Table 2. Difference Between Insulin Discontinuation Group (Group D) and Continuous Insulin User (group C)

	Group D ($n = 3$)	Group C ($n = 3$)	*P* Value
FPG (mg/dL)	94 ± 18	90 ± 4	.742
IRI (mU/mL)	13.6 ± 8.0	44.9 ± 51.8	.406
CPR (ng/mL)	1.8 ± 0.9	0.5 ± 0.5	.109
HOMA-R	3.0 ± 1.7	10.3 ± 12.3	.411
HOMA-β	265 ± 306	537 ± 559	.513
SUIT index	96 ± 26	30 ± 30	.047*
ΔCPR (6 min after 1 mg glucagon injection)	1.83 ± 0.47	0.3 ± 0.3	.013*
Dose of insulin (U/d)	5 ± 3	28 ± 9	<.037*
Follow-up period after glucagon stimulation test (d)	464 ± 245	538 ± 252	.736

Abbreviations: FPG, fasting plasma glucose; IRI, immunoreactive insulin; CPR, C-peptide; HOMA, homeostasis model assessment; SUIT, secretory unit of islet in transplantation; ΔCPR, difference between 6- and 0-minute CPR measurement after 1 mg glucagon injection.
*$P < .05$, Student *t* test.

HOMA indices use the value of IRI, which can detect both endogenous and exogenous insulin [7]. Therefore, monitoring HOMA indices was not helpful to assess β-cell function and insulin resistance when some of the patients use exogenous insulin injection. On the other hand, we found that fasting serum CPR, SUIT index, and ΔCPR after glucagon injection were significantly different between insulin users and nonusers. These results demonstrate that the parameters using CPR, which reflects intrinsic insulin secretion [2], seem to be useful to assess insulin secretion from the pancreatic grafts.

Insulin response to a variety of secretagogues in post-transplantation subjects could be higher than before the transplantation, because immunosuppression with glucocorticoid and other drugs could increase insulin resistance and compensatory insulin hypersecretion [8]. In the present study, fasting CPR and HOMA-R in the insulin-free group was 2.0 (interquartile range 1.6–2.6) and 1.8 (interquartile range 1.4–2.5), respectively. To avoid glucotoxicity [9] on the pancreatic graft, insulin secretion must be enough to compensate for increased demand after the transplantation. Indeed, high insulin secretion predicted significantly longer pancreas graft function as compared with low insulin secretion, though there was no difference in patient survival and kidney graft function [10]. Serum CPR level is known to be affected by renal function [2], though there was no direct correlation between CPR and estimated glomerular filtration rate in this study (data not shown). To avoid the modification of serum CPR concentration due to the renal dysfunction, we performed the glucagon stimulation test and compared ΔCPR during 6 minutes and other indices to evaluate insulin secretion. We found that continuous insulin users showed significantly lower SUIT index and ΔCPR and needed larger doses of insulin than the insulin-discontinuation group during the follow-up period, while FPG, fasting CPR, fasting IRI, and HOMA-β showed no significant difference between 2 groups. These findings suggest that acute response to glucagon has predictive value to assess the necessity of future insulin treatment.

There are several limitations in this study. First, follow-up periods and the number of subjects were too small to fully evaluate statistical significance of the indices. Second, this study was performed in a single institution. Third, insulin resistance should be ideally evaluated by glucose clamp technique.

In conclusion, fasting CPR, SUIT index, and ΔCPR after glucagon injection could reflect β-cell function for the recipients of pancreas transplantation, and glucagon stimulation test could give us additional information to predict insulin-free treatment.

