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ORIGINAL ARTICLE

Hydrogen-supplemented drinking water protects cardiac allografts from inflammation-associated deterioration

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Conflicts of Interest
All authors declare no conflict of interest exists.

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Summary

Recent evidence suggests that molecular hydrogen has therapeutic value for disease states that involve inflammation. We hypothesized that drinking hydrogen-rich water (HW) daily would protect cardiac and aortic allograft recipients from inflammation-associated deterioration. Heterotopic heart transplantation with short-course tacrolimus immunosuppression and orthotopic aortic transplantation were performed in allogeneic rat strains. HW was generated either by bubbling hydrogen gas through tap water (Bu-HW) or via chemical reaction using a magnesium stick [$Mg + 2H_2O \rightarrow Mg(OH)_2 + H_2$] immersed in tap water (Mg-HW). Recipients were given either regular water (RW), Mg-HW, Bu-HW, or Mg-HW that had been subsequently degassed (DW). Graft survival was assessed by daily palpation for a heartbeat. Drinking Mg-HW or Bu-HW was remarkably effective in prolonging heart graft survival and reducing intimal hyperplasia in transplanted aortas as compared with grafts treated with RW or DW. Furthermore, T cell proliferation was significantly inhibited in the presence of hydrogen *in vitro*, accompanied by less production of interleukin-2 and interferon-γ. Hydrogen treatment was also associated with increased graft ATP levels and increased activity of the enzymes in mitochondrial respiratory chain. Drinking HW prolongs survival of cardiac allografts and reduces intimal hyperplasia of aortic allografts.

Introduction

The most recent data from the International Society for Heart and Lung Transplantation database demonstrated that 10-year survival after cardiac transplant remains approximately 50% [1]. Graft injury as a result of oxidation and tissue inflammation has been implicated as one nonimmunologic factor driving allograft rejection and allograft vasculopathy [2,3]. In particular, allograft vasculopathy has a pivotal influence on late graft failure and remains an intractable obstacle to the mid-term survival of cardiac transplant recipients, accounting for 23–36% of deaths among patients who survive longer than 1 year after transplantation [4]. Although allograft vasculopathy and graft tissue inflammation are essential components of graft failure after heart transplantation, even the most promising immunosuppressive regimens have demonstrated limited overall efficacy in clinical trials, and there are no established therapeutic or preventative strategies [5].

Recent studies have demonstrated that hydrogen possesses antioxidant, anti-inflammatory and anti-apoptotic properties and can exert variety of cytoprotective functions [6,7]. Although hydrogen, an invisible, colorless, and odorless gas, is not toxic, hazardous, or poisonous,

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care must be taken during the delivery of hydrogen gas because of the high reactivity of hydrogen in the presence of specific catalysts or heat. Oral intake of hydrogen-rich water is an alternative mode of delivery for molecular hydrogen [8–10]. Drinking water containing molecular hydrogen potently protected kidney allografts from chronic rejection by inhibition of inflammatory responses through mitogen-activated protein kinases [11]. Likewise, drinking hydrogen-rich water prevented intimal hyperplasia in arterialized vein grafts and was associated with inhibition of inflammatory cytokines and activation of matrix metalloproteinases [12].

These observations of the efficacy of hydrogen-supplemented drinking water led us to hypothesize that routine oral administration of hydrogen-rich water as drinking water may delay or prevent inflammation-associated chronic deterioration of cardiac allografts. We tested this hypothesis using our established rat heterotopic heart transplant model and an orthotopic aortic transplant model.

Material and methods

Animals

Inbred male LEW ($RT1^l$) and BN ($RT1^n$) rats weighing 200–220 g were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA) and maintained in a specific pathogen-free animal facility. All procedures were performed in accordance with 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.

Heterotopic heart transplantation

Heterotopic heart transplantation was performed as described [13,14] using allogenic rat strains (LEW donor to BN recipient) or isogenic rats (LEW to LEW). Shortly after anticoagulation with 200 units of heparin, 3 ml cold Celsior was infused into the heart. The excised graft was transplanted into the abdomen of recipient rats. Tacrolimus (FK506; Astellas Pharmaceutical Co. Ltd., Osaka, Japan) was administered intramuscularly to graft recipients for 7 days at a daily dose of 0.5 mg/kg (day 0–6). Animals were given buprenorphine (0.1 mg/kg) postoperatively to maintain analgesia. Graft survival was assessed by abdominal palpation. Graft rejection was diagnosed by the cessation of heartbeat and confirmed histologically.

