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ORIGINAL ARTICLE

Evaluating acellular versus cellular perfusate composition during prolonged *ex vivo* lung perfusion after initial cold ischaemia for 24 hours

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acellular perfusion, cellular perfusion, *ex vivo* lung perfusion, large animal model, lung physiology, lung ultrastructure.

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Summary

Normothermic *ex vivo* lung perfusion (EVLP) has developed as a powerful technique to evaluate particularly marginal donor lungs prior to transplantation. In this study, acellular and cellular perfusate compositions were compared in an identical experimental setting as no consensus has been reached on a preferred technique yet. Porcine lungs underwent EVLP for 12 h on the basis of an acellular or a cellular perfusate composition after 24 h of cold ischaemia as defined organ stress. During perfusion, haemodynamic and respiratory parameters were monitored. After EVLP, the lung condition was assessed by light and transmission electron microscopy. Aerodynamic parameters did not show significant differences between groups and remained within the *in vivo* range during EVLP. Mean oxygenation indices were 491 ± 39 in the acellular group and 513 ± 53 in the cellular group. Groups only differed significantly in terms of higher pulmonary artery pressure and vascular resistance in the cellular group. Lung histology and ultrastructure were largely well preserved after prolonged EVLP and showed only minor structural alterations which were similarly present in both groups. Prolonged acellular and cellular EVLP for 12 h are both feasible with lungs prechallenged by ischaemic organ stress. Physiological and ultrastructural analysis showed no superiority of either acellular or cellular perfusate composition.

Introduction

Ex vivo lung perfusion (EVLP) was applied clinically for the first time in 2001 [1]. Since then, this technique has emerged as an effective tool for the assessment and reconditioning of extended-criteria lungs [2] including donation after cardiac death (DCD) organs [3] prior to lung transplantation. The current gold standard for lung graft preservation is exclusive cold static preservation (CSP). In contrast, normothermic EVLP enables the potential donor lung to be metabolically active and allows for an evaluation of physiological lung parameters such as airway pressures, lung compliance, pulmonary artery pressure and oxygenation *ex vivo* [4].

Steen's group in Lund pioneered EVLP and developed the normooncotic STEEN Solution™ that was supplemented with red blood cells by his group [5]. Later, a modification of the technique was postulated using STEEN Solution™ as an acellular perfusate [6]. Nowadays, both perfusate compositions are utilized by specialized transplant centres in clinical EVLP and no consensus has been reached on a preferred technique yet [7–11]. Whereas EVLP was developed by Steen *et al.* [1] for the

purpose of organ assessment especially from DCD donors and thus tended to be of short duration at the beginning of its clinical application, current perfusion times range from 4 to 11 h [12–16], opening up the field for reconditioning and treatment of marginal lung grafts. Centres in which the technique of EVLP was introduced have experienced an increase in transplantation rates by 15–30% [8,12,17]. Therefore, EVLP might evolve as the technique to alleviate the scarcity of donor grafts for the treatment of end-stage lung diseases in the future.

The main acellular and cellular EVLP protocols being currently applied clinically and experimentally differ not only in perfusate composition but also regarding perfusion flow, venous outflow pressure and ventilation parameters. The aim of this study was to analyse solely the impact of acellular and cellular perfusate on physiological and structural preservation of lungs *ex vivo* in an identical experimental setting. Thus, the EVLP protocol of this study was developed from both acellular and cellular approaches. Using lungs moderately stressed by 24 h of cold ischaemia, EVLP was performed for 12 h at a flow rate of 70 ml/kg/min either in an acellular or in a cellular fashion. Throughout EVLP, lungs were assessed on haemo- and aerodynamic parameters followed by a histomorphological and ultrastructural analysis of lung samples.

Material and methods

Animals

Lungs of 10 female domestic pigs were included in the study (58.5 ± 6.5 kg bodyweight). Animals were either assigned to the acellular (aEVLP) or the cellular (cEVLP) perfusate composition group (n = 5/group).

All experiments were conducted in accordance with the NIH 'Principles of Laboratory animal care', the German animal protection law and the European Communities Council Directive 2010/63/EU for the protection of animals used for experimental purposes. They were approved by the responsible authority (Lower Saxony State Office for Consumer Protection and Food Safety, Animal Welfare Service).

Anaesthesia and surgery

Briefly, animals were sedated with 5 mg/kg azaperone and 0.04–0.08 mg/kg atropine i.m. and induced with 0.26 ml/kg propofol. Anaesthesia was maintained with 2–2.5% isoflurane in the inspiratory volume after endotracheal intubation and 5–10 μg/kg fentanyl i.v. every 20 min; for muscle relaxation, 0.5–2.5 mg/kg pancuronium i.v. was administered. Intra-operative ventilation was performed on pressure-controlled mode with an inspiratory pressure of 1.3 kPa and a positive end-expiratory pressure (PEEP) of 0.5 kPa after thoracotomy. For the measurement of *in vivo* peak (Paw_{peak}) and plateau airway pressures (Paw_{plat}), ventilation was changed to volume-controlled mode after maximal mobilization of lungs *in situ*.

