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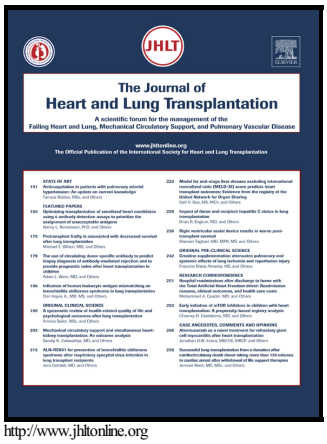

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Comparison of two strategies for ex-vivo lung perfusion

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COMPARISON OF TWO STRATEGIES FOR EX-VIVO LUNG PERFUSION

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Abstract

Background: In a porcine model, two clinically used strategies for ex vivo lung perfusion (EVLP) were compared with respect to lung function, metabolism, inflammatory response, oxidative stress and cell viability.

Methods: Porcine lungs (n=20) were preserved, harvested and kept cooled for 2 hours. After randomization, EVLP, either using a cellular perfusate and open left atrium (COA group), or an acellular perfusate and a closed left atrium (ACA group) was performed. Oxygenation (PaO_2/FIO_2), compliance, dead space, weight and perfusate oncotic pressure were registered before and after a four-hour period of reconditioning. Before and after EVLP, lung tissue samples were collected for qPCR analysis of gene expression for inflammatory markers, measurement of tissue hypoxia (HIF-alpha1) and oxidative stress (Ascorbyl radical) as well as viability (tryphan blue staining) and lung histopathology.

Results: In 3 out of 10 EVLP:s in the ACA group, EVLP was terminated prematurely due to severe lung edema and inability to perfuse the lungs. There were no significant differences in changes of lung oxygenation or pulmonary vascular resistance between groups. Compliance decreased and lung weights increased in both groups, but more in the ACA group (p=0.083 and p=0.065, respectively). There was no obvious difference in gene expression for HIF-alpha1, inflammatory markers, free radicals or lung injury between groups.

Conclusions: With both EVLP techniques, there is lung edema formation and decreased lung compliance, which were more pronounced in the ACA group. Otherwise there were no differences in lung function, inflammatory response, ischemia/reperfusion injury or histopathological changes between the EVLP techniques.

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Introduction

Lung transplantation is an established treatment in end-stage pulmonary disease, but shortage of organs remains a limiting factor. Marginal donor organs not fulfilling standard transplantation criteria can be evaluated by ex vivo lung perfusion (EVLP). Since its clinical introduction in 2001[1], an increasing amount of studies have shown that EVLP may be used as a tool to differentiate between reversible and non-reversible pathology, thereby enabling organs to be transplanted that otherwise would have been rejected[2-9].

EVLP is adopted by an increasing number of centers around the world. Most institutions have applied either one of the two more frequently published protocols for clinical use. The method originally published, was developed in Lund and initially described by Stig Steen and colleagues[10]. It uses a high pulmonary artery (PA) flow and pressure, an open left atrium and a perfusate mixed with red blood cells. The second method was developed in Toronto and employs a low PA flow and pressure, a closed atrium and an acellular perfusate[11]. There is a consensus that the perfusate should be hyperoncotic and hyperosmolar[12] but adding red blood cells to the perfusate is still under debate. Furthermore, The Toronto group claims that the use of a closed atrial technique to maintain a left atrial pressure ≈ 5 mmHg protects against lung edema and improves lung function during EVLP[13]. Another major difference between the two techniques is that the perfusate is periodically replenished with the Toronto protocol to maintain glucose levels and to provide fresh perfusate components, which is not done with the Lund model.

To our knowledge, the Lund and Toronto protocol have not been compared in a randomized study. The current experimental study was therefore undertaken to compare the Lund/Gothenburg (cellular, open atrium (COA) group)[14] with the Toronto/Vienna protocol (acellular, closed atrium (ACA) group)[2]. A number of methods were used in order to investigate whether or not there is a difference between these two protocols with respect to lung performance, pulmonary edema, cell viability, tissue hypoxia, inflammation, histopathological changes and the formation of reactive oxygen species (ROS).