Aortic transplantation

Orthotopic aortic transplantation was achieved using the technique described by Sun [15]. Briefly, the donor thoracic aorta was harvested after heparinization; a 1.5-cm section was placed in the orthotopic position. Aortic grafts taken from BN donors were transplanted into LEW recipients. No immunosuppressants were administered. Histomorphometric analysis for intimal hyperplasia was performed 60 days after transplantation.

Drinking water and experimental groups

A product consisting of metallic magnesium (99.9% pure) and natural stones in a polypropylene container (Friendear Inc, Tokyo, Japan) was used to produce hydrogen-rich water from tap water by the following chemical reaction: $Mg + 2H_2O \rightarrow Mg(OH)_2 + H_2$ [10]. Hydrogen-rich water was also generated by bubbling the tap water with hydrogen gas for 5 min [16]. Degassed control water (DW) was created by exposing Mg-HW to air for 48 h. After transplant, the rats were randomly placed in experimental groups to receive different types of drinking water (Table S1). The water bottles were changed daily and fresh hydrogen-supplemented water was prepared every day.

Measurement of hydrogen content in drinking water and blood

Hydrogen concentrations in water and blood were determined as described previously [11]. Hydrogen-rich water (3 ml) was orally administered by gavage to naïve rats. Arterial blood was taken at 15 min after oral administration of hydrogen-rich water. Administrated water or blood was placed in a glass tube and air-phase hydrogen levels were measured using gas-chromatography (Biogas analyzer BAS-1000; Mitleben, Osaka, Japan).

Real-time RT-PCR

The mRNAs for TNF-α, IFN-γ, CCL2 and CCL5, and GAPDH were quantitated in duplicate using SYBR Green two-step, real-time RT-PCR (Table S2), as previously described [17]. 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). Thermal cycling conditions were 10 min at 95 °C to activate the Amplitaq Gold DNA polymerase, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min on an ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems). Gene expression was normalized to GAPDH mRNA content.

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Histopathological analysis

Formalin-fixed hearts or aorta graft tissues were paraffin-embedded, cut into 5-μm sections, and stained with hematoxylin/eosin (H&E) or Verhoffs Van Gieson elastic tissue stain (VVG; Rowley Biochemical Institute, Danvers, MA, USA). For immunohistochemical analysis of graft-infiltrating cells, paraffin-embedded graft tissue was cut into 5 μm-sections and stained using avidin-biotin-peroxidase detection after antigen retrieval. Sections were incubated with mouse anti-rat CD68 mAb (ED1, Serotec, Raleigh, NC, USA) or with mouse anti-rat CD3 mAb (Serotec), detected by LSAB+ horseradish peroxidase (DAKO, Carpenteria, CA, USA). Positively stained cells in five random high-power fields (HPF, 400×) per section were counted with the samples' identities masked. Isotype-matched irrelevant antibodies were used as controls. Intimal and medial thickness in the aortic cross-sections were quanititated as described previously [12].

MPO activity assay

MPO activity in the cardiac graft was measured using an MPO activity assay kit (BioVision, Mountain View, CA, USA) according to the manufacturer's instructions.

MDA assay

The myocardial tissue was homogenized, and tissue MDA concentration was determined using the BIOXYTECH MDA-586 kit (Oxis International, Foster City, CA, USA), according to the manufacturer's instructions.

In vitro T cell activation

Hydrogen was dissolved in RPMI medium for 6 h under high pressure (0.4 MPa) to a supersaturated level. Hydrogen-rich medium was stored at atmospheric pressure at 4 °C in an aluminum bag with no dead volume, sterilized by gamma radiation, and freshly prepared once a week. Male wild-type C57Bl/6 mice (10–12 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Splenic T cells (2×10^5) were enriched by passage through nylon wool columns and incubated with plate-bound anti-mouse CD3ε mAb (145–2C11: 10 μg/ml; BD Pharmingen, San Diego, CA, USA) in 96-well plate for 48 h in the presence of hydrogen. Cell viability was determined by trypan blue exclusion. The media were changed every 6 h to maintain the concentration of hydrogen at 0.6 mM [18]. ^{3}H-thymidine (1 μCi/ml) was added during the last 12 h of culture. The cultures were harvested onto glass fibermats and ^{3}H-thymidine incorporation was determined with a liquid scintillation counter. IL-2, IFN-γ, IL-4, and IL-10 in culture supernatant were measured using enzyme-linked immunosorbent assays (ELISA) using commercially available kits (R&D systems, Minneapolis, MN, USA) according to manufacturer's instructions.

Measurement of tissue ATP levels

Cellular adenosine triphosphate (ATP) level was quantitated using the ENLITEN ATP luciferin/luciferase bioluminescence assay system (Promega, Madison, WI, USA) as previously described [19,20].

