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ORIGINAL ARTICLE

# Hydrogen-water ameliorates radiation-induced gastrointestinal toxicity via MyD88's effects on the gut microbiota

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**Although radiation therapy is a cornerstone of modern management of malignancies, various side effects are inevitably linked to abdominal and pelvic cancer after radiotherapy. Radiation-mediated gastrointestinal (GI) toxicity impairs the life quality of cancer survivors and even shortens their lifespan. Hydrogen has been shown to protect against tissue injuries caused by oxidative stress and excessive inflammation, but its effect on radiation-induced intestinal injury was previously unknown. In the present study, we found that oral gavage with hydrogen-water increased the survival rate and body weight of mice exposed to total abdominal irradiation (TAI); oral gavage with hydrogen-water was also associated with an improvement in GI tract function and the epithelial integrity of the small intestine. Mechanistically, microarray analysis revealed that hydrogen-water administration upregulated miR-1968-5p levels, thus resulting in parallel downregulation of *MyD88* expression in the small intestine after TAI exposure. Additionally, high-throughput sequencing showed that hydrogen-water oral gavage resulted in retention of the TAI-shifted intestinal bacterial composition in mice. Collectively, our findings suggested that hydrogen-water might be used as a potential therapeutic to alleviate intestinal injury induced by radiotherapy for abdominal and pelvic cancer in preclinical settings.**
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## INTRODUCTION

After the bone marrow, the gastrointestinal (GI) tract ranks as the second-most sensitive organ to irradiation injury during cancer therapy.[1] During radiotherapy of abdominal and pelvic malignancies, ionizing radiation destroys the mucosal surface of the GI tract, thus leading to symptoms that may impair the course of treatment and even cause death.[2] Even for healthy populations, unexpected irradiation exposure also leads to severe life-threatening intestinal injury.[3] Therefore, irradiation-induced GI tract toxicity remains a conundrum that urgently requires effective therapy.

A growing body of evidence indicates that hydrogen ($H_2$), as a novel antioxidant, scavenges hydroxyl radical and peroxynitrite.[4] In contrast to other antioxidants, gaseous molecular hydrogen efficiently penetrates cytoplasmic membranes and targets intracellular organelles, largely owing to its small size and neutral electricity.[5] Intracellularly, it selectively neutralizes cytotoxic reactive oxygen species (ROS) such as OH in living cells instead of reacting with other ROS that possess physiological roles. As a result, hydrogen has been considered an ideal therapeutic agent for a wide array of diseases, including cardiovascular, cerebrovascular, metabolic disorders and certain types of cancer.[6] Hydrogen can be administered in many different ways, among which hydrogen-water is a promising hydrogen usage, owing to its convenience and safety. Moreover, hydrogen-water has been reported to mitigate the hematological injury induced by irradiation through the suppression of radiation-induced caspase 3 activation beyond rescuing the radiation-induced depletion of platelets.[7] To date, it has been unknown whether hydrogen-water might confer protection against radiation-mediated intestinal toxicity in preclinical experimental settings.

Toll-like receptors (TLRs), one of the most well-characterized pattern recognition receptors, recognize

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microbial pathogens for the innate immune system through recognizing pathogen-associated molecular patterns.[8,9] MyD88 (myeloid differentiation primary response gene 88) is a central adaptor protein for many TLRs, and it serves as an essential modulator of the innate immune response to microbial pathogens.[10,11] MyD88 has been reported to mediate immunopathology and gut microbiota dynamics in intestinal graft-versus-host disease involving TLR9.[12] Animals deficient in *MyD88* within the T-cell compartment experience perturbed microbiota (dysbiosis) in the mucosal compartment and develop severe intestinal inflammation,[13] thus suggesting an important role of MyD88 in maintaining the mutualism between hosts and microbiota in healthy conditions.[14] However, whether hydrogen-water affects the expression of MyD88 in irradiated animals remains enigmatic.

In this study, we sought to investigate whether hydrogen-water might ameliorate radiation-mediated small intestinal toxicity by using mouse models. We found that oral gavage with hydrogen-water improved GI tract function and the epithelial integrity of small intestine tissue, thus resulting in an increase in the survival rate and body weight of mice after TAI. Administration of hydrogen-water increased the level of miR-1968-5p targeting *MyD88* in small intestine tissues and preserved the intestinal bacterial composition structure in irradiated mice. Thus our findings provide new insights into the therapeutic potential and protective mechanism of hydrogen-water in ameliorating TAI-induced GI toxicity in preclinical settings.

## MATERIALS AND METHODS

### Animals

All experiments were carried out in accordance with procedures approved by the Daegu-Gyeongbuk Medical Innovation Foundation Institutional Animal Care and Use Committee. All procedures and animal handling were performed by following the ethical guidelines for animal studies. Male C57BL/6 mice (approximately 20 g) were housed under standard conditions (ambient temperature $22 \pm 2$ °C, air humidity 40–70% and a 12/12-h light/dark cycle) and were given continuous access to a standard diet and water according to the guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All mice in this study were male and were of a pure C57BL/6 genetic background.

### Irradiation study

A Gammacell 40 Exactor (Best Theratronics Ltd., Kanata, ON, Canada) was used for all experiments. In this study, male mice were treated with a single gamma-ray dose of 15 Gy at a rate of 1.0 Gy $min^{-1}$ total abdominal irradiation (TAI). The weight of the mice treated with TAI or TAI with hydrogen-water was assessed for 5 days.

### Experimental protocol

The animals were separated into two groups. (1) TAI group: mice were treated with 15 Gy TAI. Normal water (0.2 ml) was force-fed into each mouse's stomach using gavage two times per day; and (2) TAI +HW (hydrogen-water) group: mice were treated with 15 Gy TAI. Hydrogen-water (0.2 ml) was force-fed into each mouse's stomach using gavage two times per day.

### Hydrogen-water administration

$H_2$ gas was generated from a hydrogen gas generator (SHC-300, Saikesaisi HW Energy, Shandong, China) and bubbled into 500 ml of sterile water at a rate of 150 ml $min^{-1}$ for 20 min. The concentration of $H_2$ in the water was detected with a dissolved hydrogen meter (Trustlex ENH-1000, Osaka suita, Japan). Each day, we used a fresh preparation of hydrogen-water, aiming to reach the overall saturation level of 0.8 mM.

### Formed fecal collection

For this study, six mice (6–8-week-old male or female C57BL/6J mice) were fed in one cage. They were kept under the same environment as the other experimental mice. Formed feces were collected every 2 days from the first day of irradiation. Then we counted the number of formed stools in every group.

