

Preservation of intraalveolar surfactant in a rat lung ischaemia/reperfusion injury model

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ABSTRACT: Ischaemia/reperfusion (I/R) injury, a major problem in clinical lung transplantation, is associated with surfactant dysfunction. The present study aimed to test the hypothesis that preservation related improvements in post-ischaemic lung function are associated with improved ultrastructural preservation of pulmonary surfactant.

Rat lungs were flush perfused with modified Euro-Collins solutions (ECS), stored for 2 h at 4°C, and reperfused for 40 min. Lungs were preserved with conventional (ECS 115: 115 mmol·L⁻¹ K⁺), medium-K⁺ (ECS 40: 40 mmol·L⁻¹ K⁺), or low-K⁺ (ECS 10: 10 mmol·L⁻¹ K⁺) ECS. Functional parameters were monitored during reperfusion (n=10 per group). After reperfusion, left lungs were prepared for electron microscopical and stereological analysis of surfactant (n=5 per group).

In all three experimental groups notable I/R injury developed which was lowest in ECS 40 as indicated by significantly less intraalveolar oedema, higher perfusate oxygenation, and lower peak inspiratory pressure. This was associated with a significantly superior preservation of the ultrastructure of the surface active surfactant subtype tubular myelin in ECS 40 compared with ECS 115 and ECS 10. Stereological analysis revealed that the relative amount of tubular myelin was highest in ECS 40 (mean±SEM; 6.2±0.8%) compared with ECS 115 (3.0±1.0%) and ECS 10 (2.7±1.6%).

Analysis of surfactant in its natural location within the organ showed that the severity of ischaemia/reperfusion injury correlates with differences in intraalveolar surfactant composition. Improved post-ischaemic respiratory function achieved by medium-K⁺ Euro-Collins solution is associated with superior ultrastructural preservation of tubular myelin. It is concluded that the integrity of surface active tubular myelin represents an important criterion for the assessment of lung preservation quality.

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Pulmonary surfactant prevents alveolar atelectasis by a surface area dependent reduction of the alveolar surface tension and has additional immunomodulatory functions [1, 2]. Surfactant material, which covers the alveolar epithelial surface at the air-liquid interface, is synthesized and secreted by type II pneumocytes of the alveolar epithelium [1]. An intracellular surfactant compartment consisting of the lamellar bodies within the type II pneumocytes and an intraalveolar surfactant compartment can, therefore, be distinguished. Only surfactant from the intraalveolar compartment can be collected by bronchoalveolar lavage (BAL) [3]. Two main subfractions of intraalveolar surfactant are obtained after centrifugation of BAL material: heavy large aggregates (LA), representing surface active forms that ultrastructurally largely correspond to tubular myelin, and light small aggregates (SA), representing degraded and inactive forms that ultrastructurally largely correspond to small unilamellar vesicles [4].

Abnormalities in surfactant composition and function are associated with a variety of lung diseases such as acute lung injury or acute respiratory distress syndrome (ARDS) [4–7]. Similar surfactant alterations have been reported to

be present after ischaemia and reperfusion (I/R) in experimental and clinical lung transplantation [8–13]. In patients with ARDS [14] as well as in experimental I/R injury [9], the reduced biophysical activity of surfactant is reflected by an increase in the SA/LA ratio of BAL subfractions [4]. Whereas all of these studies analysed only intraalveolar surfactant material obtained by BAL, the current authors have recently shown that an ultrastructural and stereological approach permits the distinct analysis of both surfactant compartments in lipopolysaccharide-induced lung injury [15] as well as the analysis of intraalveolar surfactant from oedematous and nonoedematous lung regions in an I/R injury model [16].

I/R injury, which is characterized by pulmonary oedema formation associated with an increase in pulmonary artery pressure and hypoxaemia [17], is the major reason for early graft dysfunction in clinical lung transplantation [18]. The clinically relevant prevalence of early graft dysfunction has been estimated to be 15–35%, with a severity ranging from very mild acute lung injury to ARDS [18], and it is clearly stated that the quality of lung preservation is a key determinant of initial graft dysfunction [18, 19].

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The purpose of the present study was therefore to test the hypothesis that an improvement in post-ischaemic lung function is associated with improved ultrastructural preservation of pulmonary surfactant. In previous studies, the current authors have established an isolated perfused rat lung model to investigate the effects of I/R on pulmonary structure and function [16, 20–22]. Using this model, the authors studied the effects of lung preservation, subsequent ischaemic storage and reperfusion on intraalveolar surfactant composition by means of transmission electron microscopy and stereology. Lung preservation was performed with conventional Euro-Collins solution (ECS), which is the solution used by most lung transplant centres [23], in comparison to ECS-based solutions with reduced K⁺ concentrations, which are known to result in different degrees of I/R injury [21, 24].

