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

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Footnotes

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Abbreviations

ATP: adenosine triphosphate

BAL: bronchoalveolar lavage

DCD: donation after cardiac death

EVLP: ex vivo lung perfusion

FiO_2: inspired oxygen fraction

HMP: hypothermic machine perfusion

MDA: malondialdehyde

PawP: peek airway pressure

PEEP: positive end-expiratory pressure

ROS: reactive oxygen species

SCS: static cold storage

TBA: thiobarbituric acid

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℃. 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.

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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,

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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, PO_2 and PCO_2 levels) were stable during 120 min of HMP. The temperature was maintained at a mean of 9.26± 0.88℃, ranging from 7.9 to 10.5℃. 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, Ca^{2+} 0.85±0.03 mmol/L). The PH, PO_2 and PCO_2 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/cmH_2O and 26.46 ± 7.10 ml/cmH_2O, 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**).

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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・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・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

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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 TNF-α and IL-6 levels in the BAL fluid were measured 4 h after reperfusion. The TNF-α 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

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

Discussion

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

This study found that short-term HMP could be performed safely for DCD lungs, not inducing any significant amount of oxidative damage. We recently developed a reliable and reproducible technique for lung HMP in a large animal model, which demonstrated stable machine perfusion characteristics and excellent lung performance during 8 h of HMP (data not shown). The current study revealed that this technique could be used for reconditioning of ischemically damaged DCD

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lungs. None of the influent valuables showed spikes, and the dynamic pulmonary compliance was also maintained during the entire period of HMP. Oxidative damage under the exposure to oxygen at hypothermia has been demonstrated in studies on isolated cell systems (14), while several studies in animal models demonstrated that liver HMP resulted in minor oxidative damage (15,16). The present study found that short-term lung HMP did not cause oxidative stress during the perfusion, which was indicated by the fact that the malondialdehyde levels in the perfusate did not increase during HMP.

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

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

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

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

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

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

In conclusion, short-term HMP could resuscitate DCD lungs injured by prolonged ischemia, and ameliorate ischemia-reperfusion injury. First, short-term HMP washed-out residual microthrombi in the donor lungs. Second, short-term HMP improved the ATP production by the mitochondrial electron transport chain, which led to the significant decrease in oxidative damage and production of 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℃ 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℃ 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

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reinflated and mechanically ventilated with FiO_2 of 1.0, and then reperfused for 4 h to evaluate the posttransplant lung functions. The right pulmonary artery was occluded with a tourniquet 45 min after reperfusion to specifically evaluate the functions of the transplanted lung. The pulmonary arterial pressure and peak airway pressure (PawP) were continuously monitored throughout the experiments. Dynamic pulmonary compliance was defined as tidal volume/(PawP − PEEP) (ml/cmH_2O). A blood gas analysis was performed using blood collected from the femoral artery at selected time points. Lung tissue biopsy samples collected from the left middle lobe 4 h after reperfusion were weighed to obtain the wet lung weight, placed in an oven at 180℃ for 24 h, and then reweighed to obtain the dry lung weight. The wet to dry lung weight ratio was calculated to evaluate the presence of pulmonary edema.

Hypothermic machine perfusion (HMP)

The lungs were placed in an XVIVO chamber (Vitrolife, Denver, CO). The pulmonary artery was cannulated directly and then connected to the perfusion circuit. The left atrium was left open, so that the left atrial pressure was always 0 mmHg. The trachea was intubated and connected to the ventilator. Mechanical ventilation was started with FiO_2 of 0.25, tidal volume of 10 ml/kg, frequency of 10 breaths/min and PEEP of 5 cmH_2O. The perfusate, which contained STEEN solution (1,500 ml) with methylprednisolone (500 mg) and heparin (10,000 IU), was driven by a centrifugal pump at a constant flow rate of 10% of the estimated cardiac output (CO = 100 ml/kg). Deoxygenation of the perfusate was started with a gas mixture of nitrogen (86%), carbon dioxide (8%), and oxygen (6%) to maintain the

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influent PCO_2 of around 40 mmHg. The temperature of influent was continuously monitored, and was maintained around 10 ℃ (31). The influent solute concentrations, PO_2, and PCO_2 levels were recorded every hour. The pulmonary arterial pressure and peak airway pressure were continuously monitored, and the physiological lung functions (dynamic pulmonary compliance and pulmonary vascular resistance) during HMP were evaluated every 30 min. Recruitments were performed to ensure a peak airway pressure of 25 cmH_2O every 30 min prior to each evaluation. Dynamic pulmonary compliance was defined as described above. Pulmonary vascular resistance was defined as (pulmonary arterial pressure − left atrial pressure)/ pulmonary arterial flow (mmHg/L).

