

**Fig. 13.** Hepatocyte-protective effect of WHP. (A, B) Hepatocyte-protective effect in vivo for isolated hepatocytes from rats fed a WHP diet. The propidium iodide-positive ratios of cultured hepatocytes were significantly lower in the WHP diet group compared with the control diet group. (C) Hepatocyte-protective effect in vitro for hepatocytes with WHP-added medium. The propidium iodide-positive ratios were markedly lower in the D+W+ group compared with the D+P- group. \* $P < 0.05$ ; NS, not significant ( $P \geq 0.05$ ). PI, propidium iodide; WHP, whey-hydrolyzed peptide.

## Conclusion

Our results clearly demonstrated that a WHP-enriched IMD effectively prevented DMN-induced liver fibrosis/cirrhosis in rats, possibly via a direct hepatocyte-protective effect and antioxidant effect through GSH synthesis. WHP may have therapeutic potential and be useful as an antifibrotic agent in the liver.

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**Original Article**

## Effects of oral intake of hydrogen water on liver fibrogenesis in mice

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**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 treating liver fibrosis. Hydrogen water is reported to have potential as a therapeutic tool for 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.

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

**Key words:** hydrogen, hydrogen water, hydroxyl radical, liver cirrhosis, liver fibrosis, liver injury

### INTRODUCTION

LIVER FIBROSIS IS a common cause of death worldwide.<sup>1</sup> 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.<sup>2</sup> Sustained hepatocyte injuries such as those caused by hepatitis viruses and alcohol, lead to the activation of HSC and hepatocyte death, which is induced by the activation of inflammatory mediators

such as transforming growth factor (TGF)-β1.<sup>3</sup> HSC are activated to differentiate into myofibroblast-like cells, which promote collagen deposition.

Reactive oxygen species (ROS) play an important role in hepatic fibrosis.<sup>4</sup> Galli *et al.* reported that the superoxide anion ( $O_2^{\cdot-}$ ) produced by xanthine and xanthine oxidase induced the proliferation of HSC.<sup>5</sup> On the other hand, Sandra *et al.* reported that rather than directly inducing HSC proliferation, hydrogen peroxide ( $H_2O_2$ ) and  $O_2^{\cdot-}$  inhibited the proliferation of these cells.<sup>6</sup> 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  $O_2^{\cdot-}$  is generated in the mitochondria by electron leakage from the electron-transport chain and the Krebs cycle.<sup>7,8</sup>  $O_2^{\cdot-}$  is also generated by metabolic oxidases, including nicotinamide adenine dinucleotide phosphate oxidase and

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xanthine oxidase.<sup>5,9</sup> Superoxide dismutase converts  $O_2^{\cdot-}$  into  $H_2O_2$ . Excess  $O_2^{\cdot-}$  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.<sup>10</sup>

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  $OH\cdot$ .<sup>11</sup> 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.<sup>12</sup> Sun *et al.* reported that i.p. injection with hydrogen-rich saline was also effective in a model of carbon tetrachloride ( $CCl_4$ )-induced liver fibrosis.<sup>12</sup> 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

**M**ALE 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 i.p. with 0.5  $\mu$ L of  $CCl_4$  per gram mouse weight twice a week for 6 weeks ( $n = 20$  for hydrogen water and control water group).<sup>13</sup> Corn oil was injected as a control ( $n = 3$  for each group). In the TAA model, the mice were injected i.p. with escalating dose of TAA twice a week for 6 weeks (first dose, 100  $\mu$ g/g; weeks 1–2, 200  $\mu$ g/g; week 3, 4300  $\mu$ g/g; weeks 4–6, 400  $\mu$ g/g) ( $n = 6$  for each group).<sup>14</sup> 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 (50 mg/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 killed 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

Hydrogen water at the concentration of 1.24 mg/L and control water were provided in 300-mL aluminum pouches supplied by Irom Pharmaceutical (Tokyo, Japan). The mice were provided water ad libitum through closed glass vessels (Irom Pharmaceutical) equipped with an outlet line containing two 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.<sup>15</sup> 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.<sup>16</sup> The isolated hepatocytes were cultured on six-well plates coated with type 1 collagen, at a cell density of  $5 \times 10^5$  cells/well, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin at 37°C in a humidified atmosphere of 5%  $CO_2$  and 95% air.

### Isolation and culturing of HSC

Hepatic stellate cells were isolated from mouse livers by a method described previously.<sup>17,18</sup> 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 and antibiotics. The isolated HSC was incubated in a  $CO_2$  incubator at 37°C. The purity of HSC was consistently more than 98% as judged by presence of lipid droplets and autofluorescence.

To analyze whether the hydrogen-rich medium suppresses HSC activation, the isolated HSC 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, 1 µg/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 bisbenzimidazole H33342 fluorochrome dihydrochloride dimethylsulfoxide solution (Hoechst 33342; Nacalai Tesque, Kyoto, Japan) and propidium iodide (PI; Calbiochem, San Diego, CA, USA) and examined under a fluorescence microscope. Five non-overlapping 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<sub>2</sub><sup>·-</sup> and H<sub>2</sub>O<sub>2</sub>, the cells were incubated with MitoSOX, 2',7'-dichlorodihydrofluorescein (CM-H<sub>2</sub>DCFDA) and hydroxyphenyl fluorescein (HPF; Nikken Seil, Shizuoka Japan), respectively.<sup>19,20</sup>

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, 1 µg/mL AMA (Sigma-Aldrich) 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 optimal cutting temperature 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 immunoglobulin (Ig)G 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, Burlingame, CA, USA), and then incubated sequentially with 3-nitrotyrosine-recognizing primary antibody (Mab5404; Merck) at 1:100 dilution overnight at 4°C, MOM biotinylated antimouse 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.<sup>21</sup> Images of five non-overlapping 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 microliters of 50% tricarboxylic acid (TCA) was added and the homogenates were further incubated on ice for 30 min. The precipitated pellets were hydrolyzed for 24 h at 110°C in 6 N HCL. Samples were filtered and neutralized with 10 N NaOH. Hydrolysates were then oxidized with chloramine-T (Sigma, St Louis, MO, USA) for 25 min at room temperature. The reaction mixture was incubated in Ehrlich'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 malondialdehyde (MDA) level was determined according to the thiobarbituric acid method using an NWLSS Malondialdehyde Assay Kit (Northwest Life Science Specialties, Vancouver, WA, USA).

Glutathione (GSH) level was determined with a 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.<sup>16</sup> The primary antibodies and dilutions were as follows: anti-α-SMA antibody (no. ab5694; Abcam) at 1:1000; and anti-α-tubulin antibody (no. CP06; Calbiochem) at 1:1000.

