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Effects of Oral Intake of Hydrogen Water on Liver Fibrogenesis in Mice

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Running head

Effecs of hydrogen on liver fibrogenesis

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Abstract

Aim: Liver fibrosis is the universal consequence of chronic liver diseases. Sustained hepatocyte injury initiates an inflammatory response, thereby activating hepatic stellate cells, the principal fibrogenic cells in the liver. Reactive oxygen species are involved in liver injury and are a promising target for the treating liver fibrosis. Hydrogen water is reported to have potential as a therapeutic tool for the reactive oxygen species-associated disorders. This study aimed to investigate the effects of hydrogen water on liver fibrogenesis and the mechanisms underlying these effects.

Methods: C57BL/6 mice were fed with hydrogen water or control water, and subjected to carbon tetrachloride, thioacetamide and bile duct ligation treatments to induce liver fibrosis. Hepatocytes and hepatic stellate cells were isolated from mice and cultured with or without hydrogen to test the effects of hydrogen on reactive oxygen species-induced hepatocyte injuries or hepatic stellate cell activation.

Results: Oral intake of hydrogen water significantly suppressed liver fibrogenesis in the carbon tetrachloride and thioacetamide models, but these effects were not seen in the bile duct ligation model. Treatment of isolated hepatocyte with 1 μg/mL antimycin A generated hydroxyl radicals. Culturing in the hydrogen-rich medium selectively suppressed the generation of hydroxyl radicals in hepatocytes and significantly

suppressed hepatocyte death induced by antimycin A; however, it did not suppress hepatic stellate cell activation.

Conclusions: we conclude that hydrogen water protects hepatocytes from injury by scavenging hydroxyl radicals and thereby suppresses liver fibrogenesis in mice.

Introduction

Liver fibrosis is a common cause of death worldwide[1]. Advanced liver fibrosis disrupts normal liver architecture, causing hepatocellular dysfunction and portal hypertension. To date, no effective hepatic antifibrotic therapies are available.

The hepatic stellate cell (HSC) is the major fibrogenic cell type in the liver[2]. Sustained hepatocyte injuries such as those caused by hepatitis viruses and alcohol, lead to the activation of HSCs and hepatocyte death, which is induced by the activation of inflammatory mediators such as transforming growth factor (TGF)-$\beta 1$[3]. HSCs are activated to differentiate into myofibroblast-like cells, which promote collagen deposition.

Reactive oxygen species (ROS) play an important role in hepatic fibrosis[4]. Galli et al. reported that the superoxide anion ($O\cdot_2^-$) produced by xanthine and xanthine oxidase induced the proliferation of HSCs[5]. On the other hand, Sandra et al reported that rather than directly inducing HSC proliferation, hydrogen peroxide (H_2O_2) and $O\cdot_2^-$ inhibited the proliferation of these cells[6]. To date, it is unclear how ROS contribute to the development of liver fibrogenesis.

Oxidative stress arises from the strong cellular oxidizing potential of ROS, or

free radicals. Most of the $O\cdot_2^-$ is generated in the mitochondria by electron leakage from the electron-transport chain and the Krebs cycle[7, 8]. $O\cdot_2^-$ is also generated by metabolic oxidases, including NADPH oxidase and xanthine oxidase[5, 9]. Superoxide dismutase converts $O\cdot_2^-$ into H_2O_2. Excess $O\cdot_2^-$ reduces transition metal ions, such as Fe^{3+} and Cu^{2+}, and the reduced forms can react with H_2O_2 to produce hydroxyl radicals $(OH\cdot)$ by the Fenton reaction. $OH\cdot$ is the strongest ROS and reacts indiscriminately with nucleic acids, lipids, and proteins[10].

Recent studies have shown that hydrogen acts as an antioxidant. A study by Ohsawa et al. on a model of brain ischemia-reperfusion injury revealed that hydrogen was a selective antioxidant for $OH\cdot$[11]. The beneficial effect of hydrogen was also reported recently in various models of liver injury, such as ischemia-reperfusion injury and injury induced by galactosamine-lipopolysaccharide[12]. Sun et al reported that intraperitoneal injection with hydrogen-rich saline was also effective in a model of carbon tetrachloride (CCl_4) -induced liver fibrosis[12]. However, they examined the effects only in a hepatic injury model, and the pathophysiological mechanism by which hydrogen-rich saline attenuates liver fibrosis remains to be elucidated. Further, the applicability of hydrogen would be more widespread if orally ingested hydrogen-water (H_2-water) could effectively suppress liver fibrogenesis.

This study aimed to investigate the effects of H_2-water on various liver injury models and the mechanisms underlying these effects.

Methods

Animals and treatment

Male C57BL/6 mice were purchased from Clea Japan (Tokyo, Japan). The mice were treated with CCl_4, thioacetamide (TAA) or bile duct ligation (BDL) to induce liver fibrosis. The mice were fed with H_2-water or control water while liver fibrosis was induced. In the CCl_4 model, the 7-week-old mice were injected intraperitoneally with 0.5 µl of CCl_4 per gram mouse weight twice a week for 6 weeks (n = 20 for hydrogen water and control water group)[13]. Corn oil was injected as a control (n = 3 for each group). In the TAA model, the mice were injected intraperitoneally with escalating dose of TAA twice a week for 6 weeks (first dose, 100 µg/g, week 1–2, 200 µg/g; week 3–4,300 µg/g; week 4–6, 400 µg/g) (n = 6 for each group)[14]. Normal saline was injected as a control (n = 3 for each group). BDL was performed by surgical ligation of the common hepatic bile duct with 6-0 nylon under pentobarbital anesthesia (50mg/kg) (n = 6 for each group). Sham-operated mice underwent a laparotomy without ligation of the common bile duct (n = 3 for each group). The animals were euthanized on postoperative day 21. The

animal protocols were approved by the Animal Research Committee of Kyoto University, and all experiments were conducted in accordance with *Guidelines for the Care and Use of Laboratory Animals* promulgated by the National Institute of Health.

H_2-water and hydrogen-rich medium

H_2-water at the concentration of 1.24mg/L and control water were provided in 300 mL aluminum pouches supplied by I'rom Pharmaceutical Co., Ltd. (Tokyo, Japan). The mice were provided water *ad libitum* through closed glass vessels (I'rom Pharmaceutical Co., Ltd.; Tokyo, Japan) equipped with an outlet line containing 2 ball bearings, which prevented the degassing of the water. The vessel was freshly refilled with H_2-water every day.

