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The title: Reconditioning lungs donated after cardiac death using short-term hypothermic machine perfusion ¹

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1 **Footnotes**

2 ¹ Part of this work was presented at the XXIV International Congress of The
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6
7 Daisuke Nakajima, Fengshi Chen, Kenji Okita, Hideki Motoyama, Kyoko Hijiya,
8 Akihiro Ohsumi, Jin Sakamoto and Tetsu Yamada participated in the performance
9 of the research.

10 Daisuke Nakajima, Fengshi Chen, Kenji Okita, Hideki Motoyama, Kyoko Hijiya,
11 Akihiro Ohsumi, Jin Sakamoto, Tetsu Yamada, Masaaki Sato, Akihiro Aoyama,
12 Toru Bando and Hiroshi Date participated in the research design.

13 Daisuke Nakajima, Fengshi Chen, Toru Bando and Hiroshi Date participated in the
14 writing of the paper.

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16 All authors declare no potential conflict of interest.

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6 1 **Abbreviations**
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8 2 ATP: adenosine triphosphate
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10 3 BAL: bronchoalveolar lavage
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12 4 DCD: donation after cardiac death
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14 5 EVLP: ex vivo lung perfusion
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16 6 FiO₂: inspired oxygen fraction
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18 7 HMP: hypothermic machine perfusion
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20 8 MDA: malondialdehyde
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22 9 PawP: peek airway pressure
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24 10 PEEP: positive end-expiratory pressure
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26 11 ROS: reactive oxygen species
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28 12 SCS: static cold storage
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30 13 TBA: thiobarbituric acid
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32 14 TLR: Toll-like receptor
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Abstract

Background. Hypothermic machine perfusion (HMP) is widely used to preserve kidneys and livers for transplantation. This study investigated whether short-term HMP could improve the quality of lungs donated after cardiac death (DCD).

Methods. In a clinically relevant uncontrolled DCD model, beagles were divided into 2 groups (n=5 each): 4 h of warm ischemia + 14 h of static cold storage (SCS group) or 4 h of warm ischemia + 12 h of static cold storage, followed by 2 h of HMP (HMP group). HMP was performed using centrifugal perfusion with STEEN solution at around 10°C. In both groups, the left lungs were then transplanted and reperfused for 4 h to evaluate the posttransplant lung functions.

Results. HMP was performed safely, not inducing any oxidative damage. The dynamic pulmonary compliance was stable during HMP, while the pulmonary vascular resistance significantly decreased. HMP microscopically eliminated residual microthrombi in the donor lungs just before transplantation. The lung tissue adenosine triphosphate (ATP) levels 4 h after reperfusion were significantly higher in the HMP group compared with the SCS group. The serum malondialdehyde levels and proinflammatory cytokine levels in the bronchoalveolar lavage (BAL) fluid 4 h after reperfusion were significantly lower in the HMP group than in the SCS group. The physiological lung functions during reperfusion were significantly better in the HMP group compared to the SCS group. HMP also significantly reduced ischemia-reperfusion injury in the microscopic findings.

Conclusions. Short-term HMP could resuscitate ischemically damaged DCD lungs, and ameliorate ischemia-reperfusion injury.

1 Introduction

Lung transplantation has become a mainstay of therapy for end-stage lung diseases. However, there has been a progressive increase in the number of patients on the waiting list, which continually exceeds the number of available organs. The use of uncontrolled DCD donors has been employed to resolve this problem (1-3). Warm ischemia inevitably occurs in uncontrolled DCD donors, and may cause ischemia-reperfusion injury after transplantation. Severe ischemia-reperfusion injury leads to primary graft dysfunction, and remains a significant cause of early morbidity and mortality after lung transplantation (4). The inhibition of ischemia-reperfusion injury is, therefore, crucial to facilitate lung transplantation from uncontrolled DCD donors.

Warm ischemia impairs the mitochondrial electron transport chain, resulting in decreased ATP production, and also decreases the efficacy of the mitochondrial antioxidant system (5,6). Depending on its severity, the reintroduction of oxygen at reperfusion can lead to a significant production of reactive oxygen species (ROS), which induces the upregulation of molecules on the cell surface and the release of proinflammatory mediators (4,7).

Hypothermic machine perfusion (HMP) has been used to preserve kidneys and livers for transplantation, with better results than static cold storage (SCS) (8,9). HMP is associated with a reduced risk of delayed graft function and improved graft survival, compared with SCS. HMP is based on the concept that the oxidative energy production by the mitochondrial electron transport would be sustained under hypothermia (10). We previously demonstrated that short-term HMP, which helped recover the ATP production by the mitochondrial electron transport chain,

ameliorated ischemia- reperfusion injury with decreased oxidative damage during reperfusion in an isolated rat lung perfusion model (11).

In the present study, we used a canine transplantation model mirroring the clinical situation to investigate whether short-term HMP could improve the mitochondrial function damaged by warm ischemia, and decrease the oxidative damage and production of proinflammatory cytokines during reperfusion, thereby reducing ischemia- reperfusion injury.

Results

Physiological lung functions during HMP

The influent variables (temperature, solutes, PO₂ and PCO₂ levels) were stable during 120 min of HMP. The temperature was maintained at a mean of 9.26±0.88°C, ranging from 7.9 to 10.5°C. There was little variation in any solute during the HMP time (Na⁺ 144.93±0.70 mmol/L, K⁺ 5.49±0.28 mmol/L, Ca²⁺ 0.85±0.03 mmol/L). The PH, PO₂ and PCO₂ levels were also maintained at means of 7.20±0.04, 113.73±1.03 mmHg and 37.67±5.67 mmHg, respectively.

The dynamic pulmonary compliance was stable during HMP. The dynamic pulmonary compliance at baseline and after 120 min of HMP were 25.77±7.18 ml/cmH₂O and 26.46 ± 7.10 ml/cmH₂O, respectively (P=0.76; **Fig. 1A**). The pulmonary vascular resistance gradually decreased during HMP. The pulmonary vascular resistance after 60, 90, and 120 min of HMP significantly decreased, in comparison to that at the baseline of HMP (P<0.05; **Fig. 1B**).

Microthrombi in the donor lungs just before transplantation

The biopsy specimens were collected from 5 donor lungs in the HMP group and 4 donor lungs in the SCS group. Residual microthrombi in the donor lungs just before transplantation were microscopically assessed to prove the wash-out effects of HMP. Residual blood cells or blood clots in the capillaries were observed more often in the SCS group (4/4 specimens; **Fig. 1C**) compared with the HMP group (0/5 specimens; **Fig. 1D**).

