主論文

Intermittent Ex Vivo Lung Perfusion in a Porcine Model for Prolonged Lung Preservation

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Background. Ex vivo lung perfusion expands the lung transplant donor pool and extends preservation time beyond cold static preservation. We hypothesized that repeated regular ex vivo lung perfusion would better maintain lung grafts. **Methods.** Ten pig lungs were randomized into 2 groups. The control underwent 16 h of cold ischemic time and 2 h of cellular ex vivo lung perfusion. The intermittent ex vivo lung perfusion group underwent cold ischemic time for 4 h, ex vivo lung perfusion (first) for 2 h, cold ischemic time for 10 h, and 2 h of ex vivo lung perfusion (second). Lungs were assessed, and transplant suitability was determined after 2 h of ex vivo lung perfusion. **Results.** The second ex vivo lung perfusion was significantly associated with better oxygenation, limited extravascular water, higher adenosine triphosphate, reduced intraalveolar edema, and well-preserved mitochondria compared with the control, despite proinflammatory cytokine elevation. No significant difference was observed in the first and second perfusion regarding oxygenation and adenosine triphosphate, whereas the second was associated with lower dynamic compliance and higher extravascular lung water than the first. Transplant suitability was 100% for the first and 60% for the second ex vivo lung perfusion, and 0% for the control. **Conclusions.** The second ex vivo lung perfusion had a slight deterioration in graft function compared to the first. Intermittent ex vivo lung perfusion created a better condition for lung grafts than cold static preservation, despite cytokine elevation. These results suggested that intermittent ex vivo lung perfusion may help prolong lung preservation.

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INTRODUCTION

Lung grafts have a limited preservation time because prolonged ischemia can result in severe ischemic–reperfusion injury after lung transplantation. Ex vivo lung perfusion (EVLP)—an emerging technology—has recently been clinically used to expand the donor pool.[1,2] The preservation time, calculated by adding cold ischemic time (CIT) before and after EVLP and EVLP duration, can be extended without compromising the lung graft function by constantly providing oxygen and nutrients during EVLP.[3,4] A longer preservation time with EVLP than the present standard preservation time (6–8 h) might overcome the donor lungs' current ischemic time and geographic limitations.[5]

Cypel et al reported that in a pig lung transplant model, 12 h of acellular EVLP after 12 h of CIT provided better acute outcomes than 24 h of CIT to extend preservation time further.[6] Recently, studies have investigated the optimal perfusate composition to maintain homeostasis by exchanging perfusate with amino acids, vitamins, or lipids, to prolong EVLP duration.[7–10] However, in these studies, given the worsening trend of physiological parameters without nutrients, avoiding machine-induced lung injury remains challenging.

Therefore, we hypothesized that the lung preservation time could be extended by intermittently performing a second EVLP (EVLP2), following the first EVLP (EVLP1), and subsequent CIT. This study investigated whether intermittent EVLP potentially benefits lung preservation by comparing lung function between lungs that underwent intermittent EVLP and conventional cold static preservation. We also examined whether EVLP can reset lung graft quality by comparing lung physiological and metabolic parameters between EVLP1 and EVLP2 in the intermittent EVLP group.

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MATERIALS AND METHODS

Study Design

Ten lungs were randomized into 2 groups, as illustrated in Figure 1. The control group (n = 5) underwent EVLP after 16 h of CIT. The intermittent group (n = 5) underwent EVLP1 after 4 h of the first CIT (CIT1) and then EVLP2 following 10 h of the second CIT (CIT2). Transplant suitability was determined 2 h after the EVLP. At the end of the EVLP, lung tissue samples were collected from nondependent areas in the lower right lobe for the wet/dry ratio, biomarker analysis, histopathology, and transmission electron microscopy (TEM). Perfusate samples were obtained after 2 h for cytokine analysis. The Cleveland Clinic Institutional Animal Care and Use Committee approved this study.

Animal Preparation

Yorkshire female pigs were sedated with ketamine/xylazine (20 mg/2 mg/kg intravenously), followed by intubation and general anesthesia with isoflurane (1.0%–2.0%). Heparin was intravenously administered at 300 units/kg. Lungs were procured in a standard manner as described previously.[11] The lungs were flushed with 1.5 L of 4 °C PERFADEX PLUS (XVIVO Perfusion Inc., Englewood, CO), then flushed retrograde with another 1.5 L of 4 °C PERFADEX PLUS and packed on ice. Lung tissues, collected using a linear cutter stapler prior to placement on ice, were sham lungs for the biomarker analysis. Regarding blood preparation, whole blood was obtained from dedicated blood donors, stored in a blood bag (TERUFLEX; Terumo, Lakewood, CO), and washed in a cell saver (autoLog; Medtronic Inc., Minneapolis, MN). In the intermittent group, blood for EVLP1 and EVLP2 was collected from a single blood donor pig.

Ex Vivo Lung Perfusion

This study utilized Lund-type EVLP protocol (LS1, XVIVO Perfusion Inc., Englewood, CO).[12] LS1 consisted of a roller pump, reservoir, membrane oxygenator, heat exchanger, and leukocyte filter. The system was primed with 2.0 L of STEEN solution, 10 000 IU heparin, 100 mg imipenem-cilastatin, and 500 mL of packed red blood cells resulting in 10%–15% hematocrit. The pH was corrected to 7.35–7.45 using isotonic trometamol. The left atrium was open, and a temperature probe was sutured inside. Perfusion was initiated at 0.2–0.3 L/min, and the lungs were gradually warmed to 37 °C. The flow rate was gradually increased to 70 mL/kg/min, and the pulmonary artery pressure (PAP) was maintained at < 20 mm Hg.

When the left atrial temperature reached 32 °C, lung ventilation (Servo-i; Maquet Critical Care, Solna, Sweden) was initiated starting with a 4 mL/kg tidal volume (TV), 7 breaths/min (respiratory rate), positive end-expiratory pressure (PEEP) of 5 cm H_2O, and fraction of inspired oxygen (FiO_2) of 0.4. When the lung temperature became 37 °C, the ventilator settings were adjusted to TV 6 mL/kg, 7 breaths/min (respiratory rate), PEEP of 5 cm H_2O, and FiO_2 of 0.4. Blood gas analysis of blood from the left atrium at 1 and 2 h at FiO_2 of 1.0. Airway parameters and PAP were documented at 1 and 2 h, respectively. Pulmonary vascular resistance (PVR) was estimated as 80 × (PAP–LAP)/circuit flow, where LAP = left atrial pressure (assumed 0 with an open left atrium). Dynamic compliance was estimated as TV/(peak inspiratory pressure – PEEP). Perfusion was maintained for 2 h. At the end of EVLP1 in the intermittent group, the lungs were cooled via retrograde flushing with 1.5 L of 4 °C PERFADEX PLUS and stored in another 1.5 L of 4 °C PERFADEX PLUS on ice for 10 h until EVLP2.

