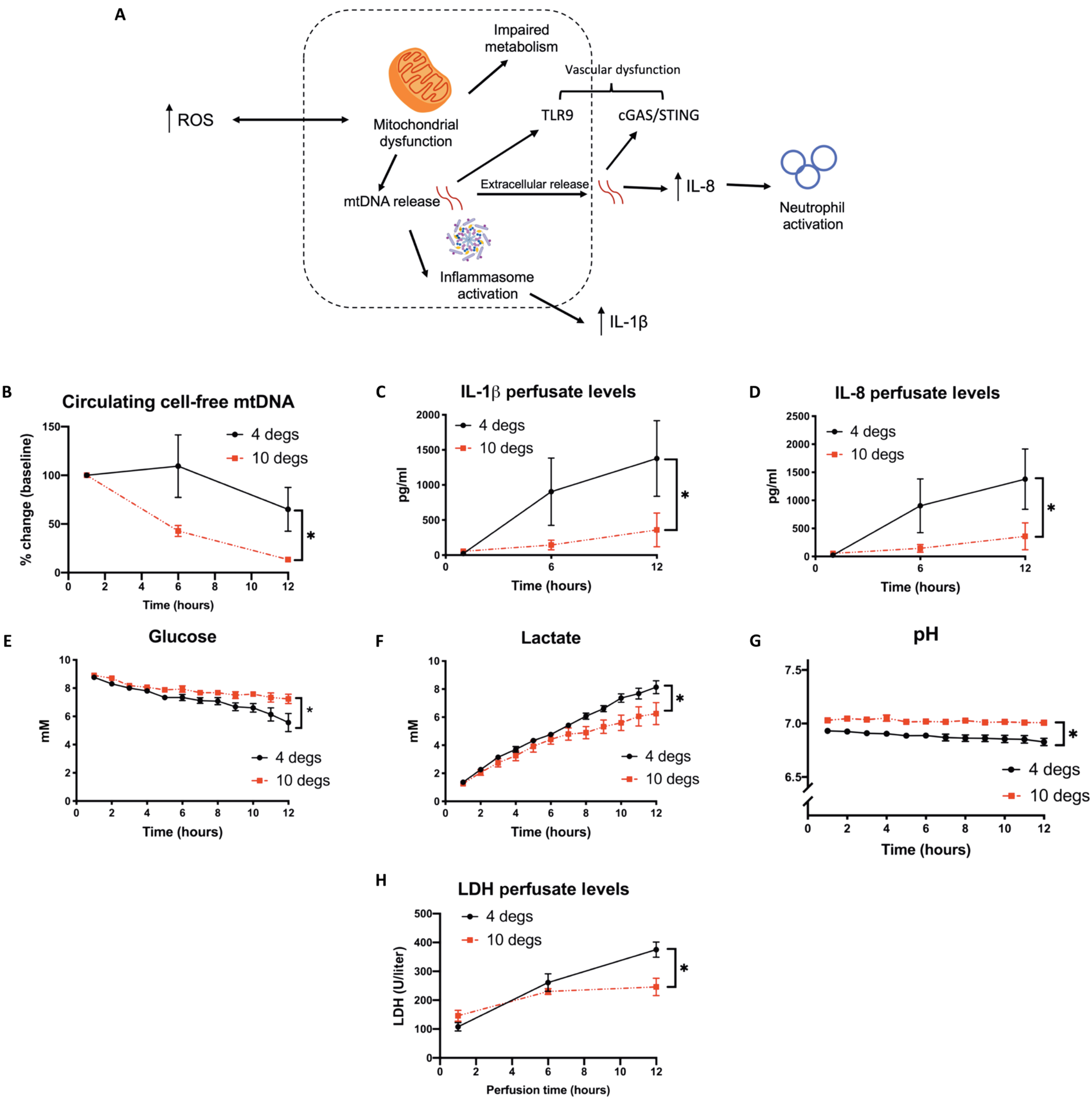

Fig. 3. Assessment of mitochondrial health between pig lungs stored at 4°C versus 10°C. (**A**) Graphical representation of biological consequences of mitochondrial dysfunction. Increased oxidative stress leads to mitochondrial dysfunction, in turn leading to mtDNA release and metabolic impairment. mtDNA can lead to increased production of IL-1β through inflammasome activation and increased release of IL-8. (**B**) ccf-mtDNA extracted from EVLP perfusate and quantified using qPCR during lung perfusion. Fold changes from baseline concentrations (1-hour perfusion) were calculated and plotted. (**C** and **D**) Perfusate inflammatory cytokine profiles. (**E** to **G**) Perfusate (E) glucose, (F) lactate, and (G) pH. (**H**) LDH activity within EVLP perfusate, a measure of cellular necrosis. *$P < 0.05$, two-way ANOVA; data expressed as means ± SEM. mtDNA, mitochondrial DNA; IL, interleukin; TLR9, Toll-like receptor 9; cGAS, comprising the synthase for the second messenger cyclic guanosine 3′,5′-monophosphate (GMP)–adenosine 3′,5′-monophosphate (AMP), STING (cyclic GMP–AMP receptor stimulator of interferon genes).