Following full heparinization, the pulmonary artery (PA) was cannulated and the ascending aorta was punctured to collect blood gas samples at FiO_2 = 1. Before flush preservation with calcium-enriched buffered Perfadex™ (60 ml/kg, XVIVO Perfusion, Gothenburg, Sweden), airways were recruited on a Paw_{peak} of 2.5 kPa and 500 μg of prostaglandin E_1 (Alprostadil, Pfizer, Berlin, Germany) was administered into the PA [18,19]. For the collection of erythrocytes, 1.5 l of blood were obtained from the inferior caval vein during Perfadex™ flush, leucocyte depleted, washed (Cell Saver, Fresenius, Schweinfurt, Germany) and centrifuged to augment the haematocrit.

Finally, lungs were clamped at an airway pressure of 1.5 kPa for organ recovery. The explanted lungs were immersed in cold Perfadex™ and stored at a temperature of 4 °C for 24 h to induce moderate stress by an ischaemia model [20,21].

Set-up of EVLP circuit, commencement of perfusion and hourly assessment

A centrifugal pump (Medtronic, Minneapolis, MN, USA) generated the flow through a leucocyte filter to the PA. The circuit reservoir was combined with a membrane oxygenator and heat exchanger (Sorin, Saluggia, Italy). Air, CO_2 and nitrogen (Linde, Leuna, Germany) were titrated on demand for a prelung pO_2 of 100 mm Hg [22] and a pCO_2 of 35–45 mmHg [23]. PA (P_{PA}) and left atrium pressure (P_{LA}) were measured online at the level of lung hilus. In both groups, the circuit was primed with 1.5 l of STEEN Solution™ (XVIVO Perfusion), 500 mg methylprednisolone, 1000 mg meropenem, 10 000 IU heparin and 12 ml trometamol buffer [5,23] to obtain a prelung pH of 7.35–7.45 at set pCO_2. In the cEVLP group, autologous erythrocyte concentrate was added to create a perfusate haematocrit of 12–13%. PA and LA were cannulated (XVIVO Perfusion).

Anterograde perfusion was initiated at 10% of full flow and increased in defined increments during the first hour adapting to the perfusate temperature [23]. Maximal flow was set at 70 ml/kg/min on normothermia [22]. P_{LA} was maintained at 0.25–0.60 kPa by adjusting the reservoir height relating to the LA outflow level.

After the commencement of perfusion, lungs were deflated to an airway pressure of 0.5 kPa. At a perfusate temperature of 32 °C, that is 30 min after perfusion start, volume-controlled ventilation was initiated with a tidal volume (V_t) of 7 ml/kg, a ventilation frequency of 10 breaths/min, FiO_2 = 0.21, PEEP = 0.5 kPa and an

upper pressure limit of 2.5 kPa (Evita 2, Dräger, Lübeck, Germany).

Every hour, P_{PA}, Paw_{peak} and Paw_{plat} were recorded and static lung compliance (C_{stat}) as well as pulmonary vascular resistance (PVR) were calculated (Table 1). The unit kPa was used for all pressure values to allow direct comparison between airway and vascular parameters (1 kPa = 10 mbar = 10.197 cm H_2O = 7.501 mm Hg). After ventilating lungs on FiO_2 = 1 for 5 min, postlung pO_2 was analysed (ABL 700 radiometer, Willich, Germany). After assessment, lungs were recruited during an inspiratory hold on 2.5 kPa for three consecutive breaths. Hourly STEEN Solution™ was replenished to keep the circuit reservoir at a constant level.

Fixation, embedding and microscopy

After 12 h of EVLP, the circuit was cooled down to 20 °C, perfusion was terminated, and lungs were clamped on an airway pressure of 1.5 kPa. The organ was fixed by an antegrade perfusion of 4 litres of cold 1.5% glutaraldehyde/1.5% paraformaldehyde solution with a perfusion pressure of 3 kPa [24].

Using a systematic uniform random sampling protocol, at least 12 samples of both lungs were obtained for histological analysis per animal and the same number was obtained for ultrastructural assessment [25]. For light microscopy (LM), samples were embedded in Technovit 8100™, and sections were stained with toluidine blue and analysed with a Leica DM 6000 B (Wetzlar, Germany) light microscope [26]. For transmission electron microscopy (TEM), samples were embedded in Epon, and sections were stained with lead citrate/uranyl acetate and analysed with a Morgagni (FEI, Eindhoven, the Netherlands) transmission electron microscope [27].

Statistics

Data were analysed with IBM SPSS Statistics 22 (Gesellschaft für wissenschaftliche Datenverarbeitung mbH Göttingen, Göttingen, Germany). After testing for normal distribution, analysis of variance for repeated measurements was utilized for comparisons of the effect of time, group and interaction on physiological parameters. All results are expressed as mean ± SD, and differences were considered significant at the level of $P < 0.05$.

Table 1. Calculation of static lung compliance and pulmonary vascular resistance

C_{stat} [l/kPa] = V_t [l]/(Paw_{plat} [kPa] – PEEP [kPa])*
PVR [dyn*s*cm^{-5}] = (P_{PA} [kPa] – P_{LA} [kPa])/Perfusion flow [l/min] * 600)†

C_{stat}, static lung compliance; V_t, tidal volume; Paw_{plat}, plateau airway pressure; PEEP, positive end-expiratory pressure; PVR, pulmonary vascular resistance; P_{PA}, pulmonary artery pressure; and P_{LA}, left atrium pressure.