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ORIGINAL ARTICLE

Clinical application of ET-Kyoto solution for lung transplantation

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Abstract Because of the severe donor shortage in Japan, even after the revision of the Organ Transplant Law in 2010, the frequency of recovery of extended criteria lungs has increased in Japan. We developed a new lung preservation solution, "ET-Kyoto solution," to enhance lung preservation, to minimize primary graft dysfunction (PGD) and to improve the post-transplant outcomes. In this study, we retrospectively analyzed our results of lung transplantation using the ET-Kyoto solution. From 2002 to 2012, 26 patients underwent transplantation of lungs preserved with ET-Kyoto solution from brain-dead donors. We retrospectively reviewed the post-transplant pulmonary function and long-term survival. The graft performance was assessed by the PGD grading system. The mean graft ischemic time was 483.8 ± 19.0 min. The oxygenation capacity after reperfusion and recovery of respiratory function were both acceptable despite the long ischemic time. The survival rate at 5 years after transplantation was 85.1 %. Lungs preserved by ET-Kyoto solution had satisfactory postoperative lung function, despite the long preservation time, with excellent long-term survival. The results were acceptable for the use of grafts with a long ischemic time.

Keywords Deceased donor · ET-Kyoto solution · Japan · Long ischemic time · Lung transplantation · Primary graft dysfunction

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Introduction

Lung transplantation is an accepted therapeutic option for patients with various respiratory diseases at the terminal stage. However, the donor organ shortage continues to be a critical problem worldwide. Particularly in Japan, the problem has been much more serious, as the annual number of deceased donors was only 0.9 per million population in 2010, whereas in most European countries and the USA, the numbers were 10–30 per million, and were five to 10 per million in other Asian countries [1]. To relieve this problem, the Japanese Organ Transplant Law was amended in 2010. After the revision, the number of brain-dead donors dramatically increased, but remained insufficient for the needs of the waiting candidates [2]. Therefore, Japanese lung transplant programs have aggressively accepted extended criteria donor lungs, and the recovery rate from brain-dead donors rose to as high as 63 % [2]. Moreover, lung grafts have been subjected to long ischemic times, with the average being 472 min [3]. In such an unfavorable situation, improvements in lung preservation were crucial to minimize the primary graft dysfunction (PGD) resulting from ischemia–reperfusion injury. We developed an original lung preservation solution, "ET-Kyoto solution", which contains trehalose, that has a protective effect on preserved lungs [4], and have used it for lung preservation in our lung transplant program [5]. In this

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study, we retrospectively examined the results of lung transplantation using the ET-Kyoto solution for lung preservation.

Patients and methods

During the first 11 years after the lung transplantation program started in Kyoto University Hospital, from April 2002 to December 2012, 63 patients underwent lung transplantation, and the ET-Kyoto solution was used for preservation of the lungs transplanted into 61 patients. Of the 61 patients, 26 received lung transplantations from brain-dead donors and 35 from living lung donors. Because this study was focused on the preservative effect of ET-Kyoto solution on pulmonary grafts after long ischemia, we excluded living-donor lobar lung transplantation from the analysis because of the short ischemic times, which ranged from 77 to 252 min. We evaluated the post-transplant pulmonary function and long-term survival in the 26 recipients of brain-dead donor lung transplants. We reviewed and collected data from the medical records of the patients retrospectively. All patients had previously provided consent for use of their clinical data for research. This study observed the procedures outlined in the 2000 Declaration of Helsinki and the 2008 Declaration of Istanbul, and was approved by the Institutional Review Board of Kyoto University.

The severity of PGD at 12, 24, 48, and 72 h after transplantation was graded according to the grading system defined by the International Society of Heart and Lung Transplantation [6]. The oxygenation capacity was evaluated by the arterial oxygen tension/inspired oxygen fraction (P/F ratio). We also analyzed the preoperative, surgery-related and postoperative factors. The preoperative factors included the donor and recipient age, indications for transplantation, time on the waiting list prior to the transplant and the donor P/F ratio before the recovery of the donor lung. The surgery-related factors were the procedure types, length of the operation, duration of extracorporeal circulation (ECC), such as cardiopulmonary bypass (CPB) and extracorporeal membrane oxygenation (ECMO), and the graft ischemic time. The postoperative factors included the P/F ratio immediately after reperfusion or after weaning from ECC when used, and the length of stay in the intensive care unit (ICU). All recipients received postoperative immunosuppression with standard triple drug therapy consisting of cyclosporine or tacrolimus, azathioprine or mycophenolate mofetil, and corticosteroids. Acute rejection was treated with a bolus injection of methylprednisolone.

