Surfactant pretreatment ameliorates ischemia-reperfusion injury of the lung

Niels P. van der Kaaija,b,*, Jack J. Haitsmac, Jolanda Kluina, Bart N. Lambrechtd, Burkhard Lachmanna, Ron W.F. de Bruinb, Ad J.J.C. Bogersa

aDepartment of Cardio-Thoracic Surgery, Erasmus MC Rotterdam, Rotterdam, The Netherlands
bDepartment of Surgery, Erasmus MC Rotterdam, Rotterdam, The Netherlands
cDepartment of Anesthesiology, Erasmus MC Rotterdam, Rotterdam, The Netherlands
dDepartment of Pulmonary Diseases, Erasmus MC Rotterdam, Rotterdam, The Netherlands

Objective: To investigate whether surfactant pretreatment provides lung protection in an animal model of lung ischemia-reperfusion injury (LIRI). Methods: Male Sprague-Dawley rats (n = 100) were randomised to receive intratracheally administered surfactant or no pretreatment. One hour thereafter, animals underwent 120 min of warm ischemia of the left lung, or were sham-operated. A third group served as healthy untreated controls. Animals were killed on day 1, 3 or 7. Blood gas values were measured and lung compliance was recorded. Broncho-alveolar lavage fluid (BALF) was obtained to assess the amount of alveolar protein, the ratio of small to large aggregate surfactant phospholipids (SA/LA ratio), and leukocyte infiltration (granulocytes, macrophages and lymphocytes, measured by Flow Cytometry). Results: LIRI resulted in a mortality rate of 17% and significantly decreased lung compliance and PaO2 (day 1 and 3 P < 0.001, day 7 P < 0.05) as compared to sham-operated and healthy controls. On day 1 more protein was present in the alveoli of ischemic lungs (P < 0.001) than in sham-operated and healthy controls. Furthermore, LIRI resulted in an increased SA/LA ratio in the left lung on day 1 (P < 0.05) and caused infiltration of granulocytes (day 1, 3 and 7 (P < 0.01)), macrophages (day 3 (P < 0.05) and 7 (P < 0.01) and lymphocytes (day 3 and 7 (P < 0.01)) in the BALF as compared to sham-operated and healthy controls. Surfactant pretreatment improved survival, lung compliance (day 3 P < 0.001) and PaO2 (day 1, 3 (P < 0.01 and 7 (P < 0.05)). It also reduced protein leakage (P < 0.05) and prevented an increase in the SA/LA ratio (P < 0.01). Although the number of macrophages and granulocytes in the BALF was increased on day 1 and 3 (P < 0.01) after surfactant pretreatment as compared to all other groups, the number of lymphocytes was reduced on day 3 (P < 0.05). Conclusions: The present study shows that surfactant pretreatment enhances recovery of lung function and lung mechanics after LIRI, resulting in normal parameters from day 3 onwards. Surfactant pretreatment in this LIRI model may provide useful information to improve donor lung function after lung transplantation.

Keywords: Ischemia-reperfusion injury; Lung; Pulmonary surfactant; Animal models

1. Introduction

Since 1966, experimental data generated in lung transplantation models have provided evidence for surfactant abnormalities and depletion after lung ischemia-reperfusion injury (LIRI) [1–6]. In 1998, Hohlfeld and colleagues demonstrated surfactant alterations in human lung transplant recipients, more than 1 year after transplantation [7]. Active pulmonary surfactant is composed of phospholipids and surfactant associated proteins and is essential for normal lung physiology, since it lowers the surface tension at the air-fluid interface inside the alveolus [8]. Surfactant hereby prevents alveoli from collapsing at the end of expiration and preserves the fluid homeostasis across the alveolo-capillary membrane. Inactivation of surfactant occurs due to the formation of reactive oxygen species (ROS), proteolytic enzymes, eicosanoids and lipases after LIRI and leads to a disturbed fluid balance homeostasis, resulting in pulmonary edema, decreased lung compliance and impaired gas exchange [8,9]. Severe LIRI occurs in 15–30% of lung transplant recipients and contributes to morbidity and mortality after lung transplantation resulting in a one-year survival rate of less than 80% [10]. Because the endogenous surfactant pool is damaged by LIRI, the effect of surfactant replacement therapy has been investigated using experimental models of LIRI [1–4]. Most of the studies investigating surfactant treatment have


Corresponding author. Address: Erasmus MC Rotterdam, Postbus 1738, 3000 DR Rotterdam, The Netherlands. Tel.: +31 10 4087693/624816437; fax: +31 10 4089471.

