

Plasmin administration during ex vivo lung perfusion ameliorates lung ischemia-reperfusion injury.

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Abstract

Background: Donor lung thrombus are considered a significant etiology for primary graft dysfunction (PGD). We hypothesized that thrombolysis in an *ex vivo* lung perfusion (EVLP) before lung transplantation could alleviate ischemia–reperfusion injury (IRI), resulting in the reduction of primary graft dysfunction.

Methods: Rats were divided into control (n = 5), non-plasmin (n = 7), and plasmin (n = 7) groups. In the non-plasmin and plasmin groups, cardiac arrest was induced by withdrawal of ventilation without heparinization. After 120 minutes of warm ischemia, the lungs were ventilated and flushed. Heart and lungs were excised *en bloc*. The lungs were perfused and ventilated in the EVLP for 30 minutes, and plasmin or placebo was administered on EVLP initiation. The lungs were then stored at 4°C for 90 minutes and finally, perfused with rat blood for 80 minutes. The physiologic and histologic findings during reperfusion and the correlation between physiologic data during EVLP and that after reperfusion were investigated.

Results: Physiologic results were better in the plasmin group than in the non-plasmin group. The lungs in the plasmin group had fewer signs of histologic injury. Caspase-3 and -7 activity in the plasmin group was lower in the non-plasmin group. Pulmonary vascular resistance (PVR) during EVLP correlated with that at the end of reperfusion.

Conclusions: Plasmin administration during EVLP protected the donor lungs after reperfusion. We also found that several physiological values in EVLP may be predictive markers for the lung function after reperfusion.

Introduction

The preservation and evaluation of donor organs using normothermic *ex vivo* lung perfusion (EVLP) before transplantation represents a new and active frontier in lung transplantation.¹⁻³

In addition to preservation and evaluation, reconditioning of the marginal lungs is also being investigated.⁴⁻⁸ In previous work, we focused on the thrombi formation in donor lungs, considered to be among the major causes of primary graft dysfunction^{9,10} and demonstrated that direct fibrinolytic agent (plasmin) administration to the EVLP perfusate could recondition the graft function.⁸ Although we showed that plasmin administration during EVLP could protect lungs damaged by thrombus, the effects after reperfusion are still unclear. Unlike under *in vivo* condition, the perfusate used in acellular EVLP does not contain white blood cells, red blood cells, platelets, and so on. At the time of reperfusion, reactive oxygen species and cytokines produced in the ischemic period are known to activate neutrophils and platelets contained in the recipient's blood, resulting in ischemia-reperfusion-induced vascular damages and lung edema.^{11,12} Thus, under *in vivo* condition, donor lungs might be severely damaged by white blood cells and platelets after reperfusion.

The aim of this study was to ascertain the effect of plasmin administration in EVLP against ischemia reperfusion injury in donor lungs in more clinically simulated settings. We also investigated that the physiologic data at the end of EVLP could predict the functions of the donor lungs after reperfusion.

Methods

Animals

Male Lewis rats (Japan SLC, Hamamatsu, Japan) were used in this study. All animals received humane care in compliance with the Principles of Laboratory Animal Care, formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996; Bethesda, MD). The study protocol was approved by the ethics committee of the Faculty of Medicine at Kyoto University, Kyoto, Japan.

All rats were randomly assigned to one of the following three groups: heart-beating donor (control; n

= 5), untreated thrombosis (non-plasmin; n = 7), or treated thrombosis (plasmin; n = 7). The body weight of the rats was similar in all three of these groups.

Heart–lung bloc preparation

Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg), intubated after tracheotomy, and ventilated during surgery. In the non-plasmin and plasmin groups, rats did not receive heparin and underwent warm ischemia after occlusion of their airways for 120 minute at 23°C. After 120 minutes of warm ischemia, the pulmonary artery and left atrium were directly cannulated. The lungs were flushed with 20 ml of Steen solution (Vitrolife, Uppsala, Sweden) (temperature 4°C; pressure 20 cm H₂O), with drainage through an incision of the left ventricle. After flushing period, a cannula was inserted into the left atrium. The pulmonary artery cannula and left ventricle cannula were connected to the perfusion circuit. Low-flow perfusion (1 mL/min) with Steen solution was initiated. Finally, the heart and lungs were excised *en bloc*.

