Supplementary material

Beneficial effects of a synthetic KL_4-surfactant in experimental lung transplantation

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Supplementary Materials and Methods

Animals and Experimental Groups. Single left-lung transplantation procedures were performed in weight-matched pairs of large (17 to 23 kg) Landrace x Large White pigs, as described previously [1, 2]. A total of 28 pigs was divided randomly into two groups: 1) Untreated group (n = 12, 6 donors and 6 recipients), without any treatment, and 2) KL_4-surfactant-treated group (n = 16, 8 donors and 8 recipients). KL_4-surfactant was provided by Discovery Laboratories (Warrington, PA, USA). Animals (donors and recipients) were sedated with ketamine (20 mg/kg body weight), diazepam (0.1 mg/kg body weight) and atropine (0.02 mg/kg body weight). Anaesthesia was induced with intravenous administration of propofol (2 mg/kg body weight), midazolam (0.6 mg/kg body weight), and fentanyl (5 µg/kg body weight). After endotracheal intubation, anaesthesia was maintained with a continuous intravenous infusion of propofol (9 mg/kg/h), midazolam (0.6 mg/kg/h), fentanyl (5 µg/kg/h,), and pancuronium bromide (0.4 mg/g/h). A ventilator (Adult Star, Infrasonics Inc., San Diego, CA) was used for mechanical ventilation, with the following ventilatory setting: oxygen inspired fraction of 1, inspiratory/expiratory ratio of 1:2, tidal volume of 10 ml/kg, peak airway pressure of 12-20 cm H_2O, and positive end-expiratory pressure of 5 cm H_2O. The initial respiratory rate was 12 breaths/min, and was adjusted to obtain an end-tidal CO_2 between 30 and 40 mmHg. All animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals [National Academies Press, Washington, DC, 1996]

Donor Operation and KL_4-surfactant Treatment. In donors, sternotomy, thymectomy, and anterior pericardectomy were performed, and both venae cavae, aorta, pulmonary artery, and trachea were prepared. Before preservation, the right pulmonary hilum was clamped and tidal volume was reduced by half. Then KL_4-surfactant (or saline in the untreated group) was instilled into the left lung through a closed suction system (Ballard Trach Care Elbow, ET ADPTS (2 mm, 2.5 mm), 5 FR/1.66 mm O.D., 12"/30.5 cm length; Kimberly-Clark, USA) connected between the endotracheal tube and the Y-piece of the ventilator. The instillation of KL_4-surfactant took 5-7 minutes. KL_4-surfactant was instilled at 37 ºC at a dose of 25 mg/kg body weight (2.5 ml/Kg body weight; 10 mg phospholipids/ml) through a continuous flow to the donor left lung, while animals were permanently ventilated. The same volume of saline (2.5 ml/kg body weight) was instilled in the donor left lung of the untreated group. Once the hilum clamp was released and after heparinization (3 mg/kg body weight), the aorta artery was clamped and the heart arrested with a crystalloid cardioplegic solution instilled into the aortic root. Then the lungs were flushed retrogradely with cold (4 ºC) University of Wisconsin solution (UW) (60 ml/kg body weight, under a perfusion pressure not exceeding 30 cm H2O). Continuous ventilation was maintained through the perfusion procedure while both pleural cavities were opened and copiously irrigated with cold saline to

further enhance graft preservation. The trachea was clamped after the lungs were moderately inflated to a pressure of 20 cm H_2O, and the heart-lung block was then removed. Both lungs were immersed in UW solution at 4 ºC for 22 h until implantation. Afterwards, the donor left lung was transplanted into the recipient animal.

Recipient Operation. Weight-matched recipients underwent a left posterolateral thoracotomy through the fourth intercostal space. The hemiazygos vein was ligated and divided, and the pericardium was opened anterior to the pulmonary veins. The three anastomotic stumps were prepared after clamping of the left pulmonary artery at the point of its bifurcation and the left main bronchus proximal to the last branch. Finally, the left atrial appendage was isolated with a side-biting clamp. The left lung removed from recipient pigs and used as control lung. The donor left lung was anastomosed to the recipient. Bronchial anastomosis was performed with a monofilament polypropylene 4–0 suture. Anastomosis of the pulmonar artery and atrial cuff were performed with nonabsorbable 5-0 running sutures leaving the last one untied. The pulmonary artery clamp was removed first to eliminate air and the preservation solution through the venous anastomosis. Then the pulmonary artery clamp was closed, and the venous clamp was removed to allow for backflow. Finally the pulmonary artery clamp was progressively opened. Ventilation was resumed with the ventilatory setting describe above. Upon ventilation of the donor lung, reperfusion was started and time was set to zero.

