



Post-reperfusion hydrogen gas treatment ameliorates ischemia reperfusion injury in rat livers from donors after cardiac death: a preliminary study

Takahisa Ishikawa¹ · Shingo Shimada¹ · Moto Fukai¹ · Taichi Kimura² · Kouhei Umemoto¹ · Kengo Shibata¹ · Masato Fujiyoshi¹ · Sunao Fujiyoshi¹ · Takahiro Hayasaka¹ · Norio Kawamura³ · Nozomi Kobayashi¹ · Tsuyoshi Shimamura⁴ · Akinobu Taketomi¹

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Abstract

Background and purpose We reported previously that hydrogen gas (H₂) reduced hepatic ischemia and reperfusion injury (IRI) after prolonged cold storage (CS) of livers retrieved from heart-beating donors. The present study was designed to assess whether H₂ reduced hepatic IRI during donation of a cardiac death (DCD) graft with subsequent CS.

Methods Rat livers were harvested after 30-min cardiac arrest and stored for 4 h in University of Wisconsin solution. The graft was reperfused with oxygenated buffer, with or without H₂ (H₂ or NT groups, respectively), at 37° for 90 min on isolated perfused rat liver apparatus.

Results In the NT group, liver enzyme leakage, apoptosis, necrosis, energy depletion, redox status, impaired microcirculation, and bile production were indicative of severe IRI, whereas in the H₂ group these impairments were significantly suppressed. The phosphorylation of cytoplasmic MKK4 and JNK were enhanced in the NT group and suppressed in the H₂ group. NFκB-p65 and c-Fos in the nucleus were unexpectedly unchanged by IRI regardless of H₂ treatment, indicating the absence of inflammation in this model.

Conclusion H₂ was observed to ameliorate IRI in the DCD liver by maintaining microcirculation, mitochondrial functions, and redox status, as well as suppressing the cytoplasmic MKK4–JNK-mediated cellular death pathway.

Keywords Hydrogen gas · Donation after cardiac death · Liver · Rat · Ischemia reperfusion

Abbreviations

| | |
|-----|------------------------------|
| ATP | Adenosine triphosphate |
| ALT | Alanine aminotransferase |
| AST | Aspartate aminotransferase |
| CPA | Cardiopulmonary arrest |
| DCD | Donation after cardiac death |

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| DBD | Donation after brain death |
| ECD | Expanded criteria donor |
| GSSG | Oxidized glutathione |
| GSH | Reduced glutathione |
| HPFs | High power fields |
| H ₂ | Hydrogen gas |
| 4-HNE | 4-Hydroxy-2-nonenal |
| IRI | Ischemia and reperfusion injury |
| IPRL | Isolated perfused rat liver |
| JNK | c-jun N-terminal kinase |
| KHB | Krebs–Henseleit bicarbonate buffer |
| LDH | Lactate dehydrogenase |
| LPO | Lipid peroxidation |
| MDA | Malondialdehyde |
| MKK4 | Mitogen-activated protein kinase 4 |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NMP | Normothermic machine perfusion |
| OCR | Oxygen consumption rate |

✉ Moto Fukai
db7m-fki@hotmail.co.jp

¹ Department of Gastroenterological Surgery I, Graduate School of Medicine, Hokkaido University, N-15, W-7, Kita-Ku, Sapporo 060-8638, Japan

² Laboratory of Cancer Research, Department of Pathology, Graduate School of Medicine, Hokkaido University, Sapporo, Japan

³ Department of Transplant Surgery, Graduate School of Medicine, Hokkaido University, Sapporo, Japan

⁴ Division of Organ Transplantation, Central Clinical Facilities, Hokkaido University Hospital, Sapporo, Japan

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| PNF | Primary non-function |
| PVP | Portal vein pressure |
| PVR | Portal vein resistance |
| ROS | Reactive oxygen species |
| SCD | Standard criteria donor |
| SDS-PAGE | SDS polyacrylamide gel electrophoresis |
| TUNEL | Terminal deoxynucleotidyl transferase |

