

Experimental research

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

Satomi Iwai^{1,2}, Shou Kobayashi³, Eiji Kobayashi^{2,3,4}, Kazuhisa Sugai⁵, Yoji Hakamata^{4,5}, Kohnsuke Shirakawa^{4,6}, Yoshinori Katsumata^{4,6}, Motoaki Sano^{4,6}

¹Laboratory of Small Animal Surgery 2, School of Veterinary Medicine, Kitasato University Towada City, Aomori, Japan

²Endowed Chair for the Promotion of Minipig Research, School of Veterinary Medicine, Kitasato University, Towada City, Aomori, Japan

³Kobayashi Regenerative Research Institute, LLC, Wakayama-shi, Wakayama-ken, Japan

⁴Center for Molecular Hydrogen Medicine, Keio University, Minato-ku, Tokyo, Japan

⁵Department of Basic Sciences, School of Veterinary Nursing and Technology, Faculty of Veterinary Sciences, Nippon Veterinary and Life Science University, Musashino-shi, Tokyo, Japan

⁶Department of Cardiology, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan

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Corresponding authors:

Eiji Kobayashi
Kobayashi Regenerative
Research Institute
LLC, Wakayama-shi
Wakayama-ken, Japan
E-mail: eijkoba@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 (H₂) 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 (H₂)-rich hot water to assess the absorption of H₂ through the skin. An H₂-rich water generation line was developed to maintain a high concentration of H₂ 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 H₂-dissolved bath. After a washout period, one pig was bathed in an H₂-dissolved bath and the other in a bath containing no H₂ 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 H₂ concentration.

Results: The H₂ concentration at the IVC of the pig in the H₂-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) (H₂ saturation level: 3%) after 20 min. The blood H₂ concentration levels of the pig in the non-H₂ 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 H₂ 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 H₂ is the wide range of ways it can be administered, including the inhalation of H₂ gas [3–7],

drinking of H₂-dissolved water [8, 9], injection of H₂-dissolved saline solution [10], and H₂-rich hot water bathing [11]. Furthermore, an important characteristic of H₂ 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 H₂ diffuses rapidly in ischaemic tissues and removed organs in a blood flow-independent manner [4, 12–14]. Given these potential benefits, bathing in H₂-rich hot water has been introduced to the healthcare field with great enthusiasm. However, no study has verified whether H₂ can be absorbed through the skin during H₂-dissolved 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 H₂ can reach the dermal vasculature or further into the body's circulatory system even after immersion in a H₂-dissolved 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 H₂ absorbability through the skin that cannot ethically be conducted using human participants.

This study analysed the dynamics of H₂ absorption through the skin of hairless mini pigs during H₂-rich hot water bathing. First, an H₂-rich water generation line that can saturate the warm water in a bathtub with H₂ was developed, then a blood sampling system to minutely analyse the dynamics of H₂ absorbed from the skin during immersion in H₂-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 H₂-dissolved water. After a washout period, a second experiment was conducted in which one pig was bathed in an H₂-dissolved bath and the other pig was bathed in warm water without H₂.

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.

H₂-rich water generation line

The H₂ concentration in the 80-litre bath was gradually increased by circulating the H₂-rich wa-

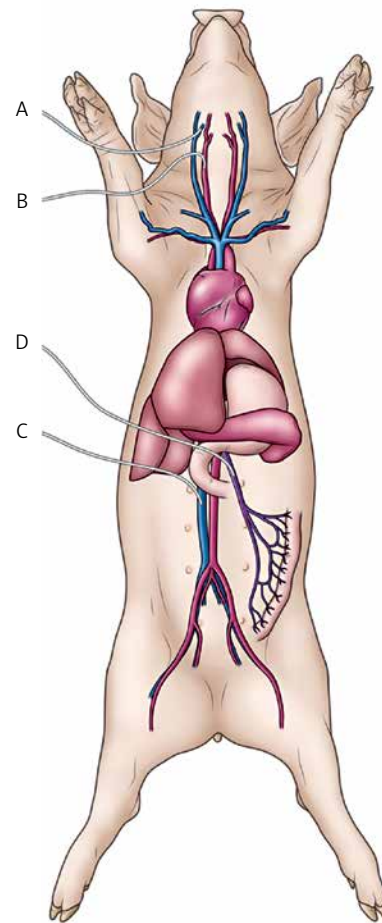


Figure 1. Location of blood collection ports. Blood H₂ concentrations during bathing in hot water containing H₂ or no H₂ were measured at the following 4 locations. A. Carotid artery. B. Jugular vein. C. Inferior vena cava. D. Portal vein

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 H₂ generator (H2J1 [33]) was used to continuously supply 100% H₂ at a pressure of 0.15 Mpa to 6000 H₂-permeable membranes bundled into cylindrical tubes [33]. Due to the pressure differential between the water pressure and H₂ pressure caused by water flowing inside the H₂-permeable membranes, the H₂ dissolved into the water at a stable concentration in the form of ultrafine bubbles (Flash Bubbling System: Doctors Man Co., Ltd., Kanagawa, Japan).