Lung tissue ATP levels

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

Malondialdehyde levels

Malondialdehyde levels were measured with the NWLSS Malondialdehyde Assay kit from Northwest (Northwest Life Sciences Specialties, Vancouver, Canada) following the manufacture's protocol. Malondialdehyde (MDA) reacted with thiobarbituric acid (TBA), forming an MDA-TBA_2 adduct that was measured at a

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wavelength of 532 nm.

Cytokine levels in BAL fluid

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

Histological evaluation of microthrombi and ischemia-reperfusion injury

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

Statistical analysis

All data are presented as means ± standard deviation. The statistical analysis

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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. TNF-α 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

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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. PaO_2 (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.

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FIGURE 1.

A

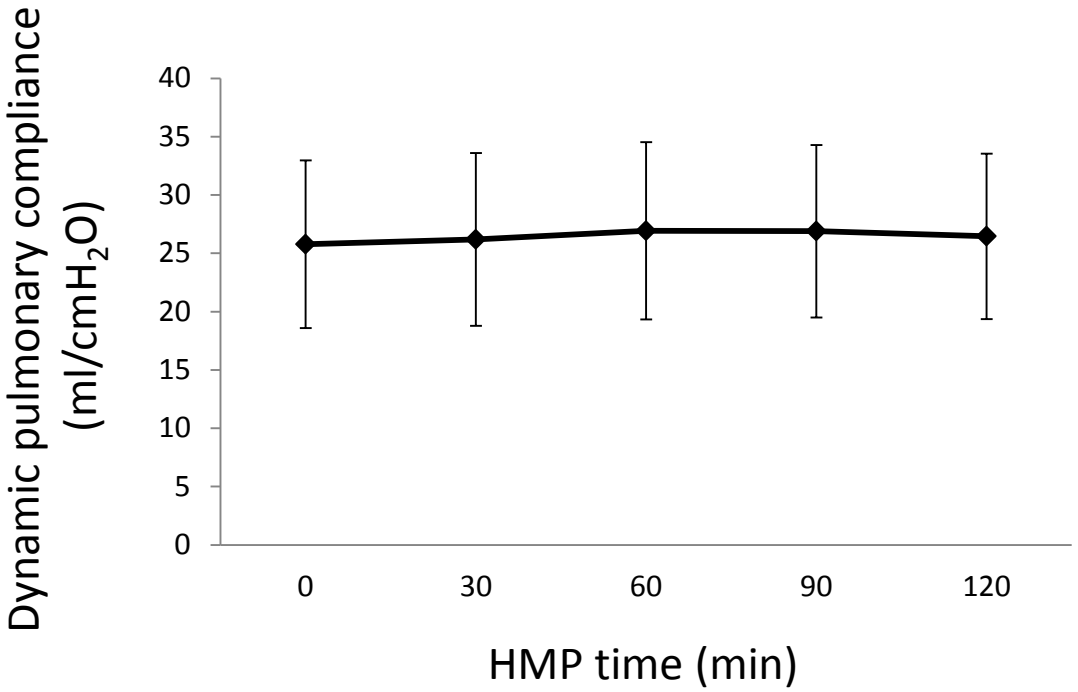
Dynamic pulmonary compliance (ml/cmH2O)
40
35
30
25
20
15
10
5
0
0
30
60
90
120
HMP time (min)

FIGURE 1.

B

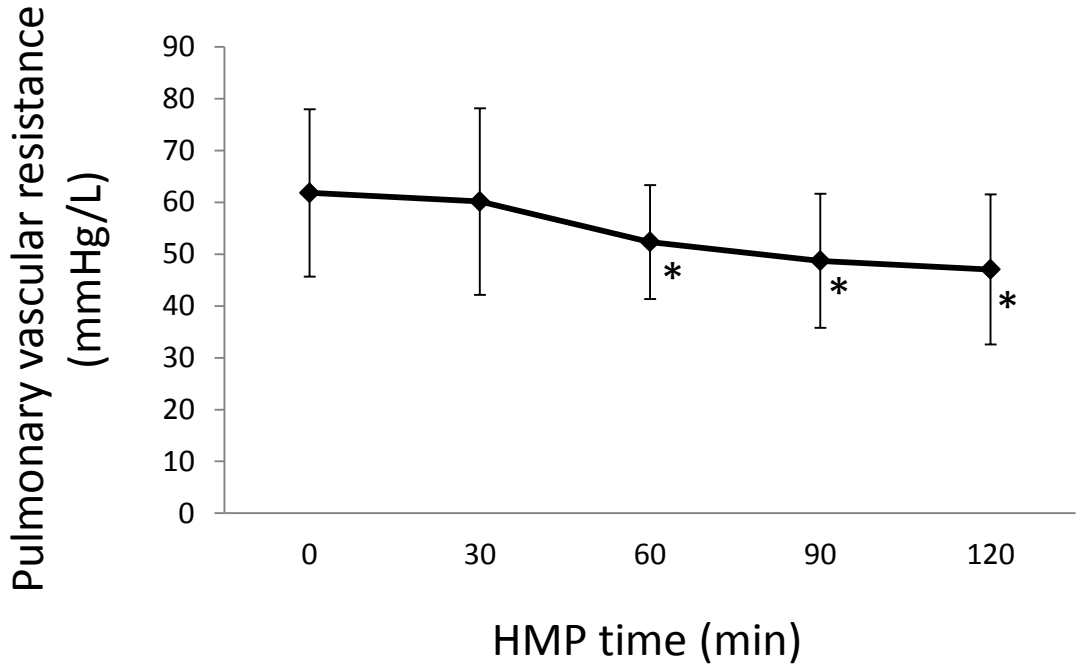
Pulmonary vascular resistance (mmHg/L)
90
80
70
60
50
40
30
20
10
0
0
30
60
90
120
*
*
*
HMP time (min)

