Convenient methods for ingestion of molecular hydrogen: drinking, injection, and inhalation

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Abstract
Molecular hydrogen (H₂) is clinically administered; however, in some hospitals, H₂ is given to patients without consideration of its safe use. In the present study, we prepared convenient and safe devices for the drinking of super-saturated H₂ water, for intravenous drip infusion of H₂-rich saline, and for the inhalation of H₂ gas. In order to provide useful information for researchers using these devices, the changes in H₂ concentration were studied. Our experimental results should contribute to the advance of non-clinical and clinical research in H₂ medicine.

Keywords: Hydrogen water, Hydrogen-rich saline, Hydrogen gas

Background
Molecular hydrogen (H₂) is a medical gas with beneficial effects on oxidative stress [1], inflammation [2], apoptosis [3], lipid metabolism [4], and signaling pathways [5]. More than 280 articles, including 24 articles on clinical studies, have demonstrated that H₂ ameliorates the pathological conditions in numerous human diseases [6] or disease models in animals [7], since Ohsawa et al. reported that H₂ could be used in antioxidant therapy [8].

H₂ is clinically administered through the oral intake of H₂ water [9–12], intravenous drip infusion of H₂-rich saline [12–15], or inhalation of air with 2-4 % H₂ gas [12]. However, in some hospitals, H₂ is given to patients by intravenous drip infusion and/or inhalation without consideration of its safe use. We have developed and provided various devices for the ingestion of H₂ to solve this problem. Furthermore, the beneficial effects of H₂ using our devices have been reported in 7 human diseases [9–16].

In the present study, we prepared convenient and safe devices for drinking super-saturated H₂ water, for intravenous drip infusion of H₂-rich saline, and for the inhalation of H₂ gas. We examined the changes in H₂ concentrations in these devices in order to provide useful information for researchers. Our experimental results reported in this article should contribute to the advance of non-clinical and clinical research in H₂ medicine.

Methods/design
Materials
A pressure-resistant 500 mL PET bottle (e.g., a Coke bottle) was used. H₂-generating agent (0.65 g) was prepared by mixing aluminum powder and calcium hydroxide at a ratio of 76 to 24 by weight. The agent was entirely wrapped with bags, namely, a gas-permeable film or non-woven fabric. The wrapped agent was then reacted with water to generate H₂ as follows:

\[ \text{2Al} + \text{Ca(OH)}_2 + 6\text{H}_2\text{O} \rightarrow \text{Ca[Al(OH)}_4]_2 + 3\text{H}_2. \]

Preparation of super-saturated H₂ water for drinking Method I
As shown in Fig. 1a and b, a pressure-resistant PET bottle (500 mL), in which gas-permeable film had been directly inserted, was filled with water and then tightly closed. Water in the bottle reacted with the H₂-generating agent (0.65 g), and the H₂ gas produced was emitted into the water in the bottle through the gas-permeable film. Thus, during this procedure, the H₂-generating agent as well as the water for the reaction did not come into contact with the drinking water. During the reaction, the H₂ gas reduced the height of the water level in the standing bottle, which was gradually
pressurized to approximately 4.5 atmospheric pressures by the gas after 24 h at room temperature. After the reaction was terminated, the H₂ gas was dissolved by shaking the bottle for about 30 s.

**Method II**

Similarly, H₂ water was obtained by the use of non-woven fabric. As shown in Fig. 1c and d, the non-woven fabric containing H₂-generating agent (0.65 g) was first inserted into an acrylic resin tube, and 0.5 mL of water was added. The tube was tightly closed with a cap attached to a check valve, and inserted into a pressure-resistant PET bottle filled with water. H₂ generated in the tube was then transferred to the bottle through the valve. In about 5 min at room temperature, the agent started a reaction in the wet fabric. The H₂ gas produced was emitted into the water through the check valve attached to the acrylic resin tube. During the reaction, the PET bottle was gradually pressurized to approximately 6 atmospheric pressures due to the generation of H₂ gas. After 24 h, the H₂ gas was dissolved by shaking the bottle for about 30 s.