The graft ischemic time and P/F ratio were expressed as the mean ± standard error of the mean (SE). Survival curves were calculated by the Kaplan–Meier method from the day of the operation until death or the day of the most recent follow-up (censored). Fisher's exact test was used for comparisons of categorical variables, and an unpaired two-tailed t test was used for continuous data. The statistical analysis was performed using the StatView 4.5 software program (Abacus Concepts, Berkeley, CA, USA).

Results

The characteristics of the transplant recipients are shown in Table 1. Their mean age was 38.8 years (range 17–56 years), which was younger than that cited in the international registry report [7]. The main indications for deceased donor lung transplantation were emphysema, lymphangioleiomyomatosis, bronchiolitis obliterans, and bronchiectasis. The number of patients with pulmonary fibrosis was small because most of the fibrosis patients had died while on the waiting list. The mean waiting time to the transplant was 852 days (range 295–2574 days). The mean age of the deceased donors ($n = 26$) was 44.5 years (range 21–68 years), and 35 % ($n = 9$) of the deceased donors were older than 50 years. The deceased donors in Japan were older than in other countries [7].

Table 2 shows the perioperative factors. The average operation lasted about 8 h, and half of the recipients required ECC during transplantation, with a mean time of 290 min. Although the mean P/F ratio of the donors was 458.4, 22 out of 25 donors (88 %) were extended criteria donors who did not meet the standard criteria for lung donation. The mean graft ischemic time (38 grafts in 26 patients) was 483.8 ± 19.0 min, and 18 % ($n = 7$) of the grafts were subjected to a long ischemic period of more

Table 1 Characteristics of the lung transplant recipients

Age (years)	38.8 (17–56)		
Sex			
Male	16		
Female	10		
Diagnosis	Single lung ($n = 12$)	Bilateral ($n = 14$)	Total ($n = 26$)
Emphysema	5	1	6
Lymphangioleiomyomatosis	3	2	5
Bronchiolitis obliterans	2	2	4
Bronchiectasis	0	4	4
Pulmonary fibrosis	3	0	3
Pulmonary hypertension	0	2	2
Other	1	1	2
Waiting time to transplant (days)	852 (295–2574)		

Table 2 The perioperative factors

	n	Mean (range)
Length of operation (min)	26	489.9 (283–1116)
ECC time (min)	13	290.3 (113–677)
Graft ischemic time (min)	38[a]	483.8 (294–823)
Donor P/F ratio[b]	25	458.4 (292–603.4)
P/F ratio after reperfusion	24[c]	347.3 (157–525)
ICU stay (days)	24[d]	9.7 (3–23)

ECC extra corporeal circulation

[a] Number of grafts

[b] P/F ratio: arterial oxygen tension/inspired oxygen fraction

[c] Excluding two patients who required ECC support after reperfusion

[d] Excluding two patients who died in the ICU

than 10 h (Fig. 1). Despite the long ischemic period, the mean P/F ratio after reperfusion in the 24 recipients who were weaned from ECC after implantation was 347.3, and 65 % ($n = 17$) of the recipients had a satisfactory oxygenation capacity with a ratio greater than 300 (Fig. 2). In addition, the P/F ratios of six recipients whose graft ischemic time was longer than 10 h were also relatively good, and there were no significant differences in the P/F ratios between recipients with an ischemic time less than 10 h (360.3 ± 24.3, $n = 18$) and those with an ischemic time longer than 10 h (308.0 ± 30.0, $n = 6$; $p = 0.37$).