The hydrogen-rich medium was prepared according to a method described previously [15]. In brief, hydrogen gas was dissolved directly into the media by bubbling the gas into the medium.

Isolation and culture of hepatocytes

Hepatocytes were isolated from mouse livers by a previously described method[16]. The isolated hepatocytes were cultured on 6-well plates coated with type 1

collagen, at a cell density of 5×10^5 cells/well, in DMEM supplemented with 10%FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Isolation and culturing of HSCs

HSCs were isolated from mouse livers by a method described previously[17, 18]. In brief, liver cells were dispersed with 0.025% PronaseE (Merck, Darmstadt, Germany) and 0.025% collagenase (Wako, Osaka, Japan). The cell suspension was centrifuged through 9.7% Nycodenz (Nycomed Pharm, Oslo, Norway) cushion. The HSC-enriched band was transferred into DMEM, supplemented with 10% fetal bovine serum (FBS) and antibiotics. The isolated HSC was incubated in a CO_2 incubator at 37 °C. The purity of HSCs was consistently >98% as judged by presence of lipid droplets and autofluorescence.

To analyze whether the hydrogen-rich medium suppresses HSC activation, the isolated HSCs were cultured in hydrogen-rich medium for 5 days, during which the medium was changed every day.

ROS induction with antimycin A and detection of ROS

To examine the effects of ROS induction for hepatocytes, the medium was replaced with serum-free DMEM 6 h after plating. Twelve hours later, 1μg/mL antimycin A (AMA; Sigma-Aldrich, St Louis, MO, USA) was added, and the cells were incubated for 1 h. Then, the cells were stained with bisbenzimide H33342 fluorochrometrihydrochloride DMSO solution (Hoechst 33342) (Nacalai Tesque) and propidium iodide (PI; Calbiochem, San Diego, CA) and examined under a fluorescent microscope. Five nonoverlapping fields at × 200 magnifications were photographed at random. PI-positive cells were considered as dead cells, and the PI-positive ratio was calculated as the percentage of PI-positive cells among the Hoechst 33342-positive cells. For the detection of OH·, $O \cdot_2^-$, and H_2O_2, the cells were incubated with MitoSOX, 2',7'-dichlorodihydrofluorescein (CM-H_2DCFDA), and hydroxyphenyl fluorescein (HPF) (Nikken Seil, Shizuoka Japan), respectively [19, 20].

To examine whether HSC activation is accelerated by ROS, the medium was replaced with serum-free DMEM 24 h after plating. Twenty-four hours later, 1μg/mL AMA (Sigma-Aldrich, St Louis, MO, USA) was added, and the cells were incubated for 1 h. The cells were observed with a BIOREVO BZ-9000 digital microscope (Keyence, Osaka, Japan) at × 200 magnifications.

Immunohistochemistry

The specimens were fixed in 4% paraformaldehyde and embedded in OCT compound. Slices of the specimens at 4-μm thickness were obtained, and the antigen was retrieved by incubation in citric acid buffer at 90°C for 20 min. After being blocked, the sections were incubated with primary antibody-recognizing α-smooth muscle actin (α-SMA; no. ab5694; Abcam, Cambridge, UK) at 1:200 dilution overnight at 4°C and then incubated with antibody-recognizing rabbit IgG labeled by Alexa594 (Invitrogen, Tokyo, Japan) at room temperature for 1 h.

For the detection of 3-nitrotyrosine, the sections were incubated for 1 h in a working solution of mouse IgG blocking reagent from the MOM Immunodetection kit (Vector, CA, USA), and then incubated sequentially with 3-nitrotyrosine-recognizing primary antibody (Mab5404, Merck, Germany) at 1:100 dilution overnight at 4°C, MOM biotinylated anti-mouse IgG reagent for 10 min and VECTASTAIN ABC reagent for 5 min. The sections were examined after incubation with Liquid DAB Substrate Chromogen System (Dako, Glostrup, Denmark) for 3 min.

Liver histology

Formalin-fixed, paraffin-embedded specimens were sliced at 4-μm thickness

and mounted on silanized glass slides. The slides were stained with Sirius red[21]. Images

of 5 nonoverlapping fields were randomly selected and captured at × 200 magnifications.

Sirius red staining was quantified by image analysis with National Institutes of Health

image (Image J).

Hydroxyproline contents

Liver tissues were homogenized in ice-cold distilled water (1 mL). One hundred

and twenty-five microliter 50% TCA was added and the homogenates were further

incubated on ice for 30 min. The precipitated pellets were hydrolyzed for 24 h at 110℃

in 6 N HCL. Samples were filtered and neutralized with 10 N NaOH. Hydrolysates were

then oxidized with chloramine-T (Sigma, St. Louis, MO) for 25 min at room temperature.

The reaction mixture was incubated in Ehrich's perchloric acid solution at 65°C for 20

min, and then cooled at room temperature. Sample absorbance was measured at 560 nm.

Purified hydroxyproline (Sigma) was used to set a standard. Hydroxyproline content

was expressed as nanograms of hydroxyproline per milligram of liver.

ROS analysis for the liver specimens

Accumulation of ROS in the liver was assessed by staining freshly frozen liver

sections with HPF. In brief, the freshly frozen liver specimens were sliced at 4 µm

thickness and were incubated with HPF diluted at 1:200 for 15 min at 37°C.

The MDA level was determined according to the thiobarbituric acid (TBA)

method using NWLSS Malondialdehyde Assay Kit (Northwest Life Science Specialties,

USA).

GSH level was determined with GSH quantification assay kit (Dojindo, Tokyo,

Japan).

Biochemical measurements were carried out at room temperature using a

spectrophotometer (Molecular Devices, Tokyo, Japan).

Western blotting

The protocol for western blotting was described previously[16]. The primary

antibodies and dilutions were as follows: anti α-SMA antibody (no. ab5694; Abcam,

Cambridge, UK) at 1:1000; and anti-α-tubulin antibody (no. CP06; Calbiochem) at

1:1,000.

