Title: Impact of the cardiac arrest mode on cardiac death donor lungs

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(2) Drafting the article or revising it critically for important intellectual content,

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Abstract

Background: Donation after cardiac death (DCD) organs could alleviate the shortage of donor lungs. This study aimed to assess the influence on lung injuries of the way in which cardiac arrest was induced and to investigate the mechanisms leading to any differences.

Materials and Methods: Male rats were allocated into 3 groups: sham (no warm ischemia), ventricular fibrillation (VF), and asphyxia group. Cardiac arrest was induced by either VF by way of a fibrillator or asphyxia caused by withdrawal of ventilation, which reflected uncontrolled and controlled DCD situations, respectively. The impact on lung flushing after 60 minutes of warm ischemia time was evaluated (n = 5, in each group). The physiological functions of the lungs in an isolated lung perfusion circuit were also evaluated with warm ischemia time prolonged to 150 minutes (n = 8, in each group). mRNA expression levels of surfactant proteins and inflammatory cytokines, pathological findings, and high-energy phosphates of the lung tissues were investigated.

Results: In the asphyxia group, flushing as well as physiological functions in the isolated lung perfusion circuit were the most severely affected. Reverse transcription-polymerase chain reaction and pathological findings revealed depletion of surfactant protein-C in lung tissues of the asphyxia group after reperfusion. In the VF group, elevation of pulmonary vascular resistance was characteristic.

Conclusions: Lung injuries were mainly attributed to alveolar wall damage and depletion of surfactant protein in the asphyxia group, and perivascular area prominent edema in the VF group. DCD donor lungs were affected differently by the way in which cardiac arrest was induced.

Key words: lung transplantation, donation after cardiac death, donor, ischemia-reperfusion injury

Introduction

Lung transplantation has been established as the treatment of choice for selected end-stage respiratory diseases; however, a shortage of donor lungs remains a crucial limitation associated with this modality in clinical practice [1, 2]. To alleviate this limitation, donor lungs from marginal donors including donation after cardiac death (DCD) donors have been utilized for lung transplantation [3]. DCD donor lungs are, however, affected by many factors, which differ from the brain death scenario. For instance, premortem hypotension and the mode of death in DCD donors have previously been reported to affect lung functions after reperfusion [4, 5], but the influence on lung injury by the way in which cardiac arrest is induced remains unclear.

In the present study, we hypothesized that during asphyxia in the agonal phase, as seen in a controlled DCD scenario, intra-alveolar oxygen is decreased, which contributes to the pathophysiology of lung injuries. The objectives of this study were therefore to assess the influence on lung injuries of the way in which cardiac arrest is induced, and to investigate the mechanisms leading to any differences.

Materials and Methods

Animals and types of treatment

Specific pathogen-free inbred male Lewis rats (weight, 290–310 g) were used (Japan SLC Inc., Hamamatsu, Japan). All animals received humane care in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication 85-23, revised 1996; Bethesda, MD) and the EU directive 2010/63/EU for animal experiments. http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm. The current study protocol was approved by the Graduate School of Medicine Ethical Committee at Kyoto University.

Statistical analysis

All statistical analyses were performed using the StatView 5.0 software program (Abacus Concepts, Berkeley, California) on an AT-compatible computer. All values are expressed as mean ± standard deviation (SD). The data were evaluated using one-way analysis of variance and Scheffe's post-hoc multiple comparison test for multiple group analysis. A probability (p) value <0.05 was considered to be statistically significant.

Experiment 1

Experimental protocol

The animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg), intubated after tracheotomy, and ventilated with room air under the following conditions: tidal volume of 0.01 mL/g; respiratory rate of 60 cycles/min; positive end-expiratory pressure of 3 cmH_2O; and a ratio of inspiratory duration of 50%. The animals were not heparinized. The animals were then randomly allocated into 3 groups: sham, ventricular fibrillation (VF), and asphyxia groups (n = 5 each).

In the sham group, the pulmonary artery was directly cannulated while the hearts were kept beating. In the VF group, VF was induced and continued for five minutes by way of a fibrillator attached directly to the right atrium and apex of the heart meanwhile lungs were ventilated with room air, and then the ventilator was disconnected. In the asphyxia group, the ventilator was disconnected while the tracheal tube was kept open to induce asphyxia. After cardiac arrest was confirmed, the chest was closed with skin staplers and the rats were placed at room temperature for 60 minutes. The pulmonary artery was then cannulated and the lungs were flushed with 50 mL of trypan blue (Sigma

Chemical Co., St. Louise, Mo) solution dissolved in normal saline (0.2 mM, 4°C) at a pressure of 20 cmH_2O, and drained through a left atrium incision. The time required for the flushing of each lung was measured, and the gross appearance of lungs dyed with trypan blue was also evaluated.

Experiment 2

Experimental protocol

The animals were randomly allocated into sham, VF, and asphyxia groups as described in experiment 1 (n = 8 each). The animals were not heparinized. In the sham group, the pulmonary artery was cannulated while the heart was beating and flushed with 20 mL of 4°C low potassium dextran solution (Perfadex®, Vitrolife, Uppsala, Sweden) at a pressure of 20 cmH_2O. In the VF and asphyxia groups, animals were placed at room temperature for 150 minutes after cardiac arrest was confirmed. The pulmonary artery was then cannulated and the lungs were flushed in the same way as in the sham group.

Ventilation with room air was initiated 5 minutes before flushing under the following conditions: positive pressure-control mode of +8 and +4 cm H_2O; respiratory rate, 60 cycles/min; and ratio of inspiratory duration of 50%. After flushing, the total lung capacity (TLC) maneuver using 30 cm of H_2O for 1 minute was performed [6]. Heart-lung blocks were then extracted from the animals (Figure 1).

Reperfusion in an isolated lung perfusion circuit

Reperfusion of the rat lungs was performed in an isolated rat lung perfusion circuit (Model 829; Hugo-Sachs Elektronik Harvard Apparatus, March-Hugstetten, Germany) as reported previously [7-9]. The perfusate was heparinized rat blood obtained from 2 donor rats diluted with saline containing 4%

bovine serum albumin and was delivered by 2 pumps. No leukocyte filter was used and the circuit was water-jacketed to maintain the temperature at 37°C.

