J. Radiat. Res., 51, 741–747 (2010)

Regular Paper

The Potential Cardioprotective Effects of Hydrogen in Irradiated Mice

Liren QIAN[1,2*†], Fei CAO[1†], Jianguo CUI[1†], Yicun WANG[3], Yuecheng HUANG[1], Yunhai CHUAI[1], Luqian ZAHO[1], Hao JIANG[1] and Jianming CAI[1*]

Ionizing radiation/Cardioprotection/Myocardium/Hydrogen.

Most ionizing radiation-induced damage is caused by hydroxyl radicals, and the selective reduction of hydroxyl by hydrogen in vitro has been demonstrated previously. Irradiation of the heart can cause chronic cardiac disease. This study was designed to test the hypothesis that hydrogen-rich water (pure water saturated with molecular hydrogen), which is easy to use, induces cardioprotection against ionizing irradiation injury in mice. In this paper, we demonstrate that hydrogen can protect myocardium degeneration from radiation-induced injury, decrease myocardium malondialdehyde (MDA), 8-hydroxydeoxyguanosine (8-OHdG) levels, and increase myocardium endogenous antioxidants in vivo. We suggest that hydrogen has a cardioprotective effect against radiation induced injury.

INTRODUCTION

Irradiation of the heart can cause chronic impairment of cardiac pump function and cardiac disease.[1)] The most significant type of radiation-induced heart disease (RIHD) appears to be that of myocardial damage, which may result from loss of alkaline phosphatase activity of capillary endothelial cells 6–10 weeks after irradiation.[2,3)] In addition to myocardial degeneration, perivascular and interstitial fibrosis are seen.[4)] Fibrosis is a sequel of both radiotherapy and accidental over exposures and has been described in many tissues, including skin,[5)] lung,[6)] heart,[7)] and liver.[8)] And fibrosis could be defined as a wound in which continuous signals for tissue repair are emitted.

At present, thiol compounds is the most effective class of radioprotectors. Previously, the WR-2721's protection against heart irradiation has been reported.[9)] However, they have significant shortcomings including relatively high toxicity and unfavorable routes of administration, which negatively affect their application and efficacy.[10)] Therefore, there is a need for safer and even more effective radioprotective treatments.

Detrimental effects of ionizing radiation (IR) on biological tissues are, in major part, mediated via increased production of hydroxyl radical. Ohsawa *et al.*[11)] found that molecular hydrogen could selectively reduce cytotoxic reactive oxygen species, such as •OH and ONOO– in vitro. Previously, we have demonstrated that hydrogen has potential radioprotective effects.[12)] In the current study, we investigated whether hydrogen-rich water exerted cardioprotective effect in irradiated mice. We demonstrated here that hydrogen treatment has a cardioprotective effect.

MATERIALS AND METHODS

Hydrogen-rich water production

Hydrogen was dissolved in pure water 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 water was stored under atmospheric pressure at 4°C in an aluminum bag with no dead volume. Hydrogen-rich water 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

*Corresponding author: Phone: +86-21-81871101,
Fax: +86-21-81871101,
E-mail: qlr2007@126.com (Liren QIAN)
E-mail: cjm882003@yahoo.com.cn (Jianming CAI)
[1]Department of Radiation Medicine, Faculty of Naval Medicine, Second Military Medical University, Xiangyin Road, 200433, Shanghai, PR China; [2]Department Of Hematology, Navy General Hospital; [3]Department Of Orthopaedics, Changhai Hospital.
†Authors contribute equally to this paper.

Abbreviations: RIHD, radiation-induced heart disease; MDA, malondialdehyde; 8-OHdG, 8-hydroxydeoxyguanosine; SOD, Superoxide dismutase; GSH, Glutathione.

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

doi:10.1269/jrr.10093

content of hydrogen in water by the method described by Ohsawa *et al.*.[11]

Irradiation

^{60}Co-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.

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). A total of 90 male BALB/c mice 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. Food and water were provided ad libitum. For experiments, mice were provided with pure water or Hydrogen-rich pure water as described by Sato *et al.*[13] 24 h before radiation. Mice were irradiated in a holder designed to immobilize unanaesthetized mice with a dose rate of 0.8 Gy/min.

Survival assays

For evaluation of radioprotective activity, mice were irradiated with a dose of 7 Gy. After irradiation they were returned to the animal facility and routinely cared for 30 days after irradiation. Survival was checked and scored daily for 30 days.

