The healing effect of hydrogen-rich water on acute radiation-induced skin injury in rats

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ABSTRACT

This study aimed to determine the healing effect of hydrogen-rich water (HRW) on radiotherapy-induced skin injury. Rats were irradiated with a 6 MeV electron beam from a Varian linear accelerator. After skin wound formation, rats were individually administrated with distilled water, HRW (1.0 ppm) or HRW (2.0 ppm). We measured the healing time and observed the healing rate of the wounded surface. After irradiation, the malondialdehyde (MDA) content and the superoxide dismutase (SOD) activity in the wounded tissues were evaluated, as determined using an MDA and SOD assay kit. Interleukin-6 (IL-6) and epidermal growth factor (EGF) levels were assessed by enzyme-linked immunosorbent assay (ELISA). Models of skin damage were successfully established using a 44 Gy electronic beam. The healing time was shortened in the two HRW-treated groups (P < 0.05). Furthermore, interventions of HRW resulted in a marked reduction in the MDA (P < 0.05) and IL-6 levels (P < 0.01). Additionally, the SOD activity in the two HRW-treated groups was higher than that in the distilled water group at the end of the 1st, 2nd and 3rd weeks (P < 0.001). The EGF level was also significantly increased at the end of the 1st and 2nd weeks (P < 0.05). Compared with the HRW (1.0 ppm) group, the healing rate was higher and the healing time was reduced in the HRW (2.0 ppm) group. A significant decrease was observed in the IL-6 level at the end of the 1st, 3rd and 4th weeks (P < 0.05) and in the EGF content at the end of the 1 week after the HRW administration (P < 0.01). Collectively, our data indicate that HRW accelerates wound healing of radiation-induced skin lesions through anti-oxidative and anti-inflammatory effects, suggesting that HRW has a healing effect on acute radiation-mediated skin injury, and that this is dependent on the concentration of the hydrogen.

Keywords: hydrogen; radiotherapy; skin; dermis; radiation-healing agent

INTRODUCTION

Radiotherapy is one of the three main methods of treatment for malignant tumors. According to the literature, 60–70% of patients with cancer receive radiotherapy [1]. Since the skin is a biological defense barrier for any irradiated area, radiation-induced skin damages, such as erythema, epilation, desquamation, hyperpigmentation and erosion, are the most common complications [2]. Approximately 85% of patients treated with radiotherapy suffer a moderate-to-severe skin reaction [3]. Some patients are even forced to terminate the radiotherapeutic course due to severe skin injury [4]. The injury mechanism is closely related to release of a large number of free radicals and reactive oxygen, deficiency in antioxidative enzymes and growth factors, and abnormal expression of a variety of cytokines during radiotherapy [5–7]. In order to mitigate the adverse effects on the skin, many strategies have been developed to prevent radiation-induced damage in the skin barrier. For instance,
Injury of the heart, lung, intestine and kidney from rheumatoid arthritis, metabolic syndrome, and ischemia-reperfusion effects were dependent on the concentration of H2. Additionally, we determined whether the healing effects of hydrogen (H2) were dependent on the concentration of H2. HRW is a convenient candidate for use as a potential protectant without known toxic side effects. Despite the beneficial effects, long-term inhalation of H2 is rarely applied in the clinical setting because of its flammable and explosive characteristics. To overcome this drawback, we attempted a new approach, namely application of hydrogen-rich water (HRW) with a high concentration of H2. HRW promotes wound healing in rats. The amount of wound healing was observed after the intervention at intervals of 2 days for 40 days in the three groups. We measured the healing time and calculated the healing rate of the various wounds. The wound healing rate (% to the manufacturer's instructions).

MATERIALS AND METHODS

Hydrogen-rich water

HRW was purchased from the Beijing Dynamic Hydrogen Source Biotechnology Limited Company (Beijing, China). The two H2 concentrations used were 1.0 ppm and 2.0 ppm.

Animals

All of the protocols were approved by the institutional ethics committee of the Hainan Medical University. Adult Wistar male rats (3-months-old, average 250 ± 15 g) were purchased from Guangdong Medical Laboratory Animal Center. The animals were housed in individual cages in a temperature-controlled room with a 12 h light/12 h dark cycle.