Transplant Suitability

Transplant suitability was evaluated based on the current standard criteria,[3] when the oxygen administration in the circuit was turned off at the end of the perfusion according to the Lund protocol. Lungs were judged unsuitable for transplantation when PaO_2/FiO_2 was <300, and a significant deterioration in the airway and vascular parameters was observed. The lungs also deteriorated with massive airway fluid or abnormal edema.

Lung Weight Assessment

The lungs were weighed at 0 and 2 h after EVLP. The lung weight ratio was lung weight at 2 h/0 h. Regarding the wet/dry ratio assessment, the samples were dried in an oven at 60 °C for 24 h to measure the dry weight.

CLUE

This technique was used to monitor extravascular lung water (EVLW) at 2 h, as previously reported.[13,14] The grade was determined using the percentage of B-lines in the ultrasound images. The CLUE (direct lung ultrasound evaluation) score was calculated using the established formula.[14]

Perfusate Free Fatty Acid Levels

Free fatty acid (FFA) levels in the perfusate were determined using a colorimetric assay kit (Ab65341, Abcam, Cambridge, United Kingdom). The FFA ratio was estimated as the FFA at 2 h/0 h.

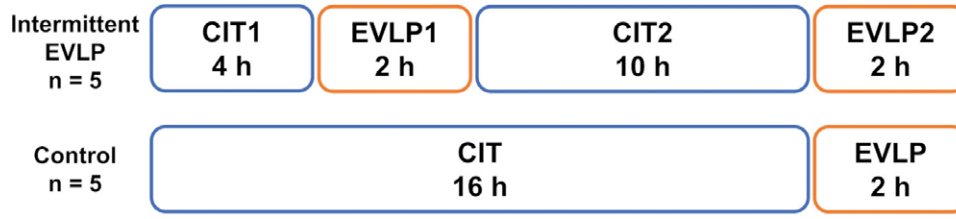

FIGURE 1. Study design. Lungs were procured in a standard fashion with cold flush and randomized into 2 groups: the control group (n = 5), which underwent EVLP following 16 h of CIT, and the intermittent group (n = 5), which included 4 h of CIT1, EVLP1, 10 h of CIT2, and EVLP2. CIT, cold ischemic time; EVLP, ex vivo lung perfusion.

Tissue Adenosine Triphosphate Levels

Frozen lung tissue (50 mg) was homogenized in 1000 µL of precooled 0.5% trichloroacetic acid. The homogenate was centrifuged at 2300*g* for 10 min at 4 °C. The supernatant was removed, and 0.002% xylenol blue and tris-acetate buffer neutralized the pH to 7.4. The adenosine triphosphate (ATP) concentration in the supernatant was measured using an ENLITEN ATP assay system in a bioluminescence detection kit (FF2000, Promega, Madison, WI). The results were normalized using the lung tissue's dry weight to minimize the effect of edema.

Tissue Hypoxia-induced Factor-1α Levels

Frozen lung tissue (50 mg) was homogenized in 500 µL of phosphate-buffered saline. The homogenate was centrifuged at 1000*g* for 20 min, and the supernatant was assayed for pig hypoxia-inducible factor-1α (HIF-1α, MBS282326, MyBioSource, San Diego, CA). The results were normalized using the lung tissue's dry weight to minimize the effect of edema.

Perfusate Cytokine Analysis

Perfusate samples at 2 h were assayed in duplicate for Interleukin (IL)-1β, IL-6, IL-8, and IL-10 in multiplex using the Luminex Platform (PCYTMAG-23K, R&D Systems, Inc., Minneapolis, MN). The samples were compared with their individual curves based on the linear range defined by the standard and low- and high-quality controls.

Histopathological Analysis

Formalin-fixed, paraffin-embedded lung tissues were divided into 5-mm sections and stained with hematoxylin and eosin. A pulmonary pathologist (C.F.) blindly assessed the pathological findings for interstitial edema, intra-alveolar edema, arteriolar thickening, vascular thrombosis, hemorrhage to congestion, acute inflammation, and intra-alveolar fibrin deposition (0, absent; 1, mild; 2, moderate; 3, severe). The acute lung injury (ALI) score was determined by adding up the pathological finding scores.

Transmission Electron Microscopy

Lung tissue samples were fixed via immersion in triple aldehyde-dimethyl sulfoxide (DMSO).[15] After rinsing in 0.1 M phosphate buffer (pH 7.3), the samples were postfixed in ferrocyanide-reduced osmium tetroxide.[16] Another water rinse was followed by soaking overnight in acidified uranyl acetate. After rinsing in distilled water again, the tissue blocks were dehydrated in ascending ethanol concentrations, passed through propylene oxide, and embedded in Embed 812 resin (Electron Microscopy Sciences, PA). Thin sections were mounted on formvar on PELCO single slot grids (Ted Pella, CA) and sequentially stained with acidified uranyl acetate. This was followed by a modification of Sato's triple lead stain.[17] The prepared sections were coated on a Denton DV-401 carbon coater (Denton Vacuum LLC, NJ) and examined using an FEI Tecnai Spirit (T12) TEM with a Gatan US4000 4kx4k CCD. The TEM finding scores were blindly assessed for damage to endothelial cells, epithelial cells, and the mitochondria based on a previous report.[18]

Statistical Analysis

All statistical analyses were performed using JMP, Version 14.2.0 (SAS Institute, Inc., Cary, NC). All data are expressed as median and range (minimum–maximum). A paired *t*-test was used for continuous data, and the Wilcoxon signed-rank test was used to compare the EVLP1 and EVLP2 groups. To compare the EVLP2 and control groups, the Student *t*-test was used for continuous data, and the Mann–Whitney *U* test was used for scoring. Transplant suitability was analyzed using the Fisher exact test to compare the EVLP2 group with the control group, and the EVLP1 group with the EVLP2 group, respectively. Statistical significance was set at $P < 0.05$.