Materials and methods

Animals

Sprague-Dawley rats (n=30) weighing 325–465 g were used in this study. All animals received humane animal care according to the Helsinki convention for the use and care of animals.

Study design

The animals were randomly assigned to three experimental groups: ECS 115 (n=10), ECS 40 (n=10), and ECS 10 (n=10). The preservation solutions, the composition of which is shown in table 1, differed in their potassium and sodium concentrations, respectively. In an isolated perfused rat lung model, lungs were flush perfused with the respective K⁺-modified ECS, stored for 2 h at 4°C, and reperused for 40 min. Haemodynamic and respiratory parameters were monitored during reperfusion. At the end of reperfusion, right lungs were used to determine wet/dry weight ratios. Left lungs of five animals per group were prepared for transmission electron microscopic and stereological analysis to quantify ultrastructural alterations in intraalveolar surfactant composition.

Methods

General anaesthesia in rats was induced by intraperitoneal injection of pentobarbital (Nembutal, 1 mg·kg⁻¹ body weight). Laparotomy was performed followed by heparinization *via* the inferior vena cava (100 IU). After thoracotomy the right and left superior vena cava, the inferior vena cava and the left azygos vein were clipped prior to careful removal of the organ block.

Table 1. – Composition of modified Euro-Collins based preservation solutions (ECS; mmol·L⁻¹)

Components	ECS 115	ECS 40	ECS 10
K ⁺	115.0	40.0	10.0
Na ⁺	10.0	85.0	115.0
Cl ⁻	15.0	15.0	15.0
PO ₄ ²⁻	57.5	57.5	57.5
HCO ₃ ⁻	10.0	10.0	10.0
Glucose %	3.5	3.5	3.5
Osmolarity mOsm·L ⁻¹	355	370	380

An isolated lung model previously described in detail was employed [20]. The isolated lungs were flush perfused *via* the pulmonary artery with 20 mL of the respective K⁺-modified ECS, and inflated with 10 mL air before hypothermic storage for 2 h at 4°C in the respective preservation solution. After storage, the right atrial cannula was connected to a double head roller pump (PA21-A; Cole Parmer Co., Chicago, IL, USA), and the lungs were reperused for 40 min starting with a flow rate of 1 mL·min⁻¹. During the first 10 min the flow rate was gradually increased to 7–8 mL·min⁻¹ and then kept constant. Reperfusion was performed using a Krebs-Henseleit buffer supplemented with washed bovine erythrocytes (haematocrit level 38–40%) and deoxygenated by means of deoxygenator gassed with 95% N₂ and 5% CO₂. Leukocytes were removed by means of a leukocyte removal filter (RC 100E, Pall Europe Ltd., Portsmouth, Hampshire, UK). During reperfusion, the lungs were mechanically ventilated with room air at a tidal volume of 4 mL and a respiratory frequency of 40 breaths per minute using a small animal respirator (Mod. No. 4601, Rhema Labor Technik Ltd., Hofheim, Germany). A positive end-expiratory pressure of 3 cmH₂O was maintained.

Blood gases were determined at 10 min intervals. The partial pressure of oxygen (PO₂) measured in the perfusate collected from the left atrium was defined as P_{a,O₂}, and the PO₂ of the deoxygenated perfusate was defined as P_{v,O₂}. The perfusate oxygenation (ΔPO₂), defined as the arteriovenous oxygenation difference (P_{a,O₂} - P_{v,O₂}) was used to assess the capability of the lung for gas exchange. Peak inspiratory pressure (PIP) was measured every 10 min by means of the small animal respirator used for mechanical ventilation. At the end of reperfusion, the right lung was used to determine the wet/dry weight ratio while the left lung was fixed for ultrastructural analysis.

A fixation and processing protocol was performed, which was developed to ensure stabilization and retention of intracellular as well as intraalveolar surfactant in place [25].