Ventilatory and Hemodynamic Measurements. Ventilatory and hemodynamic parameters were measured in basal situation (presurgery) and after lung transplantation at 0, 1, and 2 h of reperfusion. A 7-F double-lumen Swan-Ganz thermistor catheter (Edwards Swan-Ganz, Baxter Healthcare Corporation, Deerfield, IL) was introduced into the pulmonary artery through the rigth external jugular vein for direct measurement of cardiac output by thermodilution, central venous pressure, pulmonary artery pressure and wedge pressure. A catheter was inserted into the left carotid artery for direct measurement of blood arterial pressure and heart rate, and for collection of arterial blood for analysis of arterial oxygen tension, carbon dioxide, arterial saturation, hemoglobin, and pH. The arterial catheter and Swan-Ganz catheter were connected to a Vitara PM6080 Monitor (Drager, Madrid, Spain). Arterial blood samples were analyzed by a 1306 pH/Blood Gas Analyzer (Instrument Laboratory, Spain).

Lung Groups, Bronchoalveolar Lavage (BAL) Processing, and Citometric Assays. After 2 h of reperfusion, individual lung lavages were obtained from the left lung removed from the recipient (control group) and the donor left lung transplanted into the recipient in both KL4-surfactant-treated (KL4 group) and untreated (UNT group) animals. Each lung was lavaged twice with 4 ºC saline (50 ml/kg body weight). Lavages were pooled and immediately centrifuged at 400g for 10 min at 4 ºC to collect BAL cells. The volume of the remaining cell-free BAL was recorded. Cells were washed twice with cold saline and resuspended in a final volume of 5 ml. The total number of BAL cells was determined, and cell viability was assessed using trypan blue dye exclusion. BAL cell suspension was used to analyze cell population (alveolar macrophages, neutrophils, and lymphocytes). Cytometric analyses were done in a Fluorescence Flow Cytometer (FACSort, Becton-Dickinson). Cells were incubated with different monoclonal antibodies directed against porcine leukocyte differentiation antigens, labeled with either fluorescein isothiocyanate or R-Phycoerythrin. After incubation with different monoclonal antibodies, cells were washed with cold saline and treated with FACS lysing solution in order to remove erythrocytes. After washing with cold PBS buffer, cells were fixed with 2 % paraformaldehyde in PBS buffer. Generally, 10,000 were counted and fluorescence was measured using a FACScan.

Isolation of Pulmonary Surfactant Aggregates. BAL was centrifuged at 48,000g for 1h at 4 ºC to obtain a pellet of large surfactant aggregates (LA), which is the active form of surfactant (8). The small surfactant aggregates (SA) remained in the 48,000g supernatant. SA contains small surfactant phospholipid vesicles and proteins present in the alveolar fluid. Lipid extracts of LA and SA were obtained by chloroform and methanol extraction [3] to quantify total phospholipids in these fractions by phosphorus analysis as described by Rouser et al. [4].

Biochemical and Biophysical Analysis in Cell-free BAL and Surfactant Fractions. The content of total proteins was determined in cell-free BAL, LA, and SA by Lowry's method modified by adding sodium dodecyl sulphate [5]. Tumour necrosis factor-α (TNF-α) was measured in BAL by a colorimetric ELISA kit from Pierce Endogen (Rockford, IL, USA). The limits of detection for TNF ELISA were 31.3-2000 pg/ml. C-reactive protein (CRP) was measured in BAL using a pig CRP ELISA kit from GenWay (Biotech, Inc., San Diego, USA). The limits of detection for CRP ELISA were 6-200 ng/ml. Lipid hydroperoxides were quantified in the lipid extracts of LA and SA by measuring the oxidation of ferrous iron by hydroperoxides (FOX method) [6] using a lipid hydroperoxide (LPO) assay kit (Cayman Chemical Company, Ann Arbor, MI, USA).

The limits of detection for TNF and CRP ELISAs were 6-200 ng/ml for CRP ELISA and 31.3-2000 pg/ml for TNF-alpha ELISA

Protein Oxidation Assay. The oxidation of proteins was estimated on the basis of their protein carbonyl contents as determined by the dinitrophenylhydrazine (DNPH) spectrophotometric assay [7]. DNPH reacts with protein carbonyls forming the corresponding hydrazone, which is analyzed spectrophotometrically. Briefly, similar amounts of protein (0.5 mg) from cell-free BAL, SA, or LA from control and transplanted lungs were reacted with 10 mM DNPH in HCl 2 M for 1 h at room temperature. Then proteins were precipitated with 6 % of trichloroacetic and washed three times with ethanol/ethylacetate (1:1, v/v). Proteins were then solubilized in guanidine 8 M (in 50 % formic acid) and centrifuged at 16,000g during 5 min to remove traces of insoluble material. Carbonyls were measured at 366 nm (ε=21,000 M−1 cm−1). Assays were performed in triplicate. A protein blank incubated with 2 M HCI not containing DNPH was run for each sample. The results were expressed as nanomoles of DNPH incorporated/mg protein.

