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The effects of hydrogen gas inhalation during *ex vivo* lung perfusion on donor lungs obtained after cardiac death[†]

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Abstract

OBJECTIVES: Lung transplantation is a well-established treatment of end-stage lung disease; however, it is limited by a shortage of donor lungs. To overcome this problem, donation after cardiac death (DCD) and *ex vivo* lung perfusion (EVLP) are being widely investigated. In this study, the effect of hydrogen gas, a known antioxidant, was investigated on a DCD lung model during EVLP.

METHODS: Ten pigs were randomized into either a control ($n = 5$) or a hydrogen group ($n = 5$). After fibrillation by electric shock, no further treatment was administered in order to induce warm ischaemic injury for 1 h. The lungs were then procured, followed by 4 h of EVLP. During EVLP, the lungs were ventilated with room air in the control group, and with 2% hydrogen gas in the hydrogen group. Oxygen capacity (OC), pulmonary vascular resistance (PVR) and peak airway pressure (PAP) were measured every hour, and the expressions of interleukin-1 beta (IL-1β), IL-6 (IL-6), IL-8 (IL-8) and tumour necrosis factor-alpha (TNF-α) were evaluated in lung tissue after EVLP. Pathological evaluations were performed using lung injury severity (LIS) scores and the wet/dry ratio was also measured.

RESULTS: The OC in the hydrogen group was higher than in the control group, but the difference was not statistically significant ($P = 0.0862$). PVR ($P = 0.0111$) and PAP ($P = 0.0189$) were statistically significantly lower in the hydrogen group. Compared with the control group, the hydrogen group had a statistically significantly lower expression of IL-1β ($P = 0.0317$), IL-6 ($P = 0.0159$), IL-8 ($P = 0.0195$) and TNF-α ($P = 0.0159$). The LIS scores ($P = 0.0358$) and wet/dry ratios ($P = 0.040$) were also significantly lower in the hydrogen group.

CONCLUSIONS: Hydrogen gas inhalation during EVLP improved the function of DCD lungs, which may increase the utilization of DCD lungs.

Keywords: Hydrogen • Warm ischaemic injury • Donation after cardiac death • *Ex vivo* lung perfusion

INTRODUCTION

Lung transplantation (LTx) is the most effective treatment option for patients with end-stage lung disease. However, a shortage of donor lungs has resulted in the death of many patients on waiting lists for LTx. Attempts to address the donor shortage issue include the utilization of marginal donor lungs and living lobar transplantations. Steen *et al*. [1] performed the first successful transplantation of a donation after cardiac death (DCD) lung, procured from a patient who did not respond to cardiac resuscitation. Since then, several institutions have reported successful cases of LTx using DCD lungs [2, 3]. Therefore, DCD lungs may be a viable solution for increasing the number of available donor lungs; however, there is still controversy relating to their function, morbidity and mortality, compared with the lungs from heart-beating donors [4]. Furthermore, variations in warm ischaemic times lead to difficulties in accurately predicting the peritransplant function of DCD lungs [5].

Ex vivo lung perfusion (EVLP) is a system that allows the evaluation and recovery of an *ex vivo* donor lung by perfusion using normothermic perfusate. Steen *et al*. [1] reported a case in which EVLP was applied to evaluate the pretransplant condition of a DCD lung and resulted in successful transplantation. However, while EVLP is a useful method for evaluating the pretransplant function of a potentially incompatible lung, EVLP alone cannot

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alter the pathology of the donor lung [6]. Therefore, the application of various modalities to the EVLP system has been investigated in order to improve graft function.

In recent studies, hydrogen has been shown to have antioxidant and anti-inflammatory effects; in particular, it has a demonstrated ability for protecting cells, tissues and organs from oxidative injury [7]. Hydrogen, a potent free-radical scavenger, selectively reduces hydroxyl radicals and has been shown to have therapeutic antioxidant effects in the brains, hearts, livers and intestines, which have been affected by ischaemic reperfusion injury [7].

The aim of this study was to investigate whether EVLP, during simultaneous ventilation with hydrogen gas, can improve the function of donor lungs procured from a pig DCD model.

MATERIALS AND METHODS

Animals

All surgical procedures and animal care were carried out in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Guidelines and Policies for Swine Survival Surgery, provided by the Institutional Animal Care and Use Committee of the Yonsei University Health System.