Briefly, the left lungs were fixed immediately after reperfusion by vascular perfusion *via* the pulmonary artery at a hydrostatic pressure of 15 cmH₂O while airway pressure was adjusted to 12 cmH₂O. The primary fixative used was a mixture of 1.5% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.35; buffer osmolarity 300 mOsm·L⁻¹). A systematic random sampling procedure was performed in order to obtain tissue blocks representative of the whole organ [25]. Tissue processing included post-osmication, *en bloc*-staining in aqueous uranyl acetate overnight, dehydration and embedding in Araldite [25]. Semithin sections were stained with methylene blue and examined by means of light microscopy (Leitz Laborlux 11; Leitz, Wetzlar, Germany). Transmission electron microscopical examination of ultrathin sections was conducted using an EM 900 (LEO, Oberkochen, Germany).

Analysis

To assess the severity of reperfusion injury, semithin sections of three tissue blocks per lung were analysed for intraalveolar oedema estimation. An unbiased collection of test fields was analysed by light microscopy. Point counting was performed to calculate the volume density of intraalveolar oedema per gas exchange region, which was

recently shown to be a valuable parameter to assess the severity of reperfusion injury [22].

For stereological analysis at the electron microscopical level, ultrathin sections of five tissue blocks per lung were examined. An unbiased collection of test fields was obtained by a systematic quadrats subsampling scheme. Each test field was analysed at a final magnification of $\times 26,000$ employing an image analysis software package (analySIS 2.1; Soft Imaging System, Münster, Germany). Following earlier studies [15, 16], the main intraalveolar surfactant subtypes, *i.e.* tubular myelin, unilamellar, multilamellar, and lamellar body-like forms, as well as intracellular surfactant represented by the lamellar bodies within type II pneumocytes were quantified by means of point counting. The relative composition of intraalveolar surfactant was determined by calculation of the volume fractions of the distinct surfactant subtypes in relation to total intraalveolar surfactant volume. Differences in intracellular surfactant were determined by calculation of the volume density of lamellar bodies per type II pneumocyte volume. In addition, the degree of cell damage to type I pneumocytes was estimated by intersection counting as the fraction of fragmented surface areas relative to the total surface area of type I pneumocytes [22]. A mean of 180 test points on intraalveolar surfactant and a mean of 250 intersections with type I pneumocytes were counted per individual lung.

Data are given as mean \pm SEM. Normality and equal variance given ($p>0.05$), statistical analysis was performed using analysis of variance (ANOVA) followed by *post hoc* multiple comparison (Student-Newman-Keuls test) or Student's *t*-test. Otherwise nonparametric ANOVA on ranks or Kruskal-Wallis rank sum test were used. All statistical analyses were performed using the software program SigmaStat 2.0 (Jandel Scientific, Erkrath, Germany). A *p*-value <0.05 was considered significant.

Results

Functional data

Haemodynamic and respiratory data are shown in table 2. After 40 min of reperfusion, perfusate oxygenation was significantly higher in lungs preserved with medium- K^+ ECS 40 in comparison to lungs preserved either with conventional high- K^+ ECS 115 or with low- K^+ ECS 10. Furthermore, lungs preserved with either ECS 115 or ECS 10 exhibited a significantly higher peak inspiratory pressure compared to lungs preserved with ECS 40. Since the tidal volume and the positive end-expiratory pressure were constant in the experimental settings, this increase in PIP indicates a decrease in lung compliance [19]. The wet/dry ratio data did not show significant differences between the three groups.

Structural findings

Lungs preserved with medium- K^+ ECS 40 developed less severe intraalveolar oedema, especially in comparison to lungs preserved with high- K^+ ECS 115 (table 3), which, in conjunction with the functional data, indicates a lower degree of I/R injury in the ECS 40 group. However, stereological analysis of the surface fraction of fragmented type I pneumocytes failed to show significant differences between the three groups (table 3). There were no signifi-

Table 2. – Lung function after 2 h ischaemia and 40 min reperfusion

Groups n=10, each	Perfusate oxygenation mmHg	Peak inspiratory pressure cmH ₂ O	Wet/dry ratio
ECS 115	10.8 \pm 1.8	30.9 \pm 1.9	10.2 \pm 0.8
ECS 40	22.4 \pm 3.4*:#	24.8 \pm 1.3*#	11.7 \pm 0.4
ECS 10	11.8 \pm 1.4	30.9 \pm 1.2	11.5 \pm 0.8

Data are presented as mean \pm SEM. ECS: Euro-Collins based preservation solutions. *: $p<0.05$ ECS 40 *versus* ECS 115; #: $p<0.05$ ECS 40 *versus* ECS 10.

cant differences in the volume densities of intracellular surfactant (table 3).