Introduction

Critical donor shortage has prompted the safe implementation of extended-criteria donor (ECD) grafts, including DCDs. Although simple cold storage in University of Wisconsin (UW) solution has contributed to the development of liver and kidney transplantation [1], a gold standard for the use of DCD liver has not yet been established because of the associated severe IRI, which leads to primary non-function, biliary complications, and eventual graft loss [1, 2]. A retrospective analysis of DCD liver transplantation showed that primary non-function (PNF) and bile duct complications were uncommon when there were few risk factors [3]. A recent encouraging report demonstrated that the outcome of DCD livers subjected to normothermic machine perfusion (NMP) was better than that of donation after brain death (DBD) with NMP, when donors and recipients were appropriately selected [4].

DCD graft injury after transplantation progresses in three phases, namely, warm ischemia, subsequent cold preservation (low temperature and hypoxia), and reperfusion (rewarming and reoxygenation). Unlike the standard criteria donor (SCD) liver, refrigeration under energy deficiency leads to the accumulation of sodium and calcium ions, resulting in reperfusion injury [5]. Furthermore, warm IRI caused mainly by reactive oxygen species (ROS) results in the production of inflammatory cytokines, tissue injury and microcirculatory disturbance [6, 7]. However, if the cold preservation time is within certain limits, it will not enhance injury significantly [6, 7]. Based on these factors, if the degree of impairment in each phase can be suppressed, then the IRI of DCD liver could be suppressed. Accordingly, we chose the safe limit for warm ischemia in the DCD liver graft as 30 min [3] and sought the shortest clinically applicable cold preservation time. We then looked for ways to reduce IRI by starting treatment at the initiation of reperfusion.

We focused on the medical gas H₂ to assess its ability to reduce IRI in DCD liver grafts. Ohsawa et al. reported that hydrogen gas acts as a therapeutic antioxidant, and could play significant roles in radical scavenging and anti-inflammation [8]. Some authors have reported that H₂ reduces reperfusion injury in the brain [8], lung [9], heart [10], intestine [11], kidney [12] and liver [13, 14]. We felt that these factors

could be potentially applicable to the amelioration of IRI in DCD liver grafts.

In hepatic IRI, signaling molecules such as c-jun N-terminal kinase (JNK) are activated by inflammatory cytokines and various stressors including oxidative stress [15, 16]. Furthermore, prolonged JNK activation promotes the mitochondrial release of cytochrome c, cleavage of caspase 3, and then apoptosis [17], as well as necrosis caused by impaired mitochondrial energy production [16]. Although the protective effect of H₂ against hepatic cold IRI in an isolated perfused rat liver (IPRL) has been reported [18], the precise molecular mechanisms remain unclear. The aims of this study were to investigate whether H₂ ameliorates reperfusion injury of the DCD graft, and to elucidate the mechanism of its protection.

Materials and methods

Chemicals and reagents

Chemicals and reagents were purchased from Wako Pure Chemical Co. Ltd (Osaka, Japan) and antibodies were purchased from Cell Signaling Technology Japan (Tokyo, Japan) unless otherwise noted.

Animals

All experiments were conducted with the approval of the Institutional Guide of Hokkaido University in accordance with the Care and Use of Laboratory Animals. Male Sprague–Dawley rats (6–7 weeks old, 220–260 g) were purchased from Sankyo Labo Service Corporation, Inc (Tokyo, Japan), and used for the experiments after being raised for 5–7 days in our animal facility. The feed and other laboratory conditions were as described previously [19].

Operation

Animals were operated on without being fasted and were anesthetized by isoflurane inhalation. Cardiopulmonary arrest (CPA) was initiated by phrenotomy. After 30 min of CPA, the portal vein was cannulated with a 16-G catheter (NIPRO, Osaka, Japan). The liver was flushed via the portal vein with 50 ml saline (room temperature) containing 1000 IU of sodium heparin (Mochida pharmaceutical CO., LTD, Tokyo, Japan) and with 10 ml of ice-chilled UW solution thereafter. The bile duct was cannulated with a PE-10 catheter (Intramedic, Clay Adams, NJ, USA) and the liver was removed.

