Oral hydrogen water prevents chronic allograft nephropathy in rats

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Reactive oxygen species (ROS) contribute to the development of interstitial fibrosis and tubular atrophy seen in chronic allograft nephropathy (CAN). As molecular hydrogen gas can act as a scavenger of ROS, we tested the effect of treatment with hydrogen water (HW) in a model of kidney transplantation, in which allografts from Lewis rats were orthotopically transplanted into Brown Norway recipients that had undergone bilateral nephrectomy. Molecular hydrogen was dissolved in water and recipients were given HW from day 0 until day 150. Rats that were treated with regular water (RW) gradually developed proteinuria and their creatinine clearance declined, ultimately leading to graft failure secondary to CAN. In contrast, treatment with HW improved allograft function, slowed the progression of CAN, reduced oxidant injury and inflammatory mediator production, and improved overall survival. Inflammatory signaling pathways, such as mitogen-activated protein kinases, were less activated in renal allografts from HW-treated rats as compared with RW-treated rats. Hence, oral HW is an effective antioxidant and antiinflammatory agent that prevented CAN, improved survival of rat renal allografts, and may be of therapeutic value in the setting of transplantation.

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KEYWORDS: chronic rejection; hydrogen; inflammation; kidney transplantation; oxidative stress; rats

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Chronic kidney disease is the ninth leading cause of death in the United States accounting for over 40,000 deaths annually.[1] Despite advances in renal replacement therapy, transplantation remains the preferred treatment for suitable candidates.[2] However, in spite of improved postoperative immunosuppression regimens, the 10-year graft survival rates are 55 and 75% for cadaveric and live donor kidney allografts, respectively. The vast majority of late failures are attributable to chronic allograft nephropathy (CAN), recently reclassified as interstitial fibrosis and tubular atrophy with unknown etiology.[3–6] The clinical course of CAN is characterized by a progressive deterioration in renal function, manifested by increasing renal hypertension and proteinuria. Presently, no specific treatment is available for chronic rejection in clinical transplantation despite a number of successful approaches in animal models, including the use of macrophage inhibitors, angiotensin converting enzyme inhibitors, and endothelin A receptor antagonists.[7–10]

A number of factors contribute to the development of CAN, including immunological (for example, acute rejection) and nonimmunological (for example, ischemia-reperfusion injury) factors.[11,12] Oxidative stress is believed to be a common pathway that leads to both immunological and nonimmunological stress in the setting of kidney transplantation and, ultimately, to the development of CAN.[13] Markers of oxidative stress, such as plasma lipid peroxidases, are increased, whereas antioxidant markers, including glutathione, superoxide dismutase, and glutathione peroxidase, are decreased in the setting of CAN.[14–17] Despite this association, few studies have attempted to examine the effect of antioxidants on kidney allograft outcomes and those that have yielded mixed results. Vitamin E (α-tocopherol) supplementation did not prevent allograft injury in a model of CAN;[16] however, in the same model, L-arginine did attenuate proteinuria and glomerulosclerosis.[18] Furthermore, a clinical trial using recombinant human superoxide dismutase resulted in significantly decreased acute and chronic rejection.[19] Therefore, based on the seemingly conflicting results of these few studies, there is a need for additional investigations into the applicability of antioxidants for the prevention of CAN.

Molecular hydrogen has recently been shown to have therapeutic value as an antioxidant through its ability to selectively reduce cytotoxic reactive oxygen species (ROS).[20] Inhaled hydrogen gas (~4% H_2 in air) can reduce infarct size in rat models of focal cerebral and myocardial ischemia-reperfusion injury.[20,21] More recently, our group reported that perioperative hydrogen inhalation (2%) significantly ameliorates intestinal transplant injury and prevents remote organ inflammation through its antioxidant effects.[22] Drinking water containing a therapeutic dose of hydrogen (hydrogen water; HW) represents an alternative mode of delivery of molecular hydrogen. The primary advantages of HW are that it is a portable, easily administered, and safe means of delivering molecular hydrogen. Therefore, it may be of potential therapeutic value in the treatment of oxidative stress-induced pathologies. Interestingly, drinking HW, as well as inhaling hydrogen gas, can alleviate cisplatin-induced nephrotoxicity, which is known to be mediated, in part, by the accumulation of ROS that occurs secondary to the ability of cisplatin to inhibit the reducing form of glutathione[23] Consumption of HW *ad libitum* prevents the development of atherosclerosis in apolipoprotein E knockout mice, in part, through its ability to limit the amount and deleterious effects of oxidative stress in the blood vessels of these mice.[24] Furthermore, a clinical trial in type II diabetic patients given supplemental HW led to improved lipid and glucose metabolism compared with controls.[25] The aim of the present study was to determine the efficacy of HW in preventing CAN after allogeneic kidney transplantation in rats.