FIGURE 1.

C

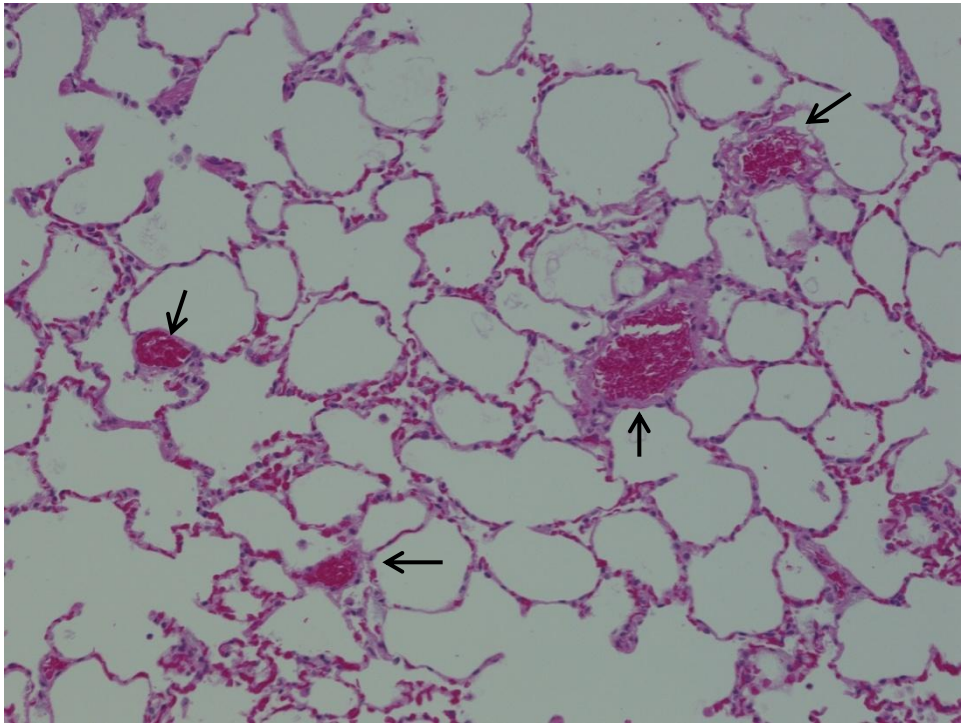

FIGURE 1.