Lung tissue ATP levels

The lung tissue ATP levels were measured before cardiac arrest, after warm ischemia, and 4 h after reperfusion to evaluate the mitochondrial function. In the HMP group, the lung tissue ATP levels, which decreased during warm ischemia, were significantly improved 4 h after reperfusion ($P < 0.05$; **Fig. 2**). Moreover, the lung tissue ATP levels 4 h after reperfusion were significantly higher in the HMP group than in the SCS group ($P < 0.05$; **Fig. 2**). The ATP levels before cardiac arrest and after warm ischemia were 6.33 ± 0.79 and 2.68 ± 1.07 nmol/mg $\cdot$ dw, respectively. The ATP levels 4 h after reperfusion in the HMP group and in the SCS group were 4.53 ± 0.38 and 3.07 ± 0.94 nmol/mg $\cdot$ dw, respectively.

Oxidative damage during HMP and reperfusion

Malondialdehyde is one of the most commonly used markers for lipid peroxidation (12). The malondialdehyde levels in the perfusate were measured at baseline and after 120 min of HMP to assess the oxidative damage that occurred during HMP. HMP did not increase the malondialdehyde levels in the perfusate; the

malondialdehyde levels at baseline and after 120 min of HMP were 2.23 ± 0.49 and 2.06 ± 0.45 nmol/ml, respectively ($P=0.69$; **Fig. 3A**). The serum malondialdehyde levels were measured 4 h after reperfusion to evaluate the oxidative damage that occurred during reperfusion. The serum malondialdehyde levels were significantly lower in the HMP group compared with the SCS group (HMP group: 1.55 ± 0.74 nmol/ml, SCS group: 3.63 ± 1.15 nmol/ml, $P<0.05$; **Fig. 3B**).

Proinflammatory cytokine levels in BAL fluid after reperfusion

The $\text{TNF-}\alpha$ and IL-6 levels in the BAL fluid were measured 4 h after reperfusion. The $\text{TNF-}\alpha$ levels were significantly lower in the HMP group than in the SCS group (HMP group: 5.83 ± 3.22 pg/ml, SCS group: 54.15 ± 29.36 pg/ml, $P<0.01$; **Fig. 3C**). The IL-6 levels were also significantly lower in the HMP group compared with the SCS group (HMP group: 1.55 ± 0.74 pg/ml, SCS group: 3.63 ± 1.15 pg/ml, $P<0.05$; **Fig. 3D**).

Physiological lung functions during reperfusion

The lung oxygenation and dynamic pulmonary compliance were significantly better in the HMP group than those in the SCS group ($P<0.01$; **Figs. 4A and B**). The wet to dry lung weight ratio, indicating the severity of pulmonary edema, 4 h after reperfusion was significantly lower in the HMP group than that in the SCS group (HMP group: 7.09 ± 0.77 , SCS group: 12.03 ± 4.05 ; $P<0.05$; **Fig. 4C**).

Histological findings of ischemia-reperfusion injury

Severe interstitial and intra-alveolar edema, hemorrhage, infiltration of

1 inflammatory cells in the air space or vessel wall, and hyaline formation were
2 detected in the SCS group 4 h after reperfusion. The acute lung injury score was
3 significantly lower in the HMP group in comparison to the SCS group (HMP group:
4 22.6 ± 6.80 , SCS group: 44.6 ± 4.45 , $P < 0.01$; **Fig. 5**).

5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 **Discussion**

20 The current study utilized a clinically relevant uncontrolled DCD model. We chose 4
21 h of warm ischemia to possibly expand the donor pool for lung transplantation,
22 although the Madrid groups reported a maximum warm ischemic time of 2 h (3,13).
23 The retrieval of lungs after cardiac death requires an intermediate period to be
24 transported to the transplant center, so we added 12 h of SCS right before HMP.
25 Dutkowski et al. suggested that 1-2 h of HMP should be performed during the
26 recipient preparation without delay of the transplant procedure (10). We previously
27 reported that 1 h of HMP significantly improved the rat lung tissue ATP levels,
28 which had decreased during warm ischemia (11). In the current study, DCD lungs,
29 which were injured by 4 h of warm ischemia and additional 12 h of cold ischemia,
30 could be resuscitated by 2 h of HMP.

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45 This study found that short-term HMP could be performed safely for DCD
46 lungs, not inducing any significant amount of oxidative damage. We recently
47 developed a reliable and reproducible technique for lung HMP in a large animal
48 model, which demonstrated stable machine perfusion characteristics and excellent
49 lung performance during 8 h of HMP (data not shown). The current study revealed
50 that this technique could be used for reconditioning of ischemically damaged DCD
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1 lungs. None of the influent valuables showed spikes, and the dynamic pulmonary
2 compliance was also maintained during the entire period of HMP. Oxidative damage
3 under the exposure to oxygen at hypothermia has been demonstrated in studies on
4 isolated cell systems (14), while several studies in animal models demonstrated that
5 liver HMP resulted in minor oxidative damage (15,16). The present study found
6 that short-term lung HMP did not cause oxidative stress during the perfusion,
7 which was indicated by the fact that the malondialdehyde levels in the perfusate
8 did not increase during HMP.

9 Intravascular microthrombus formation, which results in an increase of
10 intrapulmonary shunting and pulmonary vascular resistance, is one of the major
11 causes of reperfusion injury in lung transplantation from DCD donors. The benefits
12 of additional retrograde flushing have been shown in experimental lung
13 transplantation (17-19). In the current study, a histological examination of the
14 donor lungs just before transplantation revealed fewer microthrombi in the HMP
15 group compared with the SCS group. This indicated that most of the residual
16 microthrombi wedged in the capillaries after the flushes were eliminated by HMP
17 (9,20). Ventilation during perfusion results in better distribution of the preservation
18 solution. A reduction of minute ventilation decreases the total amount of elastic
19 stress imposed on cooled lungs (21). Therefore, the current study adopted the
20 ventilation mode reduced respiratory rate and tidal volume during HMP, which
21 resulted in stable dynamic pulmonary compliance and the elimination of residual
22 microthrombi.