Two of the 26 patients died in the ICU: one of them died of uncontrollable bleeding on the first postoperative day, and the other died from serious brain damage caused by intraoperative cerebral ischemia on the 217th postoperative day. The average length of ICU stay of the remaining 24 patients was 9.7 days. The PGD grades at twelve, 24, 48 and 72 h after transplantation are shown in Fig. 3. At 12 h after the operation, 65 % ($n = 17$) of the patients had PGD grade 0, while 19 % ($n = 5$) had grade 3. Thereafter, the graft function improved gradually, and PGD grade 0 was

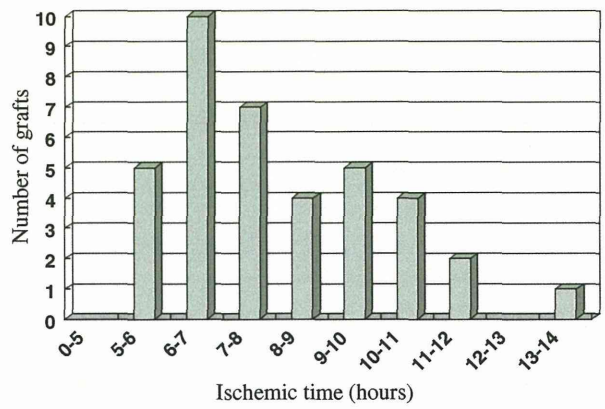

Fig. 1 The number of grafts divided according to the ischemic time

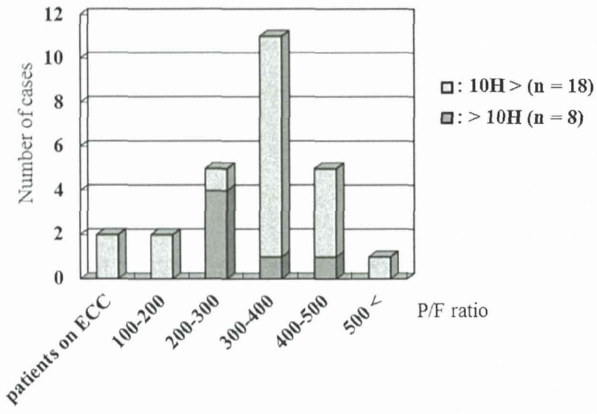

Fig. 2 The number of recipients divided according to the arterial oxygen tension/inspired oxygen fraction (P/F ratio) after reperfusion. Two patients were unable to be weaned from extracorporeal circulation (ECC). The P/F ratios of six recipients who had a graft ischemic time longer than 10 h are shown by *open bars*

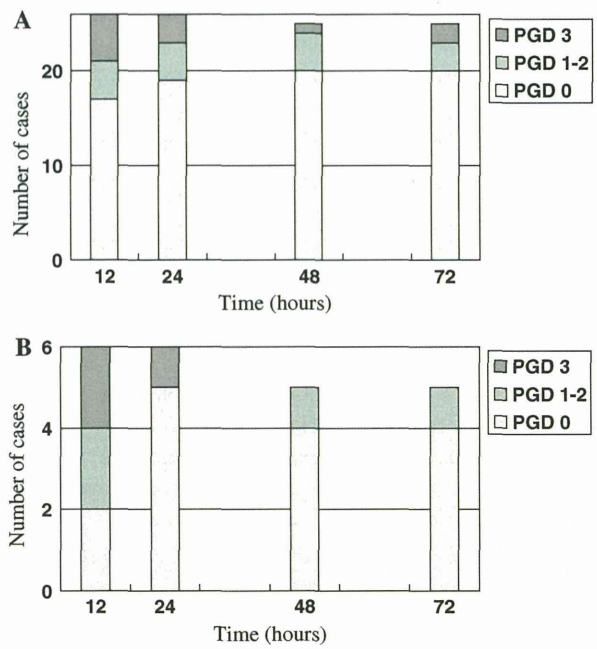

Fig. 3 The primary graft dysfunction (PGD) grade distribution among recipients at specific postoperative time points (**a**). The PGD grade distribution among the six recipients who had a graft ischemic time longer than 10 h (**b**)

