

Hydrogen as a Comprehensive Therapy For Ischemia/Reperfusion Injury after Lung Transplantation

Tomohiro Kawamura^{1*}, Toru Kimura¹, Soichiro Funaki¹, Yasushi Shintani¹, Masato Minami¹, Atsunori Nakao² and Meinoshin Okumura¹

¹Department of General Thoracic Surgery, Osaka University Graduate School of Medicine, Japan

²Department of Emergency, Disaster and Critical Care Medicine, Hyogo Medical College of Medicine, Japan

***Corresponding author:** Tomohiro Kawamura, Department of General Thoracic Surgery, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka, Japan, Tel: +81-6-6879-3152; Fax: +81-6-6879-3164; Email: tkawamu@thoracic.med.osaka-u.ac.jp

Published Date: January 30, 2016

INTRODUCTION

Lung Transplantation (**LTx**) has been established as a last resort treatment for end-stage lung disease under refractory conditions [1]. However, despite the increasing number of LTxs performed annually, overall outcomes remain unsatisfactory compared to reports of other solid-organ transplants [2]. Reactive Oxygen Species (**ROS**)-induced oxidative stress is thought to be involved in various disease states associated with organ transplantation, such as Primary Graft Dysfunction (**PGD**) or Ischemia/Reperfusion (**I/R**) injury and activation of inflammation and other immune responses, resulting in potential organ rejection and bronchiolitis obliterans syndrome, among other postoperative complications [3,4]. Therefore, development of a novel, efficient, and comprehensive therapeutic strategy to successfully abrogate the effects of oxidative stress and I/R injury could significantly improve LTx recipients' short- and long-term outcomes.

The therapeutic medical gas hydrogen has been studied in various clinical and experimental disease models in several biomedical fields. Hydroxyl radicals are the most harmful ROS in I/R injury and indiscriminately and aggressively damage cellular macromolecules, including lipids, proteins, and DNA, and lead to transplant-associated alterations in morphology and graft function. Hydrogen scavenges selectively for peroxy nitrite and hydroxyl radicals and may also act as a gaseous signaling molecule similar to nitric oxide [5,6].

Our laboratory has investigated the use of inhaled hydrogen to treat acute lung injuries and found that inhaled hydrogen treatment diminishes lung injury induced by mechanical ventilation (MV) [7] and LTx [7,8] (Figure1). We found that inhaled hydrogen gas induces Heme Oxygenase (HO)-1 through the kelch-like ECH-associated protein (Keap) 1/Nuclear factor E2-related factor (Nrf) 2/ Antioxidant Response Element (ARE) pathway [9]. Successful translation of this novel inhaled hydrogen therapeutic approach to clinical LTx may tremendously impacts LTx outcomes as well as a wide range of clinical applications for acute lung injury.

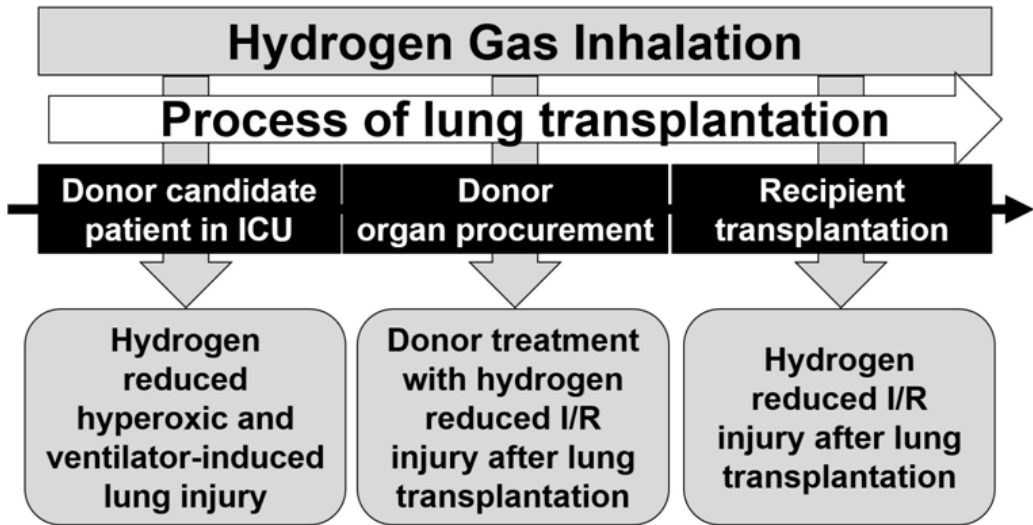


Figure 1: The therapeutic effect of hydrogen on lung transplantation.