### Quantitative reverse transcription polymerase chain reaction analysis

Total RNA was extracted from the liver sample or isolated HSC 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 polymerase chain reaction (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 glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for liver samples, and to 18S ribosomal RNA for HSC samples, as the internal control. The primer sequences used were: Col-1 $\alpha$ 1, forward 5'-GCT CCT CIT AGG GGC CAC T-3', reverse 5'-CCA CGT CTC ACC ATT GGG G-3';  $\alpha$ -SMA, forward 5'-GTC CCA GAC ATC AGG GAG TAA-3', reverse 5'-TCG GAT ACT TCA GCG TCA GGA-3'; TGF- $\beta$ 1, forward 5'-CCG CAA CAA CGC CAT CIA TG-3', reverse 5'-CCC GAA TGT CTG ACG TAT TGG AAG-3'; GAPDH, forward 5'-AGG TCG GTG TGA ACG GAT TTG-3', reverse 5'-TGT AGA CCA TGT AGT TGA GGT CA-3'; and 18S ribosomal RNA, forward 5'-AGT CCC TGC CCT TTG TAC ACA-3', reverse 5'-CGA TCC GAG GGC CTC ACT A-3'.

### Statistical analysis

Each quantitative dataset was presented as mean  $\pm$  standard error of the mean, and statistically analyzed with two-tailed Student's *t*-test followed by a post-hoc Fisher's protected least significant difference test.

## RESULTS

### Oral intake of H<sub>2</sub>-water suppressed liver fibrogenesis in models of CCl<sub>4</sub>- and TAA-induced liver injury

**I**N THE CCl<sub>4</sub> model, hepatic hydroxyproline content was significantly lower in the H<sub>2</sub>-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<sub>2</sub>-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<sub>2</sub>-water group ( $P < 0.0001$ ) (Fig. 1b). The inhibitory effect of H<sub>2</sub>-water on liver fibrogenesis were further evaluated by RT-PCR for collagen1 $\alpha$ 1 and  $\alpha$ -SMA. In the H<sub>2</sub>-water group and control water group, the mRNA levels of collagen1 $\alpha$ 1 were 4.77 and 11.55 times that in the control ( $P < 0.05$ ),

while those of  $\alpha$ -SMA were 2.15 and 3.92 times that in the control ( $P < 0.05$ ) (Fig. 1c), respectively. Western blotting showed that the  $\alpha$ -SMA levels in the H<sub>2</sub>-water group and control water group was 1.64 and 2.87 times that in the control, respectively ( $P < 0.01$ ) (Fig. 1d). H<sub>2</sub>-water also suppressed the mRNA level of TGF- $\beta$ 1 (3.03 vs 1.74 times as control;  $P < 0.05$ ) in the liver.

In the TAA model, H<sub>2</sub>-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 collagen1 $\alpha$ 1 (7.88 vs 59.4 times that in the control;  $P < 0.01$ ) and  $\alpha$ -SMA (2.24 vs 26.62 times that in the control;  $P < 0.01$ ) (Fig. 2c), and western blotting for  $\alpha$ -SMA (4.86 vs 9.96 times that in the control;  $P < 0.01$ ) (Fig. 2d). H<sub>2</sub>-water suppressed the mRNA level of TGF- $\beta$ 1 (3.37 vs 2.07 times in the control;  $P < 0.05$ ) in the liver.

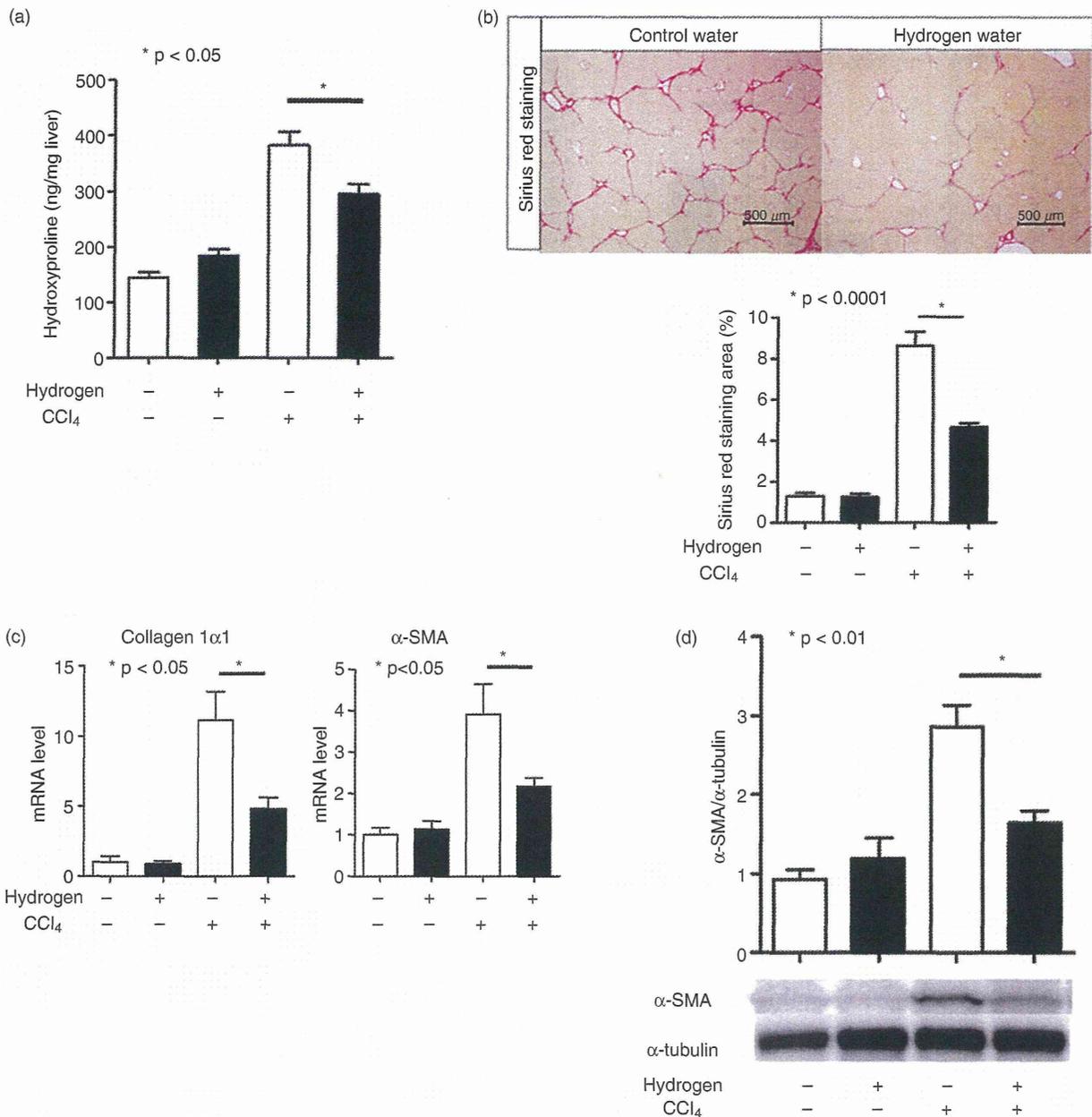
### H<sub>2</sub>-water oral intake suppressed generation of hydroxyl radicals in the liver

Hydroxyphenyl fluorescein staining for the detection of OH $\cdot$  on freshly frozen liver sections in the CCl<sub>4</sub> model showed a significantly smaller area of HPF staining in the H<sub>2</sub>-water group (Fig. 3a) than in the control water group; this indicated that orally ingested H<sub>2</sub>-water had a scavenging effect on OH $\cdot$  in the liver. These results demonstrated that CCl<sub>4</sub> injection induced the release of OH $\cdot$  and that H<sub>2</sub>-water scavenged OH $\cdot$ . In the TAA model, HPF staining was present in a significantly smaller area in the H<sub>2</sub>-water group (Fig. 3b).

