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Title: Warm retrograde perfusion can remove more fat from lung grafts with fat embolism in a porcine model

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Abstract:

Objective: In lung transplantation, unexpected pulmonary emboli, including thrombi and fat, have been observed with high probability and are associated with potential primary graft dysfunction. We evaluated a new perfusion method using warm retrograde flushing that removes more fat than conventional cold retrograde flushing. Methods: We developed a novel porcine donor model for pulmonary fat embolism by administering autologous fat in the left pulmonary artery. The left pulmonary artery and the left superior and inferior pulmonary veins were cannulated for flushing and collecting these solutions. After flushing, the left lung was reperfused under observation for three hours. Two groups underwent warm and cold additional retrograde flush (WS; warm solution group, CS; cold solution group). Results: The fat removal rate in the antegrade flush was equal in both groups (3.0±0.6% vs 3.0±0.4%, p = 0.46); however, the rate was significantly greater in the WS group in retrograde flush (25.2±3.2% vs 8.0±1.4%, p = 0.01). Histology with Oil Red O staining and its software analysis showed more residual fat in the CS group (0.12±0.01% vs 0.38±0.07%, p = 0.01). There was no significant difference in

the pulmonary function and hemodynamics during the 3-hour period after reperfusion. Conclusion: Warm retrograde perfusion can remove more fat from lung grafts with fat embolism in a porcine donor model.

Introduction

Brain dead and non-heart beating donors for lung transplantation (LTx) are primarily obtained from cases of head trauma, cerebrovascular accident, anoxia, and tumors of the central nervous system. Trauma accounts for over 40% causes of death among brain-dead donors for LTx [1] and fat embolism occurs in 68% of donors with blunt trauma or bone fractures [2, 3]. These donors often have unexpected vascular embolization with fat, which contributes to primary graft dysfunction (PGD) after LTx [4, 5]. Symptomatic manifestations of fat embolism appear in a minority of brain-dead donors; however, the incidence of macroscopic unexpected pulmonary embolism is more common [6, 7]. Indeed, unexpected pulmonary emboli including thrombi and fat were observed with high probability, in over 30% of lung transplant recipients, and associated with potential PGD [7-10]. In terms of embolic materials, fat rather than thrombi has more deleterious effects on grafts and, interestingly, has more impact on post-transplant outcomes in the lungs than in the other organs, such as the heart and kidney [9].

A recent lung flushing technique offers better graft preservation by retrograde flushing

through the pulmonary veins in addition to antegrade flushing through the pulmonary artery. Previous reports have revealed effective elimination of intravascular microthrombi in the context of lung procurement [11, 12]. However, few reports have assessed residual fat in lung grafts from trauma donors. In this study, we developed a novel porcine donor model of pulmonary fat embolism (Fig. 1) and evaluated the new perfusion method that uses warm retrograde flushing. We hypothesized that a warm flushing solution could remove more embolized fat, via the melting fat effect and decreased vasoconstriction, than the conventional cold solution. To the best of our knowledge, this is the first study to reveal the effect of warm retrograde flush in removing embolized fat.

Material and methods

To investigate the usefulness of retrograde perfusion with a warm solution in lung procurement, the experiments were conducted in two groups: a group with warm additional retrograde flush (WS; warm solution group) and a group with cold additional retrograde flush

71 (CS; cold solution group).

72

73 *Operative Procedures*

74 Landrace domestic pigs were premedicated with an intramuscular injection of

75 ketamine hydrochloride (10 mg/kg) (Ketaral; Daicdrichi Sankyo, Tokyo, Japan) and atropine

76 sulfate (0.025 mg/kg) (Fuso, Osaka, Japan). The animals were then anesthetized using

77 sevoflurane (Fluothane; Takeda, Osaka, Japan); vecuronium bromide (0.2 mg/kg) (Musculax;

78 Schering Plough, Osaka, Japan) was administered before intubation. They were mechanically

79 ventilated at a tidal volume of 12 mL/kg, a respiratory rate of 12 breaths/min, and a positive

