Protective Effects of a Hydrogen-Rich Preservation Solution in a Canine Lung Transplantation Model

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Background. **Molecular hydrogen (H_2) has protective effects against ischemia-reperfusion injury in various organs. Because they are easier to transport and safer to use than inhaled H_2, H_2-rich solutions are suitable for organ preservation. In this study, we examined the protective effects of an H_2-rich solution for lung preservation in a canine left lung transplantation (LTx) model.**

Methods. **Ten beagles underwent orthotopic left LTx after 23 hours of cold ischemia followed by reperfusion for 4 hours. Forty-five minutes after reperfusion, the right main pulmonary artery was clamped to evaluate the function of the implanted graft. The beagles were divided into two groups: control group (n = 5), and H_2 group (n = 5). In the control group, the donor lungs were flushed and immersed during cold preservation at 4°C using ET-Kyoto solution, and in the H_2 group, these were flushed and immersed using H_2-rich ET-Kyoto solution. Physiologic assessments were performed during reperfusion. After reperfusion, the wet-to-dry ratios were determined, and histology examinations were performed.**

Results. **Significantly higher partial pressure of arterial oxygen and significantly lower partial pressure of carbon dioxide were observed in the H_2 group than in the control group ($P = .045$ and $P < .001$, respectively). The wet-to-dry ratio was significantly lower in the H_2 group than in the control group ($P = .032$). Moreover, in histology examination, less lung injury and fewer apoptotic cells were observed in the H_2 group ($P < .001$ and $P < .001$, respectively).**

Conclusions. **Our results demonstrated that the H_2-rich preservation solution attenuated ischemia-reperfusion injury in a canine left LTx model.**

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Lung transplantation (LTx) is the final option to save the lives of patients with end-stage pulmonary diseases.[1,2] However, severe donor shortage remains a serious problem. Another important issue is that lungs are vulnerable to ischemia, resulting in the limitation of the ischemic time. In deceased-donor LTx, the graft lungs are exposed to ischemia during transportation. A long ischemic time often leads to ischemia-reperfusion (I/R) injury after LTx.[3,4] Therefore, developing better methods of lung preservation during the ischemic time is required to improve the treatment outcome after LTx.

Since Ohsawa and associates[5] first reported the beneficial effects of molecular hydrogen (H_2) for brain I/R injury in a mouse brain infarction model in 2007, H_2 has been reported to have protective effects against I/R injury in various organs.[6-9] Although the precise mechanism remains uncertain, the antioxidative, antiinflammatory, and antiapoptosis effects of H_2 have been reported.[10-12] Our group has previously reported the protective effects of an H_2-rich solution against lung I/R injury in a rat left hilar clamp model[13] and those of an H_2-rich lung preservation solution in a rat orthotopic left LTx model.[14] To examine the diffusion of H_2 in lungs of larger animals and evaluate the protective effects of H_2-rich preservation solution in larger animals having much more complicated immune systems than rats, we performed this preclinical study using a canine orthotopic left LTx model.

Material and Methods

Animals

Ten pairs of weight-matched Toyo beagles (Kitayama Labes, Hongo Farm, Yamaguchi, Japan) weighing 8.95 to 11.1 kg were used in this experiment. All animals

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Abbreviations and Acronyms

cDNA = complementary DNA
H2 = molecular hydrogen
IL = interleukin
I/R = ischemia-reperfusion
LTx = lung transplantation
mRNA = messenger RNA
PCR = polymerase chain reaction
ssDNA = single-strand DNA
TNF = tumor necrosis factor

received humane care in accordance with the *Guide for the Care and Use of Laboratory Animals* prepared by the National Institutes of Health (publication no. 86-23, revised 1996). The study protocol of this experiment was approved by the Ethics Committee of the Graduate School of Medicine at Kyoto University, Japan (MedKyo 19276).