is 24 days (*36*). The 30-day survival of the five recipients was 100%, and none of the patients required any use of oxygen for exertion at that time. Table 2 summarizes donor and recipient characteristics for these patients, as well as outcomes. Gross lung images after organ retrieval for each individual case is shown (fig. S8) alongside chest x-ray images taken of the donor, and during admission to the intensive

Table 1. Cell death gene expression in porcine lung tissue stored at 10°C relative to 4°C (control). Gene expression normalized using housekeeping genes *ACTG1* and *ACTA1*. Post-cold storage refers to tissue analyzed after 36 hours of storage; Post-EVLP refers to tissue analyzed after 12 hours of EVLP, which occurred subsequent to cold storage. See Fig. 1A for schematic ($n = 5$ per group). *P* values were calculated on the basis of Student's *t* test of the replicate $2^{(-\Delta Ct)}$ values for each gene in each experimental group.

	Post-cold storage		Post-EVLP	
Symbol	Fold regulation	*P*	Fold regulation	*P*
GDC	−1.1	0.91	−1.14	0.74
FASLG	−1.93	0.0013	−1.49	0.32
TP53	−1.33	0.14	−1.34	0.21
MPO	1.01	0.86	−1.14	0.74
CASP1	−1.19	0.40	1.59	0.30
CASP3	−1.36	0.13	1.60	0.44
CASP8	−1.26	0.21	−1.04	0.75
CASP9	−1.82	0.078	−1.85	0.0066
GPX4	−1.17	0.47	−1.10	0.58
NFKB1	−1.39	0.11	−1.33	0.44
BID	−1.39	0.12	−1.15	0.44

care unit (ICU) after transplantation, at 24 hours after transplantation, 48 hours after transplantation, and 72 hours after transplantation of the patient (Fig. 5). All patients are currently alive and doing well at a median follow-up of 330 days.

DISCUSSION

Here, we provide support for 10°C lung storage by evaluating its efficacy during a 36-hour preservation period, using a simple and practical method of storage and a preclinical large animal model. Functional and biological assessment after prolonged ischemia was performed to compare this approach to the current standard of 4°C. We found that, when stored at 10°C for a period of 36 hours, lungs showed superior physiologic function and developed less edema during the perfusion period as compared to lungs stored using the current standard of preservation. Important metabolites related to mitochondrial protection were present at significantly higher intensities, demonstrating a dynamic relationship between metabolism and ability to respond to cellular stress. Biological analysis of lung tissue and EVLP perfusate identified mitochondrial health as a differentiating factor between the two storage temperatures, which may explain the physiologic differences that we found. These findings are consistent with studies suggesting mitochondria as a therapeutic target during organ preservation and that hindrance of mitochondrial function leads to worsening lung function (*37*–*40*). Furthermore, this is also corroborated by several studies, which have shown the prevention of post-transplant organ dysfunction through the protection of mitochondrial health and mtDNA integrity (*41*–*43*).

During the metabolomic analysis, we discovered that lung tissue stored at 10°C had significantly higher intensities of the metabolite itaconate after the cold preservation period compared to lungs stored at 4°C. Itaconate is a mitochondrial-derived immunometabolite, which has been described as an endogenous metabolic regulator of the NLR family pyrin domain containing 3 (NLRP3) inflammasome, in which it serves to inhibit its activation (*44*). Experimental studies have shown that exogenous administration of itaconate mitigates cerebral ischemia-reperfusion injury and reduces lung injury by regulating lung fibrosis (*24*, *45*). Considering this literature, the metabolic production of itaconate during 10°C may have played a role in protecting lungs from ischemic damage during preservation.

We also identified a potential protective role of the Na^+/K^+ ATPase during the preservation period. Previous reports have shown an abolishment of fluid clearance and sodium transport during 4°C cold static storage, leading to the intracellular accumulation of Na^+ and cell swelling (*46*). Furthermore, inhibition of the Na^+/K^+ ATPase has been shown to impair mitochondrial energetics by promoting mitochondrial Ca^{2+} retention and enhanced oxidative phosphorylation (*12*). Analysis of Na^+/K^+ pump function during preservation confirmed lower pump activity during the 4°C cold static storage period versus 10°C. Moreover, the addition of ouabain during cold flush with preservation solution impaired the protective effects of 10°C storage and was associated with mitochondrial injury. Together, these findings suggest that impaired function of the Na^+/K^+ ATPase during cold lung preservation is detrimental.

