**Static Inflation Attenuates Ischemia/Reperfusion Injury in an Isolated Rat Lung In Situ**

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**Study objectives:** Ischemia (I)/reperfusion (R) lung injury is an important clinical issue in lung transplantation. In the present study, we observed the effects of lung static inflation, different perfusates, and ventilatory gas with nitrogen or oxygen on the I/R-induced pulmonary damage.

**Design and setting:** A total of 96 male Sprague-Dawley rats were used. The lung was isolated in situ.

**Methods:** In an isolated lung, the capillary filtration coefficient (Kfc), lung weight gain (LWG), lung weight (LW)/body weight (BW) ratio, and protein concentration in BAL fluid (PCBAL) were measured or calculated to evaluate the degree of lung injury. Histologic examinations with hematoxylin-eosin staining were performed.

**Results:** I/R caused lung injury, as reflected by increases in Kfc, LWG, LW/BW, and PCBAL. The histopathologic picture revealed the presence of hyaline membrane formation and the infiltration of inflammatory cells. These values were significantly attenuated by static lung inflation. The I/R lung damage appeared to be less in the lung perfused with whole blood than in the lung perfused with an isotonic solution. Therapy with ventilatory air (ie, nitrogen or oxygen) did not alter the I/R lung damage.

**Conclusions:** The data suggest that lung inflation is protective to I/R injury, irrespective of the type of ventilatory air used for treatment. The preservation of the lung for transplantation is better kept at a static inflation state and perfused with whole blood instead of an isotonic physiologic solution.

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**Key words:** ischemia/reperfusion; lung injury; microvascular permeability; perfusate; static inflation; ventilatory air

**Abbreviations:**

- BW = body weight
- HM = hyaline membrane
- I = ischemia
- ICI = inflammatory cell infiltration
- Kfc = capillary filtration coefficient
- KHS = Krebs-Henseleit solution
- LAP = left atrial pressure
- LW = lung weight
- LWG = lung weight gain
- LAP = pulmonary arterial pressure
- PCBAL = protein concentration in BAL fluid
- R = reperfusion

Ischemia (I)/reperfusion (R) lung injury occurs in many pulmonary disorders such as pulmonary artery thromboendarterectomy,1 thrombolysis after pulmonary embolism,2 and lung transplantation.3 In particular, I/R lung injury is a serious problem in lung transplantation because of the large area involved and the long duration of the procedure. I/R lung injury is postulated to be one of the causes of reimplantation response, a transient depression of...
graft function without rejection that occurs after the transplantation of donor lungs to the recipient. In addition, infection and rejection are two major causes of failure in current lung transplant technology. Previous studies have indicated that poor early graft function, presumably due to I/R injury, may contribute to the high rate of rejection. Accordingly, I/R injury is an important clinical issue in lung transplantation. It remains a difficult task to preserve the transplanted lung during the surgical procedure and to achieve good respiratory function in the recipient.

Previous studies have explored different ways to reduce the severity of I/R lung injury. Because the lung is an organ for receiving oxygen from the air, many studies have investigated the effects of different oxygenation in the protection of lung I/R injury. Other studies focused on the beneficial effect of vascular distension and continuous ventilation on the I/R lung injury. Shen et al. have found that certain agents that reduce oxygen free radicals and increase lung oxygenation could protect the lung from I/R injury.

It remains controversial whether the isolated lung leads to a better outcome in the inflated or collapsed state. In the present study, we assumed that static inflation of the lung may exert a protective effect through a mechanism not related to oxygenation. The isolated rat lung in situ was inflated with a nitrogen or oxygen mixture. In addition, we compared the I/R lung injury in the isolated lung perfused with whole blood or Krebs-Henseleit solution (KHS). The purpose was to elucidate the effect of different perfusates on the I/R lung injury. The rationale for this protocol is that we have found that various lung perfusates affect hypoxic pulmonary vasoconstriction.