RESULTS

Oral administration of HW increased local and systemic levels of hydrogen

To determine whether oral administration of HW results in increased local or systemic levels of molecular hydrogen, unoperated naïve Lewis (LEW) rats were given HW to drink and, subsequently, the concentration of molecular hydrogen was measured in the kidney and in the serum. Both local and systemic concentrations of molecular hydrogen peaked ~15 min after ingestion (Figure 1a), proving that HW is an effective mode of delivery of molecular hydrogen. We also tested whether transplant recipients given long-term, daily HW had increased concentrations of circulating hydrogen. Similar kinetic changes in hydrogen concentration were observed in both LEW recipients with LEW grafts (control rats; syngeneic transplantation prevents CAN) and Brown Norway (BN) recipients with LEW grafts treated with HW for 60 days (Figure 1b). The baseline levels of the hydrogen detected in circulation after 60 days of HW treatment was comparable to that of naïve animals, suggesting that there was no hydrogen accumulation during long-term HW administration.

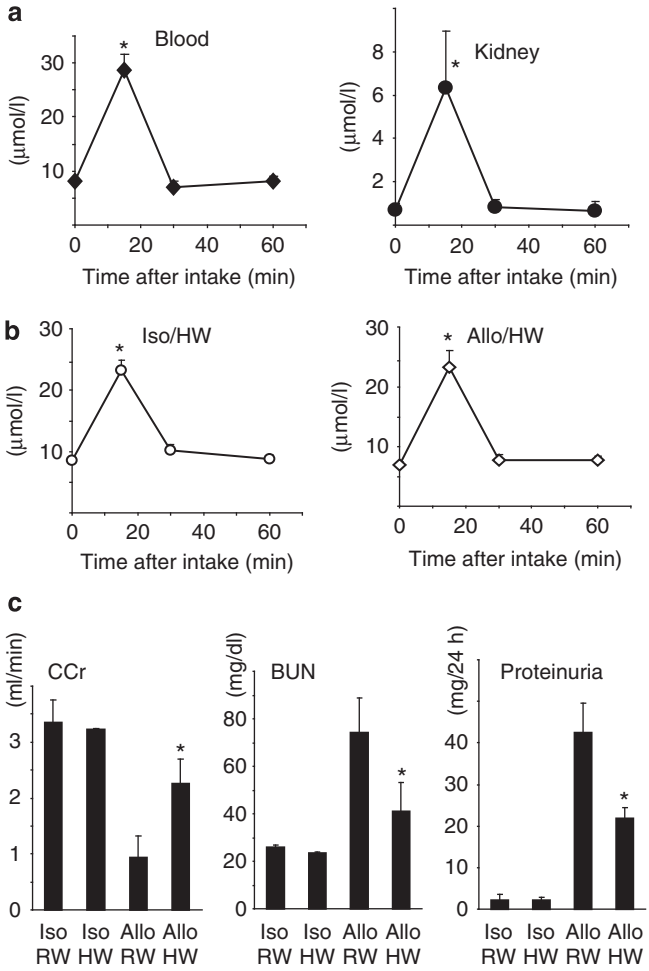

Figure 1 | Hydrogen water (HW) administration improves kidney allograft function. (**a**) Hydrogen-rich water (HW) (3 ml) was orally administered to naïve Lewis (LEW) rats by gavage. Arterial blood and kidney tissue were taken at 15, 30, and 60 min after oral administration of HW. Hydrogen concentration in blood and homogenized kidney tissue increased within 15 min and then returned to the basal levels ($n = 3$). (*$P < 0.05$ vs 0 min). (**b**) Kinetic analysis of the blood hydrogen levels after HW (3 ml) administration in the transplanted recipients that had been given HW for 60 days were performed. Both isograft recipients ($n = 3$) and allograft recipients ($n = 3$) showed similar changes in circulating hydrogen concentrations. (**c**) Male LEW rats were used as donors for either syngeneic (LEW recipient) or allogeneic (Brown Norway recipient) renal transplantation. Recipients were given either regular water (RW) or HW *ad libitum* after transplantation, resulting in a total of four experimental groups: isograft given RW (Iso/RW, $n = 8$) or HW (Iso/HW, $n = 8$); or allograft given RW (Allo/RW, $n = 18$) or HW (Allo/HW, $n = 17$). The recipients receiving daily HW had improved allograft function, as measured by blood urea nitrogen (BUN), creatinine clearance (CCr), and 24 h urinary protein excretion, as compared with those receiving RW at 60 days posttransplantation. (*$P < 0.05$ vs Allo/RW).

Oral administration of HW improves kidney function after allotransplantation

We then sought to determine the effect of HW administration on kidney function after allotransplantation. Isograft recipients (LEW donor and LEW recipient) were included as controls, as these rats do not develop CAN. Using a model of kidney transplantation followed by bilateral nephrectomy, we found that rats receiving daily HW *ad libitum* had improved allograft function, as measured by blood urea nitrogen, creatinine clearance, and proteinuria, compared with those

receiving regular water (RW) at 60 days posttransplantation (Figure 1c). These results suggest that HW can improve kidney function after allotransplantation.

Oral administration of HW improves weight gain and overall survival after allotransplantation

To determine whether the improved allograft function that was observed in HW-treated recipients correlated with global parameters of well being, we measured the weights of transplant recipients and found that the majority of animals that had undergone allotransplantation followed by administration of RW began to lose body weight ~40 days after transplantation. This process was significantly diminished in the allograft recipients that received HW (Figure 2a). Furthermore, we found that weight loss proved to be a harbinger of allograft failure and, ultimately, of death. HW-treated recipients exhibited a significant increase in survival (median survival > 150 days) compared with RW-treated controls (median survival 78 days) (Figure 2b). These results show that the improved allograft function that was observed at 60 days posttransplantation in HW-treated recipients, as compared with RW-treated controls, also leads to improved survival outcomes.