In the present chapter, we provide an overview of our following recent research findings:

1. Recipient treatment with hydrogen gas reduces I/R injury after LTx.
2. Donor treatment with hydrogen gas reduces I/R injury after LTx.
3. Hydrogen gas reduces hyperoxic lung injury via the Nrf2 pathway.

RECIPIENT TREATMENT WITH HYDROGEN GAS REDUCES I/R INJURY AFTER LUNG TRANSPLANTATION (FIGURE2)

Although investigators have proposed disparate mechanisms that lead to tissue and organ dysfunction after I/R injury, abundant amounts of ROS are generated during vascular reperfusion

and undoubtedly play important roles in the I/R process [10]. A fundamental idea underlying our studies of hydrogen's therapeutic effects is that hydrogen functions as a radical scavenger [6] (Figure 2).

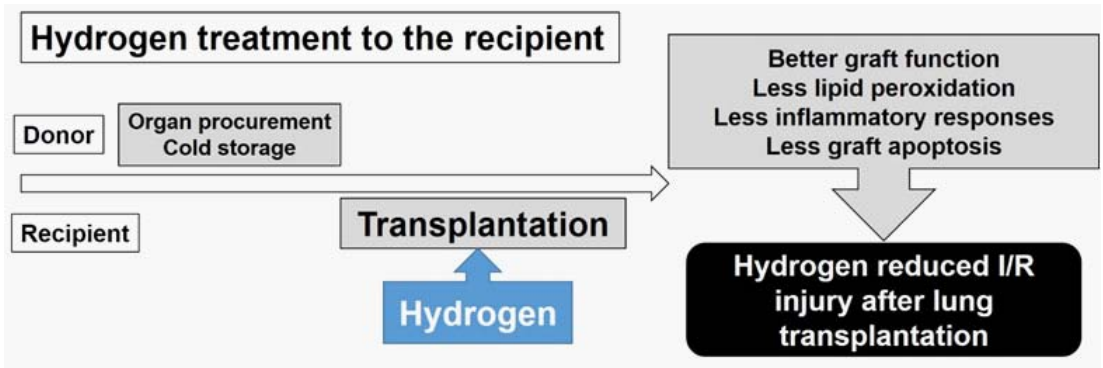


Figure 2: Recipient treatment with hydrogen gas reduces I/R injury after lung transplantation.

We hypothesized that hydrogen could lessen lung LTx-associated I/R injury. To test our hypothesis, we conducted a study utilizing an orthotopic LTx rat model to which inhaled 2% hydrogen was applied for less than two hours.

Hydrogen Inhalation Alleviated Graft Dysfunction after Reperfusion

Orthotopic syngenic left LTx (Lewis to Lewis rats) was performed with six hours cold ischemia time in cold low potassium dextran. The recipients were ventilated with 100% oxygen (O_2) or mixed gas with 98% O_2 plus 2% nitrogen (N_2) or 2% hydrogen (H_2) from the time of intubation to one hour after reperfusion. Prolonged cold storage in low potassium dextran disrupted pulmonary graft function on ventilation with 100% O_2 and resulted in a remarkable decrease in partial pressure of oxygen (pO_2) in the graft pulmonary vein two hours after reperfusion compared with sham-operated rats. Inhalation of 2% N_2 in 98% O_2 did not improve graft lung gas exchange. However, graft function was significantly better in the presence of 2% H_2 .

Hydrogen Inhalation Ameliorates Inflammatory Response-Associated I/R Injury and Reduces Graft Lipid Peroxidation

Lung cold I/R injury is accompanied by an increased expression of several proinflammatory cytokines [11]. The mRNAs for Intracellular Adhesion Molecule (ICAM)-1, Tumor Necrosis Factor (TNF)- α , Interleukin (IL)-1 β , and IL-6 were significantly unregulated compared with sham-operated animals when the recipients received nitrogen ventilation. Treatment with 2% hydrogen significantly reduced upregulation of these inflammatory mediators.