23 The current study demonstrated that short-term HMP could improve the
24 mitochondrial function following injury due to warm ischemia, and decrease the

1 oxidative damage and production of proinflammatory cytokines during reperfusion.
2 Unlike other tissues that are transplanted, lung cells are able to maintain aerobic
3 metabolism using the oxygen present in the alveoli during SCS (22). In the SCS
4 group, the lung ATP levels, which decreased during warm ischemia, were improved
5 a little, but the improvement was significantly lower than that in the HMP group.
6 HMP could continue to provide the essential substrates for cell metabolism and
7 restore the lung tissue ATP levels. The reintroduction of oxygen to impaired
8 mitochondria at reperfusion leads to a significant production of ROS, which damage
9 proteins, lipids and DNA (6). The serum malondialdehyde levels after reperfusion
10 were significantly lower in the HMP group compared with the SCS group. HMP
11 possibly prevented the overload of oxygen upon reperfusion for the mitochondrial
12 electron transport chain by recovering the mitochondrial function before
13 reperfusion, and thus decreased production of ROS. Physical alterations of the
14 plasma membrane caused by ROS activate Toll-like receptors (TLRs), which are
15 expressed in endothelial cells and respiratory epithelial cells (7). The signal
16 transduction mediated by TLRs results in the activation of $\text{NF-}\kappa\text{B}$, inducing the
17 production of proinflammatory cytokines and chemokines (7). Therefore, the
18 significantly increased levels of $\text{TNF-}\alpha$ and IL-6 in the SCS group might have
19 resulted from TLRs signaling in the pulmonary parenchymal cells, activated by the
20 significant increase in lipid peroxidation.

21 Normothermic perfusion has already been studied and proved to enable
22 organ viability assessment before transplantation, prolonged preservation, and
23 resuscitation from injuries (23-27). It has been unknown which is more suitable for
24 organ preservation, hypothermic perfusion or normothermic perfusion. The organ is

1 metabolically active under normothermic conditions, and thus normothermic
2 perfusion might allow better reconstitution of the lung tissue ATP stores. However,
3 normothermic perfusion requires that the physiological environment is completely
4 recreated with full nutritional support. Hypothermia decreases the metabolic rate
5 of the organ and could be used as a means for lung rest in the acutely injured lung
6 (21). This study demonstrated that HMP could continue to provide the essential
7 substrates for cell metabolism and restore the lung tissue ATP levels under the
8 slow-metabolic-rate conditions.

9 This study had several limitations. First, although we simulated a clinically
10 relevant uncontrolled DCD model, cardiac arrest was induced by intravenous
11 injection of potassium chloride. Such an abrupt cardiac arrest may have been
12 removed from clinical reality, in that there was not an agonal phase, which is an
13 important variable component of DCD (28). Second, the lung tissue ATP levels were
14 measured after warm ischemia and reperfusion. It might be easier to prove the
15 metabolic benefits of HMP if the ATP levels were measured just before and after
16 HMP.

17 In conclusion, short-term HMP could resuscitate DCD lungs injured by
18 prolonged ischemia, and ameliorate ischemia-reperfusion injury. First, short-term
19 HMP washed-out residual microthrombi in the donor lungs. Second, short-term
20 HMP improved the ATP production by the mitochondrial electron transport chain,
21 which led to the significant decrease in oxidative damage and production of
22 proinflammatory cytokines after reperfusion compared to SCS.

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Materials and Methods

Animals

Beagles weighing from 9 to 13 kg (Kitayama Labes Co. Ltd., Hongo Farm, Yamaguchi, Japan) were used in this study. There was no significant difference in the beagles' body weights between the two groups. All animals received humane care in compliance with the Principals of Laboratory Animal Care, formulated by the United States 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 Institutes of Health (NIH Publication 85-23, revised 1996). The study was approved by the Ethics Committee of the Faculty of Medicine at Kyoto University, Japan.

Study design

The donor procedures, including anesthesia, induction of cardiac arrest, and antegrade and retrograde flushes of the lungs, were described in detail in a separate publication (29). Cardiac arrest was induced by the intravenous injection of potassium chloride (0.5 mEq/kg) without heparinization. Four hours after cardiac arrest, the donor lungs were retrieved, and then they were divided into 2 groups (n=5 each). The lungs in the SCS group were stored in an inflated state with oxygen fraction of 0.5 at 4°C for 14 h using ET-Kyoto solution (Otsuka Pharmaceutical Factory Inc, Tokushima, Japan) (30). The lungs in the HMP group were stored in an inflated state with oxygen fraction of 0.5 at 4°C for 12 h using ET-Kyoto solution, and then reconditioned by 2 h of HMP. In both groups, the left lung was then transplanted to a recipient as previously described (29). The transplanted lung was

1 reinflated and mechanically ventilated with FiO_2 of 1.0, and then reperfused for 4 h
2 to evaluate the posttransplant lung functions. The right pulmonary artery was
3 occluded with a tourniquet 45 min after reperfusion to specifically evaluate the
4 functions of the transplanted lung. The pulmonary arterial pressure and peak
5 airway pressure (PawP) were continuously monitored throughout the experiments.
6 Dynamic pulmonary compliance was defined as tidal volume/(PawP – PEEP)
7 (ml/cmH₂O). A blood gas analysis was performed using blood collected from the
8 femoral artery at selected time points. Lung tissue biopsy samples collected from
9 the left middle lobe 4 h after reperfusion were weighed to obtain the wet lung
10 weight, placed in an oven at 180°C for 24 h, and then reweighed to obtain the dry
11 lung weight. The wet to dry lung weight ratio was calculated to evaluate the
12 presence of pulmonary edema.

13
14 ***Hypothermic machine perfusion (HMP)***

15 The lungs were placed in an XVIVO chamber (Vitrolife, Denver, CO). The
16 pulmonary artery was cannulated directly and then connected to the perfusion
17 circuit. The left atrium was left open, so that the left atrial pressure was always 0
18 mmHg. The trachea was intubated and connected to the ventilator. Mechanical
19 ventilation was started with FiO_2 of 0.25, tidal volume of 10 ml/kg, frequency of 10
20 breaths/min and PEEP of 5 cmH₂O. The perfusate, which contained STEEN
21 solution (1,500 ml) with methylprednisolone (500 mg) and heparin (10,000 IU), was
22 driven by a centrifugal pump at a constant flow rate of 10% of the estimated cardiac
23 output (CO = 100 ml/kg). Deoxygenation of the perfusate was started with a gas
24 mixture of nitrogen (86%), carbon dioxide (8%), and oxygen (6%) to maintain the

1 influent PCO_2 of around 40 mmHg. The temperature of influent was continuously
2 monitored, and was maintained around 10 °C (31). The influent solute
3 concentrations, PO_2 , and PCO_2 levels were recorded every hour. The pulmonary
4 arterial pressure and peak airway pressure were continuously monitored, and the
5 physiological lung functions (dynamic pulmonary compliance and pulmonary
6 vascular resistance) during HMP were evaluated every 30 min. Recruitments were
7 performed to ensure a peak airway pressure of 25 cmH₂O every 30 min prior to each
8 evaluation. Dynamic pulmonary compliance was defined as described above.
9 Pulmonary vascular resistance was defined as (pulmonary arterial pressure — left
10 atrial pressure)/ pulmonary arterial flow (mmHg/L).