Irradiation

A Varian linear accelerator (Clinac CX, Varian, American) emitting a continuous 6 MeV electron beam was used to induce the skin damage. Adult Wistar male rats were briefly anesthetized by an intraperitoneal injection of chloral hydrate at 300 mg/kg. The buttock skins of rats were single-dose and locally irradiated. The irradiation areas were 3 cm × 4 cm, and unirradiated areas were shielded by lead plate. The intensity of the electron beam was 400 cGy/min. The exposure time was 11 min, and the total cumulative dose of the electron beam irradiation was 44 Gy. After irradiation, the rats were all fed normally. The degree of skin injury was observed at intervals of every 2 days for 40 days.

Hydrogen-rich water treatment

After wound formation on the damaged skin, 60 male SD rats were divided randomly into three groups consisting of 20 animals per group: (i) the control group: receiving a spray of distilled water (1 ml/time, three times a day); (ii) two HRW-treated groups: receiving a spray of HRW (1.0 ppm) or HRW (2.0 ppm) (1 ml/time, three times a day).

Wound healing time and healing rate

The amount of wound healing was observed after the intervention at intervals of 2 days for 40 days in the three groups. We measured the healing time and calculated the healing rate of the various wounds. The wound healing rate (%) = (original wound area – unhealed wound area)/original wound area.

Measurement of MDA content and SOD activity in wound tissue

Before administration and at the end of the 1st, 2nd, 3rd and 4th weeks after administration, rats (4 rats per week) were sacrificed by cervical dislocation under anesthesia with 10% chloral hydrate. Skin samples from the buttock were frozen in a −70°C cryogenic refrigerator for subsequent MDA content and SOD activity detection. The MDA content was measured by the thiobarbituric assay (TBA) method using a commercially available MDA assay kit. SOD activity was tested by using a commercially available SOD assay kit (the hydroxylamine method). Both the MDA and SOD assay kits were purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu province, China, and used according to the manufacturer's instructions.

Measurement of the IL-6 and EGF content in serum

Blood samples were drawn from the heart of the rats before administration of the atomized water, and at the end of the 1st, 2nd, 3rd and 4th weeks following administration, and centrifuged at 1084 g for 15 min to obtain the serum. The IL-6 and EGF content in the serum were determined using highly sensitive ELISA kits according to the manufacturer’s recommendations. (The IL-6 and EGF assay kits were purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu province, China.)

Statistical analysis

All data were analyzed by SPSS 22.0 software. The statistical data for each group were expressed as mean ± S.E.M, and the differences between groups were analyzed by one-way analysis of variance. A P value of <0.05 was considered to be statistically significant.
RESULTS

The establishment of acute radiation skin injury models
In the present study, rat models of radioactive skin injury were successfully obtained using a single irradiation dose of 44 Gy from a 6 MeV electron beam. After 6 ± 0.9 days of irradiation, the rats experienced hair loss, inappetence and weakness. After 14 ± 0.9 days, they had large pieces of dry skin peeling. After 19 ± 0.6 days, the pieces of peeling skin were wet, follicles, and there was erythema. After 30 ± 0.6 days, erosion and ulcers appeared and gradually increased. At 35 days after the commencement of the irradiation, the ulcerated areas were no longer increasing, but the rats were experiencing acute radiation skin damage (Fig. 1).

The effect of hydrogen-rich water on wound healing
After formation of the skin injury, one experimental group was administered HRW (1.0 ppm) and the other HRW (2.0 ppm) spray (1 ml/time, three times a day), and the control group was treated with distilled water (1 ml/time, three times a day). The wounds in the three groups gradually healed. The healing rate in two HRW-treated groups was significantly faster than that in the control group at the end of the 2nd, 3rd and 4th weeks (P < 0.05) (Table 1). The increase in the healing rate in the two HRW-treated groups was statistically significant at the end of the 1st, 2nd and 4th weeks (Table 1) (P = 0.016, P = 0.00014, P = 0.00035). The skin lesions were completely healed at 34.92 ± 2.23 days in the distilled water group, at 31.76 ± 2.04 days in the HRW (1.0 ppm) group, and at 26.24 ± 1.60 days in the HRW (2.0 ppm) group.

