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ORIGINAL ARTICLE

Therapeutic effect of surfactant inhalation during warm ischemia in an isolated rat lung perfusion model

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Conflicts of Interest
None.

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Summary

Warm ischemia-reperfusion injury related to donation after cardiac death donors is a crucial and inevitable issue. As surfactant function is known to deteriorate during warm ischemia, we hypothesized that surfactant inhalation during warm ischemia would mitigate warm ischemia-reperfusion injury. We used an isolated rat lung perfusion model. The rats were divided into three groups: sham, control, and surfactant. In the control and surfactant groups, cardiac arrest was induced by ventricular fibrillation. Ventilation was restarted 110 min later; subsequently, the lungs were flushed, and heart and lung block was recovered. In the surfactant group, a natural bovine surfactant Surfacten® was inhaled for 3 min at the end of warm ischemia. Then, the lungs were reperfused for 80 min. Surfactant inhalation significantly improved graft functions, effectively increased lung tissue ATP levels, and significantly decreased mRNA levels of IL-6 and IL-6/IL-10 ratio at the end of reperfusion. Histologically, lungs in the surfactant group showed fewer signs of interstitial edema and hemorrhage, and significantly less neutrophilic infiltration than those in the control group. Our results indicated that surfactant inhalation in the last phase of warm ischemia maintained lung tissue energy levels and prevented cytokine production, resulting in the alleviation of warm ischemia-reperfusion injury.

Introduction

Lung transplantation is the last established therapeutic option for patients with end-stage respiratory failure, but the shortage of brain-dead donors remains a critical problem. To resolve this problem, various approaches have been considered [1]. Among them, successful use of lungs from donation after cardiac death (DCD) donors in clinical lung transplantation was reported in 2000 [2]. Thereafter, in many institutions worldwide, lung transplantation from DCD donors has been performed. For lung transplantation from DCD donors, it is crucial to perform *ex vivo* lung perfusion to understand the effect of warm ischemia on graft lungs [3].

A pulmonary surfactant is mostly composed of phospholipids and surfactant proteins (SP-A, -B, -C, and -D), and forms a monolayer at the air-lipid interface, reducing the surface tension in the alveoli. This

mechanism prevents alveolar collapse [4,5] and protects the lung.

Surfactants, as prophylactics, have been administered in clinical settings to patients with severe primary graft dysfunction after transplantation [6–9] and for donor lungs [10,11]. Recently, it was reported that surfactant function deteriorated with increasing warm ischemic time intervals [5]; however, no study has been conducted to date on the protective effect of surfactant administration against pulmonary warm ischemia–reperfusion (I–R) injury.

We hypothesized that pulmonary function would deteriorate during warm ischemia, and that therapeutic surfactant administration during warm ischemia would improve graft function and ameliorate warm I–R injury.

Materials and methods

Animals

Specific pathogen-free inbred male Lewis rats weighing 285–310 g (Japan SLC, Hamamatsu, Japan) were used in these studies. All animals received humane care in compliance with the Principles of Laboratory Animal Care, formulated by the US National Society for Medical Research, and the ''Guide for the Care and Use of Laboratory Animals,'' prepared by the US Institute of Laboratory Animal Resources and published by the National Institute of Health (NIH Publication 85–23, revised 1996; Bethesda, MD, USA). The current study protocol was approved by the Ethical Committee of the Graduate School of Medicine at Kyoto University, Japan.

Surfactant and aerosol delivery

In this study, we used beractant (Surfacten®, Mitsubishi Tanabe Pharma Corporation, Osaka, Japan). Beractant is a natural bovine lung extract containing phospholipids, neutral lipids, fatty acids, and the two hydrophobic, surfactant-associated proteins: SP-B and SP-C. A 30-mg/ml surfactant solution was obtained by dissolving Surfacten® with normal saline (0.9%). The surfactant was aerosolized using a nebulizer (AGAL1000, Aerogen, Ireland), placed in the inspiratory loop of the ventilator. In this system, the diameter of about 90% of the aerosolized particles was maintained below 10 μm, and that of about 60% of the aerosolized particles was maintained below 3.0 μm. In each experiment of the surfactant group, 0.4 ml fluid was loaded and aerosolized for 3 min.

Isolated rat lung perfusion model

Isolated rat lung perfusion (Model 829; Hugo-Sachs Elektronik-Harvard Apparatus) was performed as previously reported [12–17]. Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg), intubated after tracheotomy, and ventilated with ambient air at positive pressure. After a median sternotomy, the pulmonary artery was cannulated, and drainage cannula was placed in the left atrium. The cannulae were connected to the perfusion circuit. Subsequently, heart and lung block was recovered and placed in the artificial thorax. The lung was then ventilated with ambient air at negative pressure under the following conditions: respiratory rate = 60 cycles/min; peak inspiratory and expiratory chamber pressures = −8 and −4 cm H_2O, respectively; ratio of inspiratory duration = 50%. The perfusate comprised heparinized whole blood obtained from two donor rats diluted with saline containing 4% bovine serum albumin. The pH was maintained between 7.25 and 7.35 using sodium bicarbonate. The perfusate was driven by two pumps. No leukocyte filter was used. The perfusate circuit and airway were water-jacketed to maintain the temperature at 37 °C throughout the experiment. The effluent perfusate from the left atrium was deoxygenated in a glass deoxygenator with anoxic gas (nitrogen, 92%; carbon dioxide, 8%), and then pumped into the pulmonary artery of the lung. Using a pressure-equilibration vessel, the pulmonary venous pressure was maintained at +2 cm H_2O against the hilum.

