Experimental research

Direct evidence of hydrogen absorption from the skin – a pig study

Satomi Iwai[1,2], Shou Kobayashi[3], Eiji Kobayashi[2,3,4], Kazuhisa Sugai[5], Yoji Hakamata[4,5], Kohsuke Shirakawa[4,6], Yoshinori Katsumata[4,6], Motoaki Sano[4,6]

[1]Laboratory of Small Animal Surgery 2, School of Veterinary Medicine, Kitasato University Towada City, Aomori, Japan
[2]Endowed Chair for the Promotion of Minipig Research, School of Veterinary Medicine, Kitasato University, Towada City, Aomori, Japan
[3]Kobayashi Regenerative Research Institute, LLC. Wakayama-shi, Wakayama-ken, Japan
[4]Center for Molecular Hydrogen Medicine, Keio University, Minato-ku, Tokyo, Japan
[5]Department of Basic Sciences, School of Veterinary Nursing and Technology, Faculty of Veterinary Sciences, Nippon Veterinary and Life Science University, Musashino-shi, Tokyo, Japan
[6]Department of Cardiology, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan

Corresponding authors:
Eiji Kobayashi
Kobayashi Regenerative
Research Institute
LLC. Wakayama-shi
Wakayama-ken, Japan
E-mail: eijikoba@jikei.ac.jp

Motoaki Sano
School of Medicine
Keio University
Shinjuku-ku, Tokyo, Japan
E-mail: msano@a8.keio. jp

Abstract

Introduction: It has not been experimentally proven whether hydrogen gas (H_2) is absorbed into the body through the skin by hydrogen-rich hot-water bathing.

Material and methods: In this study, Hairless mini pigs, whose skin closely resembles that of humans, were bathed in hydrogen (H_2)-rich hot water to assess the absorption of H_2 through the skin. An H_2-rich water generation line was developed to maintain a high concentration of H_2 via the circulation of hot water in an 80-litre bathtub. Two hairless mini pigs (14.2 ±1.4 kg, 60 days old, 1 male and 1 female) were first placed in the H_2-dissolved bath. After a washout period, one pig was bathed in an H_2-dissolved bath and the other in a bath containing no H_2 for 20 min. During the experiment, blood was collected from the pigs' jugular vein, carotid artery, inferior vena cava (IVCs), and portal vein to measure the blood H_2 concentration.

Results: The H_2 concentration at the IVC of the pig in the H_2-dissolved bath increased from 0.733 ±0.636 ppb (w/w) to 16.9 ±4.46 ppb (w/w) after 2 min, 37.2 ±13.8 ppb (w/w) after 10 min, and 45.7 ±7.73 ppb (w/w) (H_2 saturation level: 3%) after 20 min. The blood H_2 concentration levels of the pig in the non-H_2 bath remained below the detection limit of 0.3 ppb.

Conclusions: Bathing in water with a high concentration of dissolved hydrogen was considered an effective means of supplying H_2 to skin tissues and beyond.

Key words: dermatitis, skin, pharmacokinetics, hairless mini pigs, hydrogen-rich hot water bath, hydrogen generator.

Introduction

Hydrogen has antioxidant, anti-inflammatory, anti-allergic, and anti-apoptotic effects, and it exhibits protective functions against a variety of diseases [1, 2]. One of the greatest advantages of H_2 is the wide range of ways it can be administered, including the inhalation of H_2 gas [3–7],

Satomi Iwai, Shou Kobayashi, Eiji Kobayashi, Kazuhisa Sugai, Yoji Hakamata, Kohsuke Shirakawa, Yoshinori Katsumata, Motoaki Sano

drinking of H_2-disolved water [8, 9], injection of H_2-disolved saline solution [10], and H_2-rich hot water bathing [11]. Furthermore, an important characteristic of H_2 is its powerful capacity for diffusion. As a non-polar molecule with minimal molecular weight, it can diffuse quickly in both hydrophilic and hydrophobic regions. In addition, while the action of most drugs is limited to regions reached by blood flow, animal studies have reported that H_2 diffuses rapidly in ischaemic tissues and removed organs in a blood flow-independent manner [4, 12–14]. Given these potential benefits, bathing in H_2-rich hot water has been introduced to the healthcare field with great enthusiasm. However, no study has verified whether H_2 can be absorbed through the skin during H_2-disolved hot water bathing.

The skin consists of 3 layers: the epidermis on the surface, the dermis in the middle, and the hypodermis. The stratum corneum, the top surface of the epidermis, has a lamellar structure consisting of several to several dozen layers of dead cells called corneocytes. This layer plays an important role as a barrier to the outside world. The dermis is the inner tissue of the epidermis, bordered by the basement membrane, and is composed of about 80% water and the rest collagen fibres, elastic fibres, mucopolysaccharides such as hyaluronic acid, and fibroblasts. The dermis contains blood vessels, lymph ducts, nerves, and auxiliary organs such as sebaceous glands and sweat glands. Blood vessels are distributed throughout the dermis and connect to the arteries and veins in the hypodermis. The skin prevents water outside the body from entering and prevents water inside the body from evaporating. Therefore, it is unclear whether H_2 can reach the dermal vasculature or further into the body's circulatory system even after immersion in a H_2-disolved hot water bath.