Ten 40-kg Yorkshire female pigs (XP Bio, Anseong, Korea) were randomly assigned to either the control group (Group I, $n = 5$) or the hydrogen group (Group II, $n = 5$). In the control group, lung grafts were ventilated using only room air during EVLP, while in the hydrogen group they were ventilated with a mixture of 2% hydrogen and room air.

The pigs (XP Bio, Anseong, Korea) were sedated intramuscularly with 5 mg/kg of tiletamine/zolazepam (Zoletil;Virbac, Carros, France) and 2 mg/kg of xylazine (Rompun; Bayer, Seoul, Korea). Endotracheal intubation was performed using an 8-mm diameter tube, and a Foley catheter was inserted. The pigs were then anaesthetized with isoflurane (Forane; JW Pharmaceutical, Seoul, Korea). The ventilator was set in a volume control mode with a tidal volume of 10 ml/kg, a positive end-expiratory pressure (PEEP) of 5 cm, an H_2O respiratory rate of 16–18/min and a fraction of inspired oxygen (FiO_2) of 1.0. After fibrillation was induced by a 9-V electrical shock through a vertical subxiphoid incision, the pigs were left to progress to cardiac arrest. Cardiac arrest was defined as the state when the difference between systolic and diastolic pressure was zero, and when there was an absence of electrical activity in the electrocardiogram. After declaration of cardiac death, the pigs were left untouched at room temperature for 1 h; however, mechanical ventilation was maintained with the aforementioned settings.

One hour after declaration of cardiac death, the sternum and pericardium were opened and 15,000 U of heparin (JW Pharmaceutical, Seoul, Korea) was injected into the main pulmonary artery (MPA). Cardiac massage was performed to circulate the heparin into the lung. A Prolene 4-0 (Ethicon, Peterborough, Canada) purse string suture was placed in the MPA followed by insertion of a 20 Fr. Foley catheter. After ligating the superior and inferior vena cava, the aorta was cross-clamped, and the left atrial appendage was incised. Lung preservation solution (Perfadex; Vitrolife, Göteborg, Sweden), 60 ml/kg at 4°C, was flushed into the MPA from a height of 30 cm. After the flush, the heart was excised and a retrograde perfusion of 500 ml of Perfadex into the left atrium (LA) was performed. While maintaining airway pressure at 15 cmH_2O and a FiO_2 of 0.5, the trachea was clamped and the lungs were excised to keep the lungs inflated. An LA cuff was designed to match the size of the funnel-shaped LA cannula (Vitrolife, Göteborg, Sweden) and was secured with a Prolene 4-0 suture. A pulmonary artery (PA) cannula (Vitrolife, Göteborg, Sweden) was inserted into the MPA and tied with heavy silk (Ethicon, Peterborough, Canada). A tracheal tube was placed in the airway to prevent collapse of the lungs.

Preparation of the EVLP system

The EVLP system was prepared and managed in accordance with the Toronto protocol [8]. The EVLP system consisted of a mechanical ventilator (Hamilton-C2, Hamilton Medical AG, Bonaduz, Switzerland) and a centrifugal pump (Rotaflow, Maquet Cardiopulmonary AG, Hirrlingen, Germany) to circulate perfusate through the system. Mixed gas (6% O_2, 8% CO_2 and 86% N) was administered while the perfusate passed through the membrane oxygenator (Quadrox PLS oxygenator, Maquet Cardiopulmonary AG); this allowed deoxygenation before the perfusate was re-circulated into the PA. A leukofilter was placed immediately before entry to the PA and a heat exchanger (HU 35, Maquet Cardiopulmonary AG) was connected to the membrane oxygenator.

The perfusate comprised 1500 ml of Steen solution (Vitrolife, Göteborg, Sweden) mixed with 10,000 U of heparin, 500 mg of cefazolin (Yuhan Corporation, Seoul, Korea) and 500 mg of methylprednisolone (Dong-A pharmaceutical, Seoul, Korea).