Mitochondrial activity assays

Mitochondria were prepared using a mitochondria isolation kit (Thermo Fisher Scientific, Rockford, IL, USA). Myocardial tissue was finely minced and then homogenized with ice cold mitochondrial isolation buffer. Citrate synthase activity was determined by measuring the rate of 5,5′-dithiobis-(2-nitrobenzoic acid)-reactive reduced coenzyme A (412 nm). Complex I activity was determined spectrophotometrically (340 nm) by monitoring the oxidation of 100 μM NADH in the presence of 10 μM coenzyme Q2 and in the presence and absence of 25 μM rotenone [21]. Complex II/III activity was determined spectrophotometrically (600 nm) by monitoring the oxidation of 120 μM dichlorophenolindophenol (DCPIP) in the presence of 10 μM coenzyme Q2 and in the presence and absence of 1 mM 2-thenyltrifluoroacentone (TTFA). Complex IV activity was determined spectrophotometrically (550 nm) by monitoring the oxidation of reduced cytochrome c. Complex V activity was determined spectrophotometrically (340 nm) by coupling the production of ADP to the oxidation of NADPH via the pyruvate kinase and lactate dehydrogenase reaction.

Statistical analyses

Graft survival was plotted using the Kaplan–Meier method, and the differences between groups were analyzed using the log-rank test. Other data were expressed as mean ± standard deviation (SD) and statistical analysis performed using analysis of variance (ANOVA). When ANOVA indicated a significant overall effect, differences among individual means were assessed using the Bonferroni post hoc test for multiple comparisons. A probability level of $P < 0.05$ was considered statistically significant.

Results

Oral intake of hydrogen-rich water elevated blood hydrogen levels

Hydrogen-rich water was produced either by placing a magnesium stick into tap water (Mg-HW) or by bubbling

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hydrogen gas through tap water (Bu-HW). As the magnesium reaction to generate hydrogen elevates the pH of the water, we also generated degassed control water (DW) by exposing Mg-HW to air for 48 h [18]. Regular tap water (RW) served as an additional control (Table 1). Oral administration of Mg-HW (H_2 concentration: 0.6 mM) or Bu-HW (H_2 concentration: 0.5 mM) by gavage significantly elevated blood H_2 levels from 8.1 ± 0.4 μM in naïve rats to 28.6 ± 2.9 μM in rats given Mg-HW and 19.2 ± 3.4 μM in rats given Bu-HW 15 min after intake.

Oral administration of hydrogen-rich water enhanced cardiac allograft survival

To assess the potential protective effects of hydrogen during heart transplantation, heterotopic heart transplantation was performed using allogenic rat strains [Lewis (LEW) donor to Brown Norway (BN) recipient]. Transplantation was also performed in isogenic rats (LEW to LEW). Graft rejection was diagnosed by the cessation of heartbeat (assessed by abdominal palpation) and confirmed histologically. All isogenic grafts survived >100 days regardless of supplementation of hydrogen in the drinking water. The median survival of the LEW allografts in the BN recipients was 49.5 days in rats given RW and 51.5 days in rats given DW. Supplementation of hydrogen in the drinking water, either by reaction with the Mg stick or by bubbling with hydrogen gas was remarkably effective in prolonging heart graft survival (median survival was 100 days for rats given Mg-HW and 90 days for rats given Bu-HW) without adverse effects. These results suggest that hydrogen plays key roles in prolonging the viability of cardiac allografts (Fig. 1a).

Orally given hydrogen-rich water reduced intimal hyperplasia as a result of chronic rejection

The efficacy of oral intake of hydrogen-rich water was also evaluated using an allogenic orthotopic aortic transplantation model. Aortic grafts taken from BN donors were transplanted into LEW recipients, and histomorphometric analysis for intimal hyperplasia was performed 60 days after transplantation. Drinking hydrogen-rich water significantly reduced intimal hyperplasia in the aortic grafts ($n = 6$ for each group, Fig. 1b).

Hydrogen-rich water reduced myeloperoxidase activity

Graft rejection after heart transplantation activates the immune system and initiates an inflammatory cascade that includes myeloperoxidase (MPO) activation. MPO is part of the innate host defense and catalyzes the formation of reactive oxygen species (ROS) [22]. In the recipients given RW or DW, MPO activity was increased in the cardiac grafts 50 days after transplant. Hydrogen supplementation of the drinking water significantly attenuated increases in MPO activity ($n = 5$ for each group, Fig. 2a).

Hydrogen-rich water reduced oxidative injury

Oxidative injury is common in allografts and contributes to the development of vasculoplasty [3,23]. In the recipients given RW or DW, lipid peroxidation, indicated by tissue malondialdehyde (MDA) levels in the allografts, was significantly increased 50 days after transplantation. Hydrogen supplementation of the drinking water significantly reduced tissue MDA levels (Fig. 2b).