Human skin has anatomical structures, immunological qualities, and other characteristics distinctive of humans [15–19]. Therefore, transdermal absorption mechanisms for human subjects require *in vitro* experiments using human skin and verification of pharmacokinetics in humans, but there are ethical restrictions. Mice, rats, guinea pigs, rabbits, dogs, and non-human primates have been used as surrogates for experiments on transdermal absorption of drugs, but even the hairless skin species of these animals is known to differ from human skin [20–24]. With the exception of the palms and soles, these species have markedly thinner epidermis, lack basal cell heterogeneity, a relatively flat dermal-epidermal interface, and no dermal area enclosed between epidermal projections [25]. Tissue connections in the dermis are also loose, and vascular tissue is underdeveloped [26, 27]. Even in primates, only the skin of the palms and soles has been reported to resemble human skin [28, 29].

A previous study demonstrated that the skin properties of hairless mini pigs closely resemble those of human skin, making hairless mini pigs suitable for studies regarding the transdermal absorption of medicines [30]. Because hairless mini pigs have extremely little hair on their skin, they have been used for medical training, evaluative testing of disinfectants and vaccines, and assessment testing for skin injuries [31]. Therefore, the hairless mini pig is the best experimental animal for use in experiments measuring H_2 absorbability through the skin that cannot ethically be conducted using human participants.

This study analysed the dynamics of H_2 absorption through the skin of hairless mini pigs during H_2-rich hot water bathing. First, an H_2-rich water generation line that can saturate the warm water in a bathtub with H_2 was developed, then a blood sampling system to minutely analyse the dynamics of H_2 absorbed from the skin during immersion in H_2-rich hot water was created.

Material and methods

Research design and methods

Laboratory animals

Two 60-day-old hairless mini pigs (Zenno Hairless W, Central Research Institute of Animal Feed and Livestock, Zen-Noh, Ibaraki, Japan) (one male and one female) weighing 14.2 ±1.4 kg were used in this study. This study was approved by Kitasato University (approval No. 21-087) and conducted in accordance with the Guidelines for Laboratory Animals and the Manual for the Care and Management of Laboratory Animals. During the first experiment, both pigs were bathed in warm H_2-disolved water. After a washout period, a second experiment was conducted in which one pig was bathed in an H_2-dissolved bath and the other pig was bathed in warm water without H_2.

Experimental protocol

The pigs were intubated and administered general anaesthesia. Then, a medial abdominal incision was made and catheters for drawing blood were implanted at the internal carotid artery, internal jugular vein, inferior vena cava, and portal vein, as previously described [32].

Anaesthesia procedure

The surgery was performed under general anaesthesia. The pigs were sedated by injection of a mixed solution of medetomidine (20 µg/kg), midazolam (0.2 mg/kg), and butorphanol (0.2 mg/kg) into the gluteal muscle. After sufficient sedation

was induced, mask inhalation of isoflurane 3.0% was administered, and anaesthesia was induced until the pharyngeal reflex was absent. A 24-G indwelling catheter was inserted into the central ear vein, and lactated Ringer's solution was administered at 5 ml/kg/h. The infusion rate was changed as needed according to anaesthesia monitoring. Then, a 5.5-Fr tracheal tube was introduced, and anaesthesia was maintained with isoflurane 2.0%. All pigs were administered cefazolin sodium hydrate (30 mg/kg, intravenously twice daily) and buprenorphine (analgesic; 20 µg/kg, intravenously twice daily) from the preoperative period.

Preparation for cannulation

Four catheters were percutaneously implanted to collect blood during bathing (Figure 1).

Cervical procedure

A longitudinal skin incision (approximately 4 cm) was made approximately 1.5 cm lateral to the right midline of the neck to expose the left internal carotid artery (CA) and internal jugular vein (JV). First, tobacco-pouch sutures were applied to the outer surface of the internal carotid artery, and a 16 G (1.5 mm outer diameter) central venous catheter was cannulated approximately 5 cm to the side of the aortic arch, then ligatured and secured. A tobacco-pouch anastomosis was applied in the same manner over the internal jugular vein, and a 16 G central venous catheter was inserted 5 cm in the cephalad direction and secured.

Abdominal procedure

A median incision from below the xiphoid process to the umbilicus was made, exposing the caudal vena cava (IVC). Tobacco-pouch sutures with a diameter of approximately 5 mm were applied with 5-0 nylon, and Satinsky blood vessel clamps were used to provide haemostasis. The centre of the tobacco suture was incised with ophthalmic scissors, and a 16 G (1.5 mm outer diameter) central venous catheter was inserted and fixed through the lower side. Next, the pancreatic head vein that flows into the portal vein (PV) was exposed and the bifurcation was cut down. A 16 G (1.5 mm outer diameter) central venous catheter was inserted approximately 2.5 cm into the portal vein. After tightly suturing the incised region, a special incise drape was attached to the skin to prevent immersion of the sutures and skin areas where the catheters were implanted.