Hydrogen-rich Preservation Solution

In this study, the ET-Kyoto solution (Otsuka Pharmaceutical, Tokushima, Japan), which is clinically used for LTx at Kyoto University Hospital, was used as the preservation solution.[15] The H_2-rich preservation solution was prepared using a commercially available hydrogen-generating agent (Aquela 7.0; MiZ Company, Kamakura, Japan) consisting of metal aluminum grains and calcium hydroxide.[14,16] The concentration of hydrogen was quantified with a titrant consisting of methylene blue and platinum.[17]

Experimental Design

Figure 1 shows the experimental design of this study. After the retrieval of lung grafts from a donor dog, cold preservation at 4°C for 23 hours was performed. Thereafter, the left lung graft was prepared and orthotopic left LTx for a recipient dog was performed. We set 60 minutes as the transplantation time. After the orthotopic left LTx, the graft lungs were reperfused for 4 hours. Forty-five minutes after transplantation, the right main pulmonary artery was clamped to evaluate the function of the implanted graft.

Ten donor dogs were randomly divided into two groups: H_2 group (n = 5) and control group (n = 5). In the control group, normal ET-Kyoto solution was used both for flushing and immersing the graft lungs, whereas in the H_2 group, H_2-rich ET-Kyoto solution was used for both flushing and immersing.

Donor Procurement

The operative technique has been precisely described elsewhere.[18-20] Briefly, the beagles were premedicated with an intramuscular injection of midazolam (0.5 mg/kg), xylazine (2.0 mg/kg), and atropine sulfate (0.05 mg/kg). They were intubated and mechanically ventilated with fraction of inspired oxygen of 0.5, tidal volume of 20 mL/kg, frequency of 15 breaths per minute, and positive end-expiratory pressure of 5 cm H_2O, and maintained on 0.8% to 2.0% sevoflurane and administered an intravenous injection of rocuronium bromide (0.5 mg/kg). After median sternotomy, the main pulmonary artery was cannulated through the right ventricular outflow tract and the superior and inferior vena cavae were ligated. After the incision of the left atrial appendage, 1000 mL cold normal ET-Kyoto solution (control group) or cold H_2-rich ET-Kyoto solution (H_2 group) was perfused antegradely and retrogradely from a height of 30 cm above the heart. Ventilation was continued until the retrieval of lung grafts and recovered heart-lung block, which were semi-inflated with 50% oxygen, immersed in 2500 mL normal (control group) or H_2-rich (H_2 group) ET-Kyoto solution, and preserved at 4°C for 23 hours in an aluminum bag (Lamizip AL-34L; Seisannipponsha, Tokyo, Japan).

Recipient Preparation and Orthotopic Left Lung Transplantation

Recipient dogs were anesthetized and maintained in the same manner as the donors except for fraction of inspired oxygen of 1.0. A Swan-Ganz catheter and an arterial catheter were inserted before the operation. Before the orthotopic left LTx, the left lung graft was separated from the recovered heart-lung block and flushed again antegradely and retrogradely with 500 mL normal (control group) or H_2-rich (H_2 group) ET-Kyoto solution. After left pneumonectomy, orthotopic left LTx was performed as described elsewhere.[18-21] After reperfusion, a 2.0-mm catheter was directly inserted into the left atrium to monitor the left atrial pressure. Thereafter, the right main pulmonary artery was clamped 45 minutes after reperfusion to evaluate the function of the implanted graft, and the implanted lung grafts were reperfused for 4 hours in total.

Lung Function After Transplantation

Systemic arterial pressure, pulmonary arterial pressure, and peak airway pressure were continuously monitored throughout the experiment. Arterial blood gas analysis was performed before the recipient operation and at 30, 60, 120, 180, and 240 minutes after the implantation using an automatic blood gas analyzer (ABL 80 FLEX; Radiometer, Tokyo, Japan). At the same time, cardiac output was also measured. Pulmonary dynamic compliance was defined as tidal volume divided by ([peak airway pressure] minus [positive end-expiratory pressure]).[18,21]

Evaluation of Lung Edema

At the end of the experiment, partial resection of the left lower lobe was performed to calculate the wet-to-dry lung weight ratio for evaluating lung edema. To calculate the wet-to-dry weight ratio, wet samples were first weighed to obtain the wet lung weight; after heating the samples at 100°C for 48 hours, they were weighed again to obtain the dry lung weight.