*1 l/kPa = 100 ml/mbar.

†The formula is identical with: PVR [dyn*s*cm^{-5}] = (P_{PA} [mm Hg] – P_{LA} [mm Hg])/Perfusion flow [l/min] * 80).

Results

Macroscopic observations and functional data during EVLP

Figure 1 shows the lung conditions after 1 (Fig. 1a,c) and 12 h of EVLP (Fig. 1b,d). Lung structure was macroscopically mostly well preserved throughout EVLP. Lungs generally maintained their spongy consistency and intratracheal fluid accumulation as a sign of gross oedema formation was absent in every experiment of both groups. However, sometimes alterations like circumscribed interlobular oedema formation (both groups) or reddening of initially inconspicuous lobe tips (in particular aEVLP group) were visible towards the end of EVLP and are depicted in Fig. 1b. Lung weight gain after 12 h of EVLP was 242 ± 138 g in the acellular and 171 ± 146 g in the cellular group.

Although Paw_{peak} remained well below the upper airway pressure limit of 2.5 kPa, this parameter increased from 1.32 (aEVLP) and 1.34 (cEVLP) to 1.62 kPa (both groups) throughout EVLP ($P < 0.001$, Fig. 2a). *Ex vivo* Paw_{plat} never exceeded *in vivo* pressure but also rose from 0.96/0.98 kPa during the first hour to 1.26/1.24 kPa (aEVLP/cEVLP) at the end of EVLP ($P < 0.001$, Fig. 2b). Thus, C_{stat} declined throughout ventilation time from 0.98 to 0.57 l/kPa in the aEVLP and from 0.83 to 0.55 l/kPa in the cEVLP group ($P = 0.001$, Fig. 3). In general, *ex vivo* Paw_{peak}, Paw_{plat} and C_{stat} remained within the *in vivo* range and no significant differences were observed between the acellular and cellular group.

Regarding pulmonary vasculature, P_{PA} decreased by 0.51 kPa in the aEVLP and 0.56 kPa in the cEVLP group during perfusion time ($P = 0.044$). Mean cellular P_{PA} exceeded P_{PA} in the aEVLP group by 0.55 ± 0.10 kPa ($P = 0.001$, Fig. 4a). The observed decline in PVR over perfusion time did not reach significance level, but the addition of red cells increased the mean PVR by 90.4 ± 21.6 dyn*s*cm^{-5} ($P = 0.004$, Fig. 4b).

Ex vivo perfusate oxygenation was well within physiological range, stable and similar to *in vivo* values at all time points in both groups (Fig. 5). The mean oxygenation index was 491 ± 39 in the aEVLP and 513 ± 53 in the cEVLP group; the difference was not statistically significant.

Microscopic assessment of lung structure after EVLP

The structural organ preservation after 24 h of cold ischaemia and 12 h of EVLP is shown in Figs 6 (LM level) and 7

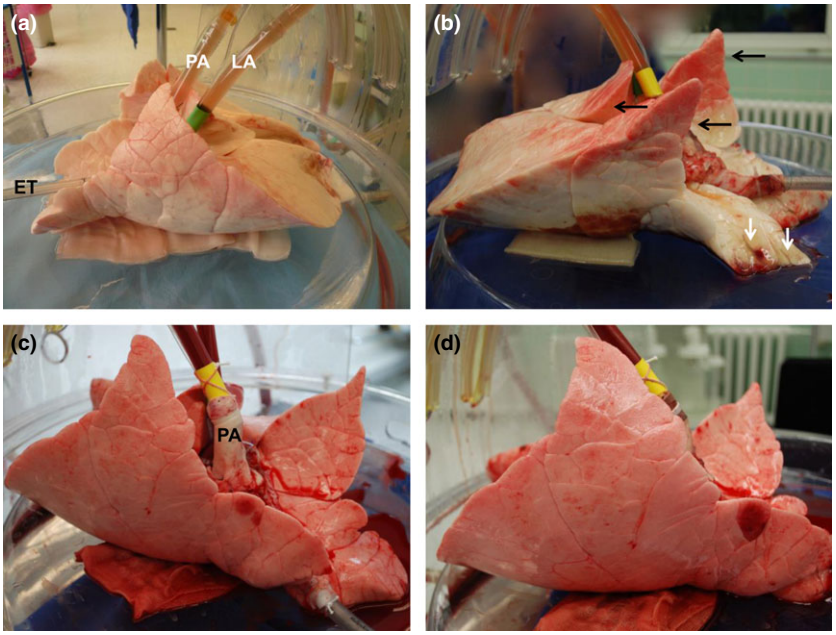

Figure 1 Ex vivo perfused lungs after 1 h of aEVLP (a), 12 h of aEVLP (b), 1 h of cEVLP (c) and 12 h of cEVLP (d). All lung lobes retained their well-inflated state throughout EVLP. Occasionally, cranial parts of superior lobes showed reduced ventilation with small areas of interlobular oedema (white vertical arrows) at the end of EVLP time in both groups (Fig. 1b). A progressive pleural reddening (black horizontal arrows) of the top portion of lobes occurred predominantly in the aEVLP group (Fig. 1b). ET, endotracheal tube; LA, cannulated left atrium; PA, cannulated pulmonary artery; EVLP, *ex vivo* lung perfusion.