H₂-rich hot water bath

To generate a large volume of highly concentrated H₂ bath water in a short period of time, a 2-by-2 arrangement was devised for the H₂ gas permeable membrane. The inlet and discharge ports were circulated through a tube hose with an inner diameter of approximately 4 mm, and

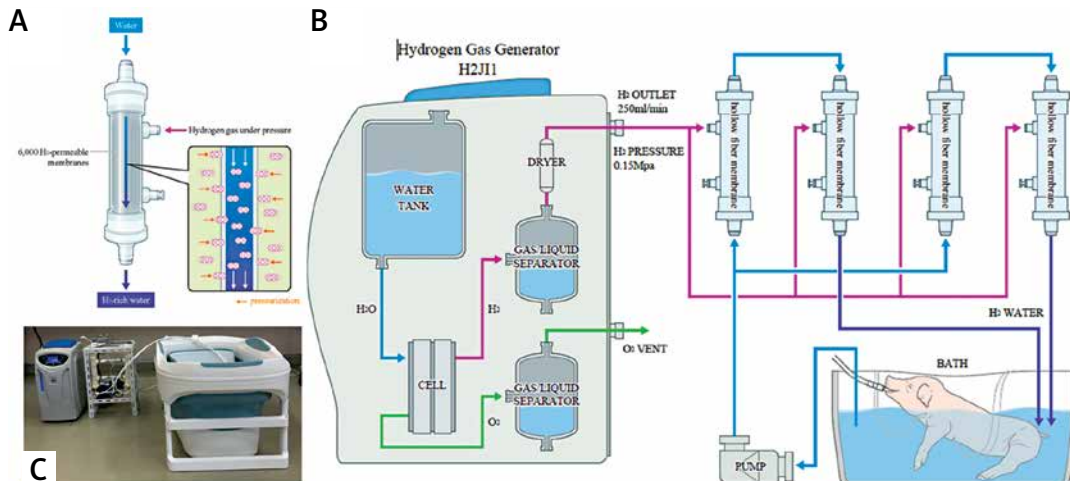


Figure 2. H₂-rich water generation line. **A** – H2J11 flash bubbling system for dissolving H₂. The bubbling system fills the bath with H₂ at a high concentration. **B** – H₂ filling circuit. This circuit circulates hot water in the bathtub to increase the concentration of dissolved H₂ in the bathtub. **C** – H2J11 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 H₂ evaporated from the water. To measure the H₂ 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 H₂-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 H₂ 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 addition,

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 H₂ Concentrations

The H₂ 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 H₂ in the blood and bath water is released into a gaseous phase when kept in sealed vials, the H₂ concentration of the blood was estimated using gas chromatography (TRiLyzer mBA-3000, Taiyo Corporation) of the air inside the vial. Calibration curves of H₂ concentrations of 0 (nitrogen gas), 5, 50, and 130 ppm were prepared. The H₂ 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

H₂ Concentration in the Bath System During the Experiment

Thirty min after starting the H₂-rich water generation line, the H₂ concentration of the bathwater was approximately 1600 ppb. Because 1.6 mg H₂ is the maximum amount that can be absorbed into 1 l of water, the bathwater contained the maximum possible amount of H₂. No significant differences in the H₂ concentration of the bathwater were observed throughout the experiment (Figure 4 A). The H₂ concentrations of the bathwater were not significantly different between the trials (Figure 4 B). When the H₂-rich water generation line was not used, the H₂ concentration in the bathwater was always below the detection limit of 0.3 ppb.

H₂ pharmacokinetics during H₂-rich hot water bathing

At all blood collection points, the concentration of H₂ 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 H₂ 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 H₂ concentration of the blood was the highest at the IVC ($n = 3$). The H₂ 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 H₂ 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 H₂ saturation level at IVC increased to as high as 3%. Due to the small number of n (3), the change over time in blood H₂ concentration in IVCs during H₂-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 H₂ 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 H₂ concentrations between blood sampling times.