80 end-expiratory pressure of 5 cm of H_2O. A femoral artery line was inserted for measuring aortic

81 pressure (AoP) and for arterial blood gas analysis. A 5F Swan-Ganz catheter was placed in the

82 main pulmonary artery from the right internal jugular vein to measure pulmonary artery

83 pressure (PAP), central venous pressure (CVP), and cardiac output (CO). A block of

84 subcutaneous fat (>25 cm^2) was dissected from the pig and shredded. Then, small pieces of its

85 fatty material were boiled into a liquid; the fluid component was extracted using heat. This fatty

86 liquid was filtered through a filter paper and collected for creating fat embolism at a later stage.

87 After median sternotomy, a 23G catheter was inserted into the left atrial appendage to

88 measure the left atrial pressure (LAP). Fat embolism was achieved by administrating autologous

89 fat (0.07 mg/kg) in the left pulmonary artery. At this time, the right pulmonary artery was

90 temporarily blocked to create unilateral fat embolism in the left lung. Subsequently, the left

91 pulmonary artery, the left superior pulmonary vein, and the left inferior pulmonary vein were

92 cannulated with 18-F, 14-F, and 16-F cannulae (femoral cannula; Toyobo, Osaka, Japan),

93 respectively, for flushing and collecting these solutions. One hour after creating fat embolism,

94 the antegrade cold flush was started at a pressure of 30 cmH_2O through the left pulmonary

95 artery with 1000 mL of low-potassium dextran glucose (LPDG) stored at 4℃. Immediately after

96 the antegrade cold flush, an additional retrograde flush at 30 cmH_2O through the left superior

97 and inferior pulmonary veins with 500 mL of LPDG at 37℃ in the WS or at 4℃ in the CS was

98 performed. Ventilation was continued through the experiments. In both antegrade and retrograde

99 flushes, the flushed solution was collected through the cannula in the pulmonary artery and
100 veins (Fig. 1). Fat was seen to be included in the supernatant liquid of the flushed solution as
101 liquid and solid. The flushed solution was the refrigerated at about 4℃ to make all the fat solid.
102 Then, the supernatant fat in the solid state was filtered, dried, and weighed. The fat removal rate
103 was calculated according to the weight of the fat collected per that of the fat injected.

104 Cannulas for the pulmonary artery and veins were then removed and reperfusion in the
105 left lung was performed. The pigs were observed for three hours after reperfusion, during which
106 time AoP, PAP, CVP, LAP, and pulmonary vascular resistance (PVR) values were continuously
107 recorded and calculated. Measurement of CO, left lower pulmonary blood gas, and femoral
108 arterial blood gas analysis were also performed three hours after reperfusion.

109 Biopsy specimens from the same part of the left lung at after creating fat embolism
110 and at after three hours from reperfusion were removed for histological examination. Lung
111 tissue samples were inflation-fixed in 10% buffered formalin and stained with hematoxylin and
112 eosin. These tissue samples were also stained with Oil Red O to detect fat and the residual fat

113 rate was calculated using software ImageJ, which can analyze specific color area of fat [13].

114

115 *Animal Care*

116 This study was conducted in accordance with the guidelines of the Institutional Animal

117 Care and Use Committee of Okayama University. The protocol was specifically approved by the

118 Institutional Animal Care and Use Committee of Okayama University (protocol #OKU-

119 2014649).

120

121 *Statistics*

122 All results are expressed as mean ± standard error of the mean (SEM). Statistical tests

123 were performed using Statcel version 4 for Excel (OMS Publishing Inc, Japan) on a

124 PC-compatible computer running Windows 10. Differences were accepted as significant if the p

125 value was less than 0.05. The Mann Whitney test was used for comparisons between groups for

126 parametric data. Repeated-measures analysis of variance (ANOVA) was used for analysis of

serial measurements.

Results

The porcine weight and baseline cardiopulmonary function of pigs were similar in both groups. All 10 animals survived the operation and the 3-hour assessment period.