Drinking hydrogen-rich water reduced infiltration of inflammatory cells into the allografts

Graft infiltration by inflammatory cells is an early hallmark of graft failure [24]. Fifty days after transplantation, there were striking increases in CD3+ T cells and CD68+ macrophages in the allografts of rats given RW or DW. Although hydrogen-rich water did not completely inhibit cellular infiltration, the number of CD3+ or CD68+ cells in the allografts of recipients given with Mg-HW or Bu-HW was significantly reduced. (Fig. 2c,d).

Hydrogen-rich water reduced inflammation-related cytokine/chemokine mRNA levels in the grafts

Recognition of foreign antigens by T cells promotes clonal expansion of the T cells; the activated T cells then secrete a variety of cytokines including interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). These cytokines stimulate the vascular endothelium to produce neutrophil- and macrophage-attracting chemokines, including chemokine (C-C motif) ligand 2 [CCL2; also known as monocyte chemotactic protein-1 (MCP-1)] and CCL5

Table 1. Drinking water for each experimental group.

Drinking water	Mg stick	Mg conc (mg/l)	H_2 conc (mM)	pH
Regular water (RW)	No	<1.0	0	6.8–7.1
Degassed water (DW)	Yes	6.2–7.9	0	9.2–9.9
Hydrogen water (Mg-HW)	Yes	6.1–7.9	0.55–0.65	9.3–9.8
Hydrogen water (Bu-HW)	No	<1.0	0.35–0.45	6.8–7.1

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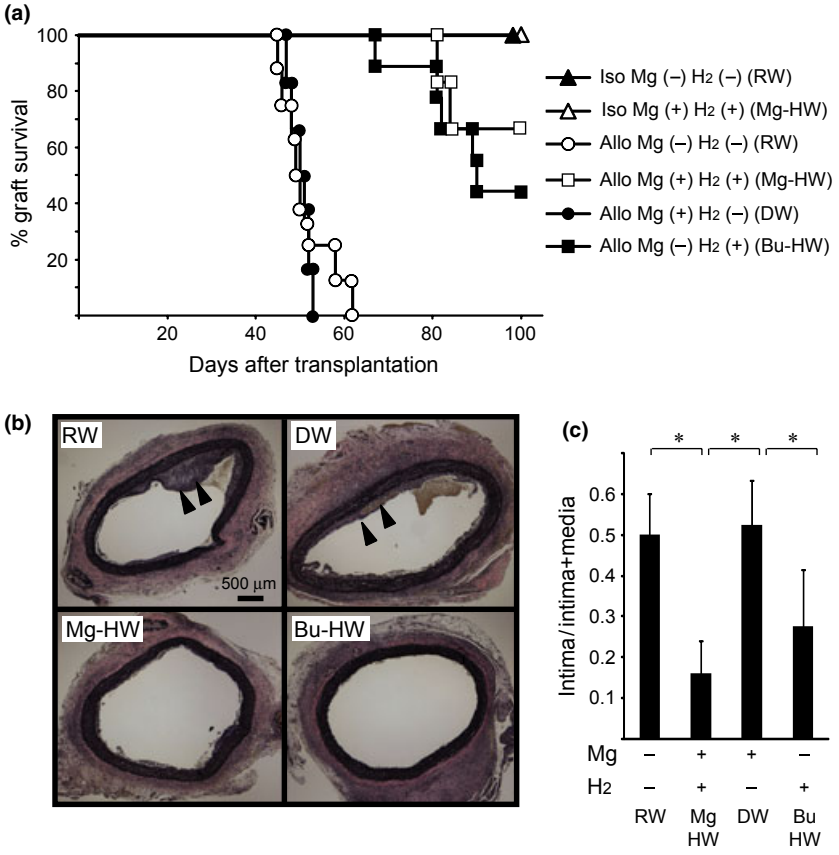

Figure 1 (a) Cardiac graft survival in isografts and allografts of recipients drinking different types of water. Isografts: RW, $n = 5$; Mg-HW, $n = 4$. Allografts: RW, $n = 9$; DW, $n = 6$; Mg-HW, $n = 6$; Bu-HW, $n = 9$. Kaplan–Meier, log-rank test, $^*P < 0.05$ versus RW. (b) Representative Verhoeff's van Gieson staining of transplanted aortas. Arrows indicate intimal hyperplasia of the aortic grafts. (c) Histogram demonstrating the ratio (%) of intimal thickness to intimal plus medial thickness ($n = 5$–6 for each group, $^*P < 0.05$).

