Hydrogen Protects Mice From Dermatitis Caused by Local Radiation

Ke Mei, Sanhu Zhao, Liren Qian, Bailong Li, jin Ni, Jianming Cai

Abstract

Background: Radiation therapy produced unwanted side effect on normal tissues, such as radiodermatitis. Hydrogen was previously shown capable of radiation protective in both animals and cell cultures. The effect of hydrogen was now to be investigated on radiation-induced cutaneous. Objective: Development of dermatitis is a frequent side effect of radiotherapy of patients with head-and-neck cancer. Here we analyzed the radioprotective efficacy of hydrogen under conditions of local, single dose or fractionated radiation treatment, and its possible molecular mechanisms.

Methods: Mice received either single-dose or fractioned irradiation of the head-and-neck area with or without subcutaneous injection of hydrogen solution before irradiation. In vitro, the effect of hydrogen medium on radiation-induced cell viability, apoptosis and biochemical assays was measured. Result: hydrogen significantly reduced the severity of dermatitis, accelerated tissue recovery, and reduced the extent of radiation induced weight loss in mice after a single dose of 15 or 20 Gy but not 25 Gy of radiation. Hydrogen was also protective from cumulative doses of 30 Gy delivered in three fractions, respectively. Hydrogen also protect HaCaT cells from radiation-induced injury, it could significantly inhibit ionizing injury.

Conclusion: These results suggest that hydrogen has a positive effect on acute radiodermatitis.
Hydrogen Protects Mice From Dermatitis Caused by Local Radiation

*Authors contributed equally to this paper

Authors’ Contribution:

A Study Design

B Data Collection

C Statistical Analysis

D Data Interpretation

E Manuscript Preparation

F Literature Search

G Funds Collection

Author:

Mei Ke\textsuperscript{AE}\textsuperscript{*}, Department of Radiation Medicine, faculty of Naval Medicine, Second Military Medical University, Xiang yin Road 800#, 200433, Shanghai, PR China. Tel: +86-21-81871150. E-mail: lunasanger@163.com

Sanhu Zhao\textsuperscript{B}\textsuperscript{*}, Department of Radiation Medicine, faculty of Naval Medicine, Second Military Medical University, Xiang yin Road 800#, 200433, Shanghai, PR China. Tel: +86-21-81871150. E-mail: zsh6021023@163.com

Liren Qian\textsuperscript{C}, Department of Hematology, Naval General Hospital, Fucheng Road, Beijing, P.R. China. E-mail: 176753457@qq.com.
Bailong Li\textsuperscript{D}, Department of Radiation Medicine, faculty of Naval Medicine, Second Military Medical University, Xiang yin Road 800#, 200433, Shanghai, PR China. Tel:+86-21-81871150, libailong2003@yahoo.com.cn

Jin Ni\textsuperscript{F}, Department of Radiation Medicine, faculty of Naval Medicine, Second Military Medical University, Xiang yin Road 800#, 200433, Shanghai, PR China. Tel: +86-21-81871150, nijin2006@yahoo.com.cn

Correspondence Author:
Dr Jianming Cai\textsuperscript{FG}, Department of Radiation Medicine, faculty of Naval Medicine, Second Military Medical University, Xiang yin Road 800#, 200433, Shanghai, PR China. Tel: +86-21-81871101, Fax: +86-21-81871101, E-mail:cjm882013@gmail.com

Keywords: Ionizing radiation; Skin; Dermis; Radiotherapy; Hydrogen

Abstract

Background: Radiation therapy produced unwanted side effect on normal tissues, such as radiodermatitis. Hydrogen was previously shown capable of radiation protective in both animals and cell cultures. The effect of hydrogen was now to be investigated on radiation-induced cutaneous.

Objective: Development of dermatitis is a frequent side effect of radiotherapy of patients with head-and-neck cancer. Here we analyzed the radioprotective efficacy of
hydrogen under conditions of local, single dose or fractionated radiation treatment, and its possible molecular mechanisms.