Fat removal rate and Histological Findings

The fat removal rate after the antegrade flush was equal between the two groups (3.0 ± 0.6% vs 3.0 ± 0.4%, p = 0.46); however, the rate was significantly higher in the WS group than in the CS group after the retrograde flush (25.2 ± 3.2% vs 8.0 ± 1.4%, p = 0.01) (Fig. 2a). Software analysis of embolized fat, detected in red microscopically, showed the higher residual fat in lung tissue in the CS group (residual fat rate; 0.12% ± 0.01 vs 0.38% ± 0.07, p = 0.01) (Fig. 2b). After retrograde flushing, the lung surface looked uniformly perfused in the both groups, macroscopically. However, the histology of samples of lung parenchyma with Oil Red

O staining showed more residual fat in the lungs in the CS group (Fig. 3).

Pulmonary function and hemodynamics

There were no apparent derangements in gas exchange before and after creating fat embolism in both groups (WR: 490.0 ± 17.1 vs 490.4 ± 30.7, p = 1.00, CR: 538.1 ± 35.8 vs 504.6 ± 28.0, p = 0.62), whereas PVR increased immediately after creating fat embolism (WR: 250.0 ± 30.0 vs 493.1 ± 54.8, p = 0.014, CR: 250.4 ± 15.8 vs 356.6 ± 62.0, p = 0.14).

During the 3 hours after reperfusion, there was no difference in the oxygen/inspired oxygen fraction (PO2/FiO2) from the left inferior pulmonary vein between the WS and CS groups (434 ± 16 vs 460 ± 15, p = 0.58, ANOVA) (Fig. 4). Mean AoP, mean PAP, CVP, LAP, CO, and PVR values were stable during the 3-hour assessment period and were not significantly different between both groups (Table 1).

Discussion

This study investigated the effect of warm retrograde flushing in removing fat emboli in porcine donor lung grafts. The results showed that the additional warm retrograde flush removed more fat in the lung grafts compared with the conventional cold retrograde flush. On the contrary, this new flushing method did not give rise to any difference in lung function and hemodynamics, including PVR, immediately after reperfusion.

Retrograde flushing is an established preservation technique for lung grafts that is used to wash out residual blood and microthrombi [11, 12, 14, 15]. This technique significantly improves lung graft function, resulting in better oxygenation, lower edema formation, and lower PVR. Although the warm temperature (23 ℃) of the flush solution reportedly offered a uniform wash out with less vasoconstriction [16], cold solution is universally used for lung flushing. In the present study, we adopted a warmer temperature for retrograde flushing to enhance the clearance effect for fat emboli through streaming and melting.

Jacob et al. performed a literature review on donor-acquired fat embolism syndrome after lung transplantation, reporting its high mortality [6]. In previous case reports,

169 donor-acquired fat embolism syndrome was mostly diagnosed after pathological examination of
170 autopsy or lung biopsy. As this review indicated, the difficult diagnosis can lead to
171 underestimation of the incidence and importance of donor-acquired fat embolism after lung
172 transplantation. Oto et al. reported that the incidence of unexpected pulmonary embolism was
173 38% (28% clot and 9% fat), showing its deleterious effect related to poor oxygenation, higher
174 PVR, prolonged intubation, longer ICU stay, and poor 1-year survival. They also reported the
175 importance of embolic materials; LTx recipients receiving lungs from donors with fat emboli
176 and thromboemboli were about 20-fold and 5-fold more likely to develop severe PGD compared
177 with those who received lungs from donors without pulmonary embolism, respectively. They
178 suggested that worse effects occur due to local toxic effects leading to negative inflammatory
179 responses increasing pulmonary vascular permeability. Fat embolism causes both mechanical
180 vascular obstruction and chemical injury, resulting in an increase in PVR and inflammatory
181 changes such as vascular hyper-permeability and pulmonary edema. According to previous
182 animal studies on fat embolism using oleic acid, alveolar edema, endothelial necrosis, and

capillary congestion were observed in the early stage within 12 hours, followed by pulmonary fibrosis in the late stage after 1 week [17-19]. In our study, warm retrograde perfusion at 37℃ could more effectively remove embolized fat originating from porcine subcutaneous self-fat, which has a melting point of 30–43℃, compared with the conventional cold flushing. Better fat removal in WS led to less residual fat in histology; however, it did not improve pulmonary function and PVR at least immediately after reperfusion. In our histological examination, no obvious lung injury like alveolar edema, endothelial necrosis, or capillary congestion were detected. Longer observation periods after reperfusion than our study could be necessary to reveal the histological effect of fat embolism on the lung.

