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Administration of Hydrogen-Rich Saline in Mice with Allogeneic Hematopoietic Stem-Cell Transplantation

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: Hydrogen, as a novel antioxidant, has been shown to selectively reduce the level of hydroxyl radicals and alleviate acute oxidative stress in many animal experiments. Hydrogen-rich saline provides a high concentration of hydrogen that can be easily and safely applied. Allogeneic hematopoietic stem-cell transplantation (HSCT) has been the most curative therapy for hematological malignancies. However, acute graft-versus-host disease (aGVHD) is the main cause of death in post-transplantation patients. In this study, we examined whether hydrogen-rich saline would show favorable effects on acute GVHD in mice.

Material/Methods: After lethal irradiation, BALB/c mice received bone marrow transplantation from C57BL/6 mice. Hydrogen-rich saline (5 ml/kg) was given to recipient mice in the hydrogen group once a day by intraperitoneal injection, and saline (5 ml/kg) was given to recipient mice in the saline group. Survival rates were monitored, clinical and pathological scores of aGVHD were determined after bone marrow transplantation (BMT), and the serum cytokine levels were examined on the 7th day after BMT.

Result: This study proves that hydrogen-rich saline increased the survival rate, reduced clinical and histopathological scores of aGVHD, promoted the recovery of white blood cells, reduced the serum cytokine levels, and reversed tissue damage after transplantation in mice.

Conclusions: Hydrogen has potential as an effective and safe therapeutic agent in aGVHD.

MeSH Keywords: **Adult Stem Cells • Allografts • Transplantation, Homologous**

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Background

Hematopoietic stem-cell transplantation (HSCT) is probably the most effective treatment of hematologic malignancies and some hereditary abnormalities [1,2]. However, one of the most common and serious complications following allogeneic HSCT is graft-versus-host disease (GVHD). Acute GVHD (aGVHD), which accounts for a substantial portion of early transplant-related morbidity and mortality, is one of the main factors that affect the long-term survival and quality of life in patients following allogeneic HSCT [3,4]. There are 3 stages in the pathophysiology of aGVHD: tissue damage and cellular activation caused by preconditioning; the activation of donor lymphocytes (T cells); and the cellular and inflammatory factors directly attack various host tissues and cause the clinical manifestation of aGVHD [5,6]. However, aGVHD primarily affects the skin, liver, and gastro-intestinal (GI) tract [4].

Recent studies have identified molecular hydrogen (H_2) as a novel antioxidant with therapeutic effects in a variety of disease models, including ischemia-reperfusion (I/R) injury, inflammatory disorders, and metabolic syndromes [7–9]. In 2007, Ohsawa et al. [8] discovered that H_2 gas has antioxidant and antiapoptotic properties that protect the brain against ischemia-reperfusion injury and stroke by selectively neutralizing hydroxyl and peroxynitrite radicals. This discovery has completely overturned the traditional view that hydrogen is a physiologically inert gas. Hydrogen is rarely used in the medical field. Since then, H_2 has come to the forefront of therapeutic medical gas research. Accumulation of oxidative damage has been demonstrated to restrict the self-renewal capacity of human hematopoietic stem cells [10].

Material and Methods

2 Hydrogen-rich saline production

The hydrogen-rich saline was prepared as described previously [11]. Briefly, hydrogen was dissolved in 0.9% saline for 6 h under high pressure (0.4 MPa) to a supersaturated level

by using a self-designed hydrogen-rich saline-producing apparatus provided by the Department of Diving Medicine, the Second Military Medical University, China. The saturated hydrogen-rich saline was stored under atmospheric pressure at 4°C in an aluminum bag with no dead volume, sterilized by gamma radiation, and freshly prepared once a week to ensure that the concentration was maintained at 0.6 mmol/L. Gas chromatography was used to confirm the content of hydrogen in saline by the method described by Ohsawa et al. [8].

Animal preparation

The recipient mice were 8–12-week-old BALB/c female mice, and the donor mice were 8–12-week-old C57BL/6 male mice. All mice were purchased from the Naval General Hospital (Beijing, China) in accordance with the Guide for Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (Publication No. 96-01). These mice were kept for 1 week to let them adapt to the environment. During this period, the mice were fed with acidified water and sterile food.

Graft-versus-host disease model

The GVHD model was performed as previously described [12]. One day after lethal total body irradiation (7.5 Gy with the rate of 170 cGy/min), BALB/c mice received BMT from C57BL/6 mice. BM inoculums were consisted of 2×10^7 BM cells and 0.5×10^7 spleen cells of C57BL/6 mice. After transplantation, the recipient mice were treated intraperitoneally with physiologic saline or hydrogen-rich saline (5 ml/kg) every day.