[also known as Regulated upon Activation, Normal T cell Expressed, and Secreted (RANTES)] [25]. Real-time RT-PCR showed that the mRNAs for these inflammatory cytokines and chemokines were significantly increased 50 days after transplantation in the allografts. Drinking Mg-HW or Bu-HW inhibited the up-regulation of these inflammatory mediators at the same time point (Fig. 3a).

Hydrogen inhibited T cell proliferation

To determine whether hydrogen directly inhibits T cell proliferation, an *in vitro* mouse T cell culture system was employed. Hydrogen significantly inhibited T cell proliferation (Fig. 3b) and the inhibition of T cell proliferation by hydrogen was associated with significantly decreased production of Th1 type cytokines, such as IL-2 and IFN-γ, as compared with control culture conditions without hydrogen. Anti-inflammatory IL-10 levels were comparable in cultures with or without hydrogen (Fig. 3c,d,e). T cell viability during 24 h in culture was also not affected by hydrogen treatment, with 78–82% of viability regardless of the presence of hydrogen in the medium (data not shown).

Oral intake of hydrogen-rich water increased tissue ATP levels in the cardiac grafts

When testing ischemia/reperfusion injury using lung or heart transplant model, we discovered that hydrogen treatment improved ATP levels in the grafts (unpublished data). Therefore, we examined tissue ATP levels in the cardiac allografts. The ability to generate ATP after transplantation and recover pretransplant tissue ATP levels is important for graft survival. Tissue ATP was measured in cardiac grafts harvested 50 days after transplantation. Although ATP content was decreased in all allografts as compared with the isografts, oral intake of hydrogen-rich water resulted in significantly higher ATP levels in the allografts as compared with allografts from rats that drank RW or DW (Fig. 4a).

Oral intake of hydrogen-rich water increased mitochondrial activity in the allografts

ATP levels directly reflect mitochondrial activity and several reports have suggested that hydrogen can protect the mitochondria from damage [7,8]. Therefore, we analyzed the effects of hydrogen-rich water on

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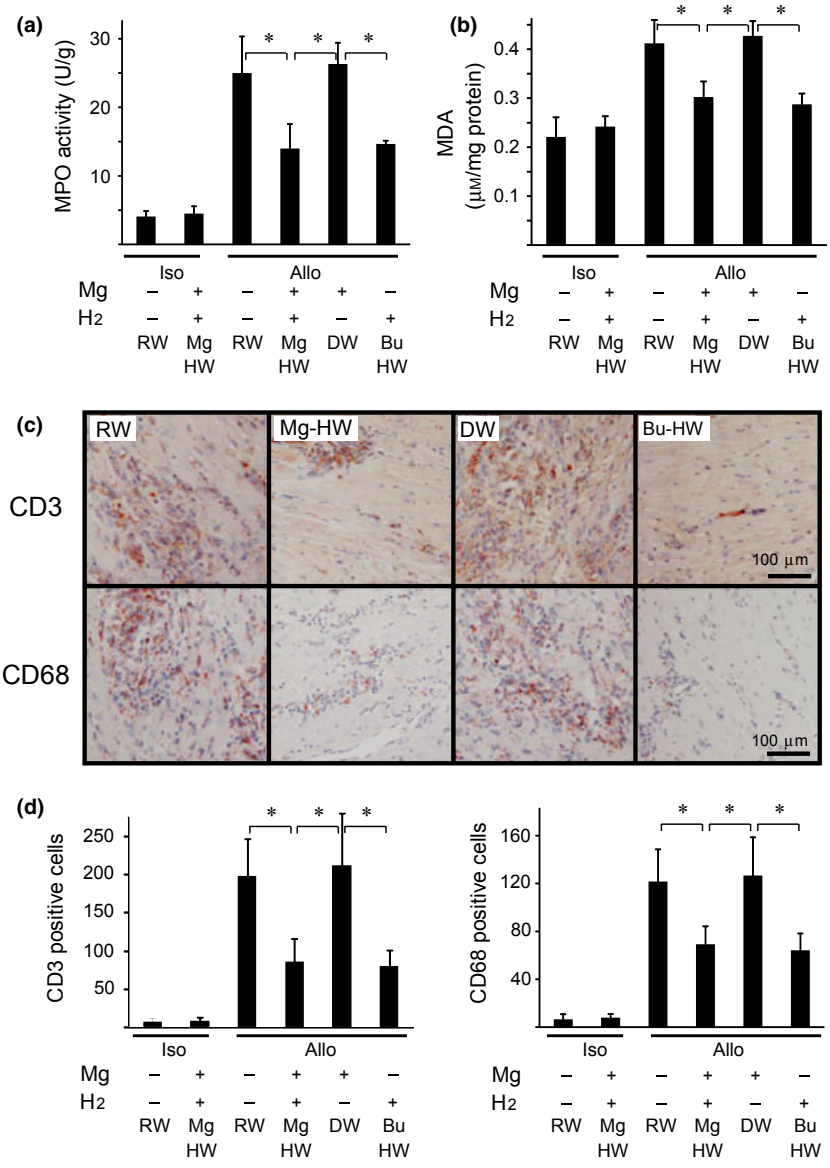