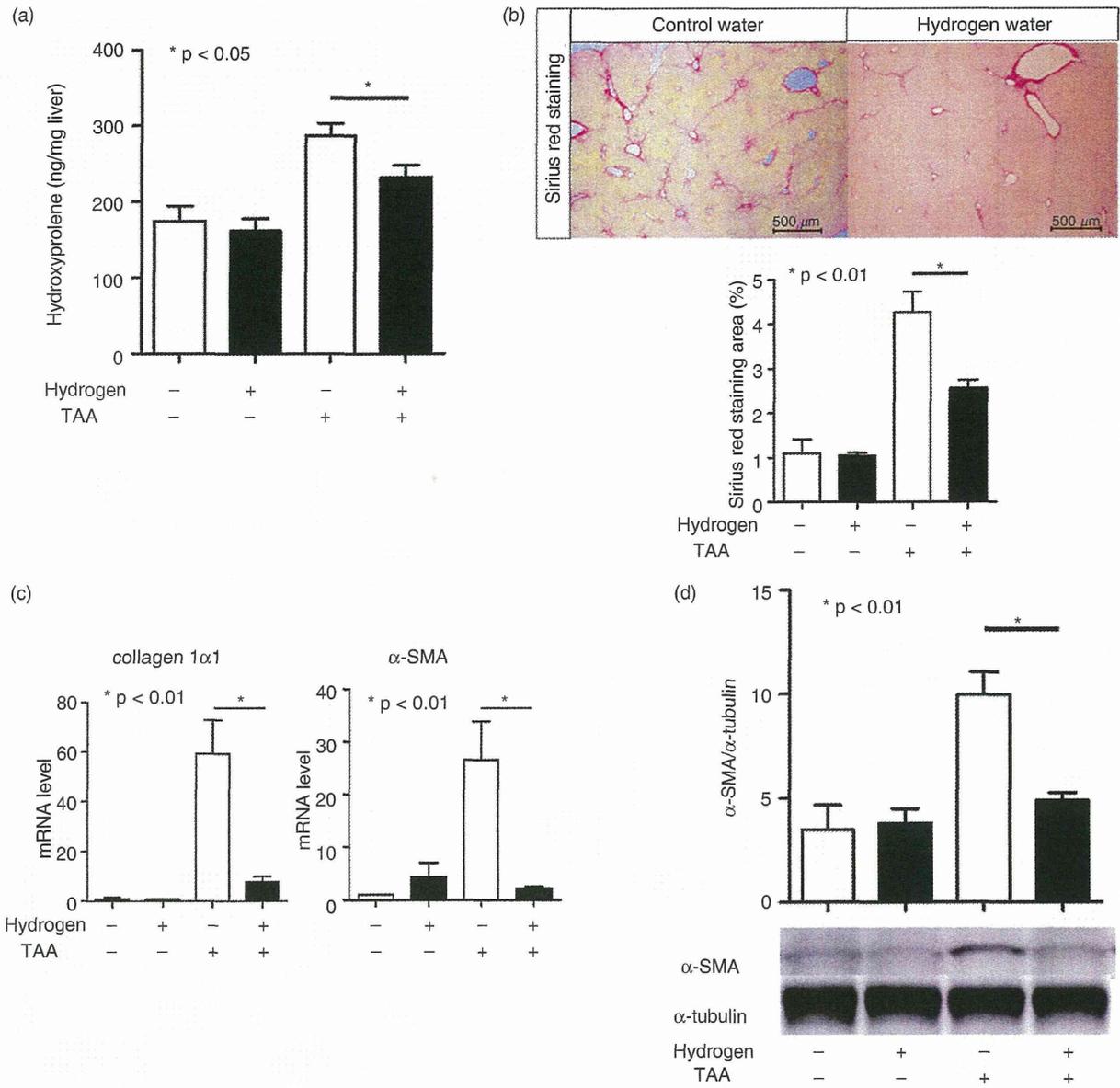
Malondialdehyde levels in the H<sub>2</sub>-water group were significantly lower in the CCl<sub>4</sub> model (3.89 vs 1.57 times that in the control) and TAA model (2.17 vs 1.41 times that in the control) (Fig. 3c). These results supports the antioxidative effect of H<sub>2</sub>-water on OH $\cdot$  which generates lipid peroxidation. H<sub>2</sub>-water did not change 3-nitrotyrosine expression (Fig. 4a,b) and GSH level (Fig. 4c).

### Inhibition of CCl<sub>4</sub>- or TAA-induced liver fibrosis by H<sub>2</sub>-water was associated with less hepatocyte damage

