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Radioprotective effect of hydrogen in cultured cells and mice

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Abstract

It has been demonstrated that hydrogen can selectively reduce hydroxyl and peroxynitrite *in vitro*. Since most of the ionizing radiation-induced cellular damage is caused by hydroxyl radicals, this study was designed to test the hypothesis that hydrogen may be an effective radioprotective agent. This paper demonstrates that treating cells with hydrogen before irradiation could significantly inhibit ionizing irradiation (IR)-induced Human Lymphocyte AHH-1 cells apoptosis and increase cells viability *in vitro*. This paper also shows that hydrogen can protect gastrointestinal endothelia from radiation-induced injury, decrease plasma malondialdehyde (MDA), intestinal 8-hydroxydeoxyguanosine (8-OHdG) levels and increase plasma endogenous antioxidants *in vivo*. It is suggested that hydrogen has a potential as an effective and safe radioprotective agent.

Keywords: *Ionizing radiation, radioprotection, intestine, hydrogen*

Introduction

Exposure to ionizing radiation (IR) can produce severe health impairments due to injury and failure to susceptible organs. Detrimental effects of IR on biological tissues are, in major part, mediated via increased production of hydroxyl radical. Hydroxyl radical produced during radiolysis of water can trigger oxidation of lipids, amino acid, and saccharides leading to formation of various secondary free radicals [1–3]. These free radicals can produce severe health impairments due to injury and failure to susceptible cells and organs.

The gastrointestinal tract is one of the most susceptible organs to radiation [4]. As low as 1 Gy of radiation induces a dramatic increase in apoptosis in mouse small intestinal crypt within 3–6 h after exposure, predominantly in the stem cell region [5].

Ohsawa et al. [6] found that molecular hydrogen could selectively reduce cytotoxic reactive oxygen species, such as •OH and ONOO− *in vitro* and exert therapeutic antioxidant activity in a rat middle cerebral artery occlusion model. Therefore, we reasoned that hydrogen might be protective against detrimental effects of radiation. However, application of H_2 gas inhalation is not convenient and may be dangerous because it is inflammable and explosive. On the other hand, H_2 gas saturated PBS/saline, which is called hydrogen-rich PBS/saline, is easy to apply and safe. In the current study, we investigated whether administration of hydrogen-rich PBS/saline exerted a radioprotective effect *in vitro* and *in vivo*. We demonstrated here that hydrogen treatment could protect cultured lymphocytes and the gastrointestinal tract from γ-radiation in mice.

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Materials and methods

Hydrogen-rich PBS/saline production

Hydrogen was dissolved in PBS/physiological saline for 6 h under high pressure (0.4 MPa) to a supersaturated level using a hydrogen-rich water-producing apparatus produced by our department. The saturated hydrogen PBS/saline was stored under atmospheric pressure at 4°C in an aluminium bag with no dead volume. Hydrogen-rich PBS/saline was freshly prepared every week, which ensured that a concentration of more than 0.6 mmol/L was maintained. Gas chromatography (Biogas Analyzer Systems-1000, Mitleben, Japan) was used to confirm the content of hydrogen in PBS/saline by the method described by Ohsawa et al. [6].

Cell culture and treatment

Human lymphocyte AHH-1 cells (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 (Invitrogen, CA) with 10% foetal bovine serum and 1% penicillin–streptomycin–glutamine at 37°C in a 5% CO_2 humidified chamber. For radioprotective studies, cells were treated with a different volume of hydrogen-rich PBS and accordingly we added a different volume of PBS in order to obtain the desired concentration of H_2 and make the final volume of the medium the same, then the treated cells were immediately irradiated with different doses of γ-ray, depending upon the requirement of the present study. After irradiation, the cells were centrifuged and cultured in RPMI 1640.

Irradiation

^{60}Co-gamma rays in the irradiation centre (Faculty of Naval Medicine, Second Military Medical University, China) were used for the irradiation purpose. Mice (with or without hydrogen pre-treatment) were exposed to different doses of radiation, depending upon the requirement of the present study.

Cell viability analyses

Human lymphocyte AHH-1 cells were seeded in 96-well plates and pre-treated with or without hydrogen-rich PBS, the treated cells were then immediately irradiated. After irradiation the cells were further cultured for 48 h. Cell viability was determined by WST assay using a Cell Counting kit (Dojindo Laboratories, Kumamoto, Japan).