The qualitative ultrastructural appearance of the intraalveolar surfactant subtypes characteristic of each experimental group is shown in figures 1–3. In the ECS 40 group, the structural integrity of the surface active tubular myelin was largely well preserved, whereas ultrastructural disintegration of tubular myelin was observed in the ECS 115 and in the ECS 10 group. In contrast to lungs preserved with ECS 40, these latter groups were characterized by loss of continuous transition of tubular myelin lattices into the osmiophilic lining layer at the air-liquid interface, and by loss of close contact to the alveolar epithelial surface. No qualitative differences were observed with regard to the other intraalveolar surfactant subtypes, *i.e.* multilamellar, unilamellar, and lamellar body-like forms. There were no differences in the ultrastructural appearance of the intracellular surfactant, *i.e.* the lamellar bodies of alveolar type II pneumocytes.

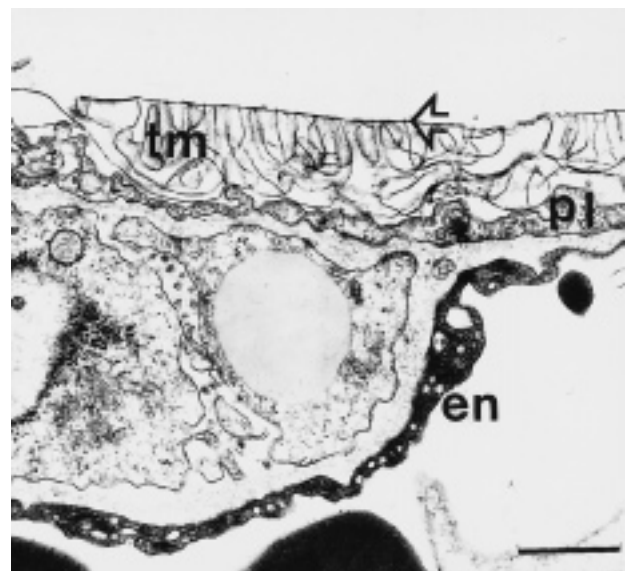


Fig. 1. – Transmission electron microscopy micrograph of intraalveolar surfactant subtypes of rat lung preserved with medium- K^+ (40 mmol·L⁻¹ K⁺) Euro-Collins solution. Well preserved tubular myelin with characteristic ultrastructure of regular lattices which are continuous with the surfactant lining layer at the air-liquid interface. The cellular components of the gas exchange barrier appear to be slightly swollen. en: endothelium; pl: type I pneumocyte; tm: tubular myelin; arrow: surfactant lining layer. Internal scale bar=1 μ m.

Table 3. – Oedema formation, epithelial damage, and intracellular surfactant after 2 h ischaemia and 40 min reperfusion

Groups (n=5, each)	Volume fraction intraalveolar oedema % parenchyma	Surface fraction fragmented type I pneumocytes % type I pneumocytes	Volume fraction intracellular surfactant % type II pneumocytes
ECS 115	31.4±4.8	49.4±10.0	7.9±2.1
ECS 40	18.8±1.7*	55.8±5.4	6.2±1.2
ECS 10	28.6±4.8	57.0±10.8	9.5±2.2

Data are presented as mean±SEM. ECS: Euro-Collins based preservation solutions. *: $p < 0.05$ ECS 40 versus ECS 115.

The quantitative analysis of intraalveolar surfactant composition confirmed the qualitative findings of a major difference between groups in the surface active tubular myelin subtype. The highest fraction of tubular myelin was observed in the ECS 40 group (table 4). In contrast, the fractions of surface inactive unilamellar forms and of freshly secreted, nontransformed lamellar body-like forms were slightly increased in the lungs preserved with ECS 115 or ECS 10 compared to the ECS 40 group, although not to a significant level (table 4).

Discussion

Analysis of surfactant alterations in clinical as well as in experimental studies is usually performed *ex situ* on material obtained by BAL. However, this procedure, which requires strictly controlled conditions to avoid experimental error [26, 27], only allows analysis of the intra-

alveolar compartment while surfactant from the intracellular compartment cannot be harvested. Additionally, the pre-existing micro-organization of the intraalveolar surfactant system might be disrupted during lavage [28]. The current methodological approach permits a detailed investigation of both surfactant compartments by analysing surfactant material preserved in its natural location within the organ by means of transmission electron microscopy and stereology.