RESULTS

Donor Characteristics

No significant differences were observed between the intermittent and control groups in terms of body weight, lung weight after procurement, or total preservation time (Table 1).

EVLP2 Versus Control Groups

Lung Evaluation During EVLP

PaO_2/FiO_2 in the EVLP2 group was significantly greater than that in the control group (397 [239–443] versus 220 [100–334] mm Hg, $P = 0.01$, Figure 2A). EVLP2 lungs had a significantly lower shunt fraction than those in the control group (Figure 2B). No differences were observed in the peak airway pressure, dynamic compliance, and PVR between the EVLP2 and control groups (Figure 2C–E). Three and 1 cases attained the target flow in the EVLP2 and control groups, respectively (Figure 2F). Lungs in the EVLP2 group were associated with significantly lower CLUE scores in the whole lung and lower lobes and relatively lower lung weight and wet/dry ratios than the control group (Figure 3A–D). Based on the standard transplant suitability criteria, 3 cases (60%) were suitable in the EVLP2 group. Contrastingly, no case was suitable in the control group (Figure 3E).

TABLE 1.

Donor characteristics

Variables	Control group (n = 5)	Intermittent group (n = 5)	*P*
Body weight, kg	52.9 (42.6–56.0)	48.6 (47.1–59.0)	0.90
Lung weight in donor OR, g	583 (465–695)	565 (432–875)	0.96
Preservation time, min	970 (957–1017)	970 (925–1034)	0.90

Continuous data are expressed as median (minimum–maximum). The preservation time was defined as CIT in the control group and CIT1 + EVLP1 + CIT2 in the intermittent group.
CIT, cold ischemic time; EVLP, ex vivo lung perfusion; OR, operation room.

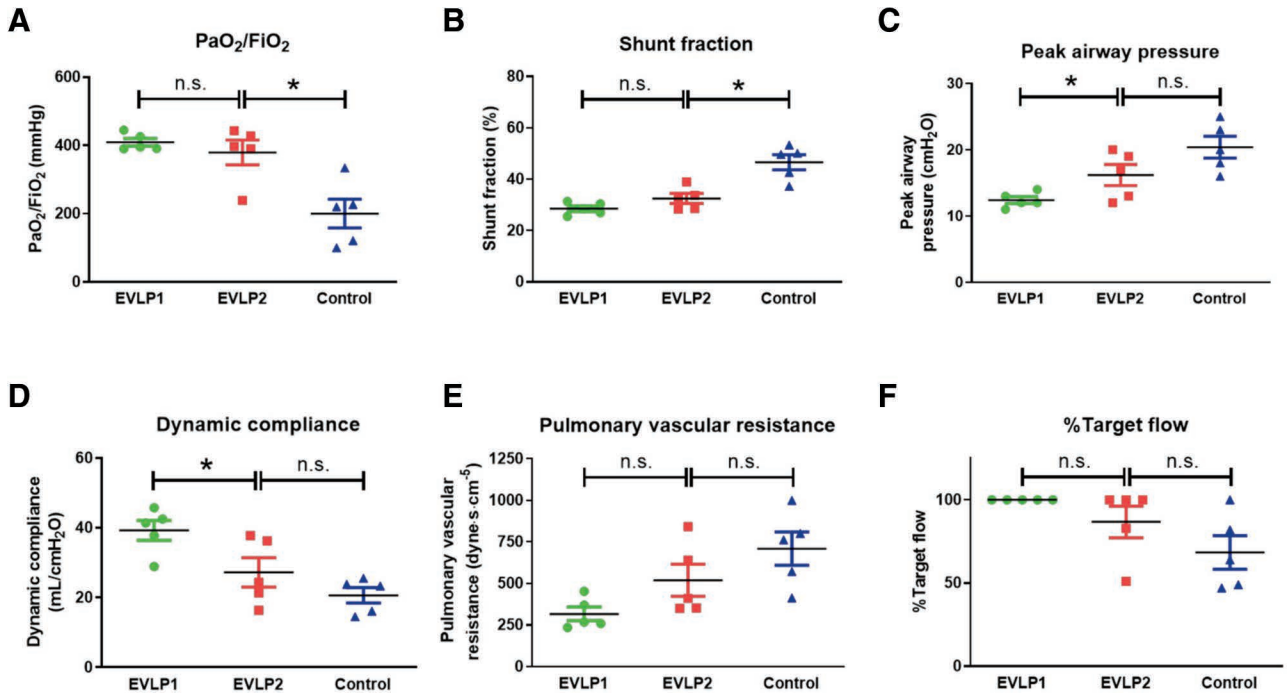

FIGURE 2. Physiological parameters of the EVLP1, EVLP2, and control groups at 2 h of EVLP represented using the scatter dot plot (n = 5, each). The middle horizontal line represents the mean, and the upper and lower lines represent the SEM. A, PaO_2/FiO_2 in the left atrium perfusate was significantly greater in the EVLP2 group than in the control group (397 [239–443] vs 220 [100–334] mm Hg, *P <0.05). In contrast, no difference exists between the EVLP1 and EVLP2 groups. B, Lungs in EVLP2 had a significantly lower shunt fraction than the control group (32.9 [28.3–38.9] vs 49.6 [37.2–53.3] %, *P <0.05). In contrast, no difference was observed between EVLP1 and EVLP2. C, Lungs in EVLP2 had significantly higher peak airway pressure than those in the EVLP1 group (19 [13–21] vs 13 [12–14] cm H_2O, *P <0.05), whereas no difference existed between the EVLP2 and control groups (19 [13–21] vs 20 [16–25] cm H_2O, P = 0.23). D, Lungs in EVLP2 had significantly lower dynamic compliance than those in EVLP1 (24.3 [16.3–37.8] vs 41.4 [28.9–45.7] mL/cm H_2O, *P <0.05), whereas no difference was observed between the EVLP2 and control groups (24.3 [16.3–37.8] vs 23.3 [14.5–25.5] mL/cm H_2O, P = 0.21). E, No difference existed in pulmonary vascular resistance among the groups. F, All cases achieved the target flow in the EVLP1 group. On the other hand, 3 cases attained the target flow in the EVLP2 group, and 1 reached the target flow in the control group. %Target flow was the final flow divided by the calculated target flow. EVLP, ex vivo lung perfusion; FiO_2, fraction of inspired oxygen; n.s., not significant; PaO_2, partial pressure of oxygen.