Management of the *ex vivo* lung perfusion system

Lungs were placed in a specially designed chamber (XVIVO chamber, Vitrolife, Göteborg, Sweden) and both the PA and the LA cannulas were connected to this system, while ensuring that no air entered into the circulation. Circulation was initiated slowly, at a rate of 150 ml/min at 20°C, and then the perfusate temperature was gradually increased to 37°C over a period of 30 min. After 20 min of circulation, ventilation was initiated and the perfusion flow rate was gradually increased. The ventilator settings comprised a tidal volume of 7 ml/kg, a respiration rate 7/min and a PEEP of 5 cmH_2O. During EVLP, the lungs in Group I (control) were ventilated with room air (FiO_2 of 0.21), while those in the Group II (hydrogen) were ventilated with 2% hydrogen mixed with room air (FiO_2 of 0.21). Simultaneously, 0.5 l/min of mixed gas was insufflated into the membrane oxygenator and the gas flow was adjusted to maintain a PCO_2 between 35 and 45 mmHg. As the perfusate temperature reached 37°C and 40% of the expected cardiac output was attained, the perfusion flow rate was increased to 1500 ml/min.

During the entire period of EVLP, LA and PA pressure was maintained at between 3 and 5 mmHg, and 10 and 15 mmHg, respectively, by adjusting the reservoir level. To keep the contents of the perfusate as constant as possible, 100 ml of Steen solution was exchanged every hour.

Evaluation of lung function

Lung functional parameters were measured every hour during EVLP. Ten minutes prior to each measurement, an airway recruitment manoeuvre was performed twice to an airway pressure of 25 mmHg, at FiO_2 1.0, in order to avoid atelectasis.

The measured functional parameters were oxygen capacity [OC, (LA perfusate PO_2–PA perfusate PO_2)/FiO_2 (mmHg)] calculated using arterial blood gas analysis, pulmonary vascular resistance [PVR (PA pressure–LA pressure) × 80/PA flow (dynes's/cm^5)] and peak airway pressure (PAP) (cmH_2O).

At the termination of EVLP 4 h later, lung specimens were excised for biological markers, pathology and wet/dry ratios from the right lower lobe. Myeloperoxidase (MPO) activities in the lung tissue were quantified using a colorimetric MPO assay kit (BioVision, CA, USA). The following cytokines: interleukin-1 beta (IL-1β), interleukin 6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor-alpha (TNF-α), were measured using a commercially available enzyme-linked immunosorbent assay (Merck Millipore Corp., MO, USA). The degree of phosphorylation of p38, c-Jun NH_2-terminal kinase (JNK) and extracellular-regulated protein kinase (ERK1/2) were measured by western blot analysis. Antibodies against total and phosphorylated p38, JNK and ERK1/2 were manufactured by Cell Signaling Technologies (Beverly, MA, USA).

Specimens were prepared for pathological evaluation by fixation in 10% buffered formalin and by haematoxylin and eosin staining. Pathological assessments were performed by a pathologist (Beom Jin Lim) without any information and in accordance to the lung injury severity (LIS) score. This score is based on four indicators: (i) alveolar capillary congestion, (ii) haemorrhage, (iii) infiltration or aggregation of neutrophils in the air space or the vessel wall and (iv) thickness of the alveolar wall/hyaline membrane formation [9]. Depending on the severity of the respective indicators, a score between 0 (minimal damage) and 4 (maximal damage) was assigned, and the sum of the scores was averaged to compare the results between the two groups.

The wet/dry weight ratios were calculated based on the weight difference of specimens before and after storage at 80°C for 72 h.