Study I: Effect of surfactant inhalation

Experimental protocol

The animals were randomly allocated to two groups: control and surfactant. In both groups, cardiac arrest was induced by ventricular fibrillation using a fibrillator attached directly to the right atrium and apex of the heart and continued for 7 min at a voltage of 2.00 V. Cardiac arrest was defined as complete immobility of the ventricles. After confirmation of cardiac arrest, the ventilator was stopped, and the lungs completely collapsed. The chest was closed using skin staplers and placed in a Styrofoam box. Lungs were completely re-expanded below the pressure of 30 cm H_2O 120 min after cardiac arrest, and positive pressure ventilation was restarted. Subsequently, heart and lung block was recovered and placed in the artificial thorax. The lung was then ventilated with ambient air at negative pressure. In the surfactant group (n = 5), surfactant was inhaled for 3 min after the beginning of negative pressure ventilation. In the control group (n = 5), there was no inhalation. Then, 135 min after cardiac arrest, the lung was connected to a flexiVent® rodent ventilator to obtain physiological data and dynamic pressure–volume (P–V) curves. After the measurement using flexiVent®, the lung was fixed by 10% formalin intratracheally. Each lung was embedded in paraffin, and stained with toluidine blue.

FlexiVent® rodent ventilator

Heart and lung block was connected to a flexiVent® rodent ventilator (Scireq, Montreal, Ontario, Canada). Prior to measurements, the volume history of the lung was standardized with two 6-s deep inflations to a pressure limit of 30 cm H_2O. After an initial 3-min run-in period of ventilation with $FiO_2 = 0.21$, tidal volume 8 ml/kg, respiratory rate 60 breaths/min, and positive end-expiratory pressure (PEEP) = 4 cm H_2O, dynamic compliance, and airway resistance were recorded. To obtain dynamic P–V curves, the lung was incrementally inflated to 30 cm H_2O and airway pressures were recorded on each volume increment.

Toluidine blue stain

To evaluate the distribution of inhaled surfactants in paraffin-embedded sections, the bilateral lobes were collected after the evaluation using flexiVent®. Surfactant particles were identified by morphology and positive staining for toluidine blue staining [18] by the pathologist. In this study, we used zinc chloride double salt of toluidine blue (Certistain®, Merck KGaA, Darmstadt, Germany).

Study II: Investigation of I–R injury

Experimental protocol

The animals were randomly allocated to three groups: sham, control, and surfactant. In the control and surfactant groups, cardiac arrest was induced and rats were left in the same way of study I. Lungs were completely re-expanded below the pressure of 30 cm H_2O 110 min after cardiac arrest, and positive pressure ventilation was restarted. The pulmonary artery was cannulated, and the lung was flushed with 20 ml of low potassium dextran (LPD; Perfadex®, Vitrolife, Uppsala, Sweden) solution (temperature 37 °C; pressure, 20 cm H_2O), with drainage through an incision of the left ventricle. A drainage cannula was placed in the left atrium. The cannulae were connected to the perfusion circuit, and perfusion was started at the minimum flow of 1 ml/min. Subsequently, heart and lung block was recovered and placed in the artificial thorax. The lung was then ventilated with ambient air at negative pressure. In the surfactant group ($n = 6$), surfactant was inhaled for 3 min after the beginning of negative pressure ventilation. In the control group ($n = 6$), there was no inhalation. In the sham group ($n = 4$), the lungs were flushed using Perfadex®, and thereafter recovered and reperfused in the same way. In the three groups, we set the initiation of the reperfusion at 25 min after re-expansion of the lungs, that is, total warm ischemic interval was 135 min. The lungs were reperfused with an initial step-by-step increase in flow for 8 min up to 10 ml/min followed by stabilization for 7 min. At the end of the stabilization period, the time was set as baseline, and evaluation time was commenced (Fig. 1).

In this experiment, physiological data, lung tissue energy levels, messenger RNA (mRNA) expressions of cytokines, and pathological examination, including hematoxylin-eosin (H–E) stain and naphthol AS-D chloroacetate esterase stain, were assessed.

Physiological data analysis

The hemoglobin level (g/dl) and blood gases (Torr) of the effluents from the deoxygenator and those from the pulmonary vein were analyzed at baseline and every 20 min after reperfusion. Shunt fraction (Qs/Qt) was calculated according to the following formula: Qs/Qt (%) = (Cc – Ca)/(Cc – Cv) × 100. Here, Cc, Ca, and Cv represent the oxygen content of the pulmonary capillary blood, pulmonary artery blood, and pulmonary venous blood, respectively. Airway resistance (cmH_2O/ml), dynamic compliance (ml/cmH_2O), pulmonary vascular resistance (PVR ($cmH_2O/ml \cdot min$), defined as [pulmonary arterial pressure–pulmonary vein pressure]/perfusate flow), and lung weight were continuously monitored.