Lactate dehydrogenase (LDH) leakage assay

LDH leakage assay was carried out using LDH cytotoxicity detection kit (Nanjing KeyGen Biotech. Co. Ltd. China) according to protocol in the user's manual. AHH-1cells were pre-treated with hydrogen-rich PBS and the final concentration of H_2 was maintained above 0.3 mmol/L. Immediately, the cells were exposed under gamma radiation and then transported to an ice bucket. After a 4 h time period we analysed the content of LDH in the cell suspension.

Apoptosis assays for cultured cells

Apoptosis was determined by Annexin V-APC and propidium iodide staining using Apoptosis Detection Kit (Bipec Biopharma, Massachusetts, MA). Treated cells were incubated with Annexin V-APC for 15 min at 4°C and propidium iodide for 5 min at room temperature. Cells were then analysed by flow cytometry. Alternatively, apoptosis was determined by Hochest33258, flourescein diacetate (FDA) and propidium iodide staining. Treated cells were washed with PBS twice and then stained with 40 mg/L flourescein diacetate, 20 mg/L Hoechst33258 at room temperature for 15 min and stained with 20 mg/L propidine iodine at room temperature for 5 min. The cellular morphology was observed using an Olympus BX60 fluorescent microscope equipped with a Retiga 2000R digital camera. Average percentage of apoptotic cells was calculated in five-to-seven randomly selected high power fields (HPF).

Mice and treatment

All the protocols were approved by the Second Military Medical University, China in accordance with the Guide for Care and Use of Laboratory Animals published by the US NIH (publication No. 96-01). Male BALB/c rats weighing 21–23 g were used in the experiments. The animals were housed in individual cages in a temperature-controlled room with a 12 h light/dark cycle and food and water were provided *ad libitum*. For experiments, mice were treated intraperitoneally (IP) with physiological saline or hydrogen-rich saline 20 min before radiation. Mice were irradiated in a holder designed to immobilize unanaesthetized mice such that the abdomens were presented to the beam.

Morphologic observation

Mice were treated intraperitoneally (IP) with physiological saline or hydrogen-rich saline 20 min before irradiation. Twelve hours after irradiation, mice were sacrificed by cervical dislocation under isoflurane anaesthesia. A 5 cm segment of small intestine which was removed at 5 cm proximal to the terminal ileum was fixed in 10% buffered formaldehyde-saline. Three 1 cm segments of intestinal specimen were embedded in paraffin and stained with hematoxylin and eosin. Morphological damages were assessed by Chiu histological injury scoring system of intestinal villi (0 = normal mucosa, 1 = slight-, 2 = moderate-, 3 = massive subepithelial

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detachments, 4 = denudes villi, 5 = ulceration) [7]. Two independent and blinded researchers performed the histological scoring.

Biochemical assays

Arterial blood samples (0.6 ml) of mice were collected 12 h after irradiation. These samples were immediately centrifuged at 2500 rpm and 4°C for 10 min. The plasma was taken for biochemical estimations (SOD, GSH and MDA). Superoxide dismutase (SOD) activity was assayed by the method of Kakkar et al. [8], based on the inhibition of the formation of NADH-PMS-NBT complex. The GSH concentration was measured by the method of Ellman [9]. This method was based on the development of a yellow colour when 5',5'-dithiobis 2-nitrobenzoic acid was added to compounds containing sulphydryl groups. MDA was assessed spectrophotometrically with the method defined by Ohkawa et al. [10] as MDA reacted with thiobarbituric acid and formed a pink, maximum absorbent complex at 532 nm wavelength.

For determination of 8-OHdG levels in DNA from the intestine of mice, DNA was extracted from the mice intestinal specimen with a DNA Extractor Kit (DNA Extractor Wb Kit, Wako Chemical, Osaka, Japan) according to the method of Nakae et al. [11]. Then the isolated DNA was digested by the method of Valls-Belles et al. [12]. The 8-OHdG levels of these samples were measured as described by Inoue et al. [13]. Briefly, the samples were added to plate wells pre-coated with mouse monoclonal anti-8-OHdG antibody (Japan Institute for the Control of Aging, Fukuroi, Japan), of which the specificity has been proved by Toyokuni et al. [14]. They were incubating for 45 min at 37°C. After being washed three times, the wells were sequentially treated with Biotinylated rabbit-anti-mouse lgG for 30 min at 37°C and Streptavidin-Horseradish Peroxidase (HRP) for 30 min at 37°C. A substrate containing 3,3',5,5'-tetramethylbenzidine (TMB) was added and the wells were incubated for 15 min at 37°C. The reaction was terminated by the addition of sulphuric acid. The absorbance was read at a wavelength of 450 nm.

