Preischemic exogenous surfactant reduces pulmonary injury

in rat ischemia/reperfusion

Christian Mühlfeld[1]*, Inga-Marie Schaefer[1]*, Laura Becker[1], Christine Bussinger[1], Marcel

Vollroth[2], Alexander Bosch[3], Ragi Nagib[2], Navid Madershahian[4], Joachim Richter[3], Thorsten

Wahlers[4], Thorsten Wittwer[4], Matthias Ochs[1]

*These authors contributed equally to this work.

[1] Institute of Anatomy, University of Bern, Baltzerstr. 2, CH-3012 Bern, Switzerland

[2] Department of Cardiothoracic and Vascular Surgery, Friedrich Schiller University,
Erlanger Allee 101, D-07747 Jena, Germany

[3] Department of Anatomy, Division of Electron Microscopy, University of Göttingen,
Kreuzbergring 36, D-37075 Göttingen, Germany

[4] Department of Cardiothoracic Surgery, University Hospital of Cologne,
Kerpener Str. 62, D-50924 Cologne, Germany

Address for correspondence:
Matthias Ochs, M.D.
Institute of Anatomy, University of Bern, Baltzerstr. 2, CH-3012 Bern, Switzerland
Phone: +41 31 631 4624, Fax: +41 31 631 3807, Email: ochs@ana.unibe.ch

Running title: Surfactant in ischemia/reperfusion injury

Word count for body of manuscript: 3038

1

Abstract

The optimal timing of exogenous surfactant application to reduce pulmonary injury and dysfunction was investigated in a rat lung ischemia and reperfusion injury model.

Lungs were subjected to flush perfusion, surfactant instillation, cold ischemia (4°C, 4h) and reperfusion (60 min). Animals received surfactant before (1), at the end (2) of ischemia, during reperfusion (3) or not at all (4). Control groups included worst case without Perfadex and surfactant (5), no-injury without (6) or with surfactant (7) and ischemia with preischemic surfactant (8). Intraalveolar edema and blood-air barrier injury were estimated by light and electron microscopic stereology. Perfusate oxygenation and pulmonary arterial pressure (PAP) were determined during reperfusion in 1 to 4.

Intraalveolar edema was almost absent in 1, 6, 7 and 8, pronounced in 2, 3 and 4, and severe in 5. Blood-air barrier injury was moderate in 1 and 8, slightly pronounced in 2, 3 and 4, extensive in 5 and almost absent in 6 and 7. Perfusate oxygenation was significantly higher in 1 compared with 2 to 4. PAP did not differ between the groups.

In conclusion, exogenous surfactant attenuates intraalveolar edema formation and blood-air barrier damage and improves perfusate oxygenation in the rat lung, especially when applied before ischemic storage.

Introduction:

Although great efforts have been made to improve lung preservation in human lung transplantation, postischemic primary graft dysfunction (PGD) is still a major problem in the early post-transplant period [1, 2]. The main reason for PGD is ischemia/reperfusion (I/R) injury, clinically manifesting within a spectrum from mild acute lung injury (ALI) to severe acute respiratory distress syndrome (ARDS). I/R injury is associated with increased short- and long-term morbidity and mortality of the host [1, 3, 4]. During I/R injury, the graft organ develops structural damage, such as interstitial and intraalveolar edema formation and loss of integrity of the blood-air barrier [5, 6]. In addition, the surfactant system of the lung is severely affected by ischemia and reperfusion, leading to the development of edema and atelectases [7-11].

Several experimental [12-21] and clinical [22-24] studies give evidence that exogenous surfactant therapy successfully supplements the imbalanced endogenous surfactant system, thus serving to attenuate I/R injury and effectively improve lung preservation and graft function [11]. The great advantage of exogenous surfactant therapy of the donor in human lung transplantation is the fact that PGD in this case can accurately be predicted and thus even be prevented, providing a promising approach for prophylactic surfactant therapy [11, 25, 26]. However, there are no systematic studies comparing preischemic surfactant treatment with application during or after ischemia in the same experimental setting. We hypothesized that the timing of exogenous surfactant application in relation to the onset of ischemia influences the structural preservation of the lung.

Therefore, the present study was performed to determine the optimal timing for exogenous surfactant treatment of the donor lung. Using an extracorporeal I/R injury rat lung model, a combined light and electron microscopic approach and design-based stereology [27, 28], we

mainly focused on the extent to which intraalveolar edema and injury of the blood-air barrier

occurred.

Materials and methods

Experimental setting

A total of 40 male Sprague-Dawley-rats (body weight 371 ± 57 g) were used in this study. All animals received humane care and were strictly treated according to the "Guide for the Care and Use of Laboratory Animals", published by the National Institute of Health (NIH publication 85-23, revised in 1996) and in compliance with the current German and Swiss laws. The bioethical committee of the district of Thuringia approved of the experiments.

After administration of pentobarbital (12 mg/100g body weight, i. p.), the trachea was exposed and the animals were intubated endotracheally. Mechanical ventilation was initiated, providing room air at a tidal volume of 5 mL, 40 breaths per minute, positive end-expiratory pressure (PEEP) of 3 cm H_2O, followed by a median laparotomy and systemic heparinization. Then a bilateral longitudinal thoracotomy and punction of the pulmonary trunk were carried out. Flush perfusion of the lung was initiated with 24 - 27 ml of Perfadex (60 ml/kg body weight) at 4° C, maintaining a perfusion pressure of 20 cm H_2O.

After that, the superior and inferior caval veins and the left azygos vein were clipped. Heart and lungs were excised en bloc with clamped trachea and stored ex situ in 30-40 mL preservation solution for 4 hours at 4°C (i.e. ischemia time). Lungs were treated with instillation of the exogenous surfactant preparation poractant alfa (Curosurf®, 200 mg/kg body weight; a kind gift from Nycomed Pharma GmbH, Linz, Austria) according to the experimental setting. Before instillation of exogenous surfactant, the lungs were once inflated at a pressure of 23 cm H_2O to recruite potentially collapsed regions of the lung [29]. Surfactant drawn up in a syringe in a volume of 4-5 mL air was then carefully instilled via the tracheal cannula. The alveolar recruitment procedure was performed again to make sure that surfactant reached the alveolar regions of the lungs and normal ventilation was restarted

afterwards. Postexperimental visualization of histological lung sections was used to confirm the even distribution of exogenous surfactant in the lung. Extracorporeal reperfusion was performed using the extracorporeal heart-lung circuit described in details in Fukuse et al. [30]. In short, the circuit consisted of a reservoir, a temperature probe (for perfusate temperature monitoring), a roller pump to raise the perfusate to the oxygenators, a blood filter and two membrane oxygenators. A perfusion pool was used at 80 cm H_2O and a preload pool at 5 cm H_2O. Mechanical ventilation was performed with a small animal respirator. The perfusate consisted of Krebs-Henseleit buffer, supplemented with bovine erythrocyte concentrate (haematocrit of 38-40 %) for 60 minutes under the same ventilation conditions as mentioned above using a quattro head roller pump (Mod-Reglo-Digital; Ismatec, Zurich, Switzerland).