Quantitative reverse transcription polymerase chain reaction analysis

Total RNA was extracted from the liver sample or isolated HSCs by RNeasy

Mini Kit with on-column DNA digestion (Qiagen, Tokyo, Japan). Total RNA was reverse-transcribed to complementary DNA by using the Omniscript RT Kit (Qiagen, Valencia, CA, USA). Quantitative real-time reverse transcription (RT)-PCR was performed using SYBR Green Master reaction mix on a LightCycler 480 II (Roche Diagnostics, Basel, Switzerland). The relative abundance of the target genes was obtained by calculating against a standard curve and normalized to GAPDH for liver samples, and to 18S ribosomal RNA for HSC samples, as the internal control. The primer sequences used were: Col-1α1-Forward: 5'- GCT CCT CTT AGG GGC CAC T-3', Col-1α1-Reverse: 5'-CCA CGT CTC ACC ATT GGG G-3', α-SMA-Forward: 5'-GTC CCA GAC ATC AGG GAG TAA-3', α-SMA-Reverse: 5'- TCG GAT ACT TCA GCG TCA GGA-3', TGF-β1-Forward: 5'-CCG CAA CAA CGC CAT CTA TG-3', TGF-β1-Reverse 5'-CCC GAA TGT CTG ACG TAT TGG AAG-3', GAPDH-Forward: 5'-AGG TCG GTG TGA ACG GAT TTG-3' GAPDH-Reverse: 5'-TGT AGA CCA TGT AGT TGA GGT CA-3', 18s ribosomal RNA-Forward: 5'-AGT CCC TGC CCT TTG TAC ACA-3', and 18s ribosomal RNA-Reverse: 5'-CGA TCC GAG GGC CTC ACT A-3'.

Statistical analysis

Each quantitative dataset was presented as mean ± standard error of the mean

(SEM), and statistically analyzed with two-tailed Student's *t*-test followed by a post-hoc

Fisher's protected least significant difference (PLSD) test.

Results

Oral intake of H_2-water suppressed liver fibrogenesis in models of CCl_4- and TAA-induced liver injury

In the CCl_4 model, hepatic hydroxyproline content was significantly lower in the H_2-water group than the control water group (295.0 vs. 382.6 ng/mg liver; $p < 0.05$) (Fig. 1a). Liver fibrosis, assessed by the intensity of Sirius red staining, was inhibited in the H_2-water group (Fig. 1b). Sirius red staining showed that the fibrotic area with collagen deposit was 8.163% in the control water group and 4.666% in the H_2-water group ($p < 0.0001$) (Fig. 1b). The inhibitory effects of H_2-water on liver fibrogenesis were further evaluated by RT-PCR for collagen1α1 and α-SMA. In the H_2-water group and control water group, the mRNA level of collagen1α1 were 4.77 and 11.55 times that in the control, ($p < 0.05$), while those of α-SMA were 2.15 and 3.92 times that in the control ($p < 0.05$) (Fig. 1c), respectively. Western blotting showed that the α-SMA levels in the H_2-water group and control water group was 1.64 and 2.87 times that in the control, respectively ($p < 0.01$) (Fig. 1d). H_2-water also suppressed the mRNA level of TGF-β1

(3.03 vs. 1.74 times as control; $p < 0.05$) in the liver.

In the TAA model, H_2-water drinking suppressed liver fibrogenesis, as assessed by the hydroxyproline content (232.7 vs. 287.1 ng/mg liver; $p < 0.05$) (Fig. 2a), Sirius red staining (2.563 vs. 4.271 %; $p < 0.01$) (Fig. 2b), RT-PCR for collagen1α1 (7.88 vs. 59.4 times that in the control; $p < 0.01$) and α-SMA (2.24 vs. 26.62 times that in the control; $p < 0.01$) (Fig. 2c), and western blotting for α-SMA (4.86 vs. 9.96 times that in the control; $p < 0.01$) (Fig. 2d). H_2-water suppressed the mRNA level of TGF-β1 (3.37 vs. 2.07 times in the control; $p < 0.05$) in the liver.

H_2-water oral intake suppressed generation of hydroxyl radicals in the liver.

HPF staining for the detection of OH· on freshly frozen liver sections in the CCl_4 model showed a significantly smaller area of HPF staining in the H_2-water group (Fig. 3a) than in the control water group; this indicated that orally ingested H_2-water had a scavenging effect on OH· in the liver. These results demonstrated that CCl_4 injection induced the release of OH· and that H_2-water scavenged OH·. In the TAA model, HPF staining was present in a significantly smaller area in the H_2-water group (Fig. 3b).

MDA levels in the H_2-water group were significantly lower in the CCl_4 model

(3.89 vs. 1.57 times that in the control) and TAA model (2.17 vs. 1.41 times that in the control) (Fig. 3c). These results supports the antioxidative effect of H_2-water on OH・ which generates lipid peroxidation. H_2-water did not change 3-nitrotyrosine expression (Fig. 4a, 4b) and GSH level (Fig. 4c).

Inhibition of CCl_4- or TAA- induced liver fibrosis by H_2-water was associated with less hepatocyte damage.

The H_2-water group showed significantly lower transaminase levels in CCl_4 model at day1 after CCl_4 injection (AST, 2532 ± 697 (H_2-water) vs. 5133 ± 908 IU/L (control water); ALT, 3441 ± 790 (H_2-water) vs. 6756 ± 1166 IU/L (control water)) and in TAA model at day1 after TAA injection (AST, 1066 ± 460 (H_2-water) vs. 2906 ± 834 IU/L (control water); ALT, 1197 ± 442 (H_2-water) vs. 4018 ± 997 IU/L (control water)).

Oral intake of H_2-water did not suppress liver fibrogenesis in the BDL-induced liver injury model, in which OH・ was not generated

In the BDL model, no difference in hydroxyproline contents was observed between the H_2-water group and the control water group (Fig. 5a). This result was further confirmed by assessment of the mRNA expression of collagen1α1 and α-SMA

(Fig. 5b) and Sirius red staining (Fig. 5c). HPF staining for the detection of OH·in freshly frozen liver sections in the BDL model showed no HPF staining in either the H_2-water or the control water groups (Fig. 5d).

Hydrogen-rich medium suppressed the generation of hydroxyl radicals and hepatocyte death induced by AMA

AMA, a complex III inhibitor in the respiratory transduction system, induces the release of $O \cdot_2^-$. Hepatocytes were isolated from C57BL/6 mice and treated with 1 µg/mL of AMA. The addition of AMA increased levels of OH·, H_2O_2, and $O \cdot_2^-$, as evaluated by the intensity of fluorescence signals emitted by the oxidized forms of HPF (Fig. 6a), CM-H_2DCFDA (Fig. 6b), and MitoSOX (Fig. 6c), respectively. Culturing in hydrogen-rich medium suppressed the generation of OH· (Fig. 6a) induced by AMA, but did not affect the increased level of H_2O_2 (Fig. 6b) or $O \cdot_2^-$ (Fig. 6c), indicating that the hydrogen-rich medium selectively scavenged OH·.