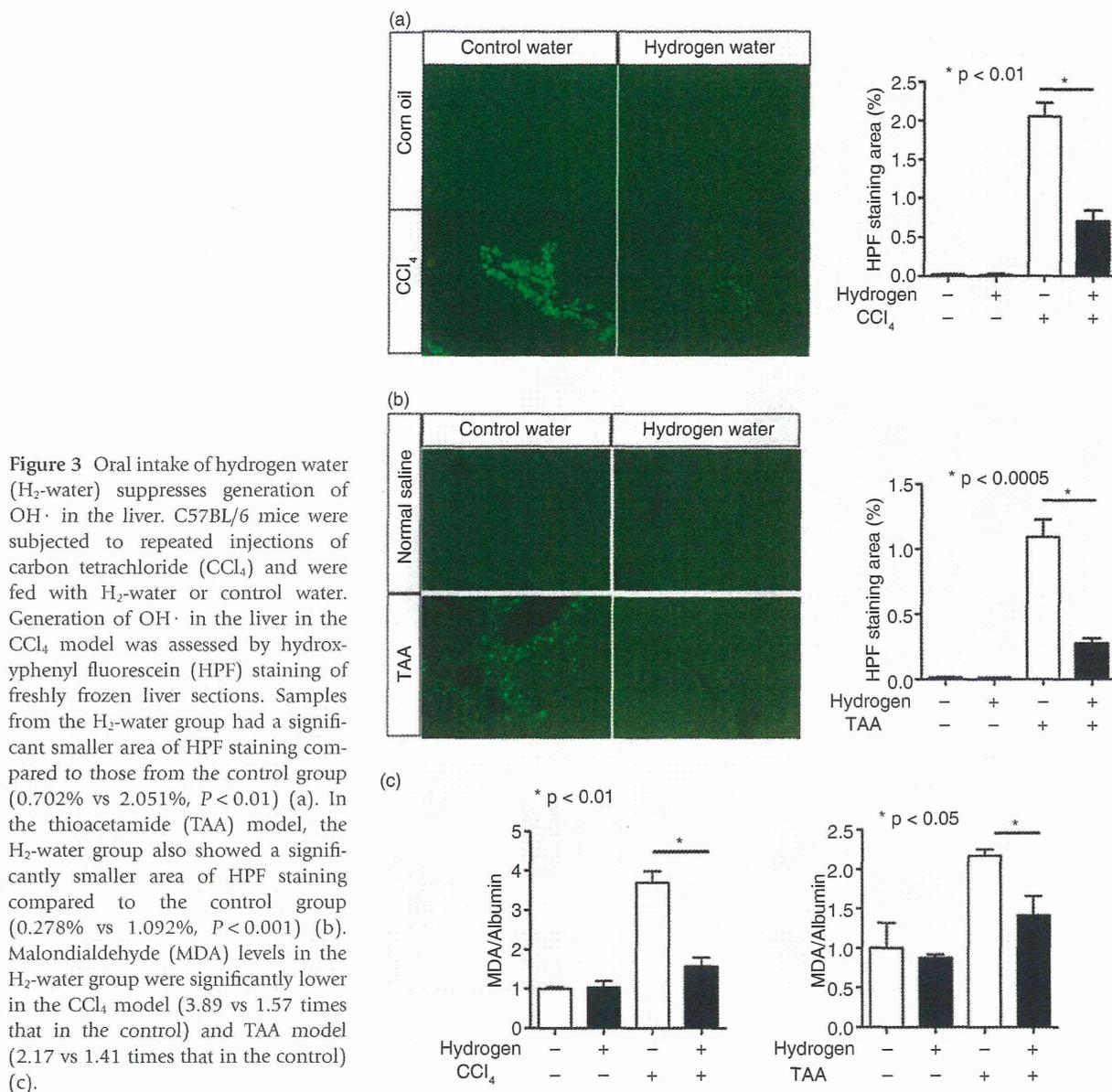
The H<sub>2</sub>-water group showed significantly lower transaminase levels in CCl<sub>4</sub> model at day 1 after CCl<sub>4</sub> injection (aspartate aminotransferase [AST], 2532  $\pm$  697 [H<sub>2</sub>-water] vs 5133  $\pm$  908 IU/L [control water]; alanine aminotransferase [ALT], 3441  $\pm$  790 [H<sub>2</sub>-water] vs 6756  $\pm$  1166 IU/L [control water]) and in TAA model at day 1 after TAA injection (AST, 1066  $\pm$  460 [H<sub>2</sub>-water] vs 2906  $\pm$  834 IU/L [control water]; ALT, 1197  $\pm$  442 [H<sub>2</sub>-water] vs 4018  $\pm$  997 IU/L [control water]).



**Figure 1** Oral intake of H<sub>2</sub>-water suppresses carbon tetrachloride (CCl<sub>4</sub>)-induced liver fibrogenesis in mice. C57BL/6 mice were subjected to repeated injections with CCl<sub>4</sub> and fed with hydrogen water (H<sub>2</sub>-water) or control water. The hepatic hydroxyproline content was significantly lower in the H<sub>2</sub>-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<sub>2</sub>-water group. Fibrotic area with collagen deposit was 8.163% in the control water group and 4.666% in the H<sub>2</sub>-water group ( $P < 0.0001$ ), as determined by Sirius red staining (b). The mRNA levels of collagen1α1 in the H<sub>2</sub>-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-smooth muscle actin (α-SMA) was 2.15 times that in the controls in the H<sub>2</sub>-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<sub>2</sub>-water group and the control water group were 1.64 and 2.87 times that in the control, respectively ( $P < 0.01$ ) (d).



**Figure 2** Oral intake of hydrogen water (H<sub>2</sub>-water) suppresses thioacetamide (TAA)-induced liver fibrogenesis in mice. C57BL/6 mice were subjected to repeated injection with TAA and were fed with H<sub>2</sub>-water or control water. Oral intake of H<sub>2</sub>-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), reverse transcription polymerase chain reaction for collagen1α1 (7.88 vs 59.4 times that in the control; *P* < 0.01) and α-smooth muscle actin (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).



**Figure 3** Oral intake of hydrogen water (H<sub>2</sub>-water) suppresses generation of OH· in the liver. C57BL/6 mice were subjected to repeated injections of carbon tetrachloride (CCl<sub>4</sub>) and were fed with H<sub>2</sub>-water or control water. Generation of OH· in the liver in the CCl<sub>4</sub> model was assessed by hydroxyphenyl fluorescein (HPF) staining of freshly frozen liver sections. Samples from the H<sub>2</sub>-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 thioacetamide (TAA) model, the H<sub>2</sub>-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). Malondialdehyde (MDA) levels in the H<sub>2</sub>-water group were significantly lower in the CCl<sub>4</sub> 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).

**Oral intake of H<sub>2</sub>-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<sub>2</sub>-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<sub>2</sub>-water or the control water groups (Fig. 5d).

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

Antimycin A, a complex III inhibitor in the respiratory transduction system, induces the release of O<sub>2</sub>·-. Hepatocytes were isolated from C57BL/6 mice and treated