On the basis of these preclinical findings, we launched a proof-of-concept clinical study to evaluate the safety and feasibility of planned semi-elective lung transplantation using a 10°C storage approach. In current practice, lungs stored using the clinical standard of preservation (4°C) are generally preserved for maximum allowable times of 6 to 8 hours (*16*). Although studies have shown that EVLP can extend preservation times, the costs and complexity of EVLP logistics makes this technique less attractive, especially when EVLP is not required for lung assessment or repair. For some lungs in our study, total preservation times exceeded 16 hours with no observable detriments to post-transplant function. Previous reports have shown that PGD grade 3 at 72 hours is associated with early mortality and the subsequent development of chronic lung allograft dysfunction (*47*–*49*). Of the five cases reported here, no patients developed PGD grade 3 at 72 hours and all patients have survived the first 30 days after being transplanted, with excellent pulmonary function. These findings support our preclinical findings in which 10°C static storage may be a viable option to support lungs for prolonged periods of time. A multicenter clinical trial will definitively address this concept, which ultimately could change the gold standard of lung preservation.

Our study has several limitations. First, lungs used in the animal experiments did not have donor-related injuries (such as aspiration, infection, ventilator injuries, contusions, or emboli). Controlling these variables allowed us to directly study the effects of our proposed strategy on quality of lung tissue preservation without the influence of these confounders. Encouragingly, 10°C storage in the setting of prolonging clinical preservation times did not appear to have any deleterious effects on what could be considered a heterogenous, albeit small, population of human donor lungs trialed. Second, in the large animal studies, EVLP was used as the primary assessment for graft performance after preservation rather than transplantation. Performing biological and functional analysis of the grafts independent of recipient factors was important to elucidate the mechanisms by which storage at 10°C is advantageous to prolonged preservation. Furthermore, physiologic performance during ex vivo lung assessments has