Experimental protocol

In the control group, the liver without CPA and cold storage was reperfused immediately after procurement on IPRL apparatus (CT group; $n = 6$). The cold preservation group was sub-divided into two groups. After CPA and subsequent cold preservation in UW solution for 4 h, livers were reperfused as an IPRL without hydrogen gas supplementation [no treatment (NT) group; $n = 6$] or with hydrogen gas treatment (H_2 group; $n = 6$).

Isolated perfused rat liver (IPRL)

Conditions of the IPRL were set in accordance with techniques described previously [20] with slight modifications [18]. Briefly, livers were perfused with 300 ml of Krebs–Henseleit bicarbonate buffer (KHB), supplemented with glucose and sodium taurocholate, at 37° and a constant pressure of 12 cmH₂O in a recirculating system. In the H_2 group, H_2 -saturated KHB in a sealed glass bottle was connected before the portal cannula, and mixed in the perfusion circuit at a flow rate of 4% versus total flow. We measured portal vein pressure (PVP; cmH₂O), portal vein resistance [PVR; cmH₂O/(ml/min/g)], bile production during 90 min of perfusion (μ l/g liver), and the oxygen consumption rate (OCR) [18].

Sample collection

At the end of perfusion, the livers were weighed and stored at -80° until use, or fixed in 10% buffered formalin and embedded in paraffin. For adenosine triphosphate (ATP) and glutathione measurements, they were snap-frozen. The effluent (KHB) was collected just after anesthesia and then 30 and 90 min after perfusion to examine activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH). They were measured with a Hitachi 7020 automatic biochemical analyzer (Hitachi, Tokyo, Japan), and liver injury was expressed as the enzyme leakage at the end of reperfusion: enzyme leakage (IU/g) = (activity_{R90}) [IU/l] \times perfusate volume [l]/liver weight [g] [18].

Histology and apoptosis

The paraffin-embedded sections were stained with hematoxylin and eosin and by the terminal deoxynucleotidyl transferase (TUNEL) method. Liver injury was assessed by a single pathologist in a blind manner. The apoptotic

index was calculated by the number of TUNEL-positive cells divided by the total cell number [18].

ATP assay

Tissue ATP was measured by the HPLC-UV method [19]. Data were expressed as micromoles per gram wet weight.

Redox status of glutathione

Tissue extract with 5% sulfosalicylic acid and reduced glutathione (GSH) quenched from the extract was applied to the NADPH-GR-DTNB-based oxidized glutathione (GSSG) recycling assay [19]. The amounts of (GSH + GSSG) and GSH are expressed as micromoles GSH equivalent per g liver. The amount of GSSG is expressed as micromoles per g liver. The redox status of glutathione was measured by the molar ratio of GSH and GSSG (GSH/GSSG).

Lipid peroxidation

Hepatic oxidative damage at the end of perfusion was assessed by the total amounts of malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) using an LPO586 kit (Oxis International, Foster City, CA) in accordance with the manufacturer's instructions [19]. Microsomal fraction was obtained from the 10% (%w/v) homogenate containing butylated hydroxytoluene (0.05%). The summation of MDA and 4-HNE contents were calculated and expressed as the nmol 4-HNE equivalent per mg of wet tissue weight [19].

Cell death and inflammatory signals

The stress-associated cell death pathway was assessed by western blot analysis as described previously (15). Frozen tissue (50 mg) was homogenized in ice-chilled lysis buffer containing EDTA-2Na, NaF, sodium orthovanadate, Nonidet P-40, and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). After the serial centrifugations at 1000 \times g for 10 min, and the resulting supernatant at 15,000 \times g for 10 min, they were stored at -80° C. Protein concentration was measured using a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

After solubilization and denaturing, 40 mg of protein was applied to Any-kD precast gel (Bio-Rad). Standard SDS polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblots were performed. The dilutions of primary antibodies were 1:1000 (pan-MKK4, phospho-MKK4, pan-JNK, phospho-JNK, Caspase3, GAPDH), and that of horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody was 1:5000. Protein bands were detected by a chemiluminescent detector Chem Doc XRS[®] (Bio-Rad) using a chemiluminescence reagent, West Dura (Thermo Scientific). The

band density of GAPDH was determined as a loading control, and the ratio of phosphorylated vs. total (pan) or that of cleaved vs. intact were calculated.