Animals were randomly assigned to 8 different groups (n=5 animals per group). Experimental groups included: group 1 which received initial surfactant instillation, flush perfusion, ischemia and reperfusion; group 2, being flush perfused, followed by surfactant instillation at the end of ischemia, and reperfusion; group 3 which was flush perfused, then stored under ischemia and finally reperfused while a bolus of surfactant was applied after 20 minutes of reperfusion; and group 4, being flush perfused, stored under ischemia and reperfused without receiving surfactant. Additional groups included: group 5, which was flush perfused with 0.9 % NaCl solution, followed by ischemia and reperfusion as worst case control; group 6 being fixed in situ immediately after flush perfusion before excision as no-injury control; group 7, treated with surfactant prior to flush perfusion and fixation; and group 8 receiving surfactant before flush perfusion, followed by ischemia (see Figure 1).

Measurement of functional data

Pulmonary arterial pressure and oxygenation were recorded during reperfusion as described previously [5]. Perfusate oxygenation (ΔPO_2), defined as the difference between oxygen tension of the perfusate collected from the left atrium after oxygenation (PO_{2ox}) and of the deoxygenated perfusate of the pre-load pool (PO_{2deox}), was used to assess the capability of gas exchange. Pulmonary arterial pressure (PAP) was determined by a pressure transducer (Statham, PVB Medizintechnik, Germany).

Fixation, sampling and tissue processing

Fixation, sampling and tissue processing were performed as described previously [27, 31, 32]. The left lung was fixed by vascular perfusion of a fixative containing 4 % paraformaldehyde and 0.1 % glutaraldehyde in 0.2 M HEPES-buffer. Afterwards, the main bronchus and artery were clamped and the left lung was excised and stored in 4°C cold fixative.

The total lung volume was determined by fluid displacement. Then the lungs were embedded in agar and sectioned from apex to base using a tissue slicer into 9-12 tissue slices of 3 mm thickness. Systematic uniform random sampling was used to obtain representative tissue blocks for stereological analysis [27]. Every other slice was used for light (LM) or transmission electron microscopy (TEM), respectively. For TEM, small specimens were sampled from the lung slices by overprojection of a transparent uniform point grid on each slice. Whenever a point hit a lung slice a tissue sample was taken at the given location and stored in the fixative for at least 24 hours.

For LM, the slices were postfixed in osmium tetroxide, washed again in sodium cacodylate and destilled water, immersed in half-saturated watery uranyl acetate overnight, dehydrated in

ascending acetone concentrations and embedded in glycol methacrylate resin (Technovit 7100®, Heraeus Kulzer, Wehrheim, Germany) overnight. From the embedded tissue blocks sections of 1 μm thickness were cut, mounted on glass slides and stained with methylene blue.

For TEM, the tissue blocks were postfixed in osmium tetroxide, stained en bloc in half-saturated watery uranyl acetate, dehydrated in an ascending acetone series and finally embedded in an epoxy resin (Araldite®, Serva Electrophoresis, Heidelberg, Germany; polymerization: 5 days at 60°C). Ultrathin sections of 40-70 nm thickness were obtained from the tissue blocks and stained with lead citrate and uranyl acetate using an Ultrostainer (Leica, Bensheim, Germany).

Stereological analysis

Design-based stereological methods were used to analyse the lung samples and obtain the quantitative data [27, 28, 33].

LM analyses were carried out using an Axioscope light microscope (Zeiss, Oberkochen, Germany) and a computer-assisted stereology system (CAST 2.0, Olympus, Ballerup, Denmark). Systematic uniform random sampling produced representative test fields for further estimations, and a point grid with defined number of test points was projected onto the slices. Subsequently the volume densities (V_V) were estimated by counting the points hitting a structure, (P_{str}), and the points hitting the reference space, (P_{ref}), with V_V (*str/ref*) := P_{str}/P_{ref}. Then the volume densities were converted to the total lung volume by multiplication with the reference volume V (*str,ref*) := V_V *(str/ref)* × *V(ref)*.

TEM was performed using an EM 900 (Zeiss, Oberkochen, Germany), supplemented with a digital camera (Megaview III, Soft Imaging System, Münster, Germany) and an image

analysis software (AnalySIS 3.1, Soft Imaging System). Systematic uniform random sampling was applied to the ultrathin sections, and digital micrographs were taken at a final magnification of $\times$ 20.000 whenever a test field included thin parts of the blood-air barrier. A test system consisting of parallel line segments and points was superimposed. By point and intersection counting, the arithmetic mean barrier thickness of the alveolar epithelium, interstitium and capillary endothelium was estimated, adopting the equation $\bar{\tau} := (l_T/2) \times (P_b/I_b)$, with $\bar{\tau}$ being the arithmetic mean barrier thickness, l_T the length of a test line, P_b the number of points hitting a barrier profile and I_b the number of intersections of the test lines with the reference surface of the barrier [27, 28]. In order to obtain further information about the extent to which the blood-air barrier was affected, semiquantitative characterization of the barrier was performed in addition. Three different categories were defined: 1 - normal, with the alveolar epithelium and capillary endothelium presenting normal electron-dense ultrastructure and a thin interstitium. 2 – swollen, with swelling of endothelial and/or epithelial structures in one or more parts of the blood-air barrier. 3 – fragmented, with disruptions of alveolar epithelium and/or capillary endothelium and denudation of the basement membrane. Thus a blood-air barrier integrity index was estimated. The surface area fractions (S_S) of normal, swollen and fragmented alveolar epithelium were estimated by relating the number of intersections of one category to the total number of intersections with the blood-air barrier [5].

The sampling and counting was designed to obtain between 100 and 200 uniformly randomly distributed counting events per lung for each parameter. This ensures that the total observed variability is dominated by the biological variability between animals and not by the variability due to the stereological procedure [33].

Statistics

Data are given as mean ± SD. Stereological data were analyzed as follows: According to the overall hypothesis of the study, comparison of the experimental groups 1-4 was performed using Kruskal-Wallis one way ANOVA on ranks. If $p < 0.05$, those groups that contributed to the overall intergroup differences were isolated by the all pairwise multiple comparison procedure (Student-Newman-Keuls Method). Control groups were compared to the corresponding experimental group (i.e. group 5 vs. group 4, group 6 vs. group 7, and group 8 vs. group 1) by the Whitney-Mann u test. Functional data between groups were analyzed by one way ANOVA, and subsequently Tukey B or Tamhane test. Differences between groups were considered statistically significant at $p < 0.05$.