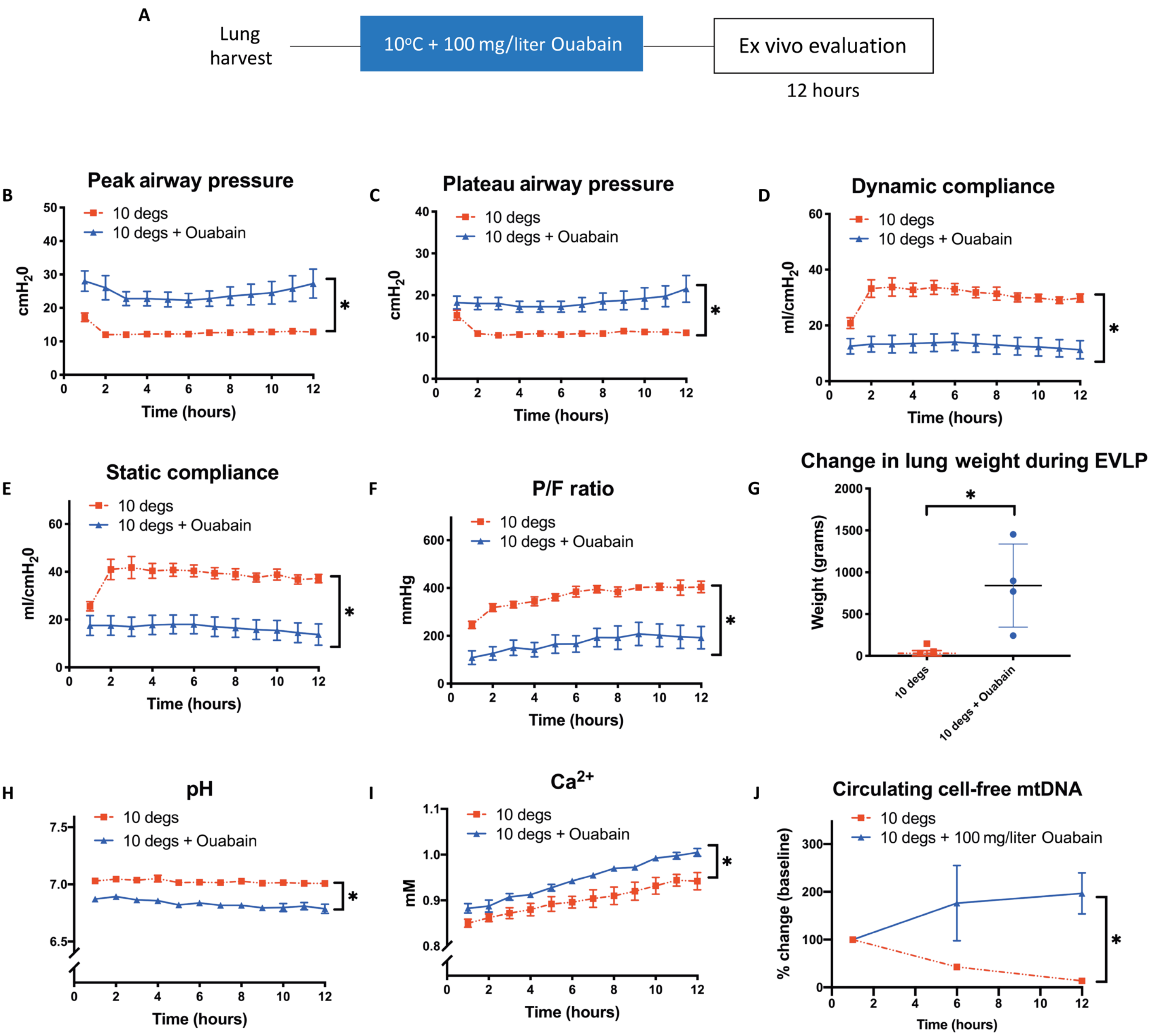

Fig. 4. Assessment of contribution of Na^+/K^+ ATPase during 10°C cold preservation. (**A**) Experimental protocol: Harvested pig lungs ($n = 4$) were flushed with Perfadex without or with ouabain (100 mg/liter) and stored for 36 hours at 10°C, followed by 12 hours of EVLP. (**B** to **G**) Results of physiologic evaluation during normothermic EVLP showing (B) peak and (C) plateau airway pressures, (D) dynamic and (E) static lung compliances, (F) P/F ratio (ratio of oxygen partial pressure to fraction of inspired oxygen), and (G) change in lung weight. (**H**) pH and (**I**) calcium during 12 hours of normothermic perfusion. (**J**) ccf-mtDNA extracted from EVLP perfusate and quantified using qPCR during lung perfusion. Fold changes from baseline concentrations (1-hour perfusion) were calculated and plotted. $^*P < 0.05$, two-way ANOVA; data expressed as means ± SEM.

been shown to correlate with post-transplant lung function (*50*). Third, direct measurement of mitochondrial function using isolated live mitochondria was not performed in this experiment due to logistic difficulties moving live cells from one facility to another. Nonetheless, the measurement of several markers of mitochondrial injury and previous reports showing the link between these markers and mitochondrial function supported a potential role of 10°C in preserving mitochondrial health.

Note that, although ccf-mtDNA measurement is widely used as a method to assess mitochondrial injury in different settings, it uses PCR technology limited by the detection of short amplicons (*51*–*53*). To increase robustness, use of two different mtDNA amplicons is suggested: Finding similar patterns on both amplicons decreases the chance of sequence variation that could impair amplification efficiency or bias toward specific mtDNA fragments (*51*). By measuring both NADH (reduced form of nicotinamide adenine dinucleotide)

Table 2. Donor and recipient information and outcomes of patients receiving lung transplants in clinical pilot study. DBD, donation after brain death; DCD, donation after cardiac death; LTx, lung transplantation; PGD, primary graft dysfunction; LOS, length of stay; ICU, intensive care unit; NO, nitric oxide; ECMO, extracorporeal membrane oxygenation; COPD, chronic obstructive pulmonary disease; IPF, idiopathic pulmonary fibrosis, n/a, not applicable.

	Case 1	Case 2	Case 3	Case 4	Case 5
Donor					
Type	DBD	DBD	DCD	DBD	DCD
Lungs donated	Double	Double	Double	Double	Double
Age	53	37	67	71	53
Sex	F	M	F	M	M
Time to arrest (min)	n/a	n/a	19	n/a	65
Warm ischemic time (min)	n/a	n/a	20	n/a	25
Preservation					
Total 10°C storage time (hours)	3.47	5.62	9.58	5.27	9.57
First lung total ischemic time (hours)	9.92	8.77	14.32	10.40	14.77
Second lung total ischemic time (hours)	11.83	10.92	16.32	12.13	16.5
Recipient					
LTx indication	COPD	IPF	IPF	IPF	IPF
Age	74	65	70	69	70
Sex	F	M	M	M	M
PGD grade admission	1	3	1	1	2
PGD grade 24 hours	1	2	1	1	2
PGD grade 48 hours	1	1	1	1	2
PGD grade 72 hours	1	1	2	1	2
Hospital LOS (days)	26	14	25	16	18
ICU LOS (days)	1	4	7	2	9
Post-transplant days of mechanical ventilation (Exact)	0.24	1.73	2.13	0.91	6.17
Post-transplant NO used?	No	No	No	No	No
Post-transplant ECMO required?	No	No	No	No	No
Currently alive?	Yes	Yes	Yes	Yes	Yes

dehydrogenase 1 (ND1) and ND4, our results demonstrated similar patterns of ccf-mtDNA release within the perfusate for both amplicons, thus solidifying the robustness of the data (fig. S9). When attempting to evaluate the degree of mtDNA oxidation through formamidopyrimidine fapy-DNA glycosylase (FPG) treatment and PCR, no significant differences between the two storage temperatures were found. Similar findings were obtained when attempting to evaluate 8-OHdG through immunohistochemistry. Results from these techniques should be interpreted with caution. For example, FPG treatment along with small amplicon PCR will not provide an overall degree of mtDNA oxidation and therefore warrants the use of Southern blot techniques. On the other hand, although 8-OHdG immunocytochemistry might represent an overall degree of nucleic acid oxidation, its presence may be involved in other biological roles such as the involvement in epigenetic events and controlling multiple steps in transcription (including expression of proinflammatory genes) (*54–59*).