Materials and Methods

Preparation of the Isolated Lung

The preparation of an isolated rat lung in situ model has been described previously. In brief, a total of 96 male Sprague-Dawley rats weighing 250 to 350 g were anesthetized intraperitoneally with sodium pentobarbital (30 mg/kg) and were intubated with an endotracheal tube. A rodent ventilator provided ventilation with a mixture of 95% room air and 5% carbon dioxide. The respiratory rate was set at 60 to 65 breaths/min, and tidal volume was set at 2 to 3 mL. The inspiratory pressure and expiratory pressure were 5 and 1 cm H2O, respectively. A vertical incision was made along the midline of the thorax and abdomen. Heparin (150 U) then was injected into the right ventricle. After 5 min, rat blood (10 mL) was withdrawn for later use. The afferent line, made from silicon tubing (internal diameter, 3 mm), was then inserted into the pulmonary trunk via the right ventricle, the efferent line, also made from silicon tubing (internal diameter, 5 mm), was inserted into the left atrium via the left ventricle.

The pulmonary trunk and the aorta then were tied off separately with cotton threads. The isolated perfused lungs were left in situ, and the whole rat was placed on an electronic balance. The digital signals of the electronic balance were converted to analog signals by a digital-to-analog converter and were recorded on a polygraph recorder. Weight changes were precalibrated on the electronic balance before preparation for the experiment. In order to verify that the changes in body weight (BW) reflected the changes in lung weight (LW), we performed a pilot test. In eight isolated lung preparations, Evan blue dye was added to the lung perfusate as a tracer. Under conditions of low pulmonary venous pressure (ie, 2 cm H2O) for 30 min and high venous pressure (ie, 10 cm H2O) for another 30 min, the dye tracer was found to be confined only in the lung. We presumed that capillary integrity was maintained throughout the body so that any change in total BW reflected the change in LW. Blue stain was not found in any other sites, including the ventricles. Before lung perfusion, the lungs were hyperinflated to avoid atelectasis. The duration of the preparation for the experiment was < 15 min.

Perfusion System

The isolated lung in situ was initially perfused with 5 mL KHS mixed with 5 mL blood and 6% albumin. The perfusion system included a venous reservoir and a roller pump. The perfusion solution was circulated via the roller pump to maintain a constant flow. The venous blood was returned via an outflow line into a reservoir. The latter was placed in a 38°C water bath for constant temperature. The lung was perfused with constant flow. Pressure transducers were connected by side arms to measure the pulmonary arterial pressure (PAP) and left atrial pressure (LAP). The PAP was set at 15 to 20 cm H2O by adjustment of the flow rate. LAP was maintained at 0 cm H2O by the height of venous outflow. Throughout the experiment, PAP and LAP were recorded continuously (Polygraph; Gould; Eastlake, OH).

Capillary Filtration Coefficient

The pulmonary capillary filtration coefficient (Kfc) was used as a marker of lung injury. The measurement and calculation procedures were employed as previously described. In brief, the LAP was raised from 0 to 10 cm H2O for 5 min and then returned to 0 cm H2O. We recorded changes in the weight of the isolated lung and plotted them against time using a sensitive electronic scale. The rise in LAP caused LW changes in a two-component fashion. The fast component reflects the rapid filling of pulmonary vessels. The slow component of the gain in LW is caused by capillary filtration. The slow component was plotted on a semilog scale against time and was extrapolated to zero time. We calculated the initial rate of LW gain (LWG) [ie, ∆Weight/∆time] when the hydrostatic forces had not exerted the effect. The calculated slow component of LWG was divided by the change in LAP to obtain the Kfc.

Protein Concentration in the BAL

After the experiment, lungs were lavaged twice with saline solution (2.5 mL per lavage). The protein concentration in BAL fluid (PCBAL) was determined with a spectrophotometer by the measurement of change in absorbance at 630 nm after the addition of bromocresol green.

Induction of Lung I/R

Lung I/R was induced essentially as described previously. In brief, the isolated lung was initially ventilated with room air
and 5% CO₂. After the stabilization of PAP and LAP, ventilation and perfusion were stopped for 75 min. When the roller pump was turned off to produce I, there was no blood drained into the reservoir. The PAP and LAP fell to zero, and the pulmonary vasculature was in a collapsed state. R then was restarted for 50 min. Ventilation was restarted simultaneously with room air and 5% CO₂.