D

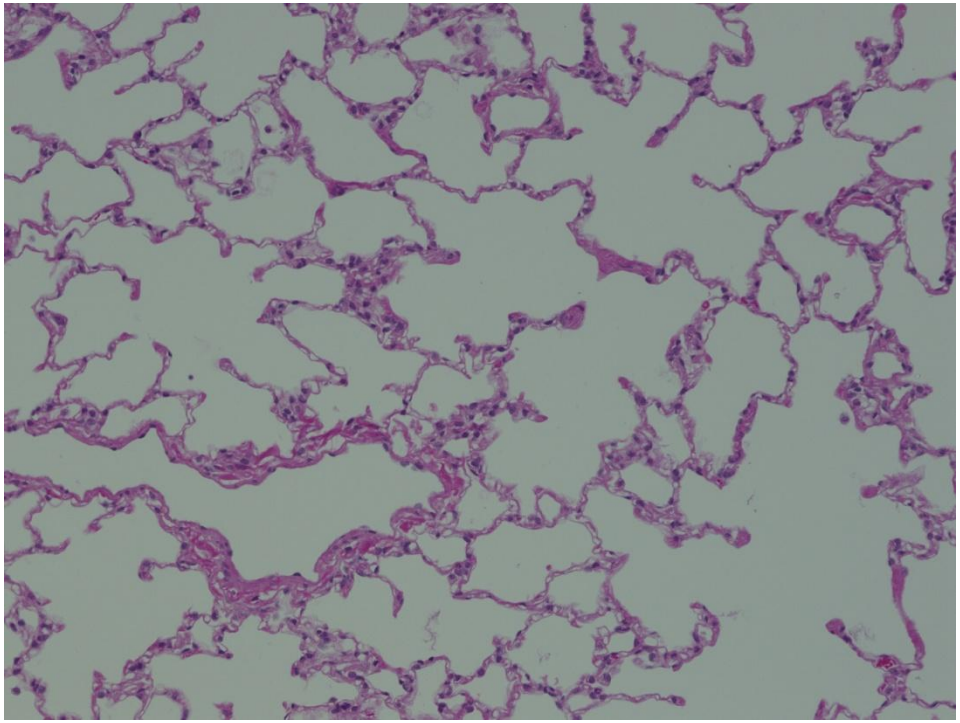

FIGURE 2.

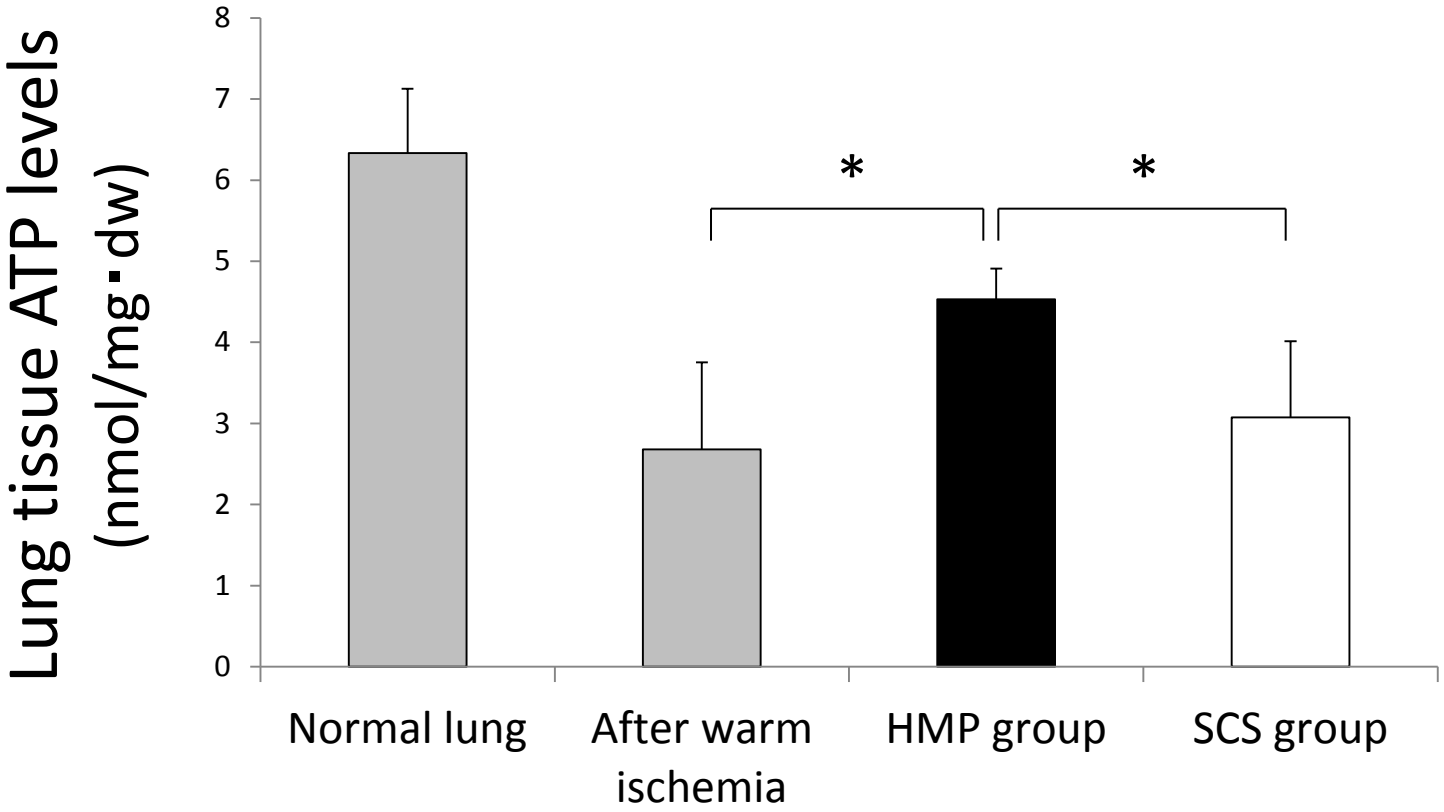
Lung tissue ATP levels
(nmol/mg▪dw)
8
7
6
5
4
3
2
1
0
*
*
Normal lung
After warm ischemia
HMP group
SCS group

FIGURE 3.