H$_2$-rich water generation line

The H$_2$ concentration in the 80-litre bath was gradually increased by circulating the H$_2$-rich wa-
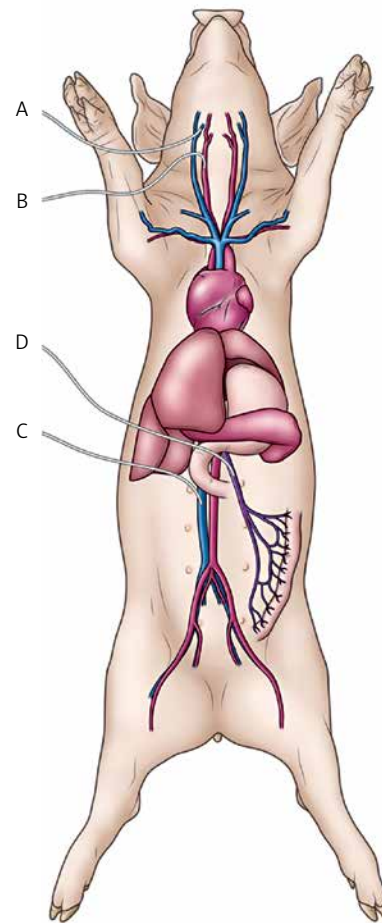

Figure 1. Location of blood collection ports. Blood H$_2$ concentrations during bathing in hot water containing H$_2$ or no H$_2$ were measured at the following 4 locations. A. Carotid artery. B. Jugular vein. C. Inferior vena cava. D. Portal vein

ter generation line through a pump with a circulation capacity of 1.5 l/min (Figure 2).

A paediatric bathtub was filled with tap water and heated to 37–38°C. Then, an H$_2$ generator (H2JI1 [33]) was used to continuously supply 100% H$_2$ at a pressure of 0.15 Mpa to 6000 H$_2$-permeable membranes bundled into cylindrical tubes [33]. Due to the pressure differential between the water pressure and H$_2$ pressure caused by water flowing inside the H$_2$-permeable membranes, the H$_2$ dissolved into the water at a stable concentration in the form of ultrafine bubbles (Flash Bubbling System: Doctors Man Co., Ltd., Kanagawa, Japan).

H$_2$-rich hot water bath

To generate a large volume of highly concentrated H$_2$ bath water in a short period of time, a 2-by-2 arrangement was devised for the H$_2$ gas permeable membrane. The inlet and discharge ports were circulated through a tube hose with an inner diameter of approximately 4 mm, and
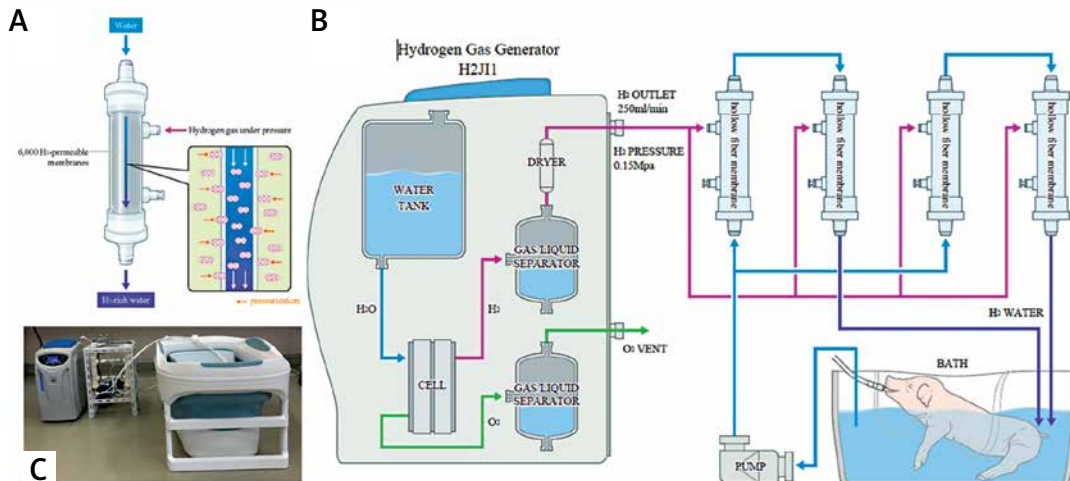
Satomi Iwai, Shou Kobayashi, Eiji Kobayashi, Kazuhisa Sugai, Yoji Hakamata, Kohsuke Shirakawa, Yoshinori Katsumata, Motoaki Sano

Figure 2. H_2-rich water generation line. **A** – H2JI1 flash bubbling system for dissolving H_2. The bubbling system fills the bath with H_2 at a high concentration. **B** – H_2 filling circuit. This circuit circulates hot water in the bathtub to increase the concentration of dissolved H_2 in the bathtub. **C** – H2JI1 bubbling system and bathtub. The bubbling system and bathtub are shown

Figure 3. Blood sampling from bathing pigs. The pig was held by both arms and bathed for 20 min to prevent the pig's head from entering the water. During the bath, the pig was intubated and fitted with a ventilator to prevent inhalation of H_2 evaporated from the water. To measure the H_2 concentrations, blood and bath water were collected simultaneously from the 4 ports and the bathtub

convection currents were created by stirring the bath by hand. The pigs were placed in the bath 30 min after the initiation of the circulation of the H_2-rich water generation line. To prevent the pigs' heads from going underwater, we used both arms to hold the pigs and bathed them for 20 min (Figure 3).