Hepatocyte death induced by AMA was suppressed in the presence of hydrogen in the medium (13.2% and 26.8% for hydrogen-rich medium and control medium, respectively; $p < 0.001$) (Fig. 6d).

Hydrogen-rich medium did not suppress culture activation of HSCs

We assessed the effect of culturing in hydrogen-rich medium on the activation of HSC, which are the principal fibrogenic cells in the liver. HSCs are known to be activated when cultured on a plastic dish. RT-PCR for collagen1α1 and α-SMA of HSCs that were cultured on a plastic dish for 5 days revealed that the hydrogen-rich medium did not suppress the activation of cultured HSCs (Fig. 7a). The activated HSCs showed traces of $O \cdot_2^-$, as detected by MitoSOX staining (Fig. 7b). The activated HSCs contained H_2O_2, as evidenced by vivid staining with CM-H_2DCFDA (Fig. 7c), suggesting that culturing in the hydrogen-rich medium did not suppress the accumulation of H_2O_2. The HSCs did not contain $OH\cdot$, as evaluated by HPF staining (Fig. 7d). These results imply that $OH\cdot$ is not associated with the activation of cultured HSCs and H_2-water does not seem to target HSCs.

Hydrogen-rich medium did not suppress or accelerate HSC activation induced by AMA

Isolated HSCs were cultured with or without hydrogen-rich medium in the presence of AMA to assess the effect of hydrogen on HSC activation under excess oxidative stress. Culturing in the hydrogen-rich medium did not affect the mRNA expression levels of the activation markers for HSC (collagen1α1 and α-SMA) (Fig. 8a).

The increased intracellular $O\cdot_2^-$ levels induced by AMA stimulation and detected by MitoSOX were not suppressed by culturing in hydrogen-rich medium (Fig. 8b). The H_2O_2 level, detected by CM-H$_2$DCFDA was not altered by AMA stimulation (Fig. 8c). Moreover, we could not detect intracellular OH$\cdot$, even after AMA treatment (Fig. 8d). These results suggest that hydrogen did not exert its antifibrotic effect through direct action on HSCs.

Discussion

In the present study, we investigated whether orally ingested H$_2$-water suppressed liver fibrogenesis. Our study demonstrated that H$_2$-water showed antifibrogenic effects in the CCl$_4$ and TAA models but did not show any difference in the BDL model. H$_2$-water exhibited an OH$\cdot$-scavenging effect in the CCl$_4$ and TAA model; however, in the BDL model, OH$\cdot$ was not detected in the liver even in the control water group. We suggest that the antifibrotic effect of H$_2$-water is associated with the scavenging of OH$\cdot$.

CCl$_4$ and TAA have been widely used to induce injury in experimental models of liver fibrosis. In both these models, ROS play a crucial role in the development of liver fibrosis[22, 23]. Intracellular ROS usually refers to the excess amounts of $O\cdot_2^-$, H_2O_2, and

OH$\cdot$. Among them, OH$\cdot$ is the strongest ROS and reacts indiscriminately with nucleic acids, lipids, and proteins[10]. To date, many reports have described the antifibrotic effect of antioxidants[24-26]. However, the mechanisms by which antioxidants suppress liver fibrosis, and the particular ROS that forms the primary target for antioxidants, remain to be elucidated. Galli et al. described the proliferation and invasiveness of HSC by O$\cdot_2^-$ induced by the action of xanthine and xanthine oxidase[5]. Therefore, we first sought to determine whether H$_2$-water exerted its antifibrotic effect through the suppression of O$\cdot_2^-$ production. The results of our in vitro experiments, however, indicated that the hydrogen-rich medium did not influence the O$\cdot_2^-$ and the H$_2$O$_2$ levels in both HSCs and hepatocytes. We examined the expression of O$\cdot_2^-$ in the injured liver by staining freshly frozen liver sections with MitoSOX, and the results showed that H$_2$-water does not influence the O$\cdot_2^-$ levels in the liver (data not shown). Instead, we found that oral ingestion of H$_2$-water resulted in the suppression of OH$\cdot$ levels in the liver and that hydrogen-rich medium attenuated the generation of OH$\cdot$ in cultured hepatocytes. These results indicated that OH$\cdot$ is the primary target for H$_2$-water.

It is well known that peroxynitrite is a reactive oxidant produced from nitric oxide and superoxide. O$\cdot_2^-$ may react with nitric oxide, which could be generated constitutively from sinusoidal endothelial cell-derived nitric oxide synthase. Aram et al.

reported that necrosis and liver fibrosis-induced by CCl4 administration were decreased in iNOS deficient mice[27]. We examined 3-nytrotyrosine in the liver. Hydrogen water did not show significant effect on 3-nytrotyrosine generation in the liver. Hydrogen seems to have less effect on peroxynitrite.

With regard to the target cells of ROS in the liver, many studies have implicated oxidative stress in the activation and proliferation of HSCs and, hence in the development of liver fibrosis[28, 29]. However, other studies have shown conflicting data as to the effects of ROS on HSC proliferation and viability[6]. Our study did not show any signs of OH· production when HSC are activated by culturing on plastic dishes. This result indicates hydrogen does not influence HSC activation directly.

On the other hand, H_2 water suppressed hepatocyte death, as demonstrated by inhibition of transaminase release in the H_2-water group. Hepatocyte injuries stimulate the phagocytosis of dead hepatocytes by Kupffer cells[30]. Activated Kupffer cells secrete profibrogenic cytokines such as TGF-β-1, which activate HSCs and eventually lead to liver fibrogenesis[31]. Our vivo study showed the decreased expression of TGF-ß1 mRNA. Decreased induction of TGF-ß1, a particularly potent profibrogenic cytokine, may be a key factor in alleviation of the fibrogenic response by H_2-water. Expression of α-SMA, an indicator for HSC activation, was also inhibited by intake of

H$_2$-water, suggesting that inhibition of TGF-β1 induction results in reduced activation of HSCs, which leads to reduced collagen deposition. Taken together, we presume that H2-water suppresses triggering profibrogenic response by scavanging hydroxyl radicals that induce hepatocyte death and subsequent activation of Kupffer cells, TGF-β1 induction, and HSC activation.