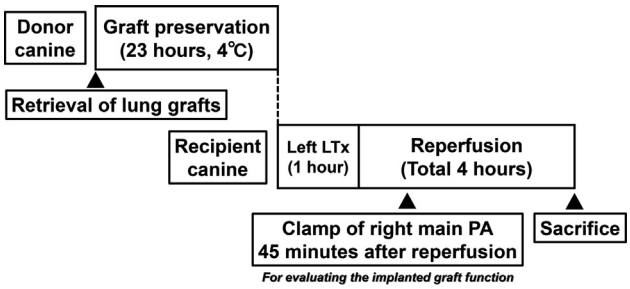

Figure 1. Design of the experiment. After retrieval of lung grafts, cold preservation for 23 hours was performed. After orthotopic left lung transplantation (LTx), graft lungs were reperfused for 4 hours. Forty-five minutes after transplantation, the right main pulmonary artery (PA) was clamped for evaluating function of implanted graft.

Macroscopic and Histologic Findings

Macroscopic appearance of the implanted lung grafts at 4 hours after reperfusion was recorded. The left middle lobe was resected at the end of reperfusion and was fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. To evaluate the degree of lung injury, the lung injury score was calculated based on the lung injury scoring system defined in the official American Thoracic Society workshop report.[22,23] Two investigators (H.K. and H.Y) blindly evaluated lung injury at 20 randomly chosen high-power fields per section at a magnification of ×400 under the supervision of a pulmonary pathologist (A.Y.).

The evaluation of apoptotic cells was performed by immunostaining for single-strand DNA (ssDNA) using rabbit polyclonal antibodies against ssDNA (IBL, Takasaki, Japan).[24] The number of ssDNA-positive cells was quantified using the average number in 10 randomly chosen fields per section at a magnification of ×200.

Quantitative Real-Time Polymerase Chain Reaction

The left upper lobe was partially resected at the end of reperfusion for quantitative real-time polymerase chain reaction (PCR) to evaluate the gene expression of interleukin-1β messenger RNA (mRNA), tumor necrosis factor α (TNF-α) mRNA, and interleukin-6 (IL-6) mRNA. The lung tissue was stabilized with RNA*later* (Qiagen, Hilden, Germany), and RNA was extracted using an RNeasy Plus Mini Kit (Qiagen) following the manufacturer's protocol. Thereafter, the total RNA was reversely transcribed to complementary DNA (cDNA) with the Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Pittsburgh, PA), and each cDNA sample was diluted to 10 μg/mL. Furthermore, 2 μL cDNA was mixed with the Thunderbird probe qPCR Mix (Toyobo, Osaka, Japan) and TaqMan Gene Expression Assay probe set (Thermo Fisher Scientific, Waltham, MA). The PCR was performed using the StepOnePlus Real-Time PCR system (Thermo Fisher Scientific). The relative quantity of each sample was calculated using the comparative ΔΔCT method. Glyceraldehyde 3-phophate dehydrogenase (GAPDH) was used as the reference gene. Each sample was analyzed in triplicate. Probes used for PCR assays were as follows: GAPDH (NM_001003142.2), IL-1β mRNA (NM_001037971.1), TNF-α mRNA (NM_001003244.4), and IL-6 mRNA (NM_001003301.1).

Statistical Analysis

All statistical analyses were performed using the EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria).[25,26] All values are expressed as mean ± SD. The Mann-Whitney *U* test was performed for the comparison of two groups. Repeated measures analysis of variance was performed for the comparison of Pa_{O_2} and Pa_{CO_2}, pulmonary vascular resistance, and pulmonary dynamic compliance. Statistical significance was defined as *P* less than .05.