12 *Lung tissue ATP levels*

13 Lung tissue biopsy specimens were collected from the right lung before cardiac
14 arrest and after warm ischemia, and then were collected from the left upper lobe 4 h
15 after reperfusion. ATP levels were measured by high-performance liquid
16 chromatography using a Shim-pack CLC-ODS column (15 cm × 6.0 mm; Shimadzu,
17 Japan) and 100 mM sodium phosphate buffer (PH 6.0) at a wavelength of 260 nm,
18 as described previously (32).

20 *Malondialdehyde levels*

21 Malondialdehyde levels were measured with the NWLSS Malondialdehyde Assay
22 kit from Northwest (Northwest Life Sciences Specialties, Vancouver, Canada)
23 following the manufacture's protocol. Malondialdehyde (MDA) reacted with
24 thiobarbituric acid (TBA), forming an MDA-TBA₂ adduct that was measured at a

1 wavelength of 532 nm.

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3 3 *Cytokine levels in BAL fluid*

4 BAL was performed with 20 ml of saline using a flexible bronchoscope wedged into
5 the left lower bronchus. Collected samples were centrifuged at 1,500g for 10 min at
6 4°C, and then the supernatant was stored at -80°C to evaluate the cytokine levels.
7 TNF- α and IL-6 levels were measured with a Quantikine ELISA kit (R&D
8 Systems Inc., Minneapolis, MN, USA) following the protocol developed by the
9 manufacture.

10
11 *Histological evaluation of microthrombi and ischemia-reperfusion injury*

12 Lung tissue biopsies were collected from the right lower lobe just before
13 transplantation and the left lower lobe 4 h after reperfusion. They were fixed in 10%
14 buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin.
15 Five sections including capillaries were examined by blinded investigators (A.O.
16 and J.S.) to evaluate the residual microthrombi in the donor lungs. The extent of
17 ischemia-reperfusion injury was scored blindly by two investigators (A.O. and J.S.)
18 using a four-point scale according to the combined assessment of edema (interstitial
19 and intra-alveolar congestion), hemorrhage, inflammatory cell infiltration, and
20 hyaline membrane formation: 0 = absent, 1 = mild, 2 = moderate, 3 = severe damage
21 (33,34).

22
23 *Statistical analysis*

24 All data are presented as means $\pm$ standard deviation. The statistical analysis

1 was performed using Student's *t*-test and a repeated-measures analysis of variance
(ANOVA). A *p* value < 0.05 was considered to be statistically significant.

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Figure legends

FIGURE 1. Physiological lung functions during HMP: Dynamic pulmonary compliance (A). Pulmonary vascular resistance (B). * $P < 0.05$ versus the baseline data. Residual microthrombi in the donor lungs just before transplantation in the SCS group (C) and in the HMP group (D). Arrows indicate residual microthrombi in the capillaries. HMP: hypothermic machine perfusion, SCS: static cold storage.

FIGURE 2. Lung tissue ATP levels before cardiac arrest, after warm ischemia, and 4 h after reperfusion. * $P < 0.05$. ATP: adenosine triphosphate, HMP: hypothermic machine perfusion, SCS: static cold storage.

FIGURE 3. Malondialdehyde (MDA) levels in the perfusate during HMP (A) and in the serum 4 h after reperfusion (B). * $P < 0.05$. $\text{TNF-}\alpha$ levels (C) and IL-6 levels (D) in the BAL fluid 4 h after reperfusion. † $P < 0.01$, * $P < 0.05$. BAL: bronchoalveolar lavage, HMP: hypothermic machine perfusion, SCS: static cold storage.

FIGURE 4. Physiological lung functions during reperfusion. The right pulmonary artery was occluded 45 min after reperfusion to evaluate the functions of the

transplanted lung only. † These data show the physiological lung functions of the bilateral lungs (native lung and transplanted lung) before the clamp of the right pulmonary artery. PaO₂ (A) and dynamic pulmonary compliance (B) were significantly better in the HMP group (solid circles) than in the SCS group (open boxes); P<0.01. Wet to dry lung weight ratio 4 h after reperfusion (C). * P<0.05. HMP: hypothermic machine perfusion, SCS: static cold storage.

FIGURE 5. Acute lung injury score: Ischemia-reperfusion injury was scored using a four-point scale according to the combined assessment of edema, hemorrhage, cell infiltration, and hyaline membrane formation. † P<0.01. HMP: hypothermic machine perfusion, SCS: static cold storage.

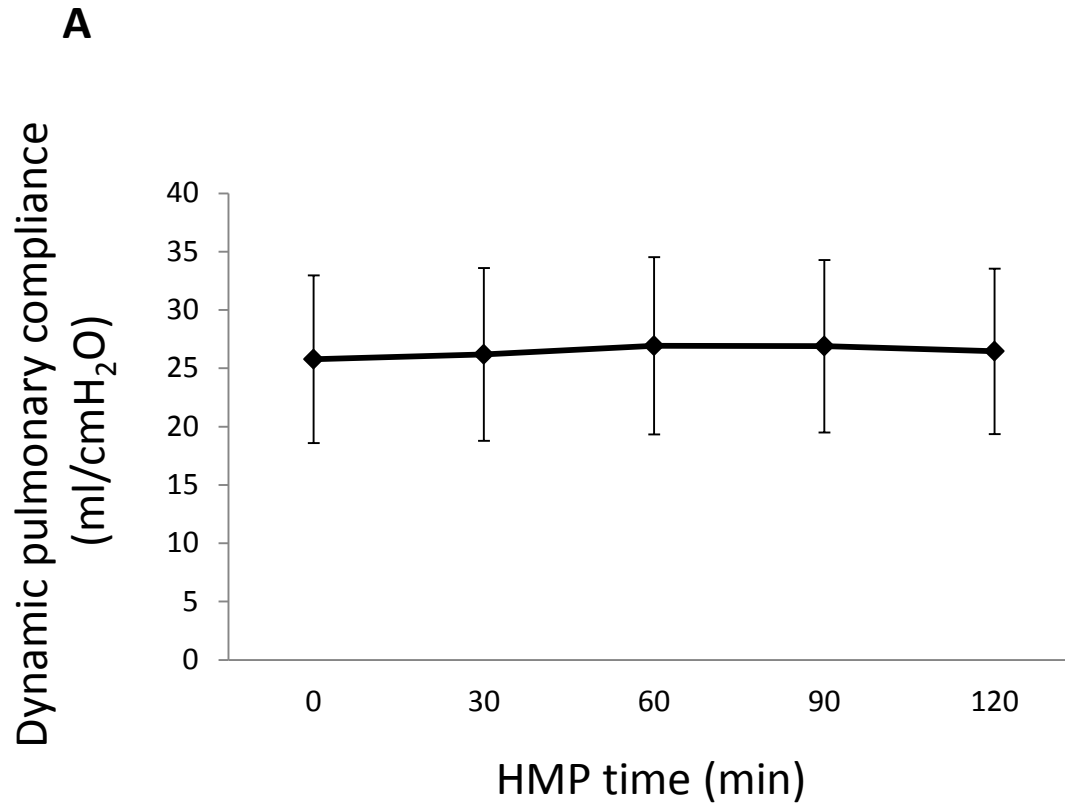
FIGURE 1.