We did not detect any suppressive effect of H$_2$-water on fibrogenesis in BDL model. This differential effect of H$_2$-water on liver fibrosis caused by varying causes is interesting. This difference may be explained by the variation in the initial step triggering the inflammatory response in the 2 models: hepatocyte injury triggers the fibrotic pathway in hepatotoxin models, whereas biliary epithelial cell injury is the primary step triggering fibrosis in the BDL model. Therefore, the absence of the antifibrotic effect of H$_2$-water in the BDL model seems to be concordant with the hepatoprotective properties of H$_2$-water. Moreover, considering our results that indicate H$_2$-water selectively scavenges OH·, the difference can be explained by the variation of ROS contribution in the models: hepatocyte injuries by CCl$_4$ and TAA are OH· dependent injuries, whereas biliary injury by BDL is independent of OH·.

Recent studies have demonstrated that H$_2$ reduces OH· and may have potential for widespread medical application as a novel, safe, and effective antioxidant

with minimal adverse effects[32]. Elimination of OH· is biologically important because although $O_2^{·-}$ and H_2O_2 are detoxified by the antioxidant defense enzymes superoxide dismutase (SOD) and peroxidase or glutathione peroxidase, no enzyme detoxifies OH·[11].

The systemic distribution of H_2 after oral intake of H_2-water has been described previously by Kamimura et al. [33]. They monitored the dynamic movement of hydrogen in rat liver after oral administration of H_2-water by introducing a needle-type hydrogen sensor into the liver. They showed that oral intake of H_2-water helps maintain a sufficient hydrogen concentration in the liver for an hour. Further, H_2-water has already been applied in humans. Clinical trials have revealed that supplementation with H_2-water reduced oxidative stress in patients with type 2 diabetes[34] and those with potential metabolic syndrome[35], thereby indicating that it influenced glucose[34] and cholesterol metabolism[35]_ENREF_38_ENREF_38_ENREF_32_ENREF_32_ENREF_32. These findings indicate that H_2-water can be administered safely to humans and has potential for use as an antifibrotic agent.

In conclusion, H_2-water protects hepatocytes from injury by scavenging OH· and thereby suppresses liver fibrogenesis in mice.

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Figure Captions

Fig. 1 Oral intake of H_2-water suppresses CCl_4-induced liver fibrogenesis in mice. C57BL/6 mice were subjected to repeated injections with CCl_4 and fed with H_2-water or control water. The hepatic hydroxyproline content was significantly lower in the H_2-water group than in the control water group (295.0 vs. 382.6 ng/mg liver; $p < 0.05$) (a). Liver fibrosis, which was assessed by Sirius red staining, was inhibited in the H_2-water group. Fibrotic area with collagen deposit was 8.163% in the control water group and 4.666% in the H_2-water group ($p < 0.0001$), as determined by Sirius red staining (b). The mRNA levels of collagen1α1 in the H_2-water group and the control water group was 4.77 and 11.15 times that in the control, respectively ($p < 0.05$). The mRNA level of α-SMA was 2.15 times that in the controls in the H_2-water group and 3.92 times that in the controls in the control water group ($p < 0.05$) (c). Western blotting for α-SMA in the H_2-water group and the control water group were 1.64 and 2.87 times that in the control, respectively ($p < 0.01$) (d).

Fig. 2 Oral intake of H_2-water suppresses TAA-induced liver fibrogenesis in mice. C57BL/6 mice were subjected to repeated injection with TAA and were fed with H_2-water or control water. Oral intake of H_2-water suppressed liver fibrogenesis, as

assessed by hydroxyproline content (232.7 vs. 287.1 ng/mg liver; $p < 0.05$) (a), Sirius red

staining (b) (2.563 vs. 4.271%; $p < 0.01$), RT-PCR for collagen1α1 (7.88 vs. 59.4 times

that in the control; $p < 0.01$) and α-SMA (2.24 vs. 26.62 times that in the control; $p <$

0.01) (c), and Western blotting for α-SMA (4.86 vs. 9.96 times that in the control; $p <$

0.01) (d).

Fig. 3 Oral intake of H$_2$-water suppresses generation of OH・ in the liver.

C57BL/6 mice were subjected to repeated injections of CCl$_4$ and were fed with H$_2$-water

or control water. Generation of OH・ in the liver in the CCl$_4$ model was assessed by HPF

staining of freshly frozen liver sections. Samples from the H$_2$-water group had a

significant smaller area of HPF staining compared to those from the control group

(0.702% vs. 2.051%, $p < 0.01$) (a). In the TAA model, the H$_2$-water group also showed a

significantly smaller area of HPF staining compared to the control group (0.278% vs.

1.092%, $p < 0.001$) (b). MDA levels in the H$_2$-water group were significantly lower in the

CCl$_4$ model (3.89 vs. 1.57 times that in the control) and TAA model (2.17 vs. 1.41 times

that in the control) (c)

Fig. 4 Oral intake of H$_2$-water did not affect 3-nitrotyrosine expression and GSH.

The 3-nitrotyrosine level in the liver did not differ in the CCl_4 model (20.1 vs. 18.6 times that in the control) (a) and TAA model (4.0 vs. 4.5 times that in the control) (b). GSH level in the liver did not show any difference between the control water group and the hydrogen water group both in the CCl_4 model (3.02 vs. 3.44 μ mol/L) and in the TAA model (3.02 vs. 2.99 μ mol/L) (c).

Fig. 5 Oral intake of H_2-water did not suppress liver fibrogenesis in the BDL-induced liver injury model, in which OH・ was not generated.

In the BDL model, hydroxyproline contents did not show any difference between the H_2-water group and the control group (a). The mRNA levels of collagen1α1 and α-SMA did not differ in the H_2-water group and the control water group (b). Sirius red staining also did not show any suppressive effect of H_2-water on liver fibrosis (c). Sirius red staining also did not show any suppressive effect of H_2-water on liver fibrosis (c). HPF staining of freshly frozen liver sections in the BDL model did not show any signs of OH・ in both the H_2-water and control water groups (d).

Fig. 6 Hydrogen-rich medium suppresses the generation of OH・ and hepatocyte death induced by AMA in hepatocytes.