The blood H₂ concentration was below the detection limit of 0.3 ppb at all blood sampling locations and times when the pigs were bathed in non-H₂ baths. This result implies that soaking in a hot tub does not stimulate intestinal bacteria to increase H₂ production.

Discussion

In the present study, we found that bathing hairless mini pigs for 20 min in hot water dissolved with H₂ in a saturated state increased the blood H₂ concentration in IVCs to 45.7 ± 7.73 ppb (w/w) (H₂ saturation: 3%). This result means that H₂ 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.

H₂ certainly has excellent diffusivity, but this feature is overestimated. If H₂ 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 H₂ is efficiently taken up into the bloodstream. In addi-

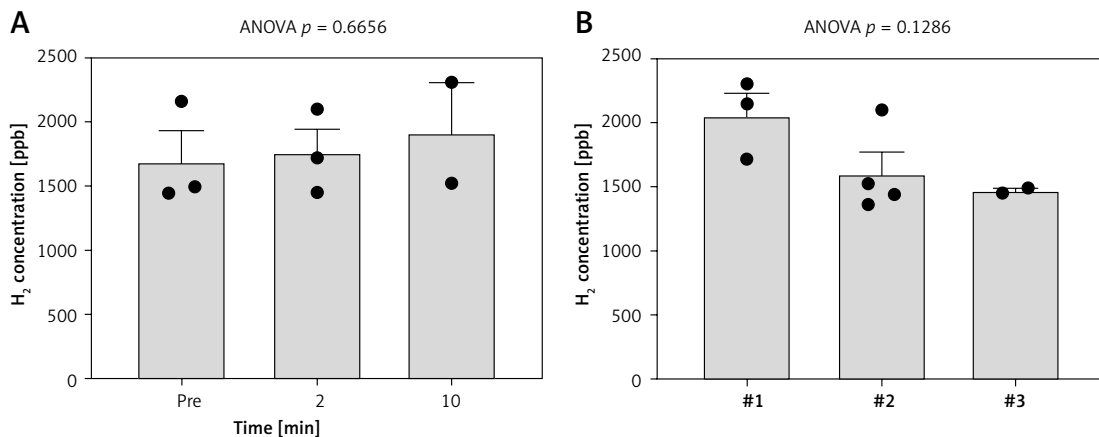


Figure 4. H₂ concentration in the H₂-rich hot water bath. **A** – The H₂ concentration in the bathtub remained stable throughout the bathing period. **B** – The H₂ concentration in the bath was stable during all 3 experimental trials