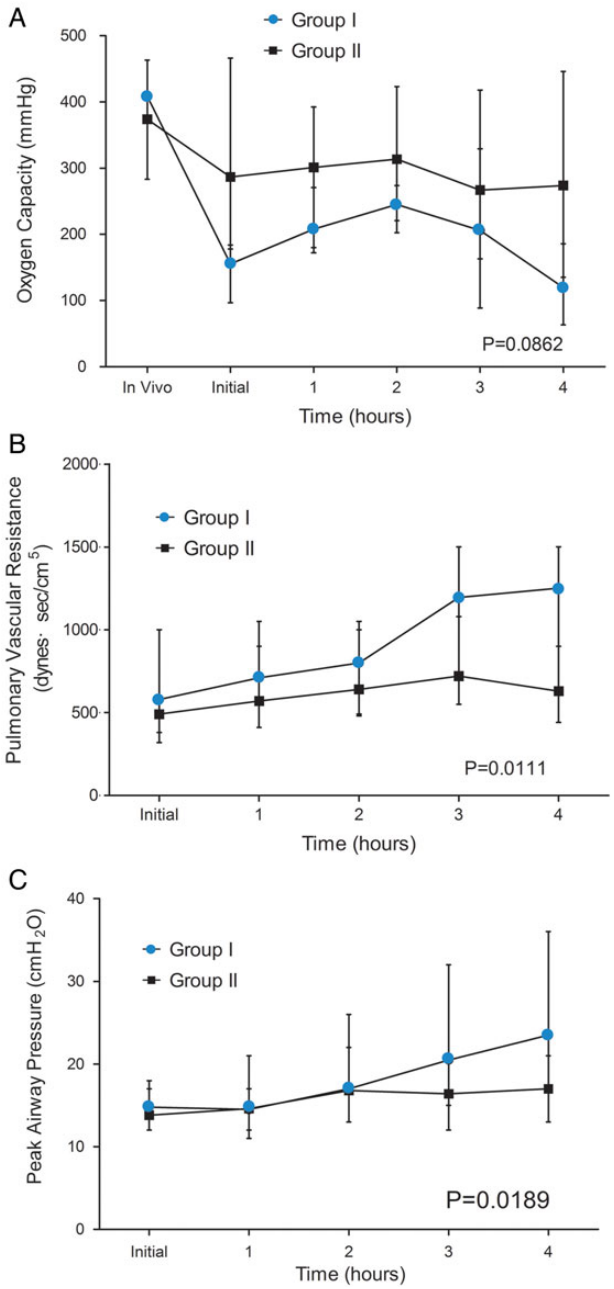

Figure 1: Changes in functional parameters during *ex vivo* lung perfusion in the control group (Group I) and the hydrogen group (Group II): (**A**) oxygen capacity, (**B**) pulmonary vascular resistance, (**C**) peak airway pressure. As time elapsed, the control group exhibited a decrease in OC and an increase in PVR and PAP, while the hydrogen group exhibited relatively stable values. The differences in the OC between groups were not statistically significant; however, the PVR and PAP were significantly lower in the hydrogen group than in the control group. PVR: pulmonary vascular resistance; OC: oxygen capacity; PAP: peak airway pressure.

Statistical analyses

Results were expressed as mean ± standard deviation. For comparison of the lung functional parameters such as OC, PVR and PAP, repeated measures analysis of variance was performed. A Mann–Whitney test was performed for the comparison of other variables (expression and phosphorylation of biological markers, the wet/dry ratio and the LIS). *P* values < 0.05 were considered statistically significant and all statistical analyses were performed using the SPSS software version 19.0 (IBM, Somers, NY, USA).

RESULTS

Functional parameters

The OC, a measurement of the oxygen transfer capacity of the lung, was higher in the hydrogen group than in the control group over the entire duration of the EVLP, although the difference was not statistically significant (Fig. 1A). PVR, which increases as lung function deteriorates, increased rapidly 2 h after the start of EVLP in the control group. Although the PVR in the hydrogen group increased slightly, it was relatively stable over the entire period. There was a statistically significant difference in PVR between the two groups (*P* = 0.0111) (Fig. 1B). The control group also exhibited a rapid increase in PAP 2 h after the start of EVLP, while the hydrogen group maintained a stable PAP; the difference between groups was also statistically significant (*P* = 0.0189) (Fig. 1C).

Myeloperoxidases

MPO activity within the lung tissue was 51.5 ± 2.3 mU/mg for the control group and 54.5 ± 1.9 mU/mg for the hydrogen group; however, this difference was not statistically significant (*P* = 0.1905).

Inflammatory cytokines

The level of expression of IL-1β, IL-6, IL-8 and TNF-α within the lung tissue was significantly lower in the hydrogen group than in the control group. The expression values in the control group and the hydrogen group, respectively, were 309.7 ± 87.2 vs 193.6 ± 85.5 ng/ml ($P = 0.0317$) for IL-1β, 4.112 ± 1.863 ng/ml vs 1.524 ± 0.753 g/ml ($P = 0.0159$) for IL-6, 40.125 ± 19.382 ng/ml vs 2.538 ± 1.833 g/ml ($P = 0.0195$) for IL-8 and 0.361 ± 0.132 vs 0.102 ± 0.059 ng/ml ($P = 0.0159$) for TNF-α (Fig. 2).