We hypothesized that the melting-fat strategy using a warm flushing solution could enhance the cleaning effect in addition to mechanical fat removal. However, warm flush can lead to an additional warm ischemic period of about 5 minutes in the clinical lung transplant setting, leading to possible negative effects especially on damaged lung grafts. Furthermore, the warm temperature of the solution seemed to induce possible negative chemical effects. The

197 deleterious effects of pulmonary fat embolism can be partly due to chemical effects causing its

198 inflammatory effect on the endothelium of the pulmonary artery [7, 18]. In this study, improved

199 fat removal in WS as seen on histology suggested positive effects of warm temperature in

200 mechanical removal of embolized fat. However, decreased PaO2 and higher PVR in WR in the

201 early stage after reperfusion likely showed possible negative effects in inducing chemical and

202 physical responses, although that improved later. We performed flushing at the same height of

203 30 cm H_2O in both WR and CR according to the usual clinical donor operation procedure.

204 Warm flushing would dilate the pulmonary vessels more effectively than cold flushing, leading

205 to a more prominent cleaning effect of embolized fat. However, in terms of preservation of the

206 pulmonary vascular endothelium, the lower pressure of the flushing and the slower lung

207 reperfusion might be desirable in WR than in CR. In general, PaO_2 does not decrease in the

208 acute period of pulmonary embolism, because of a lack of shunt blood. Therefore, a strong

209 effect of fat removal through warm flushing could not improve pulmonary function immediately

210 after reperfusion. We developed this autologous fat embolism model to mimic clinical donor fat

211 embolism, which arises from the donor's fat material rather than artificial materials like oleic
212 acid [7, 18]. However, in clinical LTx, pulmonary embolism occurs due to heterologous fat from
213 another human, which can also lead to increased inflammatory reactions. In this study, one
214 reason of no significant difference in the pulmonary function and hemodynamics during the
215 3-hour period after reperfusion may be due to autologous fat administration, rather than fat from
216 another allogenic pig. Although we used a donor reperfusion model in this study, a lung
217 transplant model may evaluate the real effects of fat emboli on LTx recipients with more
218 precision.

219 Our study has some other limitations. First, these experiments were only performed
220 with a short observation period after lung reperfusion. Thus, the three-hour-observation period
221 of this study did not allow us to draw any firm conclusions about the effect of fat embolism over
222 a longer period after graft reperfusion, which is a common clinical timeframe in LTx. The later
223 chemical damage induced by fat embolism might not be fully assessed. Second, the validity of
224 this autologous fat embolism model using large animals should be carefully evaluated. We used

autologous subcutaneous fat from porcine backs, because bone marrow suspension, which is a more realistic situation in clinical fat embolism in trauma donors, was not enough to give rise to constant pulmonary embolism in preliminary porcine experiments. Third, this is not a lung transplant model, which may evaluate the real effects of fat emboli and additional warm ischemic time on LTx recipients with more precision. Otherwise, another normothermic ex vivo lung perfusion model would provide an ideal platform to review our hypothesis.

In conclusion, the additional warm retrograde perfusion rather than the conventional cold method can remove more fat from lung grafts with fat embolism in a porcine donor model.