Results

There were no significant differences between the control and H_2 groups in donor weight (control group, 9.9 ± 0.4 kg; H_2 group, 9.9 ± 0.7 kg) or recipient weight (control group, 9.6 ± 0.8 kg; H_2 group, 9.7 ± 0.5 kg). The H_2 concentration of the H_2-rich ET-Kyoto solution was greater than 1.0 ppm before being used for immersing and flushing. The H_2 concentration of the H_2-rich ET-Kyoto solution used for immersing was maintained at more than 0.6 ppm for 23 hours after retrieval.

Lung Function and Pulmonary Hemodynamics After Transplantation

All five recipients in both groups survived until 4 hours after reperfusion. Significantly higher PaO_2 was observed in the H_2 group than in the control group ($P = .045$; Figure 2A). Furthermore, $PaCO_2$ was significantly lower in the H_2 group than in the control group ($P < .001$; Figure 2B). There was no significant difference between the two groups in pulmonary vascular resistance ($P = .412$; Figure 2C); however, pulmonary dynamic compliance in the H_2 group was significantly better than that in the control group ($P = .029$; Figure 2D).

Wet-to-Dry Lung Weight Ratio

The wet-to-dry lung weight ratio at 240 minutes after reperfusion was significantly lower in the H_2 group than in the control group (H_2 group, 9.2 ± 1.4; control group, 11.4 ± 1.6; $P = .032$; Figure 3A).

Macroscopic and Histologic Findings at 4 Hours After Transplantation

The graft lungs of the H_2 group appeared less damaged than those of the control group (Figure 3B). With respect to histologic findings, lesser neutrophil infiltration was observed in the H_2 group than in the control group (Figure 4A). Consequently, the lung injury score in the H_2 group was significantly lower than that in the control group (H_2 group, 0.30 ± 0.13; control group, 0.42 ± 0.14; $P < .001$; Figure 4B). Furthermore, significantly fewer