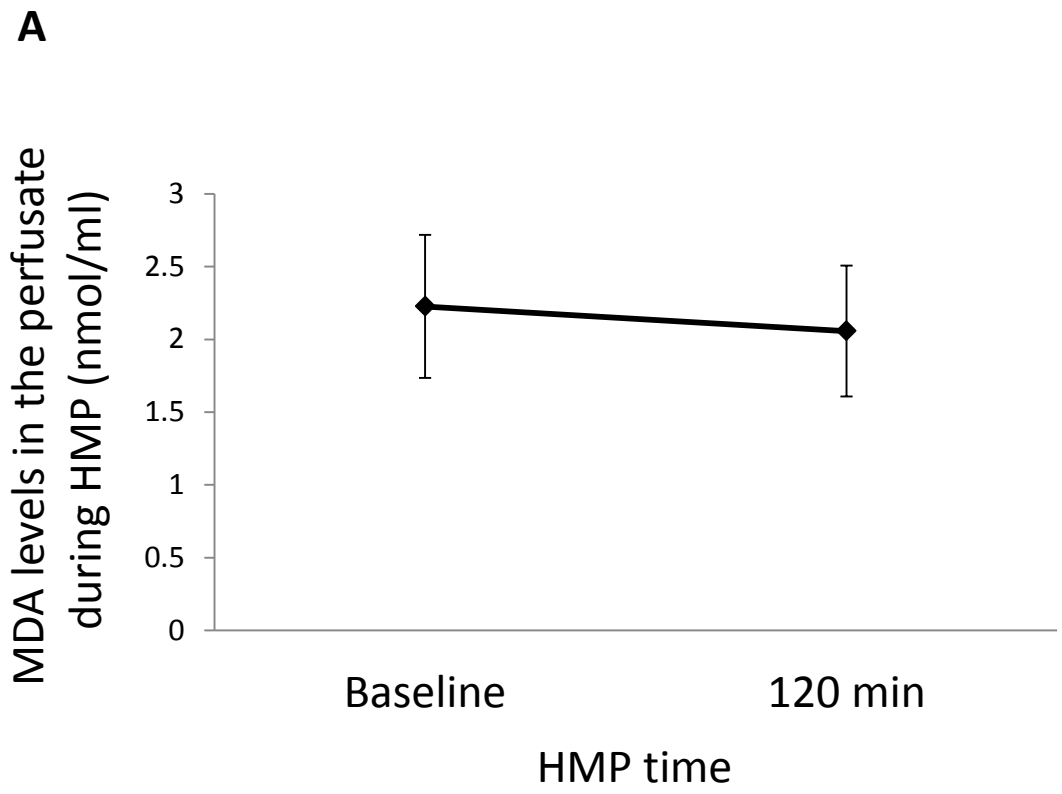
A
MDA levels in the perfusate during HMP (nmol/ml)
3
2.5
2
1.5
1
0.5
0
Baseline
120 min
HMP time

FIGURE 3.

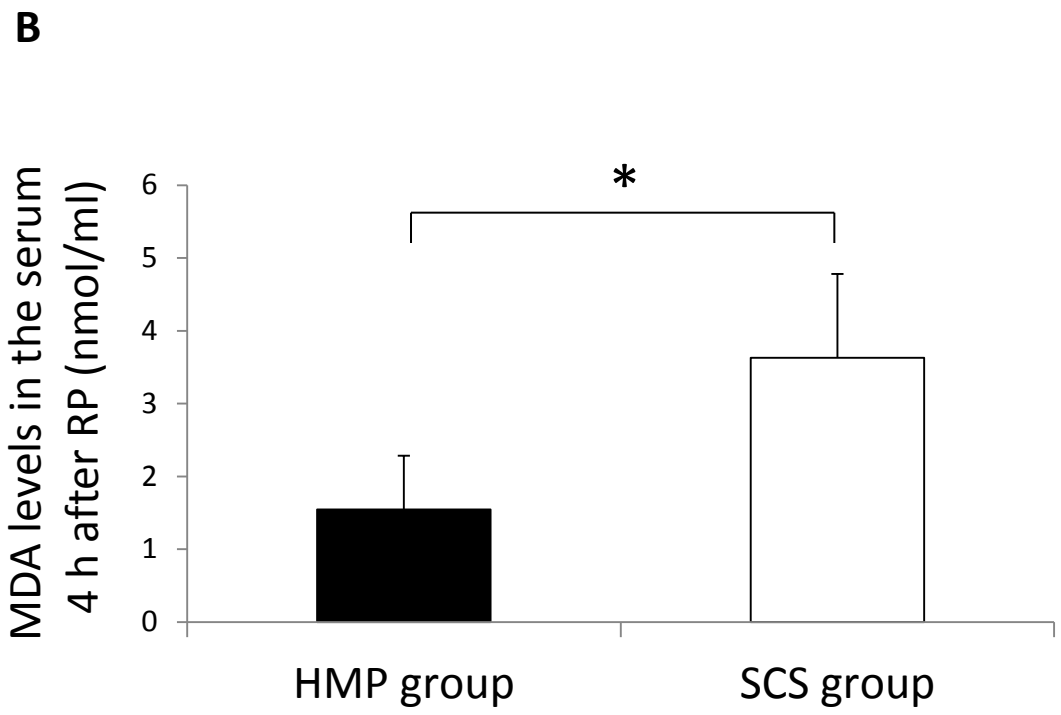
B
MDA levels in the serum 4 h after RP (nmol/ml)
0
1
2
3
4
5
6
*
HMP group
SCS group