The effluent perfusate from the left atrium was deoxygenated in a glass deoxygenator with anoxic gas (nitrogen, 92%; carbon dioxide, 8%), and was pumped into the pulmonary artery. The perfusate was stabilized by circulating outside of the lung for 10 minutes before reperfusion to maintain equal distribution. The perfusion flow was increased step-by-step for 5 minutes up to 10 mL/min.

The ventilation was changed to a negative pressure-control mode of −8 and −4 cm H_2O with room air using a chamber as an artificial thorax and continued throughout the perfusion. The zero points of time and weight of the heart–lung block were set at the end of the step up in the perfusion flow. After 75 minutes of reperfusion, lung tissues were sampled by each method below for the following evaluations.

Perfusate and physiologic data analyses

The pulmonary artery pressure and tidal volume of the lungs were measured by a controller connected to the circuit, and the weights of the heart–lung blocks were measured by a transducer. The hemoglobin level and blood gases in the deoxygenated effluent and pulmonary vein effluent were also analyzed at 0, 15, 30, 45, 60, and 75 minutes to calculate the pulmonary shunt fraction.

Evaluation of high-energy phosphates in lung tissue by high-performance liquid chromatography

After reperfusion, the pulmonary vasculature of the lungs was flushed with 20 mL of 4°C normal saline and lung tissues were stored using a freeze-drying method at −20°C. The tissues were then homogenized to measure the levels of high-energy phosphates including adenosine triphosphate (ATP),

adenosine diphosphate (ADP), and adenosine monophosphate (AMP) by high-performance liquid chromatography using a Shim-pack CLC-ODS (15 cm × 6.0 mm) column (Shimadzu Corp., Kyoto, Japan), as previously reported [9].

Evaluation of the mRNA expression using real-time reverse transcription-polymerase chain reaction

The lower part of the left lung was scraped and immersed in RNA-later (Qiagen, Tokyo, Japan) immediately after the end of reperfusion, and stored at −80°C. Total RNA was isolated from the lung tissues and purified with an RNeasy Mini kit® (Qiagen, Tokyo, Japan), and reverse transcription (RT) was performed with Ready-To-Go You prime First strand Beads® (Amersham Biosciences, Piscataway, NJ) in accordance with the manufacturer's protocol.

The forward and reverse primers used for quantitative amplification were designed using the Genefisher software program (http://bibiserv.techfak.uni-bielefeld.de/genefisher/old.html) and confirmed by electrophoresis (Table 1). Six genes were examined by real-time RT-polymerase chain reaction (PCR). A Light Cycler® thermal cycler system (Roche Diagnostics, Tokyo, Japan) was used. PCR amplification was initiated by pre-incubation for 900 seconds at 95°C, followed by 40 cycles of denaturation at 94°C, annealing at 56°C, and elongation at 72°C for 20 seconds, each with the detection of the fluorescent products. The expression levels of each gene were represented as the ratio relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene expression, which was used as an internal standard.

Pathological evaluation

A portion of the right lungs were fixed in 10% formalin and embedded in paraffin, then investigated

using hematoxylin-eosin (HE) staining. To evaluate the Surfactant Protein-C (SP-C) in lung tissues, the percentage of stained pixels in the evaluated area was calculated through immunochemical SP-C staining without a counter stain. Four of eight slides in each group, stained in uniform quality, were selected and three of 300 × 300 μm square areas in each slide (12 areas in each group) were randomly selected.

Results

Experiment 1

Lung flush analysis

Rat lungs were stained almost homogeneously in the sham and VF groups, while the lungs were stained heterogeneously in the asphyxia group, which indicated insufficient distribution of the flush solution to the areas that were not stained.

The time required for the flushing was 91 ± 31 seconds, 112 ± 15 seconds, and 205 ± 9 seconds in the sham, VF, and asphyxia groups, respectively. The flush time was significantly different between all of the groups (sham vs asphyxia, $p < 0.0001$; sham vs VF, $p = 0.022$; VF vs asphyxia, $p < 0.0001$; Figure 2).

Experiment 2

Settings of the study groups

There were no significant differences in the rat body weights, hemoglobin levels, oxygen and carbon dioxide tension, or pH of the perfusates and deoxygenator effluents at 0 minutes between the 3 groups. The characteristics of each group including the time required to induce cardiac arrest, the body temperatures of the animals when flush was initiated, and the time required for lung flushing are

described in Table 2. All the lungs underwent 75 minutes of reperfusion.

Physiological data analyses

Pulmonary shunt fraction. The pulmonary shunt fractions in the asphyxia group were significantly higher than those in the sham ($p < 0.01$) and VF ($p < 0.05$) groups. Those in the VF group were also significantly higher than those in the sham group ($p < 0.01$, Figure 3A).

Tidal volume. The tidal volumes in the asphyxia group were significantly lower than those in the sham and VF groups and those in the VF group were significantly lower than those in the sham group ($p < 0.0001$, sham vs. VF vs. asphyxia, at all points of measurement; Figure 3B).

Weight gain. Weight gains in the asphyxia group were higher than in the VF group ($p < 0.01$ VF vs. asphyxia at 15 minutes), while those in the VF group were higher than in the sham group. Weight gains in the asphyxia group were also significantly higher than those in the sham group ($p < 0.01$, asphyxia vs. sham on and after 15 minutes; Figure 3C).

Pulmonary artery pressure. At the beginning of the evaluation, pulmonary artery pressures in the asphyxia and VF groups were higher than those in the sham group ($p < 0.05$, sham vs. VF and asphyxia at 0 minutes). At and after 15 minutes of reperfusion, those in the VF group were higher than those in the asphyxia and sham groups ($p < 0.05$, sham vs. VF at 15 minutes; $p < 0.05$, VF vs. sham and asphyxia on and after 30 minutes; Figure 3D).