EVLP model

To evaluate lung function, we used the isolated rat lung perfusion setup (Model 829; Hugo-Sachs Elektronik Harvard Apparatus, Holliston, MA). The isolated heart–lung bloc was secured in an artificial thorax and ventilated with ambient air at negative pressure under the conditions as previously described.⁸ The lungs were perfused with Steen solution. EVLP was started at a low flow (1 ml/min) rate, and the flow rate was gradually increased every 2 minutes in a stepwise manner (1→3→5→7→10 ml/min). Pulmonary arterial pressure was limited carefully so that lungs in each group did not develop edema during this step-up period. Heparin was not added to the perfusate to allow clear evaluation of the fibrinolytic ability of plasmin. At the initiation of the EVLP period, 0.5 mg human plasmin (Catalog No. HCPM-0140; Hematologic Technologies, Inc., Essex Junction, VT), diluted with 0.5 ml of Steen solution, was administered from the origin of the pulmonary artery in the plasmin group. In the same manner, 0.5 ml of Steen solution was administered to the non-plasmin

group. Assessment of the isolated lungs began after 10 minutes of the step-up period and lasted for 30 minutes. PaO₂ of the perfusate was analyzed just after the step-up period and at the end of EVLP period. Fibrin/fibrinogen degradation products (FDP) in the perfusate were analyzed 10 minutes after injection of plasmin or placebo (SRL, Tokyo, Japan). Pulmonary vascular resistance (PVR; in cm H₂O/mL · min, defined as pulmonary arterial pressure–pulmonary vein pressure/perfusate flow), weight gain of the lung (mg), and dynamic airway compliance (ml/cm H₂O), were monitored continuously and recorded at 10-minute intervals during EVLP. Physiologic data from each group were continuously recorded and analyzed after the experiment.

In the non-plasmin and plasmin groups, the lungs were detached from EVLP system, inflated with air, covered with gauze dampened by cold steen solusion and stored at 4°C for 90 minutes.

Reperfusion model

In the plasmin and non-plasmin groups, lungs were reperfused for 80 minutes at 37°C using an isolated rat lung perfusion model in which physiologic lung function was evaluated. The solution used in the reperfusion model contained heparinized rat blood obtained from two donor rats and saline with 4% albumin. The hematocrit was adjusted to approximately 15%, and the pH was adjusted to 7.25–7.35 with sodium bicarbonate. The lungs were reperfused with low flow (1 ml/min) and the flow rate was gradually increased every 2 minutes, (1→3→5→7→10 ml/min) and ventilated with the same condition used in the EVLP experiment (Figure 1).

Histologic analysis

After reperfusion, the right lower lobe was retrieved and fixed using 10% formalin to evaluate the damage during reperfusion. Single-strand DNA (ssDNA) staining using hematoxylin and eosin (H&E) stain was performed in each group.

Caspase activity assay

Caspase-3 and -7 activity in lung tissue was measured using a Caspase-Glo assay kit (Promega, Madison, WI). Left lungs were stored at -80°C after 80 minutes of reperfusion. The lung tissue was homogenized with homogenizer beads at 4°C in hypotonic extraction buffer and subsequently centrifuged. The protein concentration of the supernatant was adjusted to 1 mg/ml with extraction buffer and stored at -80°C . Proluminescent substrate contained in the Caspase-GloTM Reagent (Promega) was cleaved by caspase-3, caspase-7. After caspase cleavage, a substrate for luciferase was released, resulting in the production of a luminescent signal. The luminescence of each sample was measured in a plate-reading luminometer.

Statistical analysis

All statistical analyses were performed using StatView 5.0 software (Abacus Concepts, Berkeley, CA, USA). All values are presented as mean $\pm$ standard error of the mean (SEM). Data were evaluated using repeated-measures analysis of variance, Scheffe's post hoc multiple comparison test, and Student's paired *t*-test. A probability $p < 0.05$ was considered statistically significant.

Results

Fibrinolytic ability of plasmin

FDP levels in the perfusate 10 minutes after administration of plasmin or placebo (Steen solution) are presented in Figure 2. The levels in the perfusate 10 minutes after injection were higher in the plasmin group than in the non-plasmin group (non-plasmin group: 175.9 ± 31.3 ng, plasmin group: 805.9 ± 202.9 ng, $p = 0.0097$).

Pulmonary function during EVLP and reperfusion

Pulmonary vascular resistance (PVR)

The PVR in the plasmin group was significantly lower than that in the non-plasmin group during EVLP. (non-plasmin vs plasmin, $p = 0.0062$ at 30 min; Figure 3a). PVR in the non-plasmin group during reperfusion was significantly higher than that in the plasmin and control groups (control vs

non-plasmin: $p = 0.0128$, non-plasmin vs plasmin: $p = 0.021$, plasmin vs control: $p = 0.862$, Figure 4a).