E-mail address: n.vanderkaaij@erasmusmc.nl (N.P. van der Kaaij).

1010-7940/® - see front matter © 2005 Elsevier B.V. All rights reserved.
addressed only the first few hours after reperfusion and focused mainly on treatment of the recipients. The administration of exogenous surfactant just before or after reperfusion resulted in improved lung function within hours after reperfusion [2,3]. However, treatment with surfactant before the onset of ischemia has been shown to be more beneficial for lung function than treatment just before or after reperfusion up to several hours after reperfusion [2].

Studies on the effect of surfactant treatment with longer follow-up are scarce. A study by Erasmus and colleagues demonstrated that surfactant treatment after ischemia enhanced recovery from LIRI at 1 week postoperatively [1]. Nevertheless, it is presently unknown what the effect of surfactant treatment before ischemia is on the longer term following LIRI. Therefore, the present study investigated whether surfactant treatment before the induction of ischemia ameliorates LIRI, as assessed by lung function and leukocyte infiltration on multiple time points up to 1 week after LIRI in a warm ischemia model in the rat.

2. Materials and methods

2.1. Experimental design and animals

The experimental protocol was approved by the Animal Experiments Committee under the national Experiments on Animals Act and adhered to the rules laid down in this national law that serves the implementation of “Guidelines on the protection of experimental animals” by the Council of Europe (1986), Directive 86/609/EC.

One hundred male Sprague-Dawley rats (Harlan, Horst, The Netherlands) weighing 301 ± 40 g were randomised into four groups: surfactant pretreated LIRI (n = 30), untreated LIRI (n = 30), sham-operated (n = 30), and healthy controls (n = 10). Healthy rats did not receive any treatment, sham-operated animals underwent a thoracotomy, dissection of the left lung hilus and were ventilated and anesthetized during the same period as LIRI animals, but did not receive ischemia. Untreated and surfactant pretreated animals underwent 120 min of warm ischemia of the left lung.

Exogenous natural porcine surfactant, HL-10™ (Leo Pharmaceutical Products, Ballerup, Denmark and Halas Pharma, Oldenburg, Germany), dissolved in 50 mg/ml of saline, was administered in the surfactant pretreated group intratracheally in three dosages (total dose 400 mg/kg bodyweight) over 1 h after the animals were briefly anesthetized (65% nitrous oxide/33% oxygen/2% isoflurane) and intubated. After each dosage, animals recovered from anesthesia and breathed spontaneously to allow the instilled surfactant to be adsorbed. One hour after the first dosage, animals were operated. Surfactant pretreated LIRI, untreated LIRI and sham-operated animals were killed 1, 3 or 7 days after operation.

Although the warm ischemia model used in this experiment does not fully reflect human lung transplantation, it has recently been shown that there are no major differences in injury after warm and cold ischemia, and that the use of short periods of warm lung ischemia is accepted as an accelerated model for LIRI after cold preservation [11].

2.2. Surgical procedure

Animals were anesthetized with 60 mg/kg of intraperitoneally administered Ketalin® (ketaminhydrochloride, 100 mg/ml) and a gaseous mixture of 3% isoflurane, 64% NO2 and 33% O2, whereafter they were intubated and pressure control ventilated (14 cmH2O peak inspiratory pressure (PIP), 4 cmH2O positive endexpiratory pressure (PEEP), frequency 30 breaths/min, 1.5% isoflurane, 58% NO2 and 40% O2) on a Servo ventilator 900C (Siemens Elema, Solna, Sweden). A left dorsolateral thoracotomy in the fourth intercostal space was performed. The left lung was mobilizedatraumatically and the mediastinum was dissected around the left lung hilus. Hereafter, animals of the LIRI groups underwent 120 min of warm lung ischemia by clamping the bronchus, pulmonary artery and veins of the left lung in inflated state, using one microvascular clamp. During the operation, the left hemithorax was covered by a moist tissue to minimize evaporative loss. After 120 min ischemia, the clamp was removed and the lung was recruited by a stepwise increase of PIP with a maximum PIP of 60 cmH2O until the lung was visually expanded. Recruitment of the lung, if needed, was also performed in sham-operated animals. The thorax was closed and the animals received 5 ml of 5% glucose intraperitoneally and 0.1 mg/kg of Temgesic® (buprenorphinhydrochloride, 0.3 mg/ml) intramuscularly and were weaned from the ventilator. Throughout the whole experiment, body temperature was kept within normal range by the use of a heating pad, placed underneath each animal. Postoperatively, animals were allowed to recover with additional oxygen during the first 12 h. All animals received tap water ad libitum and standard laboratory pellets, and were inspected daily.

