

## Radio-protective effect of hydrogen rich water combined with amifostine in mice

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### ABSTRACT

#### ► Original article

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**Background:** Hydrogen has been demonstrated can selectively reduce the hydroxyl, which is the main cause of ionizing radiation-induced damage. Amifostine (AM) is the only radioprotective drug approved by the U.S. Food and Drug Administration for use in radiotherapy. The purpose of the present study was to investigate the combined radio-protective effect of hydrogen rich water (HRW) and AM. **Materials and Methods:** Male ICR mice were treated intragastrically with HRW or/and intraperitoneally with AM 30 minutes prior to 9.0 Gy whole body irradiation from a <sup>60</sup>Co source (dose rate 0.96Gy/min). Then the survival rate for 30 days, the hematological parameters, the Clinical chemistry parameters and the bone marrow nucleated cells were examined. **Results:** We found that the mice treated with HRW and AM before irradiation could increase the 30-day survival rate and improve the body weight better than the HRW or AM treatment alone group and irradiation alone group. Hematological test and Clinical chemistry assays also showed the same results that HRW combined AM could better improve the recovery of hemopoietic system and alleviate the detrimental effects of radiation. **Conclusion:** The results indicate that the combined application of HRW and AM may be a better method for radiation therapy.

**Keywords:** Ionizing radiation, radioprotection, hydrogen rich water, Amifostine, mice.

### INTRODUCTION

Radiotherapy is a form of cancer treatment that utilizes the ability of ionizing radiation to induce cell inactivation and cell death. While radiotherapy destroys malignant cells, it adversely affects the surrounding normal cells<sup>(1)</sup>. Amifostine (AM), named WR-2721, which is the only radioprotective drug approved by the U.S. Food and Drug Administration for use in radiotherapy, has shown good radioprotective effects. Amifostine is now used as a radioprotectant in patients undergoing postoperative radiation therapy for head and neck cancer<sup>(2)</sup>. The molecular action mechanisms of AM are well known, involving

free-radical scavenging<sup>(3)</sup>, DNA protection and repair acceleration<sup>(4)</sup>, and induction of cellular hypoxia<sup>(5)</sup>.

In 2007, Ohsawa *et al.*<sup>(6)</sup> reported that hydrogen could selectively reduce the hydroxyl radical in vitro and exert therapeutic antioxidant activity. In recent years, hydrogen had been found had radioprotective effects in vitro and in vivo<sup>(7, 8)</sup> and drinking hydrogen rich water (HRW) could improve the quality of life of patients treated with radiotherapy for liver tumors without compromising anti-tumor effects<sup>(9)</sup>. Toxicological study found that HRW was safety for a human with a body weight of 60 kg to drink water up to at least 1.2 L/day<sup>(10)</sup>.

The aim of this study was to explore the

combined protective effect of hydrogen rich water and AM on the radiation-induced mice damage.

## MATERIALS AND METHODS

### Hydrogen rich water preparation

H<sub>2</sub> was dissolved in pure water overnight under high pressure (0.4 MPa) to a supersaturated level using a HRW-preparation apparatus produced by our department. HRW was prepared freshly to ensure that the concentration was more than 0.5 mmol/L. Dissolved hydrogen portable meter (ENH-1000, Trustlex, Japan) was used to confirm the concentration of hydrogen in water.

### Irradiation

Animals were whole-body irradiated by <sup>60</sup>Co-gamma rays in the irradiation centre (Affiliated Hospital of China Institute for Radiation Protection, China) with dose of 9.0 Gy (0.96 Gy/min, focal distance 80 cm,) at a room temperature. Mice were irradiated in a plastic multichamber device (a single mouse per chamber) designed to immobilize unanaesthetized mice.

### Animals and treatment

Male ICR mice (5 weeks old) weighing 11-13 g were obtained from the Experiment Animal Center of Academy of Military Medical science (Beijing, China; SCXK-(Military) 2012-0004). They were housed in individual cages in a temperature controlled room (SYXK-(Jin) 2008-0004) with a 12-h light/dark cycle. The mice were fed standard commercial mouse feed (Beijing Keao Xieli Feed Co, LTD) and drinking water *ad libitum*. The acclimatized mice were randomly divided into the control group ( $n=8$ ), radiation-only (IR) group ( $n=10$ ), AM (AM+IR) treatment group ( $n=10$ ), HRW (HRW+IR) treatment group ( $n=10$ ), combined action (AM+HRW+IR) group ( $n=10$ ). For experiments, mice were treated intragastrically with pure water or HRW (20 ml/kg) or/and intraperitoneally with AM (400 mg/kg, Dalian Merro Pharmaceutical Factory) dissolved in

normal saline 30 min before irradiation. The methods of administration and dosage were determined by the results of pilot study. All the experimental protocols were approved by the Bioethics Committee of the Department of Radiation Medicine and Environment Medicine, China Institute for Radiation Protection.