The influence of hydrogen-rich water on MDA content and SOD activity in wound tissue
The MDA content and SOD activity levels, as indicators of oxidative stress, are shown in Fig. 2. The three groups presented the highest levels of MDA and the lowest levels of SOD activity after the irradiation treatment and before administration of the atomised water treatment, and there were no significant differences between the groups at this stage (P > 0.05). The MDA content gradually decreased as the wound healing progressed. However, intervention of HRW led to a statistically significant reduction in the MDA content compared with the level in the control group (P < 0.05). The MDA content was 0.36 ± 0.04 mmol/ml in the control group, 0.27 ± 0.02 mmol/ml in the HRW (1.0 ppm) group, and 0.25 ± 0.07 mmol/ml in the HRW (2.0 ppm) group at the end of the 4th week after the intervention. The MDA content in the HRW (2.0 ppm) group was lower than that in the HRW (1.0 ppm) group at the end of the 1st, 2nd, 3rd and 4th weeks, with no significant difference (P > 0.05). The SOD activity gradually increased during the wound repair in all three groups. However, the degree of SOD activity increase in the two HRW-treated groups was higher than that in the control group at the end of the 1st, 2nd and 3rd weeks following the intervention (P < 0.001). There were, however, no significant differences in SOD activity at the end of the 4th week (P = 0.497, P = 0.063). Compared with the HRW (1.0 ppm) group, the SOD activity in the HRW (2.0 ppm) group was greater at the end of the 1st, 2nd, 3rd and 4th weeks; however, this elevation was not statistically significant (P > 0.05).

The influence of hydrogen-rich water on serum IL-6 and EGF content
As shown in Fig. 3, the IL-6 and EGF content in the serum were detected by ELISA and did not differ significantly between the three groups before administration of the atomized water treatments. The IL-6 concentration in the two HRW-treated groups was gradually reduced over time, whereas it fluctuated in the control group. Administration of HRW significantly lowered the irradiation-induced elevation of IL-6 compared with the level in the control group at the end of the 1st, 2nd, 3rd and 4th weeks (P < 0.01). The level of IL-6 in the HRW (2.0 ppm) group was statistically lower than that in the HRW (1.0 ppm) group at the end of the 1st, 3rd and 4th weeks (P = 0.026, P = 0.001, P = 0.032). In contrast, the EGF level increased over time in the three groups. Treatment with HRW elevated the serum EGF level: the level of EGF in the two HRW-treated groups was higher than that in the control group at the end of the 1st, 2nd, 3rd and 4th weeks after administration, and this difference was statistically significant (P < 0.05). However, the difference was not statistically significant at the end of the 3rd and 4th weeks after administration. Compared with the HRW (1.0 ppm) group, the EGF level was higher in the HRW (2.0 ppm) group at the end of the 1st week (P = 0.026).

DISCUSSION

In the present study, we demonstrated a healing effect of HRW applied by atomizer to radiation-induced skin injury in rats subjected to a single local irradiation. We revealed a reduction in the oxidative damage and in the inflammatory reaction. We utilized a rat model involving a single irradiation to demonstrate our hypothesis and chose a 6 MeV electron beam to induce reliable animal skin damage while minimizing internal organ damage. Usually, regimens of HRW are per-oral or by injection, but we used a simple spray application in our study.

With the improved quality assurance system in conventional radiotherapy, the membrane dressings on the radiotherapy site are widely applied, enabling 23% greater radiation dose to the skin surface compared to without the mask [14]. Thus, the incidence of
radiation dermatitis has been significantly increased. This affects not only the quality of life of patients but also the efficacy of the radiotherapy [14]. Currently, drugs used for the treatment of radioactive skin damage include hormones, vitamins and traditional Chinese medicines, but the effectiveness of these drugs is not ideal.

Hydrogen (H₂) is the simplest and basic element of nature, accounting for ~90% of the composition of universe. H₂, a colorless, odorless and tasteless diatomic gas, is a physiologically inert gas that has a level of reducing capacity [15]. It has been proven that H₂ is a potent free radical scavenger that selectively reduces OH and ROS. Also, H₂ possesses anti-inflammatory and anti-oxidant effects [16, 17].

As early as 1975, Dole et al. reported that breathing at 8 atmospheres of 97.5% H₂ effectively treated animal skin squamous cell carcinoma, and speculated that its mechanism related to the anti-oxidant activity of the H₂ [18]. In 2007, Ohsawa et al. also demonstrated that breathing 2% H₂ effectively removed OH and peroxynitrite (ONOO), reducing the oxidative stress injury caused by cerebral ischemia-reperfusion in rat models. After that, a number of studies have shown that H₂ can play a healing role in a variety of organ damages by anti-oxidant and anti-inflammatory mechanisms, and can be used to treat systemic diseases (in the brain, lung, heart, kidney, liver, pancreas, intestine, eye, hearing system and metabolic system) [12, 19]. In recent years, a series of studies have demonstrated that H₂ has a healing effect on radiation skin damage [12]. Yoon et al. reported that electrolytic reduction water baths assuaged radiation skin lesions caused by UVB radiation [20]. Qian et al. also illustrated that H₂ effectively reduced oxygen free radicals at the cellular level, suggesting that H₂ was a potential radiotherapy healing agent [21]. In 2012, Katoa et al. reported that hydrogen-rich electrolyzed warm water inhibited UVA-directed radioactive skin damage [22].