At the end of the experiment, all animals were anesthetized with 20 mg/kg of intraperitoneally administered Nembutal® (pentobarbital 60 mg/ml) and a gaseous mixture of 3% isoflurane, 64% NO2 and 33% O2 and weighed. A polyethylene catheter (0.8 mm outer diameter) was inserted into the carotid artery for drawing arterial blood samples and a metal cannula was inserted into the trachea. Thereafter, gaseous anesthesia was discontinued, and anesthesia was continued with 20 mg/kg Nembutal, administered intraperitoneally. Blood samples were obtained by taking 0.3 ml heparinized blood from the carotid artery to record blood gas values (PaO2 and PaCO2) in an ABL555 gas analyzer (Radiometer, Copenhagen, Denmark) before animals were pressure control ventilated. Muscle relaxation was induced by 0.7 mg/kg Pavulon® (pancuronium bromide 2 mg/ml) intramuscularly. Additional blood gas values were determined after 10 min of ventilation (12 cmH2O PIP, 2 cm H2O PEEP, frequency 30 breaths/minute and FiO2 of 1), whereafter animals were exsanguinated and euthanised by an overdose of Nembutal® (200 mg/kg), administered arterially.

2.3. Pressure-volume curve

After the animals were killed, the thorax and diaphragm were opened to eliminate the influence of chest wall compliance and abdominal pressure. A static pressure-volume curve (PVC) of the left lung was recorded using
conventional techniques [12]. This was conducted by clamping the contralateral hilum. Maximal compliance of expiration (MCE) was determined as the steepest part of the lung deflation curve. Furthermore, maximal lung volume ($V_{\text{max}}$), corrected for body weight, was recorded at a pressure of 35 cmH$_2$O.

2.4. Broncho-alveolar lavage

After recording the PVC, the left and right lung were lavaged separately with 5 ml of sodiumchloride 1.5 mM CaCl$_2$ five times. Individual lung broncho-alveolar lavage fluid (BALf) analysis was accomplished by clamping the contralateral hilum. Total recovered volume of BALf was noted.

Hereafter, the BALf of left and right lung tissue was centrifuged at 400×g and 4°C for 10 min to pellet the cells. Supernatant of left and right BALf was stored at −20°C for analysis of the surfactant and the amount of alveolar protein. The red blood cells in the pellet of the left BALf were lysed with murine osmotic lysis buffer, whereafter the suspension was washed with murine Fluorescence-Activated Cell Sorter (FACS) buffer (MFB) (phosphate buffered saline (PBS), 0.05% sodium azide and 5% bovine serum albumin), centrifuged at 400×g and 4°C for 10 min and resuspended in MFB. Cells were counted, using a Bürker-Turk cell counter (Erma, Tokyo, Japan).

2.5. SA/LA ratio and protein concentration

The supernatant of the BALf was centrifuged at 4°C for 15 min at 40,000×g to separate the surface-active surfactant pellet (large aggregates (LA)) from the non-surface active supernatant fraction (small aggregates (SA)). LA was resuspended in 2 ml NaCl, whereafter phosphorus concentration of LA and the supernatant (SA) was determined by phospholipid extraction, followed by phosphorus analysis [13]. Finally, the ratio inactive SA to active LA surfactant was calculated.

The centrifuged supernatant was also used to determine alveolar protein concentration by using a Beckmann DU 7400 photospectrometer with a wavelength set at 595 nm (Beckmann, Fullerton, California, USA), as described by Bradford (Bio-Rad protein assay, Munich, Germany) [14]. Bovine serum albumin (Sigma, St Louis, MO, USA) was used as a standard.