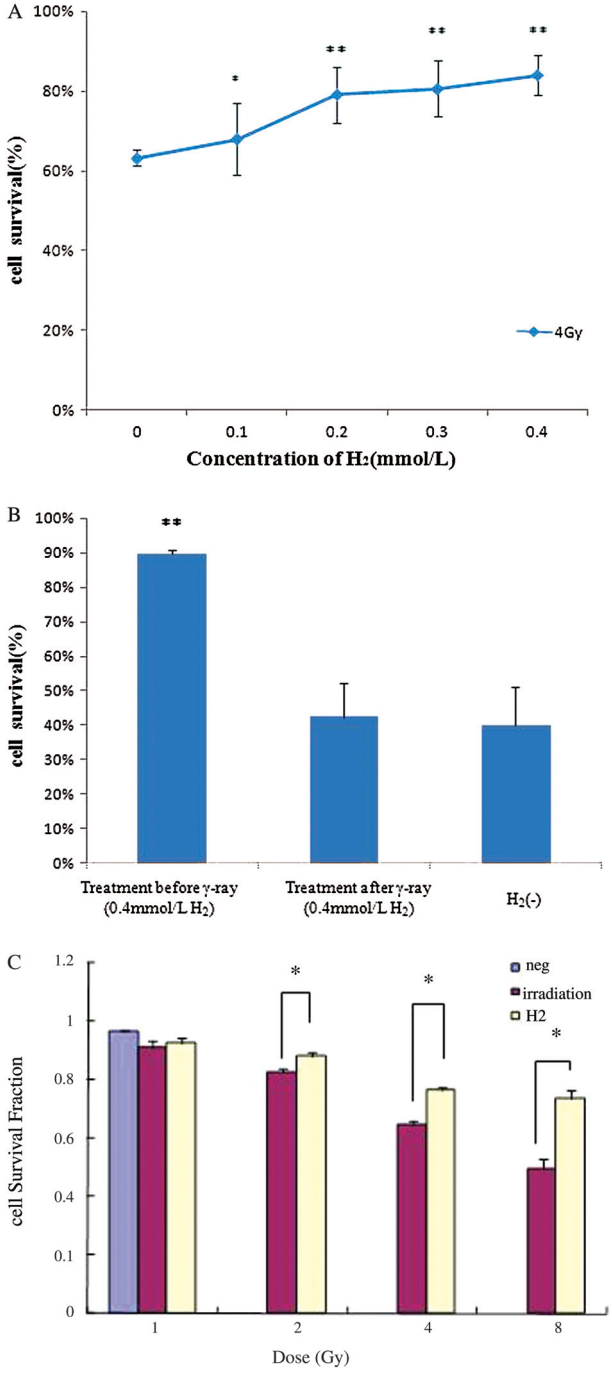

Figure 1. Dose-dependent effect of H_2 on cell viability induced by 4 Gy gamma radiation (A). Pre-treatment of 0.4 mmol/L H_2 before irradiation can increase cell survival (B). Variation of cell survival percentage pre-treated with 0.4 mmol/L H2 before different dose of irradiation (C). Values are given as mean ± SEM ($n = 6$, neg: no H_2 no radiation). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\#}p < 0.1$.

Statistical analysis

Data are expressed as means ± SEM for each experiment. The number of samples is indicated in the description

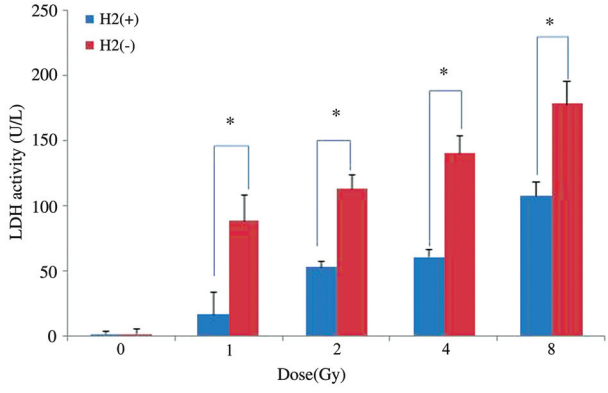

Figure 2. Changes in the levels of LDH in normal, γ-irradiated and H_2 pre-treated lymphocytes. Values are given as mean ± SEM ($n = 4$). $^{*}p < 0.01$.