Statistical analyses

Values are expressed as the means ($n=6$). The Student's *t* test or one-way ANOVA was used for evaluating statistical significance. A *p* value of <0.05 was considered significant. Statistical analyses were performed using Stat View 5.0 for Windows (SAS Institute Inc., Cary, NC). * $p < 0.05$ (NT vs. H₂), † $p < 0.05$ (vs. CT).

Results

Liver damage

In the CT group, the liver histology at the end of perfusion appeared to be almost normal. In the NT group, nuclear condensation and severe necrosis were observed, whereas in the H₂ group these changes were attenuated in Fig. 1a. Apoptosis at the end of perfusion was minimal in the CT group, whereas it was significantly augmented in the NT group, and significantly suppressed in the H₂ group (Fig. 1b, c).

In the CT group, accumulation of ALT and LDH in the perfusate during 90 min of reperfusion was 0.13 ± 0.05 and 0.08 ± 0.04 (IU/g liver), respectively. In the NT group, the ALT and LDH leakage were significantly augmented to 1.47 ± 0.61 and 4.01 ± 2.44 (IU/g liver), respectively, whereas they were significantly suppressed to 0.83 ± 0.24 and 1.25 ± 0.75 (IU/g liver) in the H₂ group, respectively

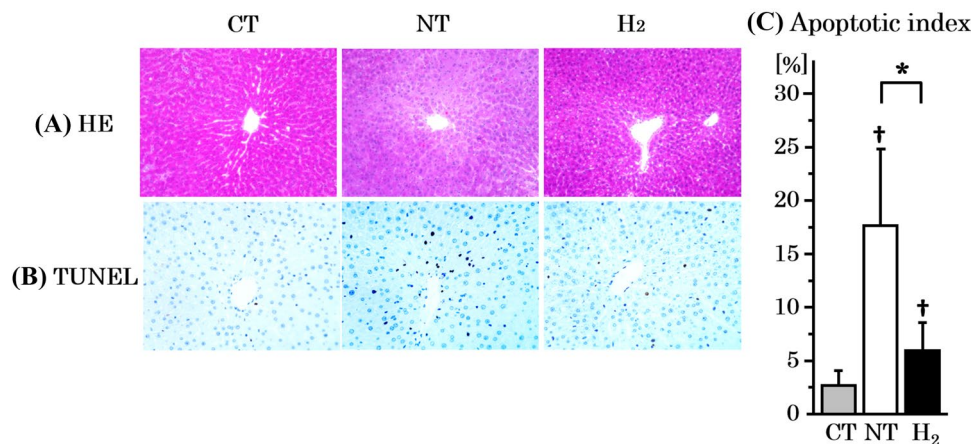


Fig. 1 Tissue damage after 90 min of reperfusion. Hematoxylin and eosin staining ($\times 20$ magnifications) revealed minimal changes in the control (CT) group. Severe vacuolization, pericentral necrosis, and nuclear condensation were observed in the no treatment (NT) group, but these changes were suppressed in the hydrogen gas (H₂) group