2.6. Flow Cytometry

Following recovery and centrifuging of the BALf as described above, pelleted cells (1×10$^6$ cells per well) were incubated on ice with 2% normal rat serum (NRS) in MFB for 15 min to prevent non-specific binding of Fc-receptors with the primary antibody. Hereafter, cells were washed with MFB twice, centrifuged for 3 min at 400×g and 4°C and surface stained for 30 min on ice with the primary mouse anti rat IgM antibody, HIS 48 (diluted 1/40 in MFB and 2% NRS). After centrifuging and washing twice with MFB, primary staining of the HIS48 antibody was revealed by secondary staining with goat anti mouse IgM, conjugated to STAR phycoerythrin (PE) (diluted 1/50 in MFB and 2% NRS) for 30 min on ice.

BALf differentiation was calculated based on morphology (Side SCatter (SSC) for granularity, Forward SCatter (FSC) for size), autofluorescence and HIS48 staining. Cells were identified as follows: lymphocytes low FSC, low SSC and no autofluorescence; granulocytes low FSC, intermediate SSC and HIS48 positive; macrophages as high SSC and FSC and autofluorescence (Fig. 1) [15].

Samples were acquired on a FACScalibur Flow Cytometer (Becton Dickinson Immunocytometry Systems, Franklin Lakes, New Jersey, USA) and were analysed using Flow Jo 6.0 software package (Tree Star, Ashland, OR, USA). The antibodies were obtained commercially (Serotec, Kidlington, UK).

Fig. 1. Flow Cytometry dot plots of cells acquired from BALf of a healthy rat (A–C) and an untreated LIRI animal (D–F). Macrophages (M) are high on FSC (large size) and on SSC (high granularity), (B) autofluorescent are the most frequently occurring cells in the BALf of healthy rats. Lymphocytes (L) are low on FSC and SSC. LIRI caused infiltration of granulocytes (G), which are intermediate on FSC and SSC, and (E) HIS48 PE positive. Plot C shows that the cells in the gate on plot B are predominantly macrophages and plot F proves that the HIS48 PE positive cells on plot E are granulocytes. BALf, broncho-alveolar lavage fluid; FSC, forward scatter; SSC, side scatter; LIRI, lung ischemia-reperfusion injury.
2.7. Statistical analysis

The results in text and tables are presented as mean ± SD and data were analysed using SPSS version 11.1 statistical software (SPSS Inc., Chicago, IL, USA). In the figures the data are displayed as mean ± SEM. If the distribution within a group was normal, as assessed by the Kolmogorov-Smirnov test, and if the condition of equal variances was met by the Levene’s test, differences between groups were tested for significance by one-way ANOVA. If the overall level of the ANOVA was significant, intergroup comparisons were made by the Bonferroni post hoc test. In the case of unequal variances or an abnormal distribution, a non-parametric Kruskal-Wallis test was performed, followed by Mann-Whitney U-tests for intergroup comparisons. The difference in mortality rate between untreated and surfactant pre-treated groups was assessed by the Fisher’s exact test. \( P \) values < 0.05 were considered to be significant.

3. Results

3.1. Survival

In the untreated LIRI group, 5 of 30 (17%) operated animals died shortly after weaning from the ventilator, due to respiratory failure. After surfactant pretreatment, only 1 out of 30 animals (3%) died due to respiratory failure. However, the difference in mortality between the untreated and surfactant pretreated LIRI groups was not significant (\( P = 0.19 \)). All sham-operated animals survived the experimental period.

3.2. PaO2 and PaCO2

\( \text{PaO}_2 \) was similar in all groups before the start of ventilation (Fig. 2). After 10 min of ventilation with an \( \text{FiO}_2 \) of 1, \( \text{PaO}_2 \) increased five-fold in healthy and sham-operated animals. However, LIRI caused a significantly lower \( \text{PaO}_2 \) on day 1, 3 and 7 after reperfusion compared to sham-operated and healthy controls. After surfactant pretreatment, \( \text{PaO}_2 \) had improved on day 1 compared to untreated LIRI animals. Normal, pre-operative values were found in surfactant pretreated animals on day 3 and 7 in contrast to untreated LIRI animals. No significant differences were seen in \( \text{PaCO}_2 \) on any day (data not shown).