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Disclosure

The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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241

242 Figures, Table and Legends for Figures

243

244 Table 1: The results of gas exchange and the hemodynamics during the 3-hour assessment period

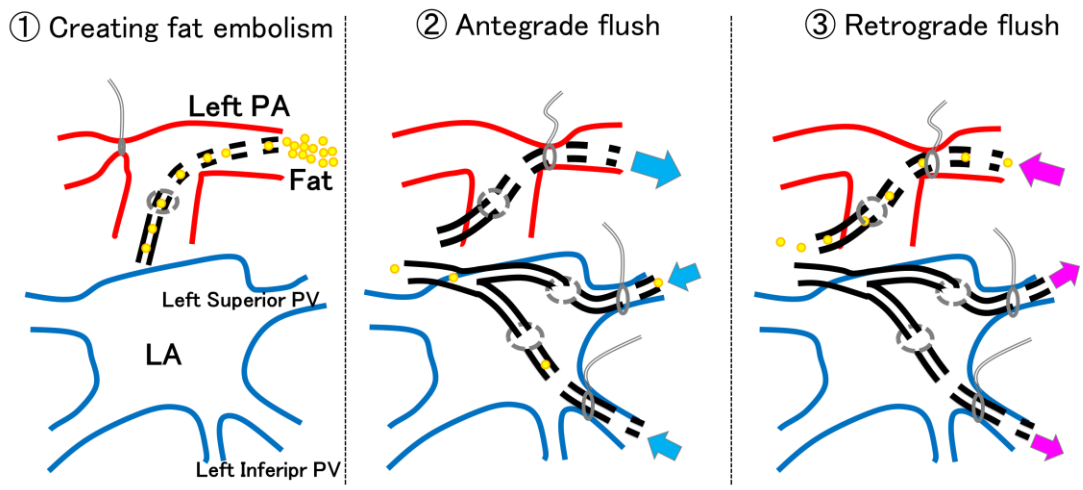
245

	pre reperfusion	after reperfusion						p value
		30m	60m	90m	120m	150m	180m	
Mean AoP (mm Hg)								
Group WS	76.0 ± 3.4	78.0 ± 5.0	72.0 ± 3.6	69.2 ± 2.2	61.4 ± 36.	72.0 ± 4.8	64.8 ± 5.7	
Group CS	74.0 ± 1.1	63.2 ± 3.9	67.0 ± 3.1	61.2 ± 2.9	63.2 ± 4.3	66.8 ± 2.6	60.0 ± 2.6	0.17
Mean PAP (mm Hg)								
Group WS	28.4 ± 1.1	27.8 ± 1.4	25.8 ± 1.1	26.4 ± 1.3	28.0 ± 2.4	28.4 ± 2.4	24.2 ± 1.4	
Group CS	21.0 ± 2.1	22.0 ± 2.3	23.2 ± 1.6	21.8 ± 2.4	22.2 ± 3.2	22.8 ± 2.4	22.0 ± 2.1	0.13
Mean CVP (mm Hg)								
Group WS	6.6 ± 0.7	6.1 ± 0.6	6.2 ± 0.5	6.7 ± 0.6	6.5 ± 0.4	6.4 ± 0.4	6.4 ± 0.4	
Group CS	5.1 ± 0.4	5.4 ± 0.5	5.6 ± 0.6	5.7 ± 0.4	5.9 ± 0.6	5.5 ± 0.6	5.7 ± 0.7	0.32
Mean LAP (mm Hg)								
Group WS	8.9 ± 0.4	8.1 ± 0.9	8.2 ± 0.9	8.6 ± 1.0	7.4 ± 0.2	8.7 ± 0.9	8.8 ± 0.9	
Group CS	8.8 ± 1.6	7.4 ± 1.0	6.5 ± 0.8	6.8 ± 0.9	6.9 ± 1.2	7.8 ± 0.9	7.3 ± 0.7	0.37

Mean CO (L/min)								
Group WS	3.3 ± 0.3	3.6 ± 0.2	3.1 ± 0.2	3.1 ± 0.3	3.0 ± 0.3	3.4 ± 0.3	3.0 ± 0.3	
Group CS	3.0 ± 0.4	2.8 ± 0.3	2.8 ± 0.3	2.6 ± 0.2	2.9 ± 0.4	3.1 ± 0.3	2.7 ± 0.2	0.38
PVR (dyne·sec·cm^{-5})								
Group WS	493.1 ± 54.8	448.7 ± 49.6	453.9 ± 25.7	483.0 ± 45.5	566.4 ± 69.9	463.3 ± 49.2	422.4 ± 39.7	
Group CS	356.6 ± 62.0	420.1 ± 48.3	497.0 ± 51.6	469.7 ± 70.3	421.0 ± 34.3	390.4 ± 27.1	431.2 ± 41.6	0.44