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of each experiment. Statistical analysis was performed by using One Way Analysis of Variance. Between groups, variance was determined using the Student-Newman–Keuls post-hoc test. A p-value of less than 0.05 was considered to be statistically significant.

Results

Hydrogen-rich PBS increases cell viability of irradiated AHH-1 cells

To study radioprotective effects of H_2 in cell culture, we examined viability of irradiated AHH-1 cells. Cells treated with or without different concentrations of H_2 were exposed under 4Gy of γ-radiation as described in the Methodssection. We demonstrated that pre-treatment of AHH-1 cells with 0.1–0.4 mmol/L H_2 before irradiation significantly increased cell survival as compared to cells treated with radiation alone at all examined doses (up to 8 Gy) (Figures 1A and C).And as shown in Figure 1, the radioprotective effect of H_2 is dose-dependent. However, as we treated cells with hydrogen-rich PBS after irradiation, the protective effect is not significant (Figure 1B).

Hydrogen-rich PBS decrease cellular lactate dehydrogenase (LDH) leakage in irradiated cells

Besides the cell viability, we also determined LDH activities to estimate cellular LDH leakage from damaged cells. The result indicated that pre-treatment with 0.3 mmol/L H_2 before irradiation significantly decreased LDH leakage of AHH-1 cells which were exposed under different doses of γ-radiation (Figure 2).