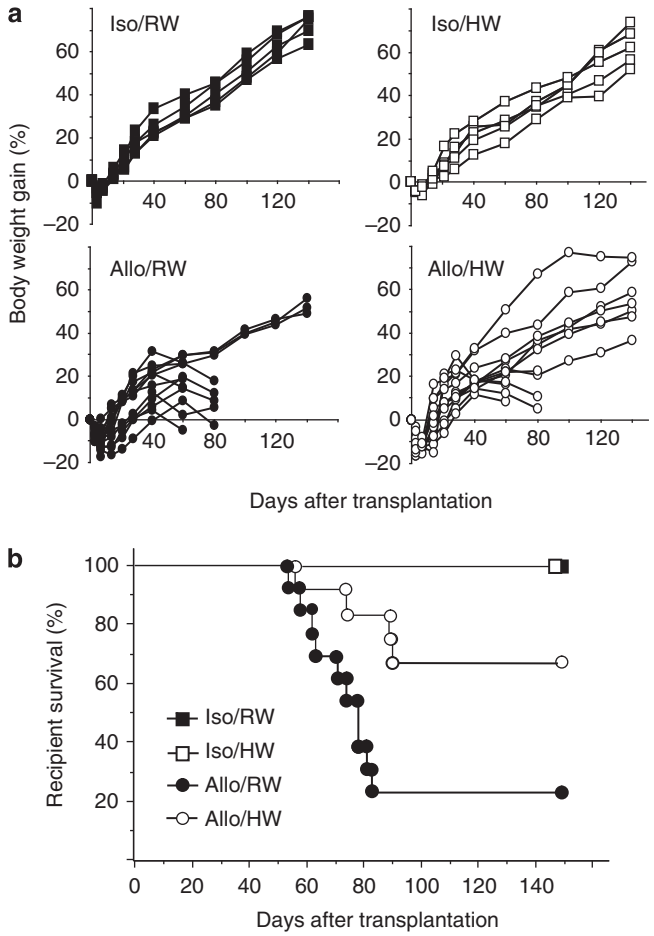

Figure 2 | Hydrogen water (HW) administration improves long-term survival after kidney allotransplantation. (**a**) Although isograft recipients showed fair body weight gains regardless of intake of HW, the recipients with allografts given regular water (RW) gradually lost body weight by 60–80 days after transplantation. The recipients given HW showed better body weight gain compared with those without HW. (**b**) In correlation with improved graft function, survival of allograft recipients was significantly prolonged with oral administration of HW. (Isograft given RW (Iso/RW), ($n = 5$); isograft given HW (Iso/HW), ($n = 5$); allograft given RW (Allo/RW) ($n = 13$); allograft given HW (Allo/HW) ($n = 12$); Kaplan-Meier, log-rank test, $P < 0.05$ Allo/RW vs Allo/HW).

Oral administration of HW prevents the progression of CAN

To determine whether the improved allograft function and overall survival observed in HW-treated animals were attributable to decreased chronic rejection, histological analysis was performed on allografts obtained 60 days posttransplantation from both RW- and HW-treated recipients. Hematoxylin and eosin staining of the allografts obtained from HW-treated recipients exhibited decreased evidence of the hallmarks of CAN, including less glomerulosclerosis and inflammatory cell infiltration, as compared with hematoxylin and eosin staining of allografts obtained from RW-treated recipients (Figure 3a). Furthermore, Masson's trichrome staining and α-smooth muscle actin (αSMA) staining on allografts obtained from HW-treated recipients showed less interstitial fibrosis and smooth muscle proliferation, respectively, compared with allografts obtained from RW-treated controls (Figure 3b and c). The expression of αSMA, indicating myofibroblast accumulation in the grafts, was mostly seen in the interstitial areas. There was no definitive, αSMA-positive staining in the tubular epithelial cells. Histopathology of the control isografts from the same time point was similar to that of normal naïve animals and was not affected by HW administration (data not shown). In addition, immunohistochemistry for both CD3 and CD68 revealed fewer graft-infiltrating T cells and macrophages, respectively, in allografts obtained from HW-treated recipients compared with those obtained from RW-treated controls (Figure 4a and b). These results proved to be statistically significant when the number of positive-staining cells per high-power field was counted for each sample (Figure 4c). Taken in total, these histological results suggest that allografts from HW-treated recipients experienced less CAN than did those from RW-treated controls.

Oral administration of HW is an effective antioxidant strategy in the setting of kidney allotransplantation

As mentioned previously, molecular hydrogen possesses potent antioxidant properties. Furthermore, oxidant stress-induced tissue damage is believed to be a common pathway in many of the pathophysiological mechanisms involved in the development of CAN. Therefore, owing to the fact that HW administration resulted in increased local and systemic concentrations of molecular hydrogen, as well as decreased histological evidence of CAN in kidney allograft recipients compared with RW-treated controls, we next determined whether the protection from CAN seen with HW administration was accompanied by a decrease in markers of oxidative tissue injury.