FIGURE 1.

B

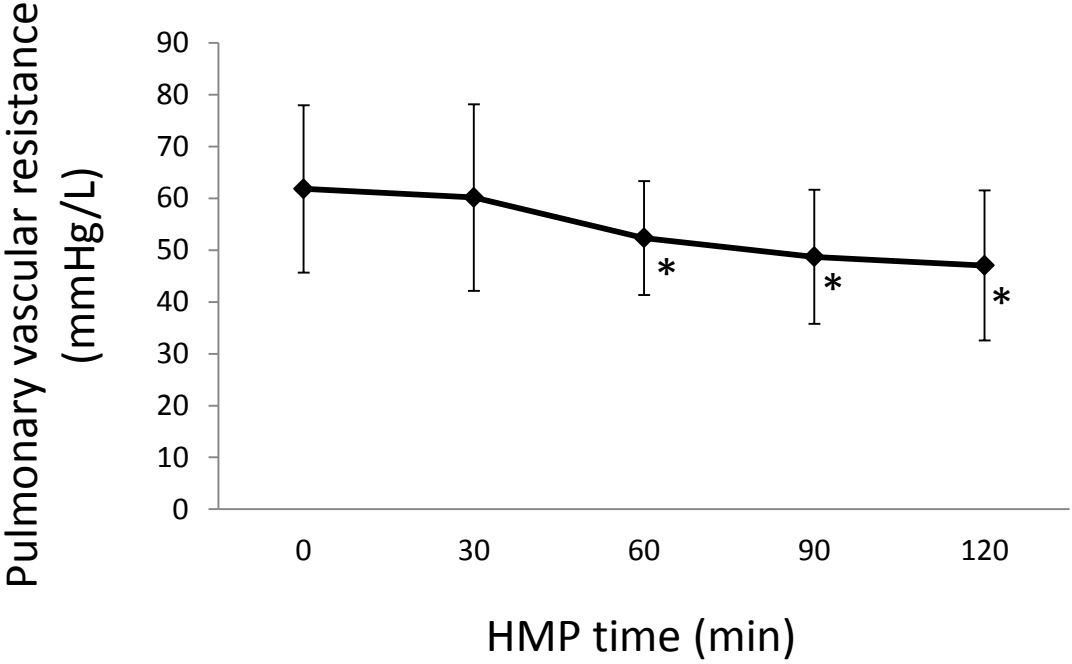


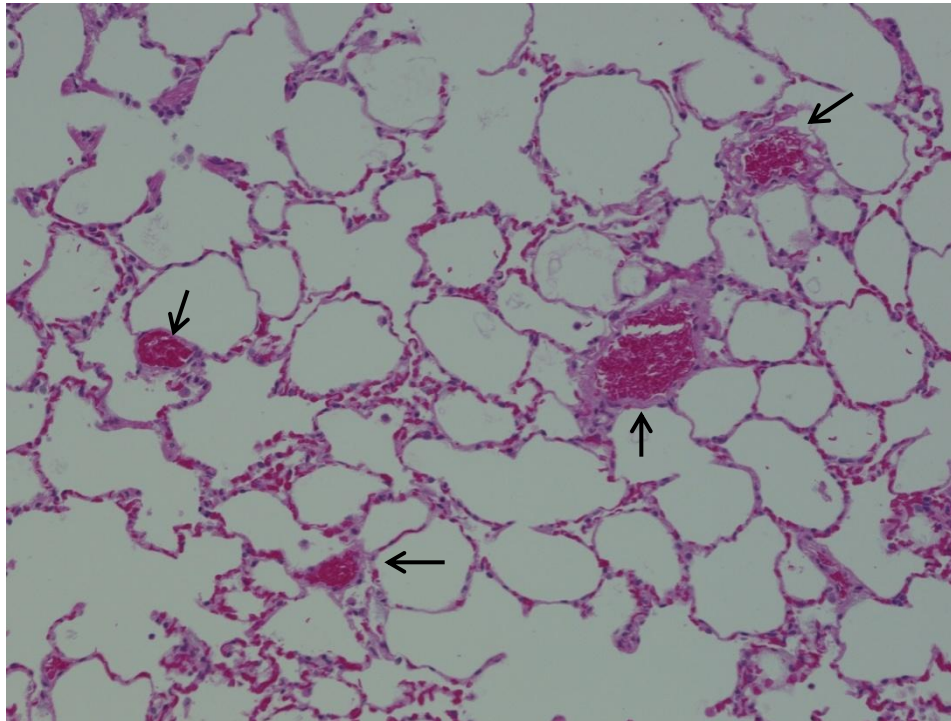
FIGURE 1.**C**

FIGURE 1.