Hepatocytes were isolated from C57BL/6 mice and treated with 1 μg/ml of AMA. Addition of AMA increased the intracellular levels of OH・, H_2O_2 and $O・_2^-$, as evaluated by measuring fluorescent signals emitted by the oxidized forms of HPF (a), CM-H_2DCFDA (b) and MitoSOX (c), respectively. Hydrogen-rich medium suppressed the generation of OH・ (a), but not of H_2O_2 (d) and $O・_2^-$ (c). Hepatocytes were stained with Hoechst 33342 and PI and observed by fluorescent microscopy (d). AMA induced hepatocyte death. The induction of hepatocyte death was suppressed by culturing in hydrogen-rich medium (13.2% in hydrogen-rich medium vs. 26.8% in control medium; p < 0.001) (d).

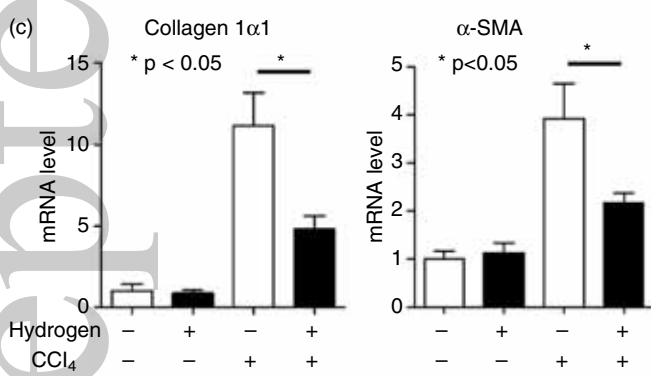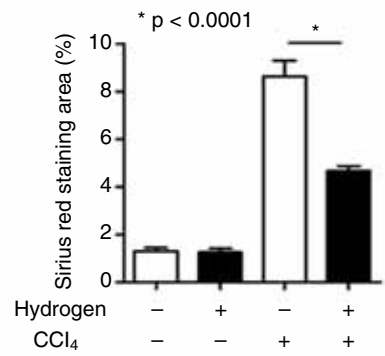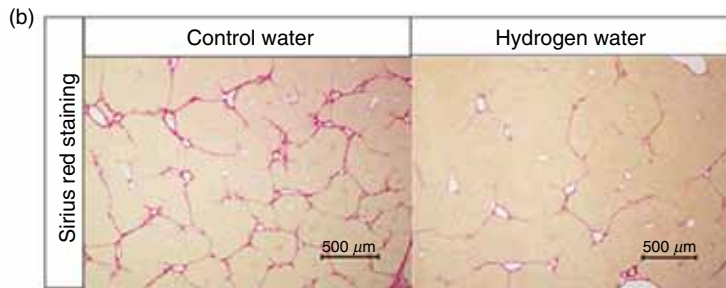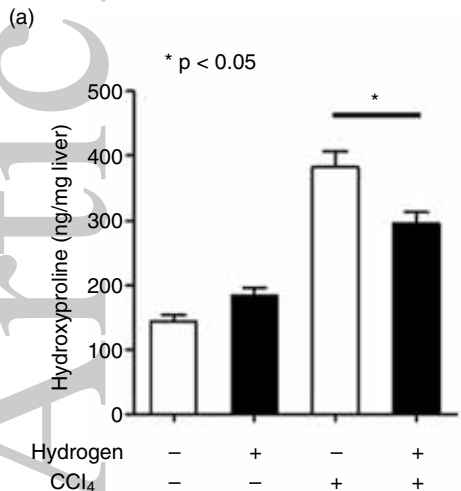Fig. 7 Hydrogen-rich medium does not suppress the activation of cultured HSCs.

HSCs were isolated from C57BL/6 mice and cultured with or without hydrogen-rich medium for 5 days on plastic dishes to allow activation. Hydrogen-rich medium did not suppress the increase in the mRNA levels of HSC activation markers (collagen1α1 and α-SMA) (a). The HSCs showed faint signals of $O・_2^-$, as detected by MitoSOX (b). HSCs activated on plastic dishes showed the production of H_2O_2, as detected by CM-H_2DCFDA (c), but culturing in hydrogen-rich medium did not show any suppressive effect on H_2O_2 production (c). The HSCs did not show the presence of OH・, as detected by HPF
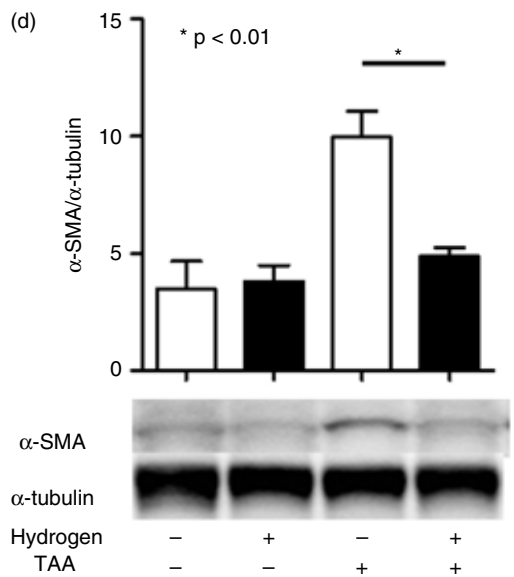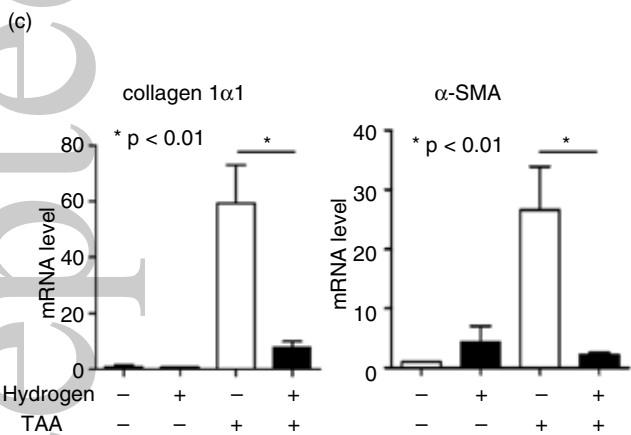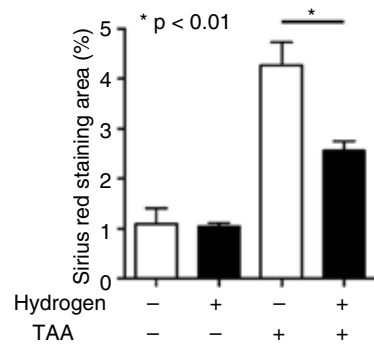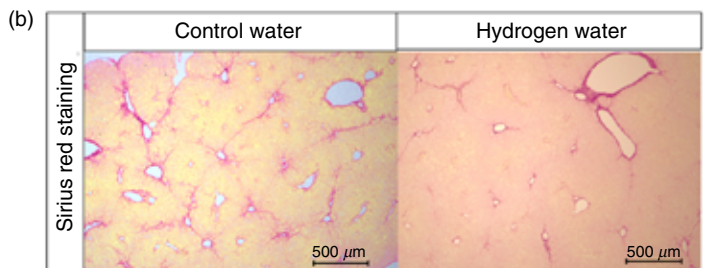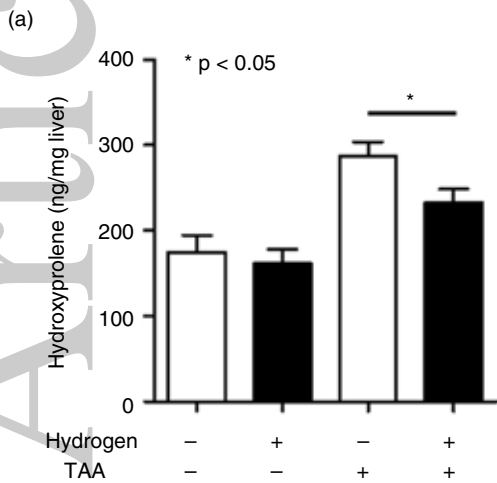
staining (d).