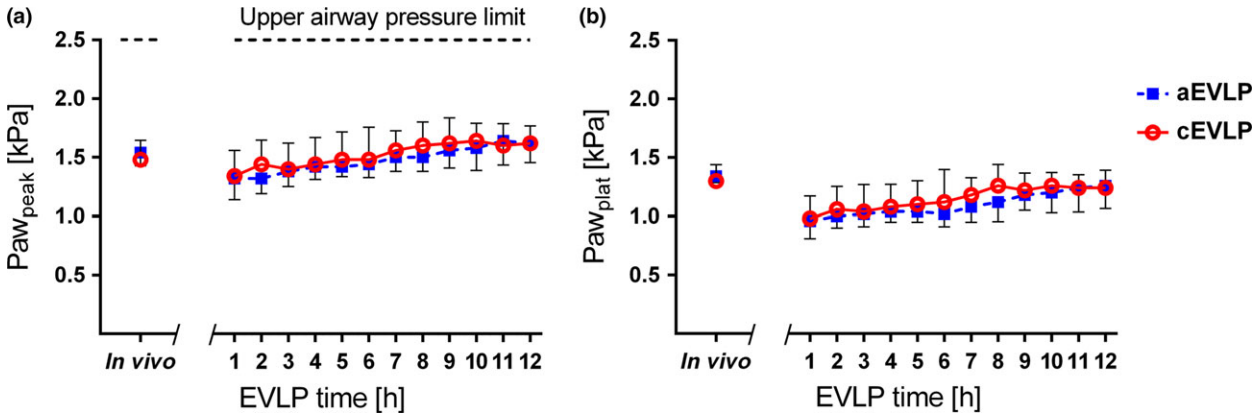

Figure 2 Airway pressures *in vivo* and during 12 h of *ex vivo* lung perfusion (EVLP). Results are expressed as mean ± SD. To increase diagram clarity, only one side of the error bar is marked. (a) Peak airway pressure (Paw$_{peak}$): *Ex vivo* Paw$_{peak}$ was clearly below the upper airway pressure limit of 2.5 kPa but increased throughout *ex vivo* perfusion time ($P < 0.001$). Groups did not differ significantly ($P = 0.76$). (b) Plateau airway pressure (Paw$_{plat}$): *Ex vivo* Paw$_{plat}$ remained below post-thoracotomy *in vivo* Paw$_{plat}$ up to 7 h of EVLP in both groups ($P < 0.01$) but rose throughout *ex vivo* ventilation ($P < 0.001$). Groups did not differ significantly ($P = 0.64$).

(TEM level). In both EVLP groups, lung integrity displayed a certain degree of heterogeneity. In most lung samples (i.e. 59 of 79 samples in the aEVLP group and 59 of 80 samples in the cEVLP group), lung histology as well as ultrastructure was unaltered and well preserved (Figs 6a-b and 7a-c). In these samples, lung parenchyma comprised well-inflated alveoli with slender alveolar septa and a continuous blood–air barrier with no signs of swelling or dissociation. Also, the surfactant system was unchanged.

However, in both aEVLP and cEVLP groups, a minority of lung samples contained small patchy alterations. The size of these lesions mainly ranged from a few μm to <1 mm

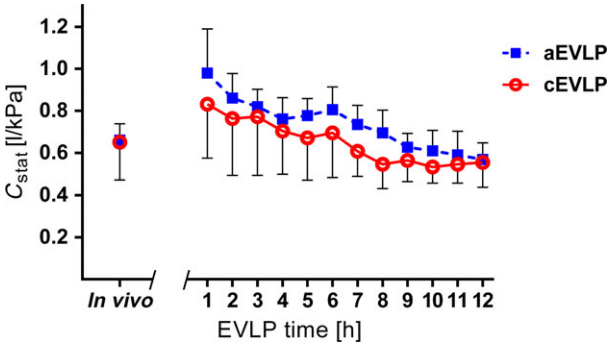

Figure 3 Static compliance (C_{stat}) *in vivo* and during 12 h of *ex vivo* lung perfusion (EVLP). Results are expressed as mean ± SD. To increase diagram clarity, only one side of the error bar is marked. *Ex vivo* C_{stat} exceeded *in vivo* (post-thoracotomy) compliance up to 2 h of EVLP ($P < 0.05$) and declined over 12 h EVLP time ($P < 0.001$). Groups did not differ significantly ($P = 0.21$).