Determination of adenosine nucleotide levels

For this, we used pieces of the right middle and lower lobes of each lung, collected immediately after flushing

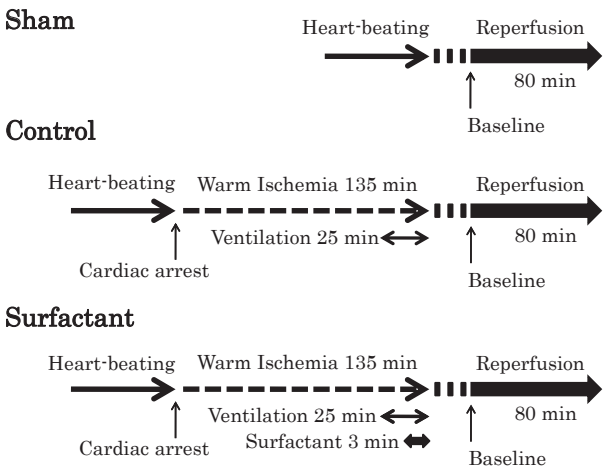

Figure 1 Experimental protocol using an isolated rat lung perfusion model. The rats were divided into three groups: sham ($n = 4$), control ($n = 6$), and surfactant ($n = 6$). In the sham group, there was no ischemia. In the control and surfactant groups, after 135 min of warm ischemia, reperfusion was started. Only in the surfactant group, the surfactant was inhaled for 3 min during the last phase of warm ischemia. All the animals were evaluated for 80 min after stabilization.

the pulmonary vascular bed with warm saline (37 °C), at the end of reperfusion. We measured levels of adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) using high-performance liquid chromatography (Shim-pack CLC-ODS column; 15 cm × 6.0 mm; Shimadzu, Japan).

Evaluation of the mRNA expression

Collected left lung specimens were stored in RNAlater TissueProtect Tubes (Qiagen, Hilden, Germany) for RNA stabilization. Total RNA was extracted from specimens using an RNeasy Mini Kit (Qiagen), and the RNA extract was incubated with RNase-free DNase I (Qiagen) to remove contaminating DNA. Reverse transcription of total RNA was performed using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) to generate complementary DNA. The forward and reverse primers used for quantitative amplification were designed using the Primer3 Input (version 0.4.0) (http://frodo.wi.mit.edu/primer3/) and confirmed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. Two genes were examined using real-time reverse transcription-polymerase chain reaction (RT-PCR). Quantitative real-time RT-PCR was performed on a LightCycler using Quanti-Tect SYBR Green PCR Master Mix (Qiagen). Polymerase chain reaction conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. Quantities of the genes of interest were calculated from corresponding standard curves using LightCycler Software (Roche, Basel, Switzerland); they are presented herein in relation to the amount of GAPDH.

Pathological evaluation

After 80 min of reperfusion, 10% formalin was instilled intratracheally into the right upper and mediastinal lobes. Each lung was embedded in paraffin, and stained with H–E.

Naphthol AS-D chloroacetate esterase stain

To assess the accumulation of activated neutrophils in paraffin-embedded sections of the right upper and mediastinal lobes collected at 80 min after reperfusion, polymorphonuclear neutrophils (PMNs) were identified by morphology and positive staining for chloroacetate esterase, a neutrophil-specific marker, according to the method described by Leder [19]. Neutrophilic infiltration was expressed by the average number of PMNs around the pulmonary artery in five randomly chosen high-power fields per section at an original magnification of 400 times. Three separate investigators (AO, JS, and DN) evaluated, interpreted, and reached a consensus, without any knowledge of the experimental groups.

Statistical analysis

All statistical analyses were performed using StatView 5.0 software (Abacus Concepts, Berkeley, CA, USA). All values are presented as the mean ± SEM. Data were evaluated using one-way analysis of variance (anova), Scheffe's *post hoc* multiple comparison test, and Student's unpaired *t*-test. A probability (*P*) value less than 0.05 was considered statistically significant.

Results

Study I

The body weights of the rats were similar in two groups (290 ± 4.8 g and 290 ± 1.6 g in the control and surfactant groups, respectively). Dynamic compliance in the surfactant group was significantly higher than that in the control group (control group versus surfactant group: 0.965 ± 0.036 vs. 1.065 ± 0.018 ml/cmH_2O; $P < 0.05$). Airway resistance in the surfactant group seemed lower than that in the control group; however, there was no significant difference between two groups (control group versus surfactant group: 0.0349 ± 0.0030 vs. 0.0309 ± 0.0031 cmH_2O/ml/s).

P–V curves

Dynamic P–V curves were obtained in both groups (Fig. 2a and b). In the surfactant group, the total lung volume at the same pressure of 30 cm H_2O was more than that in the control group.

Toluidine blue stain

In the control group, nothing was visible within alveoli (Fig. 3a). On the other hand, in the surfactant group, hollow-round or small-round brown particles were lining in contact with the alveolar wall within well-inflated alveoli (Fig. 3b and c).

