Hydrogen Inhalation Ameliorates Oxidative Stress in Transplantation Induced Intestinal Graft Injury

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Ischemia/reperfusion (I/R) injury during small intestinal transplantation (SITx) frequently causes complications including dysmotility, inflammation and organ failure. Recent evidence indicates hydrogen inhalation eliminates toxic hydroxyl radicals. Syngeneic, orthotopic SITx was performed in Lewis rats with 3 h of cold ischemic time. Both donor and recipient received perioperative air or 2% hydrogen inhalation. SITx caused a delay in gastrointestinal transit and decreased jejunal circular muscle contractile activity 24 h after surgery. Hydrogen treatment resulted in significantly improved gastrointestinal transit, as well as jejunal smooth muscle contractility in response to bethanechol. The transplant induced upregulation in the inflammatory mediators CCL2, IL-1β, IL-6 and TNF-α were mitigated by hydrogen. Hydrogen significantly diminished lipid peroxidation compared to elevated tissue malondialdehyde levels in air-treated grafts demonstrating an antioxidant effect. Histopathological mucosal erosion and increased gut permeability indicated a breakdown in posttransplant mucosal barrier function which was significantly attenuated by hydrogen treatment. In recipient lung, hydrogen treatment also resulted in a significant abatement in inflammatory mRNA induction and reduced neutrophil recruitment. Hydrogen inhalation significantly ameliorates intestinal transplant injury and prevents remote organ inflammation via its antioxidant effects. Administration of perioperative hydrogen gas may be a potent and clinically applicable therapeutic strategy for intestinal I/R injury.

Key words: Gastrointestinal motility, hydrogen, inflammation, intestinal transplantation, oxidative stress

Abbreviations: CCL2, chemokine (C-C motif) ligand 2; GC, geometric center; H_2O_2, hydrogen peroxide; IBS, irritable bowel syndrome; IL-6, interleukin-6; I/R, ischemia/reperfusion; KHBB, Krebs–Henseleit bicarbonate buffer; MDA, malondialdehyde; O_2^-, superoxide anion; $OH \cdot$, hydroxyl radical; PBS, phosphate-buffered saline; ROS, reactive oxygen species; RT-PCR, reverse transcriptase polymerase chain reaction; SITx, small intestinal transplantation; TNF-α, tumor necrosis factor-alpha.

Introduction

Hydrogen is the lightest and most abundant of chemical elements, constituting nearly 75% of the universe's elemental mass and provides the mass for stellate birth as well as the sun's source of energy by nuclear fusion with helium. In contrast, the earth's atmosphere contains less than 1 ppm of hydrogen. In concentrations over 5%, hydrogen can form explosive mixtures with air, which has been mythically typified by the 1937 Hindenburg disaster. Large inhalation concentrations of hydrogen are used as hydreliox, a mixture of hydrogen, helium and oxygen (49%, 50% and 1% mix, respectively), for very deep technical diving ventures because it shortens decompression time, prevents decompression sickness and averts nitrogen narcosis (1). Changes in neuromuscular drive with a decrease in skeleto-muscular function and bradycardia occurred during two hydroliox deep dives (HYDRA 8 and HYDRA 10). But, these changes were mainly attributed to the hyperbaric environment and not specifically to hydrogen inhalation (2,3). No reports regarding intestinal motility changes during hydrogen use in divers exist to our knowledge.

Physiologically, hydrogen gas of approximately 150 cc is produced daily by numerous strains of intestinal bacteria during fermentation of nondigestible carbohydrates primarily in the large intestine and is excreted as a natural component of abdominal gas (4), exhaled or further metabolized by colonic flora (5). Exhalation of hydrogen forms the basis for the routinely used hydrogen breath test for gastrointestinal transit and assessment of small intestinal bacterial overgrowth (6,7). It is well established that antibiotic treatment can alter results from H_2 breath tests by diminishing anaerobe H_2 producing microorganisms (8). In contrast to other gaseous bacterial byproducts in the gastrointestinal tract, hydrogen seems to have motility altering properties. In fact, irritable bowel syndrome (IBS) patients

with predominantly methane producing bacteria have a significant decrease in gastrointestinal transit compared with hydrogen producers (9). Antibiotic eradication of bacterial overgrowth in IBS dominant hydrogen producers results in symptomatic relief from diarrhea suggesting H_2 producing bacteria induced hypercontractility (10). Interestingly, the ability of the colonic flora to metabolize hydrogen has been suggested to be important in preventing the untoward effects during hydreliox diving (11,12).

Recently, it has been reported that inhaled hydrogen gas has antioxidant and antiapoptotic properties that can protect the brain and liver against ischemia/reperfusion (I/R) injury by selectively neutralizing hydroxyl radicals (13,14). I/R injury of the intestine occurs in a variety of clinical settings such as mesenteric artery occlusion, hypovolemic shock and small intestinal transplantation (SITx) (15,16). The ischemic intestine can be a major source of proinflammatory mediators that enhances not only local intestinal injury and dysfunction, but also the systemic inflammatory response leading to multiple organ failure (17,18). Although various mechanisms leading to intestinal dysfunction after I/R injury have been proposed, reactive radicals undoubtedly play a key role. Superoxide anion ($\cdot O_2^-$) radicals are generated in mitochondria by electron transport chain leakage and produced by metabolic oxidases (19). The excessive generation of $\cdot O_2^-$ radicals drives the production of H_2O_2 and subsequently hydroxyl radicals (OH·) via the Fenton reaction. OH· are the strongest of the oxidant species and react indiscriminately peroxidizing membrane lipids, oxidizing DNA and denaturing proteins (20,21).