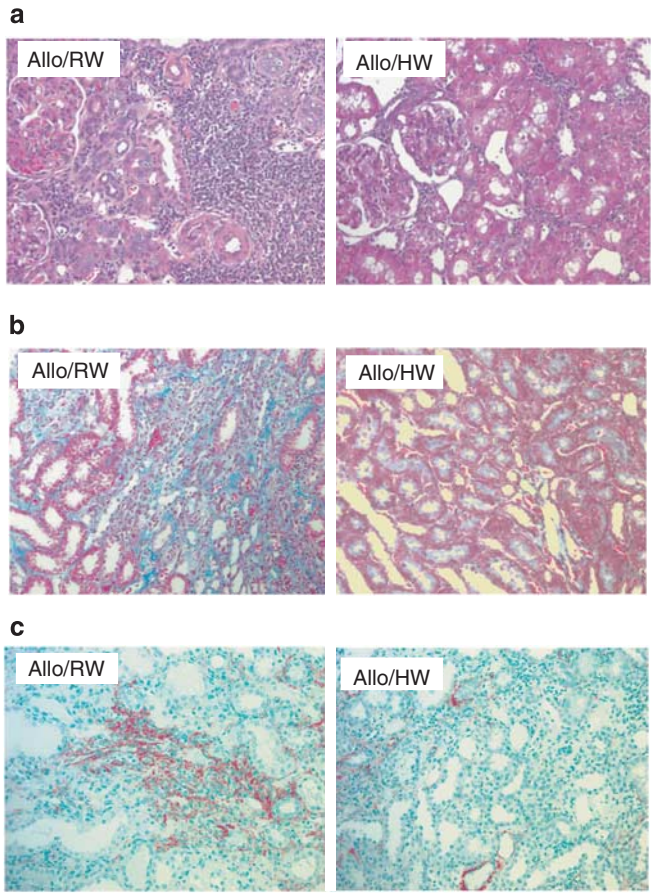

Figure 3 | Hydrogen water (HW) administration decreases chronic allograft nephropathy after kidney allotransplantation. Hematoxylin/eosin (**a**), Masson's trichome (**b**), or α-smooth muscle actin (**c**) staining was performed 60 days posttransplantation to assess histological and/or immunohistochemical evidence of chronic allograft nephropathy. Images are representative of four separate, individual grafts from each experimental group. Original magnification × 200. Allo/HW, allograft given HW; Allo/RW, allograft given RW; RW, regular water.

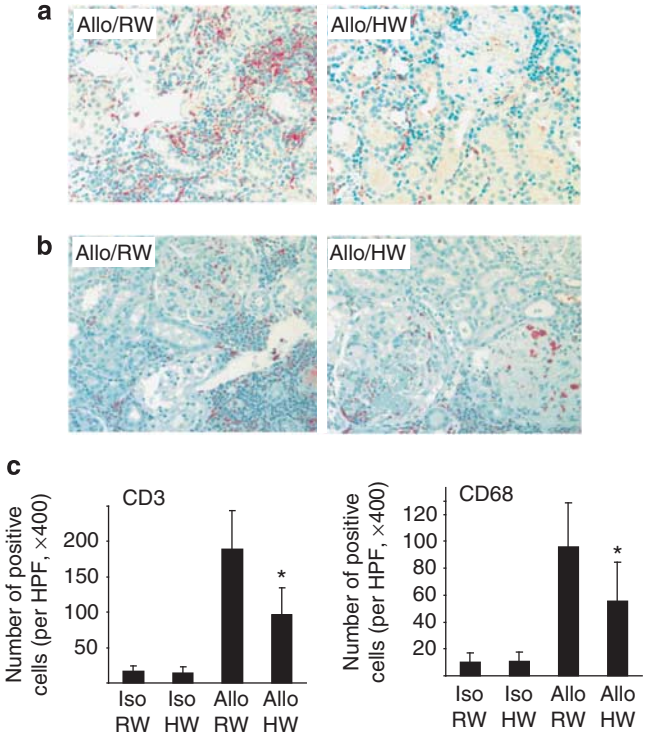

Figure 4 | Hydrogen water (HW) administration decreases intragraft inflammatory cell infiltration after kidney allotransplantation. CD3-positive (**a**) and CD68-positive (**b**) infiltrating cells were assessed by immunohistochemistry and quantitated as number of positive-staining cells per high-power field (HPF) (original magnification × 400) (**c**). Images are representative of five individual animals for each group; ($n = 5$ for each group, original magnification × 200, *$P < 0.05$ versus Allo/RW). Allo/HW, allograft given HW; Allo/RW, allograft given RW; Iso HW, isograft given HW; Iso RW, isograft given RW; RW, regular water.

Tissue malondialdehyde (MDA) levels, which indicate lipid peroxidation in cells and tissues, were significantly decreased in allografts obtained 60 days posttransplantation from HW-treated recipients compared with those obtained from RW-treated controls (Figure 5a). Furthermore, immunohistochemistry performed on allografts obtained from HW-treated recipients exhibited less 4-hydroxy-2-nonenal (HNE) and peroxynitrite staining compared with that seen in allografts obtained from RW-treated controls (Figure 5b and c). These results show that the local and systemic levels of molecular hydrogen that are achieved through the administration of HW are sufficient to effectively reduce oxidative stress-induced tissue damage in the setting of kidney allotransplantation.