seen in 80 % of the patients ($n = 20$) at 48 and 72 h, respectively, after reperfusion. In the six patients who received lungs with an ischemic time longer than 10 h, only two (33 %) of the recipients had PGD grade 0 at 12 h after reperfusion. However, 80 % or more of the patients showed PGD grade 0 after 24 h of reperfusion (Fig. 3b). There were no significant differences in the proportion of patients with grade 3 to patients with grade 0-2 at 12 h after

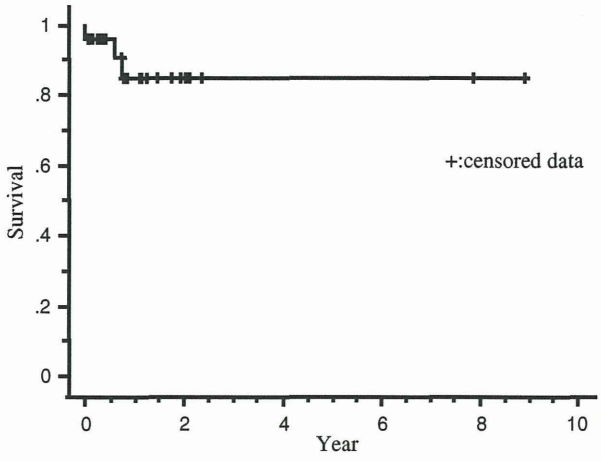

Fig. 4 The Kaplan–Meier survival curve of deceased donor lung transplantation at Kyoto University

reperfusion between the six patients with a long ischemia and the remaining 20 patients ($p = 0.56$). Three of the 26 patients developed chronic lung allograft dysfunction at 5.6, 8.4 and 16.9 months after transplantation, respectively, and were treated by augmentation of the immunosuppression, including bolus injections of methylprednisolone. The overall survival rates for 1, 3 and 5 years after lung transplantation at our institution were all 85.1 % (Fig. 4). These rates were higher than those in the international registry, which were 79, 64 and 53 %, respectively, at 1, 3 and 5 years [7].

Discussion

Although the availability of donor lungs from brain-dead donors increased after the revision of the Japanese Organ Transplant Law in 2010, a donor shortage remains a major limitation of lung transplantation. To extend the donor pool, we developed a new lung preservation solution, ET-Kyoto solution [4], after optimizing the properties of the sugar and the electrolyte contents, as well as providing additives to protect the pulmonary endothelium [8]. In 2009, the ET-Kyoto solution became commercially available (ET-K; Otsuka Pharmaceutical Factory Inc., Naruto, Japan), and has been applied for clinical lung transplantation by other Japanese transplant programs, in addition to that at Kyoto University, with excellent results. Another original lung preservation solution developed at Tohoku University, called the EP-TU solution, also has been widely applied in clinical practice in Japan, and was also reported to be associated with satisfactory post-transplant lung graft function despite a long average graft ischemic time of 483 min [9].

The severe donor shortage drove the Japanese transplant centers to accept extended criteria lungs [2]. In our series, 88 % of donors did not actually meet standard donor criteria. Moreover, the graft ischemic time is longer in Japan than in other countries, partly due to the long transportation time. According to the Transplant Registry Quarterly Data Report of the International Society of Heart and Lung Transplantation [10], the ischemic time in North America was less than 8 h in about 90 % of transplants. The average ischemic time in our series of deceased donor lung transplantation was over 8 h. Additionally, our donors were relatively older than those in North America; 35 % of the donors were over 50 years old in the current study, whereas more than 80 % of donors were reported to be less than 50 years old in North America [10]. Despite the long graft ischemic time, the present retrospective study showed that the ET-Kyoto solution resulted in excellent postoperative graft performance, as indicated by satisfactory oxygenation, PDG grades and long-term survival.