Mammalian species are lacking endogenous detoxification systems for OH· radicals; therefore, scavenging or mitigating OH· during intestinal I/R injury could have significant beneficial effects on improving patient outcomes (22,23). The protium ion of H_2 has been shown to selectively inactivate OH· by forming water. Therefore, because of the distinctly selective nature of the inactivation, H_2 is thought not to significantly alter other reactive oxygen molecular intracellular signaling pathways controlling inflammation and apoptosis. In culture, protium is reported to prevent DNA oxidation, preserve mitochondrial membrane potential and thus ATP synthesis, as well as maintain cellular morphology (14). *In vivo*, hydrogen inhalation has demonstrated promise in a model of middle cerebral artery occlusion to decrease infarct area and improve neurological outcome (14). Furthermore, in a model of hepatic IR injury hydrogen inhalation prevented hepatic cell death and reduced serum alanine aminotransferase and hepatic malondialdehyde (MDA) levels (13). In this study, we hypothesized that hydrogen inhalation could ameliorate intestinal I/R injury following SITx with prolonged cold storage. We utilized the well-established orthotopic syngeneic SITx rat model to test this hypothesis, and found that hydrogen could reduce graft oxidative injury and inflammation. Compared to endolumenal administration of hydrogen producing bacteria (12,24) exogenous supplementation of hydrogen allows the usage of the beneficial effects of hydrogen devoid of the risks and discomfort of bacterial overgrowth such as infectious enteritis within the intestinal graft.

Methods

Animals

Inbred male LEW (RT.1^l) rats weighing 200–250 g were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN), and maintained in a laminar flow animal facility at the University of Pittsburgh on a standard diet and water supplied *ad libitum*. All procedures were performed in accordance with the guidelines of the IACUC at the University of Pittsburgh.

Transplant surgery and hydrogen treatment

Orthotopic syngeneic SITx with 3 h of cold ischemia in iced lactated Ringer's solution was performed, as previously described (25,26). The donor and recipient of each transplant were treated with air or 2% hydrogen/98% air (Praxair, Danbury, CT) supplied through the anesthetic gas vaporizer utilizing a facial mask (13). Gas inhalation started 1 h before harvest or graft implantation, and the recipient continued treatment inhalation during and 1 h after surgery. Unoperated sham animals were also given air or 2% hydrogen for 2 h under general anesthesia with isoflurane. Therefore, four experimental groups were used: Sham$_{air}$, Sham$_{H2}$, SITx$_{air}$ and SITx$_{H2}$. The number of experiments per group (N) refers to the quantity of individual animals used for each endpoint; the number of observations obtained from each individual animal is stated as the number (n) where appropriate.

Gastrointestinal transit

The effects of hydrogen on gastrointestinal function were investigated *in vivo* by measuring the distribution of orally fed nonabsorbable fluorescein-labeled dextran (Molecular probes, Eugene, OR; molecular weight = 70 000) in animals 24 h postoperatively (N = 6 each group). After a period of 90 min *in vivo* gastrointestinal transit time, the animals were sacrificed and the individual gastrointestinal transit distribution histograms were constructed as previously described (27) as well as statistically analyzed using the calculated geometric center (28).

In vitro *jejunal smooth muscle contractility*

To determine the direct effects of hydrogen on the sham and transplanted intestine, jejunal circular smooth muscle mechanical activity was measured, as previously described (27,29,30). Mid-jejunal segments were harvested 24 h after SITx, a time point when intestinal motility associated with SITx is known to be maximally suppressed (27,30,31). Mucosal layers were removed by microdissection and muscle strips were prepared in a standardized manner. The muscle strips were mounted in horizontal mechanical organ chambers and contractility was monitored using an isometric force transducer (ADInstruments, Colorado Springs, CO) (N = 6 each group, n = 4 each animal). The addition of bethanechol (0.3–300 μM) elicited a concentration-dependent increase in circular muscle contractility. Contractile activity was measured by integrating the area under the contractions, which were normalized by converting the weight and length of the strip to square millimeters of tissue (1.03 mg/mm^2), and reported as g/mm^2/s.

Histopathological analysis

Six segments (two proximal, two middle and two distal) of whole intestine measuring 1 inch of length were taken 3 h after surgery (N = 6 each group, n = 6 each animal). The intestinal tissue was fixed in 10% buffered formalin, embedded in paraffin, cut into 4 μm thick sections and stained with hematoxylin and eosin. Microscopic grading of mucosal injury was performed in a blinded fashion based on the criteria reported by Park et al. (32). The

degree of mucosal injury was evaluated by a 0–5 grading scale: grade 0 equaled to healthy mucosa and grades 1–5 indicated increasing degrees of villous damage. Recipient lung tissues were also fixed in 10% formalin, embedded in paraffin, sectioned into 6 μm thickness and stained with a naphthol AS-D chloroacetate esterase staining kit (Sigma Diagnostics, St. Louis, MO) for the presence of granulocytes (N = 5 each group) (33). Positively stained cells were counted in a blinded fashion and presented as a number of positive cells/×200 high power field (HPF).

qRT-PCR

The muscularis externa was stripped from the harvested small bowel of sham animals and transplanted recipients 3 h following graft reperfusion and snap frozen in liquid nitrogen (N = 10 each group). Recipient lung tissue was also taken at sacrifice and kept at −80°C until analysis. The mRNAs for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), chemokine (C-C motif) ligand 2 (CCL2), interleukin 1β (IL-1β), interleukin 6 (IL-6), tumor necrosis factor (TNF)-α and interleukin 10 (IL-10) were quantified in duplicate using SYBR Green two-step, real-time RT-PCR, as previously described (26,34). qRT-PCR primer sequences are listed in Supplementary Table S1.