In the present study, the authors investigated ultrastructural alterations in intraalveolar surfactant in the early phase of I/R injury in an isolated perfused rat lung model. K^+ -reduced ECS (ECS 40, ECS 10) were tested against conventional high- K^+ ECS (ECS 115), which, being the preservation solution used by most lung transplant centres [23], served as standard reference. The three experimental groups exhibited different degrees of I/R injury, as demonstrated by oxygenation and PIP as well as by stereological estimation of intraalveolar oedema formation. Analysis of wet/dry ratios, however, could not detect significant differences between the three groups. A similar discrepancy between assessment of oedema formation by stereology and by wet/dry ratio determination has recently been described by the authors' group in a different series of experiments [29]. While the stereological approach allows for distinction between intraalveolar and interstitial oedema [22], both oedema forms contribute to wet/dry ratio data which also include intravascular fluid. From the present results, it can therefore be concluded that the stereological estimation of intraalveolar oedema formation better reflects the functional status of the lung than does wet/dry ratio analysis.

Although different degrees of I/R injury were clearly demonstrated, the stereological analysis of the surface

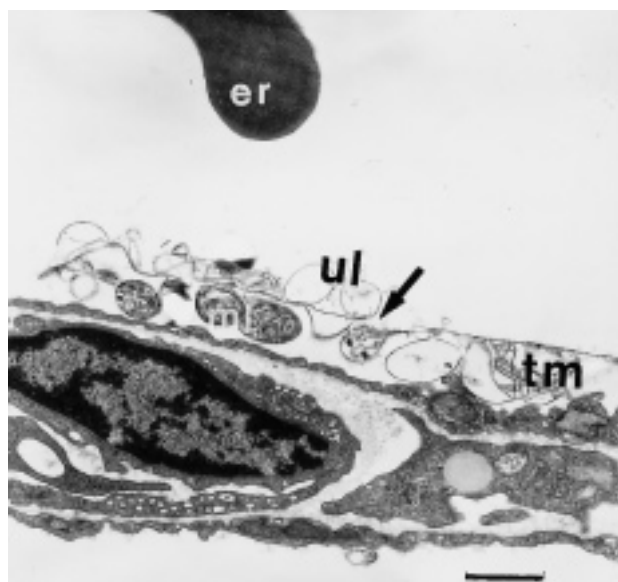


Fig. 2. – Transmission electron microscopy micrograph of intraalveolar surfactant subtypes of rat lung preserved with conventional (115 $\text{mmol}\cdot\text{L}^{-1}$ K^+) Euro-Collins solution. Only a few tubular myelin lattices are seen which have hardly any contact to the surfactant lining layer or the alveolar epithelial surface, respectively. Multi- and unilamellar surfactant forms are frequently observed. er: erythrocyte; ml: multilamellar surfactant forms; ul: unilamellar surfactant forms; tm: tubular myelin; arrow: ruptured surfactant lining layer. Internal scale bar=0.5 μm .

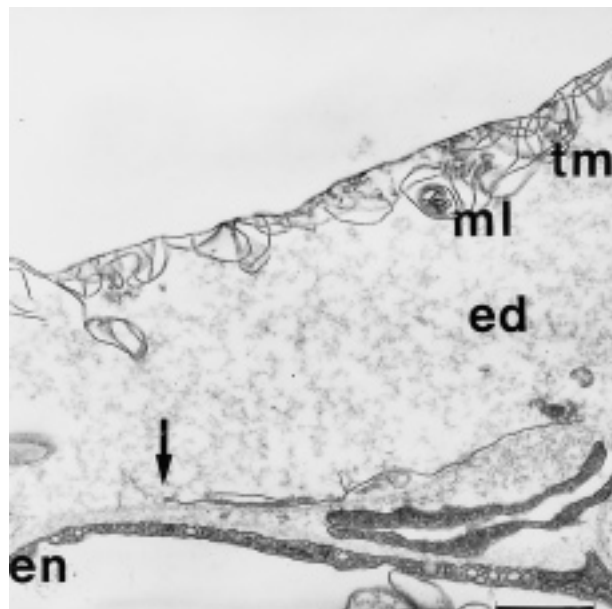


Fig. 3. – Transmission electron microscopy micrograph of intraalveolar surfactant subtypes of rat lung preserved with low- K^+ (10 $\text{mmol}\cdot\text{L}^{-1}$ K^+) Euro-Collins solution. Tubular myelin with partially disrupted lattices which are separated from the alveolar epithelial surface by intraalveolar oedema. Some uni- and multilamellar forms are seen within the oedema accumulations. ed: intraalveolar oedema; en: endo-thelium; ml: multilamellar surfactant forms; tm: tubular myelin; arrow: denuded basal lamina. Internal scale bar=1 μm .