Weight gain

In this model, weight gain reflects pulmonary edema. Although the weight gain of the heart–lung bloc increased and was higher in the non-plasmin group than in the plasmin groups ($p = 0.001$ at 30 minutes; Figure 3b), not all lungs in the non-plasmin group developed lung edema during the EVLP period. In contrast, weight gain of the heart–lung bloc significantly increased in the non-plasmin group than in the plasmin and control groups during the reperfusion period (control vs non-plasmin: $p = 0.002$, non-plasmin vs plasmin: $p = 0.0007$, plasmin vs control: $p = 0.862$; Figure 4b).

Dynamic compliance

Dynamic compliance during EVLP was slightly lower in the non-plasmin group than in the plasmin group ($p = 0.010$ at 30 minutes; Figure 3c). In contrast, dynamic compliance during reperfusion was significantly lower in the non-plasmin group than in the plasmin group (control vs non-plasmin: $p < 0.0001$, non-plasmin vs plasmin: $p = 0.0001$, plasmin vs control: $p = 0.0006$ at 80 min; Figure 4c).

PaO₂

PaO₂ in the plasmin and non-plasmin group was not high but was significantly lower in the non-plasmin group than in the plasmin group during EVLP ($p = 0.021$ at 30 minutes; Figure 4d). In contrast, PaO₂ in the plasmin group was significantly higher than that in the non-plasmin group during reperfusion (control vs non-plasmin: $p < 0.0001$, non-plasmin vs plasmin: $p < 0.0001$, plasmin vs control: $p = 0.0002$ at 80 minutes; Figure 4d).

Correlation of physiological data between EVLP and reperfusion in the plasmin and non-plasmin groups

PVR at the end of EVLP significantly correlated with that at 80 minutes after reperfusion ($r = 0.66$, $p = 0.011$, Figure 5a). Lung oxygenation at the end of EVLP slightly correlated with that at 80 minutes

after reperfusion ($r = 0.56$, $p = 0.036$; Figure 5c). However, there was no significant correlation between the dynamic pulmonary compliance at the end of EVLP and that at 80 minutes after reperfusion ($r = 0.48$, $p = 0.085$; Figure 5c).

Histologic findings after reperfusion

Lungs in the non-plasmin group exhibited stronger edema and hemorrhage than those in the plasmin group. H&E stain showed significant alveolar edema and accumulation of red blood cells in pulmonary vascular and alveolar tissue, particularly in the peripheral zone (Figure 6). ssDNA staining showed significantly fewer apoptosis cells in the plasmin group than in the non-plasmin group (Figure 6).

Caspase-3 and -7 activity assay

Caspase activity reflect early stages of apoptosis. Caspase activity of the lungs in each group was higher in the non-plasmin and plasmin group than in the control group. Caspase -3 and -7 activity in the plasmin group was lower than that in the non-plasmin group. (control: 4.1×10^5 , non-plasmin: 12.8×10^5 , plasmin: 6.5×10^5 , control vs non-plasmin: $p < 0.0001$, non-plasmin vs plasmin: $p < 0.0001$, plasmin vs control: $p = 0.030$). There was a twofold increase in caspase 3/7 activity (Figure 7) in the control group compared with the activity in the plasmin group ($p < 0.0001$).

Discussion

Thrombus formation in donor grafts is recognized as one of the most significant factors in primary graft dysfunction.^{9,10} Several studies have shown the possibility for fibrinolytic treatment of damaged donor grafts.¹³⁻¹⁷ Plasminogen activator was used in these studies as a fibrinolytic agent. However, to recondition and evaluate the donor graft in *ex vivo* lung perfusion, direct fibrinolytic agents should be used because the perfusate in EVLP does not contain plasminogen. This is why we used plasmin as a fibrinolytic agent in this study. Using plasmin in EVLP has three advantages. First, plasmin can lyse fibrin without plasminogen.^{18 19} Second, plasmin is not inactivated in EVLP because alpha-2 antiplasmin is not contained in EVLP perfusate.²⁰ Third, the risk of bleeding after reperfusion is low because plasmin is inactivated immediately by alpha-2 antiplasmin even if residual plasmin exists in