Assessment of graft oxidative injury

Flushed jejunal full thickness segments from each experimental group were harvested 3 h after reperfusion and homogenized (N = 7 each group). Tissue MDA concentration, a marker of lipid peroxidation, was determined using the manufacturer's kit direction (Kit MDA-586; Oxidresearch, Portland, OR). Full thickness jejunal samples were also analyzed for reductive capacity using the commercially available kit, Total Antioxidant Power (Oxford Biomedical Research, Oxford, MI) (N = 7 each group).

Graft wall permeability

Utilizing the well-established everted gut sac method (30,35,36), intestinal graft wall permeability was determined 3 h postoperatively as mucosal to serosal permeability of fluorescein-isothiocyanate dextran (FD4; average M.W. 4000 Da) (N = 11 each group). Briefly, the everted gut segments were prepared in the ice-cold modified Krebs–Henseleit bicarbonate buffer (KHBB; pH 7.4) and suspended in the 80 ml KHBB containing FD4 (20 μg/ml) at 37°C. Following 30-min incubation, the fluid within the gut sac was collected and the fluorescence was measured using a Perkin-Elmer LS-50 fluorescence spectrophotometer (Palo Alto, CA). Permeability was expressed as the clearance of FD4 calculated using the equations as previously described (30,37).

Data analysis

The results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using unpaired Student's t-test or analysis of variance (ANOVA) where appropriate. EZAnalyze add-in for Microsoft Excel was used to perform the F-test with Bonferroni $post hoc$ group comparisons where appropriate. A probability level of $p < 0.05$ was considered statistically significant.

Results

Blood hydrogen and methane levels

The hydrogen level in arterial blood samples were measured by gas chromatography (Biogas analyzer BAS-1000, Mitleben, Osaka, Japan). Sham animals exposed to room air inhalation had an average arterial hydrogen level of 158.5 ± 6.4 ng/mL. Inhalation of 2% hydrogen for 2 h resulted in a significant elevation in arterial hydrogen levels to 578 ± 63.6 ng/mL. These results suggest that potentially

hydrogen can be systemically delivered to rodent tissues via inhalation. A significant intra-lumenal conversion of hydrogen to methane by intestinal bacteria appeared not to occur as methane concentrations in arterial blood of air inhaled animals averaged 158.6 ± 8.9 ng/mL and hydrogen inhalation for 2 h did not alter blood methane levels (154.1 ± 8.8 ng/mL) ($p < 0.05$).

Hydrogen inhalation prevented intestinal dysmotility

Anesthesia and 2% hydrogen inhalation did not alter the gastrointestinal transit distribution histograms measured from sham animals, as the majority of the nonabsorbable fluorescent dextran was localized in the distal segments of the small intestine and cecum in both groups (Figure 1A).
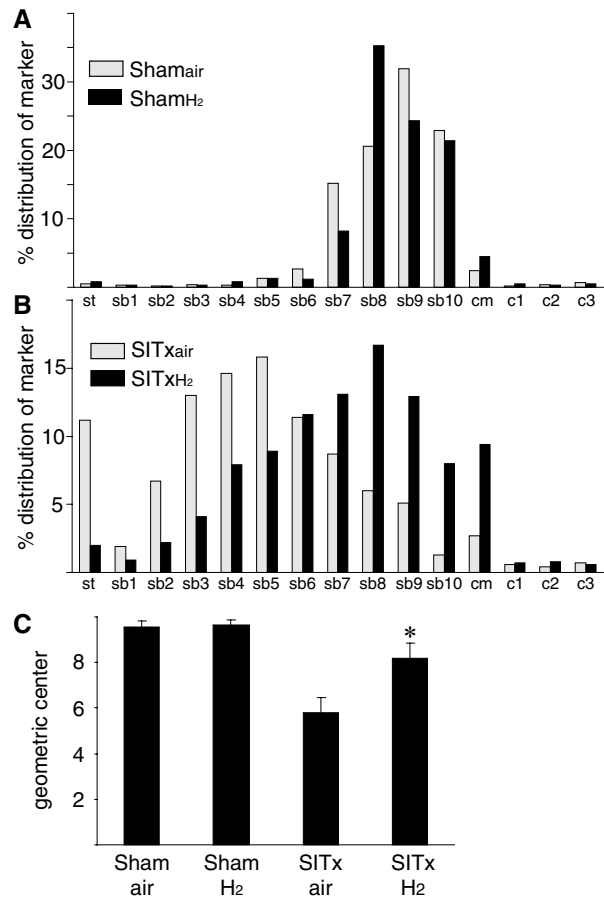
Figure 1: Gastrointestinal transit. Histogram of gastrointestinal transit showed a similar intestinal distribution of an orally fed fluorescent marker 90 min after oral administration in both Sham$_{air}$ and Sham$_{H2}$ (N = 6 each group) (A). Intestinal transplantation with prolonged cold storage caused a significant delay in gastrointestinal transit. Hydrogen inhalation significantly improved bowel motility 24 h after reperfusion (N = 6 each group) (B). The calculated transit geometric center (GC) measurements demonstrate that hydrogen inhalation therapy significantly preserves gastrointestinal function in rats undergoing SITx (*$p < 0.05$ vs. SITx$_{air}$, N = 6 each group) (C).