Recruitment and sequestration of activated alveolar macrophages play critical roles in lung I/R injury [12,13]. Immunohistochemical staining for ED1 (anti-CD68) revealed that macrophage infiltration and sequestration were significantly reduced when the recipients were ventilated with 2% hydrogen compared with nitrogen.

After I/R, nitrogen-ventilated lung grafts showed significantly higher levels of Malondialdehyde (MDA), a lipid peroxidation marker. Treatment with hydrogen gas significantly reduced tissue MDA levels two hours after reperfusion, demonstrating hydrogen treatment's antioxidant effects.

Hydrogen Mitigates I/R-Induced Graft Apoptosis

Pulmonary epithelial cell apoptosis is one of the deleterious factors leading to lung graft dysfunction [14,15]. Extended cold preservation followed by transplant and reperfusion in the presence of nitrogen increased apoptosis of epithelial cells six hours after reperfusion, as found with terminal deoxynucleotidyl transferase-mediated deoxyuridine Triphosphate Nick-End Labeling (TUNEL) staining. Inhaled hydrogen decreased apoptotic pneumocytes in the lung grafts and resulted in significant upregulation of the mRNAs and levels of the anti-apoptotic proteins B-cell lymphoma-Extra Large (Bcl-xL) and B-cell lymphoma-2 (Bcl-2) and down regulation of the mRNAs and levels of the pro-apoptotic protein Bcl-2-associated X-protein (Bax) in the grafts.

Our data demonstrated that administration of hydrogen to the recipient prevented I/R injury after LTx.

DONOR TREATMENT WITH HYDROGEN GAS REDUCES I/R INJURY AFTER LUNG TRANSPLANTATION (FIGURE 3)

Lung grafts are exposed to many harmful events prior to procurement and many factors, including practices obligatory to the transplant procedure such as donor MV and cold ischemia, predispose lungs to PGD. Notably, MV using non-injurious ventilation settings and optimized strategies for cold preservation still cause subclinical lung injury, even without pre-existing lung injury, and may generate proinflammatory cytokines [16]. Prolonged MV is one of the donor risk factors for PGD [17]. Our previous study showed that hydrogen could alleviate ventilator-induced lung injury (Figure 1). We hypothesized that hydrogen preloaded in lung allografts from donors delivered through donor airways prior to harvesting may lessen I/R injury and improve transplant outcomes.

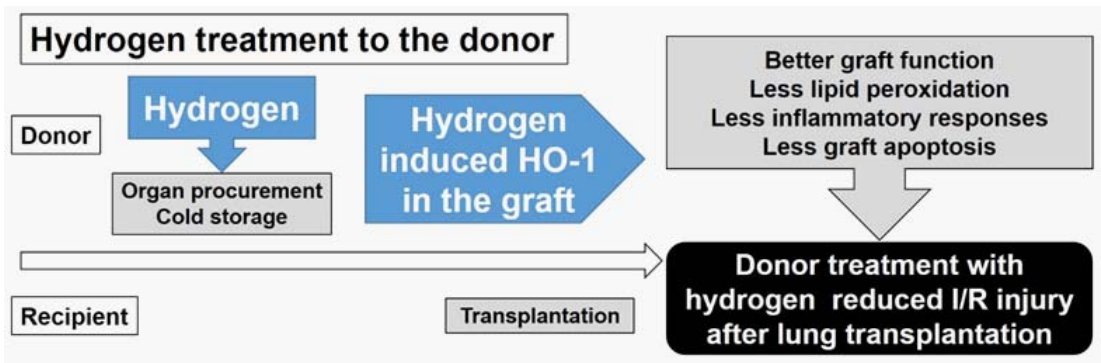


Figure 3: Donor treatment with hydrogen gas reduces I/R injury after lung transplantation.

Hydrogen Administration to the Donor Alleviates Graft Dysfunction after Reperfusion

To achieve our goals, we utilized a rat allogeneic LTx model (Lewis to Brown Norway rats), exposing donors to either 2% H₂ or 2% N₂. Donor rats underwent tracheotomy and underwent MV with a gas mixture of either 98% O₂ and 2% N₂ or 98% O₂ and 2% H₂. The gas was retained in the graft during cold storage.