Perfusate and Tissue Biomarker Analysis

The FFA ratio in the EVLP2 group was significantly higher than that in the controls (0.82 [0.8–0.86] versus 0.62 [0.37–0.70], P < 0.01, Figure 4A). EVLP2 had significantly higher ATP levels than the control group (4.7 [2.8–6.5] versus 1.5 [0.8–2.2] nmol/mg dry weight, P < 0.01, Figure 4B). HIF-1α levels were higher in the EVLP2 than in the control group without statistical significance (13.0 [9.9–15.7] versus 10.1 [8.7–11.1] pg/mg dry weight, P = 0.08, Figure 4C). The EVLP2 group had significantly higher IL-6 and IL-10 levels and relatively higher IL-1β and IL-8 levels than the control group (Figure 5A–D).

Histopathological and TEM Findings

In the histopathological analysis, the control group displayed edematous alveoli and lung injury, whereas EVLP2 findings were normal (Figure 6A). Both groups had no significant difference in the ALI score (Figure 6B). In contrast, the intraalveolar edema score was significantly lower in the EVLP2 than in the control group (Figure 6C). Regarding TEM images, EVLP2 had well-preserved morphological structures, whereas the control group had thickened endothelial cells and enlarged mitochondria (Figure 7A). These findings were limited to the sham and EVLP1 tissues. In the semiquantitative analysis of the TEM images, the epithelial cell and mitochondrial damage scores were significantly lower in the EVLP2 than in the control group (epithelial damage, 1 [0–2] versus 3 [2–4], P < 0.05; mitochondrial damage, 1 [0–3] versus 4 [2–4], P < 0.05). Contrastingly, no difference was observed in the endothelial cell damage score between the EVLP2 and control groups (Figure 7B–D).

EVLP1 Versus EVLP2 Groups

Lung Evaluation During EVLP

No difference in PaO_2/FiO_2 and shunt fraction existed between the EVLP1 and EVLP2 groups (Figure 2A and B). The peak airway pressure was significantly higher in the EVLP2 group than in EVLP1. Additionally, the dynamic compliance in the EVLP2 group was significantly lower than that in EVLP1 (Figure 2C and D). The PVR in the EVLP2 group was higher than that in EVLP1, without a significant difference (Figure 2E). All cases achieved the target flow in the EVLP1 group. In contrast, 3 cases reached the target flow in the EVLP2 group (Figure 2F).

There was no significant difference in the CLUE score in the EVLP1 and EVLP2 groups, whereas the latter had a significantly higher CLUE score in the lower lobes (Figure 3A and B). The lung weight ratio was significantly higher in the EVLP2 group than in EVLP1. Contrastingly, no difference

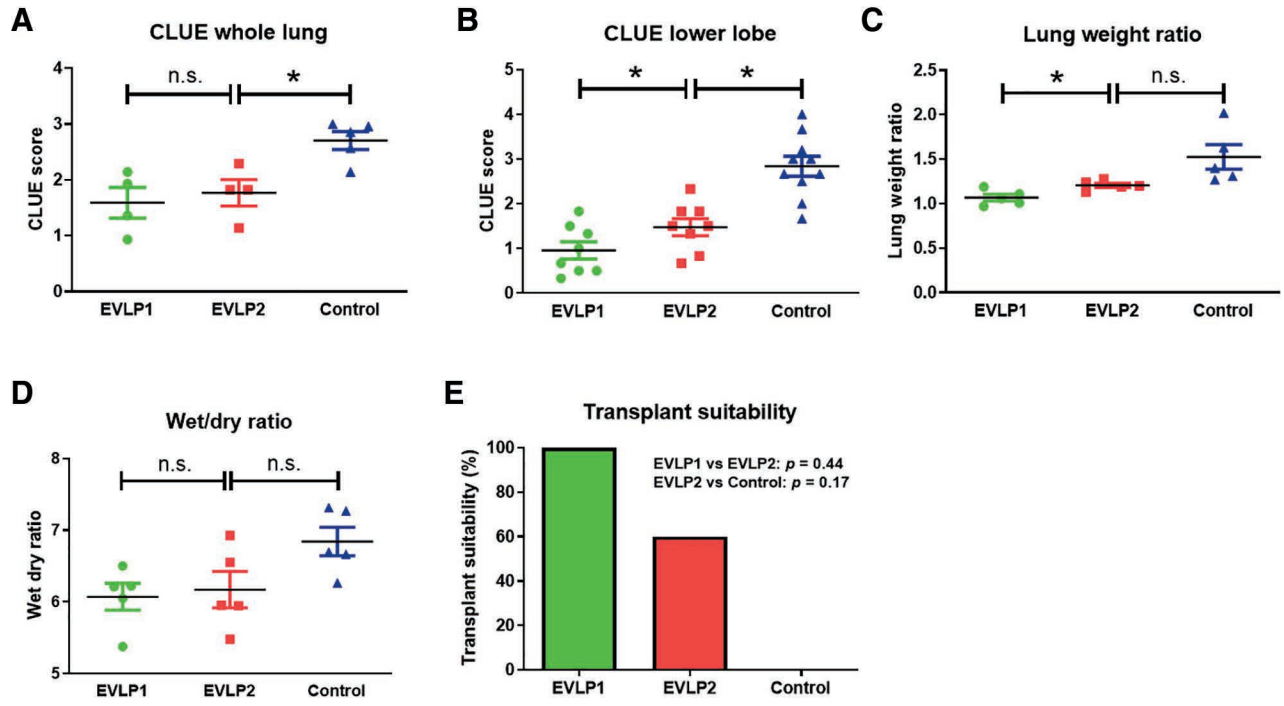

FIGURE 3. The scatter dot plot represents EVLW parameters. The bar graph represents the transplant suitability of the EVLP1, EVLP2, and control groups. A, CLUE score in the entire lung, comparing the EVLP1, EVLP2, and control group (1.6 [0.9–2.1], 1.8 [1.1–2.3], and 2.9 [2.1–3], respectively, *$P < 0.05$). B, CLUE score in the lower lobes, comparing the EVLP1, EVLP2, and control groups (0.8 [0.3–1.8], 1.5 [0.7–2.3], and 2.8 [1.7–4], respectively, *$P < 0.05$). C, Lung weight ratio, defined as lung weight at 2 h/0 h, comparing the EVLP1, EVLP2, and control groups (1.1 [1.0–1.2], 1.2 [1.1–1.3], and 1.4 [1.3–2.0], respectively, *$P < 0.05$). D, Wet/dry ratio, comparing lungs in the EVLP1, EVLP2, and control groups. There was no difference among the groups (6.2 [5.4–6.5], 6.0 [5.5–6.9], and 6.7 [6.3–7.3], respectively). E, Transplant suitability, determined via current standard criteria. There was no difference between EVLP2 and control ($P = 0.17$), and EVLP1 and EVLP2 ($P = 0.44$). In the intermittent group, CLUE score during EVLP was not obtained in 1 of 5 cases due to the nonavailability of the ultrasound device. CLUE, direct lung ultrasound evaluation; EVLP, ex vivo lung perfusion; EVLW, extravascular lung water; n.s., not significant.