Morphologic assessment of cardiac damage

For evaluation of radiation-induced cardiac damage in early and late stages, mice were killed at 24 hours and 100 days by cervical dislocation under isoflurane anesthesia after the experimental mice received a single dose of 15 Gy locally to the heart. First the thorax was opened by 2 lateral cuts through the ribs. The hearts were sampled for histologic evaluation. Tissues were embedded in paraffin wax and processed through hematoxylin-eosin or the collagen specific Masson stain in 4-μm sections. The severity and extent of histologic lesions were evaluated in the irradiated and age-matched control mice. For quantification of the degree of the myocardial degeneration in the ventricles and the interventricular septum, 5 adjacent high-power fields were evaluated as described by Tokatli *et al.*.[14] Myocardial degeneration was scored from 0 to 3: no degenerated myocytes (0-normal), a few degenerated myocytes (1-mild), about 50% degenerated myocytes (2-moderate), and more than 50% degenerated myocytes (3-severe). For quantification of total fibrillar collagen accumulation by Masson staining, the percentage of fibrillar collagen in every section was determined by a computer with Image-Pro Plus image analysis software as as described by Hui Liu *et al.*.[15] All histologic examinations were performed by two different pathologists without prior knowledge of the treatment given to the animals.

Biochemical assays

For evaluation of biochemical changes, mice were killed at 4 hours by cervical dislocation under isoflurane anesthesia after the experimental mice received a single dose of 6 Gy. The myocardial specimen was collected and homogenized in buffer, then they were centrifuged at 620 × g for 10 min at 4°C. The samples were taken for biochemical estimations (SOD, GSH and MDA). Superoxide dismutase (SOD) activity was assayed by the method of Kakkar *et al.*.[16] This method was based on the inhibition of the formation of NADH-PMS-NBT complex. Total GSH (GSH plus GSSG) concentration was measured by the method of Anderson.[17] It was measured using the 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) – oxidized GSH (GSSG) recycling assay. 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.[18]

For determination of 8-OHdG levels in DNA from the myocardium of mice, DNA was extracted from the mice myocardial specimen with a DNA Extractor Kit (DNA Extractor Wb Kit, Wako Chemical, Osaka, Japan) according to the method of Nakae *et al.*.[19] Then the isolated DNA was digested by the method of Valls-Belles *et al.*.[20] The 8-OHdG levels of these samples were measured as described by Yasuhara *et al.*.[21] Briefly, the samples were added to plate wells precoated with mouse monoclonal anti-8-OHdG antibody (Japan Institute for the Control of Aging, Fukuroi, Japan), of which the specificity has been proved by Shinya Toyokuni *et al.*.[22] They were incubating for 45 min at 37°C. After washed for 3 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 a sulphuric acid. The absorbance was read at a wavelength of 450 nm.

Comet assay

Cardiac myocytes were isolated from heart by method described previously.[23] The DNA strand breaks (DSBs) were measured by using single-cell gel electrophoresis (comet assay) based on the method of Gandhi *et al.*.[24]

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

Survival rate of mice

90% of irradiated mice without H_2 treatment before 7 Gy whole body gamma-irradiation died by the 13rd post-irradiation day (Fig. 1), while 80% of the mice pretreated with H_2 had survived. Thus, H_2 could protect mice against radiation-induced injury. Also, H_2 could mitigate radiation-induced injury.

Histology

There was no statistically significant difference among the 24-hour groups themselves (Fig. 2). On the 100th day of evaluation, there were no histopathologic changes in the group pretreated with H_2, but there was myocardial degeneration in 40% mice from the group pretreated without H_2. These degenerations were mild focal vacuolization (score 1) in the ventricles and the interventricular septum (Fig. 3), which showed a statistically significant difference.

Lesions in the irradiated mice hearts were also characterized by an increase in total collagen, as demonstrated with the Masson blue staining. In the group pretreated without H_2, the ratio of fibrillar collagen was significantly higher in 100th day postirradiation than in the group pretreated with H_2 (Fig. 4A and B). Statistical analysis suggested that there was significant difference between these two groups. There was no statistically significant difference among the 24-hour groups.