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Materials and methods

Animals

The Animal Ethical Committee of the University of Gothenburg approved the study. The animals received care in compliance with the 'Principles of Laboratory Animal Care' formulated by the National Society for Medical Research and the 'Guide for the Care and Use of Laboratory Animals' (National Institute of Health publication no 86–23, revised 1996).

After anesthesia, the animals were mechanically ventilated (for details see supplementary material). A sternotomy was performed, the lungs were harvested and after 2 hours of ischemia subjected to EVLP. The animals each day were randomized to either the ACA group (n=10) or the COA group (n=10).

Lung harvesting

After anesthesia, the animals were mechanically ventilated (for details see supplementary material). A sternotomy was performed. The pigs were exsanguinated to a cell saver in the COA group. The lungs were antegradely perfused in situ with two liters of Perfadex (XVIVO Perfusion AB, Gothenburg, Sweden), with additives as previously described.[14] The lungs were harvested, retrogradely perfused again with an additional two liters of Perfadex with additives, and weighed. Tissue samples were taken from the inferior parts of the basal lobe of either the left or right lung in a randomized fashion. From this tissue, samples were secured for quantitative PCR (qPCR) and electron-spin resonance spectrography. The lungs were stored in cold Perfadex at 8°C for two hours. After an additional retrograde perfusion with 1 liter of Perfadex, preparation for EVLP was done according to the respective protocols (see below).

Ex vivo lung perfusion

In order to validate our execution of the Toronto protocol (ACA group), we collaborated with a representative of the Vienna lung transplant group (A.S.), as practical guidance with regard to aspects of performing a certain EVLP procedure can be difficult to ascertain from scientific publications alone. We made sure to implement in the study all aspects of how EVLP is performed clinically at both our centers. Rewarming strategies differed between groups, with respect to how flow was

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increased over time and when ventilation was initiated. Maximum flow (40 ml/kg in both groups) was reached earlier in the COA group compared to the ACA group. Details regarding the conductance of the EVLP for the two groups are described in the supplementary material and in figure 1.

In both groups the system was primed with 1.5 l of Steen solution (XVIVO Perfusion AB, Gothenburg, Sweden) methylprednisolone, heparin, and in addition added salvaged red blood cells to a hematocrit of 10-15% in the COA group. After de-airing of the circuit, the lungs were gradually rewarmed to 37°C and pulmonary flow was allowed to increase gradually as temperature increased to a target of 40 ml/kg, but pressure was restricted to 15 mmHg. Ventilation was initiated at 32°C. The first lung evaluation was performed at 60 minutes after initiation of EVLP and the second after a reconditioning phase of 240 minutes. In the ACA group, perfusate was exchanged at first evaluation and then every hour during EVLP. Finally, a collapse-test was performed in conjunction with the disconnection of the tracheal tube. Lung collapse was evaluated subjectively, as normal or impaired, by the same investigator (T.N.) in all cases. At the end of EVLP the lungs were again weighed and tissue samples were secured as described above.

The accessory lobe of the lungs was selectively cannulated and perfused with a solution containing tryphan blue, followed by a formaldehyde fixative. Tryphan blue colors viable and non-viable cell nuclei differently.[15] A tissue sample was then taken from the accessory lobe for histological analysis.

Quantification of ROS in lung tissue

Ascorbyl as endogenous ROS spin trap

We used electron spin resonance (ESR), using Vitamin C as an endogenous spin probe, for detection of free radical formation generated during EVLP in lung tissue, as previously demonstrated [16] and as described in detail in the supplementary material.

Tissue gene expression

Tissue from lungs was harvested for RNA extraction to detect changes in the expression of the cytokines IL-6, IL-8, IL-10, IL-1beta, TNF-alfa, IFN-gamma, the hypoxia inducible factor 1 alpha (HIF-1alfa), and GAPDH, as described in the supplementary material.