In conclusion, the results of this study support that 10°C is a superior storage temperature compared to the current clinical gold standard of an ice cooler (4°C). In our biological analysis, we describe an underlying mechanism of lung protection related to improved mitochondrial preservation. Development of therapeutic strategies to further enhance mitochondrial protection during the preservation period may lead to further optimization of donor lung preservation and may have implications in the setting of lung injury. Preservation at 10°C is easily achievable, logistically simple to adopt, and could become the new standard of care for prolonged pulmonary

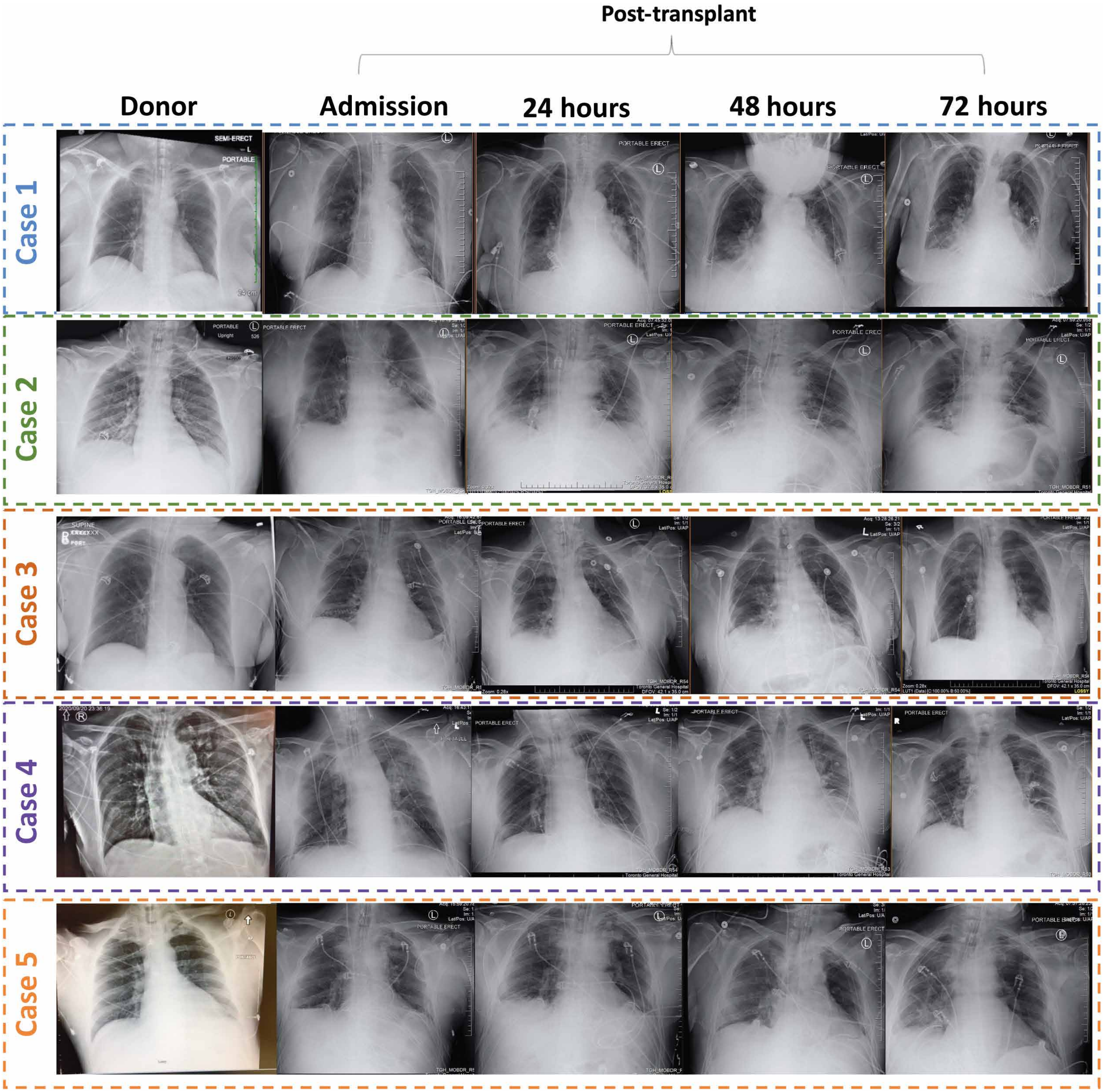

Fig. 5. Donor and recipient chest x-ray images. Chest x-ray images of the organ donors and recipients at the time of admission to the ICU and at the 24-, 48-, and 72-hour follow-up after transplant. Five patients received bilateral lung transplants using 10°C lung storage to avoid transplant surgery occurring during the night. Each case is indicated by different colored labels and dashed lines.

preservation, allowing transplants to be performed in a semi-elective fashion. This could help overcome geographic constraints faced in organ donation, reducing societal and financial costs associated with recipient relocation to the vicinity of the transplant hospital. Adopting this preservation protocol could also reduce the performance of EVLP for purely logistical purposes. Furthermore, and independent of preservation time, we speculate that 10°C cold storage may improve the quality of marginal 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 PGD. Further studies should be performed evaluating 10°C preservation for other solid organ preservation.