As previously demonstrated (27), SITx caused a significant delay in gastrointestinal transit 24 h postoperatively. In the air-treated SITx recipients ($SITx_{air}$), the fluorescent transit marker was found primarily in the stomach and proximal small bowel. In contrast, transplanted animals treated with hydrogen ($SITx_{H2}$) displayed markedly improved gastrointestinal transit with the fluorescein-labeled dextran progressing down to the distal segments of the small intestine (Figure 1B). The intestinal anastomosis sites did not appear to influence transit. The average calculated geometric centers for each group are plotted in histogram form in Figure 1C. These data demonstrate a statistically significant improvement in propulsive motility between animals transplanted with hydrogen versus air inhalation ($p < 0.05$). There was no difference in the length of small intestine between $SITx_{air}$ (53.3 ± 1.5 cm) and $SITx_{H2}$ animals (51.8 ± 0.7 cm) ($p > 0.05$).

### Hydrogen improved jejunal smooth muscle contractility of the intestinal grafts

In vitro contractile activity of jejunal circular muscle strips in response to increasing concentrations of the muscarinic agonist bethanechol was quantified. Muscle strips from $Sham_{air}$ animals generated regular spontaneous contractions and this activity was not significantly changed by hydrogen sham treatment. Although transplantation resulted in a significant decrease in spontaneous muscle contractile activity in $SITx_{air}$ animals (Figure 2A and B), jejunal muscle strips harvested from $SITx_{H2}$ animals tended to demonstrate significantly greater spontaneous contractile activity compared to transplant grafts with air inhalation. The addition of bethanechol (0.3–300 μM) to the organ bath perfusate elicited a similar concentration-dependent increase in circular smooth muscle contractility in $Sham_{air}$ and $Sham_{H2}$ animals. $SITx_{air}$ animals displayed a significant suppression in bethanechol-stimulated contractions (Figure 2A and B). In contrast, $SITx_{H2}$ animals revealed a significantly more robust bethanechol-stimulated circular smooth muscle contractile response compared to $SITx_{air}$, which neared sham muscle responses ($p < 0.05$). The average weight/length adjusted values of each group's response to bethanechol (100 μM) were $Sham_{air}$ = 2.6 ± 0.26, $Sham_{H2}$ = 2.7 ± 0.34, $SITx_{air}$ = 1.3 ± 0.33 and $SITx_{H2}$ = 2.6 ± 0.27 g/mm²/s.

Hydrogen inhibited muscularis inflammatory mRNA upregulation

Intestinal transplantation with air inhalation significantly induced the proinflammatory mRNAs of IL-1β, IL-6 and CCL2 within the isolated intestinal muscularis 3 h after transplantation. Hydrogen treatment significantly prevented this induction in the mRNA levels of these inflammatory mediators ($p < 0.05$). The level of mRNA for IL-10, known to be an antiinflammatory cytokine, was also significantly induced following transplantation at the same time point. However, hydrogen treatment did
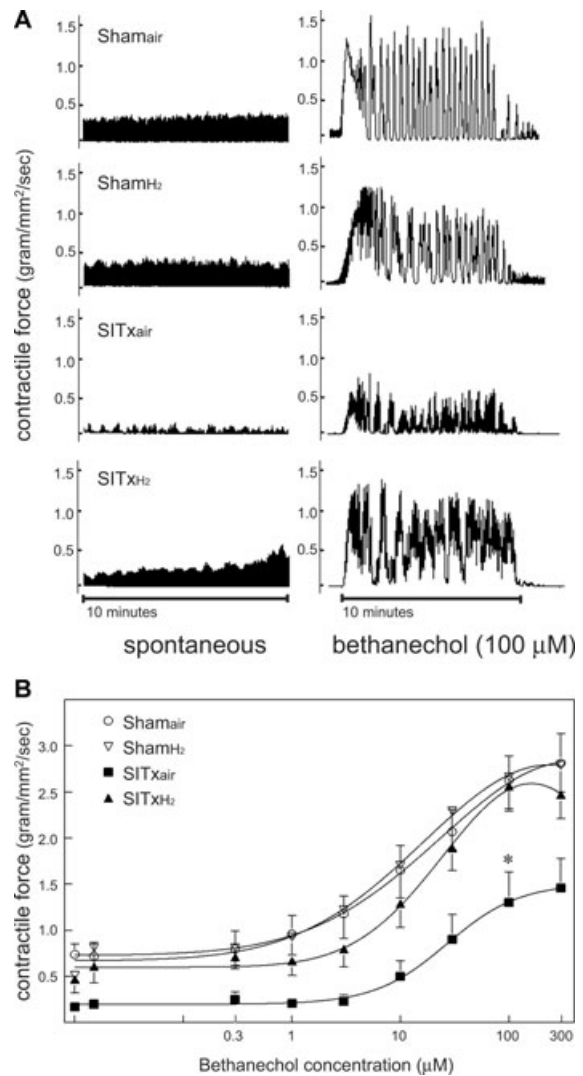
Figure 2: Representative traces of jejunal smooth muscle contractile activity (A). Representative mechanical traces of spontaneous contractility (left panels) and maximum of stimulated contractility in response to Bethanechol (100 μM) (right panels) were shown. Muscle strips from animals in both sham groups generated regular spontaneous and large phasic and tonic responses to bethanechol. Cold I/R injury with prolonged ischemic time resulted in a significant decrease of spontaneous or stimulated muscle contractile activity. Hydrogen inhalation demonstrated a greater spontaneous contractile activity 24 h after SITx in both spontaneous and bethanechol-stimulated contractile activity. Representative pictures were shown from 24 independent traces for each group. **Calculated jejunal smooth muscle contractility in response to bethanechol (B).** Jejunal circular muscle strips from sham control animals (*open circle*) and sham animals receiving hydrogen inhalation (*open triangle*) showed a dose-dependent increase in contractile area in response to bethanechol. This activity was significantly diminished in graft muscle taken 24 h following transplantation (*closed square*). Significant improvement was measured in transplanted animals treated with hydrogen treatment (*closed triangle*) (*$p < 0.05$ vs. $SITx_{air}$, N = 6 each group, n = 4 each animal).
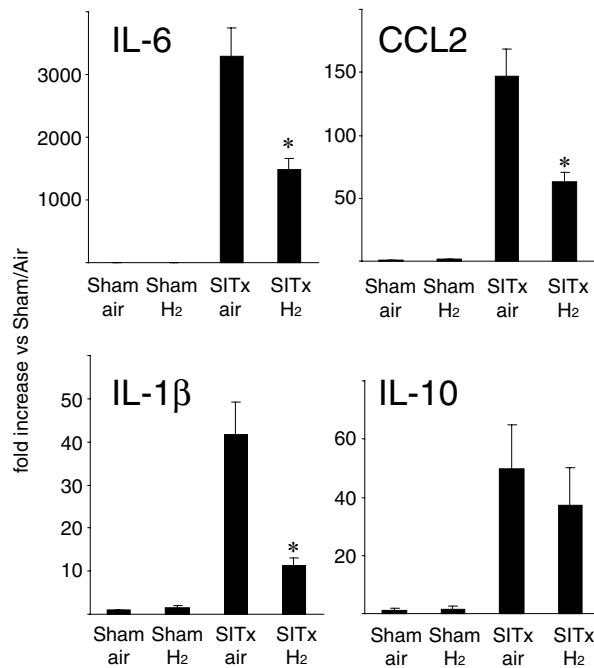
Figure 3: Real-time RT-PCR analysis of graft muscularis externa. Real-time RT-PCR analysis revealed a significant increase in mRNA expression of IL-1β, IL-6 and CCL2 in graft muscularis 3 h after transplantation compared to sham-treated animals. Hydrogen inhalation treatment resulted in significantly reduced upregulation of these inflammatory mediators (*$p < 0.05$ vs. SITx$_{air}$, N = 10 each group). IL-10 expression was upregulated over 40-fold after SITx, but hydrogen inhalation did not affect IL-10 expression 3 h after reperfusion.