High-energy phosphate levels in lungs after reperfusion

The levels of ATP, ADP, and AMP in the lung tissues after reperfusion were highest in the sham

group and lowest in the asphyxia group. The ATP levels were 6.39 ± 0.55, 4.93 ± 0.56 and 4.06 ± 0.57 nmol/mg dry weight (dw) in the sham, VF and asphyxia groups, respectively ($p < 0.05$, VF vs. asphyxia; $p < 0.01$, sham vs. VF, and sham vs. asphyxia). The ADP levels were 1.49 ± 0.14, 1.07 ± 0.17, and 0.94 ± 0.18 nmol/mg dw in the sham, VF and asphyxia groups, respectively ($p < 0.001$, sham vs. VF, and sham vs. asphyxia). The AMP levels were 0.85 ± 0.30, 0.46 ± 0.13, and 0.41 ± 0.17 nmol/mg dw in the sham, VF and asphyxia groups, respectively ($p < 0.01$, sham vs. VF, and sham vs. asphyxia; Figure 4).

RT-PCR

The mean SP-C and Surfactant Protein-A (SP-A) mRNA expression levels in the VF group were higher than those in the asphyxia group, while those in the sham group were the highest among the three groups. There was a significant difference in the mean SP-C expression level between the sham and asphyxia groups ($p = 0.019$). In contrast, there were no significant differences in the expression of inflammatory cytokines IL-1β, IL-6 and IL-10. (Table 3).

Pathological findings in the lungs after reperfusion

In the asphyxia group, the presence of intra-alveolar debris and edema, caused by destruction of alveolar capillaries, was the most remarkable among the three groups. In the VF group, edema was prominent in the perivascular area. In the sham group, the micro lung structures were well maintained (Figures 5A-C).

In immunochemical SP-C staining, the percentages of SP-C stained areas in 300 × 300 μm square areas (12 areas in each group) were 4.28% ± 1.8%, 2.02% ± 0.88 %, and 0.88% ± 0.47% in the sham, VF, and asphyxia groups, respectively. Those in the asphyxia group showed a trend toward being

lower than those in the VF group ($p = 0.078$) while those in the sham group were significantly higher than in the asphyxia ($p < 0.001$) and VF ($p < 0.001$) groups (Figure 5D).

Discussion

With regards to organ preservation, although the lungs have a unique tolerance to the absence of circulation due to oxygen supplied from residual alveoli content [10], warm ischemia affects lungs more severely than cold ischemia. While there may be various situations leading to cardiac death in DCD donors, the conditions at the beginning of warm ischemia time would influence organ preservation. Hypotension during the premortem period has previously been reported to lead to impaired lung function after reperfusion in a rat lung perfusion model [4]. In a pig lung perfusion model, hypoxic cardiac arrest impaired lung function more severely than VF or exsanguination [5]. Similarly, in Experiment 1 of the present study, differences in lung injury based on the way in which cardiac arrest was induced were also revealed. The VF group and the asphyxia groups reflected situations observed in uncontrolled and controlled DCD donors, respectively. We hypothesized that the agonal phase of asphyxia impaired the microstructure of the lungs. During the agonal phase without ventilation in the asphyxia group oxygen in the alveoli might have accrued in the pulmonary circulation, which remained after the withdrawal of ventilation, consequently decreasing oxygen content in alveoli and leading to prominent microatelectasis [11]. This unfavorable condition in the microstructure of the lungs may have been enhanced during the warm ischemia period, and might affect lung preservation. We previously reported protective effect of the ventilation during warm ischemic time against lung injury on DCD donor lungs [12]. In this study, DCD donor lungs were ventilated with room air 5 minutes before flushing, and after flushing, the total lung capacity (TLC) maneuver was performed to recruit collapsed alveoli before reperfusion in an isolated lung perfusion

circuit [6].

In Experiment 2, physiological lung functions in the isolated lung perfusion circuit were most severely affected in the asphyxia group among the three groups with regards to the shunt ratio, tidal volume, and weight gain. On the other hand, after 15 minutes of perfusion, pulmonary artery pressure in the VF group was the highest among the three groups. These results are indicative of different features in lung injuries between the asphyxia and VF groups. In the asphyxia group, lung injuries were characterized by pulmonary edema and were evident in alveolar capillaries, which were further reflected by weight gain and impaired oxygenation. In contrast, in the VF group, lung injuries were prominent in intra-pulmonary vessels rather than in the alveolar capillaries, which could have resulted in elevated pulmonary artery pressure.

Pathological evaluations showed consistent findings, namely, that the most prominent feature of lung injuries in the asphyxia group was intra-alveolar edema [13], while that in the VF group was perivascular edema. The severity of lung injuries after reperfusion was also reflected in the levels of high-energy phosphates in the lung tissue, which showed the highest depletion in the asphyxia group. Although maintained under adequate cold ischemic condition [14], the intrapulmonary ATP levels after reperfusion were affected by inflation and the amount of oxygen in the alveoli [15, 16].

Surfactant proteins also play important roles in maintaining the microstructural integrity of the lungs by providing low surface tension at the air-liquid interface and preventing alveolar collapse [17]. In this study, both the mRNA expression levels of surfactant proteins as determined by RT-PCR and pathological evaluation through immunohistochemical staining revealed the most depletion of SP-C in the asphyxia group among the three groups although protein levels were not measured [18]. Surfactant proteins are synthesized and secreted for re-uptake and recycling by type 2 pneumocytes [17, 19]. Consequently, the depletion of surfactant proteins suggests that type 2 pneumocytes in the asphyxia

group were severely injured [20].

Pre-recovery surfactant inhalation on DCD donor lungs has previously been shown to have a protective effect against warm ischemia-reperfusion injury in a canine lung transplantation model [21] and in an isolated rat lung perfusion model similar to this study [22], and exogenous surfactant instillation to donor lungs has been reported to have positive effect on the early clinical outcome of lung transplantation [23]. The results of the present experiments suggest that these transairway treatments could be suitable for lungs from controlled DCD donors.