Cold ischemia and prolonged MV harmed pulmonary function in grafts from donors treated with 2% N₂ and compared with sham rats, resulted in a significant decrease in pO₂ two hours post reperfusion. Donor hydrogen treatment significantly improved pO₂ levels two hours after reperfusion.

Hydrogen Inhalation Reduces Proinflammatory Cytokines and Graft Apoptosis

Similar to the previous study, donor treatment with 2% H₂ significantly decreased up regulation of the proinflammatory cytokines TNF- α , IL-1 β , and ICAM-1 mRNAs. TUNEL stain and real-time RT-PCR for Bcl-2 and Bax revealed that donor hydrogen treatment reduced graft apoptosis two hours after reperfusion.

Graft Characteristics Prior to Implantation and Reperfusion

Because lung allograft injury was significantly reduced when the donors received 2% hydrogen ventilation, we investigated whether the lung grafts underwent any changes after hydrogen exposure but before transplantation. Surprisingly, lung edema gas and exchange after three hours of MV were comparable regardless of inhaled gas treatment and there were no apparent histological differences. Thus, hydrogen's protective effects were not evident before transplantation and, in our experimental protocol; MV (with 98% O₂ and 2% N₂ or 2% H₂) did not result in lung graft injury in the absence of cold storage, implantation, and reperfusion.

Although no obvious alterations in graft function and morphology were found prior to transplantation, our data clearly demonstrate that donor hydrogen treatment lessened lung I/R injury. Hence, we studied lung allografts prior to implantation and found that hydrogen increased the expression of HO-1, which has antioxidant, anti-inflammatory, and cytoprotective activities. Hydrogen treatment resulted in significant upregulation of HO-1 mRNA and protein levels in the allografts after MV for three hours and cold preservation for four hours prior to implantation. This improved graft function was accompanied by an increase in HO-1 expression in the grafts.

This study demonstrated that donor hydrogen treatment alleviated I/R injury after LTx and that HO-1 induction in the grafts may be one of the mechanisms underlying hydrogen inhalation therapy's protective effects.

HYDROGEN GAS REDUCES HYPEROXIC LUNG INJURY VIA THE NRF2 PATHWAY (FIGURE 4)

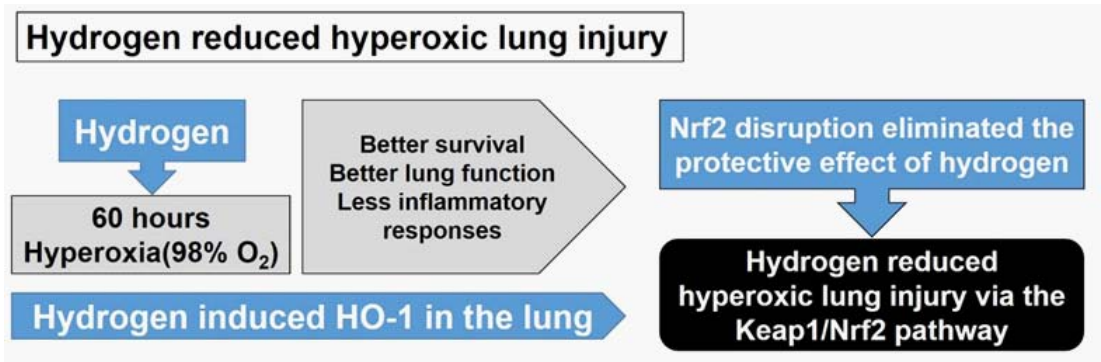


Figure 4: Hydrogen gas reduces hyperoxic lung injury via the Nrf2 pathway.

Patients in the Intensive Care Unit (ICU) for the treatment of critical conditions often require ventilator support, which can induce lung injury and worsen pre-existing lung injury, which can lead to respiratory failure. Protecting the lung is very important when treating ICU patients.

If the patient suffers an unfortunate brain death, he or she becomes a donor candidate. Therefore, protecting the lung graft should be considered in the therapeutic approach to ICU patients.