Survival assays

For the evaluation of the therapeutic effect of hydrogen, mice were returned to the animal facility and received routine care for 30 days after BMT. Survival was checked and scored daily for 30 days.

Assessment of the score of aGVHD

The recipient mice were examined daily from the start of conditioning until the indicated sampling day. Animals were

Table 1. Clinical scoring criteria of aGVHD in mice.

Score index	0	1	2
Weight loss	<10%	10–25%	>25%
Posture	Normal	Hunched post ar rest	Severly hunched posture affecting activities
Activity	Normal	Mild to moderate reduction in activity	No activity under stimulation
Fur change	Normal	Mild to moderate hair loss	Severe hair loss
Skin integrity	Normal	Local lesion at the grasping place	A large scale of lesion

Table 2. Histopathological scoring criteria of the skin, liver and gut tissue in mice.

Score index	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Skin	Normal	Vacuolar degeneration of epidermal basal cells	Spongiosis and eosinophilic degeneration of scattered individual cells	Separation of the dermo-epidermal junction	Extensive destruction of epidermis
Liver	Normal	Epithelial damage and destruction of <25% of small bile ducts	25–49%	50–74%	>75%
Gut	Normal	Single cell apoptosis of crypt or gland epithelium	Destruction of single crypts or glands	Focal mucosal ulceration	Diffuse mucosal ulceration with denudation

evaluated for 5 clinical symptoms of aGVHD: weight loss, posture, activity, fur texture, and skin integrity as described elsewhere [12,13], and all those symptoms were scored on the criteria provided in Table 1.

Histopathology

To confirm aGVHD, histopathological evaluation was performed as described previously [12]. Tissue samples were fixed in neutral buffered formalin for 24 h, transferred to 70% ethanol, dehydrated, and embedded in paraffin according to standard procedures. Sections of 4 μm were prepared and stained by hematoxylin and eosin for histopathological evaluation. And the score of histopathology was assessed according to the criteria provided in Table 2.

Analysis of cytokine secretion

Mice in the 2 groups were bled on day 7 after BMT and analyzed for the specified cytokines (IL-2, IL-6, IL-1β, and TNF-α). These cytokines were measured with a commercial enzyme-linked immunosorbent assay (ELISA) kit according to the instructions of the manufacturer.

Leukocyte counts

To evaluate the therapeutic effect of hydrogen, the peripheral blood cell count was determined regularly from the tail vein of mice on different days after BMT, as described previously [14].

Statistical analysis

Survival curves were conducted using the Kaplan-Meier product limit estimator and analyzed using the log-rank test. Other results are expressed as mean ±SD and analyzed by the 2-sample Student's T test for differences in means. P<0.05 was deemed to be significant in all experiments.

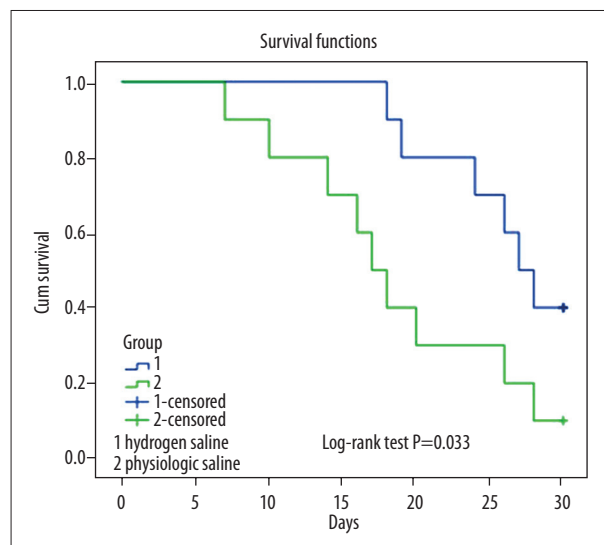


Figure 1. Survival curves by Kaplan-Meier analysis of mice in hydrogen-rich saline group and physiologic saline group. After transplantation, the two group mice were treated intraperitoneally with physiologic saline or hydrogen-rich saline every day and survival was checked daily for 30 days. Survival, P<0.05 for hydrogen-rich saline group (1) versus physiologic saline group (2).