During this observation period, the pigs were intubated and placed on a ventilator to prevent them from inhaling any H_2 that evaporated from the water.

Blood Collection

Blood samples were collected from the IJV, ICA, IVC, and PV immediately prior to bathing and 2, 10, and 20 min after entering the bath. In addi-

tion, blood samples were collected 20 min after the pigs were removed from the bath. Water samples were also collected from the bath at the same times.

Postoperative Management

Cefovecin sodium (8 mg/kg; Convenia injection, Zoetis Japan K.K., Tokyo, Japan) was postoperatively administered intramuscularly to prevent infection. Buprenorphine hydrochloride (20 μg/kg; Lepetan, Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) was administered intramuscularly twice daily for 3 days as an analgesic.

Measurements of blood and bathwater H_2 Concentrations

The H_2 concentrations of the blood were measured using previously described methods [10, 32]. Briefly, 1 ml of blood was collected in a 10-ml sealed vial and 100 μl of water was collected in a 100-ml sealed vial. Because H_2 in the blood and bath water is released into a gaseous phase when kept in sealed vials, the H_2 concentration of the blood was estimated using gas chromatography (TRIlyzer mBA-3000, Taiyo Corporation) of the air inside the vial. Calibration curves of H_2 concentrations of 0 (nitrogen gas), 5, 50, and 130 ppm were prepared. The H_2 gas concentrations of air inside vials not containing blood were also measured, and these values were subtracted from the values measured in each blood sample.

Statistical analysis

Data are presented as mean ± standard error of the mean. Repeated measures analysis of variance (ANOVA) (mixed-effects model) or one-way

ANOVA followed by Tukey's multiple comparisons test were conducted. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using GraphPad Prism, version 9.5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

H_2 Concentration in the Bath System During the Experiment

Thirty min after starting the H_2-rich water generation line, the H_2 concentration of the bathwater was approximately 1600 ppb. Because 1.6 mg H_2 is the maximum amount that can be absorbed into 1 l of water, the bathwater contained the maximum possible amount of H_2. No significant differences in the H_2 concentration of the bathwater were observed throughout the experiment (Figure 4 A). The H_2 concentrations of the bathwater were not significantly different between the trials (Figure 4 B). When the H_2-rich water generation line was not used, the H_2 concentration in the bathwater was always below the detection limit of 0.3 ppb.

H_2 pharmacokinetics during H_2-rich hot water bathing

At all blood collection points, the concentration of H_2 in the blood increased 2 min after the start of bathing and almost reached its peak 10 min later (Figures 5 A, B). Twenty minutes after the pigs were removed from the bathtub, blood H_2 concentrations at all blood collection points dropped to levels that were not significantly different from those measured before the pigs were placed in the bathtub.

Compared by blood collection site, the H_2 concentration of the blood was the highest at the IVC ($n = 3$). The H_2 concentration of the blood was significantly different between the IVC and PV and between the IVC and ICA 20 min after bathing

(Figure 5 A). The H_2 concentration of the blood at the IVC was 0.733 ±0.636 ppb (w/w) prior to bathing, 16.9 ±4.46 ppb (w/w) at 2 min, 37.2 ±13.8 ppb (w/w) at 10 min, and 45.7 ±7.73 ppb (w/w) at 20 min. In other words, 20 min after bathing, the blood H_2 saturation level at IVC increased to as high as 3%. Due to the small number of n (3), the change over time in blood H_2 concentration in IVCs during H_2-rich hot water bathing did not reach statistical significance in the analysis of variance ($p = 0.0529$) (Figure 5 B). However, the post-hoc test revealed a significant difference between the H_2 concentration of the blood at the IVC at 2 min and 20 min. For sites other than the IVC blood sampling site, the post hoc test did not reach statistical significance for blood H_2 concentrations between blood sampling times.

The blood H_2 concentration was below the detection limit of 0.3 ppb at all blood sampling locations and times when the pigs were bathed in non-H_2 baths. This result implies that soaking in a hot tub does not stimulate intestinal bacteria to increase H_2 production.

Discussion

In the present study, we found that bathing hairless mini pigs for 20 min in hot water dissolved with H_2 in a saturated state increased the blood H_2 concentration in IVCs to 45.7 ±7.73 ppb (w/w) (H_2 saturation: 3%). This result means that H_2 dissolved in the bath water permeates beyond the epidermis to the dermis, and furthermore, enters the blood vessels and reaches the systemic circulatory system at significant concentrations.