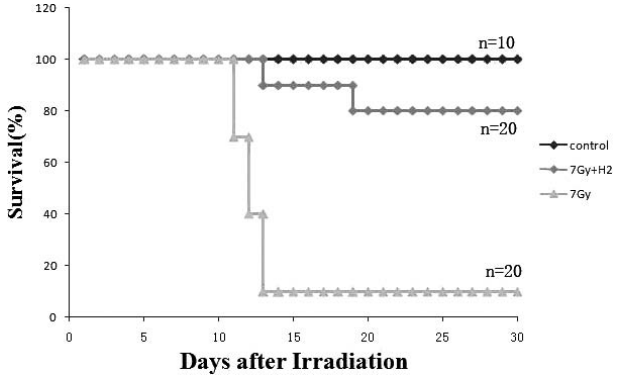

Fig. 1. Hydrogen-rich water mediated radioprotection of mice. Groups of 20 Balb/c mice were given Hydrogen-rich water or water for 24 h before irradiation. Mice in control group were normal ones which were not irradiated. Representative results from one of three independent experiments are shown. The difference in survival between the experimental groups was statistically significant (*P < 0.05).

Changes in the activities of myocardial SOD and GSH

The myocardial SOD and total GSH concentrations were measured at 4 h after irradiation (Fig. 5A and B). Myocardial SOD and total GSH concentrations at 4 h after irradiation in the H_2 group were significantly higher than that of the Control group. The results indicated that gamma irradiated cardiac muscle showed a significant decrease in the levels of both enzymatic and non-enzymatic antioxidant status when compared to hydrogen pre-treated groups which showed an increased antioxidant status, indicating that pretreatment with hydrogen restored the antioxidant status to near normal.

Changes in the levels of myocardial MDA and 8-OHdG

The myocardial MDA and 8-OHdG concentrations were measured at 24 h after irradiation (Fig. 6A and B). Myocardial MDA and 8-OHdG concentrations in the H_2 group were significantly lower than that of the Control group. Changes in the levels of MDA indicate the antioxidant potential of hydrogen. The result of 8-OHdG indicates that hydrogen could alleviate injury on DNA induced by free radicals.

Comet assay

The alteration in the levels of comet attributes is shown in Fig. 7. The irradiated mice resulted in an increase in the levels of all comet parameters (%DNA in tail, tail length, tail moment, and olive tail moment), whereas pretreatment of hydrogen prior to irradiation inhibited the increase of these parameters significantly, indicating the protective effects of hydrogen on radiation induced DNA damage.

DISCUSSION

To our knowledge, this is the first study demonstrating that hydrogen has cardioprotective effects in irradiated mice. This is also the first study revealing that H_2 reduces rate of mortality induced by γ-irradiation. The sulfhydryl compound amifostine, named WR-2721, which is the only radioprotector registered for using in human, has shown good radioprotective effects.[25] However, it has a narrow therapeutic index and is plagued by significant side effects such as hypotension, nausea, vomiting and allergy even at low doses.[26] Thus, the use of these agents limited because of their toxic effects. On the other hand, hydrogen is continuously produced by colonic bacteria in the body and normally circulates in the blood,[27] we suggest that the side effects of hydrogen must be small, and different from other antioxidants. Inhalation of hydrogen gas does not influence physiological parameters such as body temperature, blood pressure, pH, and pO2 in the blood, as shown previously.[11,28] So it is physiologically safe for humans to inhale hydrogen at a relatively low concentration. When hydrogen-rich water was placed in the stomach, hydrogen was detected in the blood, indicating the incorporation of H_2 into the body by drinking.[29] Hydrogen diffuses very rapidly into cells, and