Results

The effects of hydrogen on survival rate of recipient mice after transplantation

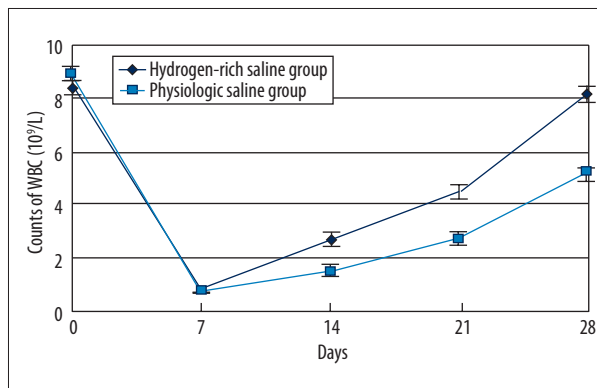
All the mice without transplantation after lethal total body irradiation died within 12 days. Forty percent of aGVHD mice with physiologic saline treatment survived to the 18th day after BMT, and 90% of the mice with hydrogen-rich saline had survived (Figure 1). This result suggests that hydrogen-rich saline can protect mice from lethal aGVHD.

Table 3. Serum IL-1 β , IL-2, IL-6 and TNF- α concentrations in mice treated with hydrogen-rich saline or physiologic saline. Data are expressed as means \pm SD. $P < 0.05$ for hydrogen-rich saline group versus physiologic saline group.

Group	IL-1 β	IL-2	IL-6	TNF- α
Hydrogen-rich saline	58.976 \pm 5.571	13.450 \pm 1.426	31.593 \pm 1.788	63.954 \pm 5.660
Physiologic saline	105.556 \pm 7.598	30.138 \pm 3.157	42.371 \pm 2.006	160.688 \pm 4.815

Table 4. The clinical score of aGVHD in mice treated with hydrogen-rich saline or physiologic saline. Data are expressed as means \pm SD. $P < 0.05$ for hydrogen-rich saline group versus physiologic saline group.

Group	7 th day	15 th day
Hydrogen-rich saline	1.8 \pm 0.249	3.9 \pm 0.526
Physiologic saline	3.9 \pm 0.314	5.9 \pm 0.433



The effects of hydrogen on serum cytokine levels in mice with aGVHD

We examined the serum cytokine levels (TNF- α , IL-1 β , IL-2, IL-6) to determine whether hydrogen could alleviate the degree of aGVHD in mice. All the serum cytokine levels in the hydrogen-rich saline group were lower compared to the physiologic saline group ($P < 0.05$) as shown in Table 3.

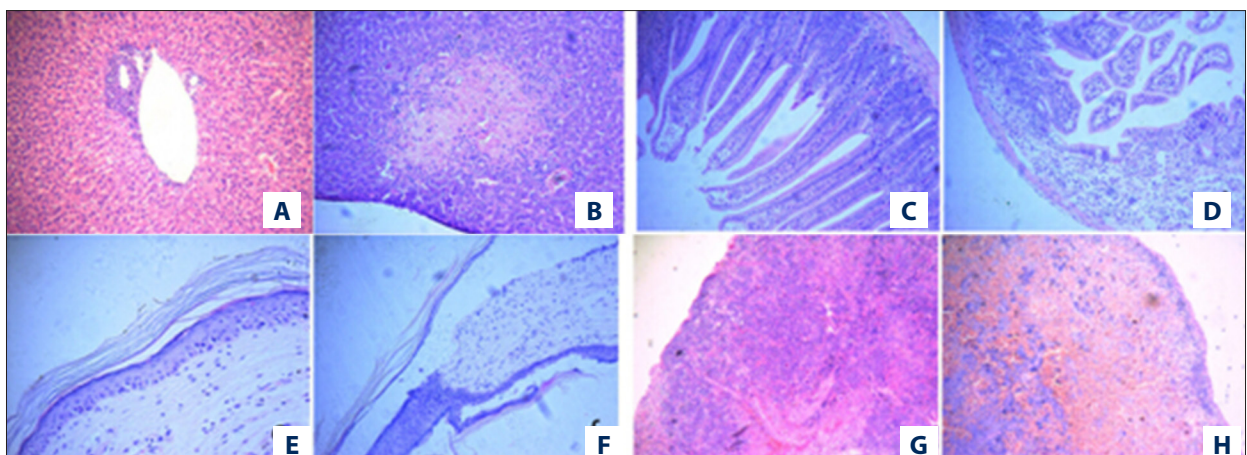
Figure 2. The change of leukocyte counts in mice treated with hydrogen-rich saline or physiologic saline after transplantation. After lethal total body irradiation and transplantation, leukocyte counts declined rapidly, and they would elevate from the 7th day after transplantation. The leukocyte counts on day 0 and day 7 were not different between two groups, and the leukocyte counts on day 14, 21 and 28 in hydrogen-rich saline group were higher than that in physiologic saline group ($P < 0.05$).**Figure 3.** Histopathological changes of the liver, gut, skin and spleen tissue in mice treated with hydrogen-rich saline or physiologic saline. Tissue stained by the hematoxylin and eosin. (A) liver in hydrogen-rich saline group (100 \times), (B) liver in physiologic saline group (100 \times), (C) gut in hydrogen-rich saline group (100 \times), (D) gut in physiologic saline group (100 \times), (E) skin in hydrogen-rich saline group (200 \times), (F) skin in physiologic saline group (200 \times), (G) spleen in hydrogen-rich saline group (40 \times), (H) spleen in physiologic saline group (40 \times). Apparent histopathological differences between mice treated with hydrogen-rich saline and physiologic saline were observed. Hydrogen-rich saline could significantly attenuate the tissue injury induced by aGVHD.

