Hydrogen-rich saline protects immunocytes from radiation-induced apoptosis

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Summary

Background: Radiation often causes depletion of immunocytes in tissues and blood, which results in immunosuppression. Molecular hydrogen (H₂) has been shown in recent studies to have potential as a safe and effective radioprotective agent through scavenging free radicals. This study was designed to test the hypothesis that H₂ could protect immunocytes from ionizing radiation (IR).

Material/Methods: H₂ was dissolved in physiological saline or medium using an apparatus produced by our department. A 2-[6-(4'-hydroxy) phenoxy-3H-xanthen-3-on-9-yl] benzoate (HPF) probe was used to detect intracellular hydroxyl radicals (•OH). Cell apoptosis was evaluated by annexin V-FITC and Propidium iodide (PI) staining as well as the caspase 3 activity. Finally, we examined the hematological changes using an automatic Sysmex XE 2100 hematology analyzer.

Results: We demonstrated H₂-rich medium pretreatment reduced •OH level in AHH-1 cells. We also showed H₂ reduced radiation-induced apoptosis in thymocytes and splenocytes in living mice. Radiation-induced caspase 3 activation was also attenuated by H₂ treatment. Finally, we found that H₂ rescued the radiation-caused depletion of white blood cells (WBC) and platelets (PLT).

Conclusions: This study suggests that H₂ protected the immune system and alleviated the hematological injury induced by IR.

key words: radioprotection • apoptosis • hydrogen • immunosuppression
**Background**

Exposure to ionizing radiation (IR) often causes immunosuppression, which enhances the probability of infection and affects the recovery from radiation sickness [1,2]. Immunosuppression also limits the further application of radiotherapy for cancer. Although it is known that IR affects functions of radioresistant immunocytes like macrophages [3], dendritic cells (DC) [4], and regulatory T cells [5], apoptosis of radiosensitive immune cells is also an important pathway for radiation-induced immunosuppression [6,7]. In different developmental stages of the immune system, cell apoptosis regulates the body’s homeostasis in physiological and pathological conditions [8]. Apoptosis induced by IR in thymocytes, splenocytes and peripheral blood lymphocytes affect the body’s immune status as well as human health.

IR causes tissue damage mainly by free radicals [9]. For decades, free radical scavengers have been studied for radioprotection of the immune system. But from thiols compounds to plant extractions, they all face significant shortcomings, including relatively high toxicity and unfavorable routes of administration, which affect their applications and efficacies. Therefore the search for safer and more effective radioprotectors continues.

Recently, Ohsawa et al. reported that molecular hydrogen (H₂) could reduce reactive oxygen species such as •OH and ONOO− etc [10]. Our department demonstrated H₂ treatment protected cultured cells and mice from radiation damage, and exerted protective effects on the gastrointestinal tract, cardiovascular system and spermatogenic epithelium from γ-irradiation [11–14]. These encouraging results prompted us to study whether H₂ treatment could protect the immune system against IR.

**Material and methods**

**H₂-rich saline/medium production**

H₂ was dissolved into physiological saline or RPMI1640 medium (Invitrogen, California, USA) for 6 h under high pressure (0.4MPa) to a supersaturated level using a H₂-rich water producing apparatus produced by our department. The saturated H₂-rich saline/medium was stored under atmospheric pressure at 4°C in an aluminum bag with no dead volume. H₂-rich saline/medium was freshly prepared 1 day before irradiation, 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 H₂ in saline/medium by the method described by Ohsawa et al. [10].

**Irradiation**

10Co-gamma rays at the Irradiation Center (Faculty of Naval Medicine, Second Military Medical University, China) were used for the irradiation. Mice and cells (with or without H₂ pre-treatment) were exposed to different radiation doses depending upon the requirement of the present study.

**Mice and treatment**

All the experiments 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 wild-type BALB/c mice, 4–6 weeks old, were purchased from the China Academy of Science (Shanghai, China). All mice were housed in individual cages in a temperature-controlled room with a 12 h light/dark cycle. Food and water were provided ad libitum. Twenty minutes before irradiation, mice were treated intraperitoneally (IP) with 0.2 ml physiological saline or saturated H₂-rich saline every 5 minutes for 4 injections. Mice were irradiated in a holder designed to immobilize unanaesthetized mice such that the abdomens were exposed to the beam. At different time points after irradiation, mice were killed by CO₂ humidified chamber. To detect cellular •OH, we treated AHH-1 cells with H₂-rich or normal medium and added 5 µM 2-[6-(4’-hydroxy) phenoxy]-3H-xanthen-3-on-9-yl] benzoate (HPF). Before irradiation, which ensured 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 H₂ in saline/medium by the method described by Ohsawa et al. [10].