H_2 certainly has excellent diffusivity, but this feature is overestimated. If H_2 is inhaled through the lungs, it is supplied to the entire body via the bloodstream. The alveolar epithelium is specially designed for gas exchange, so gaseous H_2 is efficiently taken up into the bloodstream. In addi-
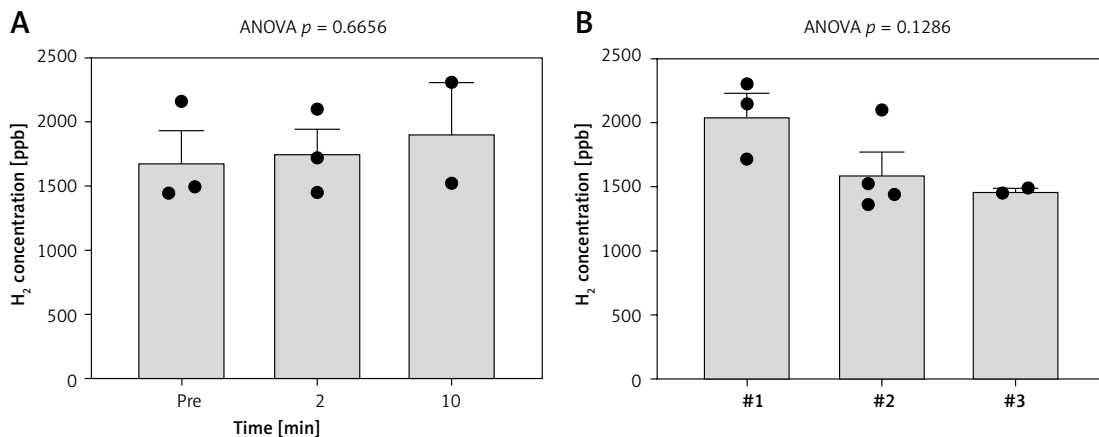
Figure 4. H_2 concentration in the H_2-rich hot water bath. **A** – The H_2 concentration in the bathtub remained stable throughout the bathing period. **B** – The H_2 concentration in the bath was stable during all 3 experimental trials
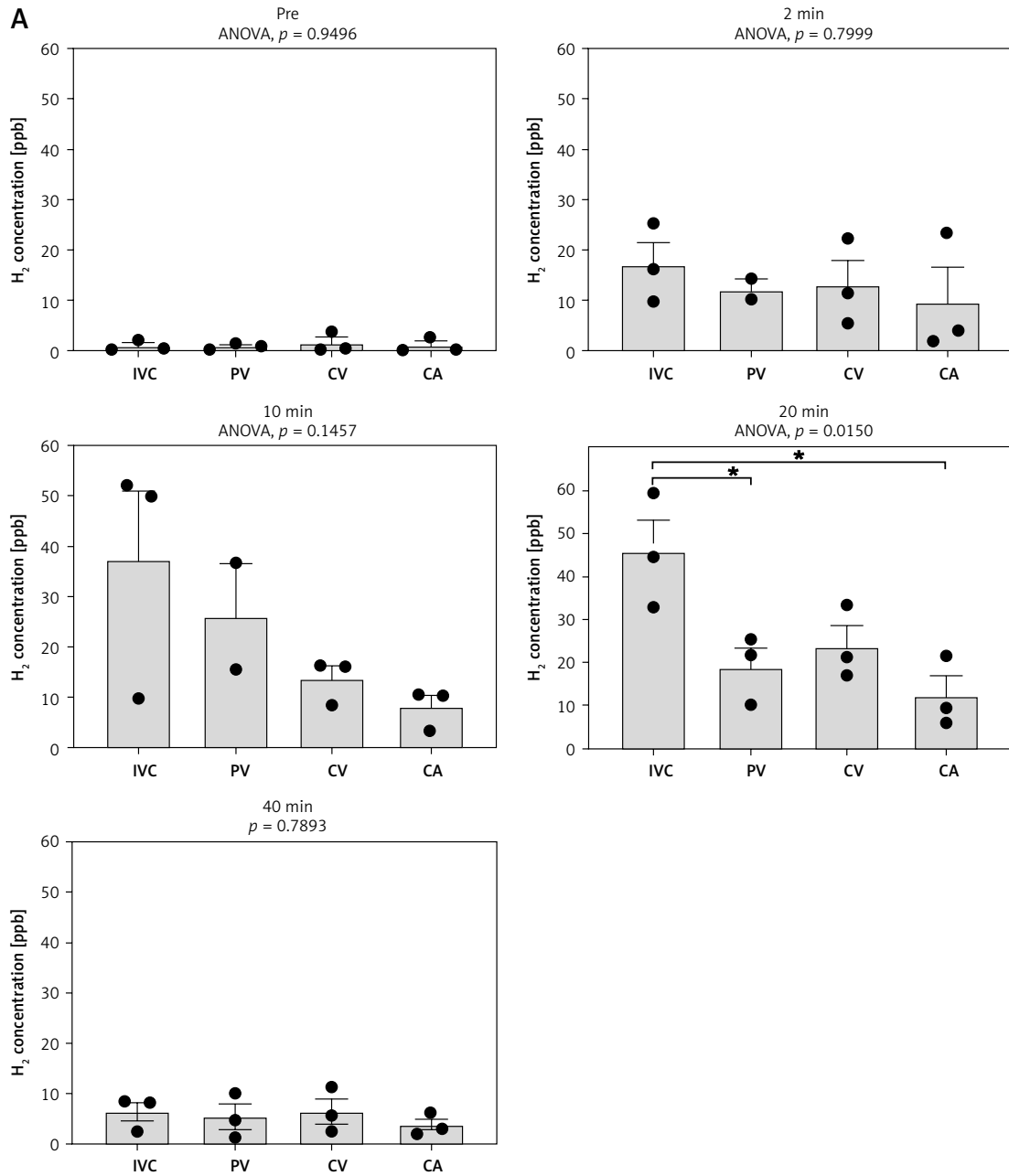
Satomi Iwai, Shou Kobayashi, Eiji Kobayashi, Kazuhisa Sugai, Yoji Hakamata, Kohsuke Shirakawa, Yoshinori Katsumata, Motoaki Sano