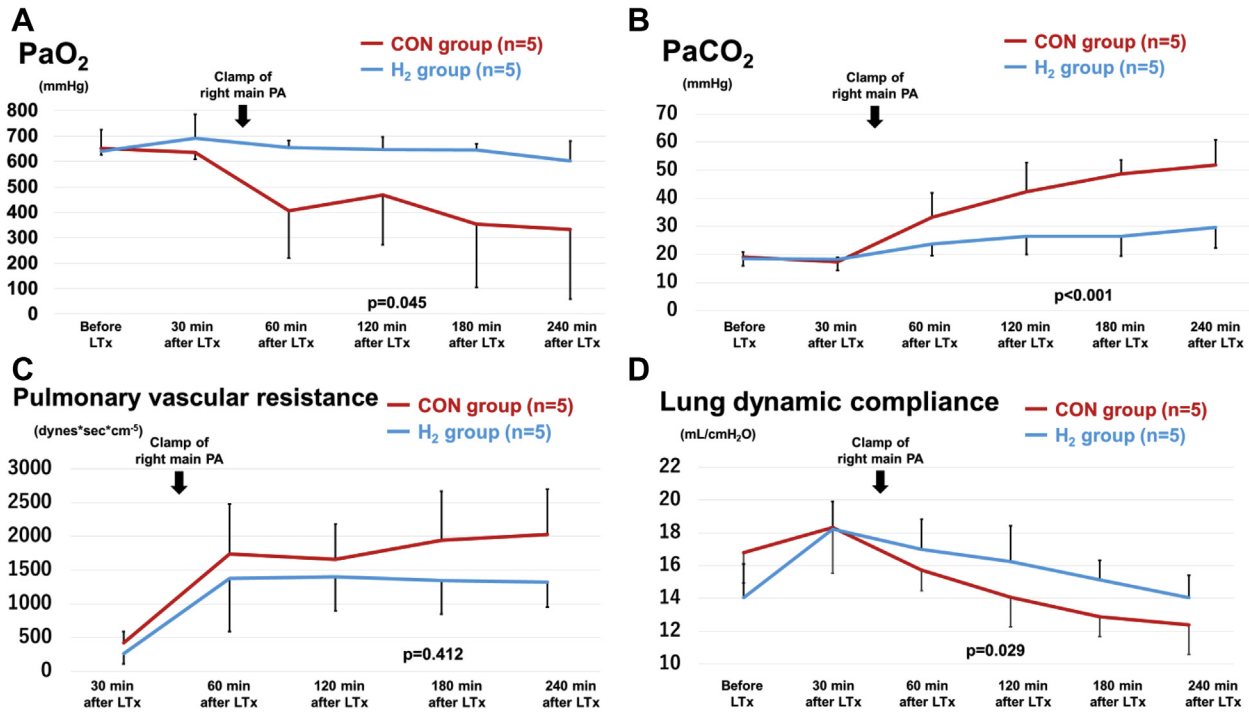

Figure 2. Physiologic data of the experiment: hydrogen-rich (H_2) group (blue line [n = 5]); and control (CON) group (red line [n = 5]). (A) Significantly higher Pa_{O_2} was observed in the H_2 group than in the control group (P = .045). (B) The PaC_{O_2} was significantly lower in the H_2 group than in the control group (P < .001). (C) There was no significant difference in pulmonary vascular resistance between the two groups (P = .412). (D) Significantly better pulmonary dynamic compliance was observed in the H_2 group than in the control group (P = .029). (LTx, lung transplantation; PA, pulmonary artery.)

ssDNA-positive cells were observed in the H_2 group than in the control group (H_2 group, 2.3 ± 1.6; control group, 6.6 ± 4.6; $P < .001$; Figure 5).

Expression of mRNA of Proinflammatory Cytokines

The expression of IL-1β mRNA in the H_2 group was lower than that in the control group, although the difference did not reach statistical significance ($P = .056$; Figure 6A). The expression of TNF-α mRNA and IL-6 mRNA was comparable between the two groups ($P = .222$ and $P = .222$, respectively; Figures 6B, 6C).

Comment

In this study, an H_2-rich solution was used for lung preservation during cold ischemia in a canine left orthotopic LTx model. By using this preservation solution, both more effective ventilation and better pulmonary compliance during 4 hours of reperfusion were achieved than with the normal preservation solution. Moreover, lung edema after LTx was attenuated with the use of the H_2-rich preservation solution, and lung injury was also attenuated macroscopically and histologically. Furthermore, this preservation solution decreased the number of

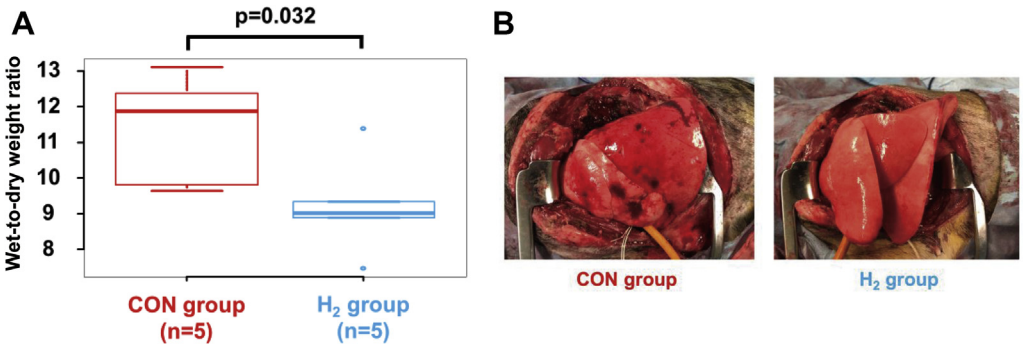

Figure 3. Wet-to-dry lung weight ratio and macroscopic findings: hydrogen-rich (H_2) group (blue [n = 5]); and control (CON) group (red [n = 5]). (A) Wet-to-dry weight ratio was measured to evaluate degree of lung edema. Wet-to-dry weight ratio in H_2 group was significantly lower than in control group (P = .032). (B) Macroscopic appearance of implanted lung grafts at 4 hours after reperfusion. These two figures were representative cases. Graft lungs of the H_2 group appeared less damaged than those of the control group.