The use of H₂ in clinical practice is feasible because of its anti-oxidant and anti-inflammatory effects. However, H₂ is a flammable and explosive gas and it is difficult to apply in the clinical situation because its explosive range in the air is ~4–74.2%. In contrast, hydrogen gas–saturated water, also called HRW, provides a steady high concentration of H₂ and is safe and easy to apply [23]. In our study, we employed HRW to apply the intervention. The study illustrated that HRW facilitated the wound repair of radioactive skin injury and shortened the healing time by increasing the activity of SOD and the EGF level and by decreasing the levels of MDA and IL-6. The first aim of our study was to establish a rat model of radioactive skin injury. Posterior buttock skins of rats were single-dose irradiated using a 6 MeV electron beam and a total dose of 44 GY. By the 5th week after radiotherapy, acute radioactive skin injury had clearly occurred and this was deemed a suitable skin-damage model. After wound formation, the rats were individually administered with distilled water, HRW (1.0 ppm) or HRW (2.0 ppm) atomization. We found that the healing time in the two HRW-treated groups was remarkably shorter and the healing rate significantly higher than in the distilled water group in the same period.

MDA is the lipid peroxide derived from oxygen free radicals being attached to polyunsaturated fatty acids in the body. The MDA level can directly reflect the degree of lipid peroxidation in

Table 1. Comparison of wound healing rate after administration between groups (%)

| Group                   | 1st week | 2nd week | 3rd week | 4th week |
|-------------------------|----------|----------|----------|----------|
| Radiation + DW          | 11.42 ± 1.20 | 34.85 ± 1.18 | 61.56 ± 2.56 | 73.92 ± 1.69 |
| Radiation + HRW (1.0 ppm) | 14.12 ± 2.50 | 42.72 ± 2.89* | 72.06 ± 2.86* | 90.70 ± 2.23*** |
| Radiation + HRW (2.0 ppm) | 19.27 ± 2.10* | 48.58 ± 1.08* | 76.56 ± 1.08** | 98.23 ± 2.10*** |

Data are presented as mean ± SEM of three experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

![Fig. 2. MDA content and SOD activity in wound tissue: hydrogen-rich water decreased the MDA content (A) and increased the SOD activity (B) in rats with acute radiation-induced skin injury. Skin samples were obtained at different experimental points (before administration, 1st, 2nd, 3rd and 4th weeks after intervention) from the distilled water group, the HRW (1.0 ppm) group and the HRW (2.0 ppm) group. Data are presented as the mean ± SEM of three experiments. *P < 0.05, **P < 0.01, ***P < 0.001.]
Our study showed that HRW reduced the MDA level in wound tissue and weakened the degree of oxidative damage to skin tissue. SOD can remove superoxide anion free radicals, protecting cells from damage [25]. It is thus an protective indicator of cell damage. In our study, HRW increased SOD activity, thus alleviating the skin injury caused by radiotherapy. IL-6 is the most important cytokine generated in the process of radioactive injury, and it has a strong pro-inflammatory effect, causing systemic and local inflammation. Our study showed that HRW reduced the serum IL-6 level, mitigating the inflammatory response. EGF is important for the growth and proliferation of epidermal cells. It can expedite the synthesis of protein, facilitate the maturation of a wound matrix, and promote the formation of granulation tissue through the EGF binding to specific receptors on the cell membrane to activate tyrosine protein kinase. Our study showed that, compared with the control group, HRW significantly increased the EGF level. Some studies have shown that H2 has a dose-dependent effect [26]. In this study, we explored the dose-dependent healing effect of HRW on radiotherapy-induced skin injury. Compared with the HRW (1.0 ppm) group, the healing rate was significantly higher and the healing time was remarkably reduced in the HRW (2.0 ppm) group. HRW (2.0 ppm) significantly downregulated the IL-6 level at the end of the 1st, 3rd and 4th weeks, and upregulated the EGF level at the end of the 1st week. Our results indicated that the higher the concentration of H2, the faster the wound healed. The results of our study suggested HRW had a healing effect on acute radiation-induced skin injury in rats, and that the effects were dependent on the concentration of H2. We speculated that its mechanism was related to reducing oxidative damage, inhibiting the inflammatory response, and promoting growth factor expression.

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CONFLICT OF INTEREST
The authors declare that there are no conflicts of interest.

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