Oral administration of HW decreases the local production of inflammatory markers in the setting of kidney allotransplantation

One mechanism by which oxidative stress leads to the development of chronic rejection is by increasing the production of inflammatory cytokines.[26,27] Therefore, because HW administration led to decreased oxidative stress and slowed the progression toward CAN in kidney allografts, we next determined whether HW treatment was also associated with a decrease in local inflammatory cytokine production. Quantitative reverse transcription PCR revealed significantly lower levels of interleukin-6, tumor necrosis factor-α, intracellular adhesion molecule-1, and interferon-γ mRNA in kidney allografts obtained from HW-treated recipients 60 days posttransplantation as compared with those obtained from RW-treated controls (Figure 6a–d). These results indicate that HW administration can attenuate the local production of inflammatory markers in the setting of kidney allotransplantation.

Oral administration of HW decreases the activation of inflammatory signaling cascades after kidney allotransplantation

Inflammatory intracellular signaling pathway activation (most notably activation of mitogen-activated protein kinases; MAP kinases) is a well-described event that contributes to the progression of kidney allografts toward chronic

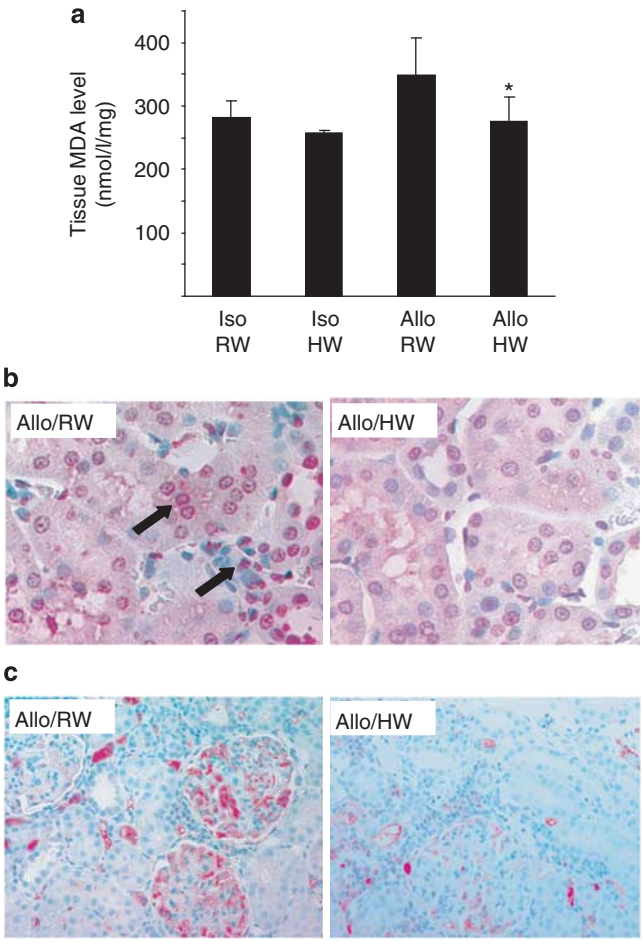

Figure 5 | Hydrogen water (HW) administration decreases intragraft markers of oxidative stress after kidney allotransplantation. (**a**) Intragraft malondialdehyde (MDA) levels were quantitated using a commercially available kit. MDA levels in the allografts treated with regular water (RW) increased 60 days after transplantation. HW significantly reduced tissue MDA levels. ($n = 5$, $^*P < 0.05$ vs Allo/RW). In addition, immunohistochemistry for 4-hydroxy-2-nonenal (HNE) (**b**) and peroxynitrite (**c**) was performed on formalin-fixed sections obtained from the allograft groups. Oxidative injuries were more prominent in the allografts given RW (Allo/RW) compared with those given HW (Allo/HW). Arrows indicate HNE-positive cells. (Original magnification $\times 400$; images are representative of three separate experiments). Allo/HW, allograft given HW; Allo/RW, allograft given RW; Iso HW, isograft given HW; Iso RW, isograft given RW.