Fig. 8 Hydrogen-rich medium does not suppress or accelerate the HSC activation induced by AMA treatment.

Isolated HSCs were cultured with or without hydrogen-rich medium in the presence of AMA to assess the effect of hydrogen on HSC activation under excess oxidative stress. Culturing in hydrogen-rich medium did not affect the mRNA expression levels of HSC activation markers (collagen1α1 and α-SMA) (a). Although AMA stimulation increased the intracellular level of $O\cdot_2^-$ as detected by MitoSOX (b), culturing in hydrogen-rich medium did not suppress this increase in the $O\cdot_2^-$ level. The H_2O_2 level, as detected by CM-H_2DCFDA, was not altered by AMA stimulation (c). Moreover, we could not detect intracellular OH· even under stimulation with AMA (d).

(a)

* p < 0.05

Hydroxyproline (ng/mg liver)

Hydrogen   −   +   −   +
CCl$_4$ −   −   +   +

(b)

Sirius red staining

Control water   Hydrogen water

500 μm   500 μm

* p < 0.0001

Sirius red staining area (%)

Hydrogen   −   +   −   +
CCl$_4$ −   −   +   +

(c)

Collagen 1α1

* p < 0.05

mRNA level

α-SMA

* p<0.05

mRNA level

Hydrogen   −   +   −   +
CCl$_4$ −   −   +   +

Hydrogen   −   +   −   +
CCl$_4$ −   −   +   +

(d)

* p < 0.01

α-SMA/α-tubulin

α-SMA

α-tubulin

Hydrogen   −   +   −   +
CCl$_4$ −   −   +   +

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(a)

* p < 0.05

Hydroxyprolene (ng/mg liver)

| Hydrogen | − | + | − | + |
| TAA | − | − | + | + |

(b)

Sirius red staining

Control water | Hydrogen water

500 μm | 500 μm

* p < 0.01

Sirius red staining area (%)

| Hydrogen | − | + | − | + |
| TAA | − | − | + | + |

(c)

collagen 1α1

* p < 0.01

mRNA level

α-SMA

* p < 0.01

mRNA level

| Hydrogen | − | + | − | + |
| TAA | − | − | + | + |

(d)

* p < 0.01

α-SMA/α-tubulin

α-SMA

α-tubulin

| Hydrogen | − | + | − | + |
| TAA | − | − | + | + |

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(a)

	Control water	Hydrogen water
Corn oil		
CCl$_4$		

* p < 0.01

HPF staining area (%)

Hydrogen	−	+	−	+
CCl$_4$	−	−	+	+

(b)

	Control water	Hydrogen water
Normal saline		
TAA		

* p < 0.0005

HPF staining area (%)

Hydrogen	−	+	−	+
TAA	−	−	+	+

(c)