Although the need for donor lungs has steadily grown, over the years the number of donors has remained constant; the current utilization of donor lungs is unacceptably low (only 15-20% of potential donors) [18]. Institutions have explored strategies to expand the criteria for the acceptance of donor lungs to ameliorate the donor shortage. Preventing organ injury through donor medical management may therefore largely impact the availability of viable organs for transplant and transplant outcomes.

In the previous experiment, we showed that hydrogen can induce HO-1 and may act as a gas signaling molecule. To elucidate the mechanisms underlying the protective effect of hydrogen, we focused on the Keap1/Nrf2/ARE signaling pathway. Nrf2 is a transcription factor that mediates a broad-based set of adaptive responses to endogenous and exogenous oxidative stressors by inducing expression of a number of antioxidant and cytoprotective factors, including HO-1, through promoter activation via the ARE [19]. Thus, it is considered an essential pathway for protection against oxidative stress and resulting forms of lung injury [19,20].

To accomplish our goals, we utilized a hyperoxic lung injury model to explore whether the protective effects of hydrogen would be omitted without Nrf2.

Hydrogen Gas Ameliorates Lung Dysfunction After Hyperoxia And Prolongs Survival from Lethal Hyperoxia in Rats

Sixty hours of exposure to a high oxygen concentration (>95%) decreased lung function, as was demonstrated by the remarkable drop in pO_2 in rats exposed to 98% O_2 , 2% N_2 (hyperoxia) compared with rats exposed to normoxia (2% N_2). Administration of 2% H_2 during hyperoxic exposure significantly improved blood oxygenation. All rats exposed to hyperoxia with 2% N_2 died within 64 hours, while rats exposed to hyperoxia with 2% H_2 survived a median of 72 hours (range 72-120 hours). After 60 hours of hyperoxic exposure, the mRNAs for ICAM-1, TNF- α , IL-6, and IL-1 β were significantly upregulated compared with rats exposed to normoxia. The 2% hydrogen treatment during hyperoxic exposure significantly lessened peak expression of the transcript factors for these inflammatory mediators.

Hydrogen Induces HO-1 and Modulates the Keap1/Nrf2 Signaling Pathway

Immunohistochemical analysis demonstrated that more cells expressed HO-1 in the hyperoxia/ H_2 rats than in the hyperoxia/ N_2 rats. Immunofluorescent analysis for HO-1 and aquaporin-5, a lung epithelial cell marker, showed that the HO-1-positive cells were lung epithelial cells. Consistent with the immunohistochemical analysis, hydrogen increased HO-1 protein and HO-1 mRNA expression after 60 hours of hyperoxia exposure. Real-time RT-PCR revealed several Nrf2-dependent, cytoprotective genes, including Glutathione S-Transferase (**GST**) A2, NAD(P)H Dehydrogenase Quinone (**Nqo**)1, Peroxiredoxin (**Prdx**)1, UDP-Glucuronosyl Transferase (UGT) 1A6, and HO-1 after 60 hours of hyperoxia exposure. Hydrogen significantly upregulated these Nrf2-dependent transcript factors in hyperoxia-exposed rats.

Disruption of Nrf2 Impairs the Protective Effect of Hydrogen Against Hyperoxic Lung Injury

To confirm the role of Nrf2 in the alleviation of hyperoxic lung injury by hydrogen, we investigated whether hydrogen protects against hyperoxic lung injury in Nrf2-deficient (*Nrf2*^{-/-}) mice. Sixty hours of exposure to a high oxygen concentration without hydrogen reduced lung function and resulted in a notable decrease of pO_2 in both wild type and *Nrf2*^{-/-} mice. Hydrogen treatment remarkably enhanced blood oxygenation in the wild type mice, as it did in the rat model. However, hydrogen did not significantly augment blood oxygenation in *Nrf2*^{-/-} mice.

Although hydrogen supplementation significantly reduced lung MDA levels in wild type and *Nrf2*^{-/-} mice, the expression of Nrf2-dependent genes (GSTA2, HO-1, Nqo1) were not induced in response to hydrogen treatment in *Nrf2*^{-/-} mice. These results suggested that impairment of the Nrf2 gene stopped hydrogen's protective effect against hyperoxic lung injury.