existed in the wet/dry ratio (Figure 3C and D). All cases (100%) were suitable in the EVLP1 group. However, 3 cases (60%) were suitable in the EVLP2 group (Figure 3E).

Tissue and Perfusate Biomarker Analysis

The FFA ratio was higher in the EVLP1 group than in EVLP2 without significance (0.98 [0.67–1.1] versus 0.82 [0.8–0.86], $P = 0.28$, Figure 4A). ATP levels did not significantly differ between EVLP1 and EVLP2 (3.2 [1.6–5.3] versus 4.7 [2.8–6.5] nmol/mg dry weight, $P = 0.19$, Figure 4B). In EVLP2, HIF-1α levels were higher than in EVLP1 without a significant difference (13.0 [9.9–15.7] versus 8.3 [5.5–11.8] pg/mg dry weight, $P = 0.11$, Figure 4C). The EVLP2 group had significantly higher IL-6 and IL-10 levels and relatively higher IL-1β and IL-8 levels than the EVLP1 group (Figure 5A–D).

Histopathological and TEM Findings

In the histopathological analysis, no significant difference was observed between the EVLP1 and EVLP2 groups

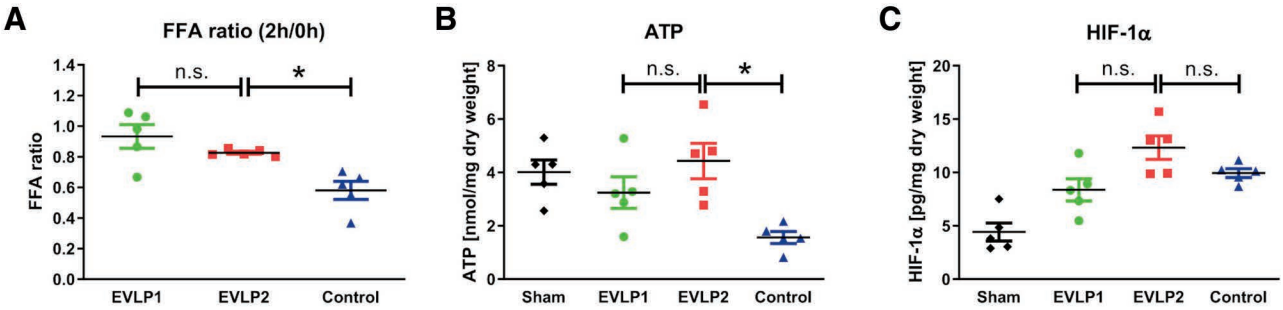

FIGURE 4. Perfusate and tissue biomarker levels at 2 h represented by scatter dot plot in each group. The middle horizontal line represents the mean, and the upper and lower lines represent the SEM. A, FFA ratio in perfusate. EVLP2 had a significantly higher FFA ratio than the control group, whereas no difference existed between the EVLP1 and EVLP2 groups (*$P < 0.05$). B, ATP levels in lung tissue. The EVLP2 had significantly higher ATP than the control. In contrast, levels of ATP in the EVLP1 and EVLP2 groups were similar to that of the sham (*$P < 0.05$). C, HIF-1α levels in lung tissue. EVLP2 had a relatively higher HIF-1α than the control and EVLP1 groups. The lung tissue in the sham group was obtained without ischemia (n = 5). ATP, adenosine triphosphate; EVLP, ex vivo lung perfusion; FFA, free fatty acid; HIF-1α, hypoxia-inducible factor-1α; n.s., not significant.