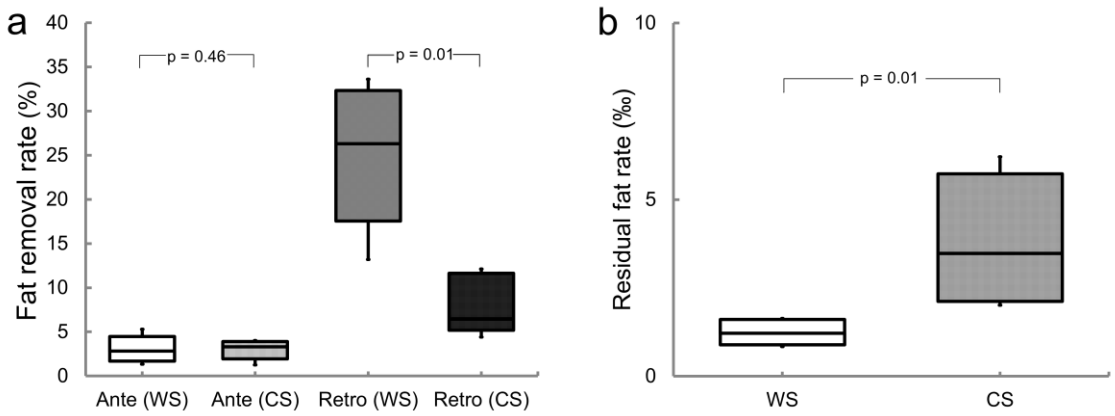
AoP: aortic pressure; CO: cardiac output; CS: cold solution group; CVP: central venous pressure; LAP: left atrial pressure; PAP: pulmonary artery pressure; PVR: pulmonary vascular resistance; WS: warm solution group

Figure 1:



The pulmonary fat embolism model used in this study ①Fat embolism was created by administrating autologous fat (0.07 mg/kg) in the left pulmonary artery (PA) with the right PA clamped. ②One hour after creating the fat embolism, the antegrade cold flush was started through the left PA with 1000 mL of low-potassium dextran glucose (LPDG) at 4℃. ③An additional retrograde flush was made through the left pulmonary vein (PV) with 500 mL of LPDG at 37℃ in the warm solution group or at 4℃ in the cold solution group. LA: left atrial

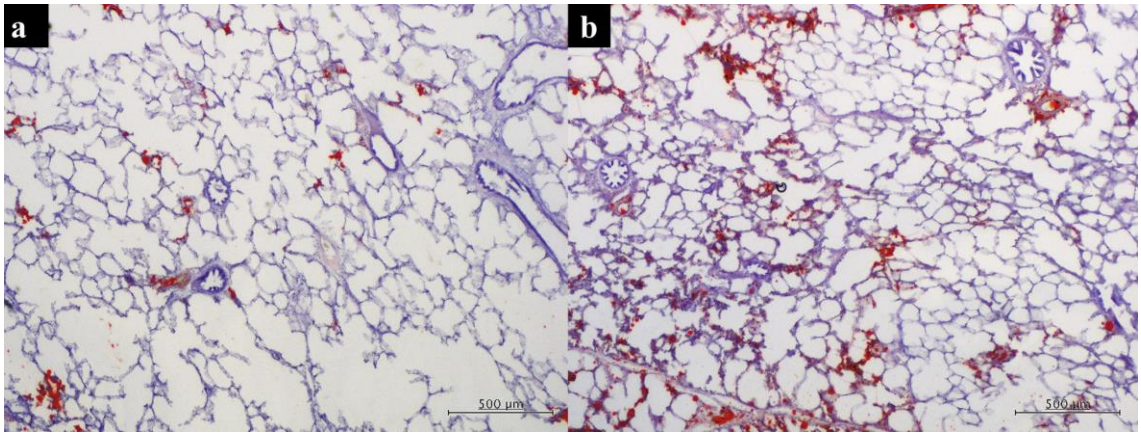
Figure 2:



(a) No statistical difference was seen in the fat removal rate in the antegrade flush ($p = 0.46$); however, the warm solution group (WS) showed significantly better removal than the cold solution group (CS) in retrograde flush ($p = 0.01$).