Table 4. – Relative intraalveolar surfactant composition after 2 h ischaemia and 40 min reperfusion

Groups (n=5, each)	Volume fraction intraalveolar surfactant subtypes % total intraalveolar surfactant			
	tubular myelin	unilamellar	multilamellar	lamellar body-like
ECS 115	3.0±1.0	83.3±2.3	13.0±2.6	0.9±0.4
ECS 40	6.2±0.8*	79.4±3.0	14.1±2.5	0.3±0.2
ECS 10	2.7±1.6	81.2±6.6	14.2±5.7	1.8±1.4

Data are presented as mean±SEM. ECS: Euro-Collins based preservation solutions. *: p<0.05 ECS 40 versus ECS 115.

fraction of fragmented type I pneumocytes failed to show significant differences between the three groups. Therefore, the degree of alveolar epithelial damage does not represent the fine structural correlate at the level of the blood-air barrier to explain differences in post-ischaemic lung function after storage in different preservation solutions. This finding confirms previous data from the authors' group where the extent of alveolar epithelial injury did not unambiguously determine the amount of intraalveolar oedema [22]. In contrast, the differences in lung function in the early reperfusion period observed in the present study correlated well with differences in intraalveolar surfactant ultrastructure and composition. Improved post-ischaemic oxygenation in the ECS 40 group was associated with superior ultrastructural preservation of the surface active tubular myelin subfraction. These findings are in line with the hypothesis that surfactant alterations contribute to lung dysfunction associated with I/R injury [4]. This hypothesis is further supported by experimental observations that exogenous surfactant therapy before reperfusion significantly improves post-ischaemic lung function [30–32].

At present, the exact mechanisms by which surfactant alterations in I/R injury occur are not completely understood. It seems likely that toxic oxidant species like free radicals, which are known to be important mediators in I/R injury [4, 33], interact with surfactant components, especially the surfactant apoproteins [34, 35]. Plasma proteins entering the alveolar space during intraalveolar oedema formation are also known to inactivate endogenous [4, 6] as well as exogenous surfactant [36], although the authors have recently shown that surfactant alterations also occur in lung regions free of oedema, indicating that these surfactant alterations are not merely a secondary consequence of intraalveolar oedema formation [16]. In addition, proteolytic damage to surfactant protein A by neutrophil elastase, experimentally demonstrated *in vitro* [37], has recently been reported to be present in patients with ARDS [38] and might therefore represent another possible mechanism by which surfactant alterations are induced in I/R injury. Detailed studies on interactions of surfactant apoproteins with mediators of I/R injury are desirable to reveal the exact mechanisms resulting in surfactant alterations in I/R injury.

Preservation of lungs for clinical transplantation aims to minimize damage resulting from ischaemic storage and reperfusion, thereby maintaining the structural and functional integrity of the organ. However, current preservation methods are only satisfactory for ischaemic periods of up to 6 h [18, 19]. Extension of tolerable ischaemic periods due to improved lung preservation would have the advantage of being able to perform lung transplantation as a nonemergency procedure, thereby allowing better match-

ing of donor and recipient [18, 19]. The enormous variation in clinical practice together with the fact that the incidence of primary graft failure has changed very little during the 1990s, make it likely that current lung preservation techniques are still suboptimal [23]. Further experimental and clinical research is therefore needed to improve the preservation methods currently employed.

While there is general agreement as to how pulmonary functional integrity should be evaluated [19], standard criteria for the assessment of the structural integrity of the preserved lung have not yet been defined. The present study has shown that the preservation of intraalveolar surfactant ultrastructure correlates with post-ischaemic lung function. Improved oxygenation in the medium-K⁺ Euro-Collins solution (40 mmol·L⁻¹K⁺) group was associated with superior ultrastructural preservation of tubular myelin. The percentage of the large aggregate subfraction in bronchoalveolar lavage studies represents a parameter that has been used as an indicator of surfactant function in acute lung injury [4, 9, 14, 30]. Based on the current observations, the authors propose that the fraction of surface active tubular myelin, which largely corresponds to the bronchoalveolar lavage large aggregate subfraction [4], represents an important criterion for the assessment of the structural integrity of intraalveolar surfactant in studies investigating lung preservation quality. It is further proposed that differences in the preservation of pulmonary surfactant are an important key to the understanding of the variability observed in the potential of different flush solutions to preserve donor lungs. Optimized lung preservation should therefore involve a procedure that maintains the structural and functional integrity of the pulmonary surfactant system before, during, and after transplantation.

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