**Preparation of H₂-rich saline for injection**

**Method III**

As shown in Fig. 2a and b, a polyethylene bag for drip infusion, dialysis fluid, or organ storage solution was immersed in a H₂-containing water tank where the water was continuously electrolyzed and circulated during the operation. The H₂ permeated through the polyethylene film and dissolved in the solution without contamination.

**Method IV**

As shown in Fig. 2c, non-woven fabric containing the H₂-generating agent was moistened with a small amount of water, and then both a drip infusion bag and the non-woven fabric were wrapped with aluminum foil under reduced pressure. The water reacted with the agent in the non-woven fabric to generate H₂, and the H₂ gas permeating through the polyethylene film in the bag dissolved into the solution.

**Preparation of H₂-containing gas for inhalation**

As shown in Fig. 3, inhalation gas was prepared by the mixing of H₂ gas and air, where the H₂ gas was
produced by the electrolysis of water, and the concentration was controlled under the detonation limit of the mixture of $\text{H}_2$ gas and air (below 4%).

**Measurement of $\text{H}_2$ concentration**

The concentration of $\text{H}_2$ gas in the water was measured using the methylene blue platinum colloid reagent-based titration method, as described previously [17], and verified using an electrochemical gas sensor (model DHD1-1, DKK-TOA Corp., Tokyo, Japan). On the other hand, the concentration of $\text{H}_2$ in the air was measured using an $\text{H}_2$ gas sensor (FIS Inc., Hyogo, Japan).

**Statistical analysis**

The concentration of $\text{H}_2$ gas in the water or air is presented as ppm (mg/L, weight/volume) or % (volume/volume), respectively. Most of the experimental data are expressed as mean ± standard deviation (mean ± SD) of more than three individual measurements. However, in the examination of $\text{H}_2$-rich saline, the $\text{H}_2$ concentration is expressed as an individual measurement to examine the differences between each bag and plastic vessel. The statistical significance was assessed by Student’s paired or unpaired $t$-test for single comparisons or by one-way analysis of variance (ANOVA) followed by Fisher’s LSD test for multiple comparisons. A $p$ value of less than 0.05 was considered to be statistically significant.

**Results/discussion**

**$\text{H}_2$ concentration of super-saturated $\text{H}_2$ water prepared by Method I**

$\text{H}_2$ concentrations in the super-saturated $\text{H}_2$ water prepared by Method I were measured at 10 °C, 15 °C, and 25 °C. As shown in Fig. 4a, at the same temperature, each $\text{H}_2$ concentration after 24 h was significantly increased compared with each $\text{H}_2$ concentration after 12 h.
After 12 h, H₂ concentration at 25 °C was significantly increased compared with the concentration at 15 °C (p < 0.001), and the concentration at 15 °C was significantly increased compared with that at 10 °C (p < 0.001). In addition, after 24 h, H₂ concentration at 25 °C showed a significant increase compared with that at 10 °C (p < 0.01). The H₂ concentration after the opening of the PET bottle was also measured at room temperature. As shown in Fig. 4b, H₂ concentration of the water was maintained at approximately 7 ppm (7.13 ± 0.22 ppm) after 24 h without opening the bottle; after the cap had been opened, the concentration after 1 h was significantly decreased compared with the concentration after immediately opening (p < 0.01). In addition, H₂ concentration after 2 h was significantly decreased compared with that after 1 h (p < 0.05). In our preliminary experiment after opening the bottle, the H₂ concentrations in the bottle after 1 and 3 h were 4.53 ± 0.15 ppm and 2.10 ± 0.10 ppm (each n = 3), respectively, when 150 mL of water was removed immediately after the termination of H₂ gas production, and the same volume of water additionally removed after 1 h (data not shown). Furthermore, to examine the stability without opening, H₂ concentration was measured after 7 days. As shown in Fig. 4c, the H₂ concentration of the water was maintained above 8 ppm (8.30 ± 0.98 ppm) after 7 days without the opening of the bottle. These results suggest that the H₂ concentration is maintained for at least 7 days without opening, but the H₂ water should be drunk within 2 h after opening. In addition, it is important that after opening, the bottle should not contain space for air in order to avoid the reduction of H₂ concentration.