the donor lung after EVLP.²⁰ As such, plasmin is tailored for *ex vivo* reconditioning of donor lungs damaged by thrombus. We have reported the efficacy of plasmin administration to the EVLP perfusate.⁸ However, the physiological data after reperfusion is still unclear. It is known that ischemia induces macrophages and endothelial cells to produce reactive oxygen species, cytokines, nicotinamide adenine dinucleotide phosphate oxidase and cell adhesion molecules.^{11 12} Activation of neutrophils and upregulation of cell adhesion molecules on endothelial cells induce neutrophils to transmigrate into the inflammatory sites.^{21,22} In addition, Platelet activation is induced after reperfusion. These sequential changes increase PVR and pulmonary capillary permeability, resulting in lung edema after reperfusion.^{11 12} The perfusate in EVLP does not contain white blood cells, platelets and red blood cells; therefore, evaluation in EVLP could differ from that after reperfusion. To simulate the clinical settings of lung transplantation, the lung was stored at 4°C for 90 minutes after warm ischemia and reperfused with rat blood for 80 minutes. Unlike our previous study, plasmin was administered during low perfusion flow because we believed that plasmin is effective administered rather in low flow than high flow. Furthermore, to prevent vascular damage, it may be important to add plasmin as early as possible. In this study, during EVLP, the difference in physiological data between the plasmin and non-plasmin groups was not very large; however, almost all data after reperfusion were significantly different between these two groups. The histological findings in the non-plasmin group showed severe edema and alveolar hemorrhage. We could find many apoptotic cells with ssDNA staining and the results of caspase activity assay corresponded with these findings. Interestingly, the peripheral area where we find large amount of residual thrombus after EVLP in our previous study, was severely damaged after reperfusion. At first, we considered that thrombus in the lung reduce the vascular bed and increase vascular pressure, which causes lung edema in normal area. However, the findings in this study strongly suggested that the peripheral lung area whose pulmonary artery was obstructed by thrombus was latently damaged by insufficient perfusion during EVLP in the non-plasmin group. However the area normally perfused was not severely damaged, which showed a small difference in the physiological data between the groups. After reperfusion, thrombus in the donor lung was resolved by plasminogen in the blood and the malperfusion areas were reperfused. Then, after reperfusion, severe damage was strongly induced by the inflow of white blood cells and

platelets in this area, which resulted in a significant difference in the variety of data between the groups .

Of note is that we observed a correlation between the physiological data at the end of EVLP and that at the end of reperfusion in this study. This precious data might give roads to the possibility that we can predict the behavior of the lung after reperfusion by physiological data during EVLP. In fact, PVR at the end of EVLP showed a significant correlation with that at the end of reperfusion, which means that PVR would be a useful parameter to detect the damage by thrombus and to evaluate the effect of fibrinolytic treatment. Although there was also a slight correlation between PaO_2 at the end of EVLP and that at 80 minutes after EVLP, the range of PaO_2 was very narrow. Because acellular solution contains no erythrocytes, the solution is saturated immediately, even in the edematous condition. Therefore, acellular EVLP cannot detect the difference in PaO_2 between the two groups. As Yeung et al also demonstrated that PaO_2 cannot detect lung damage with acellular solution.²³ We also consider that PaO_2 is not a useful parameter to predict lung function after reperfusion. Dynamic compliance is considered to be one of the most sensitive parameters to evaluate the lungs in EVLP.²³ Although dynamic compliance at the end of EVLP tended to correlate with that at 80 minutes after reperfusion, it was not significant in this study. However, the compliance after reperfusion was worse than that expected by the compliance during EVLP. As described above, after reperfusion, malperfused areas were damaged by white blood cells after reperfusion, resulting in severe edema and alveolar hemorrhage in the non-plasmin group.

There are several limitations to our study. First, to produce thrombus in the donor lungs, we used a model including 120 minutes of warm ischemia without heparinization, which represents a longer warm ischemic time than the clinical scenario of uncontrolled donation after cardiac death. A thrombosis model without dispersion is difficult to design, whereas the method used here is a simple way to initiate thrombosis in a donor lung. Another limitation with the model used here is that the lungs were ventilated with negative pressure during the EVLP period, whereas, in the clinical setting, positive ventilation is used.

In conclusion, in this clinically simulated study, our results confirmed that plasmin administration during EVLP could recondition the donor lung not only during EVLP but also after reperfusion.

Conflict of interest statement: The authors declare no conflicts of interest associated with this work.

