In vivo microelectrode monitoring of real-time hydrogen concentration in different tissues of rats after inhaling hydrogen gas

Bo-Yan Liu1,2,*, Jun-Li Xue1,2,*, Qian-Qian Gu1,3, Min Zhao1,2, Meng-Yu Zhang1,2, Ming-Yue Wang1,2, Yun Wang3, Shu-Cun Qin1,2,4
1 Taishan Institute for Hydrogen Biomedicine, Shandong First Medical University & Shandong Academy of Medical Sciences, Taian, Shandong Province, China
2 Key Laboratory of Atherosclerosis in Universities of Shandong Province, Shandong First Medical University & Shandong Academy of Medical Sciences, Taian, Shandong Province, China
*Correspondence to: Shu-Cun Qin, PhD, scqin@sdfmu.edu.cn.
#These authors contributed equally to this work.
orcid: 0000-0002-0413-3113 (Shu-Cun Qin)

Abstract

Medical effects of hydrogen have been reported in many studies. Due to difficulties in measuring hydrogen concentration in vivo after intake and high explosive risks of hydrogen, studies about dose-response relationships and tissue concentrations of hydrogen are few. Here, for the first time, we monitored real-time hydrogen concentrations in different tissues in rats including brain, liver, spleen, kidney, thigh muscle, inguinal white adipose tissue, and gonadal white adipose tissue after inhaling different concentrations of hydrogen (4%, 42%, and 67%) using an electrochemical sensor. Hydrogen concentrations in the same tissue showed a dose-dependent response. The equilibrium concentration values were highest in the brain and lowest in the thigh muscle. The saturation and desaturation curves changed more slowly in the thigh muscle and white adipose tissue than in other tissues. These results provide fundamental information for the selection of hydrogen dose applications in basic research and clinical trials. The experiments were approved by the Laboratory Animal Ethics Committee of Shandong First Medical University & Shandong Academy of Medical Sciences (No. 2020-1028) on March 18, 2020.

Key words: different tissues; dose-response; hydrogen concentration; hydrogen sensor; in vivo; inhalation; rat; real-time monitoring

doi: 10.4103/2045-9912.330694
How to cite this article: Liu BY, Xue JL, Gu QQ, Zhao M, Zhang MY, Wang MY, Wang Y, Qin SC. In vivo microelectrode monitoring of real-time hydrogen concentration in different tissues of rats after inhaling hydrogen gas. Med Gas Res. 2022;12(3):107-112.

Funding: This work was supported by the National Natural Science Foundation of China (No. 81770855); Taishan Scholars Program of Shandong Province of China (No. ts201511057); Shandong Provincial Natural Science Foundation of China (No. ZR2020HQ020), and Academic Promotion Programme of Shandong First Medical University & Shandong Academy of Medical Sciences (No. 2019QL010, 2019PT009).

INTRODUCTION

Hydrogen gas (H2) is colorless and odorless at standard temperature and pressure. Due to its small molecular weight and hydrophobic properties, H2 can easily permeate cell membranes and even enter cell organelles. H2 has long been regarded as a biochemical inert gas. In 2007, Ohsawa et al.1 demonstrated its selective antioxidant effects in a rat cerebral ischemia/reperfusion model. A large number of studies have since shown the therapeutic and preventive effects of H2 in various animal disease models.2-5,25 Meanwhile, some clinical investigations have also confirmed its beneficial effects on different diseases.6-7 Various ways have been explored to administer H2, mainly including inhaling H2, drinking H2-rich water, injecting H2-rich saline, and direct incorporation of H2 by diffusion (such as bath and eye-drops). Due to its bio-safety, countries and regions such as USA,8 Japan,9 Europe,10 and China11 have recently allow to use H2 as a food additive. At present, the therapeutic effects of H2 have attracted increasing attention worldwide.12

Despite rapid advances in understanding the biological effects of H2, the underlying mechanism is yet to be elucidated. In addition to the aforementioned hypothesis regarding selective scavenging of toxic free radicals, H2 can exert its bio-functions by reducing inflammation and apoptosis events.13 A hypothesis based on the bio-enzyme basis of H2 was recently proposed,14 and a new study showed that H2-rich water could significantly increase the activity of pepsin and change the protein structure and dynamic properties.15