Measurement of Surfactant Protein A, B and C by Westen Blot Analysis. Electrophoretic analysis of LA was performed by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using running gels of 12 %, 16 % and 18 % for surfactant proteins A, B, and C, respectively. Reducing conditions (5% β-mercaptoethanol) were used for SP-A and SP-B analysis. The same protein amount (2 μg protein) was applied for all LA samples. Either porcine SP-A, SP-B, or SP-C, previously isolated from control lungs as previously described in [8], was always applied in each gel as standard. After electrophoresis, samples were transferred to nitrocellulose (in case of SP-A and SP-B) or to polyvinylidenedifluoride membranes (PVDF) (for SP-C) using a Bio-Rad Transblot Cell (Bio-Rad, Hercules, CA). Transfer was performed at 100 V constant voltage, with a total increment of intensity of 100 mA, using 25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 20 % (v/v) methanol as a transfer buffer. Blotting was performed as previously described [9]. For SP-A and SP-B, anti-porcine-SP-A and anti-porcine SP-B polyclonal antibodies were used. As for SP-C, an anti-recombinant human-SP-C polyclonal antibody was used. In all cases, proteins were visualized using chemiluminiscence detection (Hyperfilm ECL, Amersham Pharmacia). Finally, quantification of SP-A, SP-B and SP-C was achieved by densitometric evaluation.

Surface Adsorption Assay. The ability of LA isolated from control and transplanted lungs to adsorb onto and spread at the air–water interface was tested at 25ºC in a Wilhelmy-like high-sensitive surface microbalance, coupled to a teflon dish of very small size [1, 2]. The samples were injected into the hypophase chamber of the teflon dish which contained 6 ml of 5 mM Hepes buffer (pH 7.0), 150 mM NaCl, and 5 mM CaCl2, with continuous stirring. The amount of surfactant phospholipids injected in the hypophase was the same for all LA samples. Interfacial adsorption was measured following the variation in surface tension as a function of time. For each preparation, the analysis was repeated three times.

Statistical Analysis. All data represent the mean of individual measurements ± S.D. The means were normally distributed (Shapiro-Wilk test). For statistical analysis, two-tailed unpaired

Student's t test was used for comparison of the two groups of recipients (untreated and KL_4-surfactant-treated) in the analysis of hemodynamic and gasometric parameters. In studies with different lung groups, differences in means between the three lung types (control, UNT, and KL4) were evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc analysis; a confidence level of 95 % or greater ($p < 0.05$) was considered significant.

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Supplementary Tables and Figures

Table S1. Hemodynamic parameters after lung transplantation with (KL4, n=8) and without (UNT, n=6) KL_4-surfactant treatment. Values were measured in basal situation (pre-surgery) and after lung transplantation at 0, 1, 2 h of reperfusion. Results are presented as the mean ± SD. mAP means arterial pressure; CO, cardiac output; mPAP, mean pulmonary artery pressure; PVR, pulmonary vascular resistance; HR, heart rate.

PARAMETER	GROUP	Basal	0h	1h	2h
mAP (mm Hg)	UNT	89 ± 18	87 ± 22	81 ± 24	73 ± 32
	KL4	90 ± 10	79 ± 9	72 ± 14	75 ± 24
CO (L/min)	UNT	2.6 ± 0.5	2.3 ± 0.3	1.8 ± 0.6	1.5 ± 0.6
	KL4	3 ± 0.9	2.3 ± 0.4	2.2 ± 0.2	2.1 ± 0.4
mPAP (mm Hg)	UNT	16 ± 7	17 ± 5	17 ± 8	18 ± 5
	KL4	13 ± 3	19 ± 4	18 ± 4	19 ± 4
PVR (dynes·s·cm^{-5})	UNT	347 ± 245	439 ± 241	590 ± 537	696 ± 521
	KL4	250 ± 193	416 ± 191	430 ± 150	460 ± 185
HR (bpm)	UNT	134 ± 29	116 ± 26	101 ± 23	99 ± 24
	KL4	140 ± 30	128 ± 29	117 ± 28	100 ± 13