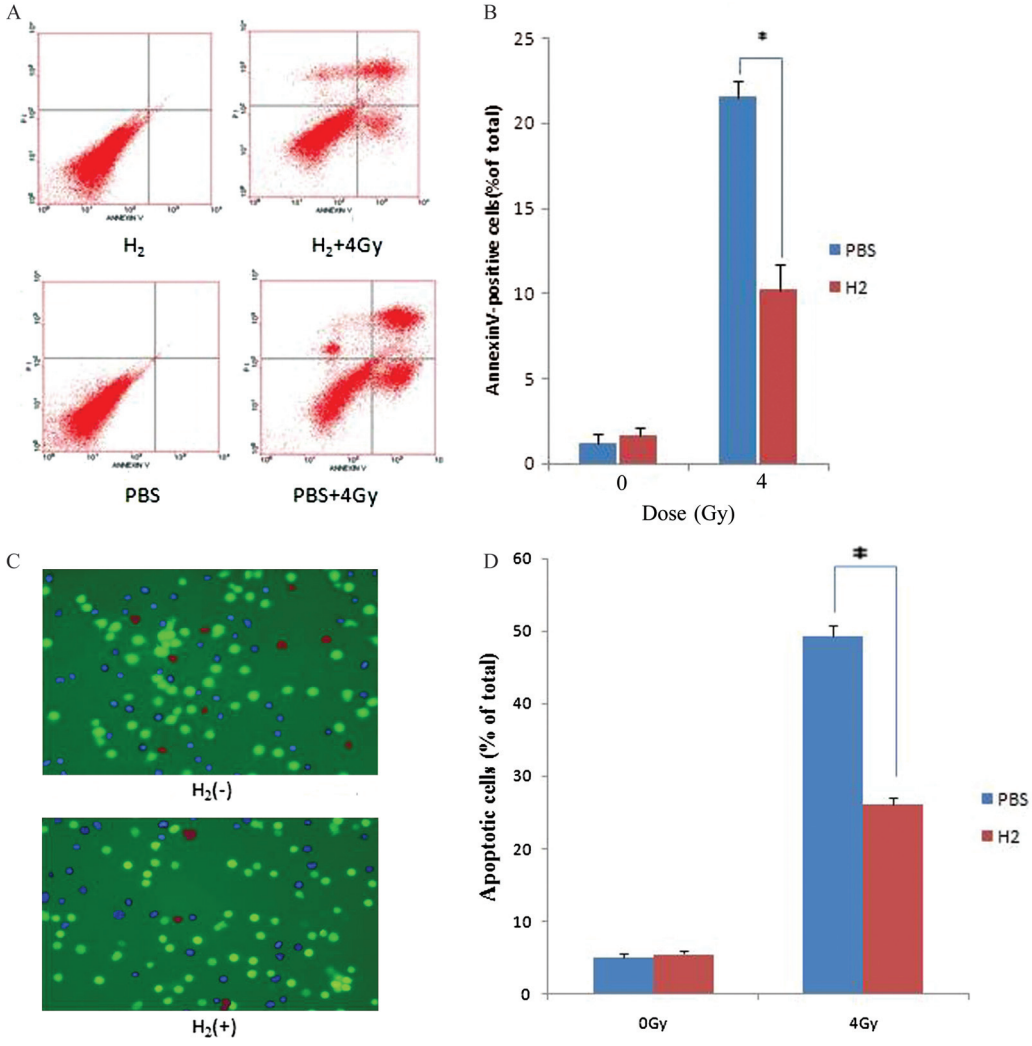

Figure 3. Hydrogen-rich PBS attenuates radiation-induced apoptosis in AHH-1 cells. Treated cells were collected 24 h after irradiation, stained with Annexin V-APC and propidium iodide and analysed by flow cytometry. Shown are representative diagrams of distribution of stained cells (A) and a bar graph of apoptotic cells expressed as a percentage of total cells. Values are given as mean ± SEM ($n = 4$). $^{*}p < 0.01$ (B). Cells were stained with FDA, Hoechst33258 and PI 24 h after irradiation and apoptotic cells were counted in multiple randomly selected fields. Shown are representative micrographs (C) and a bar graph of apoptotic cells expressed as a percentage of total cells. Values are given as mean ± SEM ($n = 4$). $^{*}p < 0.01$ (D).

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This result was consistent with the result obtained by cell viability observation.

Hydrogen-rich PBS attenuates apoptosis in irradiated AHH-1 cells

To determine the radiation-induced apoptosis of irradiated AHH-1 cells, we analysed treated cells by using Annexin V-APC and propidium iodide staining in flow cytometry assay. The early apoptotic cells decreased when pre-treated with 0.4 mmol/L H_2 as compared to cells pre-treated without H_2 (Figures 3A and B, 10.2% vs 21.5%, respectively). We further evaluated the morphology of dying cells using Hochest 33258, flourescein diacetate and propidium iodide staining. Irradiated AHH-1 cells pre-treated with hydrogen-rich PBS demonstrated a protective effect with a reduced number of apoptotic cells to 26.1% as compared to 49.3% in PBS-pre-treated irradiated cells (Figures 3C and D). These data suggest that H_2 can attenuate apoptosis in irradiated AHH-1 cells.

Hydrogen-rich saline treatment attenuates intestinal injury in vivo

We observed histological IR injuries featured by shortening of the villi, loss of villous epithelium and prominent mucosal neutrophil infiltration