Methods: Mice received either single-dose or fractioned irradiation of the head-and-neck area with or without subcutaneous injection of hydrogen solution before irradiation. In vitro, the effect of hydrogen medium on radiation-induced cell viability, apoptosis and biochemical assays was measured.

Result: Hydrogen significantly reduced the severity of dermatitis, accelerated tissue recovery, and reduced the extent of radiation induced weight loss in mice after a single dose of 15 or 20 Gy but not 25 Gy of radiation. Hydrogen was also protective from cumulative doses of 30 Gy delivered in three fractions, respectively. Hydrogen also protect HaCaT cells from radiation-induced injury, it could significantly inhibit ionizing injury.

Conclusion: These results suggest that hydrogen has a positive effect on acute radiodermatitis.

INTRODUCTION

Radiotherapy remains a widely accepted form of treatment for various types of cancer. In order to reduce the severity of adverse effects, radiation treatment is applied locally and in multiple fractions during radiotherapy. Despite the advanced therapeutic measures and technology, damage to normal tissues still can not be avoided. The skin is a biological defense barrier and a major target affected by
radiation-induced damage directly or indirectly, often showing the radiation-specific inflammation (radiodermatitis), occurs in about 95% of patients receiving radiation therapy for cancer. The irradiated areas of the skin often exhibit acute radiation damage characterized by the onset of erythema, swelling, blisters, and ulceration, followed by development of chronic inflammation, necrosis, fibrosis, and lymphedema[1]. These symptoms are not only the limiting factors during cancer radiotherapy but also public health concerns forcing the interruption or termination of the therapeutic course. In addition, most patients receiving radiotherapy treatment for head-and-neck cancers develop the highest morbidity from damage to salivary glands, including xerostomia, severe mucositis, and difficulty with swallowing[2-6]. These effects result in significant malnutrition and weight loss. During the process of undergoing radiotherapy, detrimental effects of ionizing radiation(IR) on biological tissues such as skin are mostly mediated via increased production of hydroxyl radical[7,8]. In recent years, Ohsawa et al[9] has been demonstrated that hydrogen(H2) could selectively reduce cytotoxic reactive oxygen species (ROS), such as •OH and peroxynitrite(ONOO−) in vitro, and exert therapeutic antioxidant activity in a rat middle cerebral artery occlusion model for the first time. Our department also demonstrated that H2 pretreatment could protect cultured cells, intestine and heart in mice from ionizing radiation, and improve the 30-day-survival rate in irradiated mice[10-12]. In addition, Pan Yu et al[13] suggested
that H₂ therapy could be a useful way for protecting human skin fibroblasts from high glucose and mannitol induced oxidative damage. These results show the potential of H₂ as an effective radioprotectant without known toxic side effects. But whether the H₂ can protect the radiation-induced dermatitis is not reported as yet. In this study, we hypothesized that hydrogen would reduce radiation-induced dermatitis and mucositis developed as a result of single dose or fractioned local irradiation, and can protect HaCaTs from radiation. Therefore, we evaluated the effect of hydrogen on a mouse model of radiation-induced dermatitis and mucositis of head-and-neck area, and tested the impact of hydrogen on the treated HaCaTs and investigated the mechanism of these effects.

MATERIALS AND METHODS

Hydrogen-rich saline production

Hydrogen was dissolved in physiological saline 6 hours under high pressure (0.4 MPa) to a supersaturated level using hydrogen-rich water-producing apparatus produced by our department. The saturated hydrogen PBS/saline was stored under atmospheric pressure at 4°C in an aluminum bag with no dead volume. Hydrogen-rich PBS/saline was freshly prepared every week, which ensured that a concentration of more than 0.6mmol/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.¹⁴
Cell culture and treatment

Immortalized human keratinocytes HaCaT were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, California, USA) and 1% penicillin–streptomycin–glutamine at 37°C in a 5% CO₂ humidified chamber. For radioprotective studies, cells were treated with different volume of Hydrogen-rich medium and accordingly we added different volume of medium in order to obtain the desired concentration of H₂ 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 DMEM.