FIGURE 3.

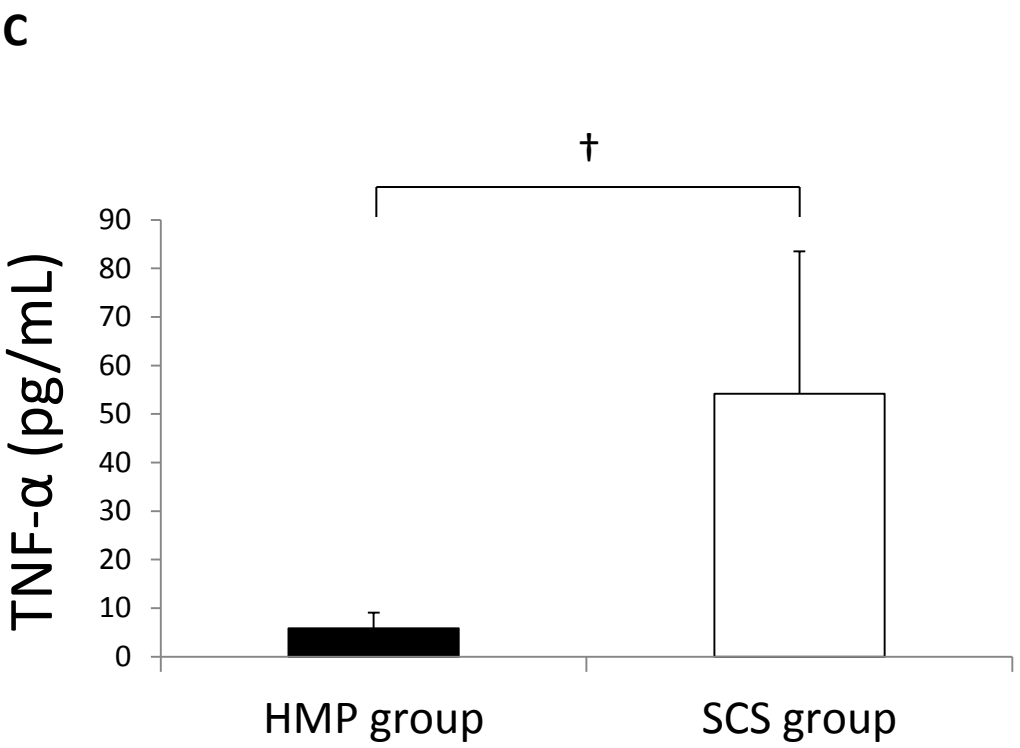

FIGURE 3.