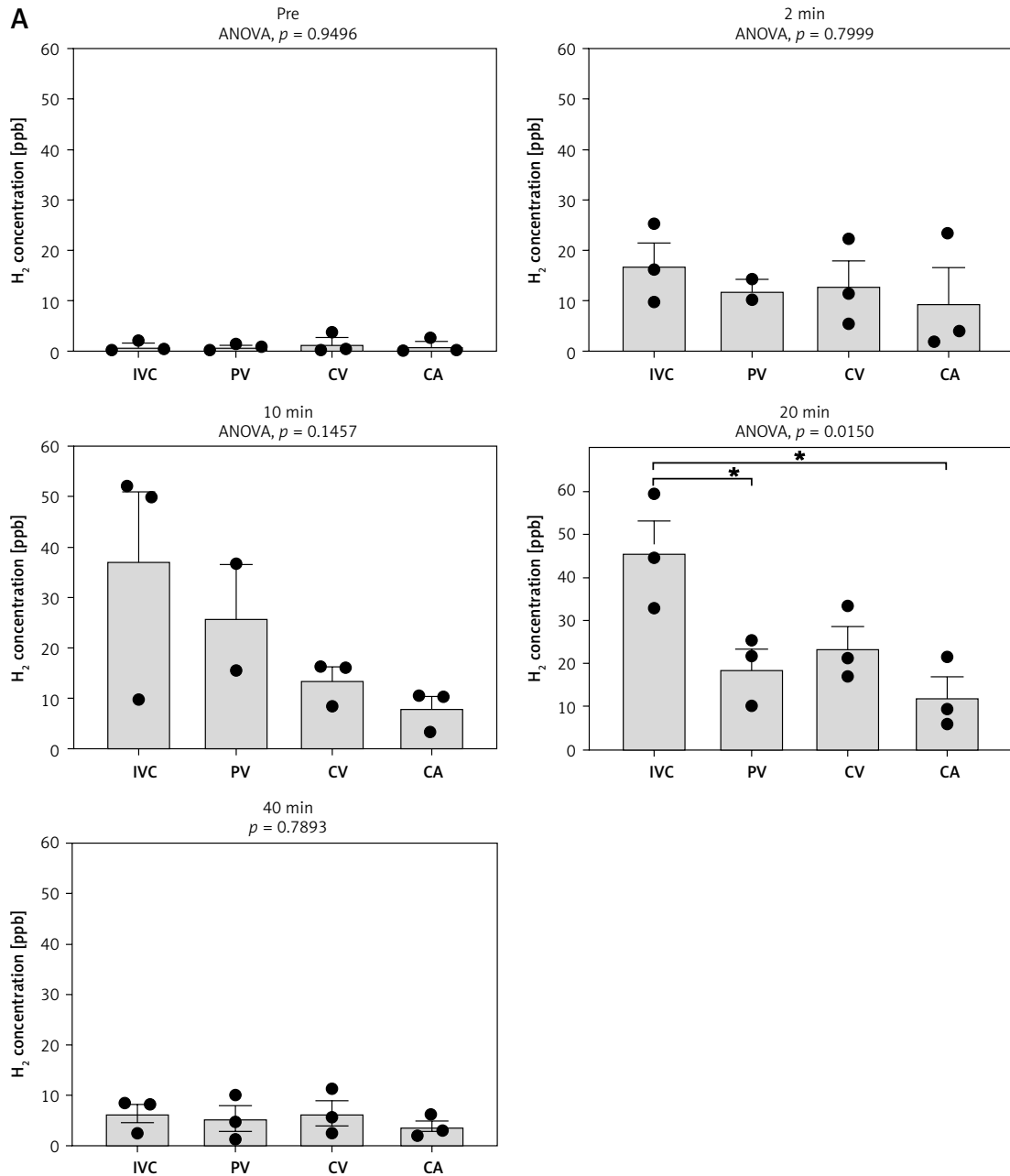


Figure 5. Pharmacokinetics of H₂ when bathing in H₂-rich hot water. **A** – Twenty min after the start of bathing, the H₂ concentration at the inferior vena cava is significantly higher than that at the internal carotid artery and the portal vein ($p < 0.05$, $n = 3$)

tion, when inhaled, the lungs, which are the largest route for H₂ to escape from the blood, can be shielded by a high concentration of H₂, making the entire body a closed space for H₂. In contrast, to get H₂ into the blood from tissues other than the lungs, H₂ must be dissolved in solution by applying pressure or other operations before contact is made.

For example, if H₂ is injected into the intestine in gaseous form, very little is absorbed. On the other hand, when H₂-rich water prepared by dissolving H₂ in water under external pressure is

injected into the small intestine, H₂ is efficiently absorbed into the blood. Using miniature pigs, we have confirmed that when H₂-rich water is injected into the small intestine, H₂ is transported from the jejunal vein to the portal vein and then to the liver. When water containing H₂ at a concentration of 5.42 mg/l (5.42 ppm) was injected directly into the jejunum over 2 min, the H₂ concentration in the portal vein was maintained at 0.05 mg/l (H₂ saturation level, ~3%) for 30 min, while the H₂ concentration in the IVC was at 1/3 that level [32]. The results of the current study