### Survival assays

Animals were observed for 30 days after the irradiation, and the number of surviving mice was checked at the same time every day. Body weight changes were measured at one day before irradiation and 3, 7, 14, 21, 30 days post-exposure.

### Hematological examination

Peripheral blood samples of mice were collected from the tail vein into dilution reagent (10  $\mu$ l to 2 ml) one day before irradiation and the 1<sup>st</sup>, 3<sup>rd</sup>, 5<sup>th</sup>, 7<sup>th</sup>, 10<sup>th</sup>, 14<sup>th</sup>, 21<sup>st</sup>, 30<sup>th</sup> post-irradiation day. White blood cell (WBC) counts, red blood cell (RBC) counts, hemoglobin (HGB) and platelet (PLT) counts were analyzed using hematology analyzer (MEK-6318, NIHON KOHDEN CORPORATION, Japan).

### Clinical chemistry assays

Blood samples of survival mice were collected from orbital sinus with ether anesthesia at the end of the observation. These samples were centrifuged at 2500 rpm for 15min after coagulation and the serums were separated. Total protein (TP), albumin (ALB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (TCHO), blood glucose (GLU), total bilirubin (TBIL), alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine (CRE), triglyceride (TG), creatine kinase (CK), lactate dehydrogenase (LDH) and uric acid (UA) were analyzed using clinical chemistry analyzer (Selectra-E, VITAL SCIENTIFIC, Netherlands).

### Bone marrow nucleated cells

One side femur was obtained from survival mice anesthetized and sacrificed by cervical dislocation, cut off the end. The bone marrow nucleated cells (BMNC) were flushed out with 1ml of 3% acetic acid by 25-gauge needle and

repeated pipetting into single cell suspension. BMNC were counted using blood cell counting plate after adjusted to proper concentration.

### Statistical analysis

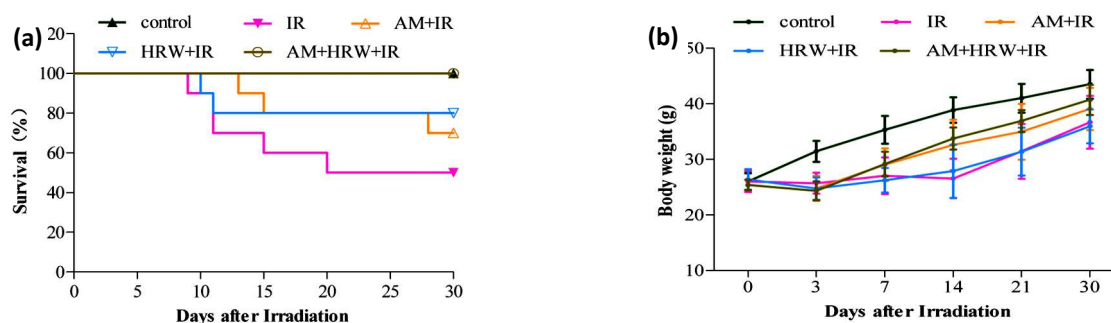
The method of Kaplan-Meier was used to estimate the distribution of the percentage of survival over time. Fisher's exact test (two-tailed) was used to estimate the statistical significance in survival difference between experimental groups. Other data were analyzed using the one-way analysis of variance (ANOVA), and the significance of differences was assessed using the Dunnett's multiple comparison test to compare the values between the IR group and other groups. A p-value of less than 0.05 was considered to be statistically significant.