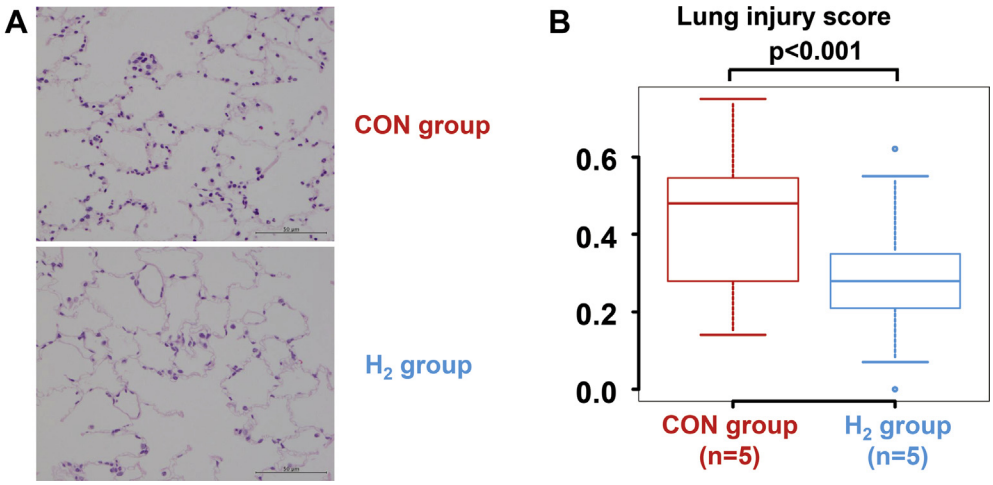

Figure 4. Histologic evaluation using the lung injury score based on the official American Thoracic Society workshop report. (A) Representative histologic findings of the two groups (original magnification ×400). Less neutrophil infiltration was observed in the hydrogen-rich (H_2) group than in the control (CON) group. (B) The lung injury score in the H_2 group (blue [n = 5]) was significantly lower than that in the control group (red [n = 5]; P < .001).

apoptotic cells and suppressed the production of proinflammatory cytokines in lung tissue. These results indicated the protective effects of the H_2-rich preservation solution against lung I/R injury after prolonged cold ischemia in a canine orthotopic left LTx model.

Since the report by Ohsawa and colleagues,[5] H_2 has been used as a therapeutic antioxidant for I/R injury attenuation and organ preservation in various medical fields.[6-11,27-32] Our group previously reported the protective effects of an H_2-rich preservation solution during cold ischemia in a rat LTx model. As a further research for clinical application, we used larger animals in this study.

In the present study, corresponding to better physiologic function of the implanted grafts, attenuation of lung edema after LTx was observed when an H_2-rich preservation solution was used for lung preservation during cold ischemia. In previous reports, when an H_2-rich preservation solution was used, the regular lining of vascular endothelial cells was well preserved based on scanning electron microscope findings in a rat LTx model[14] and a rat ex vivo liver perfusion model.[27] Furthermore, the protective effects of an H_2-rich medium on vascular endothelial permeability by the inhibition of signal pathways and downregulated expression of the adhesion molecules causing injury of the vascular endothelium in vitro were also observed in other studies.[33,34] Based on these reports and our results, we speculated that the effects of an H_2-rich preservation solution on the attenuation of lung edema in this study may be partly attributed to the reduction of vascular endothelial cell injury and vascular endothelial permeability.