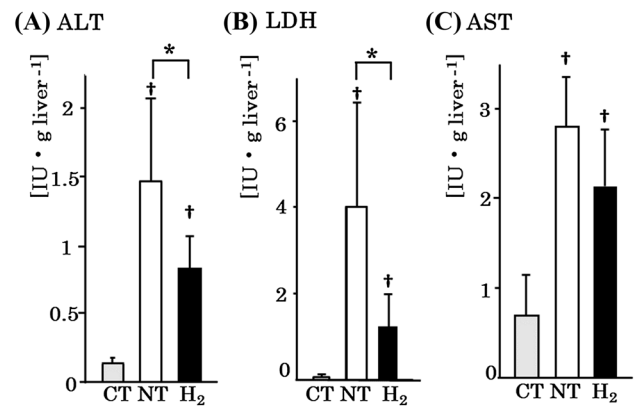


Fig. 2 The hepatic enzyme leakage after 90 min of reperfusion was minimally changed in the CT group. H₂ reduced the alanine aminotransferase (ALT) (a, $p < 0.05$), lactate dehydrogenase (LDH) (b, $p < 0.05$) and aspartate aminotransferase (AST) (c, $p = 0.08$) activities [$n=6$, means; * $p < 0.05$ (NT vs. H₂, Student's *t* test), † $p < 0.05$ (vs. CT, Student's *t* test)]

(Fig. 2a, b). AST activity showed a similar trend, but did not reach significance ($p = 0.08$) (Fig. 2c).

Hepatic microcirculation

After 30 min of equilibration perfusion, the graft was perfused at constant pressure (12 cmH₂O). In the CT group, portal vein resistance (PVR) was lowest throughout perfusion. In the NT group, it was highest throughout perfusion, whereas augmentation of PVR was significantly suppressed in the H₂ group (Fig. 3a).

(a). Few TUNEL-positive cells were observed in the CT group. These cells were increased in NT group, but were significantly suppressed in the H₂ group (b). The apoptotic index was significantly suppressed in the H₂ group (c) ($p < 0.01$) [$n=6$, means; * $p < 0.05$ (NT vs. H₂, Student's *t* test), † $p < 0.05$ (vs. CT, Student's *t* test)]

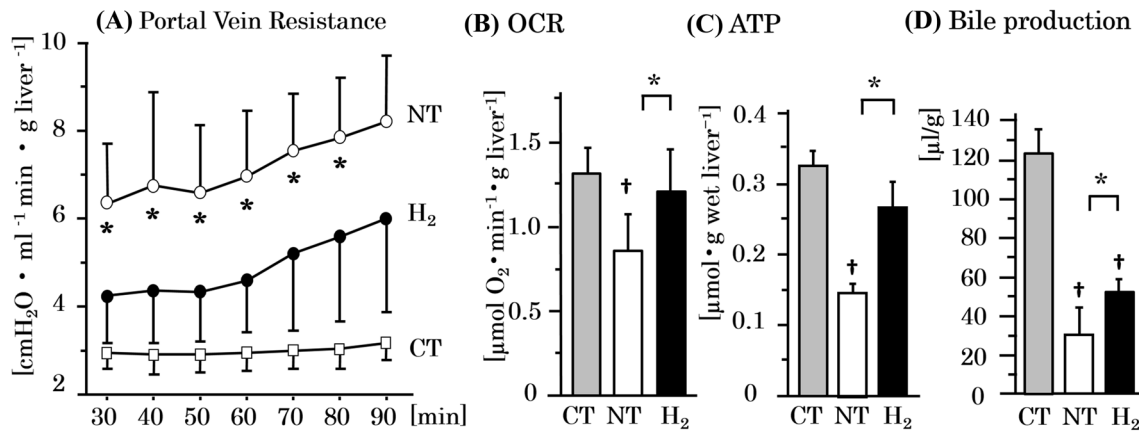


Fig. 3 Microcirculation and liver function. Portal vein resistance (PVR) was the lowest in the CT group and highest in NT group, while the elevation was significantly suppressed in the H₂ group (a) ($p < 0.05$). The oxygen consumption rate (OCR) (b) ($p < 0.05$), aden-

osine triphosphate (ATP) content (c) ($p < 0.01$), and bile production (d) ($p < 0.01$) were lowest in the NT group, but the decrease was significantly suppressed in the H₂ group [$n = 6$, means; $*p < 0.05$ (NT vs. H₂, Student's *t* test), $†p < 0.05$ (vs. CT, Student's *t* test)]