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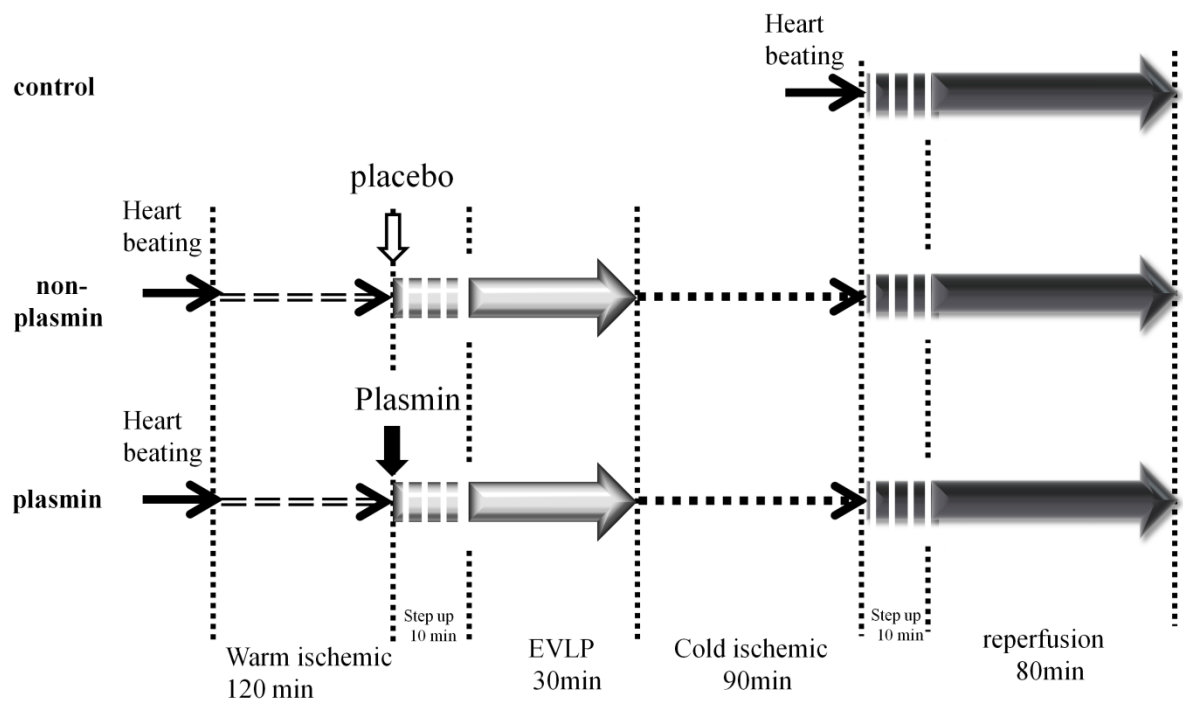


Figure 1

Experimental protocol using an isolated rat lung perfusion model

Fibrin/Fibrinogen degeneration products

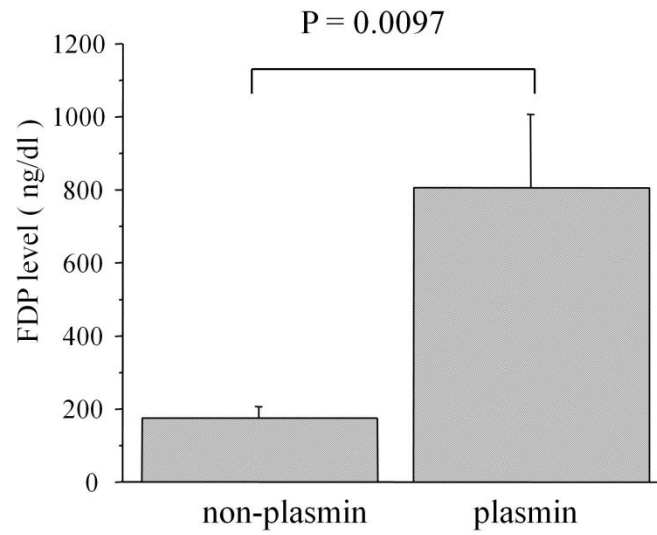


Figure 2.

FDP levels 10 minutes after administration

Non-plasmin group: 175.9 ± 31.3 ng, plasmin group: 805.9 ± 202.9 ng; $p = 0.0097$.