The present experiments have several limitations that need to be taken into consideration before these results can be applied in clinical situations. First, the sham group was not a brain death model, and lung injury during the progression of brain death was not reflected in the sham group [24]; introduction of cardiac arrest in VF group with fibrillator without respiratory arrest was different from uncontrolled DCD donor scenario; moreover, warm ischemia time without ventilation for 150 minutes is longer than that in a clinical DCD donor scenario. The isolated rat lung perfusion circuit enabled the evaluation of physiological data of the lungs in detail, but this is different from a transplantation model, and may not completely reflect the conditions in larger animals, including humans [25].

Conclusions

Lung injuries were found to be affected differently based on the manner in which cardiac arrest was induced. Lung flushing and physiological functions in the asphyxia group were more severely affected than in the VF group. The lung injuries observed in the asphyxia group were mainly attributed to pulmonary edema, and damage of the alveolar wall, which was hypothesized to be due to type 2 pneumocyte damage. These results may therefore lead to the development of better methods for obtaining donor lungs from DCD donors.

Disclosure statement The authors have no conflicts of interest to disclose.

Table 1. The forward and reverse primers designed for real-time RT-PCR

Gene name	Forward Primer	Reverse Primer
GAPDH	CCTCGTCTCATAGACAAGATGG	CATTTGATGTTAGCGGGATCTC
IL-1β	TCTGAAGCAGCTATGGCAAC	AGCCACAATGAGTGACACTG
IL-6	GTTGACAGCCACTGCCTTC	CTCCAGGTAGAAACGGAACTC
IL-10	GAGAAGCTGAAGACCCTCTG	GGCTTCTATGCAGTTGATGAAG
SP-A	AGACGTTTGTGCTGGAAGC	AGCATGGATCCTTGCAAGC
SP-C	ATGGACATGGGTAGCAAAGAG	TGCTCCTCCGATGCTCATC

Table 2. Characteristics of each group in experiment 2.

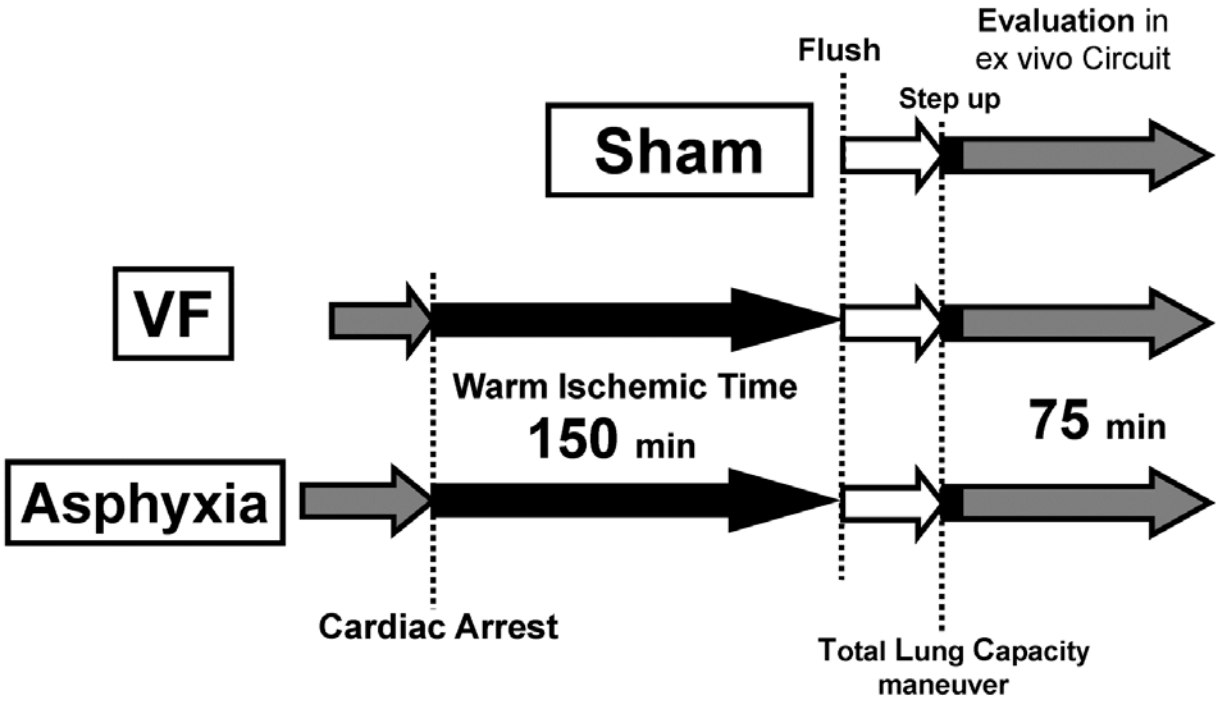
	Sham	VF	Asphyxia	p value
Number	8	8	8	
Weight (g)	300.4 ± 6.9	296.9 ± 7.5	298.5 ± 6.4	0.61
Time for cardiac arrest (min)		8.6 ± 0.5	16.1 ± 3.4	< 0.0001
Body temperature (°C)		26.7 ± 0.2	26.6 ± 0.3	0.46
Flush time (s)	65.4 ± 2.1	207.5 ± 18.8	263.6 ± 12.3	< 0.0001

Table 3. mRNA expression of each gene and IL-6/IL-10 ratio.