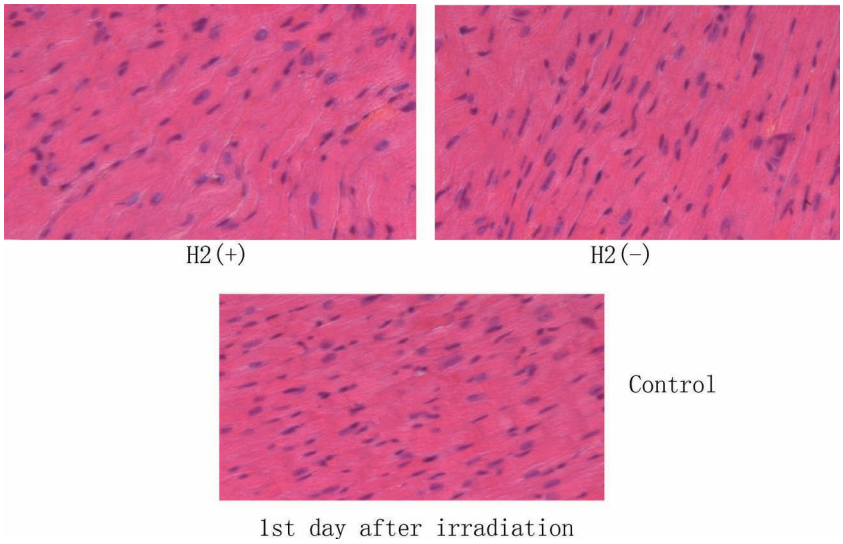

Fig. 2. Normal myocardial cells in mice at 1st postradiation day. Mice in control group were given pure water without irradiation.

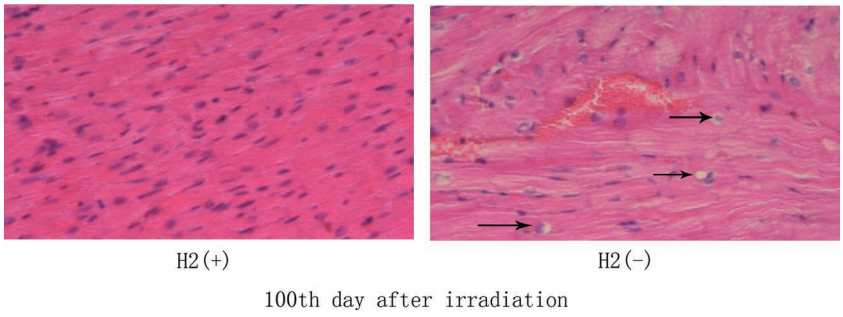

Fig. 3. Normal myocardial cells in mice pretreated with H_2 at 100th postradiation day, but myocardial cells showing vacuolization (→) in mice pretreated without H_2 at 100th postradiation day, hematoxylin-eosin (magnification × 400).

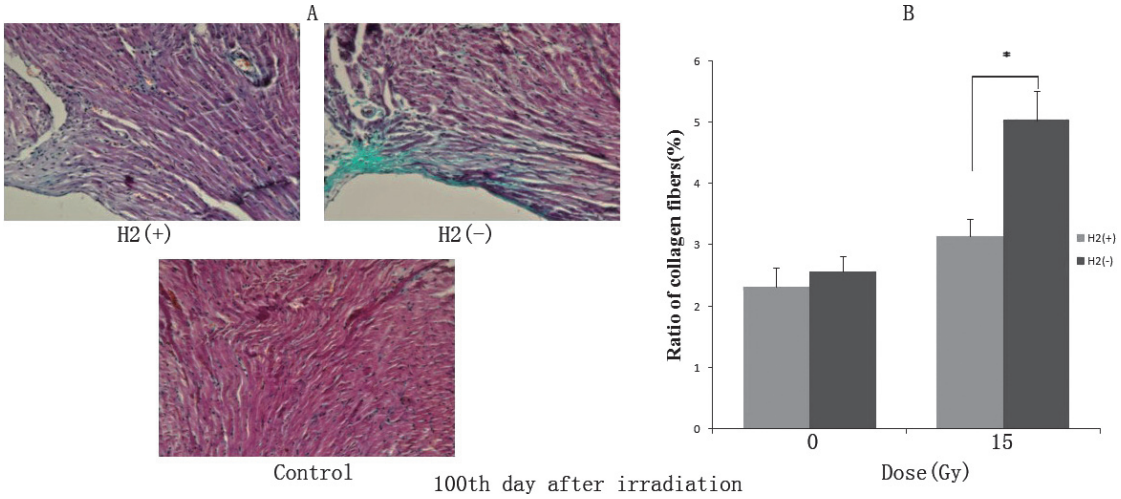

Fig. 4. Histopathologic changes of experimental groups. Mice in control group were given pure water without irradiation. Few fibers could be seen among the muscles of mice pretreated with H_2, but among the muscles of mice pretreated without H_2 fibers accumulated around the artery and invaded into the muscle, Masson (magnification × 400) (A). The percentage of fibrillar collagen accumulated at the 100th day after irradiation was shown (B) (* $P < 0.05$).