Until now, the existing molecular mechanism of the biological effect of H2 has not been fully explained due to lack of solid pharmacokinetic data. In addition, to the best of our knowledge, only a few studies have explored its concentration and distribution after intake. A previous study16 determined H2 concentrations in different rat tissues following the administration of H2 via various routes. The study revealed variable dynamics of H2 in various tissues over time and different H2 concentrations in the same tissue with different methods of H2 uptake. Another in vivo study17 monitored the sequential changes of H2 concentrations in tissues over time with continuous inhalation of 3% H2. However, conclusions of both studies were not entirely consistent. Moreover, previous studies were performed only after a single concentration of H2 intake. The dose-response curve had not been illustrated. Therefore, more accurate and detailed studies are needed to acquire the exact pharmacokinetics of H2.

This study pioneered a comprehensive and quantitative
assessments of $H_2$ distributions within various tissues in vivo after different concentrations of $H_2$ being inhaled, and obtained the dose-response curve by real-time monitoring.

**Materials and Methods**

**Gas Preparation**

Different concentrations of $H_2$ (4%, 42%, 67%; v/v) were prepared using a lab-made gas mixing device (Figure 1A). $H_2$ and oxygen gas ($O_2$) from cylinders and air from a generator (LCA-3, LICHEN-BX instrument technology Co., Ltd., Shanghai, China) with different flow rates were adjusted by flowmeters and mixed in a sealed box. The targeted concentration of $H_2$ was confirmed with a gas detector (XP-3140, New-cosmos Co. Ltd., Tokyo, Japan). Meanwhile, the $O_2$ concentration was kept at ~21% and verified with an $O_2$ detector (JR2000-02, Jingruibo Technology Co., Ltd., Beijing, China). The mixed gas was administered to a rat through a gas supply hood at a total flow rate of 3 L/min.

**Animals and Experimental Design**

Fifty 8-week-old specific-pathogen-free level male Sprague-Dawley rats weighing 180–210 g (Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China; SCXK (Lu) 20190003) were used in this study. All rats were maintained under standard conditions (21 ± 1°C; 12/12 hours light/dark cycle). Water and food were provided ad libitum. The experiments were approved by the Laboratory Animal Ethics Committee of Shandong First Medical University & Shandong Academy of Medical Sciences (approval No. 2020-1028) on March 18, 2020.

After overnight fasting, rats were sedated by intraperitoneal injection of 20% urethane (7 mL/kg, Shanghai Aladdín Biochemical Technology Co., Ltd., Shanghai, China). After losing consciousness, rats were put on a warming plate maintained at 38°C and then dissected to expose the target tissue with minimal incisions. The exposed tissue was covered with a layer of humid gauze with an opened small hole to maintain moisture in order to mimic the internal environment better, and then the microsensor tip was inserted into the tissue through the hole.

Seven tissues, including the left brain, median lobe of the liver, spleen, left kidney, thigh muscle (left hind gastrocnemius muscle), gonadal (visceral) white adipose tissue (WAT), and inguinal (subcutaneous) WAT ($n = 3–6$ per tissue), were monitored (Figure 1B). Only one target tissue was exposed at a time per rat.

**H$_2$ concentration monitoring in vivo**

The measuring device included a miniaturized Clark-type hydrogen microsensor with an internal reference electrode and a sensing anode (tip diameter 40–60 μm), a micromanipulator, and a microsensor multimeter (Unisense, Aarhus, Denmark). The signals of Clark-type sensors were controlled by target gas concentrations, sensor dimensions, temperature, and salinity. A standard curve was established by diluting the $H_2$-saturated phosphate-buffered saline at 38°C. The tip of the microsensor was inserted into the exposed tissue at a depth of ~1 mm below the tissue surface. At first, the air was administered to maintain a stable baseline. Next, a required concentration of $H_2$ was provided continuously until the $H_2$ concentration in the target tissue reached equilibrium. Then, the gas was replaced by pure air and monitoring was continued until the $H_2$ concentration returned to baseline. Predicted by Henry's law for the solubility of a gas in a liquid, and the solubility of $H_2$ at a certain temperature and salinity, the theoretical equilibrium concentrations ($C_e$) of 4%, 42%, and 67% $H_2$ in blood are about 28.5, 299.0, and 476.9 μM, respectively. The detection started before hydrogen inhalation. At the beginning, the rats were given air until reaching a stable baseline, then hydrogen was provided.