### Peripheral blood cell counts

One hundred microliters of peripheral blood was obtained from the orbital sinus by using a micro-pipette coated with the anticoagulant $K_2$EDTA 15 days after 15 Gy TAI. The cell counts included white blood cells and hemoglobin and were counted using a Celltac E hemocytometer (Nihon Kohden, Tokyo, Japan).

### Plasmid construction

A ~400 bp fragment of the *MyD88* 3′-untranslated region (3′UTR) was cloned into pGL3-control vector (Promega, Madison, WI, USA) immediately downstream of the stop codon of the luciferase gene to generate pGL3-*MyD88*. A mutant construct of the conserved seed region of *MyD88* 3′UTR (named pGL3-*MyD88*-mut), carrying a substitution of 8 nucleotides within the core seed sequence of miR-1968-5p, was generated by using overlapping extension PCR. The primers are listed in Supplementary Table S1.

### Cell culture

The 3T3 cells were maintained in DMEM (Gibco, Grand Island, NY, USA). The cells were cultured with heat-inactivated 10% fetal bovine serum (Gibco), 100 U $ml^{-1}$ penicillin and 100 mg $ml^{-1}$ streptomycin and grown at 5% $CO_2$ and 37 °C.

### Cell transfection

The cells were cultured in 6- or 24-well plates for 24 h and then were transfected with microRNA (miRNA) or small interfering RNAs, respectively. All transfections were performed using linear polyethylenimine (Sigma-Aldrich, Saint Quentin-Fallavier, France) according to the manufacturer's protocol. miR-1968-5p and anti-miR-1968-5p were synthesized by RiboBio (Guangzhou, China).

### Luciferase reporter assays

Luciferase reporter assays were performed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Cells were cultured in 24-well plates at approximately $3 \times 10^4$ cells per well. After 24 h, the cells were transiently co-transfected with the pRL-TK plasmid (Promega) containing the Renilla luciferase gene, which was used for internal normalization, and with the construct carrying the seed sequence of *MyD88* 3′UTR (or pGL3-control). All experiments were performed at least three times.

### Quantitative reverse transcriptase-PCR

Total RNA was separated from mouse tissues using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. For mature miR-1968-5p detection, total RNA was polyadenylated by poly (A) polymerase (Ambion, Austin, TX, USA), as described previously.[15] cDNA was produced by using poly(A)-tailed total RNA and reverse transcription primer with ImPro-IIReverse Transcriptase (Promega) according to the manufacturer's instructions. Quantitative reverse transcriptase-PCR was performed according to the instructions of Fast Start Universal SYBR Green Master Mix (Rox) (Roche Diagnostics GmbH, Mannheim, Germany). The primers are listed in Supplementary Table S1. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and U6 were used as controls.

### Western blotting analysis

The expression of *MyD88*, *MDA* and *HO-1* was examined using western blotting analysis. Ice-cold radio-immuno-precipitation assay buffer supplemented with phosphatase and protease inhibitors was used for protein extraction. Total protein samples were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% acrylamide gel) using a Bio-Rad Trans-Blot system (Hercules, CA, USA) and were transferred to membranes. Later, specific antibodies to *MyD88* (ab135693), *MDA* (ab27642) and *HO-1* (Proteintech Group, Chicago, IL, USA) were used at a dilution of 1:500. Additionally, membranes were incubated with anti-GAPDH (Proteintech Group), which served as the internal control. The readout was detected by using ChemiDoc XRS+ with the Image Lab Software (Bio-Rad).

### Hematoxylin and eosin

The mice in the experiment were dissected; their small intestines were immediately placed in freshly prepared 4% formaldehyde in phosphate-buffered saline and fixed overnight with rocking at 4 °C. The tissues were sectioned into 5-μm sections and stained.

### Measurement of malondialdehyde by enzyme-linked immunosorbent assay

The level of malondialdehyde (MDA) in the small intestine was determined using a detection kit from Solarbio (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) according to the manufacturer's instructions. Levels of MDA were evaluated and calculated by the following formula, according to the manufacturer's instructions:

$$\text{The levels of MDA (nmol) in the small intestine} = 25.8 \times (\text{A532} - \text{A600})$$

### Bacterial diversity analysis

Stool samples were freshly collected and stored at − 80 °C before use. DNA was extracted from the stool by using a Power Fecal DNA Isolation Kit (MoBio, Carlsbad, CA, USA). The DNA was recovered with 30 ml of buffer in the kit. The 16S ribosomal RNA (rRNA) gene was analyzed to evaluate the bacterial diversity by using the Illumina Hiseq platform (Novogene Bioinformatics Technology Co., Ltd., Madison, WI, USA).

### Microarray analysis

These samples were used to synthesize double-stranded complementary DNA (cDNA) and the cDNA was labeled and hybridized to the Affymetrix Genechip microarray 4.0 (CapitalBio Corp, Beijing, China) according to the manufacturer's instructions. The data from the microarray were used to analyze data summarization, normalization and quality control by using the Gene Spring software V11.5 (Agilent, Santa Clara, CA, USA). The differentially expressed microRNAs were selected if the change of threshold values were $>2.0$-fold and if Benjamini–Hochberg corrected $P$-values were $< 0.05$. The data were normalized and hierarchically clustered with the CLUSTER 3.0 software (Sun Microsystems, Palo Alto, CA, USA). The data visualization was performed with the Java Tree view software (Oracle, Redwood Shores, CA, USA).

### Statistical analysis

The data are presented as the mean ± s.e.m. with respect to the number of samples ($n$) in each group. Statistical significance between multiple treatment groups was determined by analysis of variance and Tukey's $t$-test. The survival rates were analyzed using Kaplan–Meier survival test. Results with $P<0.05$ were considered statistically significant.

## RESULTS

### Hydrogen-water administration protects mice against radiation-induced GI toxicity

We prepared hydrogen-rich water solution and monitored the hydrogen release kinetics under room temperature. Freshly prepared hydrogen-water showed an initial maximum $H_2$ concentration approximately 0.7 mM, but the $H_2$ concentration progressively decreased to 0.2 mM within 8 h (Figure 1a). Thus we routinely prepared fresh hydrogen-water immediately before each experiment. As shown in Figure 1b, the abdomens of TAI-treated mice exhibited radiation-induced alopecia within 2 months, thus validating the feasibility of the TAI system for inducing gut injury. After exposure to 15 Gy TAI, the animal survival rate was decreased by 50% in the control vehicle group, but it was decreased by only 10% in animals receiving the hydrogen-water (via oral gavage, Figure 1c). Moreover, hydrogen-water administration also significantly increased the body weights of the irradiated mice (Figure 1d), thus indicating that hydrogen-water protects against TAI-induced mortality and weight loss. Then the antioxidant function of hydrogen-water was explored. In irradiated animals, the oxidative stress marker MDA was significantly increased in the small intestine, but it showed a marked decrease in the hydrogen-water group (Figure 1e and Supplementary Figure 1b). Given that nuclear factor erythroid 2 related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) have important roles in the antioxidative stress response,[16] we further observed that the intestinal levels of *Nrf2* and *HO-1* increased after TAI exposure, but hydrogen-water administration slightly blunted the changes (Figure 1f and Supplementary Figure 1b).