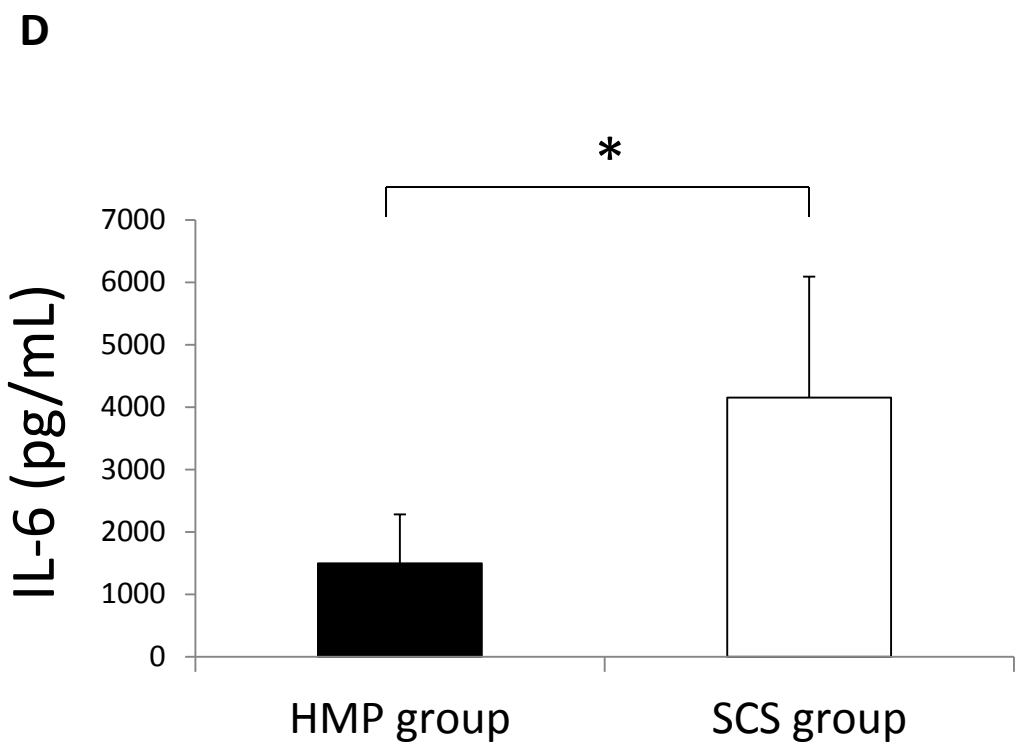
D
*
7000
6000
5000
4000
3000
2000
1000
0
IL-6 (pg/mL)
HMP group
SCS group

FIGURE 4.

A

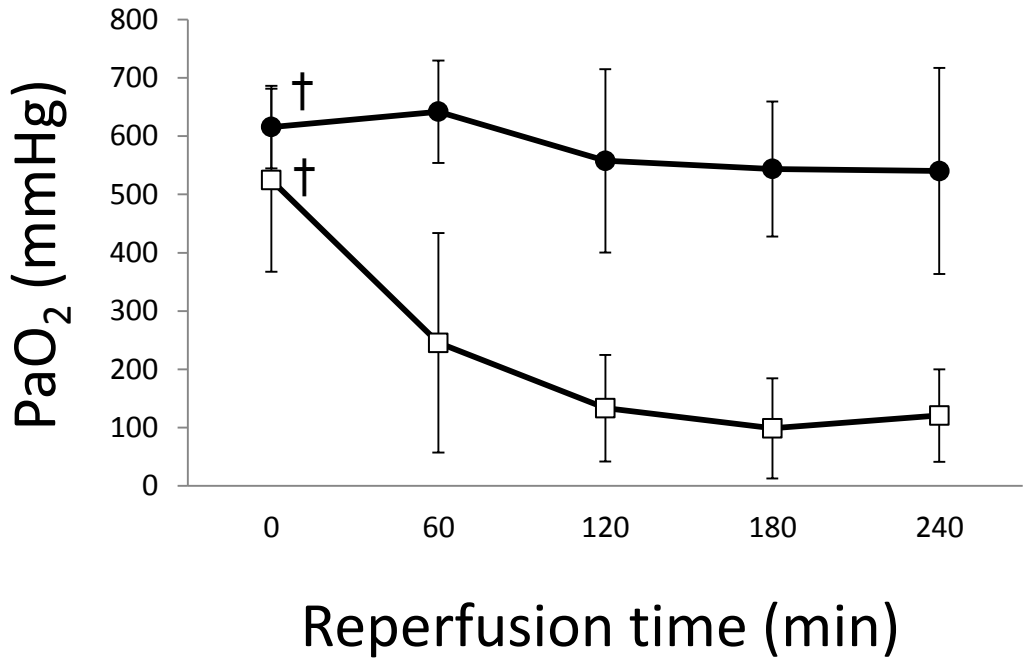
800
700
600
500
400
300
200
100
0
PaO2 (mmHg)
†
†
0
60
120
180
240
Reperfusion time (min)

FIGURE 4.