## RESULTS

Overall, 50% of the IR group animals were died by the 30<sup>th</sup> post-irradiation day (figure 1-a), while the survival rates of the AM+IR group and the HRW+IR group were 70%, 80%; especially the survival rate of the AM+HRW+IR group was 100%. There was significance between the AM+HRW+IR group and the IR group in survival rate ( $p < 0.05$ ). The significance between the survival curves was analyzed by Kaplan-Meier survival analysis and

a log-rank test. The difference in survival between the IR group and the AM+HRW+IR group was statistically significant ( $p < 0.05$ ). Body weight changes of survival mice were measured throughout the 30 days observation (figure 1-b). Body weight of the AM+HRW+IR group was significantly higher than the IR group in 14<sup>th</sup>, 21<sup>st</sup> post-irradiation day ( $p < 0.01$ ,  $p < 0.05$ ) and in 30<sup>th</sup> post-irradiation day, it still higher than the IR group, but had no significance ( $p = 0.056$ ). Except the AM+IR group in 14<sup>th</sup> post-irradiation day ( $p < 0.01$ ), no significant differences were observed when body weights of the other treatment groups were compared with the IR group throughout the 30 days observation.

The WBC counts decreased sharply up to the 7<sup>th</sup> day in the IR group, but recovered slowly and until reaching  $9.3 \times 10^9/L$  (figure 2-a). However, the AM+IR group and the AM+HRW+IR group recovered from the 3<sup>rd</sup> day. Especially, the AM+HRW+IR group increased markedly compared to the IR group up to the 5<sup>th</sup> day, the 7<sup>th</sup> day and the 10<sup>th</sup> day. The difference was statistically significant ( $p < 0.01$ ). The AM+HRW+IR group had higher RBC counts and HGB amount in comparison to the IR group from the 10<sup>th</sup> day to the 30<sup>th</sup> day (figure 2-b, 2-c). The difference was statistically significant ( $p < 0.01$ ) up to the 14<sup>th</sup> day. The PLT counts decreased from the 3<sup>rd</sup> day up to the 14<sup>th</sup> day in the IR



**Figure 1.** Kaplan-Meier curves of ICR mice subjected to hydrogen rich water and amifostine before whole body acute exposure to 9.0 Gy  $\gamma$  rays (a), body weight changes of experiment groups (b). The experimental groups were: control; IR: irradiation alone; AM+IR: treated with amifostine and irradiated; HRW+IR: treated with hydrogen rich water and irradiated; AM+HRW+IR: treated with hydrogen rich water, amifostine and irradiated. (a) A significant increase in survival was observed in hydrogen rich water and amifostine combined action group compared with the radiation-only group ( $p < 0.05$  by Fisher's exact test). No significant differences were observed when other experiment groups (AM+IR, HRW+IR) were compared with the radiation-only group [AM+IR vs IR ( $p = 0.65$ ); HRW+IR vs IR ( $p = 0.35$ )]. (b) Body weight of the AM+HRW+IR group was significantly higher than the IR group in 14<sup>th</sup>, 21<sup>st</sup> post-irradiation day ( $p < 0.01$ ,  $p < 0.05$ ). Body weight of the AM+IR group was significantly higher than the IR group in 14<sup>th</sup> post-irradiation day ( $p < 0.01$ ).

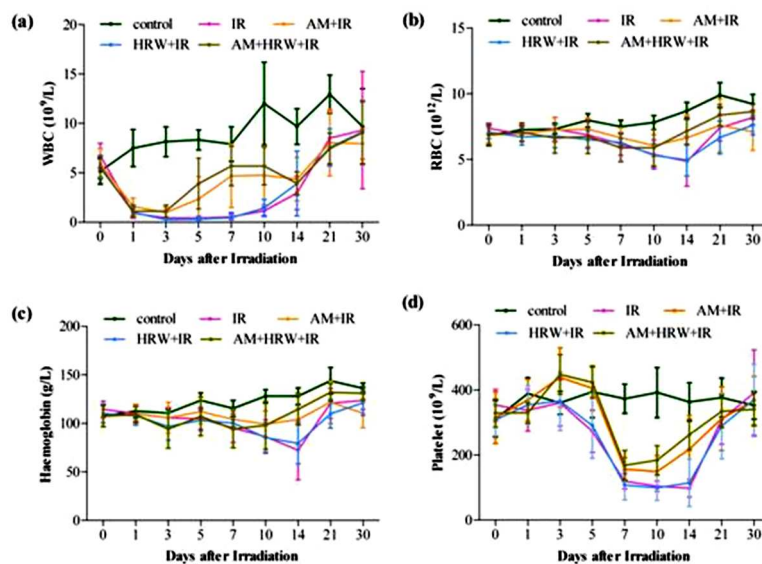
group and then slowly recovered (figure 2-d). The PLT value in the AM+IR group and the AM+HRW+IR group were significantly higher from the 3<sup>rd</sup> day to 14<sup>th</sup> day ( $p < 0.05$  or  $p < 0.01$ ). Especially, the AM+HRW+IR group showed higher value than the AM+IR group from the 7<sup>th</sup> day to 21<sup>st</sup> day, but with no statistically difference.