Exposure to a high dose of irradiation in a short time is associated with acute radiation syndrome, which manifests as severe diarrhea and fluid loss.[17] To explore whether hydrogen-water might relieve radiation-induced GI toxicity, we quantified the total amount of stool pellets in the cage bedding. As shown in Figures 2a and b, the TAI-exposed animals had fewer stool pellets than did mice treated with hydrogen-water at day 6 after irradiation. Oral gavage with hydrogen-water mitigated

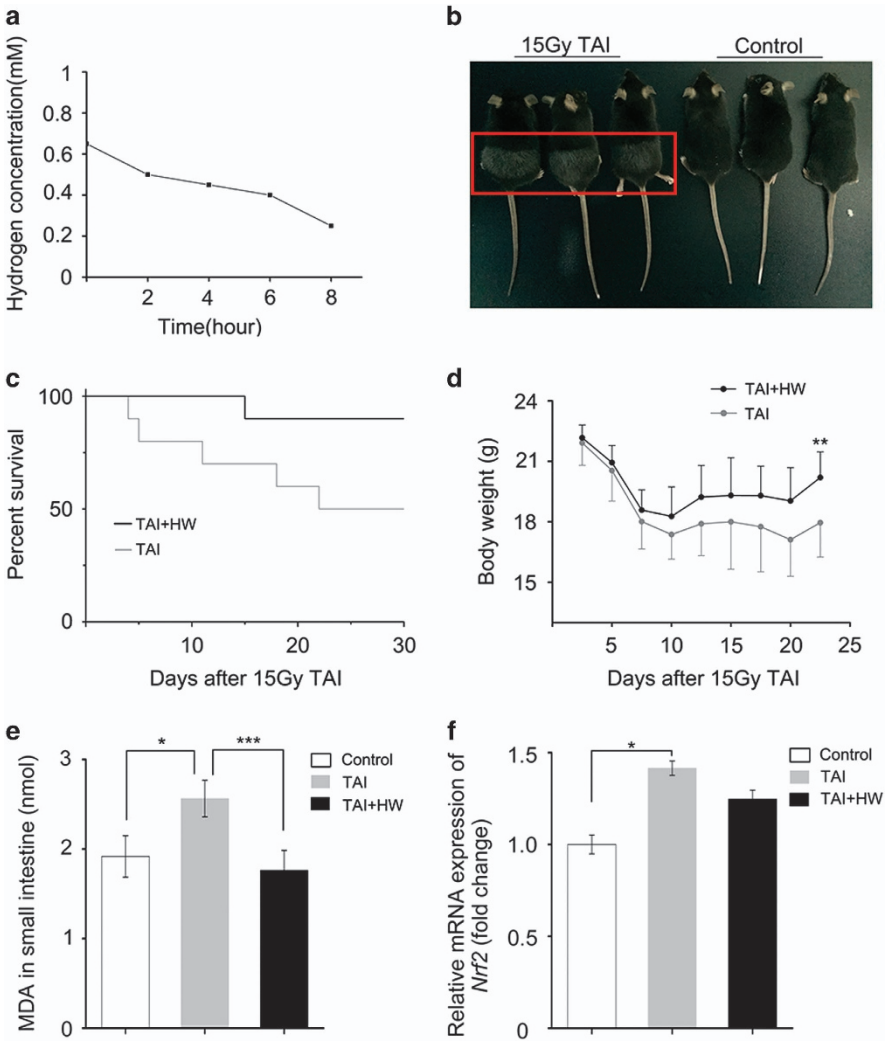


**Figure 1** Oral gavage with hydrogen-water protects mice against radiation-induced toxicity. Mice were treated with hydrogen-water 2 days before and 7 days after receiving 15 Gy TAI. (**a**) Direct examination of $H_2$ concentrations over an 8-h period. (**b**) A mouse treated with 15 Gy TAI (left) and its littermate without irradiation (right). Note the change in fur color in the irradiated lower body. (**c**) Kaplan–Meier analysis of hydrogen-water- and normal water-treated mice after 15 Gy TAI. $P<0.05$ by log-rank test between TAI-exposed mice with or without hydrogen-water treatment, $n=12$. (**d**) Body weight was compared between hydrogen-water- and saline-treated mice after 15 Gy TAI. $n=12$; $^{**}P<0.01$; Student's *t*-test. (**e**) The level of MDA in the small intestine was compared among the healthy control, 12 Gy TAI and hydrogen-water groups. $n=12$; $^{*}P<0.05$; $^{**}P<0.01$;$^{***}P<0.005$; Student's *t*-test. (**f**) The expression levels of Nrf2 were assessed in small intestine tissue from the healthy control, 12 Gy TAI and hydrogen-water groups. $n=12$; $^{*}P<0.05$; Student's *t*-test.

TAI-induced GI injury. As shown in Figure 2c, abdominal irradiation caused the intestinal contents to thin down, and watery diarrhea appeared. However, hydrogen-water treatment mitigated the injury and kept the intestinal contents close to the normal state-containing stool pellets. Hematoylin and eosin staining of small intestine further revealed a dramatic decrease in the number of intact intestinal villi in irradiated animals, and this effect was significantly rescued by oral gavage with hydrogen-water (Figure 2d), thus suggesting that hydrogen-water confers protection against irradiation injury by restoring both GI tract functions and epithelial integrity. In agreement with the essential role of solute carrier family 2 member 1(Glut 1/Slc2a1) phosphoglycerate kinase 1 (Pgk1) and multi-drug resistance protein 1 (MDR1) in maintaining epithelial integrity after toxic stimuli,[18,19] we validated that hydrogen-water treatment dramatically increased the expression levels of *Glut1*, *Pgk1* and *MDR1* in the small intestine tissues of TAI-treated mice (Figures 2e–g). In sharp contrast, oral gavage with hydrogen-water did not significantly alter peripheral white blood cell counts after TAI (Supplementary Figure 1a). Together, our observations demonstrated that hydrogen-water improves GI tract functions and epithelial integrity, thus leading to an increase in the survival rate of TAI-treated mice.