3.3. Pressure-volume curve

MCE and \( V_{\text{max}} \) did not differ between sham-operated and healthy controls (Fig. 3, Table 1). However, LIRI had detrimental effects on the static PVC: on both day 1 and 3, MCE and \( V_{\text{max}} \) of untreated LIRI animals were lower than the values found in sham-operated and healthy controls (Fig. 3A and B, Table 1). On day 7, the \( V_{\text{max}} \) was still significantly lower than in healthy and sham-operated animals (Fig. 3C, Table 1).

After surfactant pretreatment, \( V_{\text{max}} \) and MCE were still significantly lower than in sham-operated and healthy controls on day 1 (Fig. 3A, Table 1). Pretreatment with surfactant restored lung compliance to the levels observed in sham-operated and healthy animals on day 3 and 7 as compared to untreated LIRI animals. For mean and SD see Table 1. LIRI, lung ischemia-reperfusion injury.
surfactant restored lung compliance to the level observed in sham-operated and healthy animals on day 3 and 7 as compared to untreated LIRI animals (Fig. 3B and C, Table 1).

### 3.4. Alveolar protein

The total amount of alveolar protein measured in the BALF of the separately measured left and right lung was not influenced by sham operation (Fig. 4). However, 120 min of warm ischemia and reperfusion induced a significant increase in the amount of alveolar protein in the BALF of the left and right lung as compared to sham-operated and healthy controls on day 1. When animals were pretreated with surfactant, the total protein content of the left BALF was decreased on day 1 compared to the untreated LIRI animals. However, significantly more proteins were still found in the alveolar spaces than in sham-operated and healthy controls. On day 3 and day 7 no differences in the amount of alveolar protein were found between all groups.

### 3.5. SA/LA ratio

Whereas sham operation had no effect on the SA/LA ratio (Fig. 5), an increased ratio was seen after LIRI on day 1 compared to sham-operated and healthy controls. This was predominantly due to an increased total amount of SA (Table 2).

Pretreatment with surfactant prevented an increase in the SA/LA ratio on day 1. The instilled exogenous surfactant consisted predominantly of the LA form, thereby inducing an increase in the amount of LA in both the left and right BALF on day 1 and 3 as compared to all other groups (Table 2). In surfactant pretreated animals, the conversion of LA to SA after LIRI also occurred, causing a significantly higher level of SA in both lungs on day 1, 3 and 7 as compared to all other groups.

### 3.6. Flow cytometry

Macrophages were the most frequently occurring cells in the BALF of healthy and sham-operated rats on all days (Figs. 1 and 6) [11]. No significant changes were seen in the total number of macrophages, granulocytes and lymphocytes after sham operation (Fig. 6).

If animals were subjected to ischemia and reperfusion without surfactant pretreatment, granulocytes infiltrated the alveolar spaces and became the most abundant cell on day 1. Over time, the total amount of granulocytes in the BALF of untreated LIRI animals decreased, whereas the fraction of macrophages increased with a peak on day 3. Also, 3 days after reperfusion, the total number of lymphocytes increased 15-fold and was also higher on day 7 as compared to sham-operated and healthy controls (Fig. 6).

In surfactant pretreated animals, a significant increase in the total number of macrophages and granulocytes was found on day 1 and 3 as compared to all other groups.
On day 3, the number of lymphocytes in the BALf of surfactant pretreated animals was lower than in untreated LIRI animals.

4. Discussion

LIRI resulted in a mortality rate of 17%, a decreased PaO₂, and impaired lung compliance. It also caused an increase in the amount of alveolar protein, an increase in the SA/LA ratio, and an influx of granulocytes, macrophages and lymphocytes in the BALf. Surfactant pretreatment reduced mortality to 3%, and resulted in a normal lung function already on day 3 postoperatively. It further decreased alveolar protein leakage in the ischemic lung and resulted in a normal SA/LA ratio. Finally, although more macrophages and granulocytes were found on day 1 and 3 after surfactant pretreatment, the number of lymphocytes had decreased.