MATERIALS AND METHODS

Study design

The overall goal of this study involves the investigation of 10°C as a potentially superior lung storage temperature in comparison to the standard 4°C approach. Our study began with preclinical large animal studies followed by a proof-of-concept clinical study. To evaluate the physiological and biological differences between 10°C donor lung storage temperature versus the conventional 4°C approach, preclinical experiments were performed using male Yorkshire pigs. All animals received humane care, and all protocols were evaluated and approved by the Animal Care Committee, Toronto General Hospital Research Institute, University Health Network, following the Canadian Council on Animal Care Certificate of Good Animal Practices Guidelines (*60*). After explant, donor lungs ($n = 5$ per group) were randomized (using an online random generator) to storage at either 10°C in a thermoelectric cooler (accuracy of ±0.5°C) or 4°C in a walk-in cooler. This sample size is typical of large animal experiments due to the costs and logistics associated with such experiments and is usually sufficient to detect differences when the treatment effect is significant. After 36 hours of cold static preservation, the lungs were subjected to 12 hours of assessment in the normothermic EVLP platform. EVLP was performed following the Toronto Protocol, an approved method for donor lung assessment, which is currently being applied clinically (*18*). Lung assessments were performed hourly as per typical protocol (*18*), with functional outcome measures such as lung compliance, lung oxygenation, airway pressures, and change in lung weight during perfusion. Lung tissue and perfusate samples were taken for biological and metabolic evaluation. Biological and metabolic assessments were performed in a blinded fashion, with experimental group allocation concealed.

After biological analyses, we elected to further investigate the importance of Na^+/K^+ ATPase and its potential biological role during 10°C lung preservation. In a separate series of experiments, we recovered porcine lungs ($n = 4$) from male Yorkshire pigs and cold flushed them with ouabain (100 mg/liter; a known Na^+/K^+ ATPase inhibitor) supplemented into the preservation solution. Lungs were preserved for 36 hours at 10°C in a thermoelectric cooler, and EVLP was performed in a similar fashion to that of the previous experiments. Biological and metabolic assessments were also performed in a similar fashion.

On the basis of our preclinical findings, we designed a pilot clinical study to investigate the potential of 10°C to allow for semi-elective lung transplantation (specifically the avoidance of overnight transplantation) at our transplant hospital. As a proof of concept, we aimed to recruit five patients for this initial pilot study. Donor lungs that met the criteria for direct transplantation without the need of EVLP assessment were eligible for this study. Consented recipients with an age between 18 and 75 years of age were eligible through a Research Ethics Board–approved protocol. The selection of recipients for a given donor was based on blood type, total lung capacity matching, and severity of the disease (wait list status) as in usual fashion. Observed recipient outcomes included PGD at ICU admission, 24, 48, and 72 hours after transplantation; ICU and hospital length of stay; post-transplant days of mechanical ventilation; post-transplant use of NO and ECMO; and 30-day recipient survival.

Donor lung procurement

Donor male Yorkshire pigs (29 to 40 kg) were sedated with ketamine (20 mg/kg, intramuscularly), midazolam (0.3 mg/kg, intramuscularly), and atropine (0.04 mg/kg, intramuscularly) and then anesthetized with inhaled isoflurane (1 to 3%), followed by a continuous intravenous injection of propofol (3 to 4 mg/kg per hour) and remifentanil (9 to 30 μg/kg per hour). The animals were placed in supine position, intubated, and subsequently pressure control ventilated at an inspired oxygen fraction (FiO_2) of 0.5, a frequency of 15 breaths per minute, a positive end-expiratory pressure (PEEP) of 5 cmH_2O, and a controlled pressure above PEEP of 15 cmH_2O. 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 (LA) appendage was incised. A 2-liter anterograde flush (Perfadex, XVIVO Perfusion) 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 1-liter retrograde flush (Perfadex, XVIVO Perfusion) was performed. The lungs were immersed in preservation solution effluent during the static storage period.

Ex vivo lung perfusion

EVLP was performed using an established method referenced as the Toronto protocol (*18*). The lung bloc was placed in the XVIVO chamber (Vitrolife). 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 liters of an extracellular albumin solution (STEEN). 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 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 12 hours, during which physiologic assessments were taken hourly. These included ventilator parameters (dynamic compliance, static compliance, peak airway pressure, and plateau pressure) and perfusate blood gas analysis. Lungs were weighed (model CS 2000, OHAUS Corporation) before and after EVLP. The net weight gain was calculated and used to measure lung edema.

Sample collection protocol

Tissue samples were taken at specific time points 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 cold preservation and at the end of the 36 hours of preservation. During EVLP, perfusate was collected at 1, 6, and 12 hours, snap-frozen, and stored at −80°C. After EVLP, the peripheral-lateral portions of the right upper and lower lobe were sampled.