DISCUSSION

PGD, a severe form of I/R injury analogous to Acute Respiratory Distress Syndrome (**ARDS**), arises within the first 72 hours following LTx. Occurring in 10-30% of LTx recipients, it remains

a major complication of LTx and significantly contributes to the risk of early mortality [21,22]. Furthermore, PGD increases the risk of acute rejection and the risk of developing bronchiolitis obliterans syndrome, thus also contributing to late mortality [17]. Several therapeutic agents or methods have been investigated in an effort to reduce the incidence of PGD during LTx, but no standardized strategy has yet been devised [17].

Since the discovery of hydrogen's antioxidant effects [6], many experimental and clinical studies have suggested that hydrogen gas may be a useful new therapy in several biomedical fields [5,23-27]. Our lab has thoroughly investigated the use of hydrogen to treat various diseases [28-30] including acute lung injuries [7,8]. Hydrogen is a selective radical scavenger for peroxy nitrite and hydroxyl radicals [6,31]; this characteristic of hydrogen's chemistry may explain its therapeutic effects. As we showed in the "recipient" experiment, hydrogen reduced lipid oxidation and proinflammatory cytokine expression and ameliorated I/R injury after LTx (Figure 2).

However, our "donor" treatment experiment revealed that hydrogen treatment resulted in significant upregulation of HO-1 in the allografts prior to implantation and ameliorated I/R injury after LTx (Figure 3). These data suggested that hydrogen may act as a gaseous signaling molecule. HO-1 is an anti-apoptotic/anti-inflammatory rate-limiting enzyme that catalyzes the conversion of heme into equimolar amounts of carbon monoxide, iron, and biliverdin (further reduced to bilirubin through biliverdin reductase) [32]. Animal studies have shown that HO-1 plays an important protective role in many lung diseases including hyperoxic lung injury, ARDS, asthma, pulmonary hypertension, and chronic obstructive pulmonary disease [33]. Although potential therapeutic approaches to modulating HO-1 expression in patients includes the use of gene therapy or pharmacologic agents [33], hydrogen treatment to induce HO-1 might be easier than other therapies to translate into clinical practice.

To clarify the mechanisms underlying the induction of HO-1 and the protective effect of hydrogen, we utilized a hyperoxic lung injury model and focused on the Keap1/Nrf2/ARE signaling pathway.

Nrf2 is a transcription factor that mediates a broad-based set of adaptive responses to endogenous and exogenous oxidative stressors by inducing expression of a number of antioxidant and cytoprotective factors, including HO-1, through promoter activation via the antioxidant response element [19]. Under non-stressed conditions, Keap1 recruits Nrf2 and the resultant complex is rapidly diverted to a degradation pathway via ubiquitination, the so-called proteasomal pathway. Once cells are subjected to oxidative stresses, thiol moieties in the Keap1 protein are oxidized, thereby triggering the dissociation of Keap1 from Nrf2, resulting in the stabilization and accumulation Nrf2. Thus, modulators of Keap1-Nrf2 complex formation may also induce anti-oxidant activities. Nrf2 disruption completely abrogated hydrogen's protective effects against hyperoxic lung injury (Figure 4). Induction of HO-1 in response to hydrogen treatment was greatly reduced in *Nrf2*^{-/-} mice, suggesting that HO-1 induction by hydrogen at least partially

depends on Nrf2. Our study demonstrated that hydrogen protects against lung injury both by reducing the extent of oxidative injury caused by ROS, perhaps through hydrogen's free radical scavenging functions, and by inducing Nrf2-dependent protective signaling pathways. Although the molecular mechanisms underlying hydrogen's actions are largely undefined, we showed that hydrogen is a novel activator of the Nrf2 pathway and therefore a potential therapeutic strategy.

In conclusion, our findings suggest an easily applicable and potentially novel and comprehensive solution to some of the complications of LTx and provide new insight into the scientific knowledge of hydrogen. Administering hydrogen treatment by providing inhaled gas to patients is straightforward and may be feasible in clinical LTx. Although further investigations are required, hydrogen may have an impact as a novel and innovative therapy for unmet medical needs that cause considerable health burdens, in particular for patients with lung disease who are critically-ill. Hydrogen has a high potential as a therapeutic medical gas and may be beneficial at multiple stages of the LTx process.

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