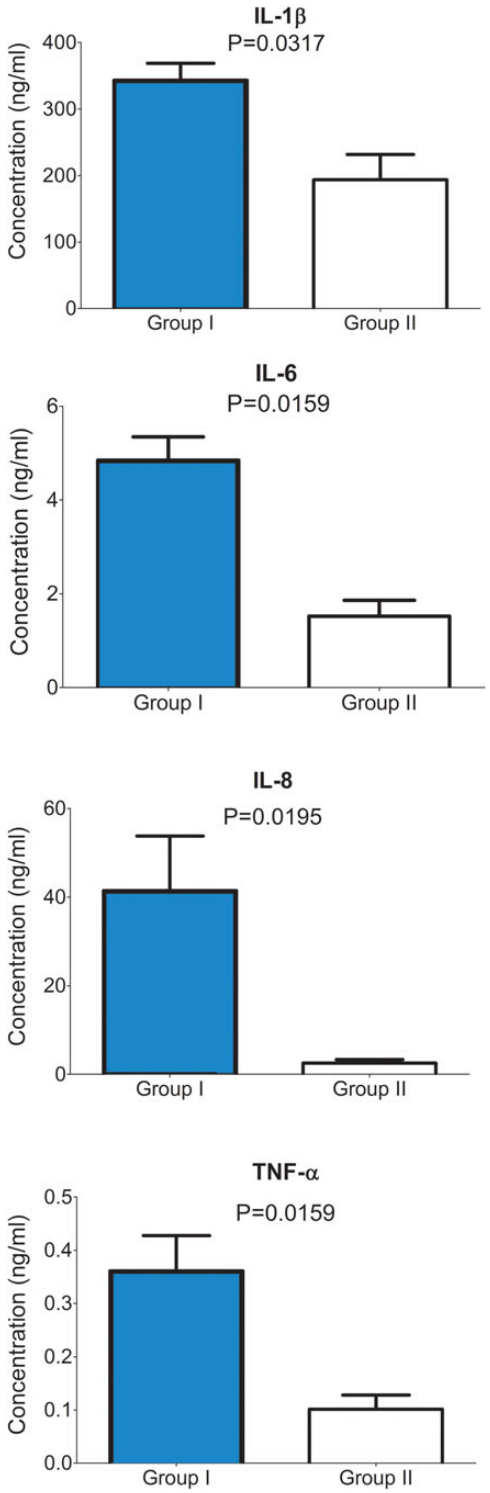

Figure 2: Expression of proinflammatory cytokines within the lung tissues. The levels of expression of proinflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α) were significantly higher in the control group (Group I) than in the hydrogen group (Group II). IL: interleukin; TNF-α: tumour necrosis factor-alpha; IL-1β: interleukin-1 beta.

Phosphorylation of MAPK-related enzymes

The degree of phosphorylation of P38 was 2.11 ± 0.42 vs 1.98 ± 0.21 ($P = 0.7302$), 1.38 ± 0.41 vs 0.44 ± 0.13 ($P = 0.1905$) for JNK and 1.36 ± 0.35 vs 0.91 ± 0.29 ($P = 0.2857$) for ERK, in the control group and the hydrogen group, respectively. Although these differences were not statistically significant, the hydrogen group had a lower degree of phosphorylation for all enzymes (Fig. 3).