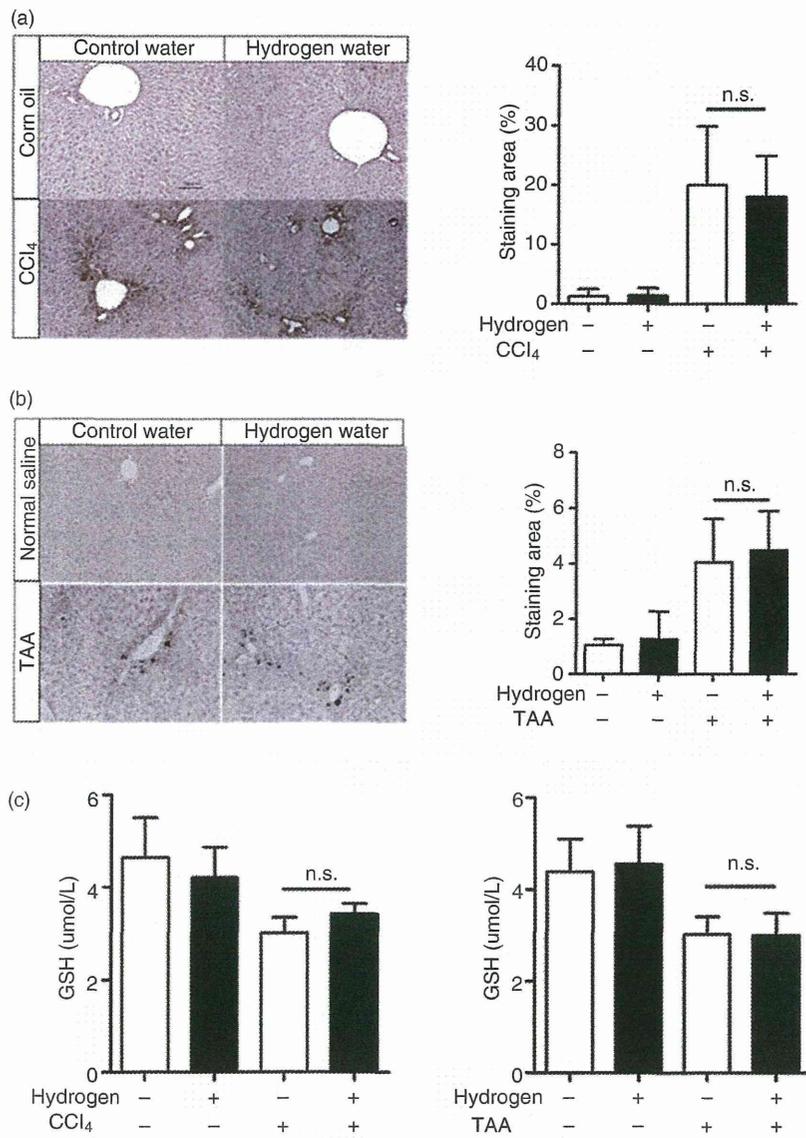


Figure 4 Oral intake of hydrogen water (H<sub>2</sub>-water) did not affect 3-nitrotyrosine expression and glutathione (GSH). The 3-nitrotyrosine level in the liver did not differ in the carbon tetrachloride (CCl<sub>4</sub>) model (20.1 vs 18.6 times that in the control) (a) and thioacetamide (TAA) 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<sub>4</sub> model (3.02 vs 3.44 μmol/L) and in the TAA model (3.02 vs 2.99 μmol/L) (c).

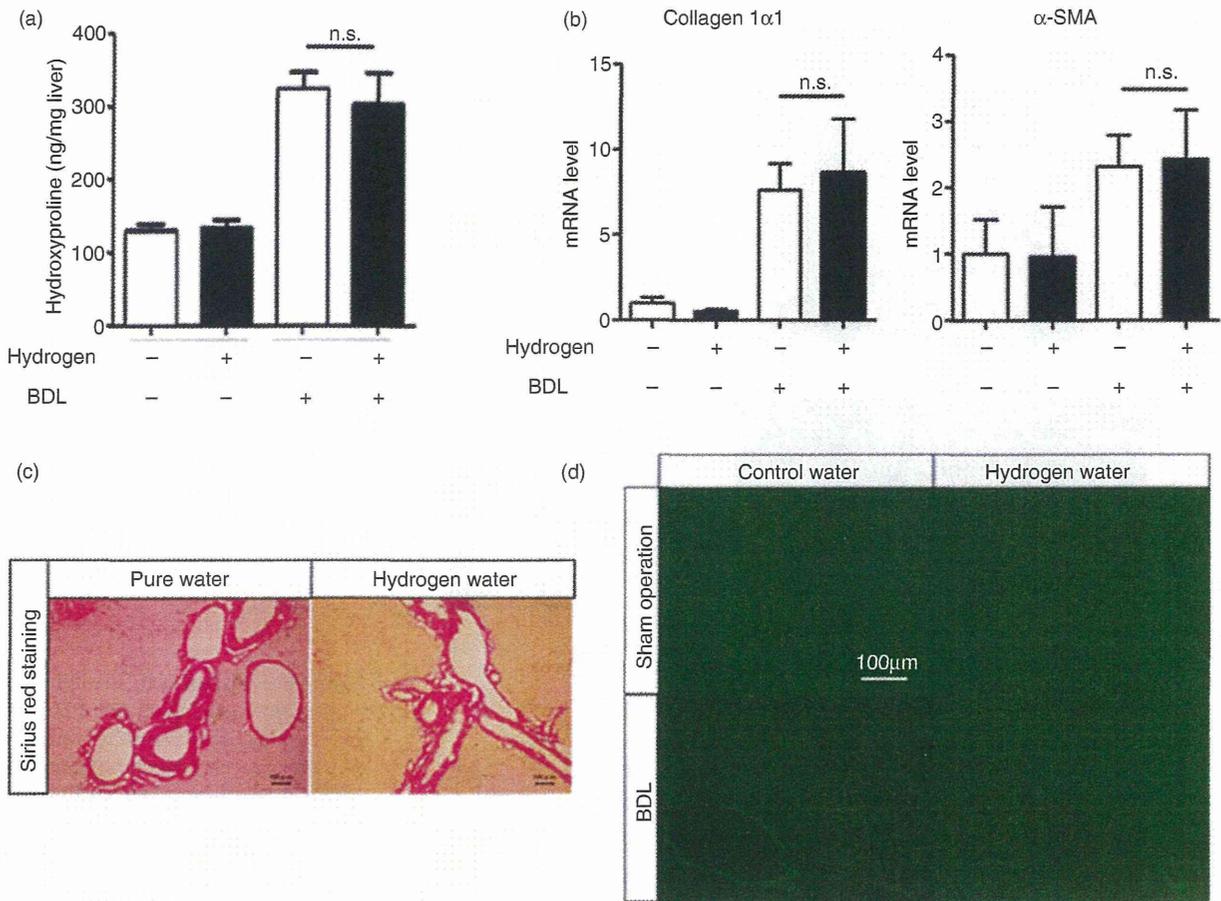
with 1 μg/mL of AMA. The addition of AMA increased levels of OH·, H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>·-</sup>, as evaluated by the intensity of fluorescence signals emitted by the oxidized forms of HPF (Fig. 6a), CM-H<sub>2</sub>DCFDA (Fig. 6b) and MitoSOX (Fig. 6c), respectively. Culturing in hydrogen-rich medium suppressed the generation of OH· (Fig. 6a) induced by AMA, but did not affect the increased level of H<sub>2</sub>O<sub>2</sub> (Fig. 6b) or O<sub>2</sub><sup>·-</sup> (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 HSC

We assessed the effect of culturing in hydrogen-rich medium on the activation of HSC, which are the principal fibrogenic cells in the liver. HSC are known to be activated when cultured on a plastic dish. RT-PCR for collagen1α1 and α-SMA of HSC that were cultured on a plastic dish for 5 days revealed that the hydrogen-rich medium did not suppress the activation of cultured