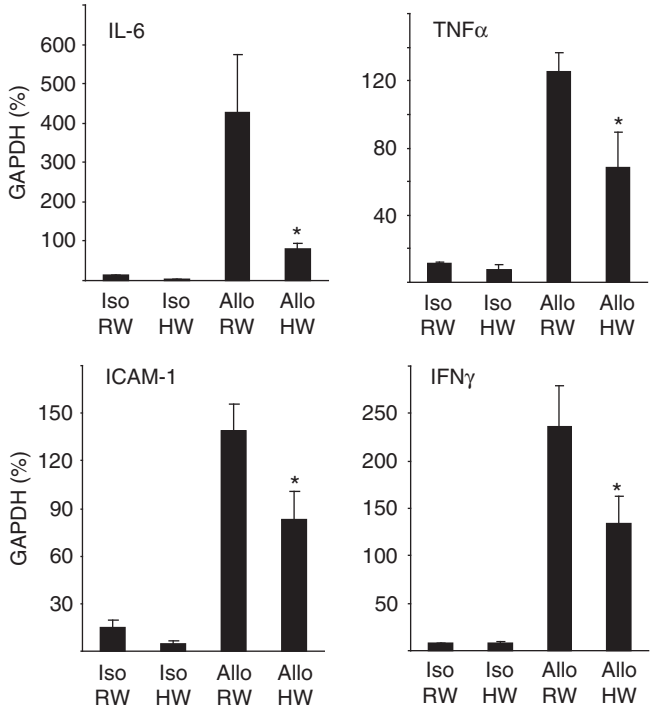

Figure 6 | Hydrogen water (HW) administration decreases the intragraft production of inflammatory cytokines after kidney allotransplantation. Interleukin-6 (IL-6) tumor necrosis factor-α (TNFα) intracellular adhesion molecule-1 (ICAM-1), and interferon-γ (IFNγ) mRNA levels in the kidney grafts taken 60 days after transplant were measured by real-time quantitative reverse transcriptase PCR analysis. Although allograft rejection caused upregulation of the mRNAs for these markers, HW significantly reduced the mRNA levels as compared with regular water (RW). ($n = 5$, $^*P < 0.05$ vs Allo/RW). Allo HW, allograft given HW; Allo RW, allograft given RW; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Iso HW, isograft given HW; Iso RW, isograft given RW.

rejection.[28–30] Oxidative stress can activate MAP kinase signaling, which ultimately contributes to the proliferation of mesangial cells in the setting of diabetic nephropathy.[31] Mesangial cell proliferation is also involved in the development of CAN. Therefore, we next determined whether HW administration could suppress MAP kinase activation in the kidney allografts. Western blot analysis showed that MAP kinases, including c-Jun N-terminal kinase, p-38, extracellular signal-regulated protein kinase as well as upstream kinase cascades (MEK-1), were less activated in allografts obtained from HW-treated recipients than in allografts obtained from RW-treated recipients (Figure 7). These results indicate that HW administration can inhibit intracellular signaling pathways that are known to contribute to the development of CAN in the setting of kidney transplantation (Supplemental data).

DISCUSSION

In this study, we found that both allograft function and overall survival were improved in rats that had been fed with a diet supplemented with HW. Allografts from HW-treated rats exhibited less infiltration of inflammatory cells and suppressed activation of intragraft inflammatory signaling pathways. The allografts from the HW-treated rats manifested fewer markers of oxidative stress and, ultimately, fewer progressed toward CAN as compared with controls. These results indicate that HW represents a potentially novel therapeutic strategy in the prevention of CAN in kidney transplantation.

Molecular hydrogen is produced continuously under normal physiological conditions, primarily during the fermentation of nondigestible carbohydrates by intestinal bacteria in the large intestine. This physiological production

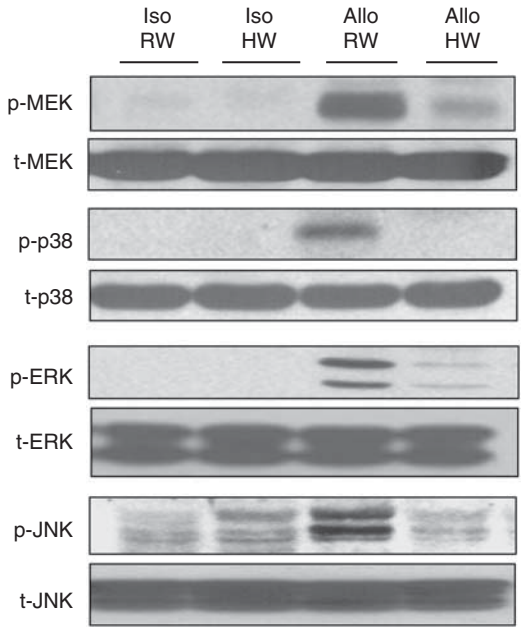

Figure 7 | Hydrogen water (HW) administration decreases intragraft inflammatory signaling cascade activation after kidney allotransplantation. Western blot analysis showed that mitogen-activated protein (MAP) kinases (JNK (c-Jun NH 2-terminal kinase), p38, ERK1/2 (extracellular signal-regulated kinase 1/2)) and upstream kinase cascades (MEK-1), were less activated in allografts obtained from recipients that were HW-treated compared with those obtained from regular water (RW)-treated recipients. Images are representative of three independent experiments. Allo, allograft recipients; Iso, isograft recipients; p, phosphorylated; t, total.

of hydrogen gas may be responsible for the baseline levels of hydrogen detected in circulation. It is excreted as flatus, further metabolized by gut flora, or exhaled as a natural component of abdominal gas. However, molecular hydrogen has known physiological roles during conditions of homeostasis. Recent evidence indicates that inhaled hydrogen gas has antioxidant and anti-apoptotic properties that can protect organs from ischemia-reperfusion-induced injury by selectively scavenging detrimental ROS. The mechanism of action of inhaled hydrogen gas in these models involves its ability to prevent oxidative damage, as indicated by decreased nucleic acid oxidation and lipid peroxidation.[20,21] Although the concentration of gaseous molecular hydrogen used in the above studies ($\sim$4%) is lower than the threshold at which it is known to be flammable (4.6%), flammability is still a realistic concern which may limit the translational applicability of inhaled molecular hydrogen. Therefore, HW represents a novel and easily translatable method of delivery of molecular hydrogen. To our knowledge, this is the first report describing the preventative effects of molecular hydrogen, delivered in water containing therapeutic doses, on the development of chronic rejection in the setting of kidney allotransplantation and, as such, represents a potentially novel and easily applicable solution to a difficult clinical scenario (that is, CAN). The major novel findings of the present study are that (i) HW improves allograft function and overall survival by preventing CAN in a rodent model of kidney transplantation, doing so in part by (ii) reducing oxidative stress-induced damage and (iii) reducing the activation of inflammatory signaling pathways and cytokine production.