Irradiation induced several indexes changes in clinical chemistry, the index of TP, ALB, ALT, GLU and BUN in the AM+HRW+IR group showed significant difference compared with the IR group ( $p < 0.05$  or  $p < 0.01$ ) (figure 3). The changes of these indexes in the AM+HRW+IR group were accordant with the control group.

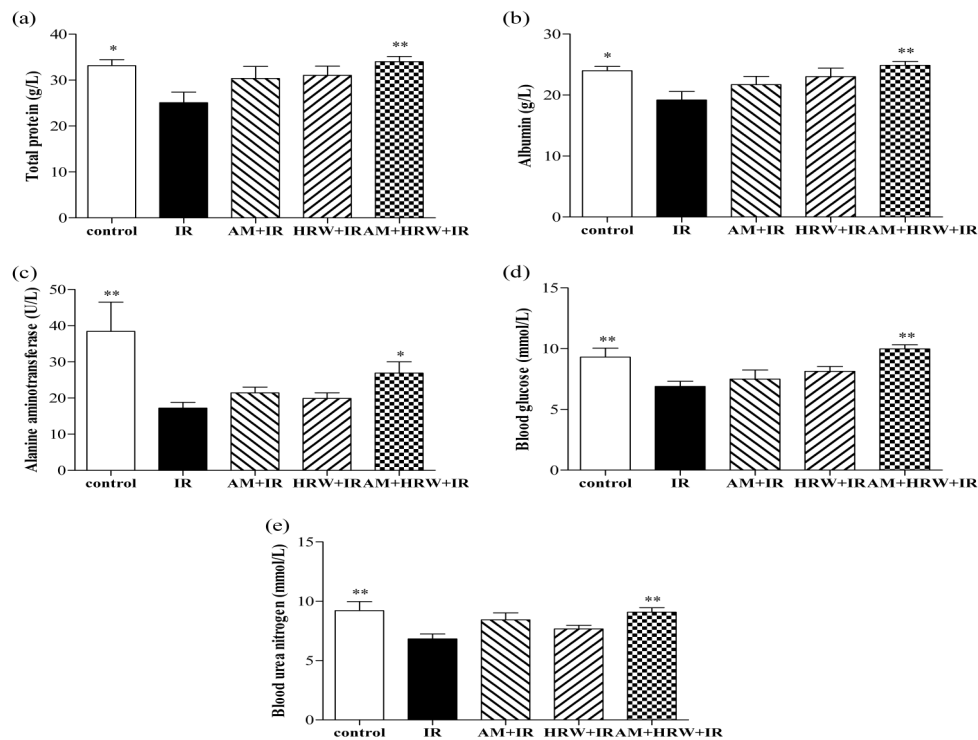
Radiation clearly decreased the numbers of BMNC and induced hematopoiesis suppression (figure 4). At day 30 after irradiation, the number of BMNC in the AM+HRW+IR group was significantly higher than the IR group ( $p < 0.05$ ) and there were no significance between the AM+IR group, the HRW+IR group and the IR group [AM+IR vs IR ( $p = 0.77$ ); HRW+IR vs IR ( $p = 0.80$ )].