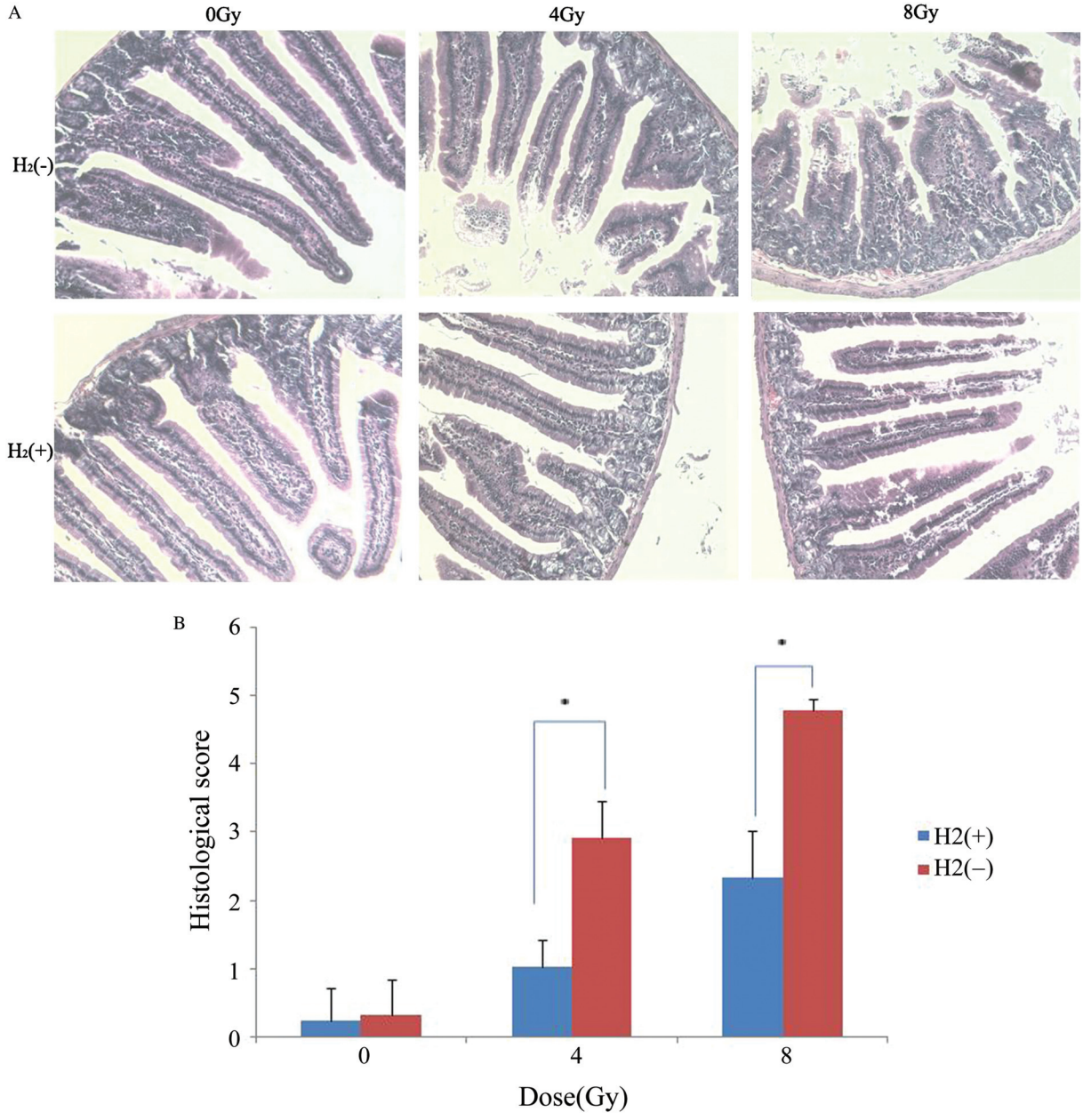

Figure 4. Morphologic observation of the intestinal tissue in normal, γ-irradiated and H2 pre-treated mice. Photomicrographs of the intestinal tissue stained by the hematoxylin and eosin (A). Intestinal mucosal injury evaluated by Chiu scoring system (B). Grading as (0 = normal mucosa, 1 = slight-, 2 = moderate-, 3 = massive sub-epithelial detachments, 4 = denudes villi, 5 = ulceration). Data are expressed as means ± SEM for at least triplicate independent experiments ($n = 8$ per group). $^{*}p < 0.01$.

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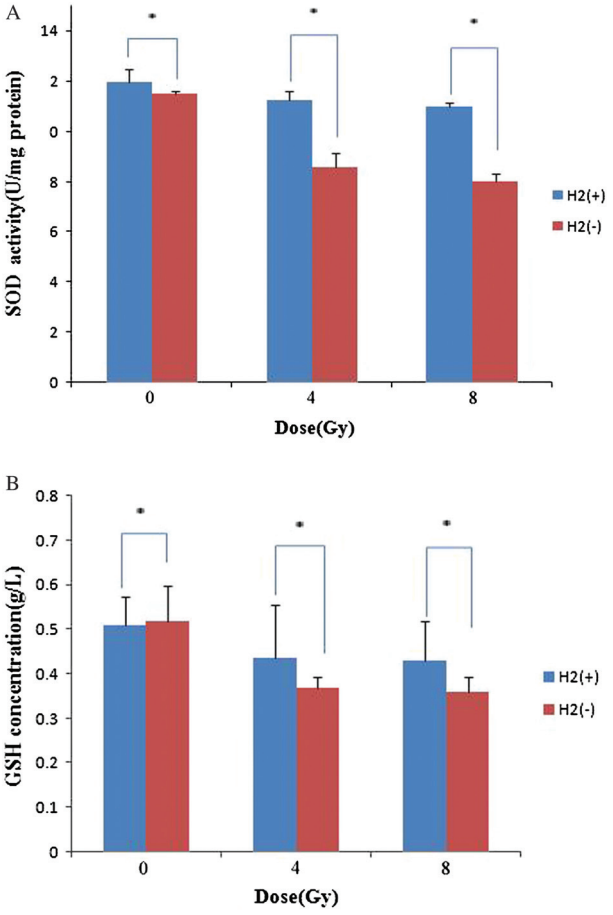

Figure 5. Changes in the activities of SOD and concentrations of GSH in normal, γ-irradiated and H_2 pre-treated mice. Values are given as mean ± SEM ($n = 4$). $^*p < 0.01$.