This study also showed that posttransplantation lung injury and production of proinflammatory cytokines were attenuated by the use of an H_2-rich preservation solution. Previous reports suggested that H_2 inhibits the activation of nuclear factor-kappa B and p38 mitogen-activated protein kinase pathway, thereby reducing the expression of proinflammatory cytokines and infiltration of neutrophils.[12,28,29,35] Furthermore, this study showed the

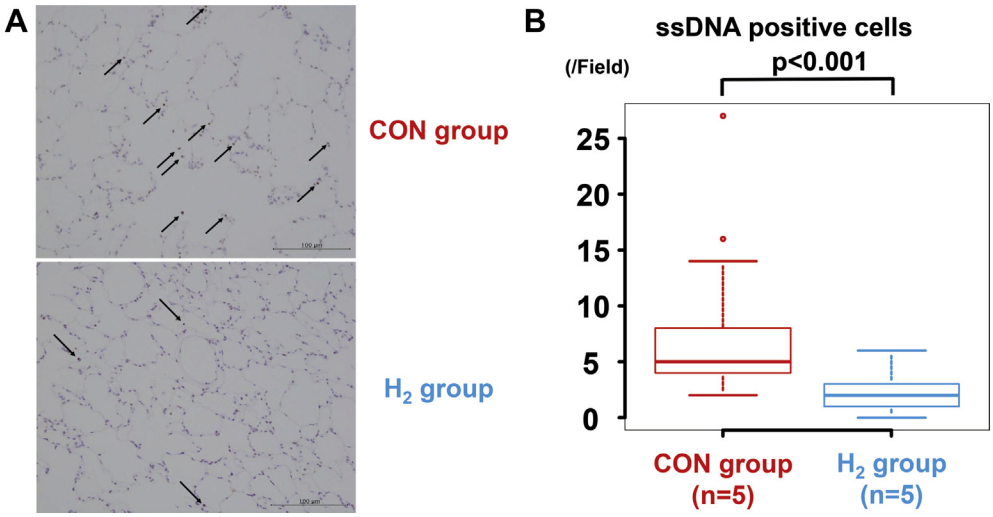

Figure 5. Evaluation of apoptotic cells using immunostaining for single strand DNA (ssDNA). (A) Representative histologic findings of the two groups (original magnification ×200). Arrows show ssDNA-positive cells. (B) Significantly fewer ssDNA-positive cells were observed in the hydrogen-rich (H_2) group (blue [n = 5]) than in the control (CON) group (red [n = 5]); P < .001).