Study II

Physiological parameters

The body weights of the rats were similar in all three groups (299 ± 9.5 g, 300 ± 5.5 g, and 299 ± 9.8 g in the sham, control, and surfactant groups, respectively). All the lungs were reperfused successfully in 80 min. Thus, consistent with previous reports [12–17], our isolated rat lung perfusion model remained stable throughout the observation period.

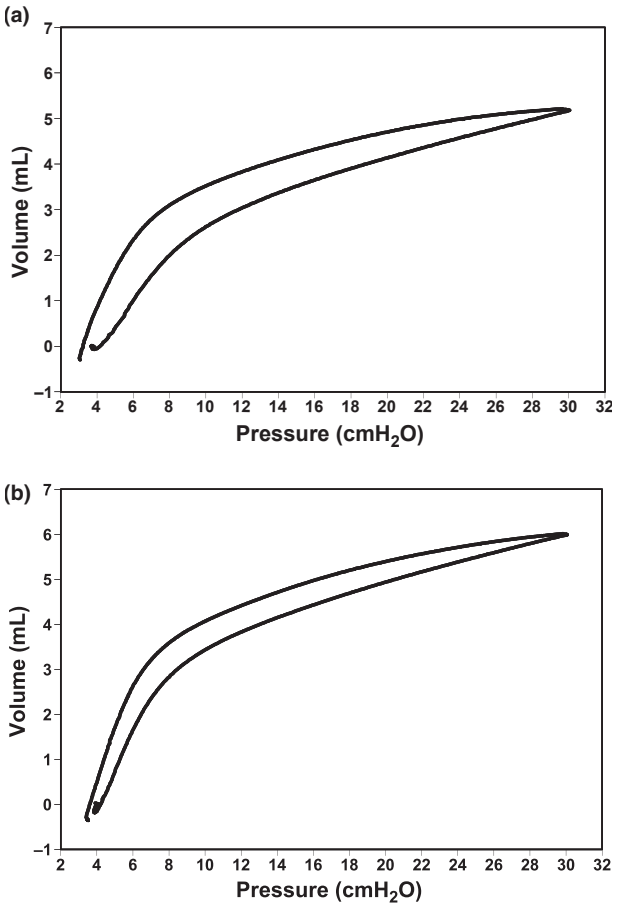

Figure 2 P–V curves. (a) the control group, (b) the surfactant group.

Airway resistance

Airway resistance in the control group increased and was significantly higher than that in the sham group ($P < 0.001$ at 80 min after reperfusion). However, in the surfactant group, airway resistance was significantly lower than that in the control group ($P < 0.01$ at 80 min; Fig. 4a).

Dynamic compliance

Dynamic compliance in the control group decreased and was significantly lower than that in the sham group ($P < 0.001$ at 80 min after reperfusion). In the surfactant group, dynamic compliance gradually increased and became the highest 20 min after reperfusion; it was significantly higher than that in the control group throughout the reperfusion period ($P < 0.001$ at 80 min; Fig. 4b).

Pulmonary vascular resistance (PVR)

There were no differences in PVR between the three groups at 80 min after reperfusion (Fig. 4c).

Weight gain

Weight gain of the lung during reperfusion indicates pulmonary edema in this model. In the control group, weight gain increased and was significantly higher than that in the sham group ($P < 0.001$ at 80 min after reperfusion). In contrast, in the surfactant group, it decreased slightly and was significantly lower than that in the control group throughout the reperfusion period ($P < 0.001$ at 80 min; Fig. 4d).

Shunt fraction

The shunt fraction in the sham group was low and increased gradually. Shunt fraction in the control group increased and was significantly higher than that in the sham group ($P < 0.001$ at 80 min after reperfusion). However, in the surfactant group, shunt fraction was dramatically lower than that in the control group, and was similar to that in the sham group ($P < 0.001$ at 80 min; Fig. 4e).

Energy levels after reperfusion

At 80 min after reperfusion, lung tissue levels of ATP, ADP, AMP, and total adenosine nucleotides (TAN; TAN = ATP + ADP + AMP) in the control group were significantly lower than those in the sham group ($P < 0.001$). Furthermore, lung tissue levels of TAN, ATP, and ADP in the surfactant