Irradiation

⁶⁰Co-gamma rays in irradiation Center (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.

Animal Care and treatment

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). Adult male Sprague-Dawley rats (8-10 weeks old; 250-300g) 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 5 mins before radiation. Adult male Sprague-Dawley rats were briefly anaesthetized by an intraperitoneal injection of chloral hydrate at 300 mg/kg. The head-and-neck areas were irradiated with single dose of 15, 20 or 25 Gy or in fractions given 24h apart with cumulative 30 Gy (3 * 10 Gy). The unirradiation field was shielded with lead blocks.

Body weight loss

Mouses survival and body weight were recorded daily or every second day, respectively. Mice which experienced weight loss > 25 % and limited motility were sacrificed. In the experiments for histological analysis, mice were sacrificed when the difference in body weight loss between the hydrogen treated and untreated groups was greatest (11-14 days after irradiation).

Early radiation dermatitis

Early radiation dermatitis was measured during weeks 2 and 3 after irradiation. The degree of early cutaneous toxicity was evaluated daily, according to skin score criteria, as previously described[1]. Briefly, a researcher presented the animals to three
observers blinded to the animal treatment group and then recorded the scores. A 5-point scoring system was used (Table 1).

Morphologic observation

Mice were treated intraperitoneally (IP) with physiological saline or hydrogen-rich PBS 5 mins before irradiation. 15 days after the first radiation treatment, mice were sacrificed by cervical dislocation under isoflurane anesthesia. Skin samples which were removed from neck were fixed in 10% buffered formaldehyde-saline.

Cell Viability Analyses

Cells were seeded in 96-well plates and pretreated with or without Hydrogen-rich medium, the treated cells were then immediately irradiated. After irradiation the cells were further cultured for 48h. Cell viability was determined by CCK-8 assay using a Cell Counting kit (Dojindo Laboratories, Kumamoto, Japan).

Apoptosis assays for cultured cells

Apoptosis was determined by Annexin V-APC and propidium iodide staining using Apoptosis Detection Kit (Bipec Biopharma, Massachusetts, USA). Treated cells were incubated with Annexin V-APC for 15 minutes at 4°C and propidium iodide for 5 minutes at room temperature. Cells were then analyzed by flow cytometry.

Biochemical assays (SOD, GSH and MDA)

Cells were collected 12h 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 [15], based on the inhibition of the formation of NADH-PMS-NBT complex. The GSH concentration was measured by the method of Ellman [16]. This method was based on the development of a yellow color when 5',5’-dithiobis2-nitrobenzoic acid was added to compounds containing sulfhydryl groups. MDA was assessed spectrophotometrically with the method defined by Ohkawa et al as MDA reacted with thiobarbituric acid and formed a pink, maximum absorbent complex at 532 nm wavelength [10].

Statistical analysis
Data are expressed as means±S.E.M. for each experiment. The number of samples is indicated in the description 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
Effects of hydrogen in mice subjected to single dose local irradiation of head and neck
The radioprotective effects of hydrogen against local radiation induced injury were first studied under the conditions of single or fractioned doses of local irradiation of the head and neck of Sprague-Dawley mice. Treatment with hydrogen alone (without
irradiation) produced no effect on body weight relative to untreated mice during the course of experiment (data not shown). Exposure to a single 15Gy dose of γ-ray brought about an approximate 5% loss in initial body weight followed by rapid recovery during Days 5 to 7 (Fig. 1A). Weight change in the group that received hydrogen 5 mins before 15 Gy irradiation was insignificant (3-5% of initial body weight variation).

In the control group that received 20 Gy, a continuous weight loss until Days 9 and 10, reaching a total loss of more than 15% of initial body weight was observed because of a dramatic reduction in food and water consumption. This was followed by a slow recovery that was still incomplete by Day 14 after irradiation. Animals treated with hydrogen showed less significant weight loss and part recovery by Day 14 indicative of protective effect of the drug (results of a representative experiment shown in Fig. 1B).