D

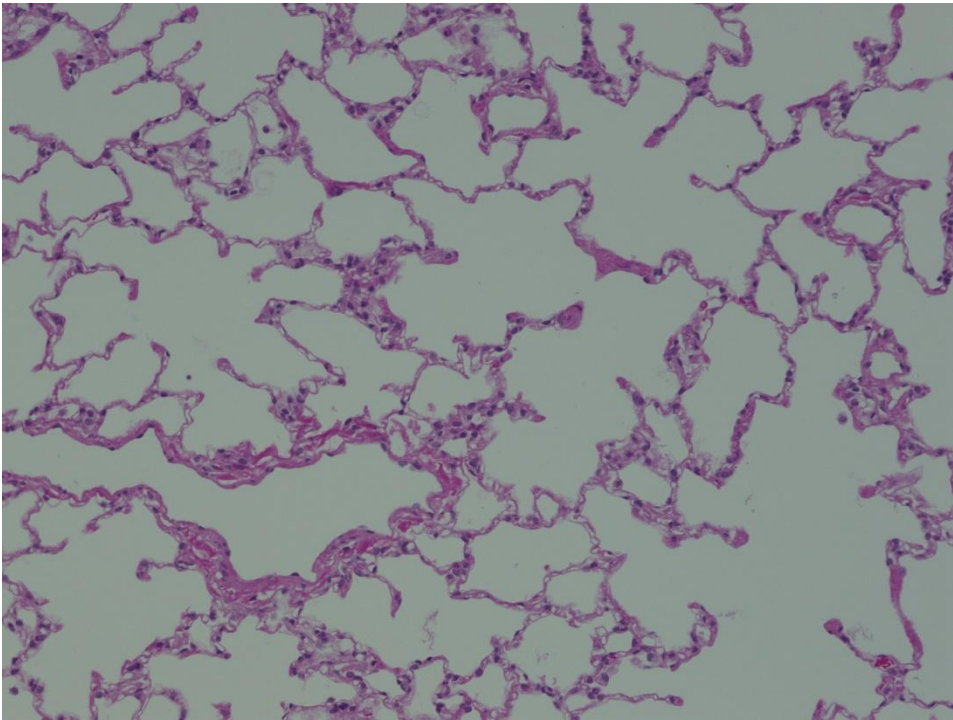


FIGURE 2.

Lung tissue ATP levels
(nmol/mg·dw)

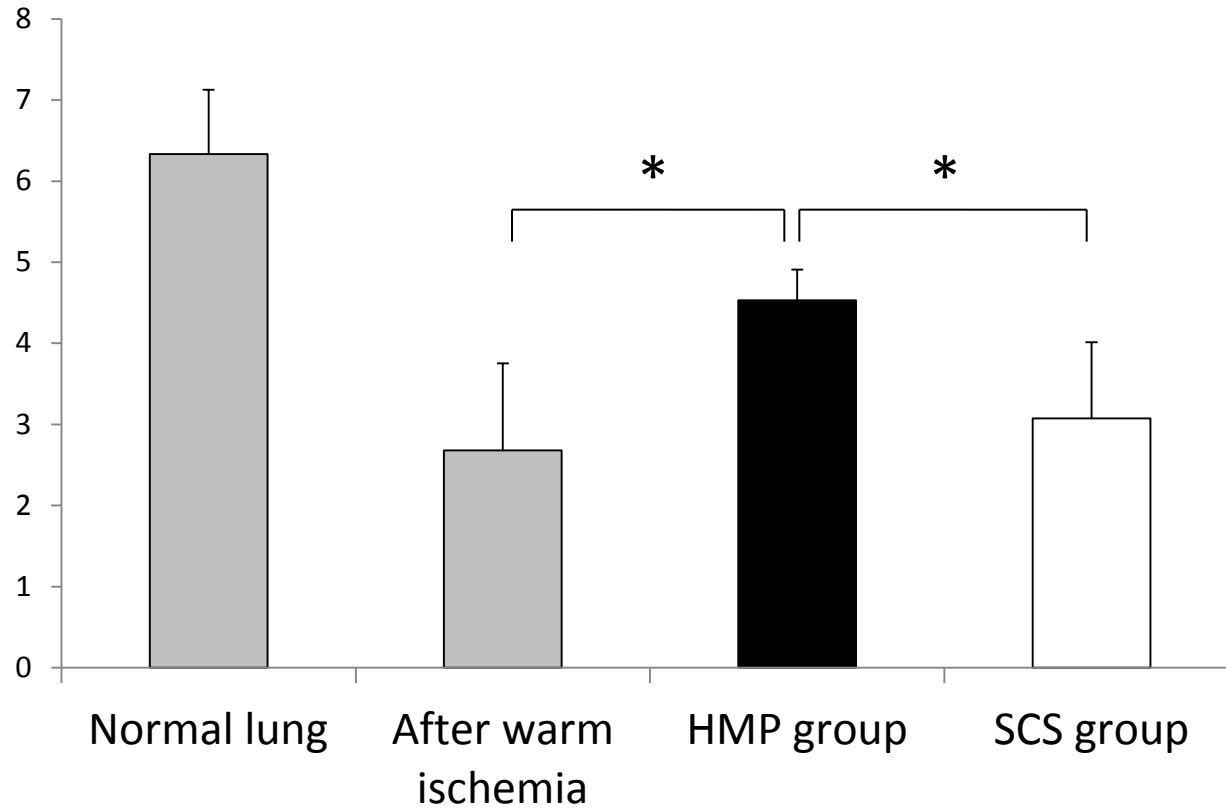


FIGURE 3.