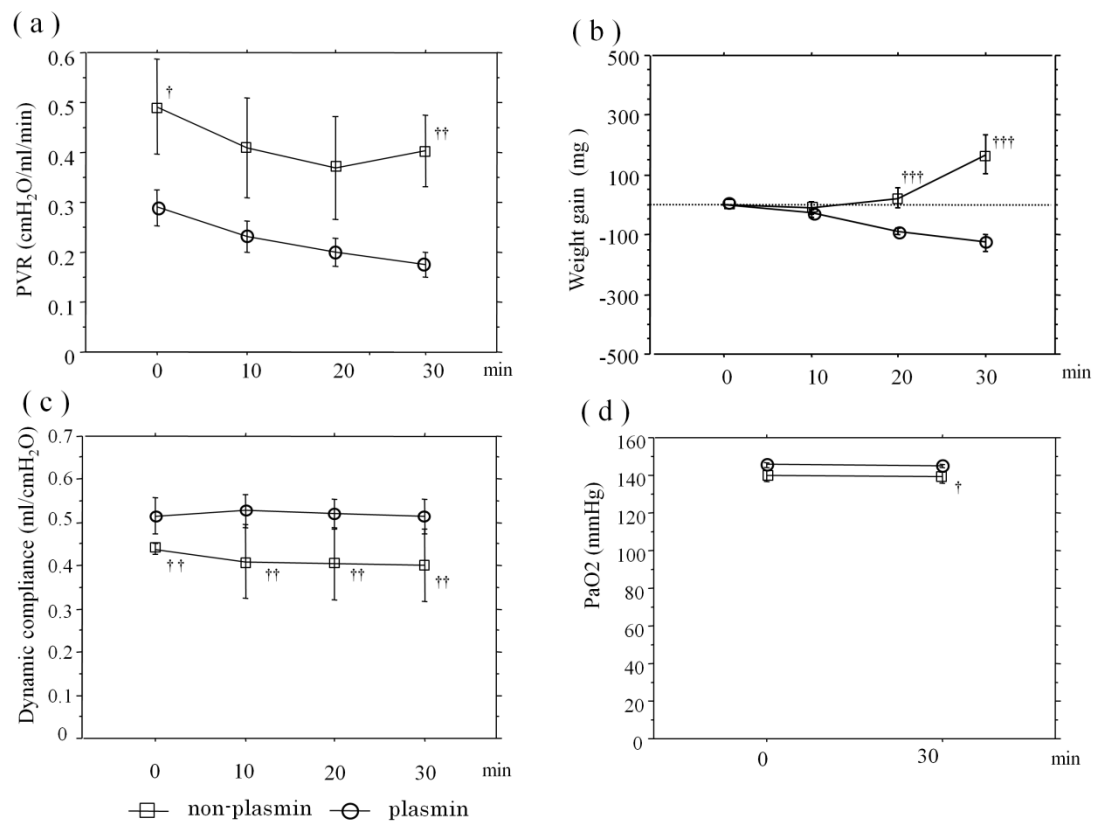


Figure 3. Physiologic lung function during EVLP

(a) Pulmonary vascular resistance, (b) Weight gain, (c) Dynamic compliance, and (d) PaO₂

† $p < 0.05$, †† $p < 0.01$ or ††† $p < 0.001$ between the non-plasmin (squares) and the plasmin groups (circles).