### Hydrogen-water decreases the level of MyD88 in the mouse small intestine

Given the important role of MyD88 in the integrity of small intestine,[20] we examined the effect of hydrogen-water administration on the expression of *MyD88* in small intestine tissues. Although TAI exposure decreased *MyD88* mRNA expression,

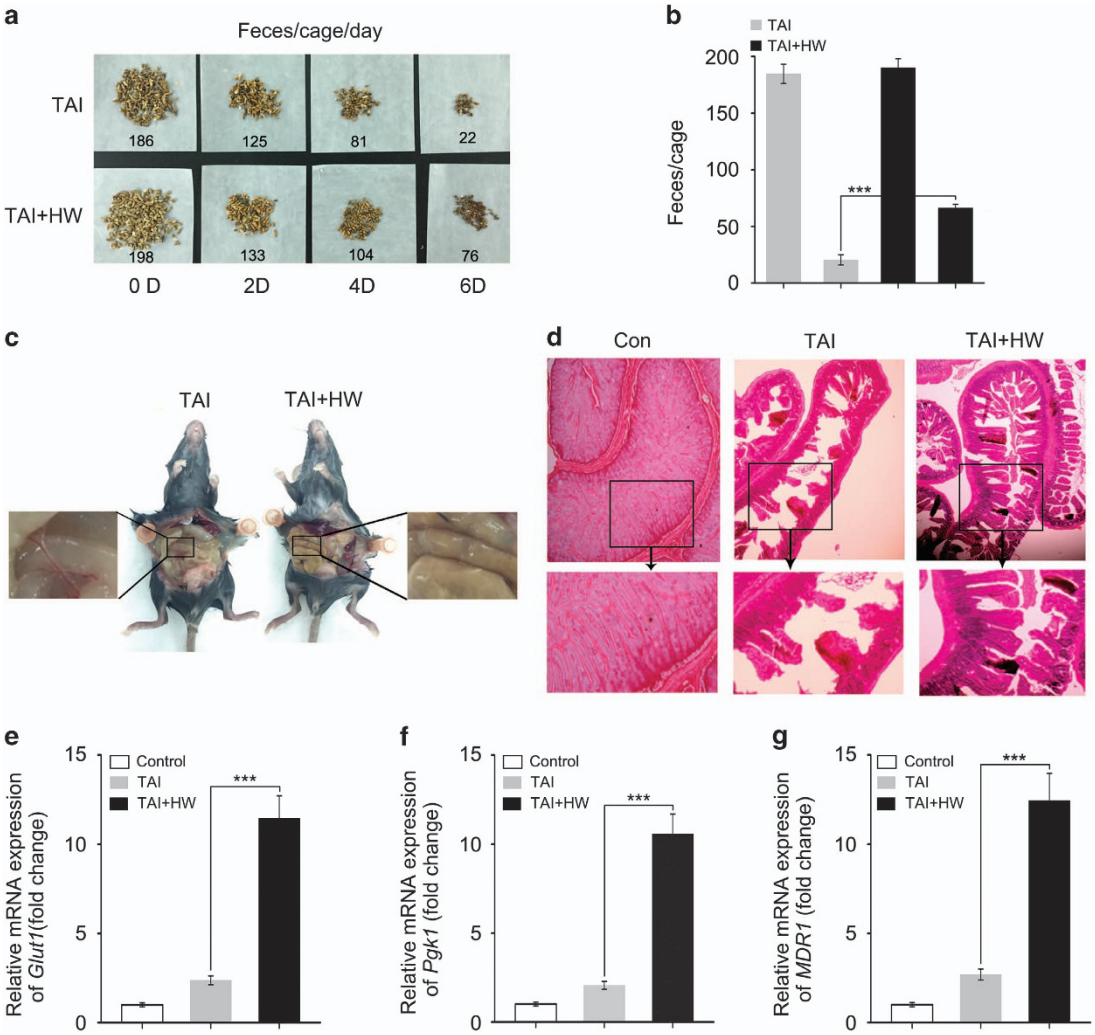


**Figure 2** Hydrogen-water administration improves GI function and epithelial integrity of irradiated mice. (**a** and **b**) Counts of droppings removed from the cage bedding each day from representative cages, $n=6$ mice per cage. Statistically significant differences are indicated: $^{***}P<0.005$; Student's *t*-test. (**c**) Examples of intestinal tract from a total abdominal irradiated mouse and a TAI-exposed mouse with hydrogen-water treatment. (**d**) The morphology of small intestine in radiation-induced mice treated with normal water or hydrogen-water is shown by H&E staining. (**e**–**g**) The expression levels of *Glut1*, *Pgk1* and *MDR1* were assessed by qRT-PCR in small intestine tissue from TAI mice without or with hydrogen-water treatment. Statistically significant differences are indicated: $^{***}P<0.005$; Student's *t*-test.

hydrogen-water administration further decreased the expression level (Figure 3a). Western blotting analysis further validated the results (Figure 3b). To elucidate the underlying molecular mechanism, we assessed the microRNA expression profile in small intestine tissues by using a microarray chip. Microarray and bioinformatics analysis (RNA 22, https://cm.jefferson.edu/rna22/) revealed that the expression of a spectrum of miRNAs had fluctuated, such as the expression of miRNA-1968-5p, which has been predicted to target a number of essential signaling molecules, including MyD88 (Figure 3c and Supplementary Figure 2). Thus the upregulation of miRNA-1968-5p by TAI and further elevation by hydrogen-water was confirmed by quantitative real-time PCR (Figure 3d), thus indicating that miR-1968-5p might be involved in the hydrogen-water-mediated protection against irradiation-induced injury. Correlation analysis between *MyD88* mRNA and miR-1968-5p levels exhibited an inverse correlation (Figure 3e), thus supporting the possibility that miR-1968-5p might target *MyD88* to inhibit its mRNA stability. Given that *TLR4* and *TLR5* are expressed in the mouse small intestine dependent on the adaptor *MyD88*,[21,22] we further evaluated the expression of *TLR4* and *TLR5* in the small intestine in this system. Our data revealed that total body irradiation decreased the expression of *TLR4* and *TLR5*. Hydrogen-water treatment further decreased their expression to a significantly different level (Figures 3f and g). Together, our data suggested that hydrogen-water ameliorates TAI-induced GI toxicity partly through upregulating miR-1968-5p, which might target *MyD88*.