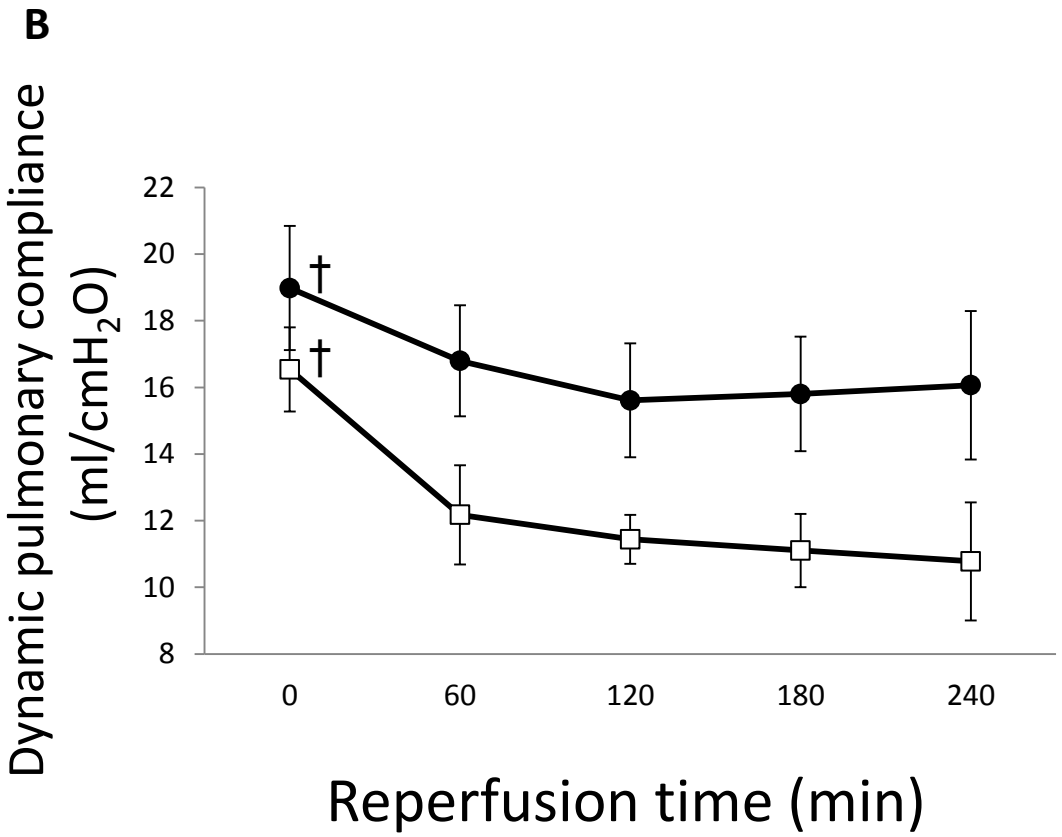
B
Dynamic pulmonary compliance (ml/cmH2O)
22
20
18
16
14
12
10
8
†
†
0
60
120
180
240
Reperfusion time (min)

FIGURE 4.

C

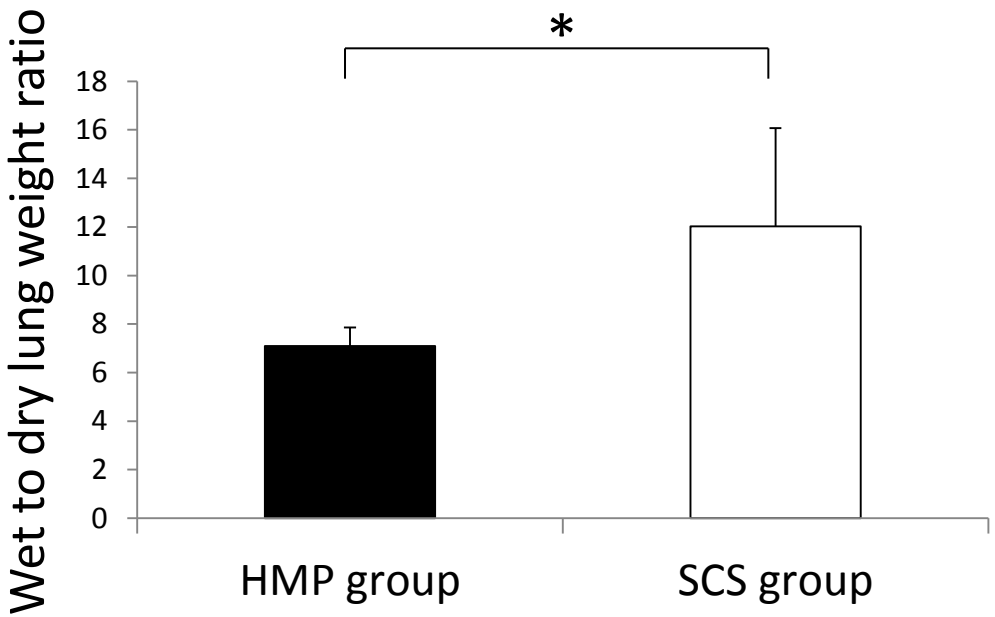
*
Wet to dry lung weight ratio
18
16
14
12
10
8
6
4
2
0
HMP group
SCS group

FIGURE 5.