Figure 2 (a) MPO activity and (b) tissue MDA levels in allografts harvested 50 days after transplantation ($n = 5$ for each group, $^*P < 0.05$). (c) Representative immunohistochemical staining for CD3 (indicating T cell infiltration) and CD68 (indicating macrophage infiltration) in heart allografts 50 days after transplant. (d) Number of cells positive for CD3 and CD68. The histograms indicate the number of positive cells per high-power field (HPF; ×400) ($n = 5$ for each group, $^*P < 0.05$).

mitochondrial activity in the grafts. Hydrogen-rich water increased citrate synthase activity in the isografts and allografts (Fig. 4b). The activities of complexes I, II/III, IV, and V were examined in mitochondria isolated from cardiac allografts harvested 50 days after transplantation. Consumption of hydrogen-rich water increased Complex I activity in the allografts. Complex II/III activity and Complex V activity were significantly compromised in the allografts; however, consumption of hydrogen-rich water resulted in partial recovery of activity as compared with control recipients. Complex IV activities were reduced in the allografts as compared with the isografts, but did not differ among the treatment groups (Fig. 4c).

Discussion

This study demonstrated that drinking hydrogen-rich water prolongs the survival of cardiac allografts and reduces intimal hyperplasia in aortic allografts. Drinking hydrogen-rich water ameliorated prolonged inflammation and the associated increases in the expression of proinflammatory cytokines and chemokines, which contribute to transplant pathology. In addition, T cell proliferation, IFN-γ secretion, and IL-2 secretion were significantly inhibited *in vitro* in the presence of hydrogen, which may contribute to enhanced cardiac allograft survival. Administration of hydrogen in the drinking water also increased mitochondrial function in the allografts.

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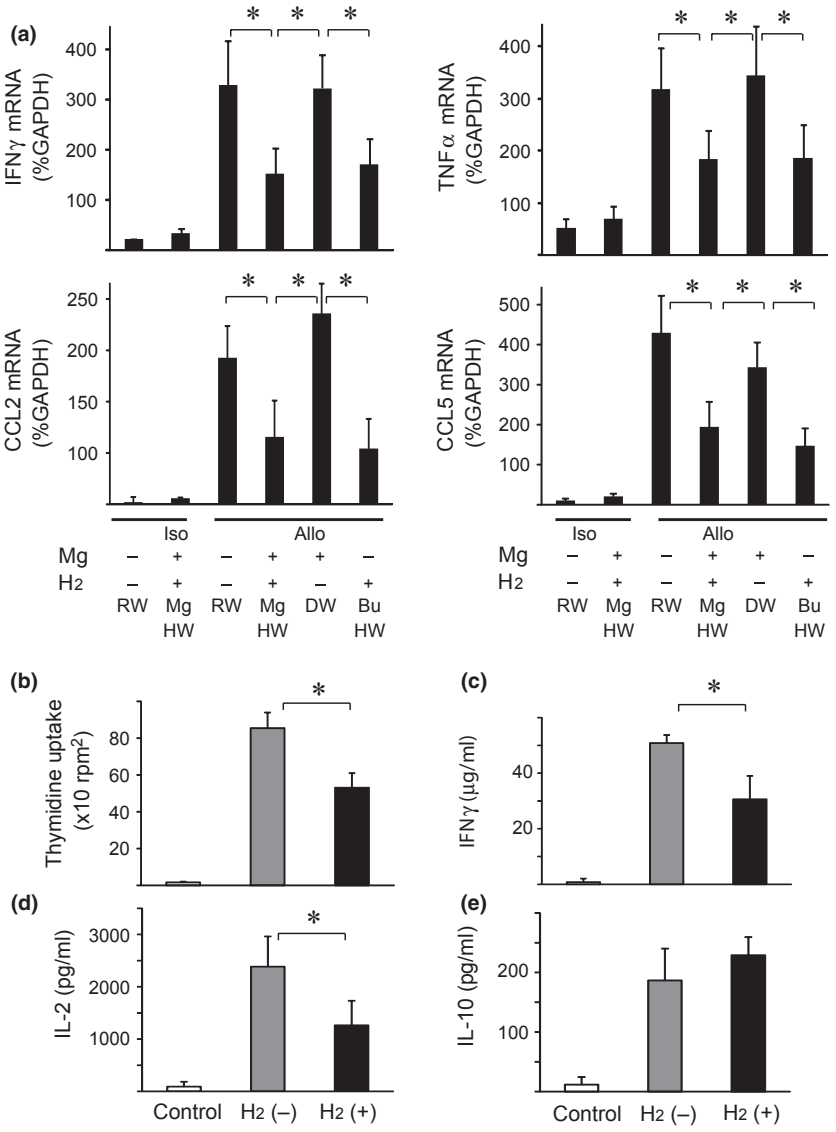