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Histology

Tissue for microscopic examination was taken for evaluation of the degree of vascular thrombosis, hemorrhage, necrosis, interstitial edema, intra-alveolar edema, intra-alveolar fibrin deposition, arteriolar thickening, cell infiltration, peribronchial edema and cell infiltrate, as described by Inci et al[17] and scored from 0 (absent) to 4 (severe). Tissue from the accessory lobe perfused with tryphan blue as described by D'Armini et al[15], for detection of viable/non-viable cell nuclei. (supplementary material)

Calculations

Pulmonary vascular resistance index (PVRI), lung compliance, dead space, the transpulmonary oxygen gradient ratio ($\Delta PO_2/FiO_2$) and the PaO_2/FiO_2 ratio were calculated according to standard formulas (supplementary material).

Statistics

Continuous data are presented as mean ± standard deviation. Changes within groups were assessed by Wilcoxon's signed rank test. The relative changes of the various variables induced in respective group were compared with the Mann-Whitney test. A p-value of <0.05 was considered statistically significant.

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Results

There were no differences in BW (58.1 ± 1.9 kg vs. 56.4 ± 2.7 kg, p = 0.165) between the two study groups.

In three lungs of the ACA group, severe edema developed during EVLP. The perfusate volume reached such low levels in these three cases that it was not possible to continue EVLP reconditioning as planned. The second evaluation in these cases was therefore not possible to conduct, and statistics are based on the seven cases in the ACA group in which the protocol could be finalized. EVLP of the lungs in the COA group was performed as planned in all experiments (n=10). Mean lung weights increased significantly in both groups during EVLP. There was a trend for a more pronounced increase in the ACA (44%) compared to the COA group (23%, p=0.065).

The transpulmonary oxygen gradient decreased significantly in the ACA group but not in the COA group. The dead space fraction increased in the ACA but not in the COA group. Compliance decreased significantly in both groups, by 44% in the ACA group, and by 25% in the COA group (Table 1) with a trend for a more pronounced decrease in the ACA group (p=0.083). PVRI increased significantly in both groups. This increase in PVRI did not differ between groups (Table 1). The oncotic pressure increased in both groups during EVLP. This increase was significantly more pronounced in the COA group (20%) compared to the ACA group (9%, p = 0.001).

All lungs in both groups developed consolidation of the inferior lobes and exhibited an impaired collapse test.

Quantification of ROS in lung tissue

We could not observe any significant difference between groups in changes in peak-to-peak amplitude measurements of pulmonary Ascorbyl radical signal in lung tissue, from the first to the second evaluation (p=0.436). ESR peak amplitudes (A.U.) were 1070 ± 234 before, and 965 ± 247 after EVLP, in the ACA group (p=0.475), compared to 917 ± 123 before, and 966 ± 257 after EVLP, in the COA group (p=0.878).

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Tissue gene expression (Table 3)

The expression of hypoxia-inducible factor (HIF)-1alpha decreased significantly in both groups during EVLP with no difference between groups. The levels of the mRNAs for the proinflammatory cytokines IL-6 and IL-8 decreased to similar extent in both groups, while TNF-alpha decreased in the ACA group and increased in the COA group (p= 0.003). The level of the mRNA for the anti-inflammatory cytokine IL-10 decreased in the ACA group but not in the COA group (p=0.005)

Histopathology

Histologic examination showed mild pathology in a few cases. Mild interstitial infiltrates were noted in two samples in each group. Mild arteriolar thickening was present in 2 samples respectively in each group. There was no apparent difference in lung histopathology between groups and no relevant statistical analysis could be performed.

Tryphan blue staining

In the ACA group, a fraction of 0.26 ± 0.45 of the examined nuclei were non-viable compared to 0.25 ± 0.29 in the COA group. There was no statistically significant difference between the groups (p=0.562).