* p < 0.01

MDA/Albumin

Hydrogen	−	+	−	+
CCl$_4$	−	−	+	+

* p < 0.05

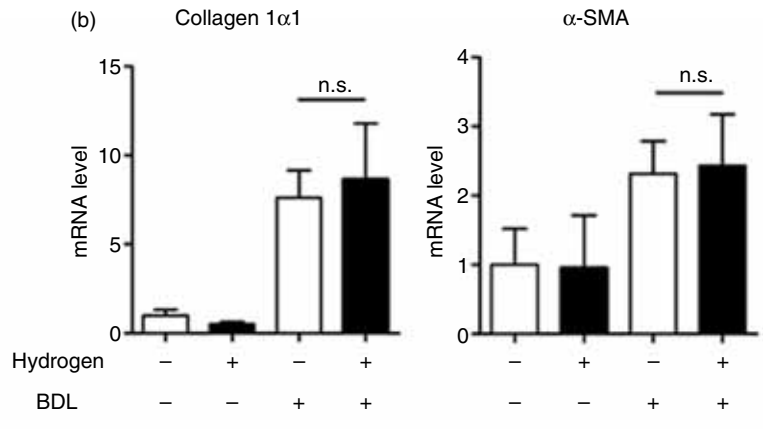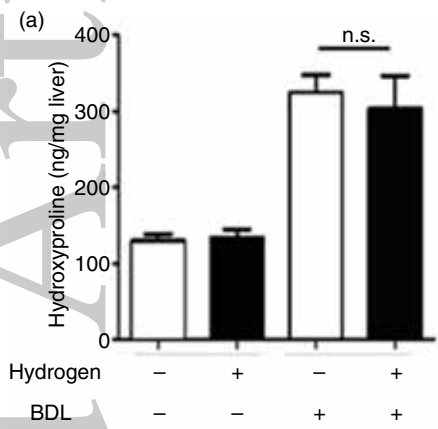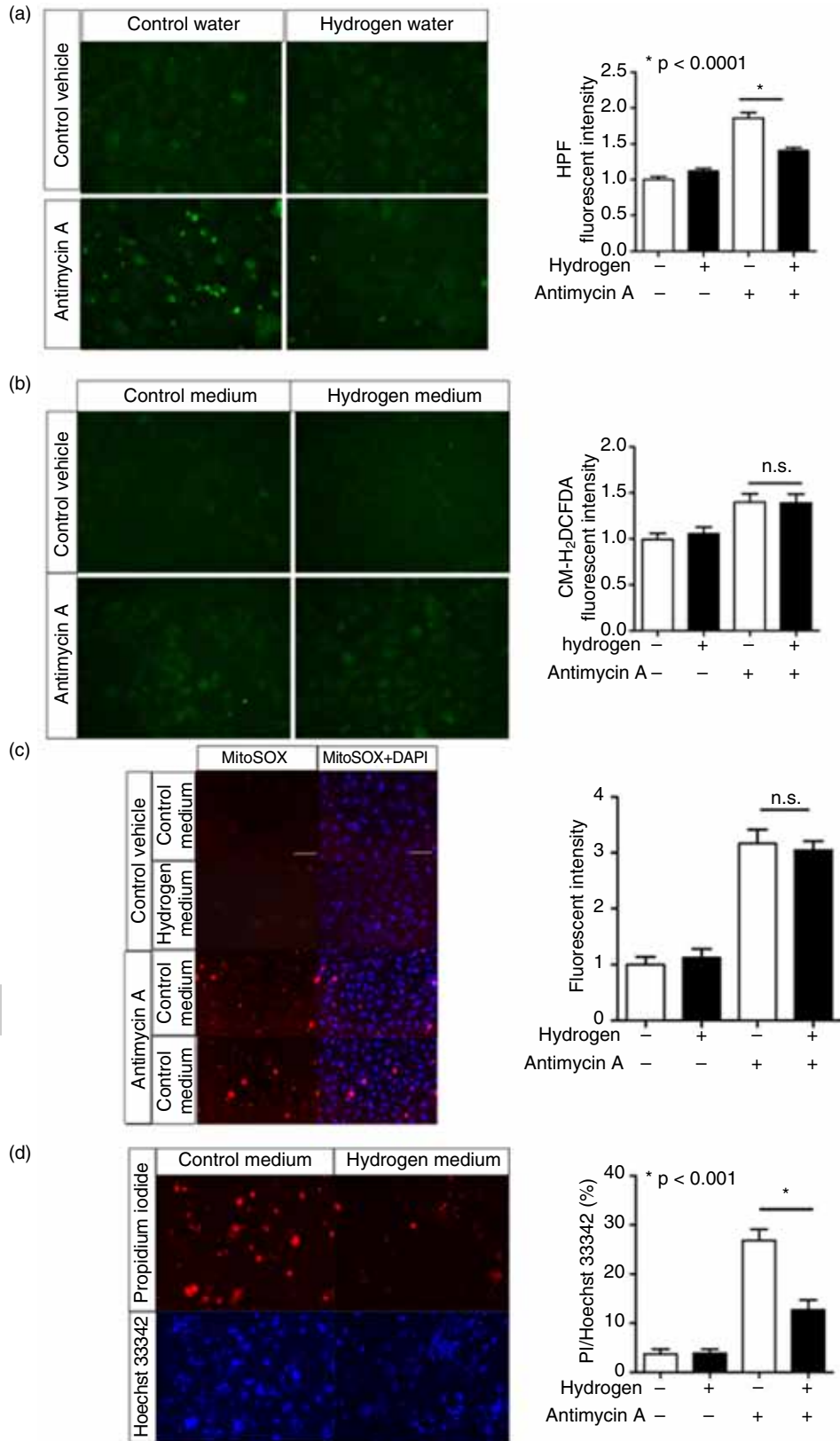MDA/Albumin

Hydrogen	−	+	−	+
TAA	−	−	+	+

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(a)

Hydroxyproline (ng/mg liver)

n.s.

| Hydrogen | − | + | − | + |
| BDL | − | − | + | + |

(b)

Collagen 1α1

mRNA level

n.s.

α-SMA

mRNA level

n.s.

| Hydrogen | − | + | − | + |
| BDL | − | − | + | + |

(c)

Sirius red staining

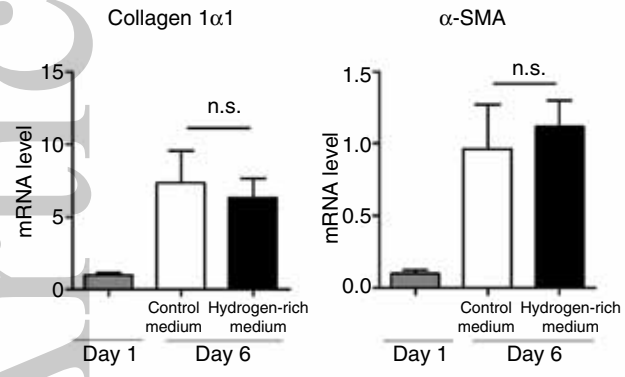| Pure water | Hydrogen water |

(d)

	Control water	Hydrogen water
Sham operation		
BDL		

100μm

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(a)

Control water   Hydrogen water

Control vehicle

Antimycin A

* p < 0.0001

2.5
2.0
1.5
1.0
0.5
0.0

HPF fluorescent intensity

*

Hydrogen   −   +   −   +
Antimycin A   −   −   +   +

(b)

Control medium   Hydrogen medium

Control vehicle

Antimycin A

2.0
1.5
1.0
0.5
0.0

CM-H$_2$DCFDA fluorescent intensity

n.s.

hydrogen   −   +   −   +
Antimycin A   −   −   +   +

(c)

MitoSOX   MitoSOX+DAPI

Control vehicle — Control medium

Hydrogen medium

Antimycin A — Control medium

Control medium

4
3
2
1
0

Fluorescent intensity

n.s.

Hydrogen   −   +   −   +
Antimycin A   −   −   +   +

(d)

Control medium   Hydrogen medium

Propidium iodide

Hoechst 33342

40
30
20
10
0

PI/Hoechst 33342 (%)

* p < 0.001

*

Hydrogen   −   +   −   +
Antimycin A   −   −   +   +

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(a)

Collagen 1α1

α-SMA

(b)

| | MitoSOX | Phase contrast |

Hydrogen water

Control water

(c)

| | DCFDA | Phase contrast |

Hydrogen water

Control water

(d)

| | HPF | Phase contrast |

Hydrogen water

Control water

(e)

CM-H$_2$DCFDA

n.s.

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(a)

Collagen 1α1

α-SMA

(b) MitoSOX / Phase contrast

Mito SOX

(c) CM-H₂DCFDA / Phase contrast

CM-H₂DCFDA

(d) HPF / Phase contrast

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