**Cell culture and detection of •OH**

Human lymphocyte AHH-1 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in RPMI1640 (Invitrogen, California, USA) with 10% fetal bovine serum and 1% penicillin-streptomycin-glutamine at 37°C in a 5% CO₂ humidified chamber. To detect cellular •OH, we treated AHH-1 cells with H₂-rich or normal medium and added 5 µM 2-[6-(4’-hydroxy) phenoxy]-3H-xanthen-3-on-9-yl] benzoate.
(HPF) (a maker of oxidation of •OH and ONOO•) (Daiichi Pure Chemicals Co., Tokyo, Japan). Immediately after being exposed to 8Gy radiation, the cells were centrifuged and washed twice with phosphate buffer saline (PBS, pH 7.4). Cell suspensions were smeared onto slides. Cellular images were obtained using an Olympus BX60 fluorescent microscope equipped with a Retiga 2000R digital camera. We quantified fluorescent signals for 100 cells for each group using ImageJ software (version 1.44p, Wayne Rasband, NIH, US).

**Apoptosis assay**

The mice were killed at 24 h after irradiation, after which spleen and thymus were immediately removed. Cells were dispersed by passage through a fine wire mesh into a 35×10mm petri dish containing 1ml PBS. Isolated splenocytes and thymocytes were washed 3 times with PBS, and then stained with Annexin V-FITC and Propidium Iodide (PI) by Apoptosis Detection Kit (Bipec Biopharma, Massachusetts, USA), according to the manufacturer’s instructions. Subsequently, cells were analyzed by flow cytometry (Beckman Coulter, California, USA).

**Detection of caspase 3 activity**

Isolated spleens were homogenized in Radio Immuno-precipitation Assay (RIPA) lysis buffer (Beyotime Biotecnology, Shanghai, China) with 1mM of serine protease inhibitor phenyl methanesulfonyl fluoride (PMSF) (Beyotime Biotecnology, Shanghai, China) at 8 h after irradiation, and they were centrifuged at 12000 g for 10 min at 4°C. Level of caspase 3 in supernatants was determined by a Caspase 3 Assay Kit (Sigma, St. Louis, MO, USA), according to the manufacturer’s instruction. The experiments were repeated 3 times.

**Hemograms analysis**

Blood samples were taken from the retro-orbital sinus/plexus using EDTA-coated blood collection tubes at 24 h after irradiation. Whole blood samples from all groups were analyzed using an automated Sysmex XE 2100 hematology analyzer (Sysmex, Kobe, Japan).

**Statistical analysis**

Data are expressed as means±SEM for each experiment. The number of samples is indicated in the description of each experiment. We used an analysis of variance (ANOVA) followed by a Student-Newman-Keuls post-hoc test for statistical analysis. We performed experiments for quantification in a blinded fashion.

**RESULTS**

**H₂-reduced radiation-induced •OH level in cultured cells**

Radiation increased the intracellular HPF fluorescence intensity compared to the control group. The HPF fluorescence...
intensity in the \( H_2 \) treated group was much lower than in the single-radiation group (Figure 1A). \( H_2 \) significantly reduced the •OH level produced by irradiation (Figure 1A, B).

\( H_2 \)-rich saline protected immunocytes from radiation-induced apoptosis

After whole body irradiation (WBI), apoptosis of thymocytes and splenocytes were significantly enhanced. The splenocyte apoptosis decreased significantly in mice pretreated with \( H_2 \)-rich saline after irradiation (Figure 2A, B). The apoptosis of thymocytes were also significantly reduced after \( H_2 \) treatment (Figure 3A, B).

\( H_2 \) attenuated radiation-induced activation of caspase 3

The caspase 3 activity in spleen tissue of irradiated mice was up-regulated compared to the controls. \( H_2 \) treatment significantly attenuated the radiation-induced activation of caspase 3 in spleen tissues (Figure 4), while single \( H_2 \) treatment had no obvious effect on the baseline activity of caspase 3.

\( H_2 \)-rich saline mitigated radiation-induced hematological injury

At 24 h after irradiation, the numbers of white blood cells (WBC) and platelet (PLT) were reduced in the irradiated mice. \( H_2 \) treatment significantly mitigated radiation-caused reduction of WBC and PLT, but had no influence on other indexes (Table 1).

| Groups | WBC \((x10^9/L)\) | PLT \((x10^9/L)\) | RBC \((x10^{12}/L)\) | HGB \((g/L)\) | MCV \((fl)\) | MCHC \((g/L)\) |
|--------|-----------------|----------------|------------------|-------------|---------|-----------|
| Control | 3.89±0.14       | 900.50±40.31   | 8.90±0.06        | 136.66±4.04 | 46.44±0.14 | 336.50±0.71 |
| \( H_2 \) | 4.18±0.54       | 872.50±31.82   | 9.28±0.12        | 138.33±7.23 | 46.12±1.13 | 333.00±5.66 |
| IR     | 0.94±0.21**     | 169.05±35.36** | 9.46±0.24        | 140.33±10.97| 45.55±1.34 | 340.50±10.60 |
| IR+\( H_2 \) | 1.81±0.16*      | 335.50±23.12*  | 8.31±0.53        | 131.67±8.51 | 46.05±0.78 | 336.00±1.41 |

\( WBC \) – white blood cells; \( PLT \) – platelets; \( RBC \) – red blood cells; \( HGB \) – hemoglobin; \( MCV \) – mean corpuscular volume; \( MCHC \) – mean corpuscular hemoglobin concentration; \( IR \) – ionization radiation. The data are expressed as mean ±SEM \((n=5)\). **P<0.01 vs. the control group and *P<0.05 vs. the irradiated control group.