(b) Residual fat rate calculated using the software ImageJ [11] was significantly lower in the warm solution group (WS) than in the cold solution group (CS) ($p = 0.01$).

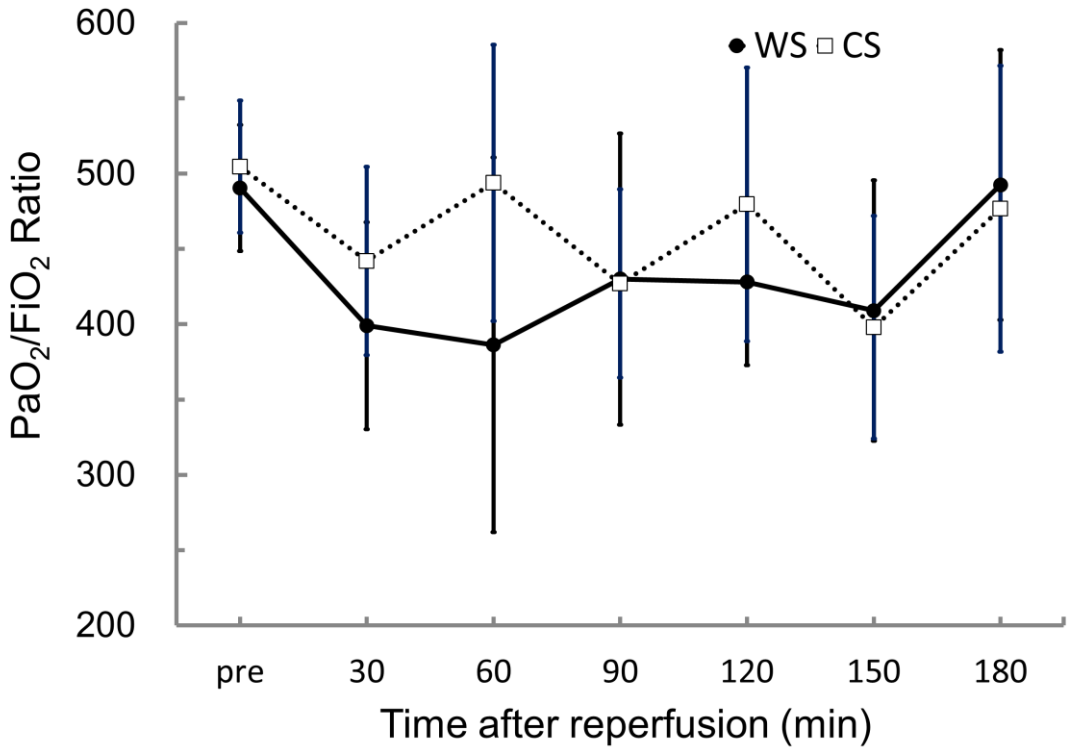
Figure 3:



Histological studies of lung samples with Oil Red O staining after 3 hours of reperfusion in the warm solution group (a) and the cold solution group (b). Less residual fat (stained with red) was observed in the warm solution group, indicating better removal of fat embolism after warm retrograde flushing.

297 Figure 4

298



299

300 PaO2/FiO2 ratio after lung reperfusion from the left inferior pulmonary vein. No statistical

301 difference was seen in the oxygen/inspired oxygen fraction during after lung reperfusion

302 between the warm solution group (WS) and the cold solution group (CS) ($p = 0.58$).

303

Conflict of interest: The authors have declared that no conflict of interest exists.

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