Results:

Perfusate oxygenation and pulmonary arterial pressure

Perfusate oxygenation was significantly higher in group 1 than in groups 2 to 4 at 20, 40, and 50 minutes. It was also significantly higher than groups 2 and 4 at 30 min, and showed a tendency to be higher than group 3 at 30 min. There were no significant differences in perfusate oxygenation between groups 2 to 4 at any point in time. The mean pulmonary arterial pressure was similar in groups 1 to 4 and did not show any significant difference at any point in time (Figure 2).

Qualitative light and electron microscopy

The lung tissue in all LM and TEM sections showed only few atelectatic areas and small amounts of erythrocytes left in capillaries and alveolar lumen, whereas most blood vessels were opened widely. Groups treated with surfactant instillation (i.e. groups 1, 2, 3, 7 and 8) presented similarly abundant amounts of surfactant in the alveoli compared to the non-treated groups, verifying that the applied substance effectively reached its intended destination. The no-injury control group 6 showed normal parenchymal lung structure and free airspaces without intraalveolar edema or erythrocytes. In general, these structural findings clearly indicate that the perfusion fixation of the lung tissue was performed successfully, leaving only very few structural alterations that might be due to the experimental procedure (Figure 3).

In the ischemia and reperfusion treated groups 1 to 5 pronounced evidence for ultrastructural injury could be detected. The extent of injury depended on whether and when surfactant was applied. In the non ischemia groups 6 and 7 lung ultrastructure was widely intact and signs of injury were missing. Group 8, which was exposed to ischemia but not to reperfusion, showed moderate lung damage due to ischemic conditions (Figure 4).

Intraalveolar edema formations as well as extravascular accumulations of erythrocytes and blood-air barrier damage were found to a variable extent, but clear differentiation between groups required a formal quantitative, i.e. stereological approach.

Stereology

In order to quantify intraalveolar edema formation at the LM level, the volumes of edema fluid, *V (ed,lung)*, and intraalveolar erythrocyte accumulation, *V (ery,lung)*, were added to the total volume of intraalveolar edema, *V (eryed,lung)* [mL per lung]. This parameter showed that intraalveolar edema formation appeared only slightly in groups 1 (0.01 mL ± 0.01) and 6 (0.01 mL ± 0.04), pronounced in groups 2 (0.07 mL ± 0.08), 3 (0.03 mL ± 0.03) and 4 (0.20 mL ± 0.23), and severe in group 5 (0.64 mL ± 0.36). In Groups 7 and 8 no intraalveolar edema or erythrocytes were found (0.00 mL ± 0.00). Peribronchovascular edema formation was estimated by the ratio of wall to luminal space of non-parenchymal vessels and airways, with groups 2, 3, 4 and 5 disposing of a higher wall/lumen ratio than the other groups. However, these findings were not significant.

A blood-air barrier integrity index was determined for each group, indicating that the alveolar epithelium was moderately swollen in groups 1 (1.80 ± 0.14) and 8 (1.72 ± 0.07), fragmented/swollen in groups 2 (2.20 ± 0.35), 3 (1.98 ± 0.15) and 4 (2.17 ± 0.20) and almost normal in groups 6 (1.26 ± 0.03) and 7 (1.33 ± 0.08) (Figure 5). Estimation of the arithmetic mean thickness of the blood-air barrier, $\bar{\tau}$ *(bab)*, resulted in the same sequence of groups, with groups 1 (367 nm ± 44) and 8 (400 nm ± 71) showing similarly moderate affection, groups 2 (518 nm ± 83), 3 (404 nm ± 88) and 4 (524 nm ± 92) with pronounced swelling, followed by group 5 being affected worst (646 nm ± 165). In groups 6 (358 nm ± 22) and 7 (358 nm ± 30) no swelling of the blood-air barrier was present. The arithmetic mean thickness of the

interstitial space, $\bar{\tau}$ *(int),* of group 5 showed a significantly increased swelling compared to all other groups. Moreover, all groups exposed to ischemia (i. e. groups 1 to 5, 8) presented considerable swelling of the blood-air barrier, distinct from the non ischemia groups (6 and 7) without swelling (Table 2).

Altogether, the results of the present study demonstrate that surfactant instillation attenuates ultrastructural injury in lungs subjected to ischemia and reperfusion, especially when applied prior to the period of ischemia.

Discussion

In human lung transplantation, I/R injury-caused PGD is a dreaded complication, remaining a significant cause of short- and long-term morbidity and mortality [1, 3, 4]. The clinical manifestation of I/R injury includes edema formation, an increase in pulmonary artery pressure and hypoxemia and ranges from mild ALI to severe ARDS [1, 10]. Several studies have identified the important role of surfactant alterations in transplantation-related I/R injury of the lung [8, 9, 34-39]. Accordingly, exogenous surfactant therapy has been applied successfully in experimental [12-21] and clinical [22-24] studies. It is therefore considered a potentially promising therapy to mitigate severe lung I/R injury, although the optimal surfactant preparation and mode of therapy still need to be determined [26, 40].

In lung transplantation, surfactant can be given before organ retrieval, i.e. it is one of the few situations in which it can be applied prophylactically [11]. Previous experimental studies indicate beneficial effects of surfactant treatment for graft lung function especially when applied before ischemia [12, 16]. In addition, experimental evidence shows that improvement of the endogenous surfactant system via application of keratinocyte growth factor successfully decreases transplantation associated I/R injury in rats [41]. A recent clinical study also suggests that exogenous surfactant therapy of donor lungs before retrieval protects post-transplantation surfactant function [23]. However, there are no systematic studies comparing preischemic surfactant treatment with application during ischemia or during reperfusion in the same experimental setting. Moreover, the structural correlate of successful surfactant therapy is not known. Therefore, the aim of the present study was to systematically define the optimal time point for exogenous surfactant instillation in I/R injured lungs using a combined light and electron microscopic and stereological approach. An established extracorporeal rat lung I/R injury model was used in this study, including the whole sequence

of transplantation-related events, namely flush perfusion, cold ischemic storage and subsequent reperfusion [5, 8, 9]. A limitation of this model is that it is not a real transplantation model. Therefore, effects resulting from the interaction between donor and host cannot be investigated. Perfadex® was used as preservation solution for its proved efficacy in previous studies [6, 42, 43], except for one group being flush perfused with NaCl to induce severe I/R injury. As for the surfactant preparation, we decided to use Curosurf® derived from porcine lung tissue, which contains the hydrophobic surfactant proteins SP-B and SP-C and which is routinely applied for the treatment of neonatal respiratory distress syndrome [44]. In our analysis we focused on the stereological estimation of intraalveolar edema formation and the ultrastructural integrity of the blood-air barrier as well as perfusate oxygenation and pulmonary arterial pressure. It has been shown previously by quantitative stereological assessment that the degree of ultrastructural injury is functionally relevant in lung I/R injury as it closely correlates with postischemic lung function [5, 8, 9]. Additionally, impaired oxygenation is one of the main features of PGD [1] and improvement of perfusate oxygenation levels as observed after preischemic surfactant therapy therefore strongly confirms the stereological data in the current study.