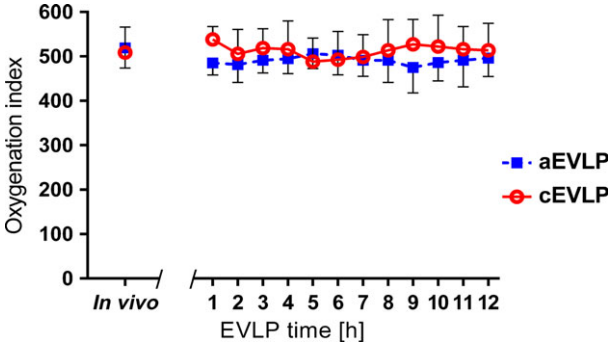

Figure 5 Oxygenation index *in vivo* and during 12 h of *ex vivo* lung perfusion (EVLP) at $FiO_2 = 1$. Results are expressed as mean ± SD. To increase diagram clarity, only one side of the error bar is marked. Oxygenation was stable over *ex vivo* perfusion time, and no difference was observed compared to *in vivo* values ($P > 0.544$). Groups did not differ significantly ($P = 0.42$).

and included areas with microatelectasis or mild ischaemia/reperfusion (IR) injury (Figs 6c-d and 7d). In the aEVLP group, 20 of 79 samples showed signs of IR injury with a range of 1 of 12 to 7/15 samples per animal. In the cEVLP group, a total of 21 of 80 samples were affected, ranging between zero of 13 and six of 15 samples per animal. Samples with IR injury were characterized by small amounts of oedema fluid in the intra-alveolar or peribronchovascular compartment. Intra-alveolar oedema fluid sometimes contained erythrocytes in the cEVLP group. In some cases in both groups, oedema formation was accompanied by swelling and vacuolization of the alveolar epithelium and/or capillary endothelium. These lesions were found disseminated throughout lung parenchyma in all lobes. Predilection sites for oedema formation were the cranial parts of the superior lobes and the part of the dorsal side of the lung which had been in direct contact with the dome plate. Lobe tips with macroscopical reddening towards the end of EVLP were characterized by an accumulation of erythrocytes exclusively in the pleural layer but not extending into the lung parenchyma.

Altogether, histological and ultrastructural lung quality was similar in the aEVLP and cEVLP group.

Discussion

The present study demonstrated the feasibility of prolonged EVLP with a physiological perfusion flow on the basis of an

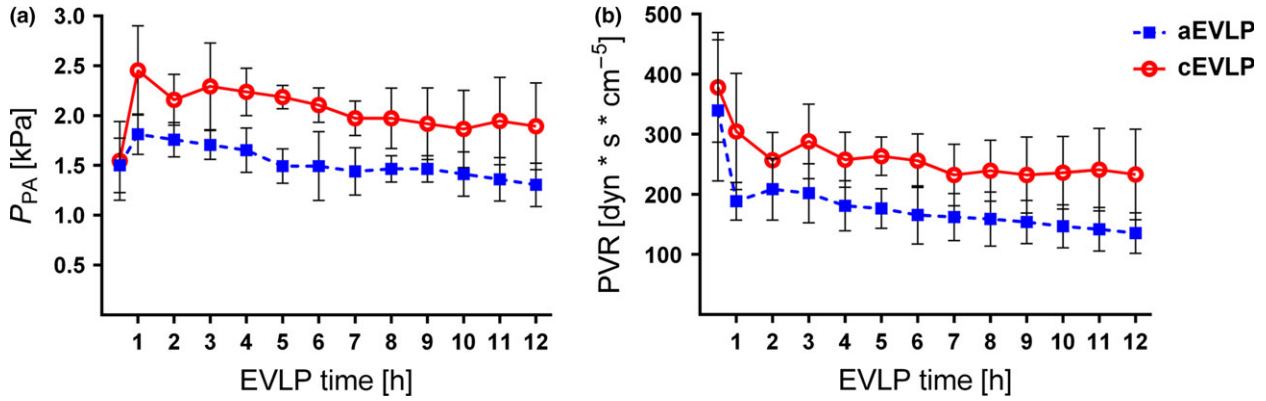

Figure 4 Parameters of pulmonary vasculature during 12 h of *ex vivo* lung perfusion (EVLP). Results are expressed as mean ± SD. (a) Pulmonary artery pressure (P_{PA}): Perfusion flow was doubled between 30 min and 1 h of EVLP, and P_{PA} also rose significantly between these time points ($P = 0.001$). Compared to the first hour of EVLP, P_{PA} decreased significantly over 12 h of perfusion in both groups ($P = 0.044$). P_{PA} was significantly higher in the cEVLP group ($P = 0.001$). (b) Pulmonary vascular resistance (PVR): PVR was significantly higher after 30 min of perfusion (50% perfusion flow) than at all other time points in both groups ($P < 0.026$). From 1 h onwards, it remained relatively stable. Values in the cEVLP group were significantly higher than in the aEVLP group ($P = 0.004$).