	Sham	**VF**	**Asp**
IL-1β	0.436 ± 0.150	0.309 ± 0.141	0.369 ± 0.185
IL-6	0.139 ± 0.049	0.283 ± 0.269	0.421 ± 0.256
IL-10	0.034 ± 0.014	0.033 ± 0.015	0.054 ± 0.029
SP-A	5.753 ± 3.832	3.165 ± 1.907	2.868 ± 2.021
SP-C	24.199 ± 14.676	12.578 ± 7.298	8.334 ± 6.031*****

Each value is shown as the GAPDH ratio, divided by GAPDH expression as the internal control (mean ± SD). ***** $p = 0.019$ vs. Sham group

Figure 1


Evaluation in
ex vivo Circuit
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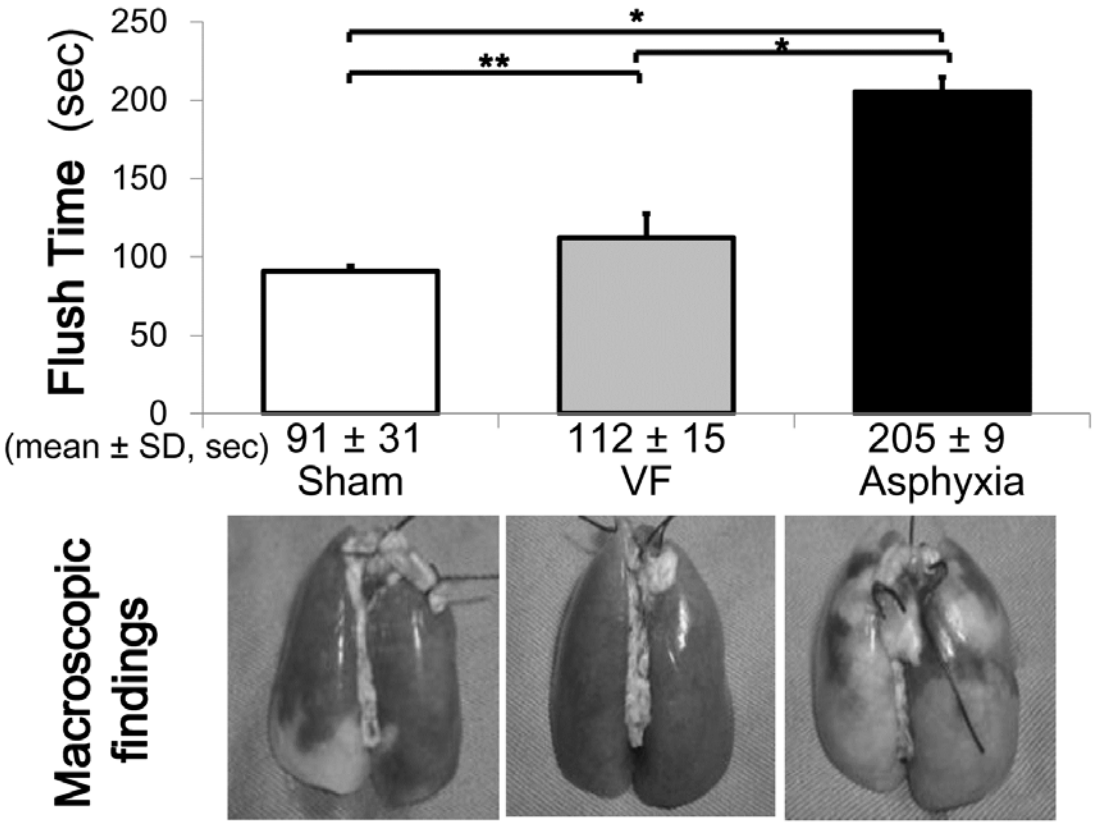
Cardiac Arrest
Total Lung Capacity
maneuver

Figure 2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91 ± 31
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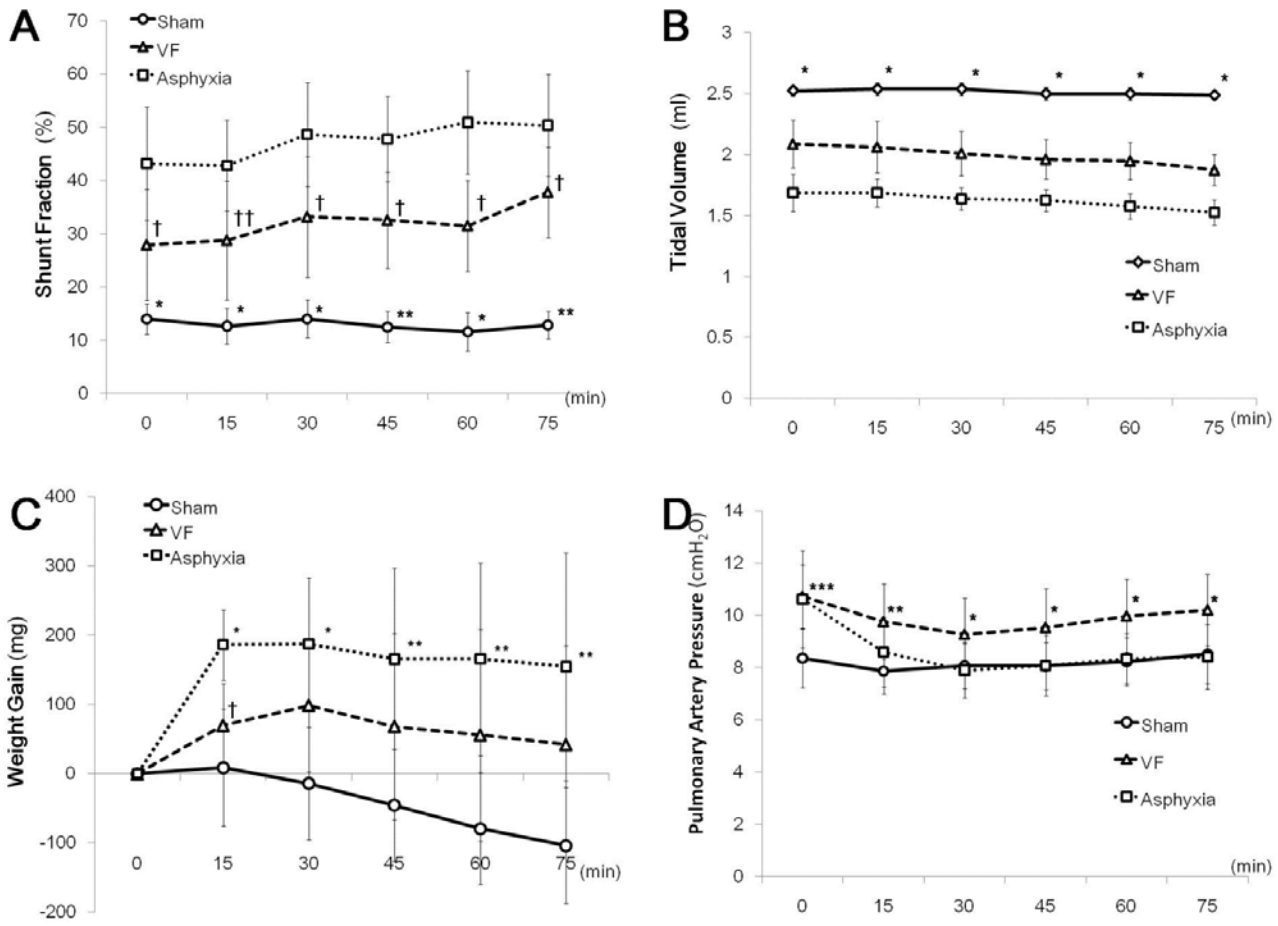
Asphyxia
Macroscopic findings