not significantly influence the expression of IL-10 mRNA (Figure 3).

Hydrogen attenuated morphological mucosal damage and prevented damage to gut barrier function

Histopathological features of the intestinal lining 3 h after reperfusion showed pronounced mucosal erosion and massive epithelial lifting in SITx$_{air}$ grafts. These mucosal alterations were significantly attenuated by hydrogen treatment (Figure 4A and B). Damage to the intestinal mucosa by I/R injury results in an increase in intestinal permeability leading to bacterial translocation (38). Transplantation injury in SITx$_{air}$ grafts led to a significant impairment of gut barrier function 3 h after surgery ($p < 0.05$ vs. Sham$_{air}$). However, hydrogen treatment significantly prevented this decrement in permeability (Figure 4A and C).

Hydrogen reduced graft oxidative injuries

The potential antioxidative properties of hydrogen inhalation were determined by the measurement of tissue MDA levels and the reductive capacities of the intestinal mucosa and muscularis. MDA levels, a marker of lipid peroxidation, increased 3 h after transplantation in tissues from SITx$_{air}$ animals (Figure 4D) ($p < 0.05$ vs. Sham$_{air}$). Periop-

erative hydrogen inhalation blunted lipid peroxidation, such that MDA levels were no longer significantly elevated over Sham$_{air}$ and Sham$_{H2}$ animals. Similarly, the reductive level in each sample was determined by evaluation of Cu$^+$ derived by reduction from Cu^{2+} due to the combined action of all antioxidants present in the sample. Hydrogen treatment did not enhance total reductive capacity in the intestine of sham-treated animals. However, transplantation injury in SITx$_{air}$ animals led to a decrease in the reductive capacity of intestinal grafts, suggesting consumption of intestinal antioxidants. Hydrogen treatment significantly forestalled the reductive capacity of the graft tissues following transplantation (Figure 4E) (*$p < 0.05$ vs. Sham$_{air}$ and $^{\delta}p < 0.05$ vs. SITx$_{air}$). These results suggested that hydrogen treatment significantly attenuated intestinal oxidative injury due to graft cold preservation and reperfusion.

Hydrogen prevented systemic inflammatory responses in recipient lung

The intestinal graft with severe transplant induced injury can be a major source of a multitude of mediators that facilitate the local intestinal inflammatory response. Furthermore, intestinal transplantation can have deleterious systemic effects on nonischemic organs including the lung. A systemic cellular inflammatory response was evident in the lung of SITx$_{air}$ animals, as the number of infiltrating neutrophils significantly increased 14- and 8-fold in the transplanted host animals 3 h after surgery compared to Sham$_{air}$ and Sham$_{H2}$ animals, respectively. Interestingly, in association with pulmonary neutrophil accumulation in SITx$_{air}$ animals, a systemic molecular inflammatory response was also evident in the host lung by a significant induction in the proinflammatory cytokines IL-6 and TNF-α, 3 h after intestinal graft reperfusion. In addition to its effects on the intestine, hydrogen inhalation significantly reduced the recruitment of neutrophils into the recipient lung by 30% (Figure 5A) and limited the upregulation in lung-derived IL-6 and TNF-α mRNAs (Figure 5B).