The basis for the present study was the fact that oxidative stress is believed to be a common pathway that leads to the development of chronic rejection in kidney transplantation. As mentioned earlier, markers of oxidative stress are elevated in kidney transplant recipients and, in contrast, markers of antioxidant pathways are diminished. Mechanistically, ROS activate inflammatory intracellular signaling pathways in vascular smooth muscle cells,[28] induce the epithelial-to-mesenchymal transition,[32,33] participate in extracellular matrix deposition by mesangial cells,[34] and contribute to renal tubular atrophy through apoptosis[35,36] and inflammation[26,27]—all of which are key processes involved in the pathogenesis of CAN.

Given the association described above between oxidative stress and the development of CAN, the finding that HW administration effectively decreases the intragraft accumulation of markers of oxidative stress, such as MDA, 4-HNE, and peroxynitrite, suggests that the antioxidant properties of molecular hydrogen are likely to be responsible for the beneficial effects on the allograft function and the prevention of progression of CAN that were observed in this rat model of kidney allotransplantation. We examined the intragraft expression of tumor necrosis factor-α, interleukin-6, interferon-γ, and intracellular adhesion molecule-1 at the mRNA level, as surrogate markers of the deleterious processes downstream from oxidative damage, and found less expression of these inflammatory cytokines in HW-treated recipients, which are well-described mediators of the fibrogenesis phase of CAN.[37] Furthermore, MAP kinase signaling, which is known to be induced by oxidative damage in the setting of kidney transplantation, was decreased in HW-treated recipients. MAP kinase signaling contributes to the development of CAN by mediating the action of growth factors, such as transforming growth factor-β,[38] participating in the proliferation of vascular smooth muscle cells[28] and the extracellular matrix,[29] and contributing to the intragraft infiltration of mononuclear inflammatory cells through the production of chemoattractants.[30]

Therefore, we conclude that HW, through its ability to act as an effective method of delivery for molecular hydrogen, can reduce the development of CAN in a rat model of kidney allotransplantation. This improves allograft function and overall survival in HW-treated recipients. The mechanism of protection afforded to HW-treated recipients likely involves the ability of molecular hydrogen to reduce oxidative stress-induced damage, which is believed to be an upstream mediator that contributes to the ultimate development of CAN. Consequently, HW may be an effective and novel tool in the clinical armamentarium against oxidative stress-induced pathologies, in general, and CAN, in particular.

MATERIALS AND METHODS

Animals

Inbred male Lewis (LEW, RT1^{l}) and Brown Norway (BN, RT1^{n}) rats weighing 200–250 g were purchased from Harlan Sprague Dawley (Indianapolis, IN, USA). Animals were maintained in cages in a specific pathogen-free facility at the University of Pittsburgh and fed a standard diet with free access to water. All procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee at the University of Pittsburgh and the National Research Council's Guide for the Humane Care and Use of Laboratory Animals.

Kidney transplantation

Orthotopic kidney transplantation was performed using a previously described technique.[39,40] In short, after intravenous heparinization (300 U), the donor's left kidney was removed with the left renal artery in continuity with a short aortic segment and the left renal vein with a patch of vena cava. The excised graft was flushed with 3 ml of UW solution (Viaspan, Du Pont, Wilmington, DE, USA). The left kidney graft was orthotopically transplanted into the recipient by end-to-side microvascular anastomoses between graft aorta and recipient infrarenal abdominal aorta, and between graft renal vein and recipient infrarenal vena cava with 10-0 Micrin suture. Both native kidneys of the recipient were removed, and end-to-end ureteral anastomosis was performed using 10-0 Micrin suture. Recipients received prophylactic antibiotics (Cefotetan disodium, 100 mg/kg, intramuscular injection) for 3 days after the transplantation.

Oral administration of HW

Hydrogen water was produced by Blue Mercury (Tokyo, Japan) using a HW-producing apparatus, by which molecular hydrogen gas was dissolved in water under a pressure of 0.4 MPa, as previously described.[23,24,41] The HW (500 ml, hydrogen concentration >0.6 mmol/l) was stored in an aluminum bag and placed in a glass vessel twice a day. Control animals were treated with RW, generated by degassing HW by gentle stirring for 24 h.

Determination of hydrogen levels in blood and tissue

Hydrogen water (3 ml) was orally administered by gavage to naïve LEW rats or transplant recipients that had already been treated with HW for >60 days. Arterial blood and kidney tissue were taken at 15, 30, and 60 min after oral administration of HW. Blood or homogenized kidney tissue was placed in a glass tube and air-phase hydrogen levels were measured by gas-chromatography (Biogas analyzer BAS-1000, Mitleben, Osaka, Japan).[22]

Experimental groups

Four experimental groups were analyzed: LEW to LEW syngeneic grafts treated with RW (Group 1), LEW to LEW syngeneic grafts treated with HW (Group 2), LEW to BN allogeneic grafts treated with RW (Group 3), and LEW to BN allogeneic grafts treated with HW (Group 4). Recipients of allografts received daily intramuscular injections of tacrolimus (FK506, Astellas Pharmaceutical, Tokyo, Japan) at a dose of 0.5 mg/kg for 7 days (days 0–6), whereas those of isografts received no immunosuppressant. Recipients were killed at 60 or 150 days after transplantation, and blood and kidney graft samples were obtained after being cleared of blood by flushing with Lactated Ringers solution. Kidney tissue was snap-frozen and stored at $-80\,^{\circ}$C until use or fixed in 10% buffered formalin for routine histopathology. For survival study experiments, recipient animals were followed until recipient death due to graft failure or for 150 days after transplantation.