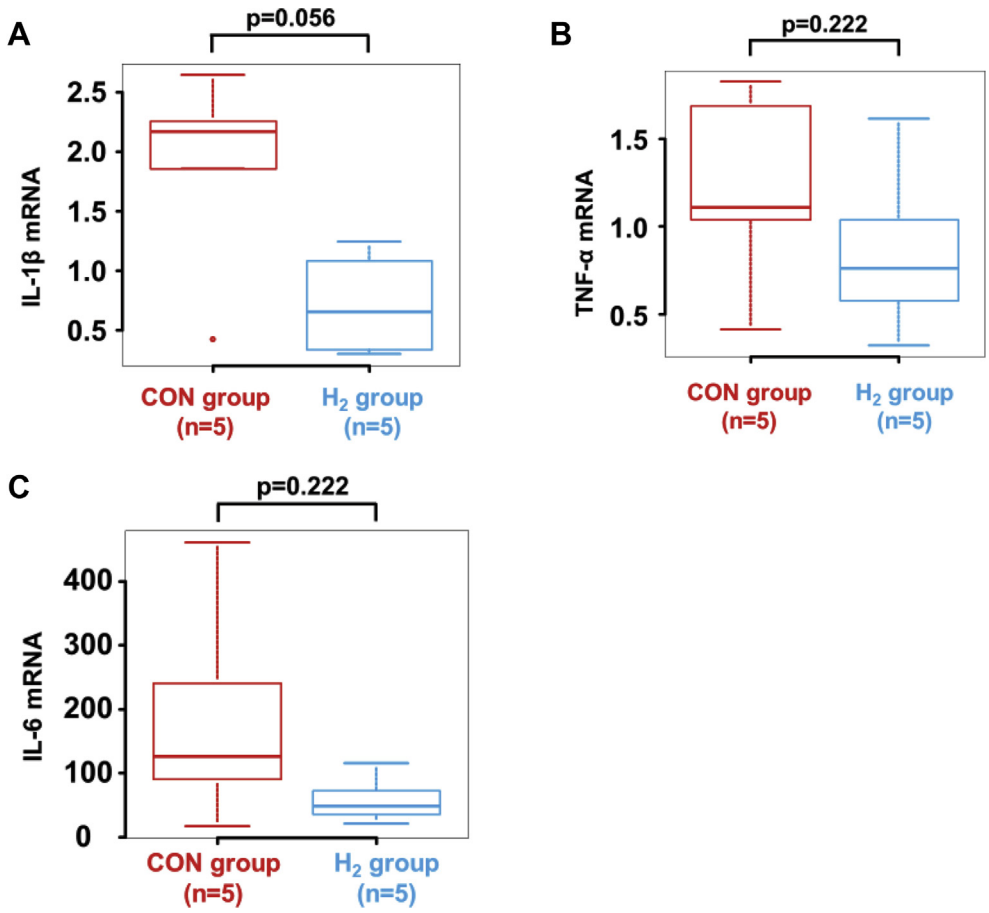

Figure 6. Quantitative real-time polymerase chain reaction results of proinflammatory cytokines in grafts at end of reperfusion. (A) Gene expression of interleukin-1β (IL-1β) in the hydrogen-rich (H_2) group (blue [n = 5]) was lower than that in the control (CON) group (red [n = 5]), although the difference did not reach statistical significance (P = .056). (B) Gene expression of tumor necrosis factor-α (TNF-α) was comparable between the two groups (P = .222). (C) Gene expression of interleukin-6 was also comparable between the two groups (P = .222). (mRNA, messenger RNA.)

antiapoptosis effects of the H_2-rich preservation solution by revealing that fewer ssDNA-positive cells were observed in the H_2 group. Apoptosis is considered a factor for early graft failure in solid organ transplantation. It was previously reported that the antiapoptosis effects of H_2 are partly derived from the suppression of proapoptosis factors such as caspase-3 and caspase-8 and upregulation of antiapoptosis factors such as B-cell lymphoma-2.[8,28,30,35,36] However, details of the mechanism remain unknown. As we could not reveal the details in this experiment, further studies using in vitro and LTx experiments in a mouse model are required.

Some of the methods used to deliver H_2 to organs include inhalation of H_2 and dissolution of H_2 into the preservation solution. In this study, we dissolved H_2 into the organ preservation solution because we considered that the use of an H_2-rich solution has some advantages over inhalation. First, an H_2-rich solution is easy to transport. Therefore, it can be used both for flushing during donor operation and for immersing the grafts before they are transported. Second, an H_2-rich solution is safer to use than H_2 gas, as more than 4% H_2 gas has flammable and explosive properties. In this study, the H_2-rich preservation solution obviously attenuated lung I/R injury in the LTx model after prolonged cold ischemia even with a low concentration of H_2, which indicated that the H_2-rich solution was efficient in delivering H_2 to organ tissues. As a future perspective, we plan to apply lung preservation with an H_2-rich preservation solution to clinical LTx.

This study has several limitations. First, the number of subjects in both groups was relatively small (n = 5) because more animal sacrifices should be avoided from the viewpoint of animal welfare. Second, we did not use a sterile method for preparing the H_2-rich preservation solution. Therefore, techniques for producing a sterile and stable H_2-rich solution are required for clinical application.

In conclusion, we demonstrated that an H_2-rich preservation solution attenuated lung I/R injury after prolonged cold ischemia in a canine LTx model. The H_2-rich preservation solutions could be a useful and effective option for cold preservation and transportation of lung grafts.

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