### Discussion

This study demonstrates that perioperative inhalation of 2% hydrogen increases arterial hydrogen levels 3.5-fold and that this elevation significantly ameliorates transplantation induced intestinal donor graft injury and a systemic inflammatory response in the recipient. Hydrogen therapy diminished the transplant-induced suppression in intestinal motility, blunted the induction of proinflammatory cytokines within the muscularis externa and protected the mucosal barrier, which was associated with a decrease in tissue MDA levels and improved antioxidant status. Additionally, hydrogen significantly abrogated the systemic inflammatory response in the host lung.

SITx has been recognized as a therapeutic option for the treatment of patients with intestinal failure, who
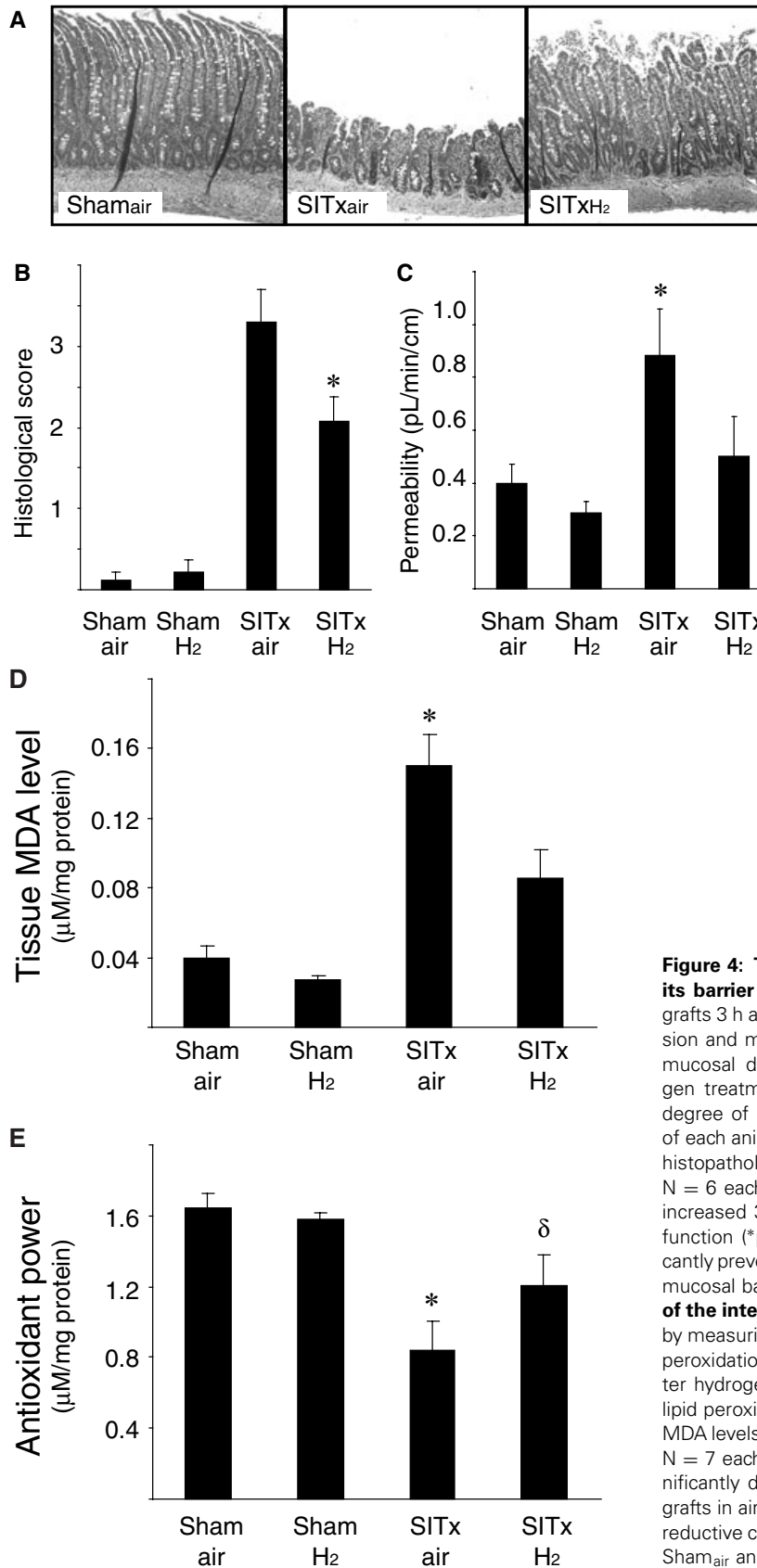
Figure 4: The effects of hydrogen on mucosal injury and its barrier function. Histopathological analysis of intestinal grafts 3 h after reperfusion showed pronounced mucosal erosion and massive epithelial lifting in air-control grafts. These mucosal damages were significantly attenuated by hydrogen treatment (H&E, original magnification ×200) (A). The degree of intestinal damage was presented as mean score of each animal. Hydrogen treatment significantly ameliorated histopathological injuries 3 h after SITx (*p < 0.05 vs. SITx$_{air}$, N = 6 each group, n = 6 each animal) (B). Gut permeability increased 3 h after SITx suggesting loss of mucosal barrier function (*p < 0.05, N = 11 each group). Hydrogen significantly prevented an increase in intestinal permeability averting mucosal barrier breakdown (C). **Marker for oxidative injury of the intestinal graft.** Graft oxidative injury was determined by measuring the tissue MDA concentration, a marker of lipid peroxidation. Demonstrating an antioxidant effect, grafts after hydrogen inhalation exhibited no significant induction of lipid peroxidation 3 h after SITx compared to elevated tissue MDA levels in air-treated intestinal grafts (*p < 0.05 vs. SITx$_{air}$, N = 7 each group) (D). Intestinal reductive capacity was significantly deteriorated 3 h after reperfusion in the intestinal grafts in air. Hydrogen-treated grafts showed maintenance of reductive capacity showing less oxidative injury (*p < 0.05 vs. Sham$_{air}$ and δp < 0.05 vs. SITx$_{air}$, N = 7 each group) (E).