In conclusion, lung transplantation using the ET-Kyoto solution resulted in good postoperative graft function with excellent recipient survival, despite the unfavorable situations presented by the severe donor shortage and the long ischemic time in Japan. Further investigations are needed to evaluate the efficacy of ET-Kyoto solution in clinical practice, because the number of cases in the current study was limited.

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Conflict of interest There is no potential conflict of interest with any companies/organizations whose products or services are discussed in this article.

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Plasmin administration during ex vivo lung perfusion ameliorates lung ischemia–reperfusion injury

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KEYWORDS:
lung transplantation;
marginal donor;
ex vivo lung perfusion;
fibrinolytic treatment;
plasmin

BACKGROUND: Donor lung thrombus is considered a significant etiology for primary graft dysfunction (PGD). We hypothesized that thrombolysis in ex vivo lung perfusion (EVLP) before lung transplantation could alleviate ischemia–reperfusion injury (IRI), resulting in a decreased incidence of PGD.

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. Hearts and both 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. We assessed physiologic and histologic findings during reperfusion and the correlation between physiologic data during EVLP and after reperfusion.

RESULTS: Physiologic results were better in the plasmin group than in the non-plasmin group. The plasmin group lungs 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 physiologic values in EVLP may be predictive markers of lung function after reperfusion.

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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 marginal lungs is also being investigated.[4–8] In previous work we focused on 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 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 conditions, the perfusate used in acellular EVLP does not

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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 damage and lung edema.[11,12] Thus, under in vivo conditions, donor lungs may 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 whether the physiologic data at the end of EVLP could predict the function of 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.[13] 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 weights of the rats were similar between the 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 minutes 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 H_2O), with drainage through an incision of the left ventricle. After a 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 both lungs were excised en bloc.

EVLP model

To evaluate lung function, we used the isolated rat lung perfusion set-up (Model 829; Hugo-Sachs Electronik Harvard Apparatus, Holliston, MA). The isolated heart–lung bloc was secured in an artificial thorax and ventilated with ambient air at negative pressure under conditions described elsewhere.[8] 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 ($1 \rightarrow 3 \rightarrow 5 \rightarrow 7 \rightarrow 10$ 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 of 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. PaO_2 of the perfusate was analyzed just after the step-up period and at the end of the 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 H_2O/ml per minute, defined as pulmonary arterial pressure – pulmonary vein pressure/perfusate flow), weight gain of the lung (mg) and dynamic airway compliance (ml/cm H_2O) were monitored continuously and recorded at 10-minute intervals during EVLP. Physiologic data from each group were recorded continuously and analyzed after the experiment.

In the non-plasmin and plasmin groups, the lungs were detached from the EVLP system, inflated with air, covered with gauze dampened by cold Steen solution, 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 2 donor rats and saline with 4% albumin. The hematocrit was adjusted to approximately 15%, and the pH was adjusted to 7.25 to 7.35 with sodium bicarbonate. The lungs were reperfused at low flow (1 ml/min), then the flow rate was increased gradually every 2 minutes ($1 \rightarrow 3 \rightarrow 5 \rightarrow 7 \rightarrow 10$ ml/min) and ventilated under the same conditions 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-Glo™ Reagent (Promega) was cleaved by caspase-3 and -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.