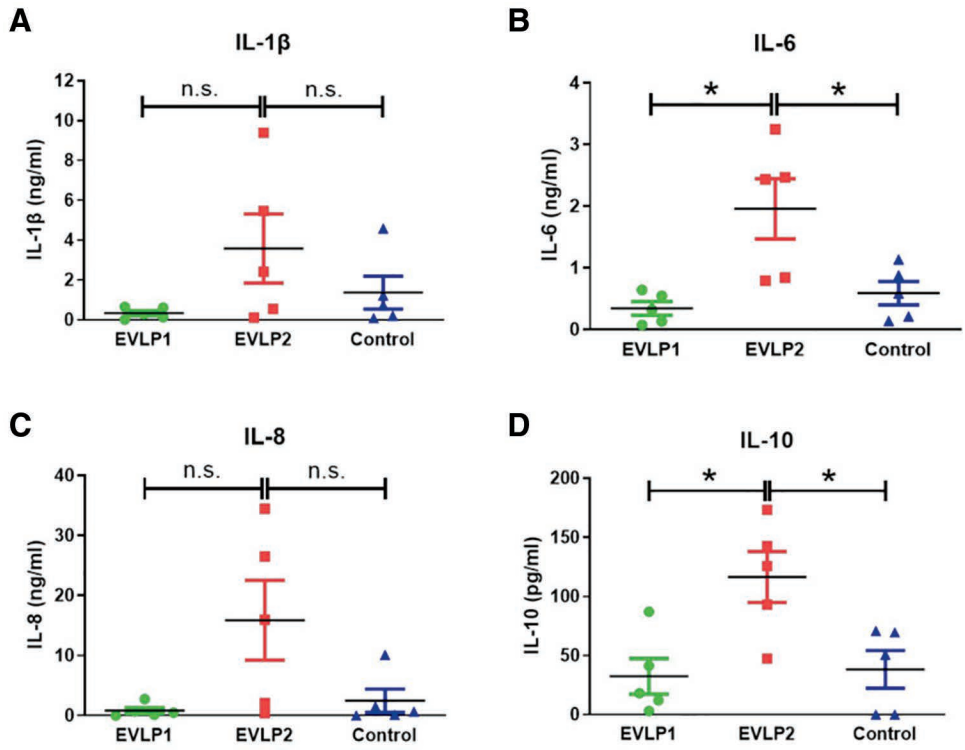

FIGURE 5. Perfusate cytokine levels at 2 h of EVLP represented by scatter dot plot. The middle horizontal line represents the mean, and the upper and lower lines represent the SEM. A, The EVLP2 group had relatively higher IL-1β than the EVLP1 and control groups. B, The EVLP2 group had higher IL-6 than the EVLP1 and control groups (*$P < 0.05$). C, The EVLP2 group had relatively higher IL-8 than the EVLP1 and control groups. D, The EVLP2 had higher IL-10 than the EVLP1 and control groups (*$P < 0.05$). EVLP, ex vivo lung perfusion; IL; interleukin; n.s., not significant.

in the ALI and intraalveolar edema scores (Figure 6A–C). In the TEM image, the morphological structure of the lung was well-preserved in EVLP1 and EVLP2 (Figure 7A). Semiquantitative analysis revealed that the EVLP1 group had significantly lower endothelial cell and mitochondrial damage scores than the EVLP2 group (endothelial cell damage, 0 [0–1] versus 1 [0–3], $P < 0.05$; mitochondrial damage, 0 [0–1] versus 1 [0–3], $P < 0.05$). However, both groups had no significant difference in the epithelial cell damage score (Figure 7B–D).

DISCUSSION

This study demonstrated that intermittent EVLP could better preserve lungs than conventional cold static preservation by comparing lung functions in a porcine model. Specifically, PaO_2/FiO_2 was significantly higher in the EVLP2 than in the control group. Furthermore, EVLP2 was significantly associated with a lower lung weight ratio and CLUE score. These parameters are indicators of EVLW—a typical finding of ischemia–reperfusion injury.[19] In addition, microscopic analyses were consistent with these results. Furthermore, EVLP2 was significantly associated with reduced intraalveolar edema and limited damage to the mitochondria, suggesting that morphological structures were well maintained in the intermittent EVLP group. Importantly, the EVLP2 group had significantly higher ATP than the controls. In previous studies, low ATP levels led to impairment of the sodium-potassium pump, cell swelling, and lung graft dysfunction during reperfusion.[20] Thus, in this study, the higher ATP levels in EVLP2 can explain the reduced ischemia–reperfusion injury compared with the controls. Additionally, the higher HIF-1α levels in EVLP2 than in the controls might suggest that intermittent EVLP could achieve protective preservation. To date, HIF-1α is a protective lung injury marker.[21,22] Eckle et al revealed that HIF-1α increased in a murine heart ischemic preconditioning model.[23] Given the repeated ischemia and perfusion nature of intermittent EVLP, our study seems consistent with this study. Overall, these findings suggest that intermittent EVLP attenuates ischemia–reperfusion injury. Therefore, when clinically applied, an intermittent perfusion strategy might achieve longer preservation to overcome the current geographical limitations for organ transportation in the future.

Many studies have been conducted on prolonged EVLP regarding extending lung preservation time. Originally, Cypel et al established a prolonged (12 h) EVLP with acellular protocol after 12 h of cold static preservation. This had better outcomes than the conventional 24 h of cold static preservation in a pig lung transplant model.[6] Following this landmark study, several others revealed that cellular perfusate with low-flow protocol could achieve better lung preservation than acellular perfusate. Sommer et al demonstrated that cellular perfusate was associated with 24 h of prolonged stable perfusion using the organ care system.[7] In addition, Loor et al reported that oxygenation after 24 h of perfusion was better in the whole blood perfusate group than in the organ care system of the control group.[10] Recent studies revealed that exchanging the perfusate or adding nutrients, such as amino acids, vitamins, and lipids, resulted in better graft functions.[8,9] However,