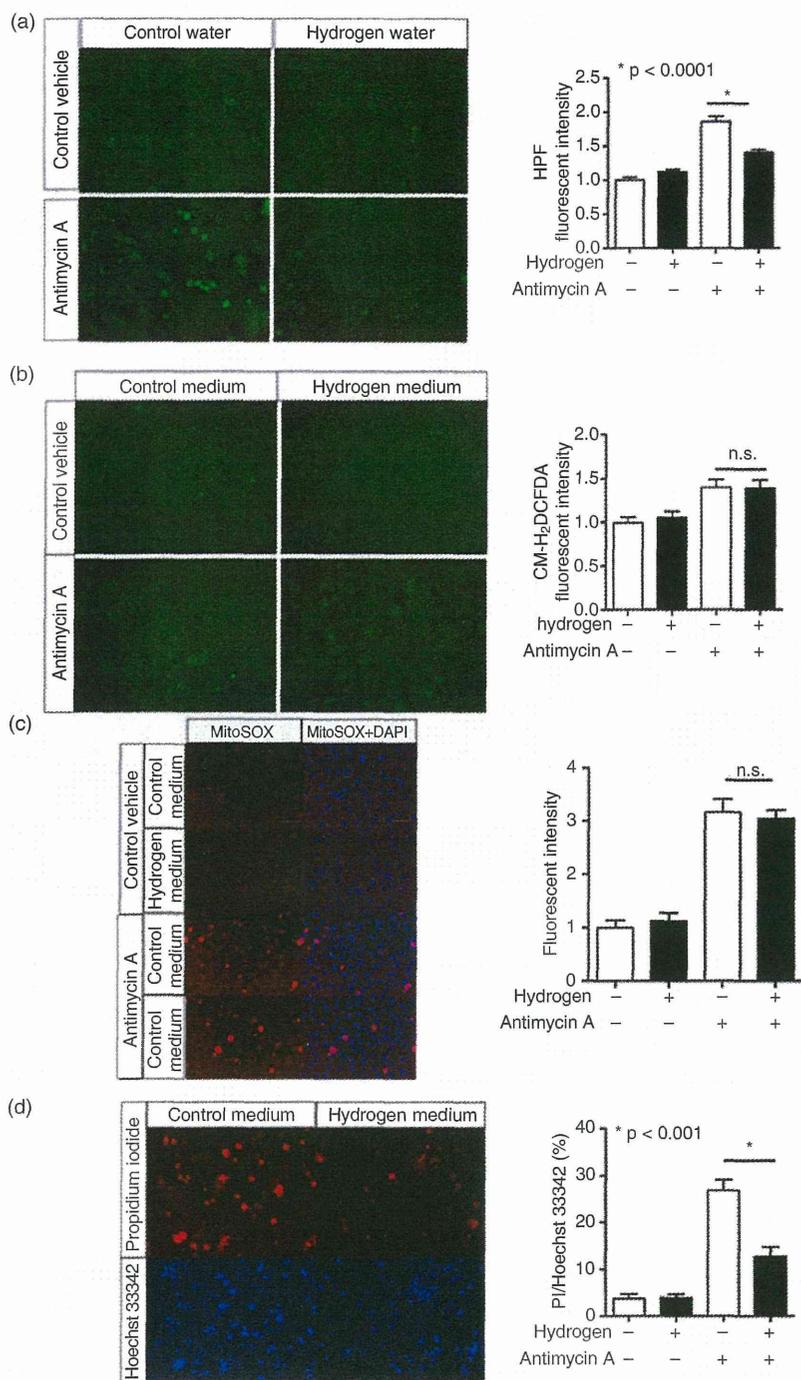


**Figure 5** Oral intake of hydrogen water (H<sub>2</sub>-water) did not suppress liver fibrogenesis in the bile duct ligation (BDL)-induced liver injury model, in which OH· was not generated. In the BDL model, hydroxyproline contents did not show any difference between the H<sub>2</sub>-water group and the control group (a). The mRNA levels of collagen1α1 and α-smooth muscle actin (α-SMA) did not differ in the H<sub>2</sub>-water group and the control water group (b). Sirius red staining also did not show any suppressive effect of H<sub>2</sub>-water on liver fibrosis (c). Sirius red staining also did not show any suppressive effect of H<sub>2</sub>-water on liver fibrosis (c). Hydroxyphenyl fluorescein staining of freshly frozen liver sections in the BDL model did not show any signs of OH· in both the H<sub>2</sub>-water and control water groups (d).

HSC (Fig. 7a). The activated HSC showed traces of O<sub>2</sub><sup>-·</sup>, as detected by MitoSOX staining (Fig. 7b). The activated HSC contained H<sub>2</sub>O<sub>2</sub>, as evidenced by vivid staining with CM-H<sub>2</sub>DCFDA (Fig. 7c), suggesting that culturing in the hydrogen-rich medium did not suppress the accumulation of H<sub>2</sub>O<sub>2</sub>. The HSC did not contain OH·, as evaluated by HPF staining (Fig. 7d). These results imply that OH· is not associated with the activation of cultured HSC and H<sub>2</sub>-water does not seem to target HSC.

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

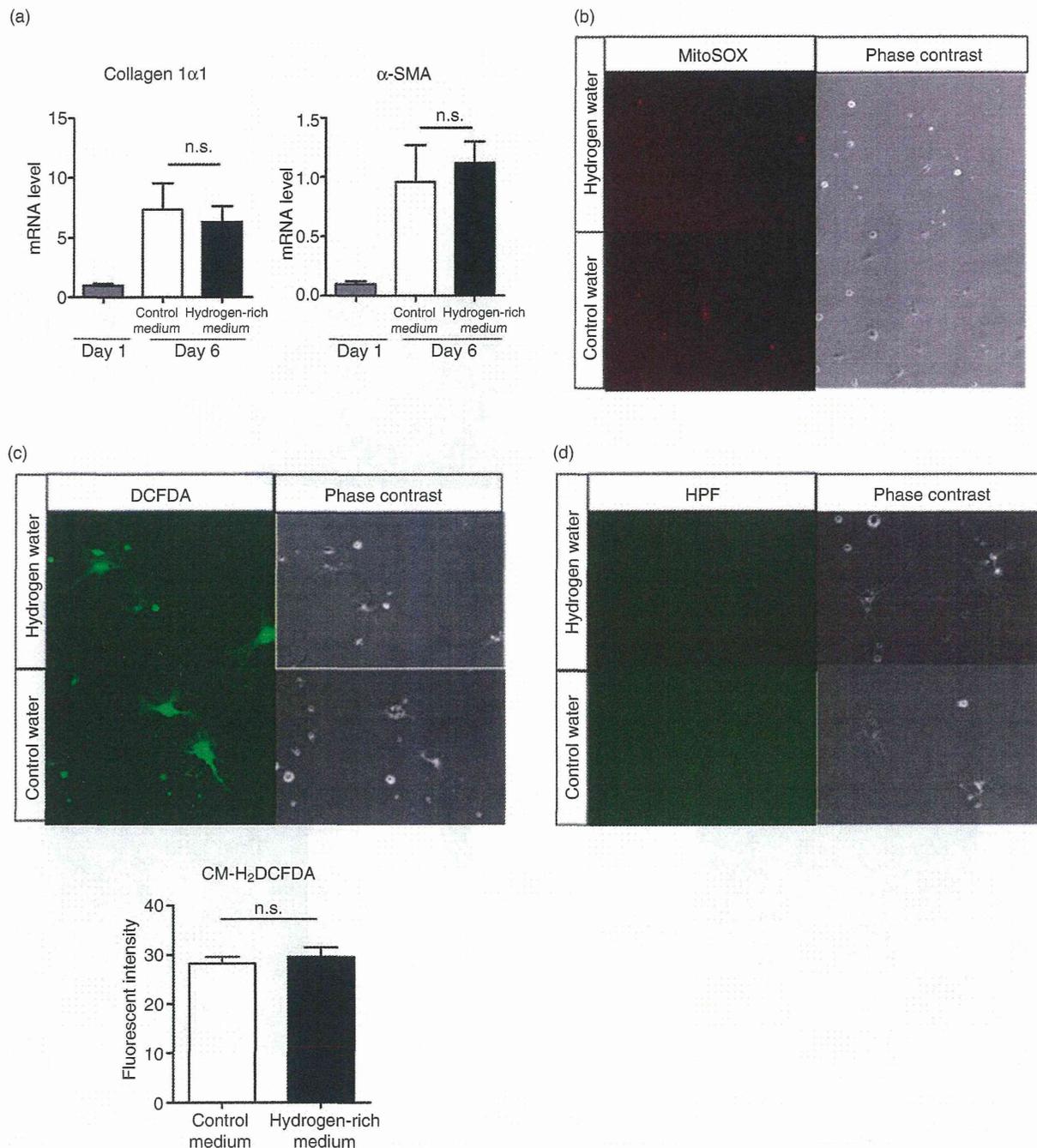
Isolated HSC were cultured with or without hydrogen-rich medium in the presence of AMA to assess the effect of hydrogen on HSC activation under excess oxidative stress. Culturing in the hydrogen-rich medium did not affect the mRNA expression levels of the activation markers for HSC (collagen1α1 and α-SMA) (Fig. 8a). The increased intracellular O<sub>2</sub><sup>-·</sup> levels induced by AMA