Figure 5. Pharmacokinetics of H_2 when bathing in H_2-rich hot water. **A** − Twenty min after the start of bathing, the H_2 concentration at the inferior vena cava is significantly higher than that at the internal carotid artery and the portal vein ($p < 0.05$, $n = 3$)

tion, when inhaled, the lungs, which are the largest route for H_2 to escape from the blood, can be shielded by a high concentration of H_2, making the entire body a closed space for H_2. In contrast, to get H_2 into the blood from tissues other than the lungs, H_2 must be dissolved in solution by applying pressure or other operations before contact is made.

For example, if H_2 is injected into the intestine in gaseous form, very little is absorbed. On the other hand, when H_2-rich water prepared by dissolving H_2 in water under external pressure is

injected into the small intestine, H_2 is efficiently absorbed into the blood. Using miniature pigs, we have confirmed that when H_2-rich water is injected into the small intestine, H_2 is transported from the jejunal vein to the portal vein and then to the liver. When water containing H_2 at a concentration of 5.42 mg/l (5.42 ppm) was injected directly into the jejunum over 2 min, the H_2 concentration in the portal vein was maintained at 0.05 mg/l (H_2 saturation level, ~3%) for 30 min, while the H_2 concentration in the IVC was at 1/3 that level [32]. The results of the current study
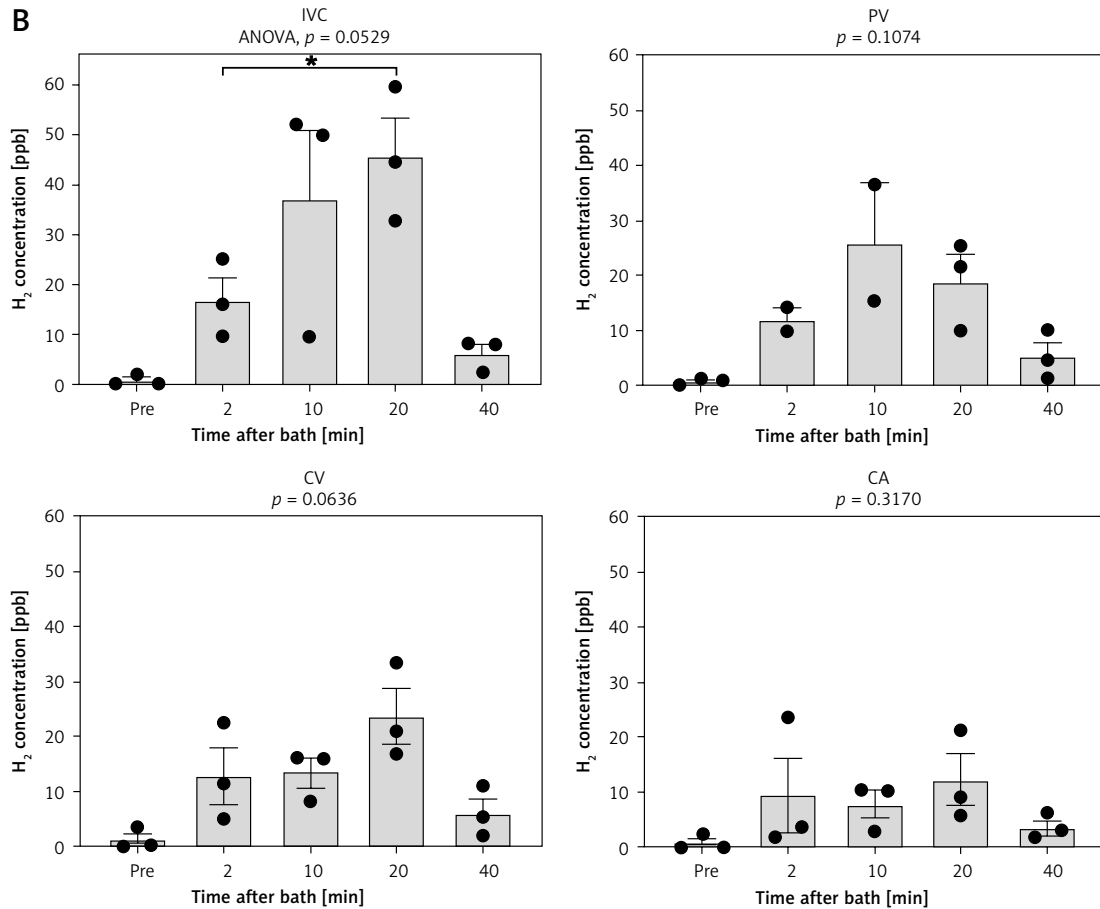
B

Figure 5. Cont. **B** – The changes in hydrogen concentration at each location during the saturated hydrogen bath are shown. The H_2 concentration at the IVC was significantly higher at 20 min than at 2 min ($p < 0.05$, $n = 3$)

suggest that introducing H_2 into the body's circulation through the skin by taking an H_2-rich hot water bath increased the internal H_2 levels to similar or greater levels as when H_2 is introduced into the portal circulation via the drinking of H_2 water.