**Experimental Protocol**

The study consisted of three series of experiments that were designed to elucidate the factors in I/R lung injury. The factors were inflation, perfusate component, and gaseous mixture. In the first series, the lung was ventilated with a mixture of room air and 5% CO₂, and was perfused with whole blood. There were the following three groups in this series: (1) a control group receiving no I/R (12 rats); (2) an I/R without inflation group (12 rats), in which the lung was kept in an expiratory state with endotracheal pressure at 1 cm H₂O; and (3) an I/R with static lung inflation group (12 rats). The lung was maintained in an inspiratory state with an endotracheal pressure of 5 cm H₂O. In the second series, the lung perfusate was changed to KHS. The grouping was the same as that in the first series (ie, control group, 12 rats; I/R with lung inflation group, 12 rats; and I/R without lung inflation group, 12 rats). In the third series, we evaluated the effect of ventilatory gas. The degree of I/R lung injury was compared between lung ventilation and inflation with 95% N₂ + 5% CO₂ (12 rats) and 95% O₂ + 5% CO₂ (12 rats).

**Pathologic Examination**

At the end of the experiment, the lung was removed, fixed in 6% formaldehyde, and dehydrated with graded concentrations of alcohol for embedding in paraffin. Paraffin slices of lung tissue were sectioned at a thickness of 5 μm and were stained with hematoxylin and eosin. The histopathologic examination of the lung tissue was performed with a light microscope. The lung pathology showed hyaline membrane (HM) formation and the infiltration of inflammatory cells. So far, there has been no grading system or scoring for these changes published in the literature. We may assess the lung pathology as follows: degree 0, no HM formation; degree 1, mild HM appearance; degree 2, moderate HM appearance; and degree 3, severe HM appearance. Various degrees were scored from 0 to 3. For inflammatory cell infiltration (ICI), the grading was as follows: degree 0, no ICI; degree 1, mild ICI; degree 2, moderate ICI; and degree 3, severe ICI. Various degrees were scored from 0 to 3. The histopathologic assessment was performed in a blinded fashion by several laboratory technicians. Each one gave a score for HM and ICI from 0 to 3. The individual scores for HM and ICI were added together to arrive at a final score, ranging from 0 to 6.

**Statistical Analysis**

Data were expressed as the mean ± SEM. The difference among groups was evaluated by analysis of variance. A Bonferroni test was used to compare the effect among groups, and a modified Student t test was used for comparison between groups.

**RESULTS**

**Kfc**

Figure 1 illustrates Kfc changes by I/R in the isolated lung perfused with whole blood or KHS. I/R caused a marked increase in Kfc. Static lung inflation significantly attenuated the increase in Kfc. The mean Kfc change in the lung perfused with whole blood (1.54 ± 0.46 g/min/cm H₂O per 100 g) was lower than that in the lung perfused with KHS (2.31 ± 0.18 g/min/cm H₂O per 100 g; p < 0.05).

**LWG and LW/BW Ratio**

Figure 2 shows that I/R increased LWG during the R period for 50 min. Static inflation significantly reduced the LWG induced by I/R. The LW/BW ratio (×10³) was markedly elevated following I/R. This change in the LW/BW ratio caused by I/R was significantly reduced by lung static inflation (Fig 3).

**PCBAL**

I/R increased the PCBAL. The change was largely attenuated by static lung inflation (Fig 4). In addition, the mean PCBAL in the isolated lung perfused with KHS (4,248 ± 109 mg/dL) appeared to be higher than that in the lung perfused with whole blood (1,221 ± 109 mg/dL; p < 0.001).
Comparison of the I/R Lung Injury in the Lung With Different Gaseous Mixture

In the lung perfused with whole blood, lung air ventilation was performed with 95% N₂ / 5% CO₂ in one group (12 rats) before I and during inflation, and with 95% O₂ / 5% CO₂ in another group (12 rats). Table 1 shows that there was no statistical difference in Kfc, LWG, LW/BW ratio, and PCBAL between the isolated lung ventilated and inflated with different mixtures of gas.