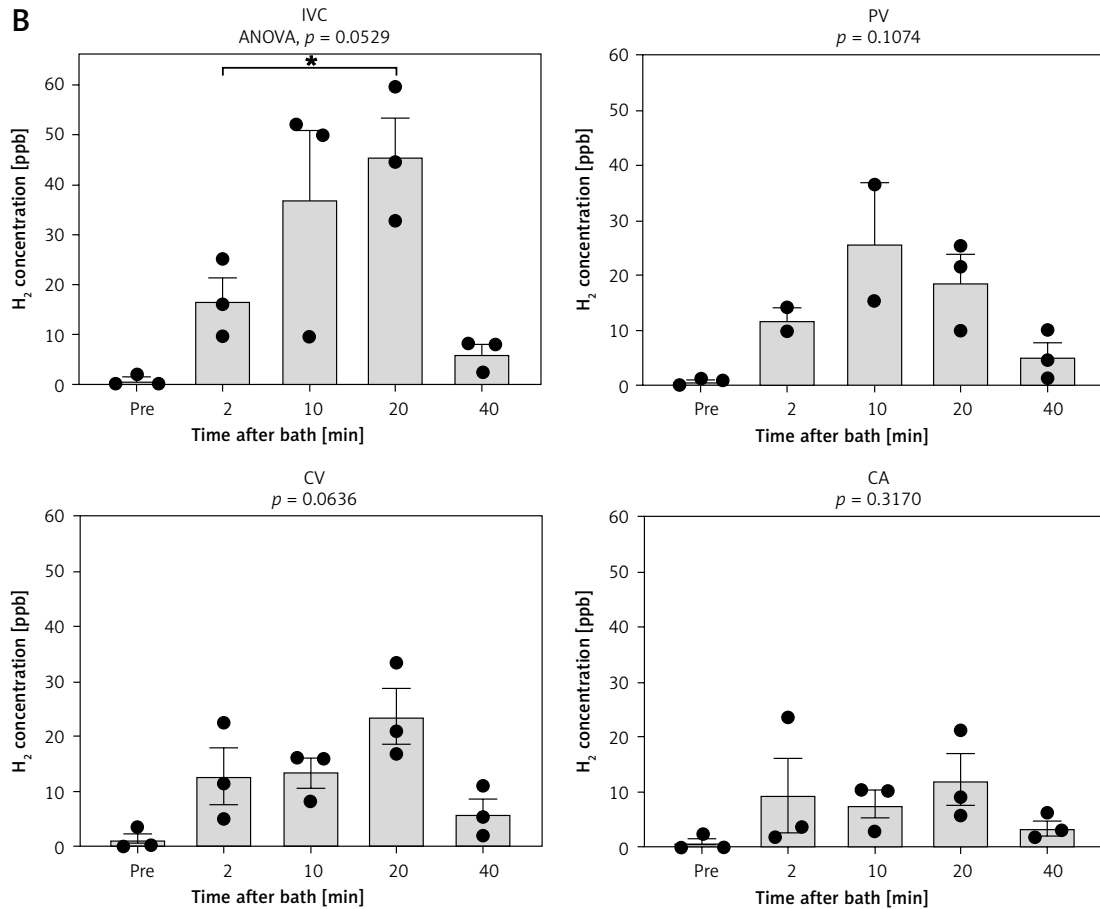


Figure 5. Cont. **B** – The changes in hydrogen concentration at each location during the saturated hydrogen bath are shown. The H₂ concentration at the IVC was significantly higher at 20 min than at 2 min ($p < 0.05$, $n = 3$)

suggest that introducing H₂ into the body's circulation through the skin by taking an H₂-rich hot water bath increased the internal H₂ levels to similar or greater levels as when H₂ is introduced into the portal circulation via the drinking of H₂ 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 H₂, an antioxidant, may be an effective therapeutic tool to prevent or treat these diseases [34]. One study regarding the effects of H₂-rich hot water bathing on skin diseases has been previously published [11]. In the previous study, patients with psoriasis took 38–42°C H₂-baths twice weekly (3 days apart). The H₂-baths lasted 10–15 min, and the dissolved H₂ concentration was 1.0 ppm. A healthy control group bathed in tap water at least twice a week. After 8 weeks, the H₂-rich water bathing group scored significantly higher than the control group on the Psoriasis Area and Severity Index, a measure of skin rash severity. H₂ 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 H₂ concentration in the ICA increased slightly during the H₂-rich water bath. Almost all the H₂ absorbed through the skin is exhaled when it reaches the lungs. Therefore, theoretically, H₂ absorbed through the skin does not reach the CA. The pigs were intubated and placed on a ventilator to prevent inhalation of H₂ evaporating from the bathtub. However, the H₂ evaporating from the bathtub may have spread into the laboratory and contaminated the air with H₂. Second, the H₂ concentration in the PV increased during the H₂-rich hot water bathing. It is possible that the pigs were able to accidentally consume H₂-rich bathwater while they were immersed up to their neck. While the possibility remains that H₂ 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 H₂ in the IVC was derived from H₂ absorbed through the skin. Similarly, the JV H₂ 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 H₂ on various diseases, few pharmacokinetic animal studies regarding H₂ have been conducted to extrapolate the results to humans. Therefore, our research group has clarified the pharmacokinetics of H₂ in the body via experiments using single and continuous inhalation of H₂ [33, 35], drinking of H₂-rich water (transcatheter injection of H₂-rich water into the jejunum) [32], and intraperitoneal injection of H₂-rich saline solutions [10].

Additionally, the pharmacokinetics of H₂ when bathing in H₂-rich hot water was clarified in the current study.

Bathing in water with a high concentration of dissolved H₂ is an effective means of efficiently and continuously supplying H₂ to skin tissues and beyond. The H₂ bathing system used in this study, which can maintain the H₂ concentration at a saturated level, is an effective health device that allows for a significant amount of H₂ absorption through the skin.

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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.

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