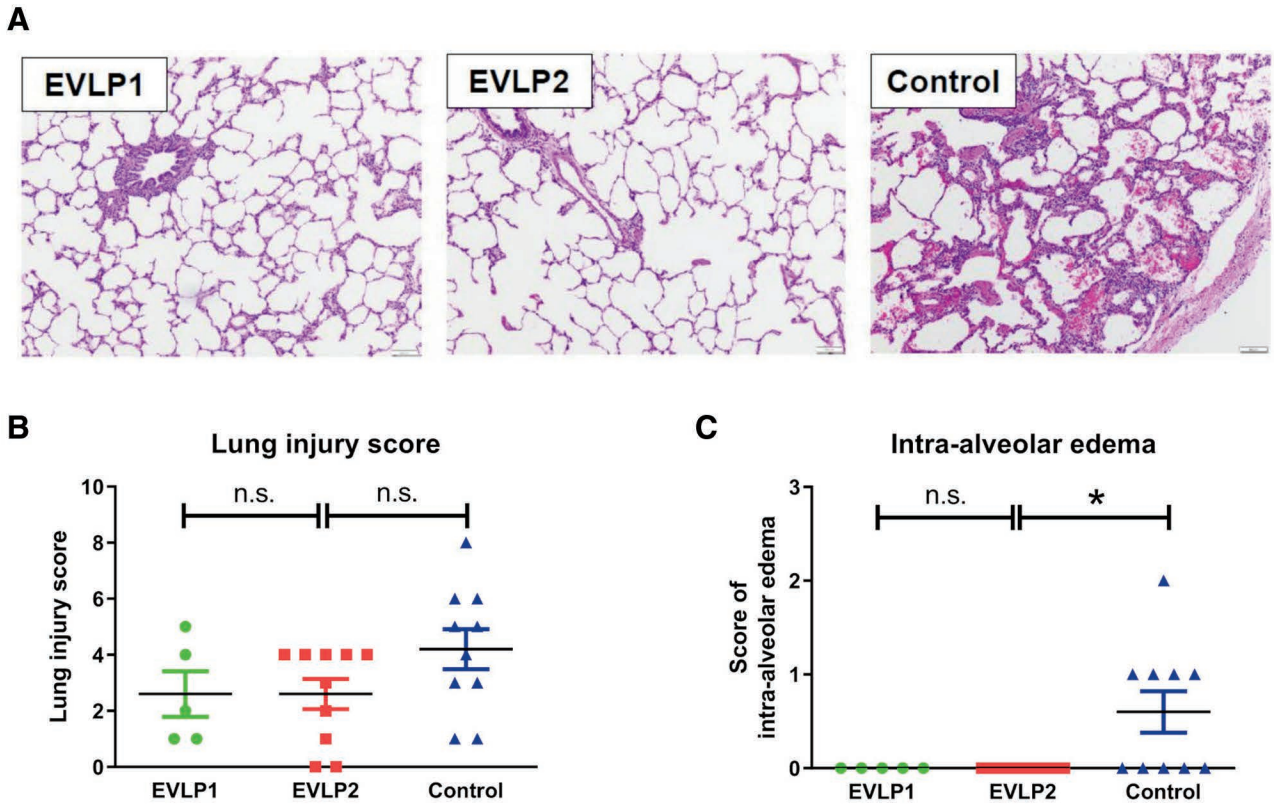

FIGURE 6. Histopathological assessment of lower lobes. A, Representative sections at 200× magnification from each group are presented. B, No significant difference was observed in the acute lung injury score among the groups. C, The score of intra-alveolar edema was significantly lower in the EVLP2 than in the control group (*$P < 0.05$). On the other hand, there no difference existed between the EVLP1 and EVLP2 groups. The scatter dot plot represents the scores. The middle horizontal line represents the mean, and the upper and lower lines represent the SEM. EVLP, ex vivo lung perfusion; n.s., not significant.

even in the low-flow EVLP protocol, prolonged perfusion leads to a time-dependent edema growth in a control group with no nutrients. To extend the preservation time using a different approach, we proposed intermittent EVLP, a new preservation strategy involving repeated ischemia and perfusion. As demonstrated in this study, intermittent EVLP could be an alternative to prolonged lung preservation. However, we did not directly compare intermittent EVLP with prolonged perfusion. Erasmus et al attempted 6 h of prolonged perfusion using Lund protocol EVLP, resulting in severe pulmonary edema with elevated PAP and deteriorated respiratory parameters.[24] This was presumably due to the injury contributed by the high flow during perfusion. Therefore, performing prolonged perfusion using the Lund protocol for lung preservation might be impractical. Recently, multiday lung preservation was achieved by repeating low-flow acellular EVLP and cold static preservation at 10 °C.[25] According to a previous large animal and a clinical study, a lung preservation temperature of 10 °C was superior to 4 °C during cold static preservation.[26,27] The temperature during cold static preservation in this recent study differed from the current clinical standard of 4 °C that we set in the present study; however, it may indicate that extending the lung preservation time by repeating short-term EVLP might be promising.

Several clinical trials and single-center studies have demonstrated that EVLP could achieve clinical outcomes comparable with lung transplantation performed without EVLP.[28–31] Following these promising reports, validating the acceptable CIT hours between EVLP termination and lung implantation has become necessary. In a porcine transplant model, Hsin et al reported that lung grafts with 10 h of CIT1 and 6 h of acellular EVLP produced equivalent graft function, cell death, and inflammation markers between the short (2 h) and long (10 h) CIT2 groups.[32] Charles et al have demonstrated that adding 6 h of CIT2 after acellular EVLP did not worsen the posttransplant lung function compared with lungs transplanted immediately after EVLP using porcine lungs from donation after circulatory death.[33] These large animal studies demonstrated that EVLP allows some time for organ transportation without compromising lung function. However, a recent multicenter study revealed that CIT2 was an independent predictor of high-grade primary graft dysfunction at 72 h and early mortality. In contrast, CIT1 did not correlate with adverse outcomes.[34] This clinical study reminds us of CIT2's importance and detrimental effects on lung graft function. Given the slight deterioration in lung function in EVLP2, followed by 10 h of CIT2, we assume that our results are relevant to a multicenter study. As indicated by the lung weight ratio and CLUE score, the EVLW degree was significantly higher in EVLP2 than in the EVLP1 group. Some impairments of the mitochondria and endothelial cells in EVLP2 may be linked to the deterioration of various physiological parameters. Furthermore, in this study, proinflammatory cytokine levels were higher in EVLP2 than in EVLP1. Cypel et al proposed that EVLP interrupts inflammation and ischemia–reperfusion injury