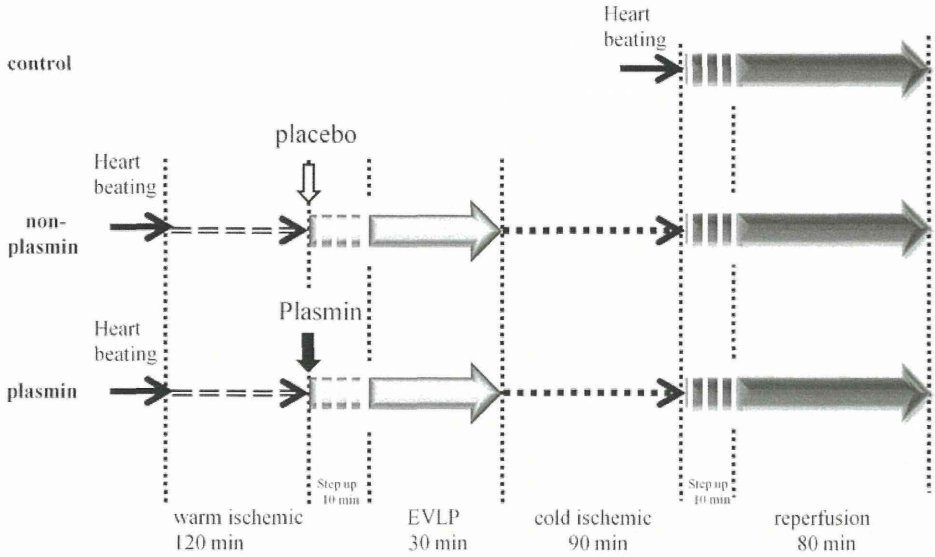

Figure 1 Experimental protocol using an isolated rat lung perfusion model.

Statistical analysis

All statistical analyses were performed using STATVIEW version 5.0 software (Abacus Concepts, Berkeley, CA). All values are presented as mean ± standard error of the mean (SEM). Data were evaluated using repeated-measures analysis of variance, Scheffé's post hoc multiple comparison test and Student's paired *t*-test. $p < 0.05$ was considered 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 (805.9 ± 202.9 ng) than in the non-plasmin group (175.9 ± 31.3 ng) ($p = 0.0097$).

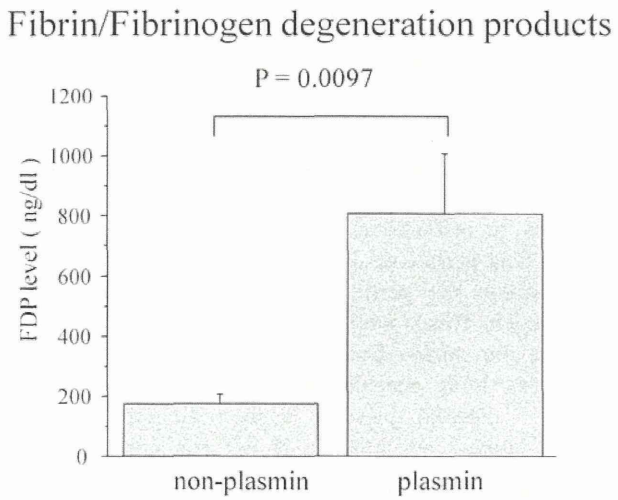

Figure 2 FDP levels 10 minutes after administration. Non-plasmin group: 175.9 ± 31.3 ng; plasmin group: 805.9 ± 202.9 ng ($p = 0.0097$).

Pulmonary function during EVLP and reperfusion

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 minutes; 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 group ($p = 0.001$ 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 increased significantly in the non-plasmin group compared with 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 minutes; Figure 4c).

PaO_2. The PaO_2 in the plasmin and non-plasmin groups was not high but was significantly lower in the latter during EVLP ($p = 0.021$ at 30 minutes; Figure 4d). In contrast, PaO_2 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).