Increasing the local radiation dose to 25 Gy led to mortality of all mice within 10 days because of weight loss greater than 25% which prompted euthanasia for ethical reasons. As indicated in Figure 1C, pretreatment with hydrogen did not alter this outcome. Although the number of radiation doses analyzed did not allow us to accurately calculate dose reduction factor (DRF) of hydrogen under applied conditions, it is clear that it is effective at least up to 20 Gy but conferred no protection at 25 Gy.
Effects of hydrogen in mice subjected to fractioned local irradiation of head and neck

Because normal human radiotherapy practice uses fractioned doses of local radiation, we next applied cumulative local γ-ray doses to 30 Gy of γ-ray radiation to the head-and-neck area of Sprague-Dawley mice as three 10Gy fractions, respectively.

Hydrogen was administered at 5 mins before each irradiation dose to the designated mice. Control mice were injected with PBS. Both radiation regimens resulted in substantial (>20%) body weight loss followed by a slow recovery, which was still incomplete by Day 14 after the first irradiation. Mice injected with hydrogen demonstrated less significant weight loss and part recovery by Day 14 (data for 30Gy regimen are shown in Fig. 2).

Macrophotographs (Fig. 2) of the irradiated region taken on Day 15 after the first irradiation offer visual evidence of the extensive skin damage and inflammation in the control irradiated vehicle-treated mice. At the histological level, the skin from the irradiated region of control mice showed atrophy of the hair follicles, hyperplasia of the epidermis with papillary thickening of its layers, hyperkeratosis, hyperemia, hemorrhage, and infiltration of the inflammatory cells in the underlying derma.

Hydrogen administrations dramatically reduced epidermal hyperplasia, hyperkeratosis, and atrophy of the hair follicles and dermal inflammation, whereas some level of hyperemia still persisted.

Observations from all analyzed mice are summarized in Table 1.
Effects of hydrogen-rich medium on cell viability of irradiated HaCaT cells
To investigate radioprotective effects of hydrogen-rich medium in cell culture, we examined viability of irradiated HaCaT cells. We demonstrated that pretreatment of HaCaT cells with 0.6mmol/L H₂ before irradiation significantly increased cell survival as compared to cells treated with radiation alone at all examined doses (up to 8 Gy) (Fig.3).

Effects of hydrogen-rich medium on cell apoptosis of irradiated HaCaT cells
To determine the radiation-induced apoptosis of irradiated HaCaT cells, we analyzed treated cells by using Annexin V-APC and propidium iodide staining in flow cytometry assay. The early apoptotic cells decreased when pretreated with 0.6mmol/L H₂ as compared to cells pretreated without H (Fig.4, 12.2% vs. 17.5%, respectively). These data suggest that hydrogen can attenuate apoptosis in irradiated HaCaT cells.

Effects of hydrogen-rich medium on cellular oxidative products and antioxidative status
The plasma SOD and GSH concentrations were measured at 12 h of irradiation (Fig.5A and B). Plasma SOD and GSH concentrations at 12 h of irradiation in the H₂ group were significantly higher than that of the Control group. The plasma MDA were measured at 12 h of irradiation (Fig. 5C). Plasma MDA at 12 h of irradiation in the hydrogen group were significantly lower than that of the Control group.
Discussion