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Discussion

In this experimental porcine model, we compared the performance of two different clinical protocols for EVLP. One group underwent EVLP according to our clinical protocol in Gothenburg with a cellular perfusate and open left atrium[8,14], very similar to what originally was described by Steen et al in Lund[10]. The second group underwent EVLP according to the clinical protocol of the Vienna/Toronto groups[2,18], which uses an acellular perfusate and a closed circuit with a closed left atrium. Our main findings of severe development of lung edema in three animals of the ACA group, necessitating premature interruption of the EVLP, together with the trend for a more pronounced increase of lung weight and decrease in lung compliance, suggest that the accumulation of interstitial/alveolar fluid was more pronounced in the ACA compared to the COA group. This was accompanied by a significant fall in lung oxygenation capacity and increase in alveolar dead space, suggesting ventilation/perfusion mismatch, during EVLP, which was not seen in the COA group.

The lung fluid accumulation and the fall in compliance in both groups during EVLP with impaired oxygenation, particularly in the ACA group, were most likely explained by an increased microvascular permeability in turn caused by the ischemia-reperfusion injury[19,20]. Such an increase in lung weight, despite the use of the hyperoncotic Steen solution, are in line with our previous clinical and experimental EVLP experiences[14,21] and has been described by others[22,23].

What is then the mechanism behind the more pronounced edema seen in the ACA group? It is not likely that we subjected the ACA group to over-perfusion as, in both groups, we achieved the same target pulmonary flow during EVLP. Furthermore, there was a progressive and similar increase in mean pulmonary artery pressure in both groups during EVLP. One could argue that the lack of oxygen carrying red blood cells in the ACA group, caused tissue hypoxia with further ischemic damage to the lungs during reconditioning. In recent experimental EVLP studies, it was shown that 4 hours of EVLP induced a 2-6 fold increase in the expression of hypoxia inducible factor (HIF)-1alpha[24,25]. In the present study, there was a similar ischemia/reperfusion-induced upregulation of the expression of (HIF)-1alpha in both the ACA and the COA group at the first lung evaluation. However, after 240 minutes of reconditioning, there was no further upregulation of HIF-1alpha, in neither of the groups,

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as the expression of mRNA for HIF-1alpha, if anything, decreased to a similar extent in both groups. This finding suggests that it is not likely that the more pronounced lung fluid accumulation in the ACA group is caused by hypoxia-induced lung injury, compared to the COA group.

In lung ischemia-reperfusion injury, immune cells are induced to generate reactive oxygen species and proinflammatory cytokines, causing an upregulation of adhesion molecules to vascular endothelial cells, which will translate into increased microvascular permeability and lung edema[19,20]. Formation of free oxygen radicals did in our model not change significantly either within or between groups during EVLP. In the present study, the proinflammatory cytokines Il-6, Il-8 and TNF-alpha were equally upregulated in both the ACA and COA group, at the first lung evaluation, to the same levels as previously demonstrated in experimental EVLP[24,25]. After reconditioning, the expression of IL-6 and IL-8 decreased in both groups. TNF-alpha decreased in the ACA group but, if anything, increased in the COA group. It therefore seems unlikely that the more pronounced extravasation of fluid in the ACA group could be attributed to a more pronounced inflammation-induced increase in capillary permeability. There was a divergent response of the expression of the anti-inflammatory cytokine, IL-10, which increased in the COA group and decreased in the ACA group. One could speculate that an IL-10 mediated limitation of the inflammatory response[26] could attenuate the inflammatory response and endothelial dysfunction in the COA group.

Included in the protocol of the Toronto technique is the use of a closed atrium to create a controlled positive left atrial pressure of 5 mmHg[13]. The Toronto group showed that a closed atrial technique led to less edema, compared to an open atrial technique during normothermic acellular EVLP[13]. It was hypothesized that a closed atrium with a positive LAP, causes a favorable distribution of perfusion away from the dependent regions of the lung. On the other hand, at a certain pulmonary artery pressure, an increase in venous outflow pressure will inevitably increase the transmural pressure of the pulmonary vascular bed, also including the pulmonary capillaries, which may, in the ex-vivo situation, cause fluid extravasation unless compensated by physiological adjustments such as increased precapillary resistance and/or reducing the fraction of capillary bed, as described in vivo[27].