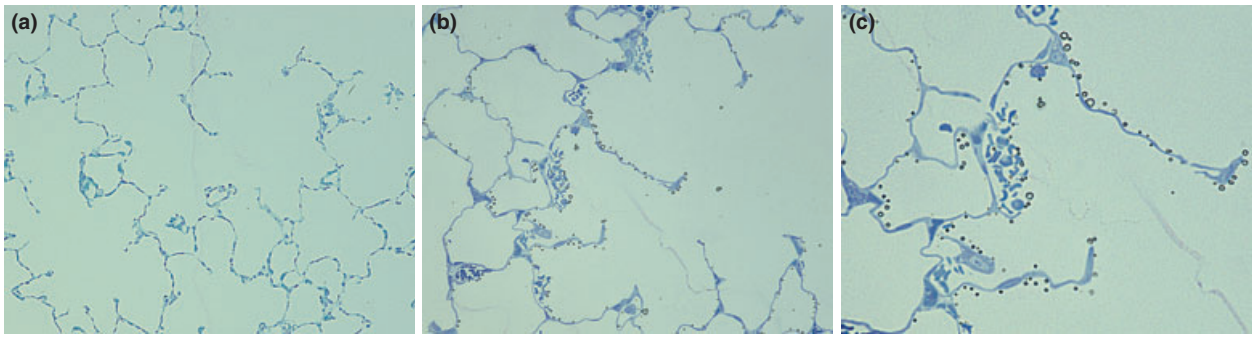

Figure 3 Histological findings just after inhalation (toluidine blue staining). (a) In the control group, nothing was visible within alveoli. (b, c) In the surfactant group, hollow-round or small-round brown particles were lining in contact with the alveolar wall (a, b: original magnification 200 times, c: original magnification 400times).

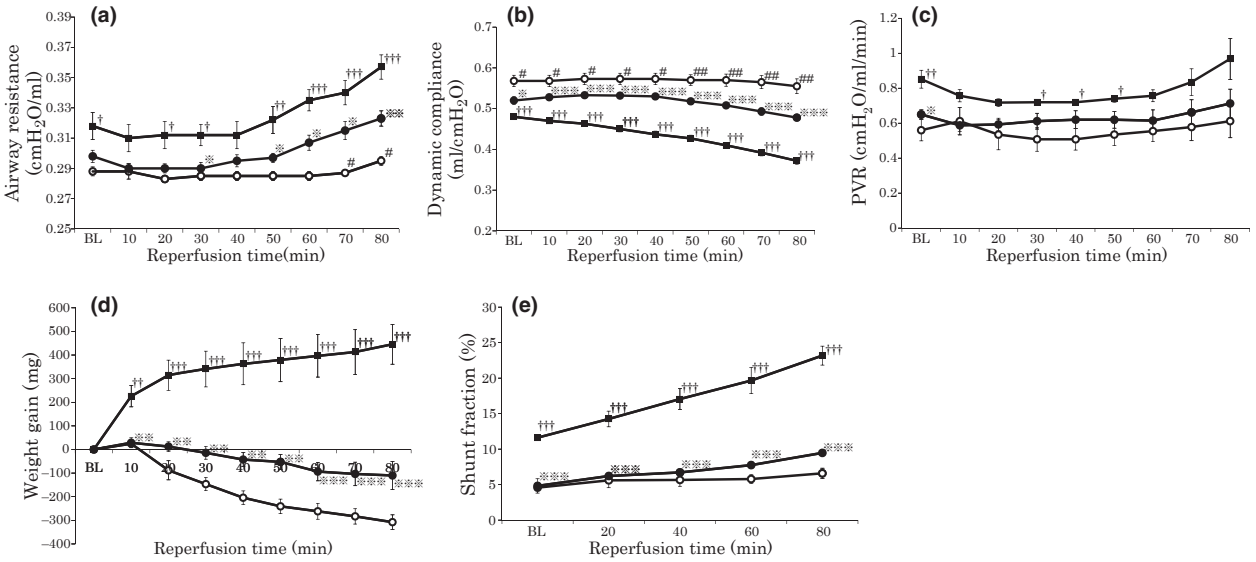

Figure 4 Physiological lung functions during reperfusion. (a) Airway resistance. (b) Dynamic compliance. (c) Pulmonary vascular resistance. (d) Weight gain of the lung. (e) Shunt fraction. $^{\dagger}P < 0.05$ or $^{\dagger\dagger}P < 0.01$ or $^{\dagger\dagger\dagger}P < 0.001$ between the sham (open circles) and control groups (boxes). ※$P < 0.05$ or ※※$P < 0.01$ or ※※※$P < 0.001$ between the control and surfactant groups (solid circles). $^{\#}P < 0.05$ or $^{\#\#}P < 0.01$ between the sham and surfactant groups. Data are expressed as mean values ± SEM (BL, baseline).

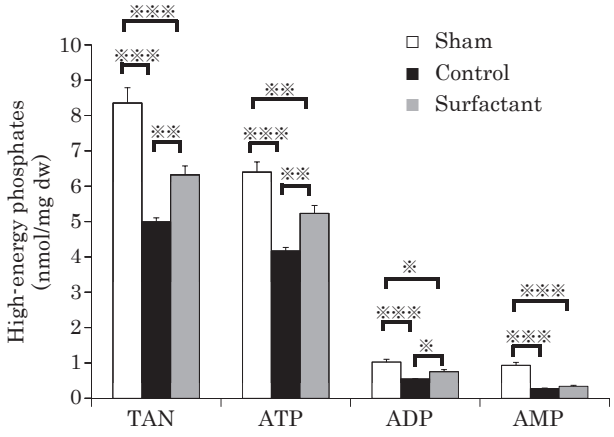

Figure 5 The levels of adenosine nucleotides (nmol/mg dry weight) were measured at 80 min after reperfusion. All values are expressed as the mean ± SEM. TAN = ATP + ADP + AMP. TAN, total adenosine nucleotides; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate. ※$P < 0.05$, ※※$P < 0.01$, and ※※※$P < 0.001$ for difference between two groups.

group were significantly higher than those in the control group (TAN, ATP: $P < 0.01$, ADP: $P < 0.05$; Fig. 5).

mRNA expressions of cytokines after reperfusion

At 80 min after reperfusion, interleukin (IL)-6 mRNA levels were significantly higher in the control group than those in the others ($P < 0.05$; Fig. 6a). On the other hand, in IL-10 mRNA levels, there were no significant differences between three groups (Fig. 6b). However, mRNA levels ratio of IL-6 to IL-10 (IL-6/IL-10) in the surfactant group were significantly lower than those in the control group ($P < 0.01$; Fig. 6c).