To our knowledge, this is the first study investigating the effects of hydrogen in mice subjected to single and fractioned dose local irradiation of head and neck. In the present result, we demonstrate the efficacy of hydrogen for protection of dermal from injury resulting from local irradiation, we also examine the preventive effects of hydrogen-rich medium against radiation-induced cell death and apoptosis and intracellular ROS in HaCaT keratinocytes. We previously demonstrated efficacy of hydrogen-rich solution in reducing severity of and lethality from acute radiation syndrome caused by total body irradiation of mice\textsuperscript{10}. Although total body radiation imitates biodefense scenarios of radiation disaster, the assessment of hydrogen’s efficacy in the context of clinically relevant scenarios (radiotherapy side effects) requires the use of models that involve fractioned irradiation. We choose a model for this study is prompted by the frequent use of radiotherapy for the treatment of head-and-neck cancer. In common use, the treatment regimens is not unusual to encounter adverse effects which predominantly affect the dermatitis, and the severity can be dose limiting\textsuperscript{6,19}. As we found that the protective of hydrogen-rich solution after irradiation is not significant and the levels of H\textsubscript{2} peaked approximately 5 mins following injection, we treated the mice 5 mins before γ-radiation\textsuperscript{10,12}. The mechanism of radioprotection by hydrogen may result from radical oxygen species (ROS) scavenging effect of molecular H\textsubscript{2}. 60-70% of the ionizing radiation-
induced tissue damage was caused by OH, ROS play a key role in by influencing dermatitis\textsuperscript{[21]}. It has been considerably reported the effect of free radical scavengers to ameliorate the oxidative injuries due to IR\textsuperscript{[22,23]}. Recent studies of our department suggest that H\textsubscript{2} has a potential as a safe and effective radioprotective agent\textsuperscript{[11,24]}. Molecular hydrogen could selectively reduces the strongest oxidant(OH and ONOO-) in a cell free system, whereas it is mild enough not to disturb metabolic oxidation-reduction reactions or to react with other ROS, such as superoxide radical and hydrogen peroxide which play important physiological roles\textsuperscript{[4]}. The rapid gaseous diffusion might make H\textsubscript{2} easily penetrate membranes and effectively target cytotoxic radicals.

Being oxidized themselves, endogenous antioxidants are a group of substances that significantly inhibit or delay oxidative processes\textsuperscript{[25]}. Antioxidant enzymes play important roles in providing protection from radiation exposure\textsuperscript{[26]}. In the antioxidant system, both SOD and GSH can protect cells from radiation induced oxidative damage. SOD can scavenge O$_2^-$, and its viability directly reflects radical scavenging ability in an organ, which is important for maintaining the dynamic balance of ROS in the body. GSH is a major intracelluar non-enzyme antioxidant. MDA is a degradation product of lipid peroxidation. The levels of MDA is elevated while the cellular oxidative defense is deficient and lipid peroxidation is increased. Membrane lipids are the major targets of ROS and the free radical chain reaction\textsuperscript{[27]}. 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 of irradiated mice. But pretreatment of hydrogen prior to radiation exposure increased the antioxidant status at both enzymic and non-enzymic levels and decreased the levels of MDA.

The approved supporting-care drug for this indication, amifostine, has shown good radioprotective effects[28], remains underused because of its toxicity and lack of specificity with respect to normal tissue protection[29]. Other radioprotectors, such as cytokines and immunomodulators, should be used with low radiation doses or in combination with radical scavengers and antioxidants[30], while thiol compounds has relatively high toxicity, 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[31]. Hydrogen is produced by colonic bacteria in the body and normally circulates in the blood[32], so it is physiologically safe for humans to inject hydrogen at a relatively low concentration. It is also a highly diffusible gas and reacts with hydroxyl radical to produce water[33]. Moreover, dissolving H$_2$ in solvent such as physiological saline or medium is easy to apply and safe. Therefore, the availability of quantitative criteria detailing the severity of dermatitis associated with radiotherapy of head-and-neck cancer makes it possible to plan and to rapidly complete clinical trials with relatively short-term endpoints. The results demonstrated
strong effect of hydrogen administration on the development and grade of dermatitis potentially broadens the clinical value of hydrogen by extending its projected applications to those radiotherapy treatments that are known to be associated with skin toxicity. These include breast cancer patients, who frequently experience skin burns during radiotherapy $^{[34]}$.