**H₂ concentration of super-saturated H₂ water prepared by Method II**

H₂ concentrations in the super-saturated H₂ water prepared by Method II were also measured at 10 °C, 15 °C, and 25 °C. As shown in Fig. 5a, at the same temperature, each H₂ concentration after 24 h was significantly increased compared with each H₂ concentration after 10 min (p < 0.001). After 10 min, H₂ concentration at 15 °C was significantly increased compared with the concentration at 10 °C (p < 0.01), and the concentration at 25 °C was significantly increased compared with that at 10 °C (p < 0.01). As shown in Fig. 5b, H₂ concentration of the water was maintained at approximately 10 ppm (10.08 ± 0.34 ppm) after 24 h without opening of the bottle; after the cap had been opened, the concentration after 1 h showed significant decrease compared with that after immediately opening (p < 0.001), and the concentration after 2 h also showed significant decrease compared with that after 1 h (p < 0.001). As shown in Fig. 5c, the H₂ concentration of the water was maintained at approximately...
10 ppm (10.10 ± 0.21 ppm) after 7 days without opening of the bottle. These results suggest that the H₂ concentration prepared by this method is maintained for at least 7 days without opening, but the water should be drunk within 2 h of the cap being opened.

**H₂ concentration of H₂-rich saline prepared by Method III**

The H₂ concentrations of H₂-rich saline prepared by Method III in the infusion bags were measured after immersion for 1, 3, 5, and 10 h (Table 1). When the 3 types of bag (No. 1–3) were immersed for 10 h, the H₂ concentration was measured after 1 or 7 days without opening. Data are presented as mean ± standard deviation (SD) for 3–5 independent measurements.

**Table 1** Details of drip infusion bag, dialysis fluid bag, and injection ampoule used in the experiment

| Experiment | No. | Trade name                      | Volume (mL) | Purpose | Vendor |
|------------|-----|---------------------------------|-------------|---------|--------|
| A          | 1   | 5 % Glucose injection           | 500         | DI      | T      |
|            | 2   | Solulact (Lactate ringer sol.)  | 500         | DI      | T      |
|            | 3   | Isotonic sodium chloride sol.   | 500         | DI      | T      |
| B          | 1   | Otsuka normal saline            | 500         | DI      | O      |
|            | 2   | Hartman’s sol. pH 8 (Lactate ringer sol.) | 500       | DI      | N      |
|            | 3   | 5 % Glucose injection (for animals) | 500     | DI      | K      |
|            | 4   | 7 % Sodium hydrogen carbonate sol. (for animals) | 500 | DI      | K      |
|            | 5   | Otsuka normal saline            | 20          | I       | O      |
| C          | 1   | Otsuka normal saline            | 500         | DI      | O      |
|            | 2   | Midperiq                        | 2,000       | DF      | T      |
|            | 3   | Isotonic sodium chloride sol.   | 100         | DI      | T      |
|            | 4   | Isotonic sodium chloride sol.   | 500         | DI      | T      |

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A: Time-dependent concentration after immersion, B: Difference between types of containers, C: Storage stability in aluminum bag, sol.: Solution, DI: Drip infusion, I: Injection, DF: Dialysis fluid, T: Terumo Corp., Tokyo, Japan, O: Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan, N: Nipro Corp., Osaka, Japan, K: Kyoritsu Seiyaku Corp., Tokyo, Japan