Histopathologic Finding

Figure 5 shows the pathologic changes in the lung after I/R without inflation. There was the appearance of HMs with the infiltration of inflammatory cells. The mean lung injury score was 5.96 ± 0.45 (six rats). These changes were essentially not observed in the I/R lung with static inflation (not shown). The mean lung injury score was 0.06 ± 0.04 (six rats). Static inflation significantly ameliorated the lung pathology (p < 0.05).

Discussion

Previous studies have reported an association between the state of lung inflation and I/R injury. Puskas and coworkers noted that donor lung storage in the inflated state during organ perfusion improved the outcome. Stevens et al also reported improved lung preservation by prevention of lung collapse. However, there have also been conflicting reports indicating that hyperinflation of the preserved lung led to a worse outcome. In fact, Aoe et al showed that immediate postpreservation lung function was satisfactory, irrespective of the state of inflation during storage. However, gas exchange deteriorated in the hyperinflated lungs in the early R period compared to the lungs stored at normal volume. Assuming that static inflation does indeed improve lung function, there are still controversies as to what type of gas mixture in the alveoli leads to a better outcome. Some investigators think that oxygen was superior to other gas mixtures, while others have shown that oxygen and nitrogen mixtures worked equally well. In the present study, we compared the effects of different gas mixtures used to inflate the preserved lung during the ischemic period. Our purpose was to show that static inflation per se, irrespective of the gas mixture, protected the lungs, probably by a mechanical mechanism rather than an aerobic mechanism.
Possible Involvement of Nitric Oxide and Oxygen Free Radicals

Koyama et al. revealed that the production of oxygen radicals mediates lung injury in the ischemic O2-ventilated canine lung. A recent study from our laboratory found that the use of endogenous and exogenous nitric oxide was detrimental to the I/R injury in the rat lung. In this study, we did not measure the levels of nitric oxide, cytokines, and oxygen radical species. However, the extent of lung injury was not different between the lung that was ventilated and inflated with 95% O2 + 5% CO2 and that ventilated and inflated with 95% N2 + 5% CO2. This finding may suggest that O2 or O2 radicals are not responsible for the I/R lung injury. In addition, the I/R lung injury was less in the lung perfused with whole blood than in that perfused with a physiologic solution. The possible explanation is that cell elements, RBCs in particular, are able to scavenge some nitric oxide and oxygen free radicals. Further studies are required to elucidate the effect of free radicals and cytokines on I/R lung injury.

Vascular Distension

Many investigators have postulated that the state of lung inflation may have an impact on preserved lung function via several mechanisms. One mechanism was the effect on vascular distension. The higher PAP in the collapsed lung was once thought to be associated with lung injury. At the onset of R, capillary recruitment occurs in the preserved lung. In the atelectatic lung, capillary recruitment was reduced because of a decreased vascular surface area. The rise in pulmonary vascular resistance in the preserved atelectatic lung might have contributed to I/R lung injury. In fact, Srinivasan et al. demonstrated evidence that in lung preparations of R vascular injury, the increase in PAP (peak PAP – baseline PAP) during R was correlated with LWG. However, in lungs protected by either mechanical ventilation or surfactant instillation, no such correlation was observed, despite similar increases in PAP. Furthermore, the vascular volume in the rat lung is approximately 0.1 mL/g LW when determined at a steady-state perfusion pressure of 10 cm

Table 1—Comparison of the I/R Lung Injury in the Isolated Lung With Static Inflation Using Different Gaseous Compositions

<table>
<thead>
<tr>
<th>Variables</th>
<th>95% N2 + 5% CO2 (n = 12)</th>
<th>95% O2 + 5% CO2 (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kfc, g/min/cm H2O per 100 g</td>
<td>1.63 ± 0.48</td>
<td>1.71 ± 0.52</td>
</tr>
<tr>
<td>LWG, g</td>
<td>3.54 ± 0.32</td>
<td>3.68 ± 0.42</td>
</tr>
<tr>
<td>LW/BW ratio, ×10^3</td>
<td>13.22 ± 0.42</td>
<td>14.02 ± 0.52</td>
</tr>
<tr>
<td>PCBAL, mg/dL</td>
<td>1,326 ± 98</td>
<td>1,286 ± 102</td>
</tr>
</tbody>
</table>