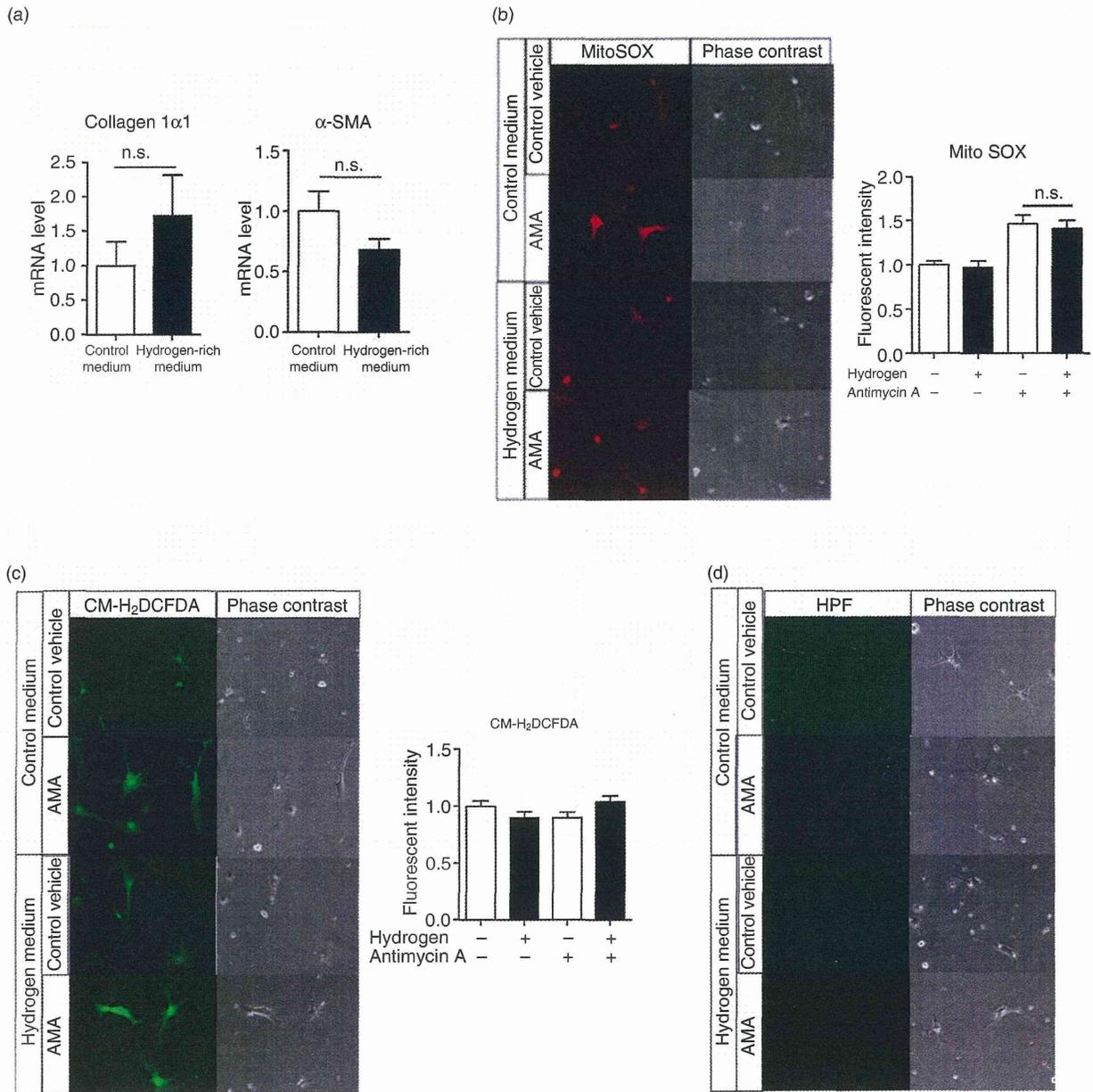
**Figure 6** Hydrogen-rich medium suppresses the generation of  $\text{OH}\cdot$  and hepatocyte death induced by antimycin A (AMA) in hepatocytes. Hepatocytes were isolated from C57BL/6 mice and treated with  $1\ \mu\text{g}/\text{mL}$  of AMA. Addition of AMA increased the intracellular levels of  $\text{OH}\cdot$ ,  $\text{H}_2\text{O}_2$  and  $\text{O}_2\cdot^-$ , as evaluated by measuring fluorescent signals emitted by the oxidized forms of hydroxyphenyl fluorescein (HPF) (a), 2',7'-dichlorodihydrofluorescein (CM- $\text{H}_2\text{DCFDA}$ ) (b) and MitoSOX (c), respectively. Hydrogen-rich medium suppressed the generation of  $\text{OH}\cdot$  (a), but not of  $\text{H}_2\text{O}_2$  (d) and  $\text{O}_2\cdot^-$  (c). Hepatocytes were stained with Hoechst 33342 and propidium iodide (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). DAPI, 4',6'-diamidino-2-phenylindole dihydrochloride.

stimulation and detected by MitoSOX were not suppressed by culturing in hydrogen-rich medium (Fig. 8b). The  $\text{H}_2\text{O}_2$  level, detected by CM- $\text{H}_2\text{DCFDA}$  was not altered by AMA stimulation (Fig. 8c). Moreover, we

could not detect intracellular  $\text{OH}\cdot$ , even after AMA treatment (Fig. 8d). These results suggest that hydrogen did not exert its antifibrotic effect through direct action on HSC.