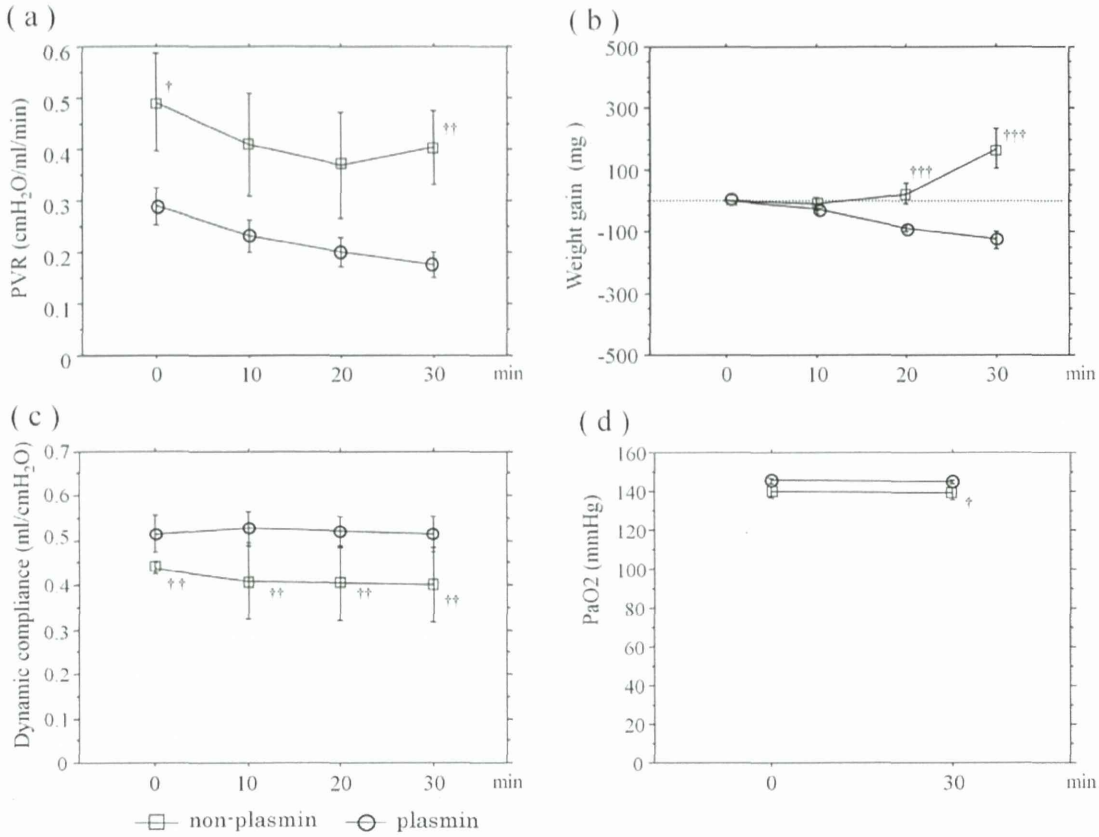

Figure 3 Physiologic lung function during EVLP. (a) Pulmonary vascular resistance, (b) weight gain, (c) dynamic compliance and (d) PaO_2. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ or $^{\dagger\dagger\dagger}p < 0.001$, between the non-plasmin (squares) and plasmin (circles) groups.

Correlation of physiologic data for EVLP and reperfusion in plasmin and non-plasmin groups

PVR at the end of EVLP correlated significantly with that at 80 minutes after reperfusion ($r = 0.66$, $p = 0.011$; Figure 5a). Lung oxygenation at the end of EVLP correlated slightly with that at 80 minutes after reperfusion ($r = 0.56$, $p = 0.036$; Figure 5c). However, there was no significant correlation between 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 reflects early stages of apoptosis. Caspase activity of the lungs in each group was higher in the non-plasmin and plasmin groups 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 2-fold increase in caspase-3 and -7 activity (Figure 7) in the control group compared with that 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.[14–18] 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 our study. Using plasmin in EVLP has three advantages. First, plasmin can lyse fibrin without plasminogen.[19,20] Second, plasmin is not inactivated in EVLP because alpha-2 anti-plasmin is not contained in EVLP perfusate.[21] Third, the risk of bleeding after reperfusion is low because plasmin is inactivated immediately by alpha-2 anti-plasmin, even if residual plasmin exists in the donor lung after EVLP.[21] As such, plasmin is tailored for ex vivo reconditioning of donor lungs damaged by thrombus.