### MiR-1968-5p inhibits the expression of MyD88 by targeting its 3′UTR

To test this hypothesis, we analyzed the effect of miR-1968-5p supplementation on *MyD88* expression through luciferase reporter assays. On the basis of bioinformatics analysis (https://cm.

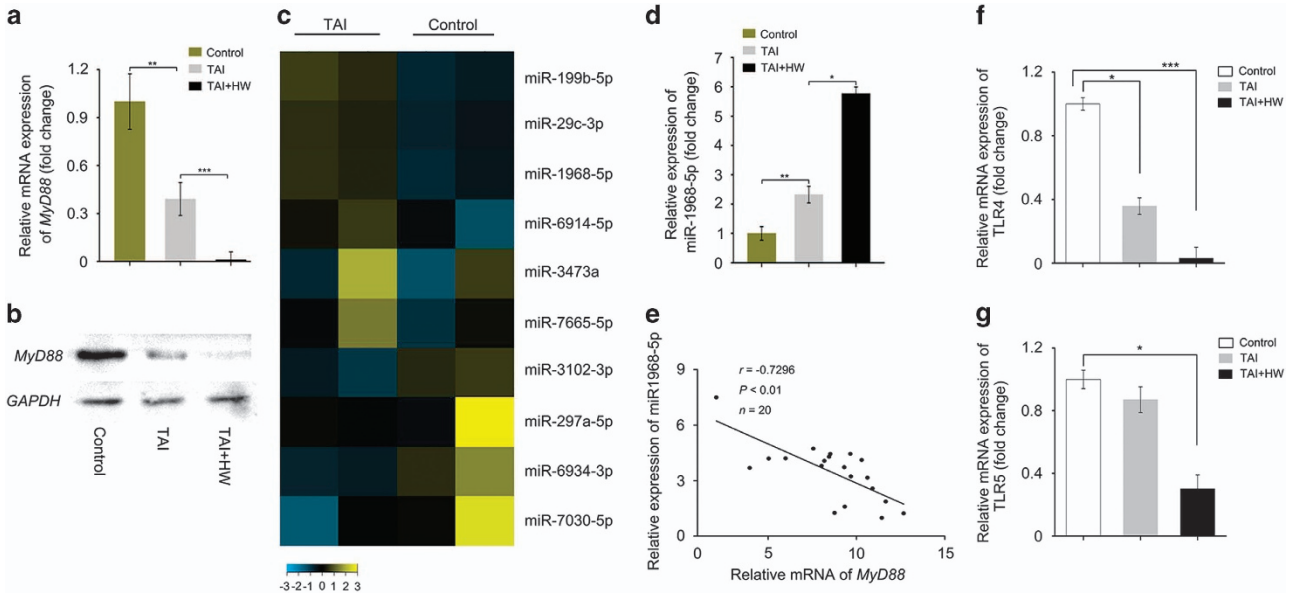


**Figure 3** Hydrogen-water upregulates the level of *MyD88*-targeting miR-1968-5p in the mouse small intestines. (**a**) The expression level of *MyD88* was examined in the aforementioned small intestine tissues by qRT-PCR. Statistically significant differences are indicated: $^{**}P<0.01$; $^{***}P<0.005$; Student's *t*-test. (**b**) The expression of *MyD88* was examined by western blotting in TAI-exposed mice with or without hydrogen-water treatment. (**c**) Alterations in miRNA expression in small intestine tissues from mice without or with TAI were assessed using microarray analysis. (**d**) The expression level of miR-1968-5p was examined by qRT-PCR in small intestine tissues from mice without TAI (Control, $n=20$), mice with TAI (TAI, $n=20$) and mice with hydrogen-water treatment after TAI (TAI+HW, $n=20$) individually. Statistically significant differences are indicated: $^{*}P<0.05$; $^{**}P<0.01$; Student's *t*-test. (**e**) The correlation between *MyD88* mRNA expression and miR-1968-5p level was examined by qRT-PCR in 20 cases of small intestine tissues from mice with hydrogen-water treatment after TAI. $^{**}P<0.01$; Pearson correlation coefficient, $r=-0.7296$. (**f**) The expression level of *TLR4* was examined in the aforementioned small intestine tissues by qRT-PCR. Statistically significant differences are indicated: $^{*}P<0.05$; $^{***}P<0.005$; Student's *t*-test. (**g**) The expression level of *TLR5* was examined in the aforementioned small intestine tissues by qRT-PCR. Statistically significant differences are indicated: $^{*}P<0.05$; Student's *t*-test.

jefferson.edu/rna22/), we predicted position 1595–1602 as one of the three possible binding sites for miR-1968-5p in the 3′UTR of *MyD88* (Figures 4a and b). Indeed, luciferase reporter assays revealed that miR-1968-5p downregulated the luciferase activity of a construct carrying the seed region of the *MyD88* 3′UTR in a dose-dependent manner (position 1595–1602, pGL3-*MyD88*) (Figure 4c). In contrast, miR-1968-5p did not decrease the luciferase activity of a construct containing the *MyD88* 3′UTR seed region with complementary sequence (mutant in same position 1595–1602, pGL3-*MyD88*-mut) (Figure 4c). Reciprocally, anti-miR-1968-5p increased the luciferase activity of pGL3-*MyD88* but not that of the pGL3-*MyD88* mutant (Figure 4d), thus suggesting that miR-1968-5p directly binds to the 3′UTR of *MyD88* mRNAs. Similarly, supplementation with miR-1968-5p led to dose-dependent suppression of *MyD88* expression in 3T3 cells (Figure 4e), whereas supplementation with anti-miR-1968-5p resulted in elevation of *MyD88* expression in these cells (Figure 4f). Together, these data confirmed that miR-1968-5p indeed suppresses the expression of *MyD88* by targeting the 3′UTR of its mRNA.

### Hydrogen-water treatment has no effect on the abundance of enteric bacteria

MyD88 'tunes' the symbiotic enteric microbes, and our previous studies have reported that gut microbiota affect the radioresistance of hosts.[23,24] Thus we performed 16S rRNA sequencing to analyze the gut bacterial community in mice after TAI exposure with or without hydrogen-water oral gavage. As shown in Figure 5a, 15 Gy gamma-ray TAI exposure significantly increased the observed species number in irradiated mice with or without hydrogen-water treatment. However, Shannon and Simpson index analysis did not indicate significant differences among controls, TAI-exposed mice and TAI-exposed mice with hydrogen-water administration (Figures 5b and c). In detail, TAI treatment caused a lower relative abundance of Bacteroidetes and higher relative abundance of Proteobacteria at the phylum level; however; oral gavage with hydrogen-water restored the abundance to a level close to that of the control group (Figure 5d). Together, our observations demonstrated that hydrogen-water has no effect on the abundance of enteric bacteria in TAI-exposed mice.