Mitochondrial respiration

Mitochondrial respiratory function was evaluated by the oxygen consumption rate (OCR). By 90 min after perfusion, it was highest in the CT group ($1.31 \pm 0.15 \mu\text{mol O}_2/\text{min/g liver}$). It fell to $0.86 \pm 0.21 (\mu\text{mol O}_2/\text{min/g liver})$ in the NT group, whereas the decrease was significantly suppressed in the H₂ group, at $1.20 \pm 0.25 \mu\text{mol O}_2/\text{min/g liver}$ (Fig. 3b). The ATP content at the end of perfusion was highest in the CT group ($0.30 \pm 0.05 \mu\text{mol/g liver}$). It was significantly lower in the NT group ($0.13 \pm 0.03 \mu\text{mol/g liver}$), but the decrease was significantly suppressed in the H₂ group ($0.24 \pm 0.08 \mu\text{mol/g liver}$; Fig. 3c).

Bile production

Integrated hepatic function was assessed by bile production during 90 min of perfusion. Bile production was highest in the CT group ($124 \pm 13 \mu\text{l/g liver}$). It was significantly lower in the NT group ($30 \pm 14 \mu\text{l/g liver}$), but the decrease was significantly suppressed in the H₂ group ($52 \pm 7 \mu\text{l/g liver}$; Fig. 3d).

Redox status and lipid peroxidation

The molecular ratio of reduced and oxidized glutathione (GSH/GSSG) was 40 ± 21 in the CT group. This was significantly reduced in the NT group (19 ± 6), but the decrease was significantly suppressed in the H₂ group (35 ± 13 ; Fig. 4a). The sum of MDA and 4-HNE at the end of the perfusion did not differ significantly among the groups (Fig. 4b).

(A) GSH/GSSG (B) LPO

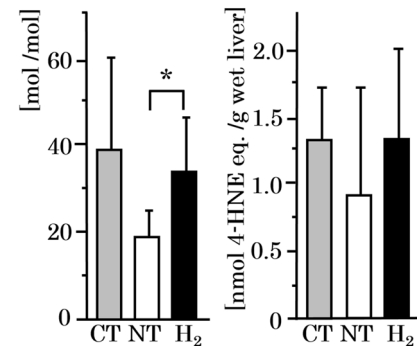


Fig. 4 Redox status and lipid peroxidation at the end of 90 min of reperfusion. The reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio decreased in the NT group, but this decrease was suppressed in the H₂ group (a) ($p < 0.05$). Lipid peroxidation (LPO) assessed by MDA+4-HNE did not change significantly among the groups (b) [$n = 6$, means; $*p < 0.05$ (NT vs. H₂, Student's *t* test)]