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In both the ACA and COA group, the oncotic pressure of the perfusate increased, as a consequence of the fluid extravasation due to increased capillary permeability. In the COA group, the oncotic pressure increased by 20%. Such a progressive increase would counteract the continuous filtration of fluid at later stages of the EVLP, according to the Starling's law of capillary exchange of fluids. According to the Vienna/Toronto protocol for clinical EVLP, 250 ml of the perfusate is exchanged every 2 hours [2 4]. This can explain why the increase in oncotic pressure was significantly less pronounced (9%) in in the ACA group. Thus, the repeated replenishment of the perfusate in the ACA group will not allow the oncotic pressure of the perfusate to increase, as in the COA group, favoring a continuous, less unopposed, filtration of fluid, in the ACA group.

In spite of the more pronounced lung edema in the ACA group, on histologic examination there were no differences between groups with respect to pathological signs of lung injury, including interstitial edema. Furthermore, in our attempt to assess cell viability, we found the same fraction of unviable cells in both the ACA and the COA group, suggesting that the two techniques exerted similar injurious effect on the lung ultrastructure during experimental EVLP.

Our results, with respect to the differential effects of cellular vs. acellular perfusates on lung function, immunologic and structural changes are, to some extent, in line with those of two recently published studies on experimental EVLP in pigs[23]. In the study by Roman et al[28], comparing cellular (n=8) vs. acellular perfusates (n=8) for four hours, the left atrial cuff was left opened in both the cellular and acellular group. There was a more stable trend for a favorable increase in lung compliance and lung oxygenation in the cellular group in their study. Electron microscopy cell characteristics were similar among the two groups. Becker et al[23] compared cellular (n=5) vs. acellular perfusates (n=5) during prolonged EVLP (12 hours) after a period of cold ischemia for 24 hours. In their study the left atrium was closed and LAP was maintained at 2-5 mmHg in both the cellular and acellular group. In both groups lung compliance decreased and lung weight increased, but lung oxygenation was maintained in both groups during EVLP. Lung histology was largely preserved in both groups.

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A limitation of this study is that we did not obtain data at the second evaluation phase from three animals exposed to ACA, for reasons described above, which bias the results of the remaining seven animals of the ACA group to a more favorable outcomes. This, in turn, will probably underestimate the true differences between the two studied protocols for EVLP. Furthermore, one could argue that the more pronounced edema formation in the ACA group was caused by the obvious fact that our group has less experience with the Vienna/Toronto protocol and therefore did not master this procedure, technically, as well as the Lund/Gothenburg protocol. On the other hand, one of the experienced members of the Vienna group (A.S.) participated and supervised our performance of the Vienna/Gothenburg protocols. Another limitation is that the organs exposed to EVLP were not transplanted and evaluated in vivo after EVLP.

The protocol for rewarming differs between the two groups. Flow is increased more gradually and from lower levels in the ACA group, compared to the COA and target flow is reached earlier in the COA group. One could argue that a more gentle increase in flow as in the ACA group may be less damaging compared to exposing the tissues to a relatively high flow early on, as performed in the COA group. On the other hand, lung edema formation was less pronounced in the COA group, suggesting that early institution of the target flow, with this technique, does not necessarily predispose the lungs to edema.

A third system, and approach to the use of EVLP, the OCS system, has not been evaluated in this study.[29,30] As initially described by Warnecke at al[31], it was introduced as a system for transportation of organs under normothermic pulsatile perfusion and ventilation, and shares features with both the Lund and Toronto protocols.[32] The present study, however, aimed to compare the two most prevailing methods and focused on EVLP in-house after cold preservation.

Conclusions

With both EVLP techniques, there are lung edema formation and decreased lung compliance, which were more pronounced in the ACA group. Otherwise there were no differences in lung function, inflammatory response, ischemia/reperfusion injury or histopathological changes between the EVLP techniques.

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Conflicts of interest and funding sources

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Table 1 –Effects of 4 hours of EVLP reconditioning on lung function, hemodynamics and weight, and on perfusate oncotic pressure.