**Fig. 5** Concentrations of H₂ in the super-saturated H₂-rich water prepared by Method II. (a) Concentrations of H₂ measured at 10, 15, and 25 °C after 10 min and 24 h (###p < 0.001, 10 min vs. 24 h at the same temperature; **p < 0.01, 10 °C vs. 15 °C after 10 min, or 10 °C vs. 25 °C after 10 min). (b) Concentrations of H₂ measured immediately after 24 h, and then measured 1 or 2 h after the cap had been opened (****p < 0.001, Immediate vs. 1 h, 1 h vs. 2 h, or Immediate vs. 2 h). (c) Concentrations of H₂ measured after 1 or 7 days without opening. Data are presented as mean ± standard deviation (SD) for 3–5 independent measurements.
approximately 1.0 ppm H₂-rich saline was obtained (Fig. 6a). There were no differences in the H₂ concentration between the types of drip infusion bag. These results demonstrated that it is necessary to immerse the drip infusion bag for at least 10 h in order to obtain 1.0 ppm H₂-rich saline. To examine the permeability of H₂ for the different polyethylene vessel materials, 5 types of vessels (No. 1–5) were immersed in the water bath for 5 h, and the change in H₂ concentration of each vessel was examined (Table 1). The H₂ concentration of various infusion bags and polyethylene vessels depends on their thickness and the content of the solution. The H₂ easily penetrated into the physiological saline (No. 1), but barely penetrated into the sodium hydrogen carbonate solution (No. 4). In addition, in the physiological saline, the H₂ more easily penetrated into the 500 mL drip infusion bag (No. 1) than the 20 mL plastic injection ampoule (No. 5) (Fig. 6b). After the infusion bags had been immersed in the bath for 3, 5, and 10 h, they were removed and the changes in H₂ concentration were measured until 5 h later. The H₂ concentration of the drip infusion bag decreased from 1.0 ppm to 0.6 ppm after 1 h of removal from the water bath after immersion for 10 h (Fig. 6c). These results suggest that intravenous drip injection with these bags should be completed within 1 h.

**H₂ concentration of H₂-rich saline prepared by Method IV**

The H₂ concentrations of 4 types of bag (No. 1–4) prepared by Method IV were also measured after 1, 3, 6, and 12 months in order to examine long-term preservation (Table 1). The H₂ concentrations in the drip infusion bags (No. 1, 3, and 4) or dialysis fluid bag (No. 2) were maintained for 12 months, suggesting that the H₂-rich saline prepared by this method could be used for 12 months (Fig. 7a).

**H₂ concentration of gas introduced by inhaler**

We examined the H₂ gas concentration for up to 3 h after starting use of the inhaler, because stability of the gas concentration is required in order to examine the performance of the gas inhaler. The H₂ gas concentration in the inhaler was 2.91 ± 0.08 % after 0.5 min, and a H₂ gas concentration of approximately 3 % was maintained for 3 h. There was no significant difference among the time points after starting (Fig. 7b). These results demonstrate that the H₂ gas could be supplied stably for 3 h using this inhaler.

In summary, we prepared two types of super-saturated H₂ water (7 or 10 ppm) for drinking. The concentrations in these waters were maintained for 7 days without opening, but the waters should be drunk within 2 h of the cap being opened. We also prepared
two types of H₂-rich saline for injection. Although intravenous drip injection with the H₂-rich saline should be completed within 1 h, H₂ concentrations in the saline prepared by aluminum foil (Method IV) were maintained for 12 months without opening. Moreover, we prepared H₂-containing gas for inhalation. The gas was controlled under the detonation limit of the mixture of H₂ gas and air, and the gas could be supplied stably for 3 h. In a recent study, we examined the H₂ concentration in rat tissue following administration of H₂ gas for inhalation. The gas was prepared by Method IV, and the H₂ gas prepared by our method are convenient and safe preparatory methods. The present results should contribute to the advance of non-clinical and clinical research in H₂ medicine.

Fig. 7 Concentrations of H₂ in the devices. a Concentrations of H₂ in the H₂-rich saline were measured at 1, 3, 6 and 12 months after preparation by Method IV. b Concentrations of H₂ in the H₂ gas inhaler were measured up to 3 h after starting. Data are presented as (a) individual measurements or (b) mean ± standard deviation (SD) for 3 independent measurements.

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