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Hydrogen-Rich Water Prevents Progression of Nonalcoholic Steatohepatitis and Accompanying Hepatocarcinogenesis in Mice

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Oxidative stress is a strong contributor to the progression from simple fatty liver to nonalcoholic steatohepatitis (NASH). Molecular hydrogen is an effective antioxidant that reduces cytotoxic reactive oxygen species. In this study, we investigated the effects of hydrogen-rich water and the drug pioglitazone on the progression of NASH in mouse models. A methionine-choline–deficient (MCD) diet mouse model was prepared. Mice were divided into three experimental groups and fed for 8 weeks as follows: (1) MCD diet + control water (CW group); (2) MCD diet + hydrogen-rich water (HW group); and (3) MCD diet mixed with pioglitazone (PGZ group). Plasma alanine aminotransferase levels, hepatic expression of tumor necrosis factor-α, interleukin-6, fatty acid synthesis–related genes, oxidative stress biomarker 8-hydroxydeoxyguanosine (8-OHdG), and apoptosis marker terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL)–positive cells in the liver were decreased in the HW and PGZ groups. The HW group showed a smaller decrease in hepatic cholesterol; however, stronger antioxidative effects in serum and lower peroxisome proliferator-activated receptor-α expression in the liver were seen in comparison with the PGZ group. We then investigated the effects of hydrogen in the prevention of hepatocarcinogenesis in STAM mice, known as the NASH-related hepatocarcinogenesis model. Eight-week-old male STAM mice were divided into three experimental groups as follows: (1) control water (CW-STAM); (2) hydrogen-rich water (HW-STAM); and (3) pioglitazone (PGZ-STAM). After 8 weeks, hepatic tumors were evaluated. The number of tumors was significantly lower in the HW-STAM and PGZ-STAM groups than in the CW-STAM group. The maximum tumor size was smaller in the HW-STAM group than in the other groups. *Conclusion:* Consumption of hydrogen-rich water may be an effective treatment for NASH by reducing hepatic oxidative stress, apoptosis, inflammation, and hepatocarcinogenesis. (HEPATOLOGY 2012;56:912-921)

Nonalcoholic fatty liver disease (NAFLD) is a common and increasing cause of chronic liver disease. Nonalcoholic steatohepatitis (NASH) is a more severe form of NAFLD and is broadly defined by the presence of steatosis with inflammation and progressive fibrosis, ultimately leading to cirrhosis and hepatocellular carcinoma (HCC).[1] NASH develops in a subset of patients with NAFLD, although the exact mechanisms remain poorly understood.

Current understanding suggests that the development of NASH is a "two-hit" process. The first hit is the development of hepatic steatosis. Insulin resistance,

Abbreviations: 8-OHdG, 8-hydroxydeoxyguanosine; AOX, acyl coenzyme A oxidase; CW, control water; FAS, fatty acid synthase; FAT, fatty acid translocase; FATP, fatty acid transport protein; HCC, hepatocellular carcinoma; HW, hydrogen-rich water; IL-6, interleukin-6; MCD, methionine-choline–deficient; mRNA, messenger RNA; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis; PCNA, proliferation cell nuclear antigen; PGZ, pioglitazone; PPAR, peroxisome proliferator-activated receptor; ROM, reactive oxygen metabolite; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

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which is almost universally present in patients with NAFLD, is thought to play a pivotal role in the accumulation of lipids in the liver.[2] Many sources of the second hit, including oxidative stress, apoptosis, and gut-derived lipopolysaccharide, trigger an inflammatory response and progressive liver damage.[3]

Oxidative stress appears to be responsible for the initiation of necroinflammation, and reactive oxygen species (ROS) are widely accepted as a source of oxidative stress. ROS are generated during the metabolism of free fatty acids in microsomes, peroxisomes, and mitochondria.[4] Emerging data suggest that ROS, lipid peroxidation products, and tumor necrosis factor-α (TNF-α) are involved in the second hit, which induces the progression of simple steatosis to NASH. Furthermore, ROS induce directional migration of resident hepatic profibrogenic cells, resulting in liver fibrosis.[5]