**Statistical Analysis**

Statistical analyses were conducted using Origin v8.5 (Origin Lab Corporation, Northampton, MA, USA) and GraphPad Prism v8.0.1 (GraphPad Prism Software, Inc., La Jolla, CA, USA). An ordinary one-way analysis of variance and Tukey’s multiple comparison test were used to assess the significance of differences in $H_2$ concentrations between various tissues. $P < 0.05$ was considered statistically significant. Data are representative of at least three independent experiments, and expressed as the mean ± standard deviation (SD).

**Results**

$C_e$ of $H_2$ in different tissues

$C_e$ of $H_2$ in different tissues after inhaling different concentrations of $H_2$ are shown in Figure 2. The figures display highest mean $C_e$ in the brain, followed by inguinal WAT and kidney, and lowest in the thigh muscle regarding the three concentrations of $H_2$. Inter-organ comparisons revealed significant differences between the brain/thigh muscle and other tissues ($P < 0.001$ or $P < 0.01$) for different concentrations of $H_2$. For 4% $H_2$, $C_e$ showed a significant difference between inguinal...
WAT and liver ($P < 0.05$). For 67% H$_2$, $C_e$ showed statistically significant differences between inguinal WAT and liver, spleen, gonadal WAT, respectively ($P < 0.05$). $C_e$ of H$_2$ in all tissues exhibited a dose-dependent increase corresponding to the concentration of inhaled H$_2$. $C_e$ values after inhaling 42% and 67% H$_2$ were about 10.5- and 16.8-time greater than 4% H$_2$ in various tissues. The theoretical $C_e$ of H$_2$ based on a blood flow model and Henry’s law for the solubility of gas are also shown in Figure 2.

**H$_2$ saturation dynamics in different tissues**

The H$_2$ dynamic curves for different tissues after inhaling various concentrations of H$_2$ are summarized in Figure 3A–F. In general, H$_2$ concentrations in the brain, liver, kidney, and spleen increased faster than in the thigh muscle, inguinal WAT, and gonadal WAT (Figure 3). For 4% H$_2$, the concentrations in the brain, liver, kidney, and spleen rose rapidly in a short time and then gradually leveled in about two minutes for the liver, kidney, spleen and three minutes for the brain (Figure 3A). However, the plots for the thigh muscle, inguinal WAT, and gonadal WAT exhibited a gradual increase after H$_2$ was inhaled and they needed much more time to reach the $C_e$ (Figure 3D). The ascending order of different tissues reaching 50% and 90% saturation concentrations was spleen, liver, kidney, brain, gonadal WAT, thigh muscle, and inguinal WAT (Figure 3G and H). For 42% and 67% H$_2$, trends similar to those of 4% H$_2$ were observed (Figure 3B, C, E, and F) and the orders were same as 4% H$_2$ (Figure 3G and H). In the same tissue, similar time was needed to reach 50% $C_e$ for different H$_2$ concentrations applied. Meanwhile, a dose-dependent relationship for the time to reach 90% $C_e$ was observed (Figure 3G and H).

**Figure 2: Equilibrium H$_2$ concentrations in the brain, liver, spleen, kidney, thigh muscle, gonadal white adipose tissue (WAT), and inguinal WAT after inhaling different concentrations of H$_2$.**

Note: (A–C) 4%, 42%, 67% H$_2$. The dotted line represents the theoretical $C_e$ of H$_2$. Data are shown as the mean ± SD ($n = 3–6$ rats per tissue). **$P < 0.01$, ***$P < 0.001$, vs. brain; ##$P < 0.01$, ###$P < 0.001$, vs. thigh muscle; &$P < 0.05$, vs. inguinal WAT (one-way analysis of variance followed by Tukey’s multiple comparison test). H$_2$: Hydrogen gas.