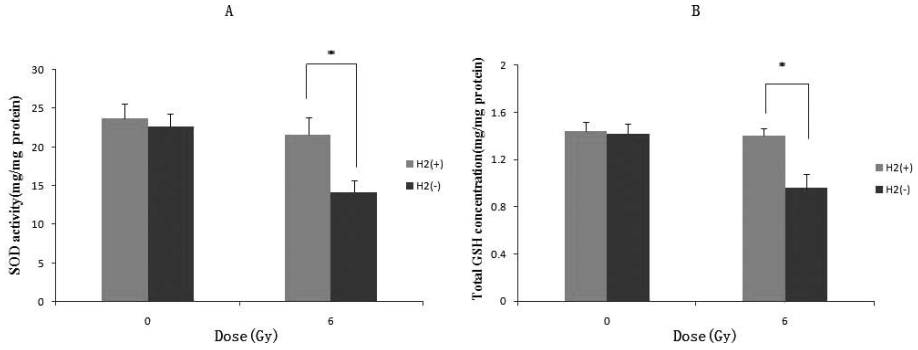

Fig. 5. Changes in the activities of SOD (A) and concentrations of total GSH (B) in normal, γ-irradiated and H_2 pretreated mice. Values are given as mean ± SEM (n = 8). *P < 0.01.

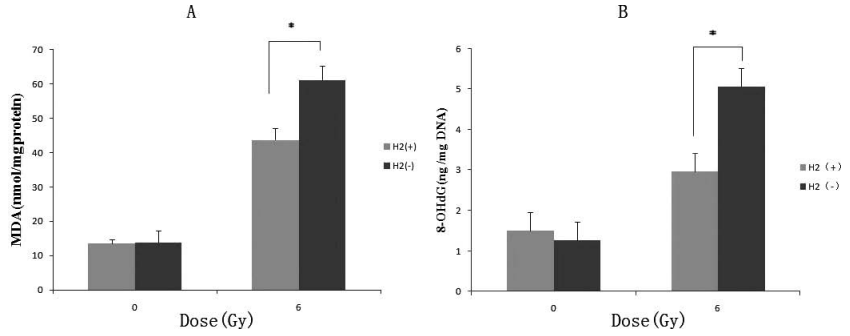

Fig. 6. Level of myocardial MDA (A) and 8-OHdG immunoreactivity (B) in normal, γ-irradiated and H_2 pretreated mice. Values are mean ± SEM (n = 6), *P < 0.01.

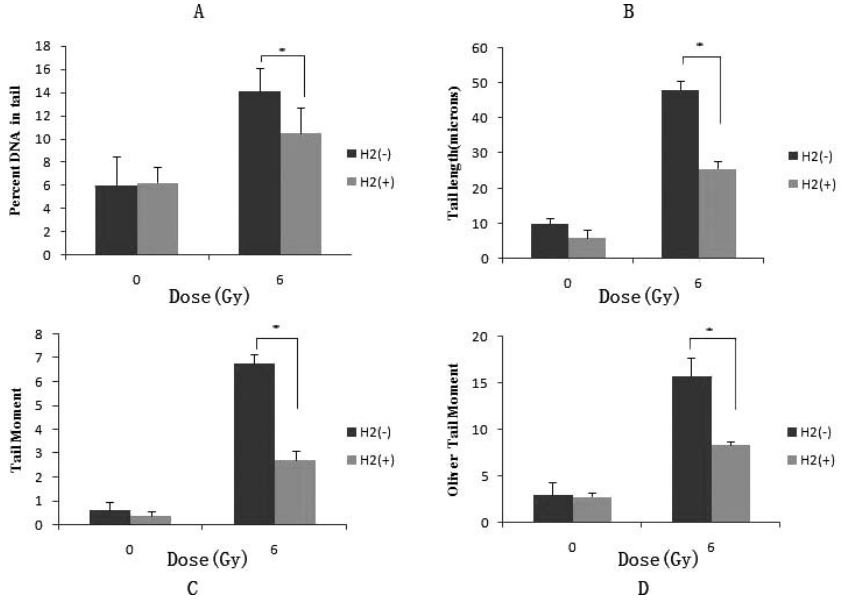

Fig. 7. Effect of hydrogen in Cardiac myocytes on DNA damage assayed by comet assay: (A) %DNA in tail, (B) tail length, (C) tail moment, (D) olive tail moment.

high efficacy is expected.[11,28] It is a highly diffusible gas and it reacts with hydroxyl radical to produce water.[30] Dissolving H_2 in solvent such as pure water, physiological saline or medium is easy to apply and safe. Therefore, it may have great potential for clinical use.