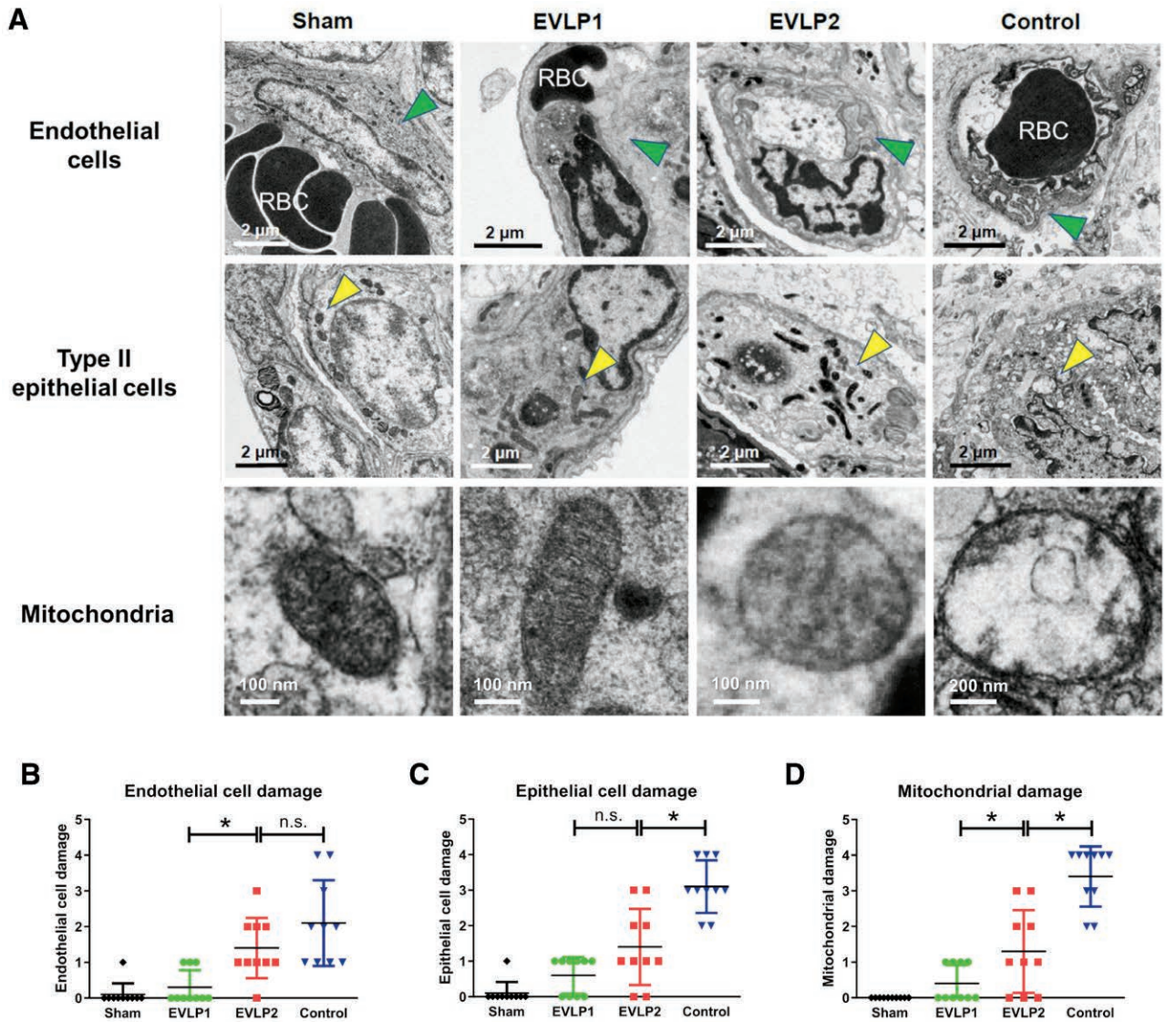

FIGURE 7. Electron microscopic assessment of lower lobes. A, Representative sections of endothelial cells (green arrow), type II epithelial cells, and mitochondria (yellow arrow) with magnified images from each group are illustrated. In the magnified images, the mitochondrial structure was preserved in the sham, EVLP1, and EVLP2 groups, whereas mitochondrial swelling (rarefied matrix) was observed in the control group. B, The EVLP2 group had a higher endothelial damage score than EVLP1 (*$P < 0.05$), whereas no significant difference existed between the EVLP2 and control group. C, The EVLP2 group had a significantly lower score of epithelial cell damage than the control (*$P < 0.05$). D, The EVLP2 group had a significantly lower mitochondrial damage score than the control, while EVLP2 had a significantly higher score than the EVLP1 group (*$P < 0.05$). EVLP, ex vivo lung perfusion; n.s., not significant; RBC, red blood cell.

and may "restart the clock" after EVLP.[35] However, our results imply that the clock hand does not return to 0, although differences exist in the EVLP protocol. We propose that intermittent EVLP might prolong lung preservation despite slight deteriorations in EVLP2, suggesting room for further improvement in maintaining lung graft quality. The proposed strategy may allow, for example, intercontinental transportation during CIT2 and overcome current geographic allocation limitations. We determined that a minimum CIT2 of 10 h was required for successful transportation across continents and subsequently set 10 h of CIT2.

The EVLP2 group had higher proinflammatory cytokine levels than the control group. In contrast, the levels of anti-inflammatory cytokine in the EVLP2 group were higher than those in the control group, indicating that these cytokines were counterbalanced. Recent reports highlighted the role of elevated cytokines in EVLP as a predictor of transplant suitability and the inflammatory signaling profile during EVLP.[36–38] According to these reports, elevations in IL-6 and IL-8 were associated with decreased oxygenation, edema, and impaired organ function. Furthermore, Davis et al highlighted that deterioration of graft function during EVLP was correlated with elevation of extracellular DNA and tumor necrosis factor-α.[39] To address these issues, various efforts have been made to minimize the effects of inflammatory cytokines. De Wolf et al investigated whether dialysis during EVLP affects lung function and pro-inflammatory gene expression but were unable to show a clear benefit of dialysis.[40] Iskender et al reported that cytokine filtration during EVLP could reduce inflammatory cytokines

and development of pulmonary edema.[41] Our data showed elevations in inflammatory cytokines in EVLP2, which may be partially associated with impaired organ function. Therefore, our data might represent the disadvantage of intermittent EVLP. Combining cytokine filtration therapy with intermittent EVLP may lead to further lung graft improvement.

This study had several limitations. First, the animal model may differ from the human lung model due to the absence of brain death and pulmonary edema. Additionally, the severity of ischemia–reperfusion injury following CIT in pig lungs may differ from that in human lungs. However, despite the species differences, the fundamental concept of intermittent EVLP might be applicable. Second, the sample size was small. Third, the results cannot be applied in clinical practice owing to the lack of lung transplantation. In this study, transplant suitability was determined based on the standard criteria of the Lund protocol. Previous studies revealed that physiological data during the Lund protocol EVLP was related to posttransplant outcomes.[30,42] However, EVLP2 could also be considered a dedicated assessment of lung function and viability. Therefore, caution is required when interpreting the results. Future experiments should include actual lung transplantation to assess lung graft function fully. Fourth, in the EVLP2 and control groups, several cases did not achieve the target flow, implying that EVLP parameters should be cautiously interpreted. Moreover, in the EVLP2 group, not all physiological parameters and biomarkers consistently changed favorably compared to the control group. Furthermore, our proposed strategy can be implemented in clinical systems such as the XVIVO perfusion and Organ Care system. However, the results may vary due to the use of the Lund protocol EVLP in this study. Steinmeyer et al observed that cellular EVLP resulted in slightly less edema than acellular EVLP using an electron microscope.[43] Lastly, based on the nature of the study design, EVLP1 and EVLP2 were compared as time-series data, and EVLP2 and control were analyzed as independent groups. The comparison did not involve all 3 to 4 groups (sham, EVLP1, EVLP2, and control). The result may represent type I statistical error due to repeated analyses.

In conclusion, this study demonstrated that intermittent EVLP was significantly associated with better PaO_2/FiO_2, less EVLW, higher ATP levels, lower intraalveolar edema histopathology score, and better preservation of TEM findings than conventional cold static preservation in the control group. Given the elevation of proinflammatory cytokines in EVLP2, further improvement is needed to optimize the outcome. These data suggest that intermittent EVLP might be feasible for prolonging the lung preservation time to overcome the current geographic allocation limitations of the donor lungs.

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