Histological examination (H–E stain)

In the sham group, mild perivascular and interstitial edema was observed (Fig. 7a). In the control group, severe perivascular edema was observed in almost all vessels with sporadic perivascular and interstitial hemorrhage (Fig. 7b). In the surfactant group, mild perivascular and interstitial edema was observed in almost all vessels, but little severe edema and hemorrhage was observed (Fig. 7c).

Naphthol AS-D chloroacetate esterase stain

Around the pulmonary artery, in the sham group, there were scarcely any PMNs (Fig. 8a), but in the control group, several PMNs were seen (Fig. 8b), and in the surfactant group, a few PMNs were seen (Fig. 8c). The neutrophilic infiltration was significantly lower in the surfactant group than in the control group ($P < 0.001$; Fig. 8d).

Discussion

In this study, we used an isolated rat lung perfusion model to investigate the effect of surfactant inhalation on I–R injury in rat lungs from DCD donors. Our results showed that surfactant inhalation during the last phase of warm ischemia improved graft functions, maintained lung tissue energy levels, and prevented cytokine production, thereby attenuating warm I–R injury in rat lungs. This is

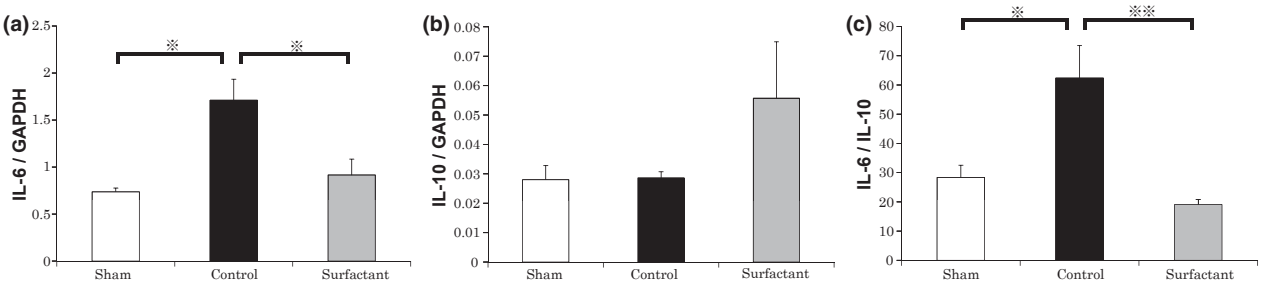

Figure 6 mRNA Expressions of cytokines after reperfusion. (a) IL-6 mRNAlevels. (b) IL-10 mRNA levels. (c) mRNA levels ratio of IL-6 to IL-10 (IL-6/IL-10). All values are expressed as the mean ± SEM. ※$P < 0.05$ or ※※$P < 0.01$ for difference between two groups.

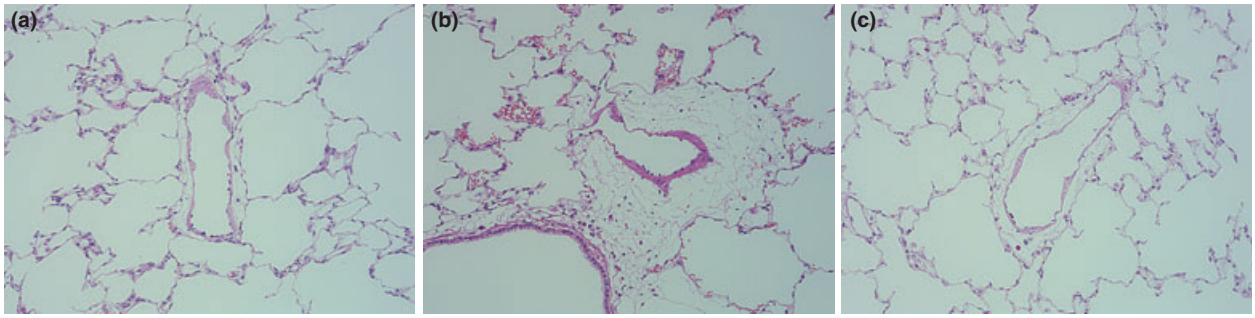

Figure 7 Histological findings after reperfusion (hematoxylin–eosin staining). (a) In the sham group, mild perivascular and interstitial edema can be seen. (b) In the control group, severe perivascular edema is seen in almost all the vessels, and perivascular and interstitial hemorrhage is visible in a few vascular areas. (c) In the surfactant group, mild perivascular and interstitial edema is visible in almost all vessels, and severe edema and hemorrhage is almost absent.

the first study to report that surfactant inhalation during warm ischemia attenuated warm I–R injury of the lung.