## DISCUSSION

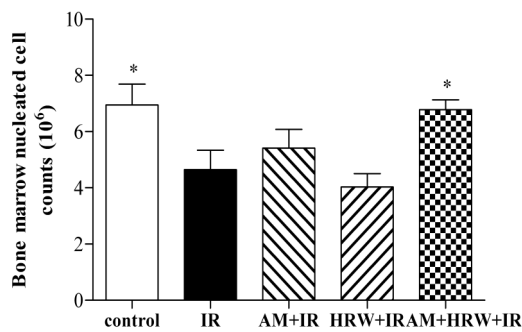
Ionizing radiation-induced tissue damage was caused mainly by hydroxyl radicals<sup>(11)</sup>. Hydroxyl radicals can easily react with cellular macromolecules such as DNA, protein and lipid, to exert strong cytotoxic effects<sup>(8,12)</sup>. Ohsawa et al.<sup>(6)</sup> found hydrogen could selectively reduce the hydroxyl radical without react with other reactive oxygen species, which possess physiological roles and effective protect cells against oxidative stress damage. Hydrogen has favorable distribution characteristics: it can penetrate biomembranes and diffuse into the cytosol, mitochondria and nucleus to reduce cytotoxic radicals. Hydrogen had been found to be a new class of radioprotective agent and it had protective effects on different systems, such as bone marrow<sup>(12)</sup>, intestine<sup>(13)</sup>, skin<sup>(14)</sup>, Testis<sup>(12, 15)</sup>, lung<sup>(16, 17)</sup>, skin<sup>(14)</sup>, and heart<sup>(18)</sup> etc. AM is taken up into the normal tissues and dephosphorylated by membrane-bound alkaline phosphatase to WR-1065, which is the active metabolite of radioprotection.



**Figure 2.** Hematological changes of experiment groups. Blood was collected from the caudal vein into dilution reagent (10 $\mu$ l to 2ml) one day before irradiation and the 1<sup>st</sup>, 3<sup>rd</sup>, 5<sup>th</sup>, 7<sup>th</sup>, 10<sup>th</sup>, 14<sup>th</sup>, 21<sup>st</sup>, 30<sup>th</sup> post-irradiation day. The data were represented as the mean value of four independent sets of experiments: (a) WBC counts, (b) RBC counts, (c) HGB content and (d) PLT counts. (a) The WBC counts of the AM+HRW+IR group was significantly different compared to the IR group up to the 3<sup>rd</sup> day, the 5<sup>th</sup> day, the 7<sup>th</sup> day and the 10<sup>th</sup> day ( $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.01$ ,  $p < 0.01$ ). (b) The AM+HRW+IR group had statistically higher RBC counts in comparison to the IR group up to the 14<sup>th</sup> day ( $p < 0.01$ ). (c) The AM+HRW+IR group had statistically higher HGB amount in comparison to the IR group up to the 14<sup>th</sup> day ( $P < 0.01$ ). (d) The PLT value in the AM+IR group and the AM+HRW+IR group were significantly higher in comparison to the IR group from the 3<sup>rd</sup> day to 14<sup>th</sup> day ( $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.05$ ,  $p < 0.01$ ;  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.01$ ,  $p < 0.01$ ,  $p < 0.01$ ).



**Figure 3.** Clinical chemistry changes of experiment groups. Blood samples of survival mice were collected from orbital sinus with ether anesthesia. The data were expressed as means  $\pm$  standard deviation. Results were compared to IR group, \* $p < 0.05$ , \*\* $p < 0.01$ .



**Figure 4.** Number of bone marrow nucleated cells at the end of 30-day observation. The data were expressed as means  $\pm$  standard deviation. Results were compared to IR group, \* $p < 0.05$ .

Radiation survival is a result of several factors, such as the prevention of damage through the inhibition of free free-radical generation; efficient scavenging of free radicals; repair of DNA, membrane and other damaged target molecules and the replenishment of severely damaged or dead cells (19). In the present study, significant radioprotection was achieved when HRW and AM combined to be administered 30 min before irradiation. The study revealed that pre-irradiation administration of HRW combined AM resulted in 100% 30-day survival in mice exposed to 9.0-Gy

whole body gamma irradiation, but irradiated mice without HRW and AM suffered 50% mortality. IR exposure directly damages hematopoietic stem cells and alters the capacity of bone marrow stromal elements to support and/or maintain hematopoiesis. Our study found the HRW combined AM could better improve the recovery of hemopoietic system and alleviate the detrimental effects of radiation. In addition, the HRW combined AM treatment group showed better radio-protective effects in the changes of body weight, clinical chemistry.

Our results demonstrated that the combined application of HRW and AM possesses the desirable properties of an ideal radioprotector. Further studies are required to reveal the best combination of dose to achieve the full potential of this combination in clinical radiotherapy.

**Conflict of interest:** Declared none.

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