### Oral gavage with hydrogen-water results in retention of the intestinal bacterial composition pattern impaired by TAI

Next, Principal Coordinate Analysis was used to further determine the role of hydrogen-water in shaping the intestinal bacterial flora profile. As shown in Figure 6a, the intestinal bacterial composition profile substantially changed after TAI treatment as well as hydrogen-water treatment. Statistically,

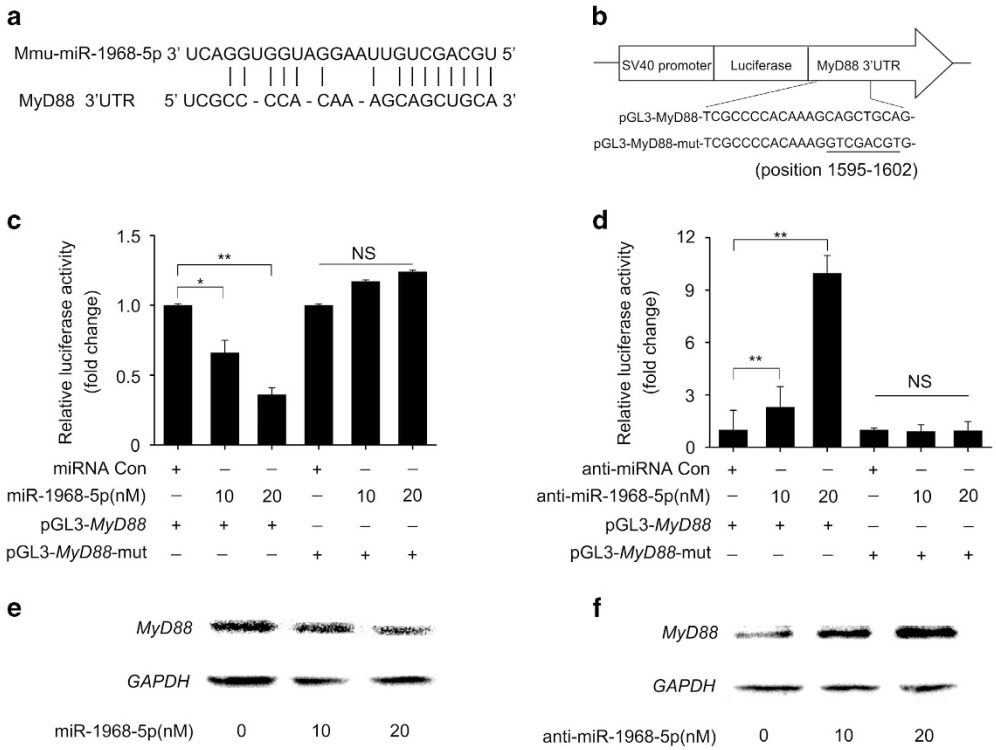


**Figure 4** MiR-1968-5p inhibits the expression of *MyD88* by targeting its 3′UTR. (**a** and **b**) MiR-1968-5p inhibits the expression of MyD88 by targeting the predicted conserved miR-1968-5p-binding site at nucleotides 1595–1602 of the *MyD88* 3′UTR. The generated mutation sites at the *MyD88* 3′UTR seed region are indicated. The wild-type *MyD88* 3′UTR (or mutant) was inserted into the downstream of luciferase reporter gene in the pGL3-control vector. (**c** and **d**) The effect of miR-1968-5p (or anti-miR-1968-5p) on pGL3-*MyD88* and pGL3- *MyD88*-mut reporters in 3T3 cells was measured by luciferase reporter assays. Statistically significant differences are indicated: $*P<0.05$; $**P<0.01$; Student's *t*-test. (**e** and **f**) The effect of miR-1968-5p (or anti-miR-1968-5p) on the expression of *MyD88* in 3T3 cells was measured by western blotting. Each experiment was repeated at least three times. NS, not significant.

unweighted unifrac analysis revealed that TAI drove a marked difference in gut microbiota composition, whereas hydrogen-water treatment eliminated the difference (Figures 6b and c), thus suggesting that hydrogen-water might preserve the TAI-shifted bacterial composition to perform radioprotection. Specifically, TAI treatment caused a lower abundance of Bacteroidia, Betaproteobacteria and Coriobacteria and a higher relative abundance of Phycisphaerae, Planctomycetia and Sphingobacteria at the genus level, whereas hydrogen-water treatment reversed these changes (Figures 6d and e). Comparing the gut bacteria at the genus level by using linear discriminant analysis effect size calculation, we found many taxa to be different in abundance between the TAI group and hydrogen-water treatment cohorts. For instance, the S24_7 was most abundant after TAI treatment compared with the hydrogen-water treatment group, which had a greater abundance of Bacteroides (Figure 6f). Together, our data indicated that hydrogen-water treatment preserves the intestinal bacterial composition shaping from TAI exposure.

## DISCUSSION

Hydrogen-water has been reported to be administered as an antioxidant in biological systems.[25] Research on the health benefits of hydrogen-water has demonstrated that hydrogen-water has long-term effects; clinical studies on humans indicate that hydrogen-water may be used to treat metabolic syndrome and diabetes and to enhance the therapeutic effect of anticancer drugs.[26–28] Radiotherapy for malignant pelvic and abdominal tumors may cause acute and late complications due to the side effects of irradiation, which are the main challenge for radiation oncologists, medical physicists and radiobiologists. On the basis of previous studies, we speculated that hydrogen-water might be a curative scheme to treat GI toxicity induced by radiotherapy for abdominal and pelvic malignancies. To test our hypothesis, we performed several experiments using mouse models. Notably, our observations revealed that oral gavage with hydrogen-water significantly increased the survival rate and body weight of TAI-exposed mice. Moreover, hydrogen-water treatment improved GI tract functions and epithelial integrity after TAI exposure. The mechanism of radiation-induced GI toxicity partly results from damage due to oxidative stress and the production of ROS.[29] Membrane lipids are the major targets of ROS and the free radical chain reaction.[30] Increases in lipid peroxidation products such as MDA are indices of lipid damage.[31] In the present study, intestinal MDA increased after TAI exposure; however, hydrogen-water administration reverted the changes, thus suggesting that hydrogen-water performed its radioprotective role partly by decreasing the production of ROS and effectively inhibiting oxidative