Figure 3 (a) Inflammation-related cytokine/chemokine mRNA levels for IFN-γ, TNF-α, CCL2, and CCL5 in the allografts at day 50 were determined using real-time RT-PCR ($n = 5$ for each group, $^*P < 0.05$). (b) *In vitro* T cell proliferation with or without hydrogen. T cell proliferation was determined by thymidine incorporation ($^*P < 0.05$). ELISA analysis for (c) IFN-γ, (d) IL-2, and (e) IL-10 in the culture supernatant ($^*P < 0.05$). Data are representative of three independent experiments.

Oxidative stress, including lipoprotein oxidation, likely contributes to allograft vasculopathy, limiting long-term survival after cardiac transplantation [3,26,27]. Transplant-induced allograft vasculopathy is an important pathology. Clinical manifestations of transplant atherosclerosis are observed in 50% of heart transplant recipients within 5 years of transplantation [28,29], with 75% of transplant recipients exhibiting significant allograft vasculopathy by 3 years post-transplant [30] and 90% exhibiting robust allograft vasculopathy within 10 years [31]. The mechanisms of graft vasculopathy remain controversial; immune injury and classic risk factors (such as hypertension, dyslipidemia, and diabetes mellitus) seem to act in concert [32]. Clinical and experimental studies have demonstrated that long-term antioxidant treatment of cardiac transplant recipients improves allograft outcomes through inhibition of graft vasculopathy [3,23,33,34]. In early research on hydrogen as a therapeutic gas, several studies attributed the beneficial effects of hydrogen to hydrogen's ability to function as a hydroxyl radical scavenger [7,35]. Undoubtedly, hydrogen acts as antioxidant and the ability of hydrogen to eliminate toxic ROS is likely an important mechanism of action in the protection of cardiac allografts from inflammation-induced deterioration and allograft vasculopathy.

Oxidative stress in cardiac allografts is also associated with structural damage and mitochondrial dysfunction, characterized by reduced energy production through oxidative phosphorylation and loss of respiratory enzyme activity of complexes I-V in the inner mitochondrial

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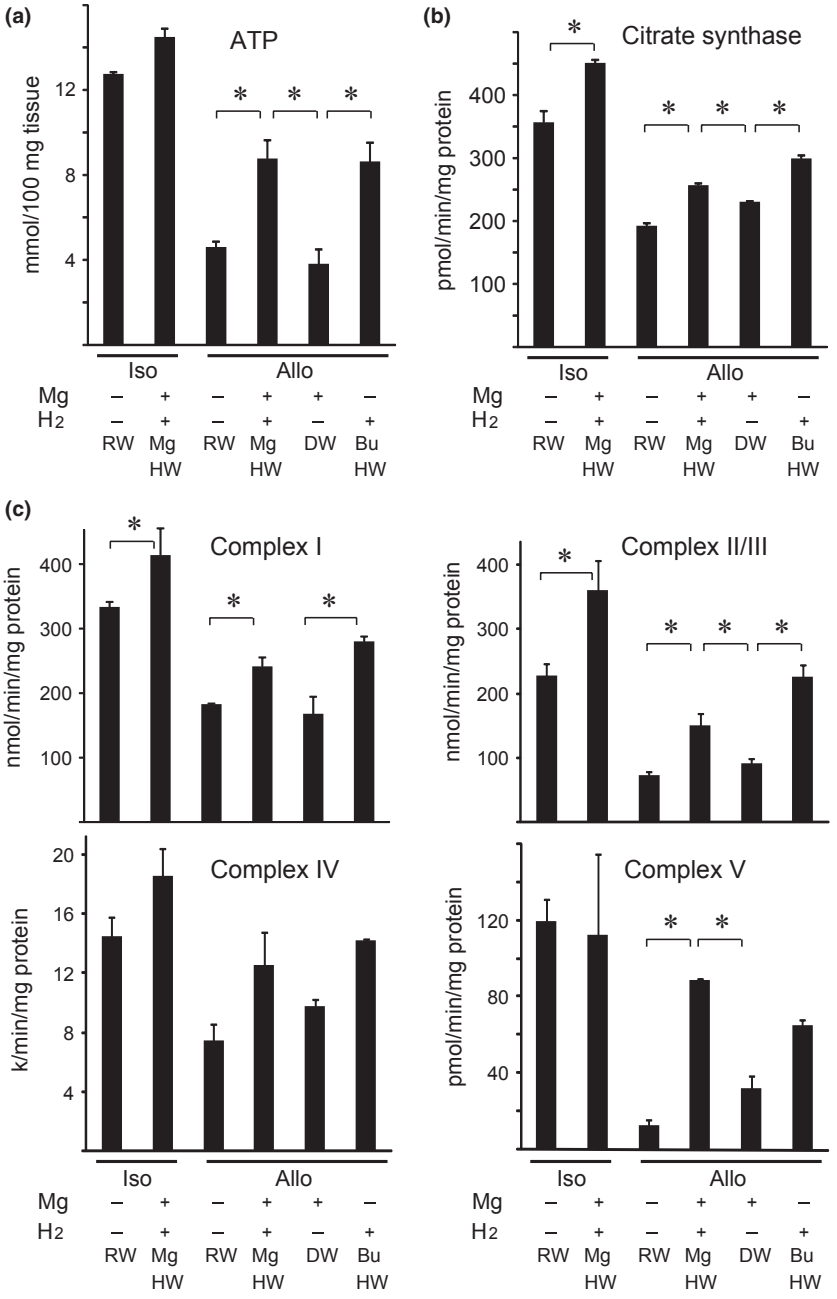