**Figure 4.** \( H_2 \)-rich saline attenuated radiation induced activation of caspase 3 in mice spleen tissues. Caspase 3 activity of each group was determined and expressed in a bar graph. Values are given as mean ±SEM \((n=5)\), *P<0.01.

**DISCUSSION**

To our knowledge, this is the first study demonstrating that \( H_2 \) has protective effects on the immune system of irradiated mice. Radiation-induced apoptosis in radiosensitive immunocytes causes depletion of cells in immune organs and blood, which leads to immunosuppression [15], but until now no ideal radioprotector for immune system has met the requirements of both efficacy and safety. We previously demonstrated that \( H_2 \) exerted a protective effect against \( \gamma \) irradiation in cultured cells and mice [12]. In this study, we found \( H_2 \) reduced the hydroxyl radical level in AHH-1 cells after radiation. We also found that \( H_2 \)-rich saline effectively reduced radiation-induced apoptosis in thymocytes and splenocytes in living mice after WBI. In the execution phase of cell apoptosis, caspase 3 was often activated and radiation-induced caspase 3 activation was also partially inhibited by \( H_2 \) treatment. Finally, we studied the hematological change after \( H_2 \) treatment and found that \( H_2 \) rescued the radiation-caused depletion of WBC and PLT, but did not find any influence on other indexes.

Recently, Ohsawa et al. reported that \( H_2 \) could effectively reduce the most cytotoxic of ROS, •OH [10]. Inhalation of \( H_2 \) was also reported to protect cerebral, myocardial and hepatic IR injury, and ameliorated oxidative stresses in lung and intestinal transplantation [16–18]. To our knowledge, 60–70% ionizing radiation-induced damage was caused by •OH [19]. •OH could easily react with DNA, proteins, lipids, etc and exert strong cytotoxic effects. In this study, our data showed that incubation of AHH-1 cells with \( H_2 \)-rich medium reduced the intracellular •OH produced by IR. The reaction between \( H_2 \) and •OH was also previously demonstrated in a cell-free Fenton system [10]. Thus, in AHH-1 cells, \( H_2 \) may react with •OH and protect cells from radiation damage, which may account for some mechanisms active in mitigating radiation damage.

IR could induce cell apoptosis in radiosensitive cells, like CD4+ T helper (Th) cells, CD8+ cytotoxic T lymphocyte (CTL), or the antibody-producing B cells [20], which act as effectors in cell-mediated and humoral immune responses [24]; thus the apoptosis or depletion of these cells directly causes immunosuppression. We found that the \( H_2 \) could effectively inhibit radiation-induced apoptosis in thymocytes and splenocytes of living mice. The reduction of apoptosis in thymocytes and splenocytes suggests that \( H_2 \) could also alleviate radiation-induced thymus and spleen damage. We also found that \( H_2 \) attenuated radiation-induced caspase 3 activation, which plays a central role in the execution phase of cell apoptosis, but the change in caspase 3 may be due to the reduction in •OH by \( H_2 \) through the ROS signaling pathway.
We then examined whether \( \text{H}_2 \) had a protective effect on hematological injury induced by irradiation. We found that \( \text{H}_2 \) attenuated the decrease in WBCs caused by radiation, which suggested a marked enhancement of immune function. The protective effects of \( \text{H}_2 \) on WBC may act similarly to the inhibition of apoptosis on lymphocytes in thymus and spleen, as \( \text{H}_2 \) is so easy to diffuse into cells in blood and tissues. \( \text{H}_2 \) also has protective effects on PLT against radiation damage, but the underlying mechanism is unclear. However, no significant change was observed in the red blood cells (RBC), hemoglobin (HBG), mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration (MCHC). This is not surprising, because the nucleus, which is absent in RBCs, is an important target of radiation.

Besides efficacy, safety is another important requirement for an ideal radioprotector. \( \text{H}_2 \) is produced by bacteria in the body and then released into the circulation \([21]\), and the reaction between \( \text{H}_2 \) and \( \text{O}_2 \) produces water \([22]\), indicating that low concentration \( \text{H}_2 \) could be safe for human health. Therefore dissolving it in PBS, physiological saline or medium would provide a convenient method of application. Immunosuppression is the most frequent consequence of routine irradiation. \( \text{H}_2 \) exhibits great potential as novel, safe and effective radioprotector for use by radiation workers, radiologists and doctors, as well as clinical uses.

**Conclusions**

We found that \( \text{H}_2 \)-rich saline or water could inhibit radiation-induced apoptosis in immune cells and ameliorate the homological injuries. The protective effect was attributed to its free radicals scavenging capacity. We suggest \( \text{H}_2 \) is a safe and effective radioprotector for the immune system. But the direct target of \( \text{H}_2 \) and the exact mechanism requires further study.

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**Declaration of interest**

The author has no conflict of interest to disclose.

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