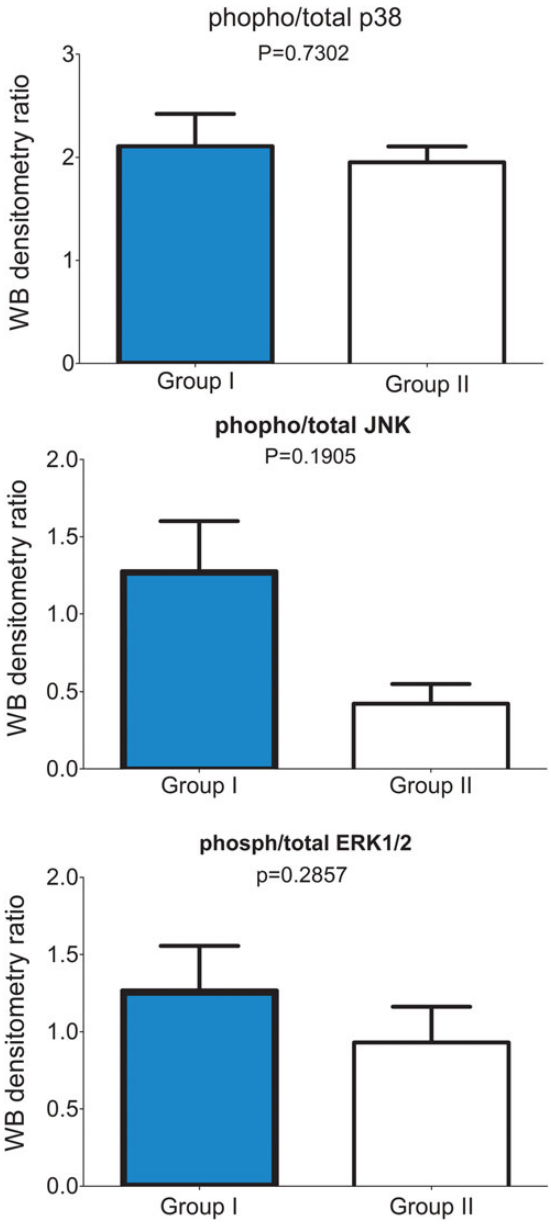

Figure 3: Degree of phosphorylation of MAPK-related enzymes. The degrees of phosphorylation of P38, JNK and ERK were lower in the hydrogen group (Group II) than in the control group (Group I); however, these differences were not statistically significant. JNK: c-Jun NH_2-terminal kinase; ERK: extracellular-regulated protein kinase.

Lung injury severity score

The LIS scores were 0.9 ± 0.1in the control group and 0.2 ± 0.2 in the hydrogen group ($P = 0.0358$), indicating that the degree of lung injury was lower in the hydrogen group.

Wet/dry ratio

The wet/dry ratio was 5.715 ± 0.557 in the control group and 5.077 ± 0.268 ($P = 0.0400$) in the hydrogen group, indicating that the extent of pulmonary oedema was lower in the hydrogen group.

DISCUSSION

Because of a shortage of brain death donors, many patients with end-stage lung disease die on the waiting list for LTx. A potential solution to the lung shortage is LTx using DCD lungs. Even after sudden cardiac arrest, lungs can contain sufficient oxygen within the pulmonary vessels and can provide this to tissues via direct diffusion. Furthermore, the lungs have a relatively low metabolic requirement, resulting in tolerance of warm ischaemia, and can maintain function for up to ∼1 h after cardiac arrest. While the safety of LTx using DCD lungs has been repeatedly reported [1, 10], the evaluation methods available for DCD lung function are insufficient compared with brain death donor lungs. Furthermore, the use of DCD lungs has been associated with ethical controversy [11].

Conventional hypothermic lung preservation can maintain lung viability during the ischaemic period; however, cold conditions inhibit cellular metabolism and prevent the recovery of previously damaged lungs. In addition, it is difficult to accurately evaluate the pretransplant function of the donor lung with the conventional lung preservation method [4]. In contrast, an EVLP system can provide both a continuous supply of oxygen and the nutrients necessary for cellular metabolism for lung grafts at normal temperatures. An EVLP system allows pulmonary function to be maintained and can accurately evaluate the pretransplant condition of the lung [8]. Furthermore, although the pulmonary function of a donor lung is marginal, there are various techniques associated with EVLP management which have been reported to improve donor lung function [12, 13]. Steen *et al.* [14] applied EVLP to a DCD lung and successfully performed an LTx, demonstrating that EVLP is a useful and accurate method for evaluating pulmonary function. Following this, numerous institutions have also reported successful results when applying EVLP to DCD lungs [12].

Although EVLP may be a useful means to detect if the donor lung has the possibility to reverse initial incompatibilities, there is controversy relating to whether EVLP alone can improve donor lung pathology [6]. Therefore, in order to improve lung function further, procedures such as nitric oxide (NO) ventilation, surfactant injections or gene therapy have been performed simultaneously during EVLP [15–17].

Recently, numerous animal experiments have demonstrated that hydrogen gas inhalation prior to lung procurement can reduce ischaemic reperfusion injury or primary graft dysfunction in LTx [18, 19]. In a rat model, Noda *et al.* [20] reported that hydrogen gas inhalation during EVLP can resolve EVLP-related adverse effects and improve post-transplant graft function. However, the effects of hydrogen on the lungs of large animals have not yet been reported. And, this study focused on the effects of hydrogen inhalation during EVLP in DCD lung.