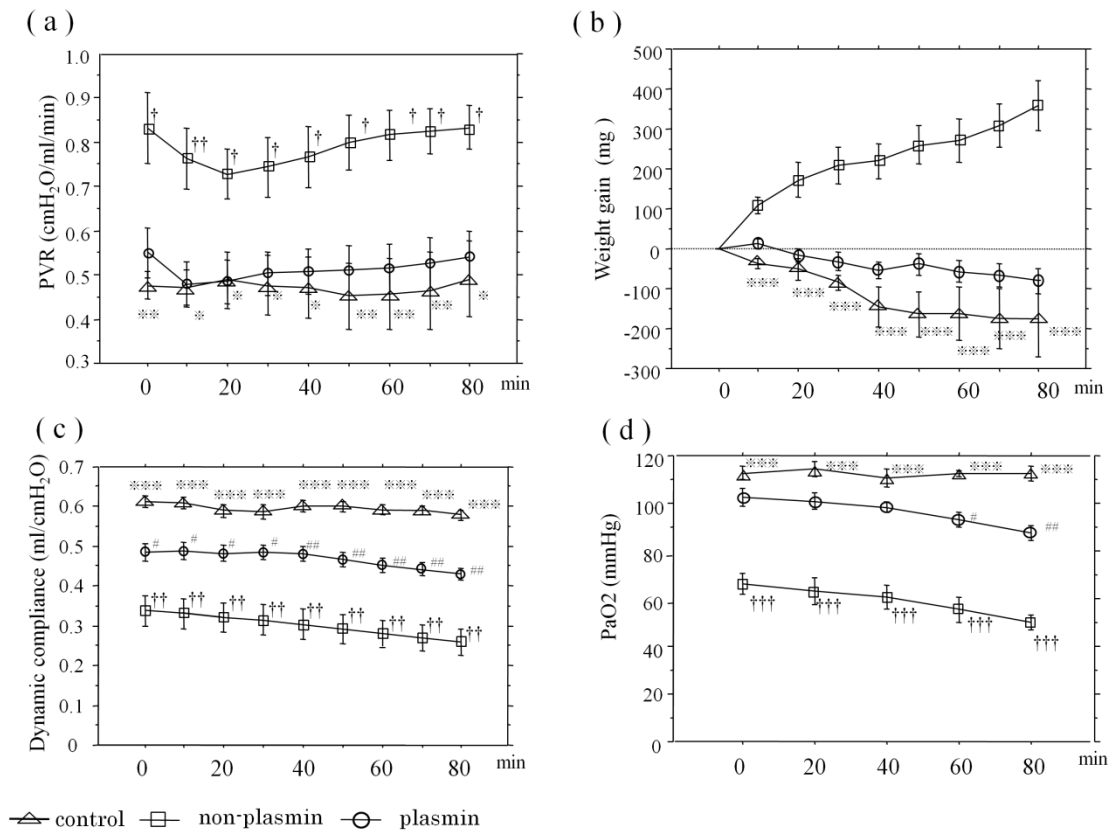


Figure 4. Physiologic lung functions during reperfusion

(a) Pulmonary vascular resistance, (b) Weight gain, (c) Dynamic compliance, and (d) PaO₂

* $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$, between the control (triangles) and non-plasmin (squares) groups. † $p < 0.05$, †† $p < 0.01$ or ††† $p < 0.001$ between the non-plasmin and plasmin (circles) groups. # $p < 0.05$, ## $p < 0.01$ or ### $p < 0.001$, between the control and plasmin groups. Data are expressed as mean values $\pm$ SEM.

Correlation between at the end of EVLP and reperfusion

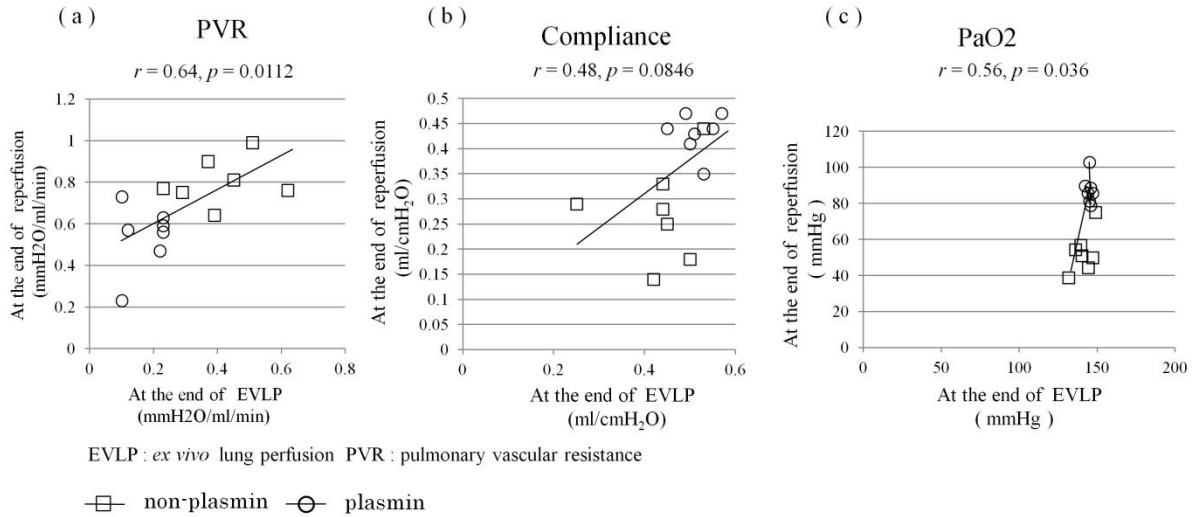


Figure 5. Correlation between physiologic lung functions at the end of EVLP and lung functions 80 minutes after transplantation. (a) Lung oxygenation at the end of EVLP was correlated with lung oxygenation 80 minutes after transplantation ($r = 0.64$, $p = 0.0112$). (b) There was no significant correlation between the dynamic compliance at the end of EVLP and that 80minutes after reperfusion ($r = 0.48$, $p = 0.0846$). (c) Although the range of PaO₂ during EVLP were very narrow (131.5 to 148.9 mm Hg), there was a slight correlation between the PaO₂ at the end of EVLP and that 80 minutes after reperfusion ($r = 0.56$, $p = 0.036$).