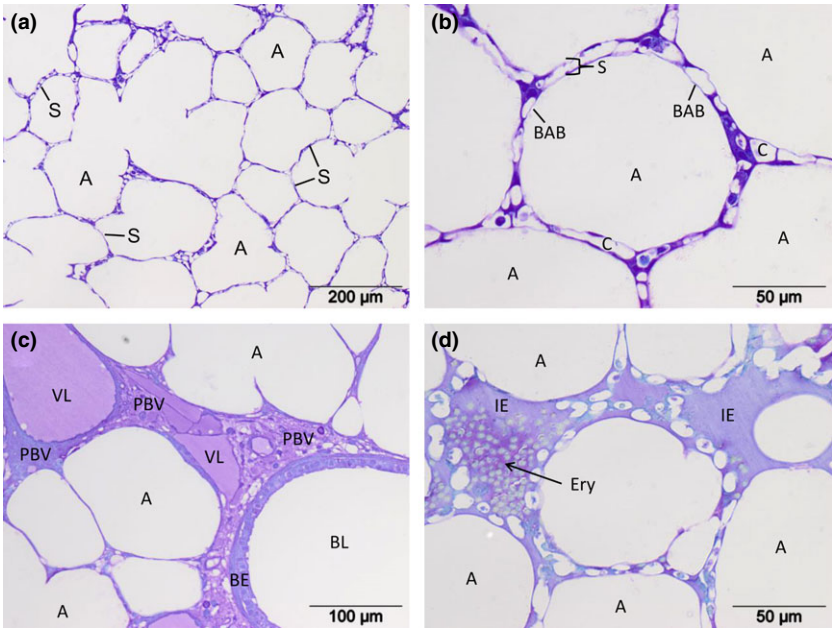

Figure 6 Histological lung structure after 24 h of cold ischaemia and 12 h of EVLP. (a) and (b) Unaltered lung structure representative for the majority of lung samples of the aEVLP (a) and cEVLP groups (b). (c) and (d) A minority of lung samples contained areas with mild ischaemia/reperfusion injury. (c) aEVLP group; lung parenchyma, pulmonary vasculature and airways. A small amount of oedema fluid is found in the peribronchovascular space. (d) cEVLP group; area of lung parenchyma exhibiting low-grade intra-alveolar oedema: alveolar lumina are partially filled with oedema fluid. A, alveolar lumen; BAB, blood–air barrier; BE, bronchiolar epithelium; BL, bronchiolar lumen; C, capillary lumen; Ery, erythrocytes; IE, intra-alveolar oedema fluid; PBV, peribronchovascular space; S, alveolar septum; and VL, vascular lumen; EVLP, *ex vivo* lung perfusion.

acellular or a cellular perfusate composition. In our ischaemia model, porcine lungs were subjected to 24 h of cold ischaemia in order to induce a moderate, well-defined degree of organ stress prone to lead to IR injury and impairment of organ function [20,28,29]. Even though good organ quality with respect to physiological function and microscopic preservation was obtained at the end of the subsequent EVLP period, this study does not recommend an extension of the generally accepted CSP duration of 6–8 h in clinical lung transplantation [30]. If extended ischaemic/*ex vivo* times are required, normothermic EVLP could be used to substitute partly the CSP duration. For this indication, the portable OCS device has been employed successfully with times of 2–5 h of CSP plus up to 11 h of normothermic EVLP [13,15,16].

Compared to the initial EVLP indications to serve mainly as an assessment platform for DCD and extended criteria lungs, duration of clinical EVLP has shifted from short term to prolonged *ex vivo* lung assessment [1,7,12–14,22,31]. Apart from the aspect of organ reconditioning and the possibility of therapeutical approaches [32], prolonged EVLP can reveal a trend in functional lung parameters towards improvement, stability or deterioration. Currently, these trends are implemented for the clinical decision-making algorithm to utilize an organ as an adequate lung graft after EVLP [12,14]. We observed an increase in airway pressures/decrease in compliance during EVLP in both groups. It was gradual within the *in vivo* range and remained below 15% compared to baseline values until 5–7 h of extracorporal ventilation (Figs 2 and 3). The development of aerodynamics is a very sensitive parameter for the assessment of lung quality. Deterioration above 15% after 4–6 h of clinical EVLP, however usually from poorer starting values, is considered as an exclusion criterion for subsequent transplantation [33,34].

Mean Paw_{peak} was 1.50 kPa (aEVLP) and 1.53 kPa (cEVLP) (Fig. 2a) in this study. Wallinder *et al.* (cellular EVLP) and Cypel *et al.* (acellular EVLP) reported moderately higher Paw_{peak} of 1.8–2.0 kPa in clinical studies on marginal lungs [12,14]. Static compliance of isolated lungs during 2–10 h clinical cellular EVLP resembled the mean compliance of approximately 0.01 l/kPa/kg in this study [14]. When comparing airway parameters, it needs to be taken into account that in these experiments, lungs were utilized which had been subjected to extended cold ischaemia as a defined model stressor, in contrast to marginal clinical donor lungs with oftentimes multiple and heterogeneous damages. Additionally, airway pressures and lung compliance are dependent on lung volume and species.