Because several skin diseases, including psoriasis, atopic dermatitis, skin aging caused by sun exposure, and carcinogenesis, are caused by oxidative stress, bathing in water saturated with H_2, an antioxidant, may be an effective therapeutic tool to prevent or treat these diseases [34]. One study regarding the effects of H_2-rich hot water bathing on skin diseases has been previously published [11]. In the previous study, patients with psoriasis took 38–42°C H_2-baths twice weekly (3 days apart). The H_2-baths lasted 10–15 min, and the dissolved H_2 concentration was 1.0 ppm. A healthy control group bathed in tap water at least twice a week. After 8 weeks, the H_2-rich water bathing group scored significantly higher than the control group on the Psoriasis Area and Severity Index, a measure of skin rash severity. H_2 bathing also significantly improved itchiness, measured using a visual analogue scale.

However, this study is not without limitations. First, although the difference was not statistically significant, the H_2 concentration in the ICA increased slightly during the H_2-rich water bath. Almost all the H_2 absorbed through the skin is exhaled when it reaches the lungs. Therefore, theoretically, H_2 absorbed through the skin does not reach the CA. The pigs were intubated and placed on a ventilator to prevent inhalation of H_2 evaporating from the bathtub. However, the H_2 evaporating from the bathtub may have spread into the laboratory and contaminated the air with H_2. Second, the H_2 concentration in the PV increased during the H_2-rich hot water bathing. It is possible that the pigs were able to accidentally consume H_2-rich bathwater while they were immersed up to their neck. While the possibility remains that H_2 may have entered the body from sources other than the skin, the fact that the concentration in the IVC was more than twice that in the CA and PV suggests that the majority of the H_2 in the IVC was derived from H_2 absorbed through the skin. Similarly, the JV H_2 was thought to be derived from the skin of the neck and occipital region.

In conclusion, although there have been many animal and clinical studies examining the efficacy of H_2 on various diseases, few pharmacokinetic animal studies regarding H_2 have been conducted to extrapolate the results to humans. Therefore, our research group has clarified the pharmacokinetics of H_2 in the body via experiments using single and continuous inhalation of H_2 [33, 35], drinking of H_2-rich water (transcatheter injection of H_2-rich water into the jejunum) [32], and intraperitoneal injection of H_2-rich saline solutions [10].

Additionally, the pharmacokinetics of H_2 when bathing in H_2-rich hot water was clarified in the current study.

Bathing in water with a high concentration of dissolved H_2 is an effective means of efficiently and continuously supplying H_2 to skin tissues and beyond. The H_2 bathing system used in this study, which can maintain the H_2 concentration at a saturated level, is an effective health device that allows for a significant amount of H_2 absorption through the skin.

Acknowledgments

Conflict of interest

Eiji Kobayashi, Shou Kobayashi and Motoaki Sano declare the financial interests/personal relationships which may be considered as potential competing interests. The rest of the authors have nothing to declare.