Figure 3


A
Sham
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Shunt Fraction (%)
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B
Tidal Volume (ml)
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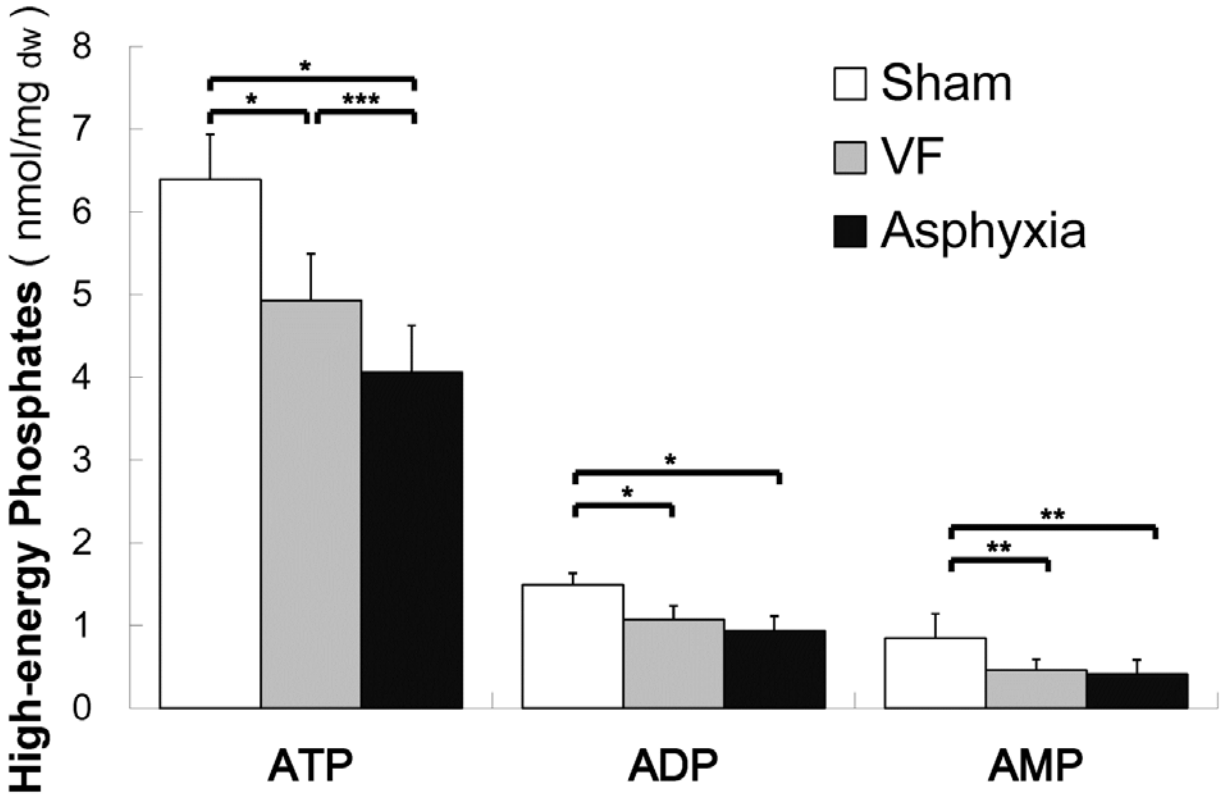
Asphyxia
(min)
C
Sham
VF
Asphyxia
Weight Gain (mg)
(min)
D
Pulmonary Artery Pressure (cmH2O)
Sham
VF
Asphyxia
(min)