In conclusion, this study showed that hydrogen significantly reduced the severity of dermatitis, accelerated tissue recovery, and reduced the extent of radiation induced weight loss in mice after a single or fractions dose. Hydrogen-rich medium also protect HaCaT cells from radiation-induced injury, it could significantly inhibit ionizing. This method is comparatively easier and safer than the direct application of hydrogen gas. The use of hydrogen significantly reduced intracellular O$_2$ levels and enhanced the antioxidative capability of cells. Thus, the application of hydrogen may provide a new method in the treatment of radiodermatitis and oxidative damage caused by radiation treatment.

**Acknowledgements**

This study was supported by grant from the National Natural Science Foundation of China (No. 30770503; No. 30500579).

**Declaration of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.


Figure legends

Figure 1. Body weight changes after radiation treatment of the head-and-neck area in mice. Mouse body weight changes after single irradiation with 15 (A, n = 6), 20 (B, n = 6), or 25 Gy (C, n = 4) with and without prior hydrogen solution injection (0.6 mmol/L H₂ 20 ml/kg per mouse). Mouse body weight changes after 30 Gy local head-and-neck radiation treatment (D, n = 6), with and without hydrogen solution (0.6 mmol/L H₂ 20 ml/kg per mouse), given in three 10-Gy fractions over 3 consecutive days. *Significant difference between irradiated groups, with and without hydrogen treatment (p < 0.05).

Figure 2. Severity of early skin reactions measured 7 to 21 days after 30 Gy irradiation (given in three 10-Gy fractions over 3 consecutive days) was less severe and progressed more slowly in hydrogen-treated animals, compared with those treated with radiation alone and radiation plus PBS. The comparative photographs were taken on Day 15 (A, B, and C). A comparison of skin scores is shown (Table 1). Hematoxylin and eosin (H&E) estained skin sections from the neck area obtained 15 days after the first of three 10 Gy fractions of 30 Gy total irradiation.

Figure 3. Dose dependent effect of H₂ on HACAT cell viability induced by 0, 2, 4, 8 Gy gamma radiation. Pretreatment of 0.6 mmol/L H₂ before irradiation can increase cell survival. Values are given as mean ± SEM ((n=6), neg: no H₂ no radiation). *P< 0.05, **P< 0.01, #P<0.1.

Figure 4. Hydrogen-rich PBS attenuates radiation-induced apoptosis in HACAT cells. Treated cells were collected 24 h after irradiation, stained with Annexin V-APC and propidium iodide and analyzed by flow cytometry. Shown are representative diagrams of distribution of stained cells and a bar graph of apoptotic cells expressed as a percent of total cells. Values are given as mean±SEM (n=4). *P< 0.05

Figure 5. Changes in the activities of SOD, concentrations of GSH and levels of MDA in normal, γ-irradiated and H₂ pretreated HaCaT cells. Hydrogen-rich medium significantly decreased levels of MDA, a marker of oxidative stress. Shown are 12 hours after irradiation. Values are given as mean±SEM (n=6), *P<0.01.
Table 1. The criteria for radiation-induced early toxicity in soft tissue

<table>
<thead>
<tr>
<th>Score</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>Normal</td>
</tr>
<tr>
<td>1.5</td>
<td>Slight erythema</td>
</tr>
<tr>
<td>2.0</td>
<td>Depigmentation with &lt;25% hair loss</td>
</tr>
<tr>
<td>2.5</td>
<td>Early dry desquamation, thickening, &gt;25% hair loss</td>
</tr>
<tr>
<td>3.0</td>
<td>Dry desquamation, mild edema</td>
</tr>
<tr>
<td>3.5</td>
<td>Dry desquamation, early moist</td>
</tr>
<tr>
<td>4.0</td>
<td>Moist desquamation, moderate &lt;50%</td>
</tr>
<tr>
<td>4.5</td>
<td>&gt;50% desquamation ± some necrosis</td>
</tr>
<tr>
<td>5.0</td>
<td>Significant necrosis and loss of dermis</td>
</tr>
</tbody>
</table>

Note: Time after irradiation <2 months; scores peaked at 3 to 4 weeks