Although lung transplantation from DCD donors has been in use globally, increased awareness is required regarding the evaluation of the availability of the grafts and the reconditioning of marginal donor lungs. However, pretreatment in DCD donors has raised ethical issues. Considering this, inhalation appears advantageous, as it is not only suitable and convenient, but also involves a lung-specific drug delivery route [12,13,15]. Previously, nebulization was chosen as a surfactant replacement therapy in clinical [6,7] and experimental settings [20]. Some studies have concluded that the tracheal instillation delivery method is superior to aerosolized surfactant [21,22]; however, we chose the inhalation technique because the distribution of aerosolized surfactant is superior to that of instilled surfactant when the pattern of ventilation is uniform [21]. In addition, our previous experiments using drug inhalation in warm ischemic-reperfused rat lungs have been successful [12,15].

Inci *et al.* showed that pulmonary functions, wet-to-dry weight ratio, and protein levels in bronchoalveolar lavage were significantly different between heart-beating and DCD donors with warm ischemia of 3 h using a porcine model of *ex vivo* lung perfusion [5]. Although our experiments differed from those of Inci *et al.*, our results were similar to theirs. The function of a pulmonary surfactant is widely known: it decreases surface tension, prevents microatelectasis, increases compliance, and reduces airway resistance. First, in the current study I, we demonstrated the efficacy of surfactant inhalation with improved graft function and deposited surfactants beneath the alveoli. Just after the inhalation, in the surfactant group, dynamic compliance was significantly higher than that in the control group. Furthermore, we also observed inhaled surfactants lining beneath the alveoli with relative uniformity. Mühlfeld reported the effect of preischemic exogenous surfactant instillation and showed instilled surfactants attached to the alveoli, which was consistent with our findings [23].

In the current study II, dynamic compliance in the surfactant group was higher than that in the control group throughout reperfusion, but during the latter half of reperfusion, dynamic compliance and airway resistance gradually deteriorated in both the control and surfactant groups. As Novick *et al.* reported previously, additional surfactant inhalation during reperfusion could improve effectiveness [20]. Furthermore, in the current study, surfactant inhalation decreased shunt fraction probably because of reduced microatelectasis and pulmonary

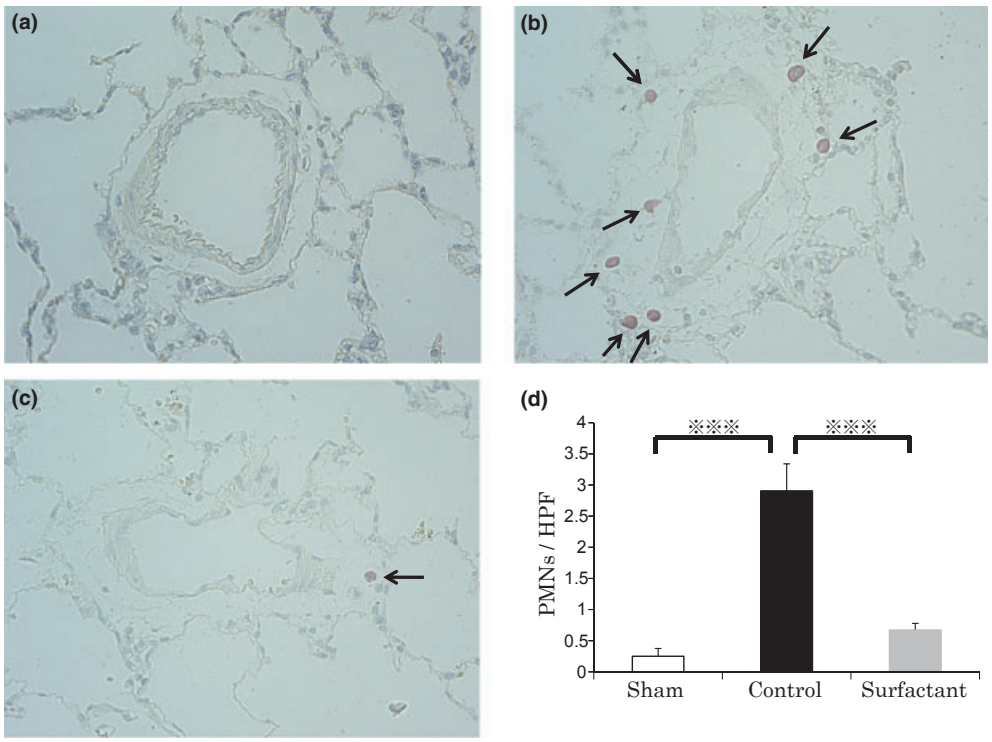

Figure 8 Naphthol AS-D chloroacetate esterase stain for investigation of neutrophilic infiltration. (a) In the sham group, PMNs are scarce. (b) In the control group, several PMNs can be seen. (c) In the surfactant group, a few PMNs are visible. Arrows indicate PMNs around the pulmonary artery. (d) Neutrophilic infiltration is expressed by the average number of PMNs counted in five high-power fields/section, and numbers are expressed as mean values ± SEM. ※※※$P < 0.001$ between the sham and control groups, and between the control and surfactant groups.

edema, leading to improved ventilation/perfusion mismatch and oxygenation. Low doses of ultrasonically delivered natural surfactant were found to be as effective as conventional doses of tracheal-instilled surfactant in reducing shunt flow in an acute lung injury model, but exert more advantageous effects on ventilation/perfusion matching [24]. According to our preliminary experiments, 1 min and half of inhalation was not effective, but 3 min of inhalation was the most effective against the I–R injury (data not shown).