Figure 4


High-energy Phosphates (nmol/mg dw)
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Sham
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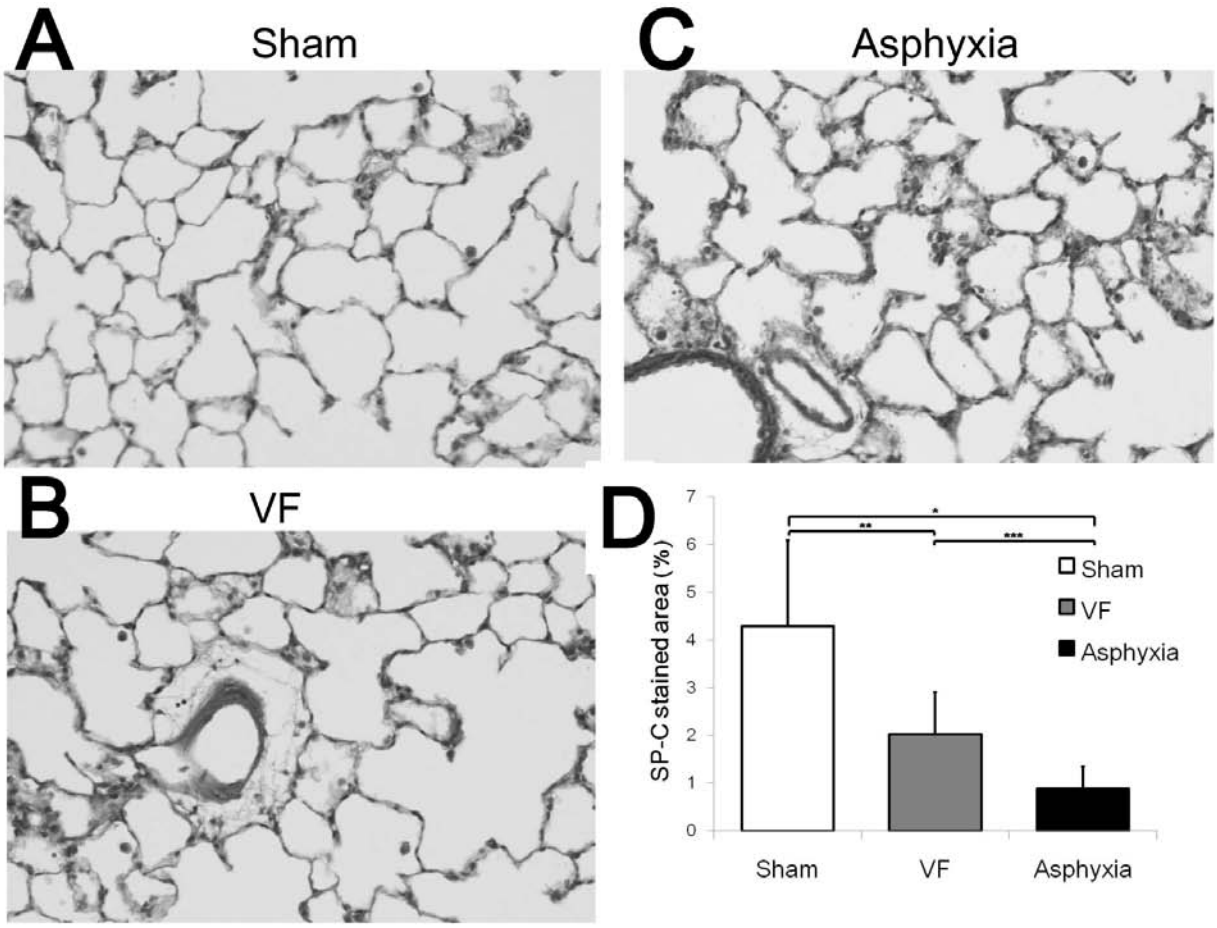
ATP
ADP
AMP

Figure 5


A
Sham
C
Asphyxia
B
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SP-C stained area (%)
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VF
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Sham
VF
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Figure Legends

Figure 1. The protocol for experiment 2. In the VF and asphyxia groups, after introduction of cardiac arrest, lungs were left in vivo for 150 minutes as the warm ischemic time. A total lung capacity (TLC) maneuver was performed before the extraction of the heart-lung block. After 5 minutes for a step-up period, perfusion at 10 mL/min was continued for 75 minutes in an ex vivo lung perfusion circuit.

Figure 2. The time required for flushing (mean ± SD) with 50 mL of trypan blue dyed normal saline at a pressure of 20 cmH2O was significantly different between the groups. ***** $p < 0.0001$, VF vs. Asphyxia, and Sham vs. Asphyxia; ****** $p = 0.022$, Sham vs. VF groups. In the macroscopic findings of the lungs after flushing, the lungs were found to be stained almost homogeneously in the sham and VF groups, while the lungs were stained heterogeneously in the asphyxia group.

Figure 3. The physiological lung function (mean ± SD) during reperfusion in the ex vivo circuit of the sham group (n = 8; circles), VF group (n = 8; triangles), and asphyxia group (n= 8; squares). (A) Pulmonary shunt fractions in the asphyxia group were significantly higher than those in the sham and VF groups. The pulmonary shunt fractions in the VF group were significantly higher than those in the sham group. ***** $p < 0.0001$, Sham vs. Asphyxia, $p < 0.01$: Sham vs. VF; ****** $p < 0.0001$, Sham vs. Asphyxia and VF; †$p < 0.01$, VF vs. Asphyxia; ††$p < 0.05$: VF vs. Asphyxia. (B) The tidal volumes in the asphyxia group were significantly lower than those in the sham and VF groups. The tidal volumes in the VF group were significantly lower than those in the sham group. ***** $p < 0.0001$, Sham vs. VF vs.

Asphyxia. (C) The weight gain in the asphyxia group was higher than in the VF group. The weight gain in the VF group was higher than in the sham group. Beginning at 15 minutes of reperfusion, the weight gains in the asphyxia group were significantly higher than those in the sham group. ***** $p < 0.001$, Asphyxia vs. Sham; ****** $p < 0.01$, Asphyxia vs. Sham; †$p < 0.01$, VF vs. Asphyxia. (D) The pulmonary artery pressure (cmH_2O) in the asphyxia and VF groups were higher than in the sham group at the start point of evaluation, and after 15 minutes of reperfusion, the pulmonary artery pressures in the VF group were higher than in the asphyxia and sham groups. * $p < 0.05$: VF vs. Sham and Asp ** $p < 0.05$: Sham vs. VF *** $p < 0.05$: Sham vs. VF and Asp

Figure 4. The levels of high-energy phosphates (nmol/mg dry weight) including adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) were measured using high-performance liquid chromatography. ***** $p < 0.001$, ****** $p < 0.01$, ******* $p < 0.05$.

Figure 5. The histological findings of the lungs after reperfusion (hematoxylin and eosin stain, original magnification ×20). (A) In the sham group, the lung structure was well maintained. (B) In the VF group, edema was prominent in the perivascular area, with less injury in the alveolar wall than in the asphyxia group. (C) The intraalveolar debris and edema in the alveolar wall, caused by destruction of alveolar capillaries, were most remarkable in the asphyxia group among the three groups. (D) The ratio of the SP-C stained area in 300 × 300 μm square areas (12 areas in each group). (***** $p < 0.0001$ Sham vs. Asphyxia, ****** $p = 0.0002$ Sham vs. VF, *******$p = 0.078$ Asphyxia vs. VF)

References

[1] Yusen RD, Edwards LB, Kucheryavaya AY, Benden C, Dipchand AI, Dobbels F *et al. The registry of the International Society for Heart and Lung Transplantation: thirty-first adult lung and heart-lung transplant report--2014; focus theme: retransplantation*. J Heart Lung Transplant 2014;**33**:1009-24.