References

1. Iuchi K, Imoto A, Kamimura N, et al. Molecular hydrogen regulates gene expression by modifying the free radical chain reaction-dependent generation of oxidized phospholipid mediators. Sci Rep 2016; 6: 18971.
2. Shirakawa K, Kobayashi E, Ichihara G, et al. H2 inhibits the formation of neutrophil extracellular traps. JACC Basic Transl Sci 2022; 7: 146-61.
3. Hayashida K, Sano M, Kamimura N, et al. Hydrogen inhalation during normoxic resuscitation improves neurological outcome in a rat model of cardiac arrest independently of targeted temperature management. Circulation 2014; 130: 2173-80.
4. Hayashida K, Sano M, Ohsawa I, et al. Inhalation of hydrogen gas reduces infarct size in the rat model of myocardial ischemia-reperfusion injury. Biochem Biophys Res Commun 2008; 373: 30-5.
5. Matsuoka T, Suzuki M, Sano M, et al. Hydrogen gas inhalation inhibits progression to the "irreversible" stage of shock after severe hemorrhage in rats. J Trauma Acute Care Surg 2017; 83: 469-75.
6. Ohsawa I, Ishikawa M, Takahashi K, et al. Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. Nat Med 2007; 13: 688-94.
7. Sugai K, Tamura T, Sano M, et al. Daily inhalation of hydrogen gas has a blood pressure-lowering effect in a rat model of hypertension. Sci Rep 2020; 10: 20173.
8. Ohsawa I, Nishimaki K, Yamagata K, Ishikawa M, Ohta S. Consumption of hydrogen water prevents atherosclerosis in apolipoprotein E knockout mice. Biochem Biophys Res Commun 2008; 377: 1195-8.
9. Zhang Y, Su WJ, Chen Y, et al. Effects of hydrogen-rich water on depressive-like behavior in mice. Sci Rep 2016; 6: 23742.
10. Hirano M, Sugai K, Fujisawa M, et al. Pharmacokinetics of hydrogen administered intraperitoneally as hydrogen-rich saline and its effect on ischemic neuronal cell death in the brain in gerbils. PLoS One 2022; 17: e0279410.
11. Zhu Q, Wu Y, Li Y, et al. Positive effects of hydrogen-water bathing in patients of psoriasis and parapsoriasis en plaques. Sci Rep 2018; 8: 8051.
12. Kobayashi E, Sano M. Organ preservation solution containing dissolved hydrogen gas from a hydrogen-absorbing alloy canister improves function of transplanted ischemic kidneys in miniature pigs. PLoS One 2019; 14: e0222863.
13. Nishi K, Iwai S, Tajima K, Okano S, Sano M, Kobayashi E. Prevention of chronic rejection of marginal kidney graft by using a hydrogen gas-containing preservation solution and adequate immunosuppression in a miniature pig model. Front Immunol 2021; 11: 626295.
14. Oharazawa H, Igarashi T, Yokota T, et al. Protection of the retina by rapid diffusion of hydrogen: administration of hydrogen-loaded eye drops in retinal ischemia-reperfusion injury. Invest Ophthalmol Vis Sci 2010; 51: 487-92.
15. Braverman IM. Ultrastructure and organization of the cutaneous microvasculature in normal and pathologic states. J Invest Dermatol 1989; 93 (2 Suppl): 2S-9S.
16. Grice EA, Segre JA. The skin microbiome [published correction appears in Nat Rev Microbiol. 2011 Aug;9(8):626]. Nat Rev Microbiol 2011; 9: 244-53.
17. Higgins JC, Eady RA. Human dermal microvasculature: I. Its segmental differentiation. Light and electron microscopic study. Br J Dermatol 1981; 104: 117-29.
18. Lavker RM, Kwong F, Kligman AM. Changes in skin surface patterns with age. J Gerontol 1980; 35: 348-54.
19. Nakamizo S, Egawa G, Honda T, Nakajima S, Belkaid Y, Kabashima K. Commensal bacteria and cutaneous immunity. Semin Immunopathol 2015; 37: 73-80.
20. Holbrook KA, Hennings H. Phenotypic expression of epidermal cells in vitro: a review. J Invest Dermatol 1983; 81 (1 Suppl): 11s-24s.
21. Kligman LH, Akin FJ, Kligman AM. The contributions of UVA and UVB to connective tissue damage in hairless mice. J Invest Dermatol 1985; 84: 272-6.
22. Kligman LH, Kligman AM. The effect on rhino mouse skin of agents which influence keratinization and exfoliation. J Invest Dermatol 1979; 73: 354-8.
23. Montagna W, Chase H, Melaragno H. The skin of hairless mice. I. The formation of cysts and the distribution of lipids. J Invest Dermatol 1952; 19: 83-94.
24. Sueki H, Gammal C, Kudoh K, Kligman AM. Hairless guinea pig skin: anatomical basis for studies of cutaneous biology. Eur J Dermatol 2000; 10: 357-64.
25. Mackenzie IC. Ordered structure of the epidermis. J Invest Dermatol 1975; 65: 45-51.
26. Bohling MW, Henderson RA, Swaim SF, Kincaid SA, Wright JC. Comparison of the role of the subcutaneous

tissues in cutaneous wound healing in the dog and cat. Vet Surg 2006; 35: 3-14.

27. Metcalfe DD, Kaliner M, Donlon MA. The mast cell. Crit Rev Immunol 1981; 3: 23-74.

28. Lavker RM, Sun TT. Heterogeneity in epidermal basal keratinocytes: morphological and functional correlations. Science 1982; 215: 1239-41.

29. Lavker RM, Sun TT. Epidermal stem cells. J Invest Dermatol 1983; 81 (1 Suppl): 121s-7s.

30. Horie M, Sekiya I, Nakamura T, et al. In vivo pharmacokinetics of ketoprofen after patch application in the Mexican hairless pig. Biopharm Drug Dispos 2009; 30: 204-8.

31. Lavker RM, Dong G, Zheng PS, Murphy GF. Hairless micropig skin. A novel model for studies of cutaneous biology. Am J Pathol 1991; 138: 687-97.

32. Ichihara G, Katsumata Y, Moriyama H, et al. Pharmacokinetics of hydrogen after ingesting a hydrogen-rich solution: a study in pigs. Heliyon 2021; 7: e08359.

33. Sano M, Shirakawa K, Katsumata Y, Ichihara G, Kobayashi E. Low-flow nasal cannula hydrogen therapy. J Clin Med Res 2020; 12: 674-80.

34. Bajgai J, Lee KJ, Rahman MH, Fadriquela A, Kim CS. Role of molecular hydrogen in skin diseases and its impact in beauty. Curr Pharm Des 2021; 27: 737-46.

35. Sano M, Ichihara G, Katsumata Y, et al. Pharmacokinetics of a single inhalation of hydrogen gas in pigs. PLoS One 2020; 15: e0234626.