Intraalveolar edema formation is a hallmark of I/R injury, resulting in an increased barrier thickness for pulmonary gas exchange. According to Fick's law of diffusion, the development of intraalveolar edema reduces the oxygenation capacity of the lung. We used design-based stereological methods to quantify the degree of edema formation in its preserved micro-organisation and localisation within the organ. These parameters are more sensitive indicators for impaired lung function and better correlate with the respiratory capacities of the organ than the wet-to-dry ratio [5, 9, 45]. This high sensitivity is important since in the present study, the amount of intraalveolar edema is rather low. A previous direct structural-functional correlation study demonstrated that with a volume fraction of more than 3%, intraalveolar

edema formation becomes functionally relevant [45]. In the present study, only the two reperfused groups that did not receive surfactant had an intraalveolar edema volume fraction of more than 3% of the parenchymal volume: group 4 (3.6%) and group 5 (7.9%) (see Table 1). In addition, the perfusate oxygenation is significantly better in group 1 than in groups 2-4 which strongly underlines the stereological data. Accordingly, the present study shows that exogenous surfactant therapy, especially when applied preischemically, effectively attenuates intraalveolar edema formation, thus contributing to improved lung preservation and the prevention of I/R injury.

I/R injury also leads to disintegration of the blood-air barrier, including swelling of the interstitium and swelling or fragmentation of epithelial and endothelial cells [5, 6]. Estimation of the arithmetic mean barrier thickness by electron microscopy yields information on the extent of interstitial as well as epithelial and endothelial edema formation [6]. The estimation of a blood-air barrier integrity index as an established method to quantify the ratio of normal, swollen and fragmented parts of the blood-air barrier provides important information on the degree of lung damage [5, 6]. Based on this approach, the present results suggest that preischemic surfactant instillation is superior to application during or after ischemia with respect to the attenuation of blood-air barrier injury (Figure 5), however, these data do not reach significance level.

In conclusion, the present study provides quantitative morphological and functional evidence that surfactant application significantly attenuates I/R injury by reducing intraalveolar edema formation and blood-air barrier injury and improving perfusate oxygenation. This observation was most pronounced when surfactant was administered before the onset of ischemia, a finding which offers a rationale for pretreatment of the donor lung with surfactant in lung transplantation in order to improve lung preservation quality.

Acknowledgements:

References

1. Christie JD, Carby M, Bag R, Corris P, Hertz M, Weill D. Report of the ISHLT working group on primary lung graft dysfunction part II: definition. A consensus statement of the International Society for Heart and Lung Transplantation. *J Heart Lung Transplant* 2005; 24: 1454-59.

2. Mulligan MS. Primary graft dysfunction following lung transplantation: pathogenesis and impact on early and late outcomes. *In*: Lynch III JP, Ross DJ, eds. Lung and heart-lung transplantation. Taylor & Francis, New York, 2006; pp. 437-464.

3. Christie JD, Kotloff RM, Ahya VN, Tino G, Pochettino A, Gaughan C DeMissie E, Kimmel SE. The effect of primary graft dysfunction on survival after lung transplantation. *Am J Respir Crit Care Med* 2005; 71: 1312-16.

4. Daud SA, Yusen RD, Meyers BF, Chakinala MM, Walter MJ, Aloush AA, Patterson GA, Trulock EP, Hachem RR. Impact of immediate primary lung allograft dysfunction on bronchiolitis obliterans syndrome. *Am J Respir Crit Care Med* 2007; 175: 507-13.

5. Fehrenbach H, Schepelmann D, Albes JM, Bando T, Fischer F, Fehrenbach A Stolte N, Wahlers T, Richter J. Pulmonary ischemia/reperfusion injury: a quantitative study of structure and function in isolated heart-lungs of the rat. *Anat Rec* 1999; 255: 84-89.

6. Mühlfeld C, Müller K, Pallesen LP, Richter J, Wahlers T, Wittwer T, Ochs M. Impact of preservation solution on the extent of edema formation and blood-air barrier damage in experimental lung transplantation. *Anat Rec* 2007; 290: 491-500.

7. Lewis JF, Novick RJ, Veldhuizen RAW. Surfactant in lung injury and lung transplantation. New York: Springer; 1997.

8. Ochs M, Nenadic I, Fehrenbach A, Albes JM, Wahlers T, Richter J, Fehrenbach H. Ultrastructural alterations in intraalveolar surfactant subtypes after experimental ischemia and reperfusion. *Am J Respir Crit Care Med* 1999; 160: 718-24.

9. Ochs M, Fehrenbach H, Nenadic I, Bando T, Fehrenbach A, Schepelmann D Albes JM, Wahlers T, Richter J. Preservation of intraalveolar surfactant in a rat lung ischaemia/reperfusion injury model. *Eur Respir J* 2000; 15: 526-31.

10. De Perrot M, Liu M, Waddell TK, Keshavjee S. Ischemia-reperfusion-induced lung injury. *Am J Respir Crit Care Med* 2003;167: 490-511.

11. Lewis JF, Veldhuizen R. The role of exogenous surfactant in the treatment of acute lung injury. *Annu Rev Physiol* 2003; 65: 613-42.

12. Novick RJ, MacDonald J, Veldhuizen RA, Wan F, Duplan J, Denning L Possmayer F, Gilpin AA, Yao LJ, Bjarneson D, Lewis JF. Evaluation of surfactant treatment strategies after prolonged graft storage in lung transplantation. *Am J Respir Crit Care Med* 1996; 154: 98-104.

13. Buchanan SA, Mauney MC, Parekh VI, DeLima NF, Binns OA, Cope JT, Shockey KS, Tribble CG, Kron IL. Intratracheal surfactant administration preserves airway compliance during lung reperfusion *Ann Thorac Surg* 1996; 62: 1617-1621.

14. Erasmus ME, Petersen AH, Hofstede G, Haagsman HP, Bambang Oetomo S, Prop J. Surfactant treatment before reperfusion improves the immediate function of lung transplants in rats. *Am J Respir Crit Care Med* 1996;153: 665-670.

15. Novick RJ, Gilpin AA, Gehman KE, Ali IS, Veldhuizen RA, Duplan J, Denning L, Possmayer F, Bjarneson D, Lewis JF. Mitigation of injury in canine lung grafts by exogenous surfactant therapy. *J Thorac Cardiovasc Surg* 1997; 113: 342-353.

16. Hausen B, Rohde R, Hewitt CW, Schroeder F, Beuke M, Ramsamooj R Schäfers HJ, Borst HG. Exogenous surfactant treatment before and after sixteen hours of ischemia in experimental lung transplantation. *J Thorac Cardiovasc Surg* 1997; 113: 1050-58.