Both experimental and clinical studies have shown the beneficial effect of surfactant treatment to lung transplant recipients within several hours after reperfusion [1–4,16]. Surfactant therapy before ischemia is thought to be more beneficial than treatment at the time of reperfusion or after reperfusion [2]. This may be explained by the fact that surfactant given to the donor results in a more homogenous distribution in the lung as compared to treatment at reperfusion, when alveolar damage has already occurred [8]. In the latter case, intratracheally instilled surfactant will predominantly accumulate in open areas of the lung instead of atelectatic areas, where it is most needed. Moreover, Erasmus and colleagues showed that the endogenous surfactant pool is inversely proportional to the time of

<table>
<thead>
<tr>
<th>Phospholipids (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Left BALf</td>
</tr>
<tr>
<td>SA</td>
</tr>
<tr>
<td>Mean SD</td>
</tr>
<tr>
<td>214 73</td>
</tr>
<tr>
<td>470 142</td>
</tr>
<tr>
<td>Healthy</td>
</tr>
<tr>
<td>175 107</td>
</tr>
<tr>
<td>677 321</td>
</tr>
<tr>
<td>Sham day 1</td>
</tr>
<tr>
<td>412 a 154</td>
</tr>
<tr>
<td>724 b 245</td>
</tr>
<tr>
<td>Sham day 3</td>
</tr>
<tr>
<td>340 c 107</td>
</tr>
<tr>
<td>553 277</td>
</tr>
<tr>
<td>Sham day 7</td>
</tr>
<tr>
<td>230 d 54</td>
</tr>
<tr>
<td>543 302</td>
</tr>
<tr>
<td>LIRI day 1</td>
</tr>
<tr>
<td>640 e 77</td>
</tr>
<tr>
<td>347 261</td>
</tr>
<tr>
<td>LIRI day 3</td>
</tr>
<tr>
<td>184 f 104</td>
</tr>
<tr>
<td>242 258</td>
</tr>
<tr>
<td>LIRI day 7</td>
</tr>
<tr>
<td>194 g 114</td>
</tr>
<tr>
<td>223 a 62</td>
</tr>
<tr>
<td>LIRI and su day 1</td>
</tr>
<tr>
<td>1008 h 406</td>
</tr>
<tr>
<td>486 d 1797</td>
</tr>
<tr>
<td>LIRI and su day 3</td>
</tr>
<tr>
<td>1251 i,j 1265</td>
</tr>
<tr>
<td>2763 d,e 822</td>
</tr>
<tr>
<td>LIRI and su day 7</td>
</tr>
<tr>
<td>459 j 172</td>
</tr>
<tr>
<td>555 f 110</td>
</tr>
</tbody>
</table>

SA and LA phospholipids were measured in the BALf of healthy, sham-operated, LIRI untreated, and surfactant pretreated LIRI groups on day 1, 3 and 7. The data are presented as mean ± SD. BALf, broncho-alveolar lavage fluid; LIRI, lung ischemia-reperfusion injury; Su, surfactant pretreatment; SA, small aggregate; LA, large aggregate.

- a P < 0.05 versus healthy animals.
- b P < 0.05 versus untreated LIRI group.
- c P < 0.01 versus healthy animals.
- d P < 0.01 versus healthy and sham-operated group on corresponding day.
- e P < 0.01 versus untreated LIRI group.
- f P < 0.05 versus healthy and sham-operated group on corresponding day.

On day 3, the number of lymphocytes in the BALf of surfactant pretreated animals was lower than in untreated LIRI animals.

Fig. 6. Total number of macrophages, lymphocytes and granulocytes in the BALf of the left lung of healthy, sham-operated, LIRI untreated, and surfactant pretreated groups on day 1, 3 and 7. LIRI caused an influx of granulocytes (day 1, 3 and 7), macrophages (day 3 and 7), and lymphocytes (day 3 and 7). In surfactant pretreated animals, very high numbers of macrophages and granulocytes were present on day 1 and 3. On day 3, the number of lymphocytes in the BALf was lower in surfactant pretreated animals than in untreated LIRI animals. LIRI: lung ischemia-reperfusion injury; BALf: broncho-alveolar lavage fluid. * P < 0.05, ** P < 0.01 versus healthy and sham-operated on corresponding day. # P < 0.05, ## P < 0.01 versus untreated LIRI on corresponding day.
ischemia because of the remaining surfactant inactivating activity of phospholipases during ischemia [5]. This results in influx of serum proteins, which further inhibit surfactant function. Because the normal endogenous surfactant pool contains approximately 10-15 mg lipid per kg, pretreatment with 400 mg lipid per kg body weight exogenous surfactant, predominantly in the LA subform, substantially enlarges the surface-active surfactant pool. The high level of the LA subform found on day 1, 3 and 7 after pretreatment illustrates this. Although, after surfactant pretreatment, conversion of LA into SA still occurred as indicated by the increased levels of SA in the pretreated group on all days, probably sufficient surface-active phospholipids remained after pretreatment and LIRI to result in normal lung function.