A

B
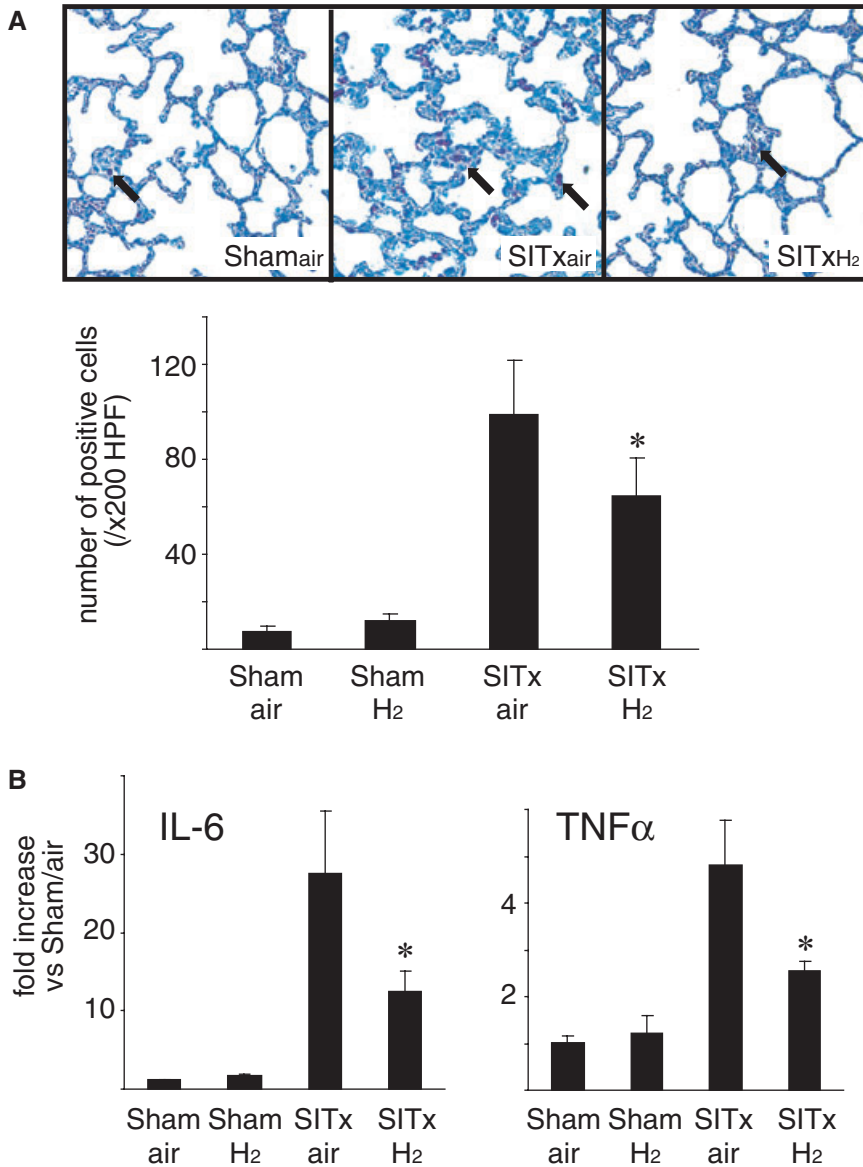
Figure 5: Inflammatory response in the recipient lung. Representative pictures of the recipient lung stained for neutrophils and a histogram quantifying infiltrating neutrophils in the recipient lung were shown. Hydrogen reduced the number of increased infiltrating neutrophils in the recipient lung 3 h after reperfusion. The MPO-positive cells were counted and presented as the number of positive cells/ $\times 200$ HPF (*$p < 0.05$ vs. $SITx_{air}$, $N = 5$ each group) (A). Recipient lung inflammation is associated with inflammatory cytokine mRNA upregulation such as IL-6 and $TNF\alpha$. Hydrogen treatment significantly inhibited these cytokine changes 3 h after reperfusion (*$p<0.05$ vs. $SITx_{air}$, $N = 5$ each group) (B).

develop life-threatening complications from total parenteral nutrition (39,40). Although intestinal graft survival has been significantly improved, to approach the 70% survival of lung allografts, due to new potent immunosuppressive regimens, preservation and ischemia/reperfusion (I/R) injury during SITx are obligatory and major causes of recipient morbidity and mortality. During cold storage, the lack of oxygen results in the decreased production of adenosine triphosphate (ATP), leading to alterations in intracellular ion concentrations and the activation of cytotoxic enzymes. Also, hypothermia itself can cause cellular injury due to suppression in Na^+/K^+ pump activity (41). Upon vascular reperfusion, copious amounts of superoxide anion ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2) are generated causing graft injury (42). At low concentrations, these ROS function as

necessary signaling molecules and critically modulate the activation of the immune system and thus participate in antibacterial defense (43). However, they become extremely detrimental when they overcome the antioxidant capacity of the host cells. At high concentrations they react with catalytic iron to produce highly reactive hydroxyl radicals ($OH\cdot$) through the Haber-Weiss reaction or Fenton reaction (20,21). Hydroxyl radicals aggressively and indiscriminately damage cellular macromolecules, including DNA, proteins and lipids, giving rise to transplant associated alterations in graft function and morphology. Superoxide and H_2O_2 are detoxified by endogenous cellular antioxidants, such as superoxide dismutase, catalase, peroxidase or glutathione-peroxidase. However, no endogenous enzymatic pathway is known to neutralize $OH\cdot$ radicals.