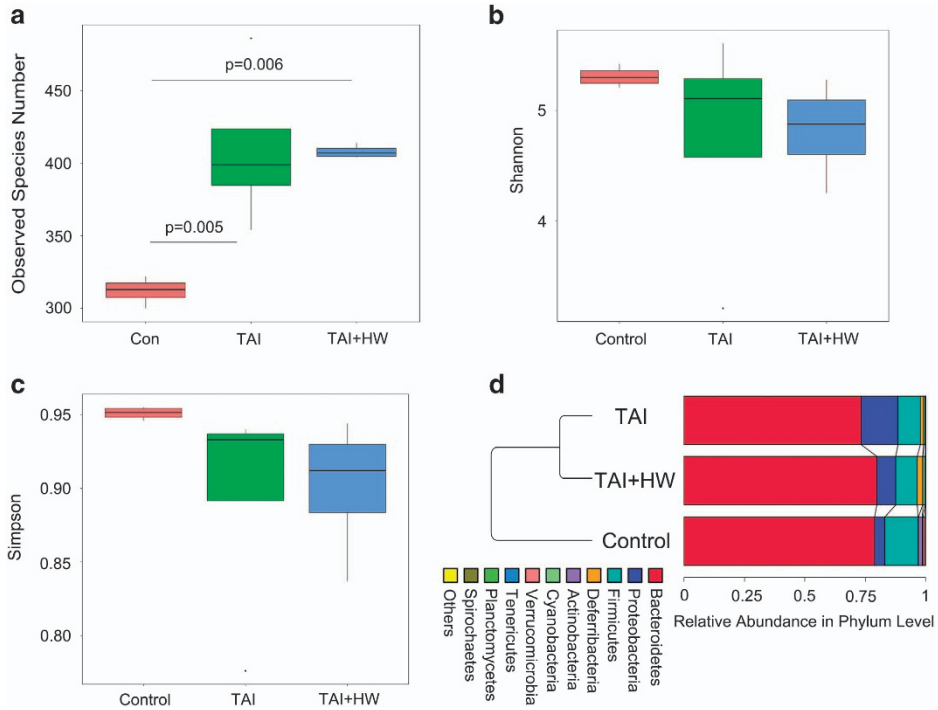


**Figure 5** Hydrogen-water treatment has no effect on the abundance of enteric bacteria. (**a**) The observed species number of intestinal bacteria in con and TAI-treated mice (with or without hydrogen-water oral gavage) was examined by 16S rRNA high-throughput sequencing after 5 days of TAI exposure. (**b** and **c**) The Shannon (**b**) and Simpson (**c**) diversity indices of intestinal bacteria in con and TAI-treated mice (with or without hydrogen-water oral gavage) were assessed by 16S rRNA high-throughput sequencing after 5 days of TAI exposure. For panels (**a**–**c**), the top and bottom boundaries of each box indicate the 75th and 25th quartile values, respectively, and lines within each box represent the 50th quartile (median) value. Ends of whiskers mark the lowest and highest diversity values in each instance. (**d**) The relative abundance of enteric bacteria at the phylum level in con and TAI-treated mice (with or without oral gavage of hydrogen-water) was assessed using 16S high-throughput sequencing after irradiation at day 5. Statistically significant differences are indicated: Student's *t*-test, $n=4$ per group.

reactions. Nrf2 is a redox-sensitive transcription factor that has an important role in cellular antioxidant defense.[32] After stimulation, Nrf2 translocates to the nucleus and initiates transcription of cytoprotective genes, such as HO-1. Hydrogen-water was protective against ROS and oxidative reactions, thereby removing stress. Thus the TAI-upregulated Nrf2 and HO-1 expression was downregulated after hydrogen-water consumption. Together, our data provided novel insights into the function of hydrogen-water in clinical application, demonstrating that hydrogen-water can be used as a therapeutic strategy during radiotherapy for abdominal and pelvic carcinoma.

The TLR family, one of the characterized families of innate immune receptors, induces innate immune responses that resist pathogens and recognize microbial components. Previous studies have identified a protective function of TLRs against irradiation injuries to the GI tract.[20] MyD88 is a key signaling adaptor for almost all TLRs and manipulates diverse physiological and pathological states, such as the development of liver and pancreatic cancer and colon carcinogenesis, as well as sarcomagenesis.[33] Conditional deletion of *MyD88* from intestinal epithelial cell renders mice more tolerant to local ischemic/reperfusion insult than wild-type controls,[34] thus suggesting an important role of MyD88 in the regulation of gut injury. Moreover, deletion of *MyD88* enhances the radioresistance in a mouse model, as compared with wild type.[35] Therefore, we focused on MyD88 to determine the molecular mechanism by which hydrogen-water mitigated TAI-induced GI toxicity. Intriguingly, our observations revealed that oral gavage with hydrogen-water predominantly downregulated the expression of *MyD88* in small intestine tissues from irradiated mice by upregulating the level of miRNA-1968-5p, thus suggesting that this radioprotection effect might depend on miRNA-1968-5p/MyD88 signaling. Interestingly, *MyD88* deletion in intestinal epithelial cells protects mice against high-fat-diet-promoted obesity, diabetes and low-grade inflammation.[36] Thus hydrogen-water might be used to treat MyD88-mediated obesity, diabetes and low-grade inflammation in clinical settings, although further study is required.

The GI tract is inhabited by a dense population of organized and highly specialized microbial flora that collectively modulate host immunity and metabolism.[37] Disturbances in host–microbe interaction are associated with many diseases, including obesity, malnutrition, inflammatory bowel disease, liver disease and cancers.[38] Given that MyD88 affects the composition of intestinal microbiota through sophisticated mechanisms,[39] and differences in initial microbial colonization are linked to the susceptibility or protection against