Evaluation of graft function

Renal graft function was assessed by measuring plasma creatinine (P(Cr)) and blood urea nitrogen levels, as well as urinary protein excretion and urinary creatinine (U(Cr)) levels using an auto-analyzer (Beckman Instruments, Fullerton, CA, USA). Creatinine clearance (CCr; ml/min) was calculated using the formula (CCr; ml/min) = (U(Cr) $\times$ urine volume)/(P(Cr) $\times$ time). Urine samples were collected using metabolic cage systems.

Histopathological analysis

Formalin-fixed graft tissues were paraffin embedded, cut into 5 µm sections, and stained with hematoxylin/eosin, modified Masson's trichrome, or Verhoeff's elastic tissue stain (Rowley Biochemical Institute, Danvers, MA, USA). Sections were also immunohistochemically analyzed using the avidin–biotin–peroxidase complex method after antigen retrieval and incubation with mouse anti-SMA (α-SMA, DAKO, Carpinteria, CA, USA), monoclonal anti-rat CD68 (ED1, Serotec, Raleigh, NC, USA), or monoclonal anti-rat CD3 (Serotec), followed by incubation with LSAB + horseradish peroxidase (DAKO). Tissue oxidative injury was also assessed by immunostaining with rabbit polyclonal anti-nitrotyrosine (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse monoclonal anti-4-HNE antibody (JAICA, Shizuoka, Japan), followed by incubation with biotinylated anti-mouse IgG antibody (Vector Laboratories, Burlingame, CA, USA) and avidin–biotin–peroxidase complex (Vector Laboratories). Diaminobenzidine was used as the peroxidase substrate. In each study, a set of sections was stained without the primary antibody as a negative control.

Assessment of tissue MDA

Tissue MDA levels were assessed using the BIOXYTECH MDA-586 kit (OxisResearch, Portland, OR, USA), as previously described.[42,43] Kidney samples were homogenized in buffer (pH 7.9) containing 5 mmol/l butylated hydroxytoluene to prevent sample oxidation and stored at $-80\,^{\circ}$C. Once all samples were collected, they were thawed on ice and 10 µg of probucol was added to further minimize oxidative reactions, then *N*-methyl-2-phenylindole (NMPI) in 25% methanol and 75% acetonitrile were added to the supernatants, followed by addition of 1 $\times$ hydrogen chloride (HCL) and incubated at 45 °C for 60 min. A standard curve using 1,1,3,3-tetra-methoxy-propane (TMOP), which generates free MDA during the acid hydrolysis step, was also prepared. All samples and standards were centrifuged (10,000 *g*, 10 min) and absorbance at 586 nm was measured using the Spectronic Biomate 3 (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

SYBR green real-time reverse transcription PCR

The levels of mRNAs for interleukin-6, intracellular adhesion molecule-1, tumor necrosis factor-α, interferon-γ, and glyceral-dehyde-3-phosphate dehydrogenase were quantified in duplicate using SYBR Green two-step, real-time reverse transcription PCR, as previously described.[42,43] Briefly, 1 µg of RNA from each sample was used for reverse transcription with oligo dT primers (Invitrogen, Carlsbad, CA, USA) and Superscript II enzyme (Invitrogen) to generate first-strand cDNA. The PCR reaction mixture was prepared using SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA, USA). Each sample was analyzed in duplicate using the conditions recommended by the manufacturer. Gene expression was

normalized with glyceraldehyde-3-phosphate dehydrogenase mRNA content.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blots

Cytoplasmic protein was isolated from the kidney grafts, and sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed using standard protocols. Total and phosphoprotein levels were determined by western blot using primary rabbit polyclonal antibodies and secondary goat anti-rabbit antibodies (1:10,000, Pierce Chemical, Rockford, IL, USA), as previously described.[44] The following primary antibodies were used: anti-phosphorylated-extracellular signal-regulated protein kinase1/2 and anti-total-extracellular signal-regulated protein kinase1/2 (Santa Cruz), anti-phosphorylated-p38 MAP kinases, anti-phosphorylated-c-Jun N-terminal kinase, anti-total-p38, anti-total-c-Jun N-terminal kinase, anti-phosphorylated-MEK, and anti-total-MEK (all from Cell Signaling Technology, Beverly, MA, USA).

Statistical analysis

Recipient survival was plotted using the Kaplan–Meier method, and the differences between groups were analyzed using the log-rank test. The other results were expressed as mean with standard deviation (s.d.). Statistical analysis was performed using analysis of variance (ANOVA) and the F-test with Bonferroni *post hoc* group comparisons, where appropriate. A probability level of $P<0.05$ was considered to be statistically significant with 95% confidence interval.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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