Inflammatory cytokine and MPO assay

Tissue lysates and perfusates were assayed using ELISA kits for porcine interleukin IL-1β (catalog no. DY6226, IL-1 beta Pig ELISA Kit, R&D Systems) and IL-8 (catalog no. P8000, Porcine IL-8/CXCL8 Immunoassay, R&D Systems) according to the manufacturer's instructions. Tissue lysates were also assayed for MPO using an ELISA kit (catalog no. MBS700708, Pig MPO ELISA Kit, MyBioSource) according to 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 before profiling analysis using gas chromatography–mass spectrometry (MS) and liquid chromatography–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 (*61*). Data were processed by imputing missing or zero values with half of the minimum value, and metabolites with more than 50% missing values were deleted from further analysis. Subsequently, data were normalized (quantile), log_2-transformed, and autoscaled. Principal components analysis, hierarchical clustering, and statistical tests were performed on normalized data.

DNA/RNA oxidation

Lung tissue was formalin-fixed, paraffin-embedded, sectioned, and stained for 8-OHdG (ab48508, anti–8-OHdG antibody) to evaluate oxidative damage and counterstained with 3,3′-diaminobenzidine (DAB) (ab64238, DAB Substrate Kit, Abcam). Slides were scanned, and average nuclear dye intensity was calculated using image analysis software (HALO Image Analysis Software).

mtDNA oxidation

mtDNA oxidation was measured with qPCR using one primer pair: MT-ND4. DNA was extracted from frozen pig lung tissues via a DNA extraction protocol, which used proteinase K digestion. The extracted DNA was diluted to a concentration of 1 ng/μl and treated with FPG (catalog no. M0240L, New England Biolabs Inc.) or with an equivalent volume of H_2O. Treatment mixtures were incubated at 37°C for 60 min, followed by an inactivation step of 60°C for 10 min, and then kept at 4°C until use. Upon use, treated DNA was plated with Bioline 2× SensiFAST SYBR No-ROX (catalog no. BIO-98050, SensiFAST SYBR No-ROX Kit, Meridian Biosciences). The qPCR was performed using CFX96 (Bio-Rad Laboratories Inc.) with the following cycling conditions: 95°C for 3 min and then 95°C for 10 s and 60°C for 30 s, repeated for 40 cycles. After the completion of qPCR, the ΔC_t of each sample was calculated [ΔC_t = (average C_t FPG treated) – (average C_t FPG untreated)].

The following primer pairs were used (*Sus scrofa*): ND4, 5′-GCAACACTAGTACCCACACTAAT-3′ (forward) and 5′-TCCTGCTAGGGTGTAGAATAGG-3′ (reverse); B2M, 5′-GAACGCTGCTCTGACCTAAA-3′ (forward) and 5′-GGTCTCTCAGAAGGTGCTACTA-3′ (reverse).

Measurement of ccf-mtDNA in lung perfusate

ccf-mtDNA was extracted from pig lung perfusate (catalog no. 51304, QIAamp DNA Mini Kit, Qiagen) following the manufacturer's protocol for DNA purification from blood or bodily fluid spin columns. A total of 150 μl of perfusate was used for the collection of ccf-mtDNA, and 50 μl of UltraPure distilled water free of deoxyribonuclease and ribonuclease was used to elute from the column. The estimated absolute value of ccf-mtDNA was quantified, in copies per microliter, by comparing against a standard curve created from an oligonucleotide of the PCR product (Integrated DNA Technologies), of known concentration, serially diluted to concentrations ranging from 1.0×10^2 to 1.0×10^8 copies per microliter. MT-ND4 primers were used to amplify mitochondrially encoded ND4 gene to represent the mitochondrial genome. qPCR was run with a total reaction volume of 20 μl. qPCR was performed using CFX96 (Bio-Rad Laboratories Inc.) with the following cycling conditions: initial denaturation at 95°C for 3 min followed by 40 cycles of 95°C for 10 s and 60°C for 20 s and fluorescence measurement. Then, it is followed by a melt curve analysis—65° to 95°C, increasing at increments of 0.5°C every 5 s and then proceeding with fluorescent read.

The following primer pairs were used: ND4, 5′-GCAACACTAGTACCCACACTAAT-3′ (forward) and 5′-TCCTGCTAGGGTGTAGAATAG-3′ (reverse); gene block: 5′-ATTCTATATCCTATTCGAAGCAACACTAGTACCCACACTAATTATCATCACACGCTGAGGAAACCAAACAGAACGACTCAATGCAGGACTTTATTTCCTATTCTACACCCTAGCAGGATCCCTACCACTGCTAGTAGCAC-3′.

To strengthen the validation of our findings, another amplicon was measured. Using similar methods, mitochondrially encoded ND1 was quantified in the perfusate.