17. Hohlfeld JM, Strüber M, Ahlf K, Hoeper MM, Fraund S, Krug N Warnecke G, Harringer W, Haverich A, Fabel H. Exogenous surfactant improves survival and surfactant function in ischaemia-reperfusion injury in minipigs. *Eur Respir J* 1999; 13: 1037-43.

18. Erasmus ME, Hofstede GJ, Petersen AH, Batenburg JJ, Haagsman HP, Oetomo SB, Prop J. SP-A-enriched surfactant for treatment of rat lung transplants with SP-A deficiency after storage and reperfusion. *Transplantation* 2002; 73: 348-352.

19. Friedrich I, Börgermann J, Splittgerber FH, Brinkmann M, Reidemeister JC, Silber RE, Seeger W, Schmidt R, Günther A. Bronchoscopic surfactant administration preserves gas exchange and pulmonary compliance after single lung transplantation. *J Thorac Cardiovasc Surg* 2004; 127: 335-343.

20. Günther A, Balser M, Schmidt R, Markart P, Olk A, Börgermann J, Splittgerber FH, Seeger W, Friedrich I. Surfactant abnormalities after single lung transplantation in dogs: impact of bronchoscopic surfactant administration. *J Thorac Cardiovasc Surg* 2004; 127: 344-354.

21. Van der Kaaij NP, Haitsma JJ, Kluin J, Lambrecht BN, Lachmann B, de Bruin RW, Bogers AJ. Surfactant pretreatment ameliorates ischemia-reperfusion injury of the lung. *Eur J Cardiothorac Surg* 2005; 27: 774-782.

22. Strüber M, Hirt SW, Cremer J, Harringer W, Haverich A. Surfactant replacement in reperfusion injury after clinical lung transplantation. *Intensive Care Med* 1999; 25: 862–64.

23. Strüber M, Fischer S, Niedermeyer J, Warnecke G, Gohrbandt B, Görler A Simon AR, Haverich A, Hohlfeld JM. Effects of exogenous surfactant instillation in clinical lung transplantation: a prospective, randomized trial. *J Thorac Cardiovasc Surg* 2007; 133: 1620-1625.

24. Kermeen FD, McNeil KD, Fraser JF, McCarthy J, Ziegenfuss MD, Mullany D, Dunning J, Hopkins PM. Resolution of severe ischemia-reperfusion injury post-lung transplantation after administration of endobronchial surfactant. *J Heart Lung Transplant* 2007; 26: 850-856.

25. Novick RJ, Gehman KE, Ali IS, Lee J. Lung preservation: the importance of endothelial and alveolar type II cell integrity. *Ann Thorac Surg* 1996; 62: 302-14.

26. Novick RJ. Innovative techniques to enhance lung preservation. *J Thorac Cardiovasc Surg* 2002; 123: 3-5.

27. Ochs M. A brief update on lung stereology. *J Microsc* 2006; 222: 188-200.

28. Ochs M. Stereological analysis of acute lung injury. *Eur Respir Rev* 2006; 15: 115-121.

29. Lachmann B. Open up the lung and keep the lung open. *Intensive Care Med* 1992 ; 18 : 319-321.

30. Fukuse T, Albes JM, Takahashi Y, Brandes H, Hausen B, Schäfers HJ. Influence of red blood cells on lung function in an ex vivo rat heart-lung model. *J Surg Res* 1995 ; 59 : 399-404.

31. Fehrenbach H, Ochs M. Studying lung ultrastructure: *In*: Uhlig S, Taylor AE, editors. Methods in pulmonary research. Birkhäuser, Basel, 1998; pp. 429-454.

32. Mühlfeld C, Rothen-Rutishauser B, Vanhecke D, Blank F, Gehr P, Ochs M. Visualization and quantitative analysis of nanoparticles in the respiratory tract by transmission electron microscopy. *Part Fibre Toxicol* 2007; 4:11.

33. Weibel ER, Hsia CCW, Ochs M. How much is there really? Why stereology is essential in lung morphometry. *J Appl Physiol* 2007; 102: 459-467.

34. Veldhuizen RA, Lee D, Sandler D, Hull W, Whitsett JA, Lewis J, Possmayer F, Novick RJ. Alterations in pulmonary surfactant composition and activity after experimental lung transplantation. *Am Rev Respir Dis* 1993; 148: 208-215.

35. Erasmus ME, Petersen AH, Bambang Oetomo S, Prop J. The function of surfactant is impaired during the reimplantation response in rat lung transplantation. *J Heart Lung Transplant* 1994; 13: 791-802.

36. Andrade RS, Solien EE, Wangensteen OD, Tsai MY, Kshettry VR, Bolman RM. Surfactant dysfunction in lung preservation. *Transplantation* 1995; 60: 536-41.

37. Casals C, Varela A, Ruano ML, Valiño F, Pérez-Gil J, Torre N, Jorge E, Tendillo F, Castillo-Olivares JL. Increase of C-reactive protein and decrease of surfactant protein A in surfactant after lung transplantation. *Am J Respir Crit Care Med* 1998; 157: 43-49.

38. Hohlfeld JM, Tiryaki E, Hamm H, Hoymann HG, Krug N, Haverich A, Fabel H. Pulmonary surfactant activity is impaired in lung transplant recipients. *Am J Respir Crit Care Med* 1998; 158: 706-712.

39. Maitra G, Inchley K, Novick RJ, Veldhuizen RAW, Lewis JF, Possmayer F. Acute lung injury and lung transplantation influence in vitro subtype conversion of pulmonary surfactant. *Am J Physiol Lung Cell Mol Physiol* 2002; 282: L67-L74.

40. Van Raemdonck D, Strüber M, Venuta F, Vlasselaers D, Wisser W, Erasmus ME. Strategies in the prevention and the treatment of ischaemia-reperfusion injury after lung transplantation. *Eur Respir Mon* 2004; 29: 66-88.

41. Sadovski J, Kuchenbuch T, Ruppert C, Fehrenbach A, Hirschburger M, Padberg W, Günther A, Hohlfeld JM, Fehrenbach H, Grau V. Keratinocyte growth factor prevents intra-alveolar oedema in experimental lung isografts. *Eur Respir J* 2008; 31: 21-28.

42. Strüber M, Hohlfeld JM, Fraund S, Kim P, Warnecke G, Haverich A. Low-potassium dextran solution ameliorates reperfusion injury of the lung and protects surfactant function. *J Thorac Cardiovasc Surg* 2000; 120: 566-572.

43. Wittwer T, Albes JM, Fehrenbach A, Pech T, Franke UF, Richter J, Wahlers T. Experimental lung preservation with Perfadex: effect of the NO-donor nitroglycerin on postischemic outcome. *J Thorac Cardiovasc Surg* 2003; 125: 1208-1216.