While differences were not statistically significant, the hydrogen group maintained a higher OC than the control group for the entire duration of the EVLP. The hydrogen group exhibited significantly lower PVR and PAP than the control group during the course of the EVLP. While the control group showed a rapid deterioration of lung function 2 h after the start of EVLP, the lung function in the hydrogen group remained stable. These findings demonstrate the protective effect of hydrogen gas on lung injuries, which may occur as time elapses during EVLP.

To quantify the degree of inflammatory response in lung tissue, phosphorylation of p38, JNK and ERK associated with the Mitogen-activated Protein Kinases (MAPK) pathway, and the expression levels of proinflammatory cytokines (including IL-1β, IL-6, IL-8, and TNF-α) were measured. While there were no significant differences between the two groups in terms of MAPK-related enzymes, the hydrogen group exhibited lower levels of phosphorylation than the control group, and also had significantly lower expression levels of all inflammatory cytokines evaluated. This may confirm an anti-inflammatory effect of hydrogen gas inhalation during EVLP. Furthermore, the degree of LIS indicated by the LIS score was lower in the hydrogen group than in the control group, and regarding the lower wet/dry ratio in the hydrogen group, it appears that hydrogen gas inhalation may also play a role in reducing pulmonary oedema.

The DCD model in this study was designed in accordance with Maastricht category III donors because these are most commonly used donors in current clinical LTx [21, 22]. Although there is no clear definition regarding the tolerable warm ischaemic time in DCD lungs, <60 min of ischaemic time is generally recommended [23]. Therefore in this study, warm ischaemic time was defined as 1 h, though 1 h may not be sufficient time to induce warm ischaemic injury. Prolonged mechanical ventilation is able to induce lung injury in the warm ischaemic period; but, in order to prevent atelectasis and to supply oxygen, immediate reintubation and ventilation after cardiac death is declared in most institutions conducting DCD LTx [21, 22].

There are several methods that provide hydrogen in a body. Hydrogen-rich water (hydrogen-bubbled water) derived from electrolysed hydrogen dissolved in pure water is easy to create, simple to use and can be administered orally or intravenously. However, with oral administration there is the possibility of hydrogen loss in the stomach or in the intestine [24]. Hydrogen can also be administered in the form of electrolysed-reduced water; this protects DNA, RNA and protein from oxidative damage, and has been shown to have a beneficial effect in patients with liver injury, infection and diabetes [24]. Similar to other medical gases such as NO and carbon monoxide, hydrogen is also available via inhalation. Since hydrogen gas immediately diffuses within the lung tissues, inhalation has the advantage of producing rapid effects. In pulmonary diseases in particular, hydrogen gas inhalation can be an appropriate and simple method because the gas is administered through a closed ventilation circuit [7].

Hydrogen gas is generally known to be flammable. However, a mixture of atmospheric air and hydrogen (<4.6%), or a mixture of oxygen and hydrogen (<4.1%) is considered to be safe. Furthermore, hydrogen at concentrations <4% is not associated with any adverse effects in animals and humans [25]. In this study, 2% hydrogen gas was administered directly to the *ex vivo* lungs via a mechanical ventilator.

The present study has a few limitations. First, although hydrogen gas inhalation during EVLP showed a positive effect in terms of maintenance and improvement of DCD lung function, it is not

known if this effect would be sustained when using these lung grafts in a real LTx recipient. Evaluating the effect of hydrogen only by its function during EVLP may be insufficient, especially since severe lung injury can occur during the reperfusion process, after a period of warm ischaemia. Therefore, to clarify the effect of hydrogen gas inhalation, further studies designed to evaluate post-transplant lung function after actual LTx are required. Secondly, the present study primarily investigated variables related to DCD lung function and did not investigate the mechanisms involved with the maintenance and improvement of lung function by hydrogen gas ventilation during EVLP. Thirdly, owing to the risk of explosion, it was difficult to perform the experiment using different concentrations of hydrogen gas; therefore, the optimal concentration of hydrogen gas inhalation for this procedure still needs to be elucidated.

In conclusion, this study demonstrated that hydrogen gas ventilation during EVLP for DCD lungs with warm ischaemic injury might have preserved and improved lung function. This procedure may provide a solution to the shortage of brain death donor lungs. Further research investigating the precise mechanisms of the action of hydrogen gas and the effects of hydrogen gas administration on DCD lungs after LTx is required.

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Conflict of interest: none declared.

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