Several studies have suggested a beneficial role for antioxidants such as vitamin E and thiazolidinediones (insulin sensitizers) in NAFLD or NASH.[6,7] In particular, one of the thiazolidinediones, pioglitazone (PGZ), is considered to be effective in improving insulin sensitivity, steatosis and inflammation. However, the effects are insufficient for histologically clear improvement.[7] Furthermore, most clinical studies on atherosclerotic diseases with dietary antioxidants have failed to show clear success. This is partly because of the nonselective effects of these antioxidative drugs and difficulties with regard to distribution into the cytosol.[8]

Molecular hydrogen has recently been shown to have therapeutic value as an antioxidant through its ability to reduce cytotoxic ROS such as hydroxyl radicals (·OH), but not superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), or nitric oxide.[9] Hydrogen is distributed into cytosol without any specific receptors or hydrophilicity.[10] Inhaled hydrogen gas can reduce infarct size in rat models of focal cerebral and myocardial ischemia reperfusion injury.[9] Drinking water containing therapeutic doses of hydrogen (i.e., hydrogen-rich water [HW]) represents an alternative model for the delivery of molecular hydrogen following treatment of ROS-induced pathologies.[11] However, there have been no reports demonstrating the efficacy of HW on oxidative stress in chronic liver diseases such as NASH.

In this study, we evaluated the effects of HW in experimental steatohepatitis induced in mice fed a methionine-choline–deficient (MCD) diet, which is a well-understood NASH model.[12] The efficacy of treatment was compared with that of PGZ. Furthermore, we evaluated the effects of HW on hepatic tumorigenesis in a streptozotocin-induced NASH-related hepatocarcinogenic mouse model.

Materials and Methods

Animals and Experimental Design. An MCD diet–induced NASH model was prepared. Eight-week-old male C57BL/6 mice were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). They were divided into three experimental groups and fed for 8 weeks as follows: (1) MCD diet (Research Diets, Inc., New Brunswick, NJ) + control water (CW group; n = 8); (2) MCD diet + HW (hydrogen level: 0.35-0.45 ppm; Blue Mercury, Tokyo, Japan) (HW group; n = 8); and (3) MCD diet mixed with 0.01% PGZ (LKT Laboratories, Inc., St. Paul, MN) + control water (PGZ group; n = 8).

After 8 weeks, the mice were killed. Blood samples were obtained from the right atrium via cardiac puncture and their livers were excised. The livers were cut into pieces and fixed in 10% formalin for histological analysis or fresh-frozen in liquid nitrogen and stored at −80°C until use.

For hepatocarcinogenesis experiments, STAM mice (Charles River Laboratories Japan Inc., Yokohama, Japan), a NASH-cirrhosis-hepatocarcinogenic model, were prepared. The STAM mouse NASH model was established according to an unpublished protocol. Briefly, pregnant C57BL/6 mice were purchased from CLEA-Japan (Tokyo, Japan) and 2-day-old male pups were injected with streptozotocin (200 μg per mouse) and fed a high-fat diet (HFD-32, CLEA-Japan) from the age of 4 weeks. This mouse model progresses from NAFLD to NASH at 8 weeks of age and develops hepatocellular carcinoma at 16 weeks of age.

Eight-week-old male STAM mice were purchased from Charles River Laboratories Japan Inc. They were divided into three experimental groups and fed for 8 weeks as follows: (1) high-fat diet (Research Diets, Inc.) + CW (CW-STAM), (2) high-fat diet + HW (HW-STAM), and (3) high-fat diet mixed with 0.01% PGZ + control water (PGZ-STAM). After 8 weeks,

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mice were killed, their hepatic tumors were counted, and tumor size was measured.

Animals had free access to water and food and were maintained in a temperature-controlled animal facility with a 12-hour light-dark cycle. All protocols and procedures conformed to the guidelines of the Okayama University Committee for Care and Use of Laboratory Animals and were approved by the Animal Experiments Ethics Committee of Okayama University.

HW Administration. HW was purchased from Blue Mercury Inc. (Tokyo, Japan). Molecular hydrogen was dissolved in the water under high pressure (0.4 MPa) to a supersaturated level using an HW-producing apparatus. The saturated HW was stored in an aluminum bag and shipped. The HW was placed twice a day into a closed glass vessel equipped with an outlet line containing two ball bearings, which kept the water from being degassed. The hydrogen preserving capacity of this method has been established (personal communication, Ohta and Ohsawa, Nippon Medical School).