**Figure 3: H$_2$ saturation dynamics in the brain, liver, spleen, kidney, thigh muscle, gonadal white adipose tissue (WAT), and inguinal WAT after inhaling different concentrations of H$_2$.**

Note: (A–F) Changes of H$_2$ concentrations in the brain, liver, spleen, kidney, thigh muscle, gonadal white adipose tissue (WAT), and inguinal WAT during inhalation of 4% (A, D), 42% (B, E), and 67% (C, F) H$_2$. (G, H) Time taken to reach 50% (G) and 90% (H) saturation. Data are shown as the mean ± SD ($n = 3–6$ rats per tissue), and were analyzed by one-way analysis of variance followed by Tukey’s multiple comparison test. H$_2$: Hydrogen gas.
**H₂ desaturation dynamics in different tissues**

The H₂ concentrations in different tissues started decreasing after H₂ administration was withdrawn (Figure 4A–F). In the beginning, the brain, liver, kidney, and spleen exhibited a similar sharp drop right after H₂ withdrawal, and then plots decreased gradually until reached the baseline (Figure 4A–C). However, the curves of the thigh muscle, inguinal WAT, and gonadal WAT exhibited a more gradual decrease after H₂ withdrawal and took more time to reach the baseline (Figure 4D–F). In ascending order of the time needed for different tissues to reach 50% and 90% desaturation, the spleen and liver took the least time, followed by kidney, brain, thigh muscle, gonadal WAT, and inguinal WAT (Figure 4G and H). In the same tissue, the times to reach 50% and 90% desaturation concentrations exhibited a dose-dependent relationship. More time was required to reach baseline for higher H₂ concentrations.

**DISCUSSION**

As a small molecular gas, H₂ can diffuse into the target tissues without any hindrance, even while passing through the blood-brain barrier. Inhalation is a common method to administer H₂. When mixed with air, the explosive range of H₂ is 4–75% (v/v). Hence, inhalation of 2–4% H₂ gas is frequently used in medical researches. The development of H₂ generator has recently led to an increasing number of studies using high H₂ concentrations. A H₂ generator produces a mixture of H₂ (67%) and O₂ (33%) by electrolyzing water. A commercial medical-grade H₂/O₂ ventilator has been shown to ameliorate different diseases in animal models and patients. However, an abnormal O₂ content may have an ambiguous effect on research results. Therefore, in some experiments, the H₂/O₂ mixture gas was diluted with nitrogen (N₂) to obtain 42% H₂ and maintain the same O₂ concentration (~21%) as in the atmosphere. The distribution of H₂ in tissues after inhaling low concentrations of H₂ has been investigated. However, how the specific molecular mechanisms of high concentrations of H₂ differ from that of low concentrations needs further investigation. In addition, the distribution or concentration differences of H₂ in vivo after inhaling different concentrations of H₂ also need further exploration. In this study, we monitored the concentrations of H₂ in different tissues in rats after inhaling 4%, 42%, and 67% H₂.

In the beginning of inhalation, H₂ enters the arterial blood through the alveoli and is then taken into tissues by a pressure gradient until reaches equilibrium. In this study, H₂ in the brain reached the highest Cₑ, followed by inguinal WAT, kidney, gonadal WAT, and spleen tissues. In the thigh muscle, the H₂ concentration was significantly lower than in other tissues. This is consistent with a previous report suggesting that the saturation concentration of H₂ in the thigh muscle is much lower than in the blood and myocardium of rats. However, the distribution of H₂ concentrations in different tissues found in this study was contradictory to results in some other literatures. Yamamoto et al. reported that after inhalation of 3% H₂, the saturation concentration was highest in the liver and lowest in the kidney. Another study revealed that the H₂ concentrations in different tissues varied with different administration methods and H₂ inhalation resulted in the highest H₂ concentration in the muscle. The reasons for the contradictory results varied and may be attributed to the different detected locations and depths chosen to represent the whole organ, the different concentrations and durations for H₂.
inhalation, or the methodological differences.

Intermittent and continuous measurements have been applied to determine the \( H_2 \) concentrations in tissues. An intermittent measurement requires taking samples at regular intervals. After homogenization, \( H_2 \) is released into an airtight tube and then the gas is collected and measured with gas chromatography. In contrast, continuous measurement leads to the ability to create a continuous concentration curve against time using a microsensor \textit{in vivo}, which has been used in biomedical fields. A continuous measurement was performed in this study to obtain a complete equilibrium curve.