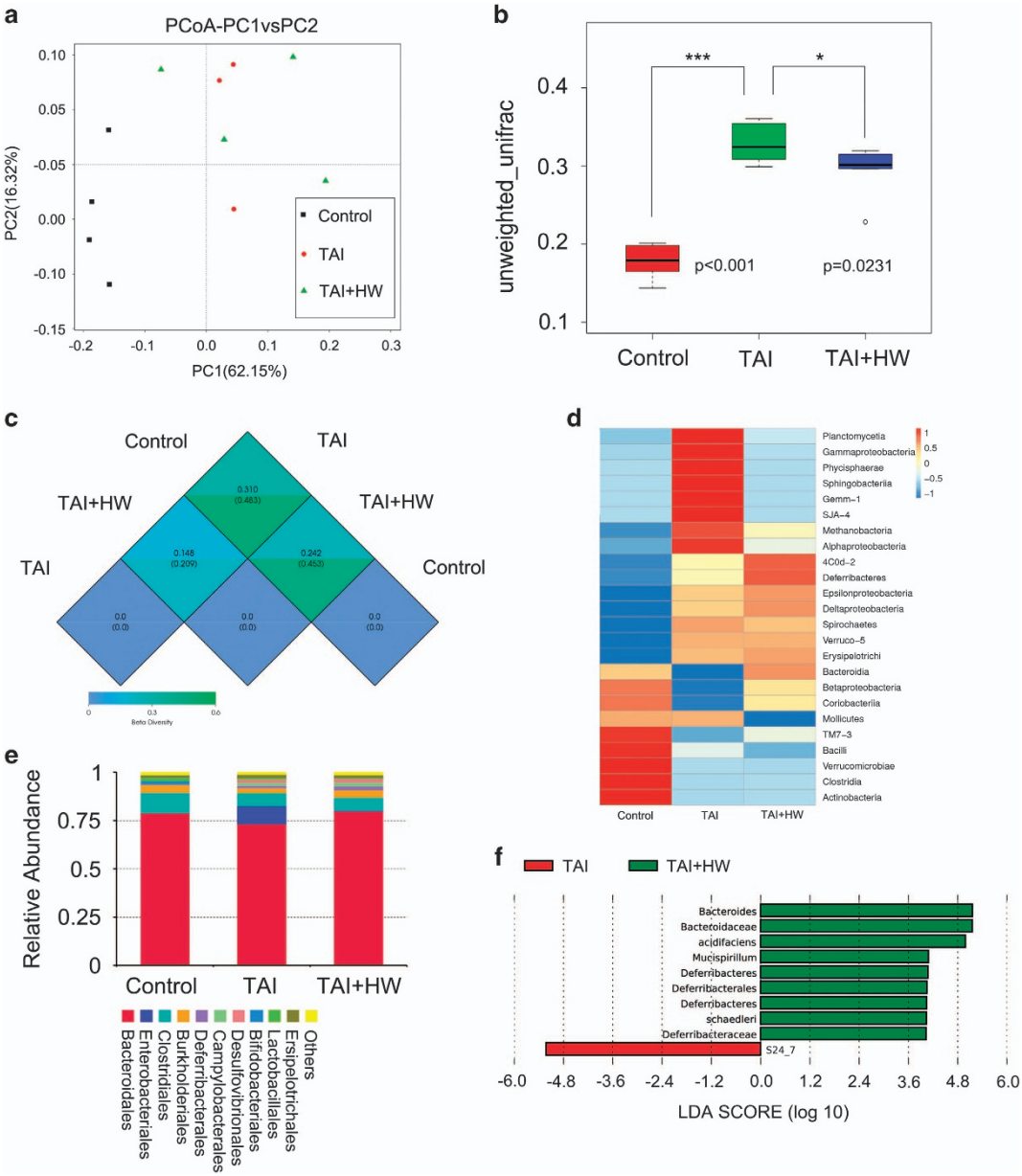


**Figure 6** Oral gavage with hydrogen-water retains the intestinal bacterial composition pattern impaired by TAI. (**a**–**c**) Principal component and $\beta$ diversity analyses were used to measure the shift in the intestinal bacterial composition profile in con and TAI-treated mice (with or without hydrogen-water oral gavage) after irradiation at day 5. Statistically significant differences are indicated: $^{*}P<0.05$, $^{***}P<0.001$; Student's *t*-test, $n=4$. For panel (**b**), the top and bottom boundaries of each box indicate the 75th and 25th quartile values, respectively, and lines within each box represent the 50th quartile (median) value. Ends of whiskers mark the lowest and highest diversity values in each instance. (**d**) Alterations in intestinal bacterial patterns at the genus level in con and TAI-treated mice (with or without hydrogen-water oral gavage) were assessed using 16S high-throughput sequencing after irradiation at day 5, $n=4$. The heatmap is color coded on the basis of row *Z*-scores. The mice with the highest and lowest bacterial levels are in red and blue, respectively. (**e**) The relative abundance of the top 10 bacteria at the genus level in con and TAI-treated mice (with or without hydrogen-water oral gavage) was assessed using 16S high-throughput sequencing after irradiation at day 5, $n=4$. (**f**) Linear discriminant analysis (LDA) effect size (LEfSe) results showed that the bacteria were significantly different in abundance between the TAI and hydrogen-water groups and indicated the effect size of each differentially abundant bacterial taxon in the small intestine ($n=4$). Statistically significant differences are indicated: Student's *t*-test.

radiotherapy,[40] we speculated that hydrogen-water might shape the intestinal bacterial community and alleviate TAI-induced GI toxicity. As expected, interrogation of microbiota composition by 16S RNA sequencing showed that oral gavage with hydrogen-water resulted in retention of the intestinal bacterial composition in irradiated mice. Manipulation of the gut microbiota has been shown to be an effective therapeutic approach to prevent radiotherapy-mediated GI toxicity.[23,41] On the basis of our findings, hydrogen-water might be a novel agent for patients after radiotherapy. Recent mounting evidence

indicates that the composition of gut microbiota is associated with the gene expression profile of hosts.[42] Thus hydrogen-water-educated gut microbiota might elicit upregulation of miR-1968-5p after TAI, a possibility that requires further study. Together, our observations provide potential mechanisms by which hydrogen-water ameliorates TAI-induced GI tract toxicity and suggest that hydrogen-water might serve as a therapy for dysbacteriosis-mediated diseases.

In aggregation, hydrogen-water alleviates and protects against TAI-induced small intestinal toxicity in a mouse model. Oral gavage with hydrogen-water markedly improved the GI tract functions and epithelial integrity after TAI exposure. Mechanistically, hydrogen-water increases the level of miR-1968-5p in the mouse small intestine, thereby down-regulating the expression of *MyD88* through directly targeting its 3′UTR. Moreover, hydrogen-water treatment results in retention of the intestinal bacterial composition structure after irradiation. Thus our findings provide novel insights into the function and mechanism of hydrogen-water mitigating TAI-induced GI injury and pave the way for use of hydrogen-water in clinical practice to improve the prognosis after abdominal and pelvic cancer radiotherapy.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Experimental & Molecular Medicine website (http://www.nature.com/emm)