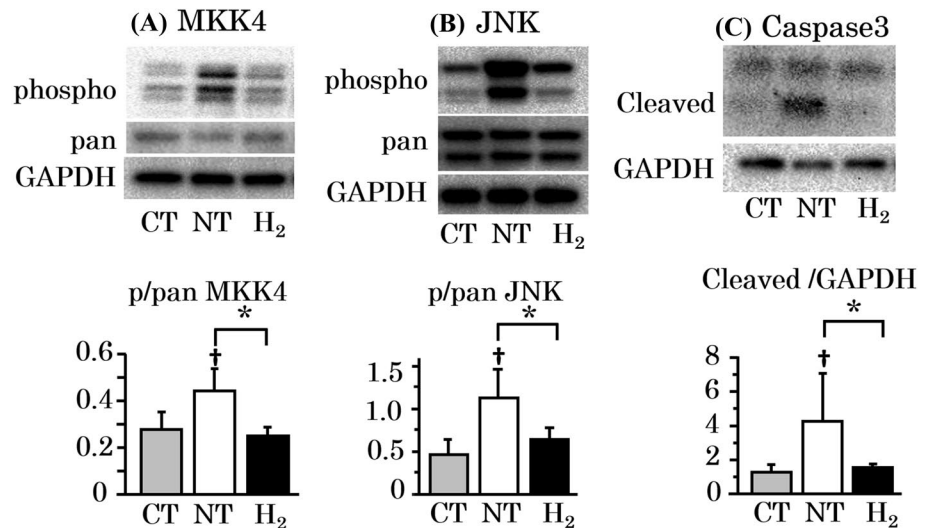
Cell death and inflammatory signals

Acute stress responses were evaluated by western blot of the cytoplasmic MKK4–JNK pathway. The NT group had significantly higher values than both the CT group and the H₂ group (Fig. 5a, b). Caspase 3, a downstream constituent of the MKK4–JNK pathway, was increased in the NT group, but was inhibited significantly in the H₂ group (Fig. 5c).

Discussion

We demonstrated the efficacy of H₂ in DCD grafts with CS, evidenced by the maintenance of microcirculation, mitochondrial function, and suppressed cellular death. Although

Fig. 5 The mitogen-activated protein kinase 4 (MKK4)–c-jun N-terminal kinase (JNK) cell death signal pathway. Cytosolic protein in the liver at the end of reperfusion was applied to the western blot (top) and the relative band intensity (bottom) was calculated. The phosphorylation of MKK4 (a) ($p < 0.001$) and JNK (b) ($p < 0.01$) and the cleavage of caspase 3 (c) ($p < 0.05$) were highest in the NT group, but were significantly suppressed in the H₂ group [$n = 6$, means; * $p < 0.05$ (NT vs. H₂, Student's *t* test), † $p < 0.05$ (vs. CT, Student's *t* test)]



the precise mechanism of H₂-mediated protection is still not fully understood, the MKK4–JNK pathway was shown to be a therapeutic target.

H₂ ameliorated hepatic reperfusion injury after warm ischemia in mice [13] and pigs [21]. H₂ administration during reperfusion reduced hepatic reperfusion injury after prolonged CS in heart-beating rat donors [18]. In the DCD liver, minimal change was observed at the end of CS and/or machine perfusion, but the injury became apparent during reperfusion [22, 23]. Accordingly, we tested whether post-reperfusion treatment by H₂ suppressed IRI in DCD grafts with CS. The H₂ concentration in the perfusion circuit is calculated as 29.3 μM theoretically, and 11.5 μM in reality [18]. Moreover, the possible amount of H₂ leaked from the perfusion circuit is sufficiently below the explosion hazard concentration of 4.7% in air [8].

In this study, the GSH/GSSG ratio was decreased in the NT group, but was augmented in the H₂ group, leading to minimization of the IRI. GSH is converted to GSSG through its function as a co-factor for antioxidant enzymes [19]. GSSG, on binding with the protein thiol, reduces free GSH levels gradually, thereby reducing the availability of GSH, antioxidant enzyme activity, and increasing oxidative stress [24, 25]. Mitochondrial function is maintained in hepatic warm ischemia, and reperfusion with NAPDH and ATP is maintained, with both the GSH and GSH/GSSG recovering early after reperfusion, suppressing subsequent impairment [19]. In this study, GSH/GSSG and ATP recovered quickly, supporting the suppression of tissue injury.

Lipid peroxidation (LPO) products, assessed by the summation of MDA and 4-HNE, did not differ significantly among the groups. This is controversial because H₂ is recognized as a radical scavenger [8, 18]. Lipid hydroperoxides, the initial products of LPO, are produced even under hypoxia, whereas MDA and 4-HNE are produced by a

cleavage of hydroperoxides in an oxygen-dependent manner. Therefore, lower values of MDA and 4-HNE do not necessarily mean the absence of lipid peroxidation, especially under conditions of low inconsistent oxygen delivery [19]. A possible explanation for the discrepancy is that H₂ administration suppressed the ROS-mediated initiation of LPO. Concomitantly, H₂ increased oxygen delivery, thereby possibly accelerating cleavage of lipid hydroperoxide. Conversely, in the NT group, the production efficiency of MDA and 4-HNE decreased with the reduced oxygen supply. These results indicate that the main effect of H₂ lies in the improvement of microcirculation rather than in radical scavenging.