	Acellular, closed atrium (ACA) group				Cellular, open atrium (COA) group				COA vs. ACA Mean change %
	Evaluation 1	Evaluation 2	Δ%	p-value	Evaluation 1	Evaluation 2	Δ %	p-value	p-value
Lung weight (g)	442 ± 39	642 ± 152	44 ± 28	0.008	442 ± 32	539 ± 76	23 ± 21	0.007	0.065
Lung weight (g/kg bw)	7.6 ± 0.5	11.0 ± 2.6	44 ± 28	0.008	7.8 ± 0.4	9.6 ± 1.5	23 ± 21	0.007	0.065
Transpulm. O_2 gradient (FiO_2 1.0) (kPa)	53 ± 5	41 ± 11	- 22 ± 24	0.042	51 ± 8	48 ± 12	- 4 ± 33	0.44	0.270
Lung compliance (ml/cm H_20)	60 ± 22	31 ± 10	- 44 ± 24	0.012	56 ± 11	41 ± 10	- 25 ± 21	0.011	0.083
Mean pulmonary artery pressure (mmHg)	11 ± 2	17 ± 6	60 ± 38	0.018	11 ± 3	19 ± 1	94 ± 70	0.005	0.360
Pulmonary flow index (L/min/m^2)	2.14 ± 0.06	1.95 ± 0.27	- 8 ± 14	NA	2.20 ± 0.09	1.95 ± 0.26	-12 ± 10	NA	NA
PVRI (dynes x sec)/cm^5)	240 ± 66	543 ± 345	122 ± 122	0.018	358 ± 106	766 ± 162	129 ± 89	0.005	0.740
Perfusate oncotic pressure (mmHg)	34.9 ± 1.6	38.3 ± 1.1	9 ± 5	0.018	27.3 ± 1.9	32.9 ± 2.5	20 ± 4	0.005	0.001
Dead space fraction	0.220 ± 0.102	0.372 ± 0.123	87 ± 78	0.018	0.181 ± 0.121	0.172 ± 0.045	53 ± 113	0.59	0.351

Data expressed as mean and standard deviation. Non-parametric statistics used for analysis. Evaluation 1 is one hour after initiation of EVLP and Evaluation 2 is after 4 hours of EVLP. NA; not applicable, bw; body weight PVRI; pulmonary vascular resistance index.

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Table 2 – Change in mRNA expression during EVLP reconditioning

Gene	Acellular, closed atrium (ACA) group				Cellular, open atrium (COA) group				COA vs. ACA Mean change %
mRNA expression level (%GAPDH)	Evaluation 1	Evaluation 2	Δ%	p-value	Evaluation 1	Evaluation 2	Δ%	p-value	p-value
HIF-1alfa	113.5 ± 0.8	110.3 ± 1.6	- 2.8 ± 1.8	0.028	113.4 ± 1.1	108.9 ± 1.7	- 4.0 ± 1.0	0.005	0.220
IL-10	135.7 ± 1.9	129.1 ± 3.7	- 4.8 ± 2.5	0.028	134.5 ± 2.2	133.9 ± 5.1	- 0.43 ± 3.1	0.333	0.005
IL-6	140.9 ± 2.1	116.4 ± 10.8	- 17.3 ± 8.0	0.028	139.7 ± 1.7	123.1 ± 10.2	- 11.9 ± 7.3	0.005	0.220
IL-8	122.0 ± 3.2	102.1 ± 10.1	- 16.1 ± 9.8	0.028	121.3 ± 4.1	99.7 ± 9.5	- 17.7 ± 8.5	0.005	0.875
TNF-alfa	124.4 ± 5.4	115.7 ± 8.3	- 5.5 ± 6.7	0.046	123.6 ± 4.4	128.5 ± 4.8	6.2 ± 5.1	0.047	0.003

Data expressed as mean and standard deviation. Non-parametric statistics used for analysis.

Evaluation 1 is one hour after initiation EVLP and Evaluation

2 is after 4 hours of EVLP.

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Legends

Figure 1: (A) Experimental protocol for EVLP. (B) Rewarming protocol (COA group). (C) Rewarming protocol (ACA group)

FIO2: fraction of inspired oxygen; PIP: peak inspiratory pressure; PEEP: positive end-expiratory pressure; bw: body weight.