A

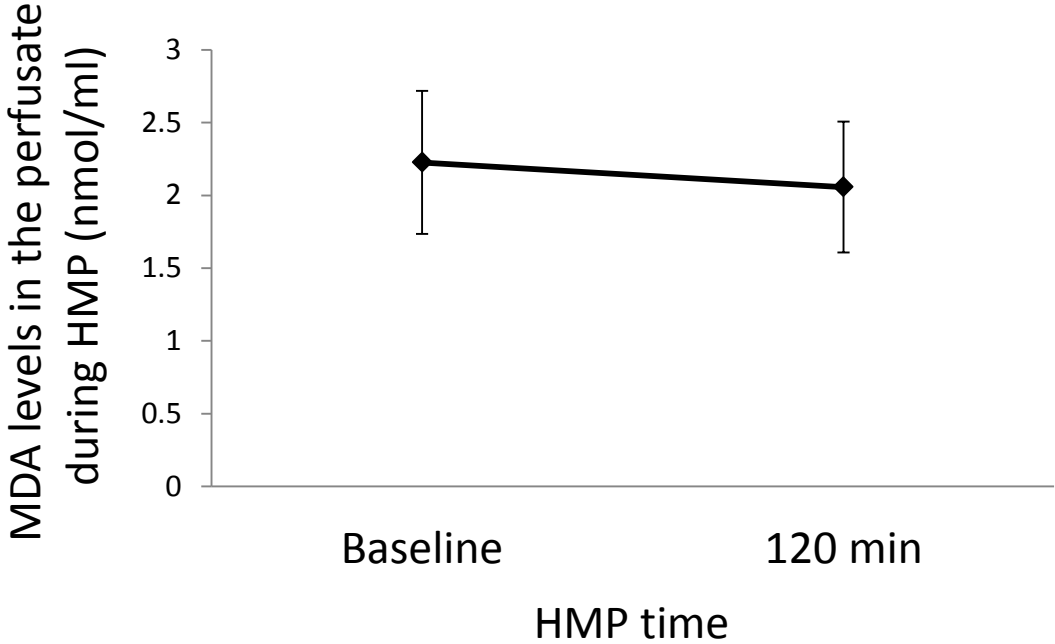


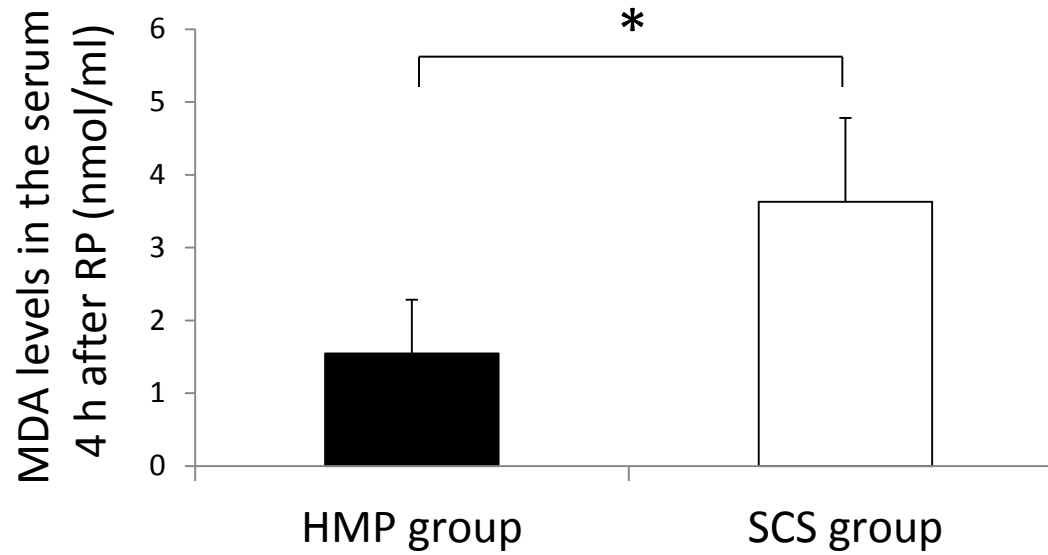
FIGURE 3.**B**

FIGURE 3.

C

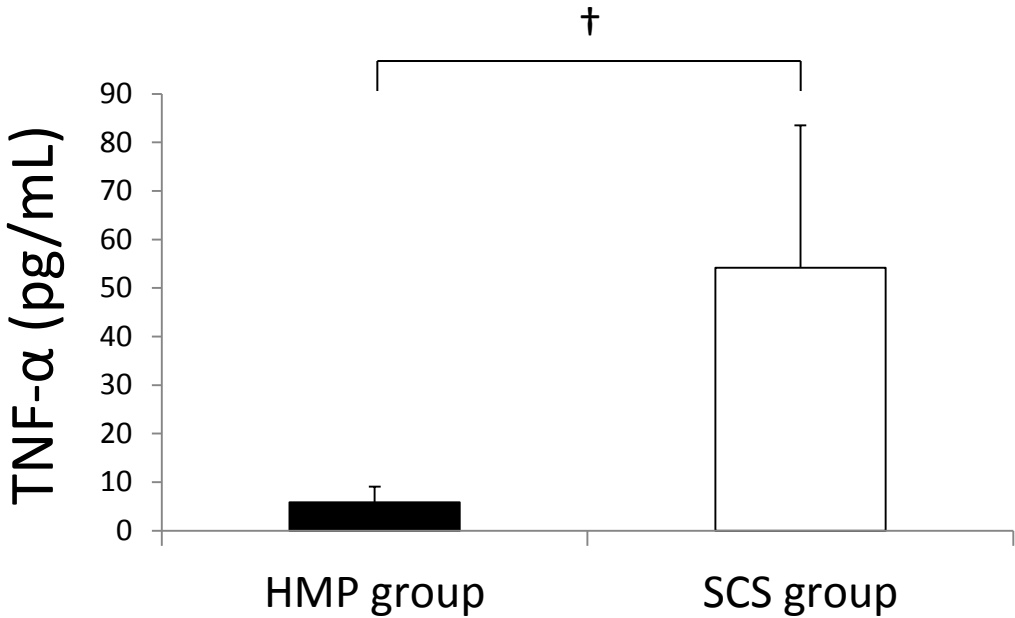


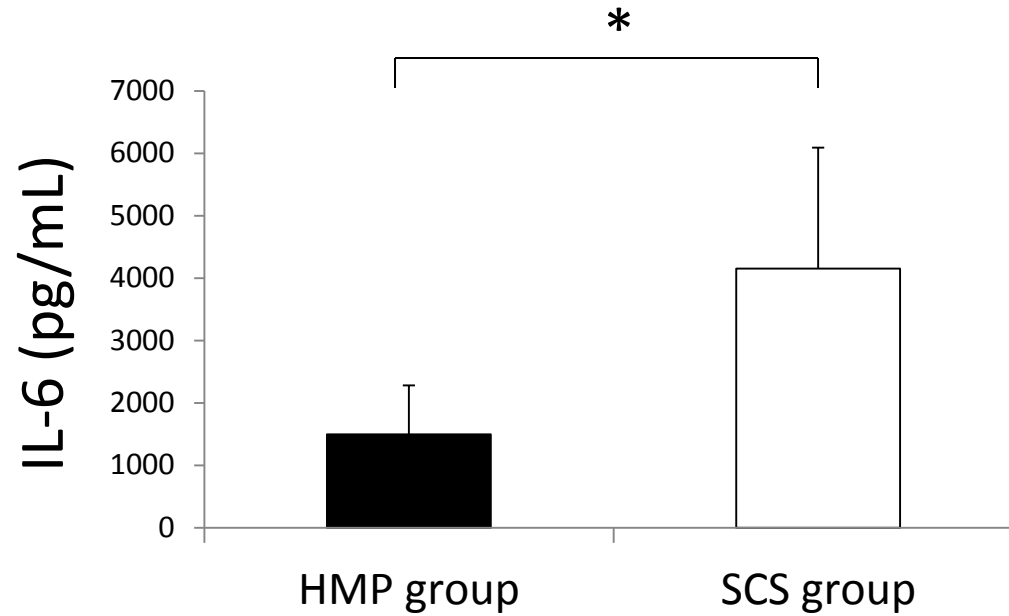
FIGURE 3.**D**

FIGURE 4.