This study indicates that late cardiac effects of irradiation

were characterized by mild degeneration (score 1) in 40% of the mice killed 100 days after irradiation and pretreated without H_2. This percentage was statistically different from the percentage of the mice treated with H_2. Also the ratio of fibrillar collagen in myocardium showed a significant difference between the mice killed 100 days after irradiation pretreated with and without H_2 by the collagen specific Masson stain. It is thought that H_2 could be a cardioprotector, because less histopathologic myocardial damage was detected in mice receiving hydrogen-rich water preirradiation as compared to mice given water only.

Antioxidant enzymes such as SOD are important in providing protection from radiation exposure.[31] The proper balance of the enzymes in the whole organism are required for maximum radioprotection..Glutathione (GSH) participates non-enzymatically in protection against radiation damage.[32] 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.[33] The increase in the levels of lipid peroxidation products, such as malondialdehyde and TBARs, points to the indices of lipid damage.[34] The research of membrane damage induced by reactive oxygen species is related to human diseases and their possible prevention by antioxidants constitutes an active area of research in recent years. Also, DNA is one of the major targets of ROS and 8-OHdG is formed from deoxyguanosine in DNA by hydroxyl free radicals.[35] 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 myocardial MDA and 8-OHdG 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 and 8-OHdG. This effect was due to its antioxidant property and showed that hydrogen acted as a good scavenger against free radical generation and thereby inhibits lipid and DNA peroxidation.

The alkaline comet assay is an effective technique to monitor the extent of the DNA damage. Our results show that pretreatment of hydrogen prior to irradiation inhibited the increase of comet parameters when compared to irradiated group. The exact mechanism is still unknown. But this may be due to the effective antioxidant potential of hydrogen. Thus hydrogen could have decreased the DNA damage during exposure to irradiation by effectively scavenging the free radicals.

Thus from the results obtained, we conclude that the effect of reducing radical oxygen species plays an important role in the radioprotective effects of hydrogen. However, the exact mechanism and signaling pathway involved in the protection role of hydrogen in ionizing radiation injury need to be studied in the future.

ACKNOWLEDGEMENTS

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

REFERENCES

1. Lee CK, *et al* (2000) The need for long-term surveillance for patients treated with curative radiotherapy for Hodgkin's disease: University of Minnesota experience. Int J Radiat Oncol Biol Phys **48**: 169–179.
2. Lauk S (1987) Endothelial alkaline phosphatase activity loss as an early stage in the development of radiation-induced heart disease in rats. Radiat Res **110**: 118–128.
3. Lauk S, *et al* (1985) Radiation-induced heart disease in rats. Int J Radiat Oncol Biol Phys **11**: 801–808.
4. Stewart JR, *et al* (1995) Radiation injury to the heart. Int J Radiat Oncol Biol Phys **31**: 1205–1211.
5. Lefaix JL, *et al* (1998) Diagnosis of acute localized irradiation lesions: review of the French experimental experience. Health Phys **75**: 375–384.
6. McDonald S, *et al* (1995) Injury to the lung from cancer therapy: clinical syndromes, measurable endpoints, and potential scoring systems. Int J Radiat Oncol Biol Phys **31**: 1187–1203.
7. Fajardo LF, *et al* (1973) Pathogenesis of radiation-induced myocardial fibrosis. Lab Invest **29**: 244–257.
8. Geraci JP, *et al* (1993) Radiation hepatology of the rat: parenchymal and nonparenchymal cell injury. Radiat Res **136**: 205–213.
9. Kruse JJ, *et al* (2003) Effects of amifostine on radiation-induced cardiac damage. Acta Oncol **42**: 4–9.
10. Maisin JR (1998) Bacq and Alexander Award lecture--chemical radioprotection: past, present, and future prospects. Int J Radiat Biol **73**: 443–450.
11. Ohsawa I, *et al* (2007) Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. Nat Med **13**: 688–694.
12. Qian L, *et al* (2010) Radioprotective effect of hydrogen in cultured cells and mice. Free Radic Res **44**: 275–282.
13. Sato Y, *et al* (2008) Hydrogen-rich pure water prevents superoxide formation in brain slices of vitamin C-depleted SMP30/GNL knockout mice. Biochem Biophys Res Commun **375**: 346–350.
14. Tokatli F, *et al* (2004) The potential cardioprotective effects of amifostine in irradiated rats. Int J Radiat Oncol Biol Phys **58**: 1228–1234.
15. Liu H, *et al* (2009) Studies on pentoxifylline and tocopherol combination for radiation-induced heart disease in rats. Int J Radiat Oncol Biol Phys **73**: 1552–1559.
16. Kakkar P, *et al* (1984) A modified spectrophotometric assay of superoxide dismutase. Indian J Biochem Biophys **21**: 130–132.
17. Anderson ME (1985) Determination of glutathione and glutathione disulfide in biological samples. Methods Enzymol **113**: 548–555.
18. Ohkawa H, *et al* (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem **95**: 351–