All mortality in our experimental study was due to development of severe pulmonary edema. The accumulation of fluid in the alveolus is predominantly due to the damaged surfactant system, illustrated by an increase in the SA/LA ratio. As a result surfactant cannot maintain its surface lowering function inside the alveolus causing further development of pulmonary edema and subsequently decreased lung compliance and gas exchange, contributing to early morbidity and mortality [1-4,8]. Although prolonged ventilation strategies could probably reduce mortality in this experimental setting, animals were ventilated postoperatively for as short periods as possible because of the confounding effect of ventilation on LIRI [17]. The conversion of LA into SA and the decrease in lung compliance and PaO2/FiO2 ratio within hours after reperfusion has been described in previous experimental studies [1-6]. We confirm that LIRI resulted in the conversion of the highly surface active LA subtype into the poor surface active SA subtype on day 1, and impaired PaO2 and lung compliance throughout the experimental period. We furthermore demonstrate that surfactant treatment before the induction of warm ischemia completely normalised lung compliance and PaO2 from day 3 onwards and prevented the increase in the SA/LA ratio. Thus, surfactant pretreatment enhances the recovery of lung function but, even more importantly, may prevent in part the damage caused by LIRI.

The high level of alveolar protein in the LIRI animals illustrates the loss of fluid homeostasis, resulting in development of high permeability edema. It is known that when serum proteins accumulate in the alveoli, they are able to further dose-dependently inhibit surfactant function, probably by competing with surfactant phospholipids for a place at the air-water interface, resulting in additional deterioration of the lung function [8,18]. Surfactant replacement therapy at the time of reperfusion interrupts this vicious circle by restoring the fluid homeostasis across the alveolocapillary membrane [18]. Our results indicate that administration of surfactant before ischemia also decreases the amount of alveolar protein on day 1 following LIRI, which may be an important mechanism in the observed amelioration of LIRI.

In the non-ischemic right lung of untreated LIRI animals, an increase in the amount of alveolar protein on day 1 was noticed. Whether this is due to spillover from the injured left lung or a direct injury of the right lung as a result of LIRI or ventilator settings cannot be determined. Other studies also showed increased levels of alveolar protein in ischemic and non-ischemic lungs in experimental transplantation models [5,6]. Friedrich and colleagues demonstrated that separate ventilation of the transplanted and non-transplanted lung reduced the level of alveolar protein in the non-transplanted lung suggesting that injury of the native lung occurs due to hyperinflation, which arises when both transplanted and non-transplanted lungs are simultaneously ventilated [4].

Besides the protective effect of surfactant pretreatment on lung architecture after LIRI, surfactant therapy may have a suppressive effect on the inflammatory process that is part of LIRI [19]. Therefore, the present study examined the influx of macrophages, granulocytes and lymphocytes in the BALF of the left lung. Reduction of the inflammatory reaction in the context of ischemia and reperfusion is important and has proven to be successful in amelioration of injury [20-22].

LIRI led to a local inflammatory reaction, characterised by an influx of macrophages, granulocytes and lymphocytes on day 1, followed by an influx of macrophages and lymphocytes on day 3. We observed a significant increase in the number of macrophages and granulocytes in the surfactant pretreated group, most prominent on day 1 with levels returning to normal at day 7. This increase in macrophages and granulocytes may be partly explained by their surfactant recycling capacity, which mainly depends on the number of alveolar macrophages, but also on alveolar type II cells and to a lesser extent granulocytes [23]. Alveolar macrophages can more easily be recruited to the lung compared to alveolar type II cells. This suggests that macrophages, and to a lesser extent granulocytes, are recruited to the lung in response to the increased demand for surfactant recycling cells.