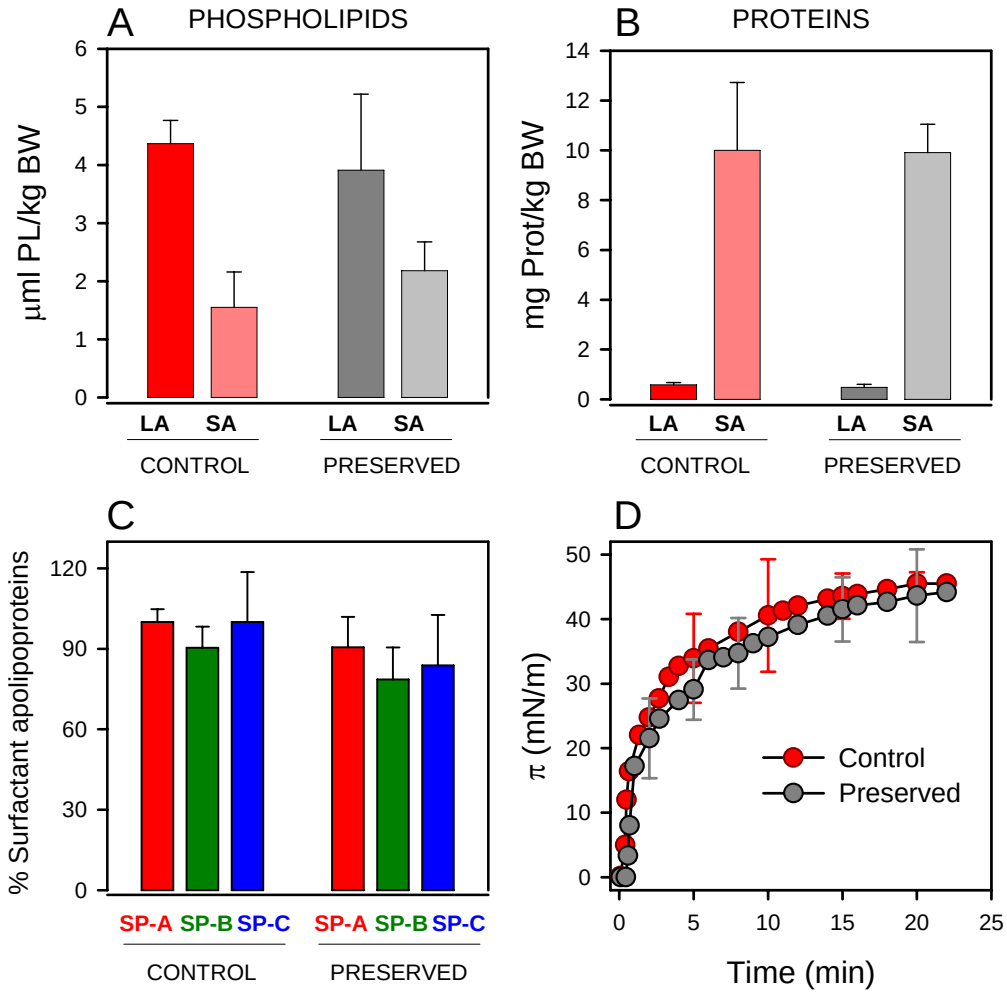

Figure S1. **Analysis of surfactant composition and function of the preserved lungs**. (**A**) Phospholipid content in large (LA) and small (SA) surfactant aggregates; (**B**) Protein content in LA and SA; (**C**) Western blot analysis for SP-A, SP-B and SP-C; (**D**) Interfacial adsorption kinetics of large surfactant aggregates. Results are presented as the mean ± SD. Control lungs (n=10) Preserved lungs (n=10)

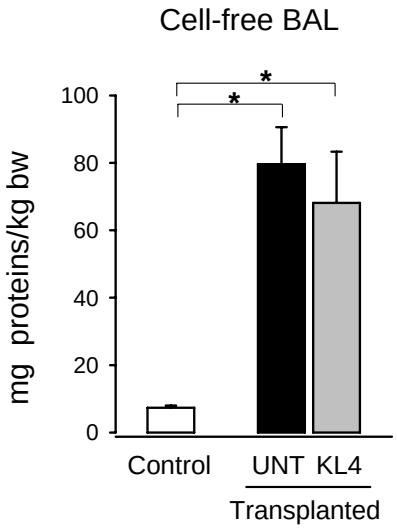

Figure S2. Total amount of proteins in cell-free bronchoalveolar lavage of control (n=10) and transplanted lungs from untreated (UNT, n=6) and KL_4-surfactant-treated (KL4, n=8) groups. Results are presented as the mean + SD. *$p < 0.001$.