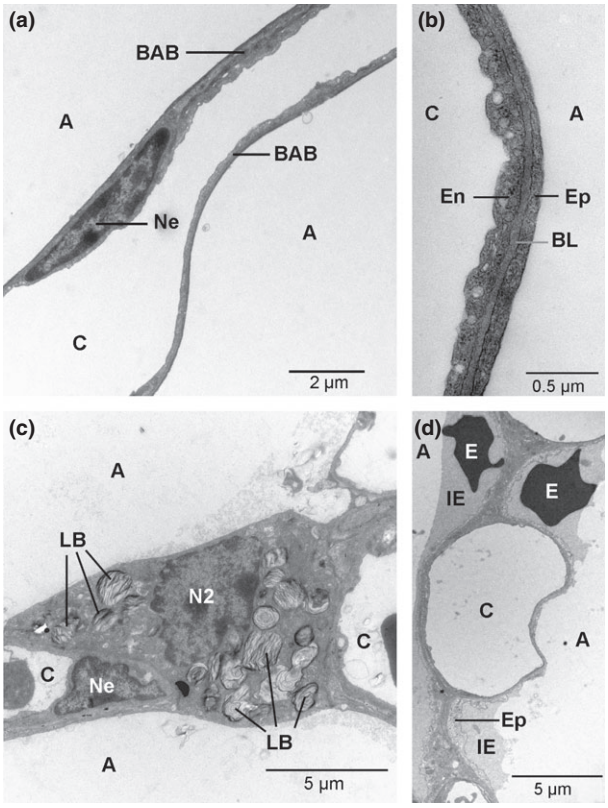

Figure 7 Ultrastructure of lung parenchyma after 24 h of cold ischaemia and 12 h of *ex vivo* lung perfusion (EVLP). (a)–(c): Unaltered ultrastructure representative for the majority of lung samples. (a) aEVLP group; alveolar septum. The septum separates two adjacent alveoli and contains an open capillary. The blood–air barrier is intact. Septum and alveolar lumina are free from oedema. (b) aEVLP group; blood–air barrier (BAB). All three layers of the BAB, that is alveolar epithelium, basal lamina and capillary endothelium, are continuous and without damage or swelling. (c) cEVLP group; type II alveolar epithelial cell within the alveolar septum. Many surfactant-containing lamellar bodies can be seen within the cytoplasm. (d) cEVLP; area of lung parenchyma exhibiting low-grade ischaemia/reperfusion injury. A small amount of intra-alveolar oedema fluid is visible, and the alveolar epithelium is swollen. Such lesions were found in a minority of lung samples from both EVLP groups. A, alveolar lumen; BAB, blood–air barrier; BL, basal lamina; C, capillary lumen; E, erythrocyte; En, capillary endothelium; Ep, alveolar epithelium; IE, intra-alveolar oedema; LB, lamellar body; Ne, nucleus of endothelial cell; and N2, nucleus of type II epithelial cell.

Mean P_{PA} was 1.49 kPa during aEVLP and 2.04 kPa during cEVLP (Fig. 4a). In accordance with these findings, other groups have also described moderately higher P_{PA} during cellular perfusion: Steen *et al.* reported a P_{PA} of 2.1–2.4 kPa in a porcine DCD model, and Wallinder *et al.* recorded similar measurements in a clinical series [5,35]. In contrast, Cypel *et al.* [12] stated a P_{PA} of 1.3–1.5 kPa during acellular perfusion of human lungs. However, these findings cannot be compared directly as protocols differed not only in perfusate composition. Cellular EVLP by Steen and co-workers was performed with a perfusion flow corresponding to 70 ml/kg/min and an open atrium [22]. Cypel *et al.* from Toronto favoured perfusion of lungs with 40% of estimated cardiac output, that is 40 ml/kg/min in pigs. Additionally, in acellular EVLP, a P_{LA} of 0.25–0.60 kPa was achieved by cannulation of the LA [12,23].

A central aspect of this study is the fact that acellular and cellular perfusate compositions were directly compared using identical settings, for example by challenging the lungs throughout normothermic perfusion with a physiological flow of 70 ml/kg/min. As overall cellular P_{PA} exceeded acellular P_{PA} by 0.55 $\pm$ 0.10 kPa (Fig. 4a), discrepancies in haemodynamics described by other groups are not only attributable to higher perfusion flow but also to the cellular perfusion itself causing a higher perfusate viscosity due to the cellular compartment [36,37].

The only other study comparing acellular and cellular perfusates following an identical protocol was recently published by Roman *et al.* [38]. Experimental EVLP was performed in a porcine model with a maximal perfusion flow of 50 ml/kg/min for 4 h after 30 min of warm ischaemia and 2 h of CSP. These experiments showed only a trend towards higher PVR during cellular perfusion. This could be due to the lower maximal perfusion flow being applied [36,37]. In our study, P_{PA} and PVR were still similar between groups after 30 min of perfusion when flow was 50% of the targeted flow, that is 35 ml/kg/min. As soon as full flow was applied, discrepancies between an acellular and a cellular perfusate in pulmonary haemodynamics manifested (Fig. 4).