The increased number of granulocytes in the pretreated group may also be explained as follows. In the untreated LIRI group, the endogenous surfactant is impaired following LIRI, thus increasing surface tension. As a result, the alveolus collapses, leading to alveolar shunting and ventilation-perfusion mismatch. To correct this, constriction of arterioles occurs. Surfactant pretreatment keeps the alveolus open, thereby preventing shunting and constriction of arterioles, thereby facilitating infiltration of granulocytes. Another reason for the higher influx of granulocytes might be that surfactant components are chemotactic for granulocytes [24]. Also, the instillation of saline, in which the surfactant phospholipids were dissolved, may have contributed to the influx of granulocytes. Saline without surfactant phospholipids was not instilled in untreated LIRI animals, because this would lead to high mortality in this group.

The infiltration of macrophages and granulocytes, the leakage of alveolar proteins and the decreased lung compliance in the pretreated group on day 1 suggest that some lung damage still occurred as a result of lung ischemia and reperfusion. However, although the number of infiltrating cells was higher than in untreated groups, PaO2 and lung compliance had improved. Thus, surfactant pretreatment did not have an effect on the number of infiltrating macrophages and granulocytes, but possibly did modulate the effect of the infiltrating cells. Surfactant may have functioned as an anti-oxidant agent or as a physical barrier between lung epithelial, endothelial cells and ROS producing cells so that surfactant itself, but not lung tissue, was
damaged after LIRI. The latter can be illustrated by the increased levels of SA in pretreated groups [8, 25]. Surfactant was able to reduce the infiltration of lymphocytes on day 3. Whether this reduction is due to a targeted response of surfactant components, less activation of antigen presenting cells or preserved lung architecture, resulting in less presentation of self-antigens, remains unanswered. However, the reduction in infiltration of lymphocytes after surfactant pretreatment likely contributes to the improved function observed in this study.

In conclusion, the present study shows that surfactant pretreatment improves animal survival and decreases LIRI. Whereas the number of infiltrating macrophages and granulocytes were increased after surfactant pretreatment, lymphocytes were decreased. The timing of surfactant administration used in this study would permit donor pretreatment in the clinical setting.

Acknowledgements

The authors thank Jaschenka Houtgraaf (Department of Surgery) and Stefan Krabbdam (Department of Anesthesiology) for their technical assistance and Laraine Visser-Isles (Department of Anesthesiology) for English-language editing.

References


Appendix A. Conference discussion

Dr Van Raemdonck (Leuven, Belgium): I have a technical question regarding the measurement of your compliance. This was a study where there was ischemia of the left lung.

Dr van der Kaaij: Yes.

Dr Van Raemdonck: How did you measure the compliance, then, of the left lung, or did you measure compliance of both lungs?

Dr van der Kaaij: We first performed pressure-volume curves of both lungs. Then we measured the compliance of both lungs. Thereafter we clamped the right lung and we measured the compliance of the left lung only. What I have presented here is the compliance of the left lung. However, the compliance of both lungs is also decreased, but we decided to show only the compliance of the left lung because that was the lung, which was clamped.

Dr Van Raemdonck: And you did this through a median sternotomy? How did you clamp the right bronchus?
Dr van der Kaaij: You mean how we induced ischemia?
Dr Van Raemdonck: No, no. What kind of incision did you use?
Dr van der Kaaij: To induce ischemia, we performed a left-sided thoracotomy, whereafter we put a clamp on the pulmonary artery, veins, and bronchus of the left lung. To measure compliance, we did a sternotomy, but then the rat was already killed.
Dr W. Klepetko (Vienna, Austria): Can you elaborate a bit about the theoretical background? Animals preoperatively, before you introduce the ischemic period, must have normal surfactant levels. Why do those animals benefit from additional surfactant? In the clinical reality, you might assume that the donor already has reduced surfactant levels and he eventually might benefit from additional surfactant, but in your experimental setting, with the starting point of normal surfactant levels, it's difficult to understand what the mechanism of action is.
Dr van der Kaaij: We also measured the inactivation of surfactant, and what we saw is that the endogenous surfactant system of the rats was altered after ischemia-reperfusion injury, probably by a production of oxygen radicals. When we gave additional surfactant to the donor, we found also an inactivation of the surfactant, but now the rat had so much surfactant that simply not all of the surfactant was inactivated. So in these pretreated animals there was a larger pool of surfactant surface active phospholipids which still could reduce the surface tension. In IRI untreated rats there was probably not enough surfactant. Therefore we think that we should give enough surfactant to prohibit the total inactivation of surfactant after IRI.