Figure 4 (a) Tissue ATP levels, (b) citrate synthase activity, and (c) the activities of mitochondrial complexes I, II/III, IV, and V were measured in the cardiac grafts harvested 50 days after transplantation ($n = 5$ for each group, $*P < 0.05$).

membrane [36]. Hydrogen can penetrate the mitochondrial membrane, prevent the decline of the mitochondrial membrane potential, and protect the mitochondria from ROS [7]. Drinking hydrogen-rich water increased mitochondrial function in the allografts and prevented the loss of mitochondrial complex activity. Thus, hydrogen may directly protect the mitochondria and, thereby, restore mitochondrial energy metabolism, especially fatty acid metabolism. Fatty acid metabolism reduces the generation of ROS and, therefore, reduces oxidative stress [8]. Our data also demonstrated that hydrogen-rich water increased citrate synthase activity in isografts and allografts. Although further investigation is needed, this result suggests that hydrogen-rich water stimulated proliferation of mitochondria in the graft because citrate synthase activity reflects mitochondrial density. Restoration of ATP levels in the allografts in recipients that consumed hydrogen-rich water likely results from increasing the number of mitochondria in the graft and sustaining mitochondrial respiratory function.

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Drinking hydrogen-supplemented water generated with a magnesium stick increases the daily intake of magnesium. The clinical signs of excess magnesium intake include changes in mental status, nausea, diarrhea, appetite loss, muscle weakness, difficulty breathing, extremely low blood pressure, and irregular heartbeat [37,38]. We did not see any animals manifesting these adverse events, nor did we see these adverse events in human subjects in a previous study [10]. The magnesium concentrations of Mg-HW were 6.8–7.1 mg/l. The recommended dietary allowance for magnesium is 80–130 mg/day for children and 350–420 mg/day for adults (http://ods.od.nih.gov/factsheets/magnesium/#en4#en4). Thus, the extra magnesium consumption from drinking Mg-HW will likely be far below the daily recommended levels. As dietary magnesium is absorbed in the small intestines and excreted through the kidneys, the risk of magnesium toxicity may increase in patients with kidney failure [39].

In conclusion, drinking hydrogen-rich water daily prevented cardiac allograft rejection and reduced aortic allograft intimal hyperplasia in a rat model. The addition of hydrogen-rich water to post-transplant therapeutic regimens may significantly improve health care for transplant recipients. Supplementation of drinking water with hydrogen is safe and cost-effective, and hydrogen-supplemented water can administered to transplant recipients without changing their lifestyle.

Authorship

KN and YT: participated in research design, research performance, data analysis, and wrote the manuscript; TK, YW and KM: participated in research performance, including the histopathological data analysis; NS, XS, YT and CAB: substantially contributed to the study concept; AN: provided the working hypothesis, participated in research design, the performance of the research and data acquisition, and wrote the manuscript. All authors reviewed and approved the final manuscript.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Experimental groups.

Table S2. Nucleotide sequences of oligonucleotide primers (RT-PCR).

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