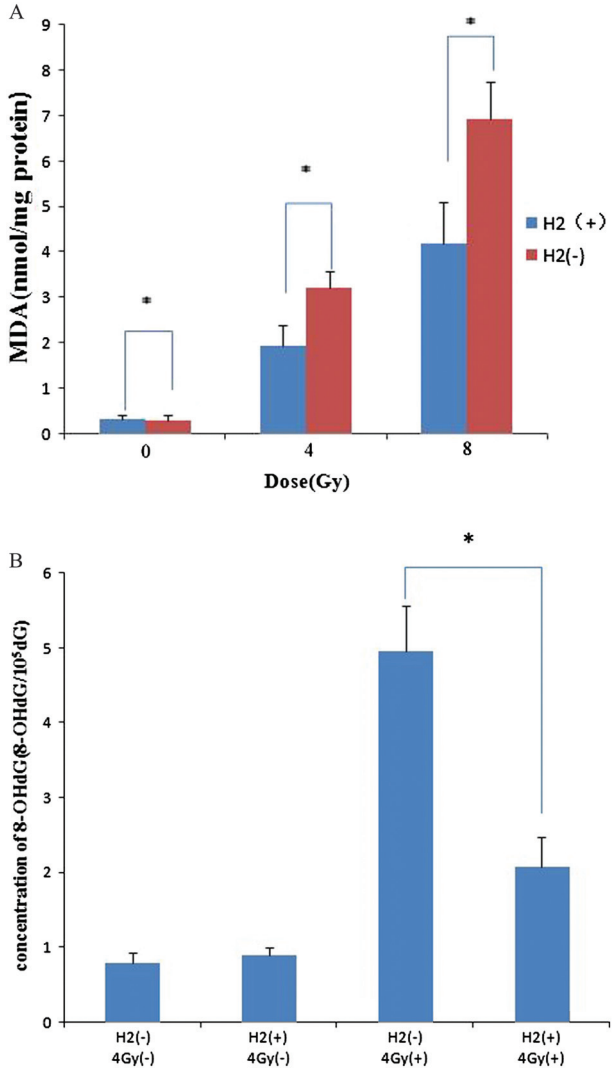

Figure 6. Hydrogen-rich saline significantly decreased levels of MDA, a marker of oxidative stress (A). Oxidative DNA damage was assessed by 8-OHdG immunoreactivity. Shown are, 12 h after irradiation, intestinal 8-OHdG concentrations in normal, γ-irradiated and H_2 pre-treated groups (B). Relative to the Control, H_2 significantly decreased the concentration of 8-OHdG. Values are mean ± SEM ($n = 6$), $^*p < 0.01$.

(Figure 4A). All of these changes were ameliorated by administration of hydrogen-rich saline. Chiu scoring and microphotographs are shown in Figure 4B. As shown in Figure 4, hydrogen-rich saline administration significantly reduced the mucosal injury caused by IR.

Changes in the activities of plasma SOD and GSH

The plasma SOD and GSH concentrations were measured at 12 h of irradiation (Figures 5A and B). Plasma SOD and GSH concentrations at 12 h of irradiation in the H_2 group were significantly higher than that of the control group.

Changes in the levels of plasma MDA and intestinal 8-OHdG

The plasma MDA and intestinal 8-OHdG concentrations were measured at 12 h of irradiation (Figures 6A and B). Plasma MDA and intestinal 8-OHdG concentrations at 12 h of irradiation in the H_2 group were significantly lower than that of the control group.