The \( C_e \) for different tissues showed different saturation and desaturation rates in this study. In general, spleen and liver tissues needed less time to reach 50% and 90% \( C_e \) than other tissues. The blood flow model has been applied to theoretical models of \( H_2 \) distributions. As per the gaseous diffusion model based on the distance between the gas supply hood and each organ, the brain reached a higher \( H_2 \) concentration due to the short distance between the face and head. In the blood flow model, different tissues reached the same \( C_e \) at different rates based on the blood flow. This study indicated that both of the models worked because highest \( C_e \) was found in the brain, and the \( C_e \) in most tissues approached the theoretical concentrations.

\( H_2 \) concentrations of different tissues showed different saturation and desaturation rates in this study. In general, spleen and liver tissues reached 50% and 90% \( C_e \) followed by the kidney and brain. In tissues of the thigh muscle, gonadal WAT, and inguinal WAT, the equilibrium rates were slower than other tissues. The blood flow of muscle and adipose tissue was slower than that of abdominal tissues. This may lead to a slower equilibrium rate. Moreover, as a fat-soluble gas, \( H_2 \) accumulated in adipose tissues. This leads to a slower rate when approaching equilibrium compared with other tissues. A previous report showed that the saturation time was significantly longer and the concentration increased more slowly in muscle than the other examined organs for 3% \( H_2 \). Another study revealed that for 2% \( H_2 \), the arterial \( H_2 \) concentration in rats reached a maximum level after 5 minutes, whereas the increasing rate of \( H_2 \) concentration was much slower in the center of the thigh muscle and it reached the maximum level after 30 minutes.

The blood flow rate in various tissues has been measured by the \( H_2 \) clearance method. Therefore, the desaturation of \( H_2 \) occurs mainly through blood circulation to the lung and then is released out of the body.

An \textit{in vitro} experiment has shown a dose-dependent relationship for \( H_2 \) in protecting cells from cell death and reacting with hydroxyl radicals. In an \textit{in vivo} experiment, inhalation of \( H_2 \) (1–4%) was applied for hepatic injury caused by ischemia-reperfusion, and 2–4% \( H_2 \) was found to work the best. Another study on myocardial ischemia-reperfusion injury revealed that inhalation of 0.5–2% \( H_2 \) significantly reduced infarct size in a dose-dependent manner with 2% \( H_2 \), providing the most prominent effects. In contrast, 4% \( H_2 \) inhalation did not show the alleviating effect. These results indicated the importance of choosing the appropriate dosage of \( H_2 \) for various tissue injuries. Considering the different \( C_e \), the optimal concentration of \( H_2 \) inhalation may vary from disease to disease. Moreover, given the different saturation rates in tissues, different inhalation times may be needed to achieve the desired effect for lesions in different tissues, especially in muscle and adipose tissue.

We used a microsensor tip inserted into the tissue at a depth of 1 mm below the organ surface. The concentrations of \( H_2 \) may vary between different locations and depths in a same tissue and it is needed to estimate in future studies. This study revealed the \( H_2 \) distribution in different tissues under anesthesia in rats. However, it is known that anesthesia results in the tendency for blood flow rates to decrease in most tissues. To confirm these results, it would be of interest to measure the \( H_2 \) distribution in different tissues while the animal is in a conscious state. However, at present, this would be difficult to achieve because of the constraints of the current measurement methods. Pharmacokinetics of \( H_2 \) \textit{in vivo} varies with methods of administration and thus influence the biomedical effects. Other \( H_2 \) intake methods, such as drinking \( H_2 \)-rich water, are also worth further exploration.

To summarize, the \( H_2 \) distribution in different tissues of rats during and after inhaling different concentrations of \( H_2 \) over time was investigated. The results provide a reference for \( H_2 \) dose selection for animal and clinical trials and promote the use of \( H_2 \) in clinical therapies.

**Acknowledgements**

The authors are grateful to technical instructors of Shanghai Weizai Technology Co., Ltd. for their help in the monitoring technology.

**Author contributions**

BYL, J LX, and SCQ designed the experiments. BYL, JLX, QQG, MYZ, MZ and MYW performed experiments. JLX and BYL analyzed the data and wrote the manuscript. YW helped with the methodology. All authors reviewed the manuscript.

**Conflicts of interest**

There is no conflict of interest.

**Open access statement**

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Date of submission: January 13, 2021
Date of decision: February 28, 2021
Date of acceptance: April 30, 2021
Date of web publication: November 29, 2021