ND1 (forward), 5′-TTATCTACACCCTAGCAGAAACC-3′

ND1 (forward), 5′-AAAGGTCCGGCTGCATATT-3′

Gene expression measurement of cell death–related genes

Gene expression was evaluated using a Custom RT^2 PCR Array for Pigs (CLAS40678D), which includes genes for necroptosis (*TNFa*, *FASLG*, *CASP8*, and *NFKB*), pyroptosis (*CASP1* and *GPX4*), apoptosis (*CASP3*, *CASP8*, *CASP9*, *TP53*, and *NFKB*), and ferroptosis (*MPO*, *GPX4*, and *TP53*). Following the manufacturer's protocols, RNA was converted to complementary DNA (cDNA) (catalog no. 330404, RT^2 First Strand Kit, Qiagen). cDNA was loaded into plates, in triplicate, mixed with RT^2 SYBR Green qPCR Master Mix (catalog no. 330503, RT^2 SYBR Green qPCR Master Mix, Qiagen). C_t values were analyzed using Qiagen RT^2 Profiler PCR Data Analysis Software. Genes were normalized using the geometric mean of housekeeping genes *ACTA1* and *ACTG1*.

Tissue analysis of cell death markers

Lung tissue samples were embedded in paraffin after fixation in 10% buffered formalin for 24 hours, followed by 5-μm-thick sectioning. To assess cellular apoptosis, tissue sections were stained with TUNEL (In Situ Cell Death Detection Kit, POD, Roche Diagnostics GmbH, Mannheim, Germany). Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted with Fluorescent Mounting Medium (IHC WORLD, catalog no. 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 quantified using image analysis software (HALO Image Analysis Software, PerkinElmer) and expressed as a percentage of total cells. Using collected perfusate samples, LDH activity was analyzed by the University Health Network Core Clinical Laboratory using standard assays.

Evaluation of Na^+/K^+ ATPase activity during preservation

To assess Na^+/K^+ ATPase activity, snap-frozen lung tissue samples were analyzed using a commercially available assay (catalog no. MBS8243226, Na^+/K^+ ATPase Microplate Assay Kit, MyBioSource, San Diego, CA). For some of the animal experiments, ouabain (catalog no. O3125, ouabain octahydrate, Sigma-Aldrich) was added at a concentration of Perfadex (100 mg/liter; low-potassium dextran lung preservation solution) in the anterograde flush (2 liters) and retrograde flush (1 liter) before lung storage.

Human lung pilot studies

Eligible human donor lungs were those meeting criteria for direct transplantation without the need for EVLP assessment. Consented recipients with age between 18 and 75 years of age were eligible through a Research Ethics Board–approved protocol. Transplants with a planned recipient anesthesia starting time between 10:00 p.m. and 4:00 a.m. (donor cross-clamps from 6 p.m. onward) were allowed to be delayed to the morning (6:00 a.m. start at the earliest). Lungs were transported in the usual fashion in a cooler of ice at 4°C, and upon arrival to the transplant hospital, they were immediately transferred to cold static preservation at 10°C within a specific incubator (myTEMP 65HC, Benchmark Scientific). The myTEMP 65HC Incubator is designed to provide accurate and uniform temperature control, with a large, digital display allowing for visual monitoring of the chamber temperature. This incubator has been approved to meet the electrical and technical specifications to be placed within the operating room. The selection of recipients for a given donor was based on blood type, lung size matching, and severity of the disease (wait list status) as in usual fashion. Observed recipient outcomes included PGD at ICU admission, 24, 48, and 72 hours after transplantation; ICU and hospital length of stay; post-transplant days of mechanical ventilation; post-transplant use of NO and ECMO; and 30-day recipient survival.

Statistical analysis

Experimental results in pigs are expressed as means ± SEM, whereas clinical data are expressed as median with range. Mann-Whitney tests were performed to compare difference between groups. For data involving a time component, two-way analysis of variance (ANOVA) for repeated measures was used, followed by a Bonferroni correction for multiple comparisons. Statistical significance was considered for $P < 0.05$. GraphPad Prism version 7 (GraphPad Software) computer software was used to conduct all statistical analyses for these studies. For the untargeted metabolomic analysis, for two-group comparisons, the *t* test was used. Statistical significance was considered for features with a false discovery rate (FDR)–corrected $P < 0.05$ (*62*). For the cell death gene expression analysis, genes with a fold change of >1.5 with a *P* value of <0.05 were considered significant. The *P* values were calculated on the basis of a Student's *t* test of the replicate $2^{(-\Delta Ct)}$ values for each gene in both experimental groups. Individual subject-level data are reported in data file S1.

SUPPLEMENTARY MATERIALS

www.science.org/doi/10.1126/scitranslmed.abf7601
Figs. S1 to S9
Tables S1 to S3
Data file S1

View/request a protocol for this paper from *Bio-protocol*.

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Static lung storage at 10°C maintains mitochondrial health and preserves donor organ function

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Prolonging pulmonary preservation
Lungs can be preserved on ice for about 6 to 8 hours before transplantation, which imparts geographic and logistical restrictions on organ use. Ali *et al*. used pig lungs and ex vivo lung perfusion to show that increasing the static storage temperature from 4° to 10°C could maintain organ function while prolonging storage time. Increased storage temperature preserved mitochondrial function and led to greater abundance of mitochondrial metabolites. Human lungs stored at 10°C were successfully transplanted into five patients after storage times of about 10 to 16 hours, supporting the clinical utility of this approach.

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