**Figure 7** Hydrogen-rich medium does not suppress the activation of cultured hepatic stellate cells (HSC). HSC 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-smooth muscle actin [α-SMA]) (a). The HSC showed faint signals of O<sub>2</sub><sup>-•</sup>, as detected by MitoSOX (b). HSC activated on plastic dishes showed the production of H<sub>2</sub>O<sub>2</sub>, as detected by 2',7'-dichlorodihydrofluorescein (CM-H<sub>2</sub>DCFDA) (c), but culturing in hydrogen-rich medium did not show any suppressive effect on H<sub>2</sub>O<sub>2</sub> production (c). The HSC did not show the presence of OH<sup>•</sup>, as detected by hydroxyphenyl fluorescein (HPF) staining (d).



**Figure 8** Hydrogen-rich medium does not suppress or accelerate the hepatic stellate cell (HSC) activation induced by antimycin A (AMA) treatment. Isolated HSC 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 (collagen1 $\alpha$ 1 and  $\alpha$ -smooth muscle actin [ $\alpha$ -SMA]) (a). Although AMA stimulation increased the intracellular level of O<sub>2</sub><sup>-</sup> as detected by MitoSOX (b), culturing in hydrogen-rich medium did not suppress this increase in the O<sub>2</sub><sup>-</sup> level. The H<sub>2</sub>O<sub>2</sub> level, as detected by 2',7'-dichlorodihydrofluorescein (CM-H<sub>2</sub>DCFDA), was not altered by AMA stimulation (c). Moreover, we could not detect intracellular OH $\cdot$  even under stimulation with AMA (d).

## DISCUSSION

IN THE PRESENT study, we investigated whether orally ingested H<sub>2</sub>-water suppressed liver fibrogenesis. Our study demonstrated that H<sub>2</sub>-water showed antifibrogenic effects in the CCl<sub>4</sub> and TAA models but did not show any difference in the BDL model. H<sub>2</sub>-water exhibited an OH·-scavenging effect in the CCl<sub>4</sub> and TAA model; however, in the BDL model, OH· was not detected in the liver even in the control water group. We suggest that the antifibrotic effect of H<sub>2</sub>-water is associated with the scavenging of OH·.

Carbon tetrachloride 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.<sup>22,23</sup> Intracellular ROS usually refers to the excess amounts of O<sub>2</sub><sup>·-</sup>, H<sub>2</sub>O<sub>2</sub> and OH·. Among them, OH· is the strongest ROS and reacts indiscriminately with nucleic acids, lipids and proteins.<sup>10</sup> To date, many reports have described the antifibrotic effect of antioxidants.<sup>24–26</sup> 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<sub>2</sub><sup>·-</sup> induced by the action of xanthine and xanthine oxidase.<sup>5</sup> Therefore, we first sought to determine whether H<sub>2</sub>-water exerted its antifibrotic effect through the suppression of O<sub>2</sub><sup>·-</sup> production. The results of our *in vitro* experiments, however, indicated that the hydrogen-rich medium did not influence the O<sub>2</sub><sup>·-</sup> and the H<sub>2</sub>O<sub>2</sub> levels in both HSC and hepatocytes. We examined the expression of O<sub>2</sub><sup>·-</sup> in the injured liver by staining freshly frozen liver sections with MitoSOX, and the results showed that H<sub>2</sub>-water does not influence the O<sub>2</sub><sup>·-</sup> levels in the liver (data not shown). Instead, we found that oral ingestion of H<sub>2</sub>-water resulted in the suppression of OH· levels in the liver and that hydrogen-rich medium attenuated the generation of OH· in cultured hepatocytes. These results indicated that OH· is the primary target for H<sub>2</sub>-water.

It is well known that peroxynitrite is a reactive oxidant produced from nitric oxide and superoxide. O<sub>2</sub><sup>·-</sup> 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 CCl<sub>4</sub> administration were decreased in inducible nitric oxide synthase deficient mice.<sup>27</sup> We examined 3-nitrotyrosine in the liver. Hydrogen water did not show significant effect on

3-nitrotyrosine 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 HSC and hence in the development of liver fibrosis.<sup>28,29</sup> However, other studies have shown conflicting data as to the effects of ROS on HSC proliferation and viability.<sup>6</sup> 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<sub>2</sub>-water suppressed hepatocyte death, as demonstrated by inhibition of transaminase release in the H<sub>2</sub>-water group. Hepatocyte injuries stimulate the phagocytosis of dead hepatocytes by Kupffer cells.<sup>30</sup> Activated Kupffer cells secrete profibrogenic cytokines such as TGF-β-1, which activate HSC and eventually lead to liver fibrogenesis.<sup>31</sup> Our *in vivo* study showed the decreased expression of TGF-β1 mRNA. Decreased induction of TGF-β1, a particularly potent profibrogenic cytokine, may be a key factor in alleviation of the fibrogenic response by H<sub>2</sub>-water. Expression of α-SMA, an indicator for HSC activation, was also inhibited by intake of H<sub>2</sub>-water, suggesting that inhibition of TGF-β1 induction results in reduced activation of HSC, which leads to reduced collagen deposition. Taken together, we presume that H<sub>2</sub>-water suppresses triggering profibrogenic response by scavenging 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<sub>2</sub>-water on fibrogenesis in BDL model. This differential effect of H<sub>2</sub>-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 two 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<sub>2</sub>-water in the BDL model seems to be concordant with the hepatoprotective properties of H<sub>2</sub>-water. Moreover, considering our results that indicate H<sub>2</sub>-water selectively scavenges OH·, the difference can be explained by the variation of ROS contribution in the models: hepatocyte injuries by CCl<sub>4</sub> and TAA are OH·-dependent injuries, whereas biliary injury by BDL is independent of OH·.

Recent studies have demonstrated that H<sub>2</sub> reduces OH· and may have potential for widespread medical application as a novel, safe and effective antioxidant

with minimal adverse effects.<sup>32</sup> Elimination of OH· is biologically important because although O<sub>2</sub><sup>·-</sup> and H<sub>2</sub>O<sub>2</sub> are detoxified by the antioxidant defense enzymes superoxide dismutase and peroxidase or glutathione peroxidase, no enzyme detoxifies OH·.<sup>11</sup>

The systemic distribution of H<sub>2</sub> after oral intake of H<sub>2</sub>-water has been described previously by Kamimura *et al.*<sup>33</sup> They monitored the dynamic movement of hydrogen in rat liver after p.o. administration of H<sub>2</sub>-water by introducing a needle-type hydrogen sensor into the liver. They showed that oral intake of H<sub>2</sub>-water helps maintain a sufficient hydrogen concentration in the liver for 1 h. Further, H<sub>2</sub>-water has already been applied in humans. Clinical trials have revealed that supplementation with H<sub>2</sub>-water reduced oxidative stress in patients with type 2 diabetes<sup>34</sup> and those with potential metabolic syndrome,<sup>35</sup> thereby indicating that it influenced glucose<sup>34</sup> and cholesterol metabolism.<sup>35</sup> These findings indicate that H<sub>2</sub>-water can be administered safely to humans and has potential for use as an antifibrotic agent.

In conclusion, H<sub>2</sub>-water protects hepatocytes from injury by scavenging OH· and thereby suppresses liver fibrogenesis in mice.

## ACKNOWLEDGMENTS

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