358.
19. Nakae D, *et al* (1997) Involvement of 8-hydroxyguanine formation in the initiation of rat liver carcinogenesis by low dose levels of N-nitrosodiethylamine. Cancer Res **57**: 1281–1287.
20. Valls-Belles V, *et al* (2008) alpha-Tocopherol, MDA-HNE and 8-OHdG levels in liver and heart mitochondria of adriamycin-treated rats fed with alcohol-free beer. Toxicology **249**: 97–101.
21. Yasuhara T, *et al* (2007) Increased 8-OHdG levels in the urine, serum, and substantia nigra of hemiparkinsonian rats. Brain Res **1133**: 49–52.
22. Toyokuni S, *et al* (1997) Quantitative immunohistochemical determination of 8-hydroxy-2'-deoxyguanosine by a monoclonal antibody N45.1: its application to ferric nitrilotriacetate-induced renal carcinogenesis model. Lab Invest **76**: 365–374.
23. Kim Y, *et al* (2003) Anthracycline-induced suppression of GATA-4 transcription factor: implication in the regulation of cardiac myocyte apoptosis. Mol Pharmacol **63**: 368–377.
24. Gandhi NM, *et al* (2004) Radioprotection of DNA by glycyrrhizic acid through scavenging free radicals. J Radiat Res **45**: 461–468.
25. Gosselin TK, *et al* (2002) Amifostine as a radioprotectant. Clin J Oncol Nurs **6**: 175–176, 180.
26. Kligerman MM, *et al* (1984) Toxicity of WR-2721 administered in single and multiple doses. Int J Radiat Oncol Biol Phys **10**: 1773–1776.
27. Reth M (2002) Hydrogen peroxide as second messenger in lymphocyte activation. Nat Immunol **3**: 1129–1134.
28. Fukuda K, *et al* (2007) Inhalation of hydrogen gas suppresses hepatic injury caused by ischemia/reperfusion through reducing oxidative stress. Biochem Biophys Res Commun **361**: 670–674.
29. Nagata K, *et al* (2009) Consumption of molecular hydrogen prevents the stress-induced impairments in hippocampus-dependent learning tasks during chronic physical restraint in mice. Neuropsychopharmacology **34**: 501–508.
30. Labiche LA, *et al* (2004) Clinical trials for cytoprotection in stroke. NeuroRx **1**: 46–70.
31. el-Nahas SM, *et al* (1993) Radioprotective effect of vitamins C and E. Mutat Res **301**: 143–147.
32. Kojima S, *et al* (2000) Effect of pre-irradiation with low-dose gamma-rays on chemically induced hepatotoxicity and glutathione depletion. Anticancer Res **20**: 1583–1588.
33. Mishra KP (2004) Cell membrane oxidative damage induced by gamma-radiation and apoptotic sensitivity. J Environ Pathol Toxicol Oncol **23**: 61–66.
34. Dubner D, *et al* (1995) Free radicals production and estimation of oxidative stress related to gamma irradiation. Biol Trace Elem Res **47**: 265–270.
35. Kasai H (1997) Analysis of a form of oxidative DNA damage, 8-hydroxy-2'-deoxyguanosine, as a marker of cellular oxidative stress during carcinogenesis. Mutat Res **387**: 147–163.

Received on July 19, 2010
Revision received on September 13, 2010
Accepted on September 15, 2010