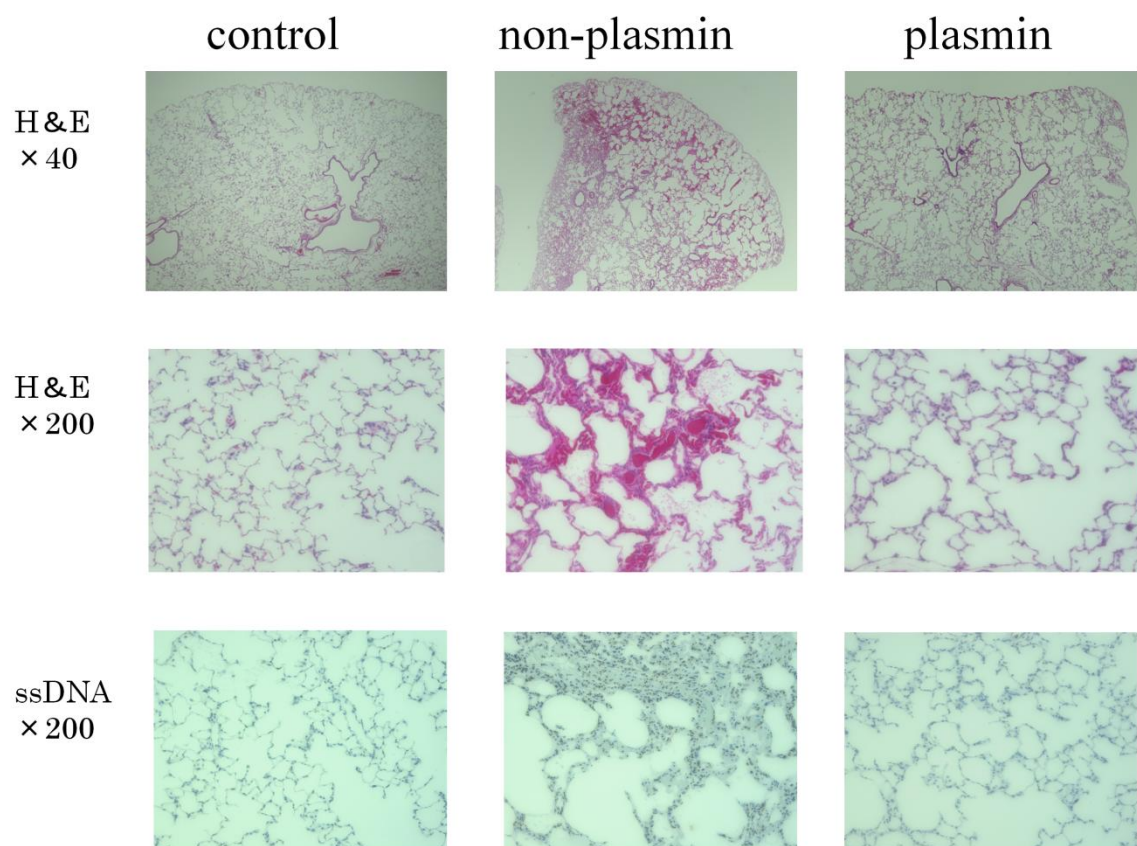


Figure 6. Histologic findings after EVLP (H&E staining and single-strand DNA staining)

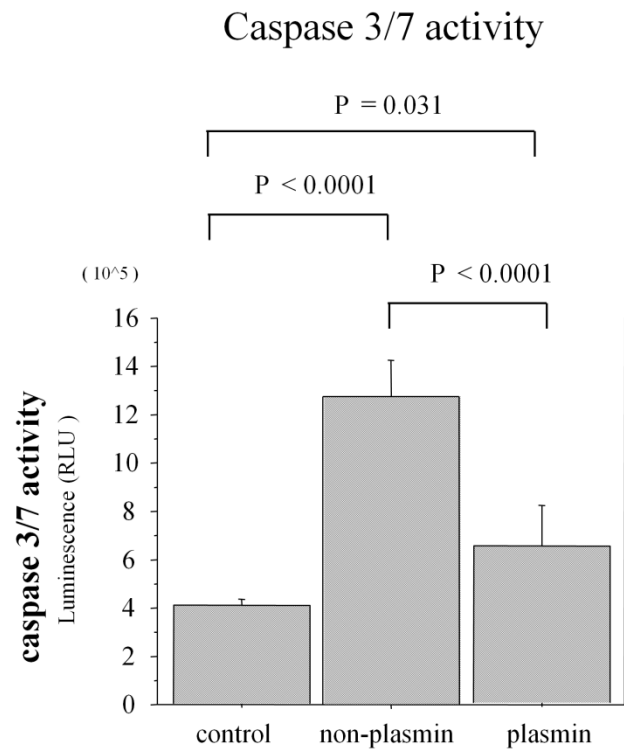


Figure 7. Caspase -3 and -7 activity assay. Caspase -3 and -7 activity of the plasmin group was lower than that of the non-plasmin group (control: 4.1×10^5 , non-plasmin: 12.8×10^5 , plasmin: 6.5×10^5 , control vs non-plasmin: $p < 0.0001$, non-plasmin vs plasmin: $p < 0.0001$, plasmin vs control: $p = 0.031$).

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