Table 5. The tissue injury evaluated by histopathological scoring system in mice treated with hydrogen-rich saline or physiologic saline. Data are expressed as means \pm SD. $P < 0.05$ for hydrogen-rich saline group versus physiologic saline group.

Group	Liver	Skin	Gut
Hydrogen-rich saline	1.0 \pm 0.333	1.1 \pm 0.233	1.1 \pm 0.233
Physiologic saline	2.3 \pm 0.335	2.6 \pm 0.221	1.7 \pm 0.153

The effects of hydrogen on aGVHD score

We determined the aGVHD score on the 7th and 15th day after BMT. As shown in Table 4, hydrogen can significantly reduce the aGVHD score ($P < 0.05$).

The effects of hydrogen on leukocyte counts

After lethal total body irradiation and transplantation, leukocyte counts declined rapidly, and then increased from the 7th day after BMT. We analyzed the leukocyte counts on day 0, 7, 14, 21, and 28 after BMT. The leukocyte counts on day 0 and day 7 were not different between the 2 groups, and the leukocyte counts on day 14, 21, and 28 in the hydrogen-rich saline group were higher than in the physiologic saline group ($P < 0.05$) as shown in Figure 2.

The effects of hydrogen on histopathology

Histopathology scoring was conducted on the basis of the criteria provided previously (liver, skin, and gut). Hydrogen-rich saline significantly reversed the damage, and the histopathology grade was lower in the hydrogen-rich saline group compared with the physiologic saline group ($p < 0.05$) as shown in Table 5 and Figure 3. The normal spleen structure was destroyed in receptors and the number of lymphocyte cells in splenic white pulp was reduced (Figure 3H) in the physiologic saline group, and the change in spleen was reversed in the hydrogen-rich saline group (Figure 3G).

Discussion

In many studies, examination of survival rate provides a criterion standard determination for experimental GVHD [15]. In our first study of hydrogen in mice with allogeneic hematopoietic stem-cell transplantation, as in the model of mixed bone marrow transplantation in mice [6], hydrogen-rich saline was shown to protect mice from lethal aGVHD. It could improve clinic grade of aGVHD, promote the recovery of white blood cells, and increase the survival rate of recipient mice.

GVHD is characterized by immune damage and is the result of an attack on the target organs of the host and the actions

of related cytokines by activated donor T cells characterized by immune damage [16]. During all 3 phases of GVHD, recipient conditioning regimen damages patient tissues and causes release of inflammatory cytokines. The T helper cell type 1 (Th1) cytokines, such as TL-2 and TL-6, could increase the risk of worsening aGVHD [17,18], and those cytotoxic molecules directly assault various host tissues and underlie the clinical manifestations of aGVHD [5,6]. Those activated cells produce free radicals, which can result in severe cell damage and play an important role in the development of aGVHD. Hydrogen as a kind of selective antioxidant [19] and has great potential in the prevention and treatment of many illnesses, with effects of anti-oxidation [20–23], anti-inflammation [20,23–25], anti-apoptosis [23], and signaling pathways regulation [26–28].

Several reports have shown that tissue damage due to conditioning is important in triggering and development of GVHD [29,30]. It is not clear why some tissues (e.g., liver and skin) are more vulnerable to GVHD damage [31]. In the animal model with GVHD, mice may show clinical symptoms such as hunched posture, hair loss, and diarrhea [32]. With those clinical symptoms, the tissue damage could not be ignored. Our findings show that hydrogen-rich saline can significantly reduce the grading of tissue histopathology, including skin, liver, and gut. There were no criteria provided in the grading of spleen histopathology in the GVHD model, but some studies have reported that in the GVHD model normal spleen structure disappeared, and the number of lymphocytes in white pulp decreased [32–37]. Our study shows that hydrogen treatment can reduce spleen damage.

Conclusions

From the results obtained, we know that hydrogen can reduce the levels of cytokines and reverse tissues damage in the aGVHD model, suggesting that, due to its high diffusibility, hydrogen could be used clinically as a novel therapeutic drug.

Conflicts of interest statement

None declared.

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