A

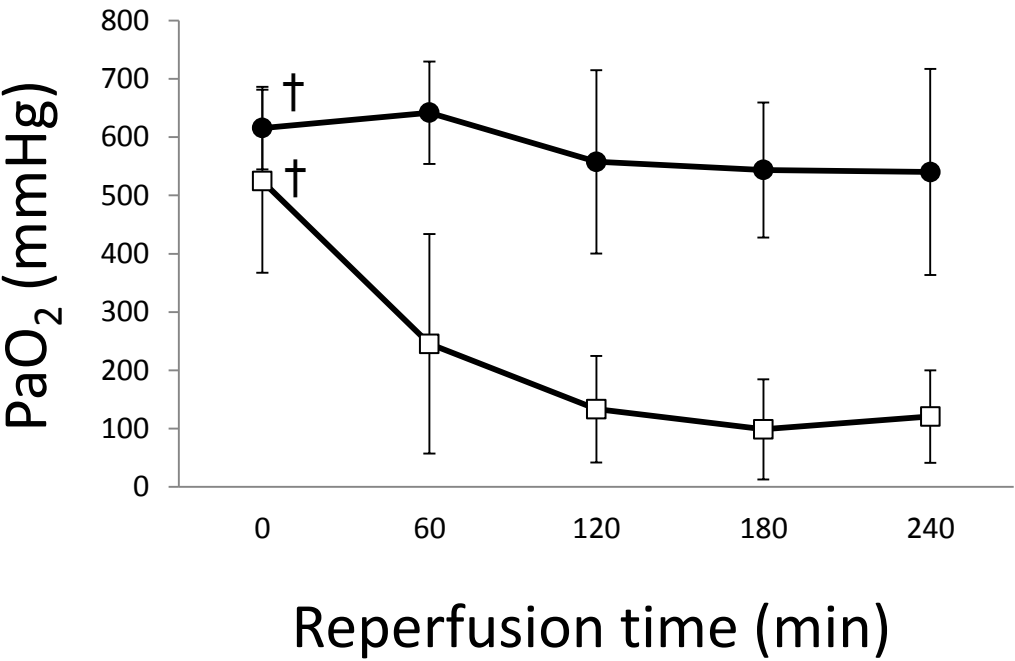


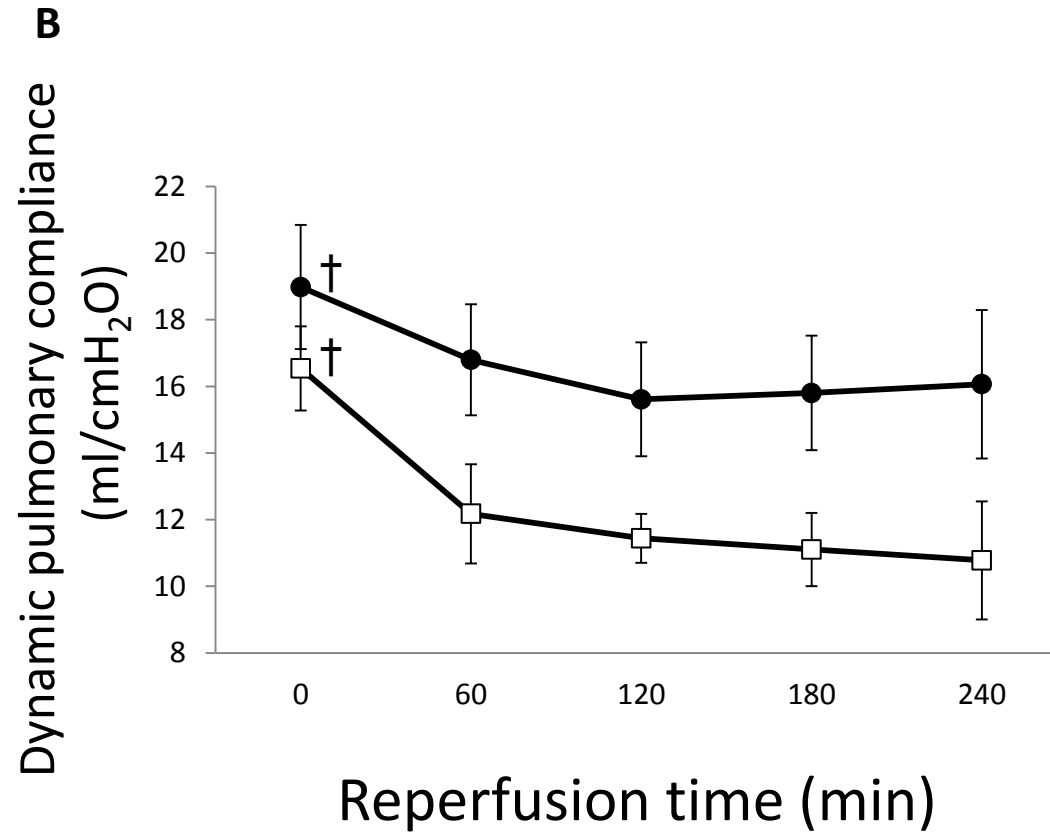
FIGURE 4.

FIGURE 4.

C

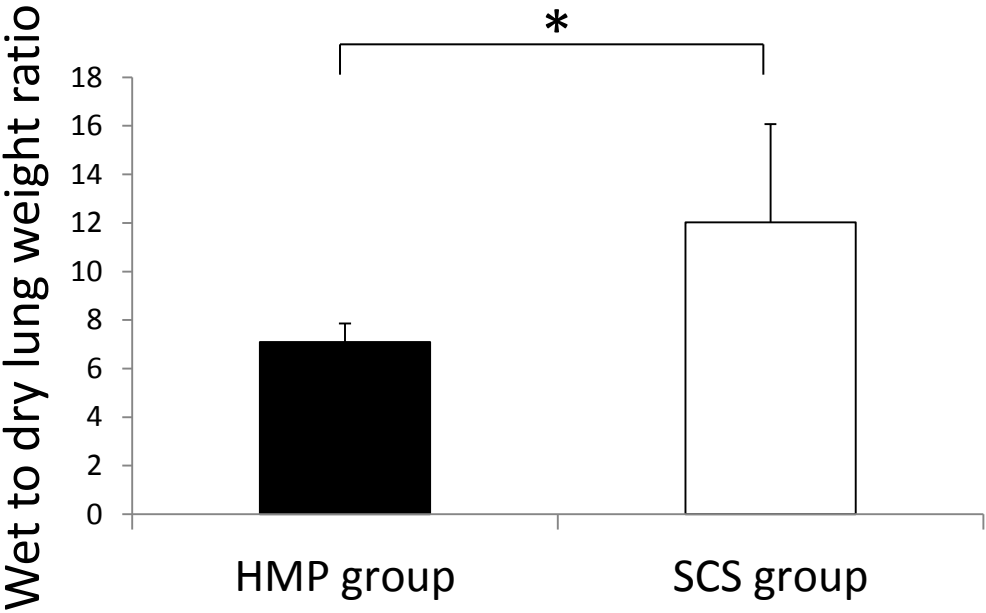


FIGURE 5.