44. Saugstad OD, Curstedt T, Halliday HL, Robertson B, Speer CP. Surfactant replacement therapy from 1986 to 2006: a 20-year success story. *Biol Neonate* 2006; 89 :282-283.

45. Fehrenbach A, Fehrenbach H, Wittwer T, Ochs M, Wahlers T, Richter J. Evaluation of pulmonary edema: stereological versus gravimetrical analysis. *Eur Surg Res* 2001; 33: 270-278.

Table 1: Light microscopic results of stereological analysis.

Group	1	2	3	4	5	6	7	8
BW rat [g]	360 (53)	368 (34)	376 (62)	366 (70)	404 (30)	430 (15)	360 (75)	304 (5)
	* vs. 8							* vs. 1
V(lung) [mL]	5.2 (0.9)	4.8 (1.5)	4.6 (0.6)	6.2 (1.6)	8.7 (1.9)	5.3 (0.6)	5.7 (.1.0)	5.6 (0.5)
				* vs. 5	* vs. 4			
V (par,lung) [mL]	4.68 (1.11)	4.06 (1.18)	4.07 (0.59)	5.57 (1.51)	8.04 (1.66)	4.91 (0.62)	5.32 (1.03)	5.02 (0.55)
				* vs. 5	* vs. 4			
V (nonpar,lung) [mL]	0.54 (0.27)	0.75 (0.41)	0.48 (0.15)	0.58 (0.26)	0.71 (0.37)	0.43 (0.29)	0.42 (0.26)	0.60 (0.39)
V (ed,lung) [mL]	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.12 (0.19)	0,22 (0.17)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	* vs. 4	* vs. 4	* vs. 4	* vs. 1-3				
V (ery,lung) [mL]	0.01 (0.01)	0.07 (0.08)	0.03 (0.03)	0.08 (0.09)	0.41 (0.25)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	* vs. 8			* vs. 5	* vs. 4			* vs. 1
V (eryed,lung) [mL]	0.01 (0.01)	0.07 (0.08)	0.03 (0.03)	0.20 (0.23)	0.64 (0.36)	0.01 (0.04)	0.00 (0.00)	0.00 (0.00)
	* vs. 2-4, 8	* vs. 1	* vs. 1	* vs. 1, 5	* vs. 4			* vs. 1
V (air,lung) [mL]	4.49 (1.08)	3.76 (1.15)	3.87 (0.54)	5.37 (1.33)	7.40 (1.46)	4.90 (0.62)	5.16 (1.14)	4.87 (0.51)
				* vs. 5	* vs. 4			
V (surf,lung) [mL]	0.18 (0.12)	0.23 (0.11)	0.17 (0.08)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.16 (0.17)	0.14 (0.06)
	* vs. 4	* vs. 4	* vs. 4	* vs. 1-3		* vs.7	* vs. 6	
V (lum,nonpar) [mL]	0.32 (0.15)	0.35 (0.11)	0.24 (0.08)	0.25 (0.11)	0,30 (0,16)	0.23 (0.12)	0.25 (0.14)	0.39 (0.25)
V (wall,nonpar) [mL]	0.22 (0.11)	0.40 (0.32)	0.24 (0.09)	0.34 (0.21)	0.42 (0.22)	0.20 (0.18)	0.18 (0.13)	0.22 (0.13)
Wall-to-lumen ratio	0.72 (0.12)	1.05 (0.55)	1.05 (0.29)	1.42 (0.97)	1.42 (0.21)	0.74 (0.43)	0.68 (0.11)	0.61 (0.11)

Stereological data are given as mean with standard deviation (SD). Abbreviations:

BW: body weight; V: total volume; par: parenchyma; nonpar: non-parenchyma; ed: intraalveolar edema; ery: erythrocytes; air: air-filled spaces within parenchyma; surf: intraalveolar surfactant; lum: lumen of non-parenchymal vessels and airways, wall: wall of non-parenchymal vessels and airways; * = p<0.05.

Table 2: Electron microscopic results of stereological analysis.

Group	1	2	3	4	5	6	7	8
$\bar{\tau}$ (endo) [nm]	160 (10)	193 (24)	161 (23)	208 (35)	246 (82)	146 (24)	151 (18)	153 (26)
$\bar{\tau}$ (int) [nm]	69 (19)	98 (19)	73 (23)	99 (16)	130 (26)	42 (9)	41 (12)	76 (14)
$\bar{\tau}$ (epi) [nm]	139 (21)	227 (62)	171 (45)	217 (59)	269 (72)	170 (25)	167 (27)	171 (45)
$\bar{\tau}$ (bab) [nm]	367 (44)	518 (83)	404 (88)	524 (92)	646 (165)	358 (22)	358 (30)	400 (71)
S_S (normal) [%]	24.6 (9.9)	15.3 (9.4)	18.4 (7.3)	10.3 (6.8)	1.6 (3.0)	74.4 (3.0)	67.4 (8.2)	28.5 (7.6)
S_S (swollen) [%]	70.6 (7.5)	49.4 (19.9)	65.0 (16.1)	62.7 (14.6) * vs. 5	27.4 (18.5) * vs. 4	25.6 (3.0)	32.7 (8.2)	71.2 (7.9)
S_S (fragmented) [%]	4.8 (4.9)	35.3 (27)	16.6 (13.6)	27.0 (16.1) * vs. 5	71.0 (21.4) * vs. 4	0.00 (0.00)	0.00 (0.00)	0.3 (0.38)
bab integrity index	1.80 (0.14)	2.20 (0.35)	1.98 (0.15)	2.17 (0.2) * vs. 5	2.71 (0.20) * vs. 4	1.26 (0.03)	1.33 (0.08)	1.72 (0.07)

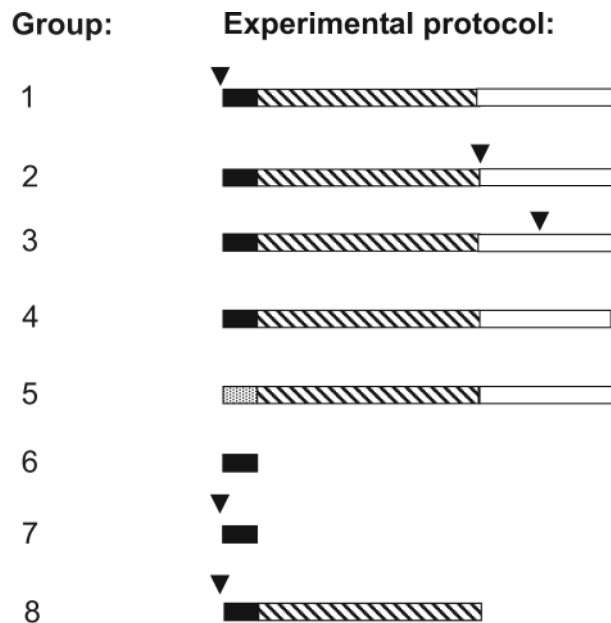Stereological data are given as mean with standard deviation (SD). Abbreviations:

$\bar{\tau}$: arithmetic mean barrier thickness; endo: endothelium; int: interstitium; epi: epithelium;

bab: blood-air barrier; S_S: surface area fraction of total surface area; * p<0.05.