Discussion

To our knowledge, this is the first study demonstrating that hydrogen has radioprotective effects *in vitro* and *in vivo*. In several recent studies, H_2 inhalation was reported to protect cerebral [6], myocardial [15] and hepatic [16] I/R injury in animal models. In addition, Buchholz et al. [17] reported that hydrogen inhalation ameliorates oxidative stress in transplantation-induced intestinal graft injury. This radioprotective effect may result from radical oxygen species (ROS) scavenging effect of molecular H_2, as previously reported in a brain injury model [6]. The effect of free radical scavengers to ameliorate the oxidative injuries due to ionizing radiation has been considerably reported [18,19]. Radical oxygen species O_2^- and H_2O_2 are

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detoxified by antioxidant defense enzymes, whereas ·OH and ONOO– could not be detoxified by antioxidant defense enzyme. It has been demonstrated that hydrogen gas selectively reduces ·OH and ONOO– [6]. The hydroxyl radical is the most reactive product of reactive oxygen species generated in cells. Hydroxyl radicals can easily react with cellular macromolecules, including DNA, proteins and lipids, to exert a strong cytotoxic effect. Since most of the ionizing radiation-induced damage is caused by hydroxyl radicals, we speculate that the radioprotective effect may result from its radical oxygen species (ROS) scavenging effect.

Endogenous antioxidants are a group of substances that significantly inhibit or delay oxidative processes while being oxidized themselves [20]. Antioxidant enzymes are important in providing protection from radiation exposure [21]. Also, glutathione (GSH) participates non-enzymatically in protection against radiation damage [22]. Therefore, a reduction in the activity of these substances can result in a number of deleterious effects. Membrane lipids are the major targets of ROS and the free radical chain reaction [23]. The increase in the levels of lipid peroxidation products such as malondialdehyde and TBARs are the indices of lipid damage [24]. Also DNA is one of the major targets of ROS and 8-OHdG is formed from deoxyguanosine in DNA by hydroxyl free radicals [25]. In our study, we observed a significant decrease in the levels of enzymatic antioxidant (SOD), non-enzymatic antioxidant (GSH) and an increase in the levels of plasma MDA and intestinal 8-OHdG of irradiated mice. However, pre-treatment of hydrogen prior to radiation exposure increased the antioxidant status at both enzymatic and non-enzymatic levels and decreased the levels of MDA and 8-OHdG.

The mechanism of protection on SOD and GSH is most likely based on the ability of H_2 to effectively inhibit oxidative reactions .It has been demonstrated that O_2- can undergo either spontaneous or enzyme-catalysed (SOD) dismutation to hydrogen peroxide (H_2O_2) or can react with nitric oxide (NO·) to form the toxic product peroxynitrite (ONOO–) [26]. Hydrogen gas can reduce ONOO– [6]. Therefore, it's possible that H2 can accelerate the reaction between O_2- and NO·. More O_2- would react with NO· while the enzyme-catalysed reaction by SOD would weaken. Thus, the SOD can be protected. Besides, hydroxyl radicals can react with themselves to form H_2O_2, which could oxidize GSH to GSSG [1]. Hydrogen gas can reduce hydroxyl radical [6], thus probably hydrogen gas could partly reduce the formation of H_2O_2 in the reaction chain which may lead to the protection of GSH. We may conclude that the protection in the antioxidant status during H_2 pre-treatment has further decreased the attack of free radicals, prevented DNA damage and decreased lipid peroxidation, thereby decreased the deleterious effects of radiation.

Some radioprotectors, such as thiol compounds, has relatively high toxicity [27], while cytokines and immunomodulators should be used with low radiation doses or in combination with radical scavengers and antioxidants [28] and natural antioxidants, such as vitamin E, flavonoids and others, have fewer toxic side-effects but also a lower degree of protection compared to thiol agents [27]. The sulphydryl compound amifostine, named WR-2721, which is the only radioprotectant registered for use in humans, has shown good radioprotective effects [29]. However, it has many side-effects limiting its clinical use such as hypertension, nausea, vomiting and others [30]. However, hydrogen is continuously produced by colonic bacteria in the body and normally circulates in the blood [31], so it is physiologically safe for humans to inhale hydrogen at a relatively low concentration. It is also a highly diffusible gas and reacts with hydroxyl radical to produce water [32]. Dissolving H_2 in solvent such as PBS, physiological saline or medium is easy to apply and safe. Therefore, it may have great potential for clinical use.

In conclusion, the effect of reducing radical oxygen species plays an important role in the radioprotective effects of hydrogen. However, the exact mechanism and signalling pathway involved in the protection role of hydrogen in ionizing radiation injury need to be studied in the future.

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