An advantage or disadvantage of a higher but still physiological perfusion pressure was not demonstrated regarding airway and oxygenation parameters during 12 h of EVLP or by subsequent LM or TEM structural analysis in this study. However, it is tempting to speculate that the higher P_{PA} in the cEVLP group might have been protective of a ventilation–perfusion mismatch. Acellular P_{PA} approached Paw_{plat} at the end of perfusion, whereas cellular P_{PA} still exceeded this airway parameter (Figs 2b and 4a). When applying West's model of lung zones [39] to an *ex vivo* lung in a supine position [40], this resulted partly in West's lung zone I conditions (alveolar pressure > P_{PA} > P_{LA}) in acellularly perfused lobes above the level of lung hilus. The irregularity in perfusion indicated by the pleural reddening of upper-located lung regions at the end of acellular EVLP (Fig. 1b) could therefore indicate areas that were ventilated but mal-perfused. Being aware of the limitations of West's model [41] and applying these considerations to marginal donor lungs in clinical EVLP with increased airway pressures [12,14], a moderately higher P_{PA} might be beneficial regarding overall lung perfusion.

Oxygenation capacities reported in acellular and cellular EVLP studies match the findings of this study: Yeung *et al.* and Ingemansson *et al.* published oxygenation indices of 530–550 and 515 at $FiO_2 = 1$, respectively [31,34]. However, it has to be taken into consideration that the partial pressure of oxygen is similar between acellular and cellular perfusates, but the total oxygen content is very different due to the lack of an oxygen carrier in an acellular perfusate [34]. This may or may not be relevant for lung evaluation and reconditioning purposes, but the comparison of oxygenation indices has to be undertaken keeping this point in mind. In this study, a prelung pO_2 of 100 mm Hg was chosen mainly to ensure the supply of precapillary lung parenchyma with sufficient oxygen especially during the no-ventilation period after commencement of EVLP and gradual warming of the organ. At set prelung pO_2, prelung erythrocytes in the cEVLP group were already saturated. Thus, oxygenation of the perfusate in both groups only depended on lung function, regardless of an acellular or a cellular perfusate composition.

Lung histology had been evaluated in different settings of acellular EVLP. Medeiros *et al.* [42] collected samples from human lungs unsuitable for transplantation before harvest and after 10 h of CSP and 1 h of EVLP. Lung histology did not show a significant difference between those time points. Cypel *et al.* [6] observed less lung injury in transplanted porcine lungs after 12 h of CSP and 12 h of acellular EVLP compared to 24 of hours CSP alone. Roman *et al.* [38] studied the ultrastructure of porcine lungs after 2 h of CSP and 4 h of EVLP. They found no difference in the degree of cell injury comparing acellular and two different cellular perfusate solutions.

In our study, samples for histological and ultrastructural evaluation were generated by systematic uniform random sampling and thus rendered an unbiased analysis representative of the condition of the whole organ [23]. From each animal, 12 or more samples were obtained for LM and for EM assessment. This large number allowed the detection of heterogeneity in tissue conditions within the lungs. The majority of samples in both groups had an intact lung structure in light microscopical and even electron microscopical assessment. This was remarkable as the total preservation time was 36 h and the lungs were substantially challenged in our ischaemia/reperfusion model. IR injury was detectable only in a minority of samples. Histological and ultrastructural analyses revealed these as mostly rather small and low-grade lesions, while oxygenation indices were still high and very similar to prechallenge *in vivo* measurements. The distribution and the low incidence of lesions emphasized the importance of taking a sufficient number of samples per lung from all organ parts to obtain results representative for the whole lung. Regarding all samples, EVLP largely prevented manifestation of IR injury during *ex vivo* reperfusion in this ischaemia model and the two perfusate types were equally effective in preserving lung structure and ultrastructure during prolonged EVLP.

Study limitations: It cannot be excluded that applying different experimental conditions could generate different results regarding the comparison of acellular and erythrocyte-enriched perfusate in EVLP. We imposed 24 h of cold ischaemia on the lungs as a model stressor of moderate intensity and good reproducibility. However, the main clinical application of EVLP is the assessment and reconditioning of marginal lungs. Injury of these organs results from a large variety of insults of different origin and severity, for example contusions, ventilator-induced lung injury, oxygen toxicity, aspiration, volume overload or the cytokine storm during brain death [43]. Each source of injury might lead to differential reactivity during EVLP and could require specific adaptations of the EVLP protocol. Even though ischaemia-related injuries become visible at least on a microscopical level within 1–4 h after the start of reperfusion [20,28,29], injuries from EVLP-dependent sources could also possibly arise later than the 12-h perfusion time of this study, as with increasing perfusion time, the risk of haemolysis or infection grows. In this study, the lungs were processed for histology directly after EVLP to study solely the influence of the *ex vivo* treatment. Thus, lung performance after implantation into recipient animals could not be evaluated.

In conclusion, the present study has demonstrated extended cellular perfusion for as long as 12 h and the feasibility to perform acellular EVLP with a physiological perfusion flow. Both perfusate compositions led to largely similar oxygenation indices, airway parameters and structural as well as ultrastructural organ preservation, but a cellular perfusate composition generated a moderately higher perfusion pressure during prolonged EVLP. Thus, we conclude that acellular and cellular perfusate compositions are both suitable for prolonged EVLP of up to 12 h to facilitate the evaluation of potential donor lungs.

Authorship

SB, MO and AS: designed the study. SB, JSt, MA, KH, JSa and AS: performed the experiments. SB and AS: analysed the data. SB: drafted the manuscript. AH, GW, MO and AS: revised and edited the manuscript.

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