Figure legends:

Figure 1:

Overview of the experimental protocol.

Figure 2:

Perfusate oxygenation and mean pulmonary arterial pressure. * = p<0.05.

Perfusate oxygenation
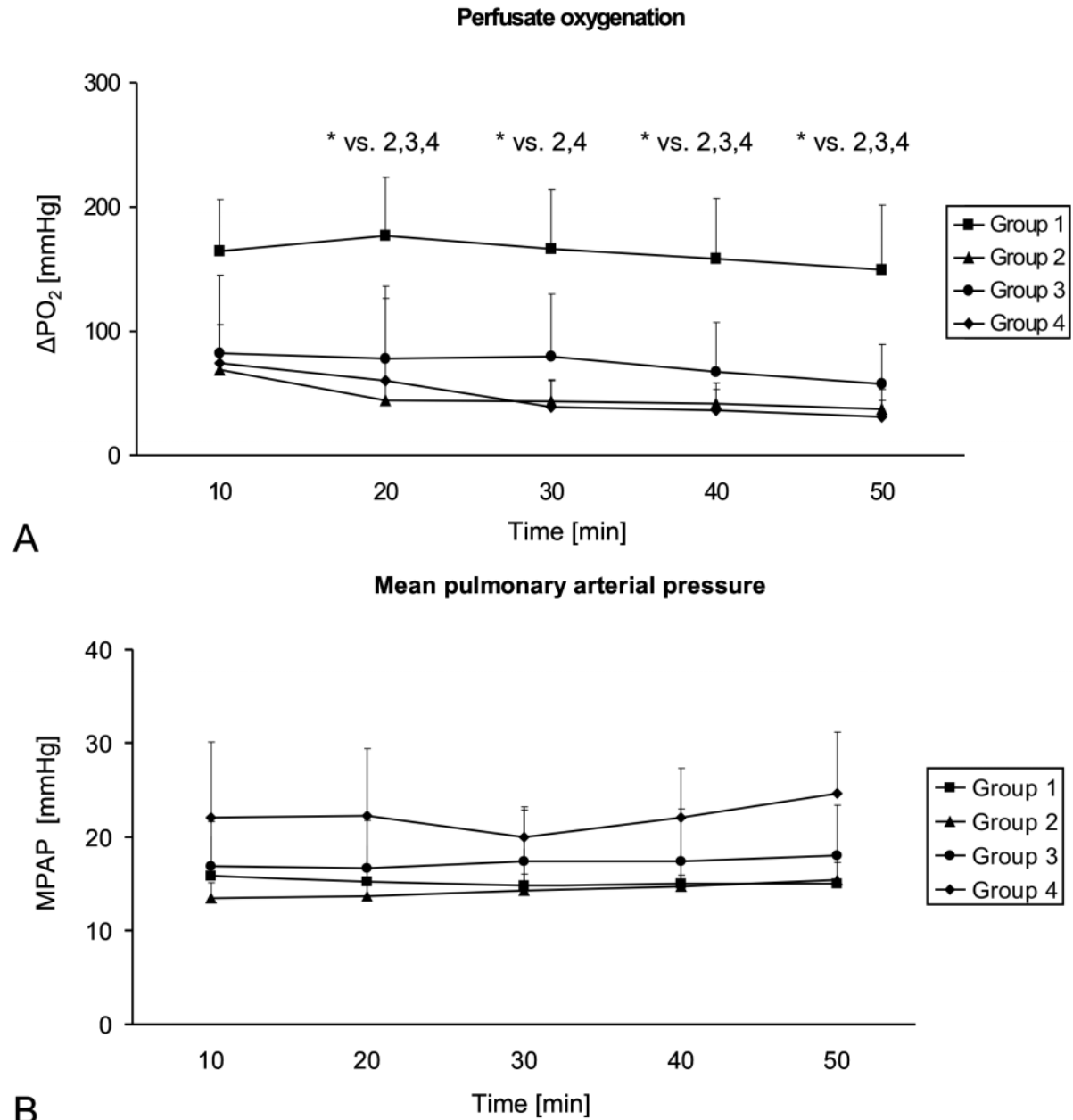

A

Mean pulmonary arterial pressure

B

Figure 3:

Light micrographs showing lung histology in groups 1 (A), 2 (B), 3 (C), 4 (D), 5 (E), 6 (F), 7 (G), and 8 (H). Surfactant treatment before ischemia and reperfusion (A) diminished intraalveolar edema (ed) formation and extravasation of erythrocytes (ery) (compare B - E), resulting in a structural preservation close to the no-injury control groups without (F) and with (G) surfactant (surf).
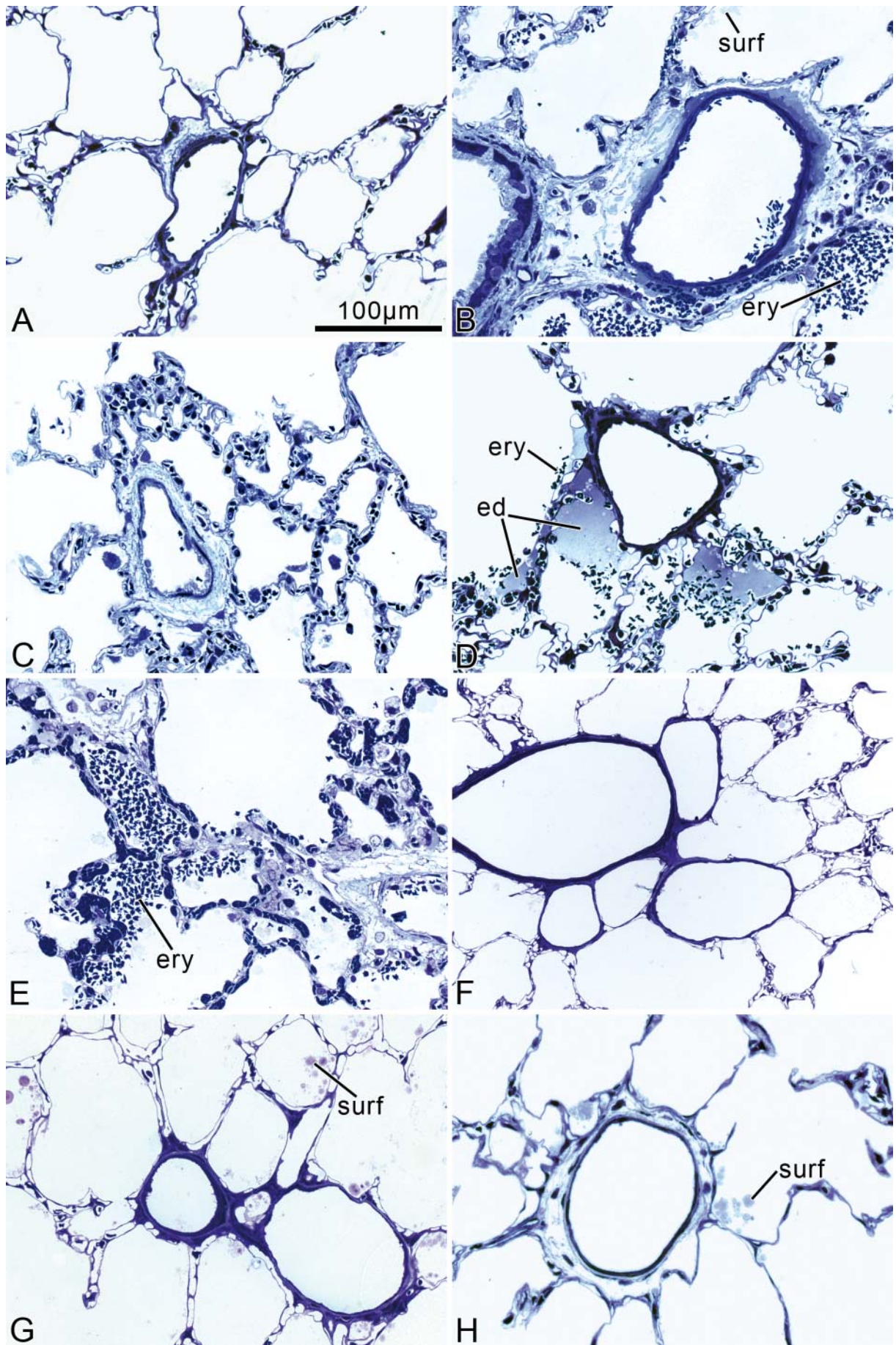
Figure 4:

Electron micrographs showing blood-air barrier ultrastructure in groups 1 (A), 2 (B), 3 (C), 4 (D), 5 (E), 6 (F), 7 (G), and 8 (H). Surfactant treatment before ischemia and reperfusion (A) diminished swelling and fragmentation of the components of the blood-air barrier (compare B - E), resulting in a structural preservation close to the no-injury control groups without (F) and with (G) surfactant. alv = alveolar lumen, cap = capillary lumen, ery = erythrocyte, epi = alveolar epithelium, int = interstitium, endo = capillary endothelium, surf = intraalveolar surfactant.
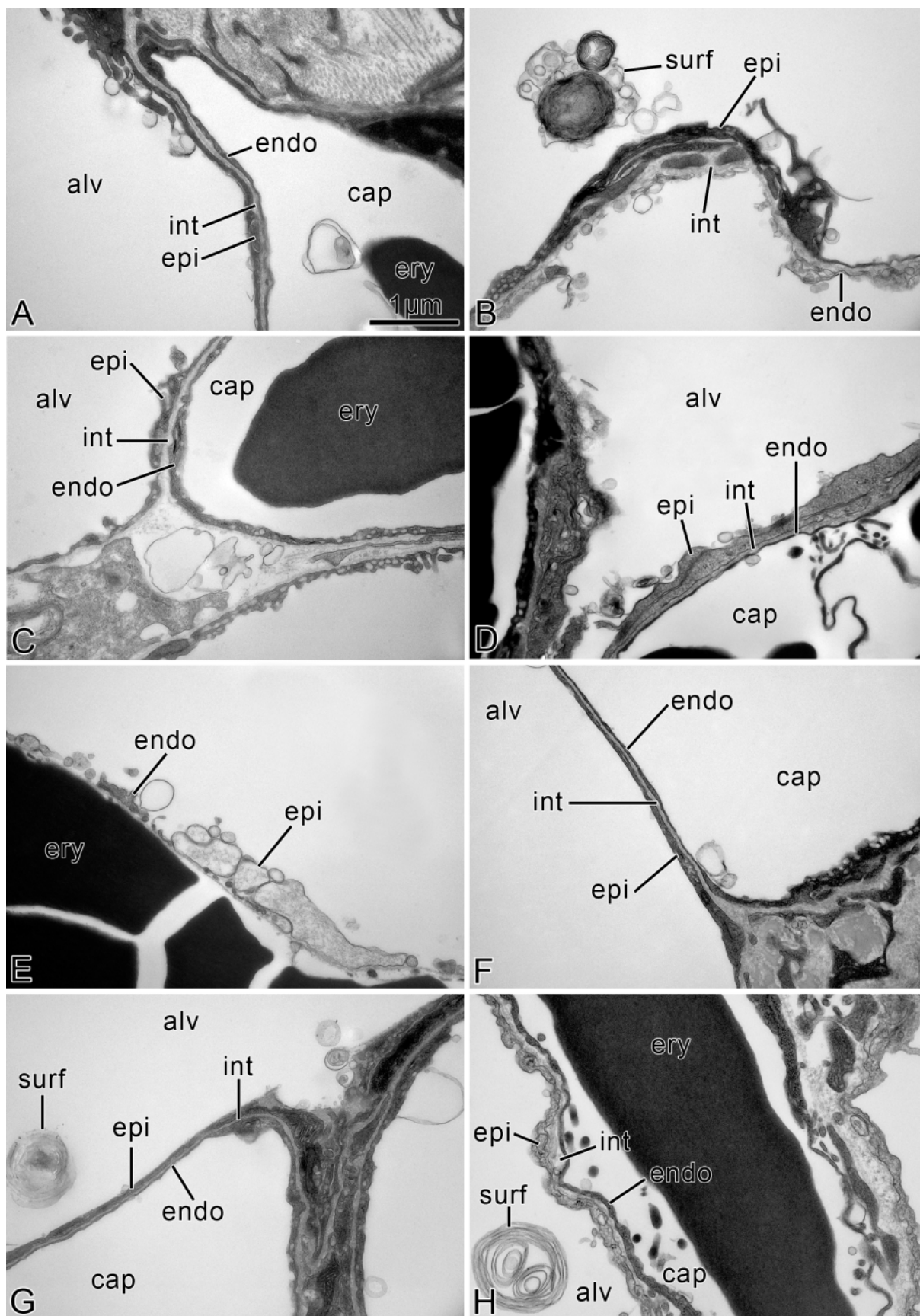
Figure 5:

Fraction of normal, swollen, and fragmented surface of the blood-air barrier. Surfactant treatment before ischemia and reperfusion (group 1) resulted in a deceased fraction of fragmented blood-air barrier surface (compare groups 2 - 5). The fraction of swollen blood-air barrier surface is comparable to the same treatment without reperfusion (group 8). Only mild swelling was present due to the experimental procedure (groups 6 and 7).