[2] Sato M, Okada Y, Oto T, Minami M, Shiraishi T, Nagayasu T *et al. Registry of the Japanese Society of Lung and Heart-Lung Transplantation: official Japanese lung transplantation report, 2014*. Gen Thorac Cardiovasc Surg 2014;**62**:594-601.

[3] Cypel M, Keshavjee S. *Strategies for safe donor expansion: donor management, donations after cardiac death, ex-vivo lung perfusion*. Curr Opin Organ Transplant 2013;**18**:513-17.

[4] Tremblay LN, Yamashiro T, DeCampos KN, Mestrinho BV, Slutsky AS, Todd TR *et al. Effect of hypotension preceding death on the function of lungs from donors with nonbeating hearts*. J Heart Lung Transplant 1996;**15**:260-8.

[5] Van de Wauwer C, Neyrinck AP, Geudens N, Rega FR, Verleden GM, Lerut TE *et al. The mode of death in the non-heart-beating donor has an impact on lung graft quality*. Eur J Cardiothorac Surg 2009;**36**:919-26.

[6] DeCampos KN, Keshavjee S, Slutsky AS, Liu M. *Alveolar recruitment prevents rapid-reperfusion-induced injury of lung transplants*. J Heart Lung Transplant 1999;**18**:1096-102.

[7] Aoyama A, Chen F, Fujinaga T, Sato A, Tsuruyama T, Zhang J *et al. Post-ischemic infusion of atrial natriuretic peptide attenuates warm ischemia-reperfusion injury in rat lung*. J Heart Lung Transplant 2009;**28**:628-34.

[8] Fujinaga T, Nakamura T, Fukuse T, Chen F, Zhang J, Ueda S *et al. Isoflurane inhalation after circulatory arrest protects against warm ischemia reperfusion injury of the lungs*. Transplantation 2006;**82**:1168-74.

[9] Chen F, Nakamura T, Fujinaga T, Zhang J, Hamakawa H, Omasa M *et al. Protective effect of a nebulized beta2-adrenoreceptor agonist in warm ischemic-reperfused rat lungs*. Ann Thorac Surg 2006;**82**:465-71.

[10] Dark JH. *Lung transplantation from the non-heart beating donor*. Transplantation 2008;**86**:200-1.

[11] Joyce CJ, Baker AB, Kennedy RR. *Gas uptake from an unventilated area of lung: computer model of absorption atelectasis*. J Appl Physiol 1993;**74**:1107-16.

[12] Sakamoto J, Chen F, Yamada T, Nakajima D, Ohsumi A, Kikuchi R *et al. Effect of preprocurement ventilation on lungs donated after cardiac death in a canine lung transplantation model*. Transplantation 2011;**92**:864-70.

[13] Morita M, Tabata N, Maya A. *Studies on asphyxia: on the changes of the alveolar walls of rats in the hypoxic state*. Forensic Sci Int 1985;**27**:81-92.

[14] Date H, Matsumura A, Manchester JK, Obo H, Lima O, Cooper JM *et al. Evaluation of lung metabolism during successful twenty-four-hour canine lung preservation*. J Thorac Cardiovasc Surg 1993;**105**:480-91.

[15] Hirata T, Fukuse T, Nakamura T, Ueda M, Kawashima M, Hitomi S *et al. Reperfusion lung injury after cold preservation correlates with decreased levels of intrapulmonary high-energy phosphates*. Transplantation 2000;**69**:1793-801.

[16] Fukuse T, Hirata T, Nakamura T, Kawashima M, Hitomi S, Wada H. *Influence of deflated and anaerobic conditions during cold storage on rat lungs*. Am J Respir Crit Care Med

1999;**160**:621-7.
[17] Rooney SA, Young SL, Mendelson CR. *Molecular and cellular processing of lung surfactant*. Faseb J 1994;**8**:957-67.
[18] Kaneda H, Waddell TK, de Perrot M, Bai XH, Gutierrez C, Arenovich T *et al. Pre-implantation multiple cytokine mRNA expression analysis of donor lung grafts predicts survival after lung transplantation in humans*. Am J Transplant 2006;**6**:544-51.
[19] Novick RJ, Gehman KE, Ali IS, Lee J. *Lung preservation: the importance of endothelial and alveolar type II cell integrity*. Ann Thorac Surg 1996;**62**:302-14.
[20] Zheng D, Limmon GV, Yin L, Leung NH, Yu H, Chow VT *et al. A cellular pathway involved in Clara cell to alveolar type II cell differentiation after severe lung injury*. PLoS One 2013;**8**:e71028.
[21] Ohsumi A, Chen F, Sakamoto J, Nakajima D, Hijiya K, Motoyama H *et al. Protective effect of pre-recovery surfactant inhalation on lungs donated after cardiac death in a canine lung transplantation model*. J Heart Lung Transplant 2012;**31**:1136-42.
[22] Ohsumi A, Chen F, Nakajima D, Sakamoto J, Yamada T, Fujinaga T *et al. Therapeutic effect of surfactant inhalation during warm ischemia in an isolated rat lung perfusion model*. Transpl Int 2012;**25**:1096-105.
[23] Struber M, Fischer S, Niedermeyer J, Warnecke G, Gohrbandt B, Gorler A *et al. Effects of exogenous surfactant instillation in clinical lung transplantation: a prospective, randomized trial*. J Thorac Cardiovasc Surg 2007;**133**:1620-5.
[24] Avlonitis VS, Wigfield CH, Kirby JA, Dark JH. *The hemodynamic mechanisms of lung injury and systemic inflammatory response following brain death in the transplant donor*. Am J Transplant 2005;**5**:684-93.
[25] Wierup P, Liao Q, Bolys R, Sjoberg T, Rippe B, Steen S. *Lung edema formation during cold perfusion: important differences between rat and porcine lung*. J Heart Lung Transplant 2005;**24**:379-85.