Endothelial damage induced by I–R injury increases vascular permeability and causes perivascular and intraalveolar edema. Dreyer reported that surfactant treatment decreased intraalveolar edema formation and protein leakage in a rat lung I–R injury model [25]. In addition, capillary leakage of plasma proteins into the alveolar space reportedly inactivated the pulmonary surfactant and reduced lung compliance [26], and therefore, the inhibition of protein leakage by the surfactant activated surfactant function. In the current study, weight gain in the control group increased markedly from baseline, whereas weight gain in the surfactant group decreased. We believe that surfactant inhalation prevents capillary leakage of plasma protein as well as inactivation of pulmonary surfactant. Inhibition of pulmonary edema can also be attributed to both A549 cells (which resemble type II pneumocytes in many features) and rat alveolar type II pneumocytes; they have been reported to exhibit a fall in transcellular Na^+ transport in hypoxic conditions [27]. Stabilization of transcellular Na^+ transport by surfactant replacement therapy might improve pulmonary edema.

The most important cellular role for oxygenation is in ATP synthesis via the mitochondrial electron transport chain [28]. Elongation of the postmortem warm ischemic period resulted in a reduction in the adenine nucleotide levels, in correlation with exacerbation of the pulmonary functions after reperfusion in a rat lung perfusion model [29]. In the current study, lung tissue levels of TAN, ATP, ADP, and AMP after 135 min of warm ischemia were significantly lower than those in the heart-beating donor, and those after 80 min of reperfusion in the surfactant group were the same as those after 135 min of warm ischemia (results not shown), whereas lung tissue levels of TAN, ATP, and ADP in the control group significantly decreased. Several reports have supported the correlation between reduction in energy levels and I–R injury. First, the reduction in the levels of intrapulmonary high-energy phosphate compounds after reperfusion may

contribute to reperfusion injury [30]. Second, energy depletion might be one of the major causes of I–R injury in warm ischemia [31]. In solid organs, such as the heart, I–R injury is regarded to partially rely on cell viability related to the levels of the high-energy phosphate compounds or mitochondrial status [32]. Therefore, the levels of ATP can be considered useful indicators of reperfusion injury [33]. On the basis of several theories, we believe that surfactant inhalation during warm ischemia mutually protected the viability of type II pneumocytes and graft functions, leading to maintenance of lung tissue energy levels.

It has been demonstrated that cytokine expression levels are associated with ischemia-reperfusion injury in lung transplantation [34,35]. Kaneda *et al.* reported that IL-6 had significantly higher expression levels in the lungs that ultimately lead to patient death within 30 days using quantitative real-time RT-PCR in clinical settings [36]. Furthermore, they also demonstrated that ratio of IL-6/IL-10 was the best overall marker to predict the recipient early outcome [36]. These observations were consistent with our results.

A surfactant is considered to have pleiotropic effects during I–R injury. It has anti-inflammatory effects, accompanied by decreased apoptosis [37]. In the current histological investigation, surfactant inhalation prevented not only pulmonary edema and hemorrhage, but also neutrophilic infiltration. Some reports emphasized that neutrophilic sequestration greatly affect the vulnerability of the lung after I–R injury [38,39]. The PMN sequestration causes tissue damage by adhering to the vasculature, infiltrating local tissues, and releasing superoxides and elastases [39]. Therefore, we measured neutrophilic infiltration and found that it was significantly reduced in the surfactant group. Prevention of neutrophilic infiltration could be related to anti-inflammation. Thus, additional clarification of the pleiotropic effects of surfactant administration is required.

There are several limitations to our study: a small animal *ex vivo* model as opposed to an actual lung transplantation model was used; no cold preservation was applied, which is always required clinically; and lungs were ventilated with negative pressure. Therefore, in the future, we will evaluate the effect of surfactant inhalation in a large animal lung transplantation model, where warm ischemia of donor lungs is induced *in vivo*, followed by cold ischemia with preservation solution.

In conclusion, we confirmed, using an isolated rat lung perfusion model, that surfactant inhalation during the last phase of warm ischemia improved graft function, maintained lung tissue energy levels, and prevented cytokine production, resulting in the attenuation of pulmonary I–R injury. Our data also showed that surfactant inhalation could be a convenient and effective method in lung transplantation from DCD donors.

Authorship

AO, DN, JS and FC: performed the research. AO, FC, DN, JS, TY, TF, TS, HS, TB and HD: designed the research. AO, FC, DN, JS and HD: wrote the paper.

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