Therefore, the therapeutic targeting of hydroxyl radicals could be critical for the amelioration of transplant induced graft injury. A number of agents have been experimentally used to scavenge free radicals in the transplant setting, including superoxide dismutase, alpha-tocopherol, glutathione, allopurinol, oxypurinol, deferoxamine, dimethylthiorea and dimethyl sulfoxide (44,45). Conceptually, the selective OH· scavenging activity of hydrogen should provide a similar beneficial effect to the iron chelator deferoxamine, dimethylthiorea and dimethyl sulfoxide in averting the damaging effects of OH· radicals (46,47). Indeed, hydrogen inhalation may have several potential advantages over current pharmacological therapies, as it is highly diffusible and could potentially reach subcellular compartments of the mitochondria and nuclei, which are the primary site of ROS generation and DNA damage, but which are also notoriously difficult to target pharmacologically. Hydrogen scavenging of OH· would preserve mitochondrial membrane potential, maintain ATP synthesis, prevent DNA damage and decrease lipid peroxidation (14). Secondly, hydrogen gas is physiological safe for human inhalation and hydrogen is continuously produced in the body by colonic bacteria and normally circulates in blood (43). Interestingly, the colon is more resistant to ischemic injury compared to the small intestine in both warm and cold I/R injury in canine models, which may be partially explained by local hydrogen's actions of eliminating hydroxyl radicals (44).

In 2001, Gharib et al. reported using a chronic infectious liver model that animals maintained in a hydrogen supplemented hyperbaric chamber were significantly protected from liver injury as assessed by less fibrosis, improved hemodynamics, increased nitric oxide synthase II activity, increased antioxidant enzyme activity, decreased lipid peroxide levels and decreased circulating TNF-α levels (48). Recently, Ohsawa et al. demonstrated that hydrogen gas selectively reduces the levels of hydroxyl radicals *in vitro* and can also exert potent *in vivo* antioxidant activity in a rat cerebral ischemia model (14). Furthermore, the same group has shown that inhaled hydrogen reduced liver warm I/R injury, which was associated with less oxidative stress (13). Our results show that hydrogen markedly prevents the transplant-induced suppression in gastrointestinal motility and jejunal muscle contractility. Mechanistically, the improved muscle function was associated with a reduction in the mRNA induction of the proinflammatory cytokines IL-1β and IL-6. Hydroxyl radicals have been shown to be a potent inducer of both IL-1β and IL-6 (49–51). Additionally, the transplant induction of chemokine CCL2 mRNA was also significantly diminished by hydrogen inhalation. Like the interleukins, CCL2 is readily induced by OH· radicals and iron chelation by dimethylthiourea and dimethyl sulfoxide (i.e. OH· scavenging) was shown to inhibit both TNF-α and xanthine oxidase induced CCL2 gene expression in endothelial cells and monocytes possibly through an NF-κB-dependent pathway (47). In contrast to proinflammatory mediators, the transplant induction of mRNAs for the antiinflammatory mediators IL-10 and HO-1 (data not shown) in

this study was not altered by hydrogen inhalation. Indeed, we actually found one citation in the literature in which a hydroxyl radical scavenger caused an increase in IL-10 induction (52). Hence, hydroxyl scavenging appears to leave intact the beneficial antiinflammatory mediators IL-10 and HO-1.

The intestinal mucosa is known to be particularly sensitive to I/R generated ROS injury. Therefore, we hypothesized that hydrogen OH· scavenging might also prevent transplantation induced mucosal damage. Histological examination of the reperfused graft demonstrated that hydrogen inhalation significantly prevented mucosal structural damage and improved intestinal barrier function. These improvements by hydrogen inhalation were associated with the abated production of intestinal MDA and improved tissue oxidant potential after bowel engraftment. A conspicuous reduction in neuronal lipid peroxidation by hydrogen inhalation was also observed by 4-hydroxy-trans-2-nonenal immunohistochemistry following cerebral ischemia (14).

Hydrogen maintenance of mucosal permeability and improved barrier function of the implanted graft could have important systemic benefits, as intestinal derived endotoxemia has been demonstrated to occur following both liver and intestinal transplantation (18,53). Therefore, along with the subdued release of proinflammatory mediators from the graft, it could be predicted that systemic inflammatory responses in distant organs would also be diminished. In our final series of experiments, we did measure a decreased proinflammatory molecular response within the host lung that was accompanied by a significant decrease in the postsurgical recruitment of neutrophils into the pulmonary alveolae. As hydrogen was systemically inhaled salutary effects on these parameters could have been due to both indirect and direct antioxidant effects on the host lung.

Medical gas therapy is a novel and relatively unexplored field of science and to our knowledge hydrogen inhalation therapy has never been tested in any model of transplantation. Clinically, hydrogen gas therapy could be delivered by a variety of systems including, simple inhalation using a ventilator circuit, a facemask or a nasal cannula. In addition, hydrogen gas therapy could be administered via a parental hydrogen-releasing moiety and/or a hydrogen enriched fluid. The results of the current study continue to validate the therapeutic potential of medical gas therapies by demonstrating that perioperative hydrogen inhalation significantly protects graft structure and function, and blunted graft and systemic molecular inflammatory responses.

Acknowledgments

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Supporting Information

The following supporting information is available for this article:

Table S1. Nucleotide sequences of oligonucleotide primers (RT-PCR).

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