*Values given as the mean ± SE. There were no significant differences in these parameters between different ventilatory gaseous mixtures (p > 0.05).
H$_2$O. Therefore, it is unlikely that vascular distension by itself can produce the degree of LWG that was observed in our study. During lung I, after stopping the perfusion pump, the pulmonary vasculature was in a state of collapse. Accordingly, vascular distension alone cannot explain the development of I/R injury. In the present study, lung inflation was maintained in an inspiratory state with an endotracheal pressure of 5 cm H$_2$O. This level may not be high enough in humans but was sufficient in the isolated rat lung because it significantly reduced the I/R lung injury.

**Surfactant Secretion**

In a previous study, it was shown that phosphatidylglycerol and surfactant-associated protein A levels were decreased after lung graft storage and R in dogs. This indicates a decrease of surfactant synthesis during I. It has also been shown that air inflation to total lung capacity is a major physiologic stimulus to the release of lung surfactant into the alveolar space. Indeed, Levine and Johnson have noted a link between atelectasis and the loss of surfactant activity. Another study hypothesized that lung microvascular injury may in part be attributable to the loss of surfactant, leading to alveolar collapse. It is known that ventilation can change surfactant secretion and that it produces a stressful condition to increase the intercellular junction width of the capillary endothelium, epithelium, and basement membrane. Wirtz and Schmidt also have shown that the secretion of pulmonary surfactant can be enhanced by distension of the lung. In fact, Akashi and coworkers suggested that inflation during storage might preserve the ability of cells to secrete surfactant. In this study, we observed that I/R injury was characterized by an appearance of HMs (Fig 5). This finding supports the idea that the surfactant level was decreased in patients with I/R lung injury. We also showed that static inflation attenuated I/R lung injury. There were no significant differences between the control group and the static inflation groups in terms of Kfc, LW, LW/BW ratio, and PCBAL after I/R. It is likely that the protection from I/R injury conferred by static inflation observed in our present study may have been mediated at least in part by surfactant production, which reduces the alveolar oxygenation.

Under physiologic conditions, the alveolar oxygenation is obtained from the vascular and ventilatory routes. Schütte et al found that continuous ventilation and vascular distension were protective in lung I/R injury. Their experiments were performed with ventilation, not static inflation. In addition, the ischemic period was as long as 240 min. In our experiment, the duration of lung I was only 75 min. It is possible that the protective effect of static inflation may become insignificant during a long I period (ie, 240 min). There also have been other reports with arguments concerning the effect of treatment with ventilatory air. In a study by Koyama et al, oxygen was found to worsen I/R injury in the canine lung. One possible explanation for this phenomenon is that the presence of oxygen free radicals may potentiate I/R injury. In a study with rabbit lung by Sakuma and coworkers, I/R injury was prevented by lung inflation with air, oxygen, or nitrogen. In our study, we also found that static inflation exerted a protective effect against I/R lung injury evaluated by the parameters of Kfc, LWG, LW/BW ratio, and PCBAL. Furthermore, the I/R lung injury was not different between the lung ventilated with N$_2$ and that ventilated with O$_2$. Therefore, it is likely that lung inflation may exert a protective effect against I/R injury through a mechanism that is independent of alveolar oxygenation.

In conclusion, the present study has demonstrated that static inflation of preserved rat lungs with a room air and carbon dioxide mixture protects the lung against I/R injury. The I/R lung injury appears to be more severe in the isolated lung perfused with KHS than in the lung perfused with whole blood. There is no difference in I/R lung injury between inflation with nitrogen/carbon dioxide and inflation with an oxygen/carbon dioxide mixture. Accordingly, it is unlikely that the protective effect works by decreasing vascular distension alone. This effect is independent of alveolar oxygenation. The stimulation of surfactant secretion resulting from alveolar distension remains a possible mechanism. Other explanations such as the stimulation of mediator release are not performed in this study, but should be explored in future research.

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