The energy state (Fig. 3c) and oxygen consumption rate (Fig. 3b) suggested protection of mitochondrial function. Mitochondrial aerobic metabolism promotes an increase in the production of ROS [26]. In this study, H₂ treatment resulted in high GSH/GSSG (Fig. 4a), a constant LPO level (Fig. 4b), and augmented bile productivity (Fig. 3d). Therefore, it cannot be assumed that rising OCR means enhancement of oxidative damage. From these data, hydrogen gas was observed to protect mitochondrial function and breaking the vicious cycle of mitochondrial dysfunction and oxidative stress. Since bile duct complications are an important issue in liver transplantation using DCD grafts, the effect of H₂ treatment on bile duct injury should be evaluated by a liver transplantation model with arterial reconstruction in future studies.

Mitochondria-dependent apoptosis in hepatic IRI [15] and transplantation [27] are promoted in part by JNK, which was also shown to amplify ROS generation after translocation to mitochondria [28]. Moreover, the inhibition of JNK reduced cardiac IRI [29] and improved outcome after hepatic cold preservation and transplantation [30]. In the present study, cytosolic phospho-JNK and

phospho-MKK4 were much lower in the H₂ group than in the NT group (Fig. 5a, b), as were apoptosis and cleavage of caspase 3 (Figs. 1b, 5c). To our knowledge, this is the first report to demonstrate the involvement of MKK4–JNK axis as a target cascade of H₂ treatment. Since MKK4 and JNK rapidly translocate to mitochondria upon oxidative stress and enhance oxidative stress thereafter [29, 31], determining whether H₂ works on the mitochondrial MKK4–JNK pathway is important. However, we could not evaluate MKK4–JNK in mitochondria, due to the difficulty in the isolation of mitochondria in severely damaged tissue. Biological responses in mitochondria are now under investigation.

H₂ reduced vascular resistance in hypertensive rats [32] and in porcine DCD lungs [33]. In this study, the H₂-mediated reduction of PVR was evident within 15 min, but increased with perfusion time, with detritus on the in-line filter, regardless of H₂ treatment and was not observed in the CS model. In fact, the liver weight was decreased in the DCD model after perfusion without affecting the water and non-water contents. Therefore, it is speculated that constant flow perfusion efficiently washes out embolization factors; however, re-entry of small particles that could pass the in-line filter may cause a gradual increase of PVR. These results imply that H₂ reduced PVR, independent of the washout and/or organ swelling factors.

The blood levels of inflammatory cytokines, such as IL-1beta and TNF alpha, were elevated within 3 h of reperfusion [34], and maintained high expression thereafter [35]. Since H₂ treatment suppressed TNF alpha and HMGB1 levels in a sepsis model [36], we evaluated pro-inflammatory transcription factors, including NF kappa B (p65 subunit) and AP-1 (c-Fos), in the nuclear fraction. These factors did not differ significantly among the groups [37]. Moreover, phosphorylation of cytoplasmic c-Fos, c-Jun, ASK1, p38 MAPK, Erk1/2, p90RSK, PI3k, PDK1, Akt, mTOR, and cPLA₂ did not change significantly with H₂ treatment (data not shown). The absence of inflammation in this model could be explained by the absence of leukocytes, the approximately 30-times dilution by perfusate (vs. circulating volume in vivo), and the end-point of 90 min. These results imply that extracorporeal acellular perfusion may become a useful tool to condition the DCD graft without enhancing the inflammatory cascade.

In conclusion, post-reperfusion treatment by H₂ gas for a DCD liver graft with cold preservation suppressed reperfusion injury, primarily by maintaining hepatic microcirculation and also by protecting mitochondria. Thus, H₂ treatment contributed to liver protection by the maintaining redox status as well as inhibiting the MKK4–JNK pathway.

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Compliance with ethical standards

Conflict of interest We have no conflicts of interest to declare.

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