Measurement of Plasma Aminotransferases, Plasma Glucose, and Insulin. Aspartate aminotransferase and alanine aminotransferase, plasma glucose, and insulin levels were determined using standard methods at Skylight Biotech Company (Akita, Japan). Insulin-resistance index was assessed as the product of plasma glucose level multiplied by insulin level in the STAM model. In the MCD model, the insulin levels were too low to measure in all groups.

Measurement of Intrahepatic Lipid Concentrations. Intrahepatic lipids were extracted using Folch's method, and concentrations were determined using standard enzymatic methods at Skylight Biotech Company.

Histological Analysis, TUNEL, and PCNA Staining of the Liver. NAFLD activity was assessed using the NAFLD activity score (NAS) as described by Kleiner et al.,[13] with separate scores for steatosis (0-3), hepatocellular ballooning (0-2), and lobular inflammation (0-3). The NAS is the sum of these scores, and values of ≥ 5 are reported to be correlated with a diagnosis of NASH. All liver specimens were assessed by two hepatologists (T. Y. and A. T.) blinded to the study groups. For analysis of apoptosis, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was performed according to the manufacturer's protocols (DeadEnd Colorimetric TUNEL System; Promega, Madison, WI). The apoptosis index was calculated as the percentage of TUNEL-positive nuclei (stained clear-brown) after at least 500 cells were counted. Results were expressed as the mean number of TUNEL-positive apoptotic hepatocytes in each group. The proliferative activity of hepatocytes was estimated by immunostaining for proliferation cell nuclear antigen (PCNA) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Results were expressed as the mean number of PCNA-positive nuclei (stained clear-brown) after at least 500 cells were counted.

Electron Microscopic Findings. Hepatocyte mitochondria were examined by transmission electron microscopy. Liver specimens were embedded in Spurr's resin (TAAB Laboratories Equipment Limited, Berkshire, UK). Thin sections were double-stained with lead and uranyl acetate, and were then observed with a Hitachi H-7650 transmission electron microscope (Hitachi High-Technologies, Tokyo, Japan) at 75 kV.

Quantitative Real-Time Polymerase Chain Reaction. Total RNA was prepared from liver tissue with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA levels corresponding to various target genes were quantified using a polymerase chain reaction (PCR)-based technique. Extracted RNA was converted into complementary DNA via reverse-transcription (SuperScript III Reverse Transcriptase; Invitrogen, Carlsbad, CA). Specific gene expression was quantified via real-time PCR performed on a LightCycler 480 Instrument (Roche Diagnostics Ltd, Rotkreuz, Switzerland). RNA expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was measured as an internal control. Relative expression levels of target genes were compared after normalization against GAPDH. TNF-α, a regulator of inflammation and apoptosis, and interleukin-6 (IL-6), a master regulator of inflammation, were determined with a LightCycler primer & probe set (Nihon Gene Research Laboratories, Sendai, Japan). Hepatic lipogenic genes were assessed with SYBR green fluorophore. Primer sequences were as follows: acyl coenzyme A oxidase (AOX), 5′-TGGTATGGT GTCGTACTTGAATGAC-3′ (forward), 5′-AATTTCTACCAATCTGGCTG-CAC-3′ (reverse); fatty acid synthase (FAS), 5′-ATCCTGGAACGAGAACACGAT CT-3′ (forward), 5′-AGAGACGTGTCACTCCTGGA CTT-3′ (reverse); fatty acid translocase (FAT), 5′-CCAAATGAAGAT-GAGCATAGGACAT-3′ (forward), 5′-GTTGACCTG-CAGTCGTTTTGC-3′ (reverse); fatty acid transport protein (FATP), 5′-ACCACCGGGCTT CCTAAGG-3′ (forward), 5′-CTGTAGGAATGGTGG CCAAAG-3′ (reverse); peroxisome proliferator-activated receptor-α (PPARα), 5′-CCTCAGGGTACCACTACG GAGT -3′ (forward), 5′-GCCGAATAGTTCGCCGAA -3′ (reverse); and PPARγ, 5′-TTGCTGAACGTGAA

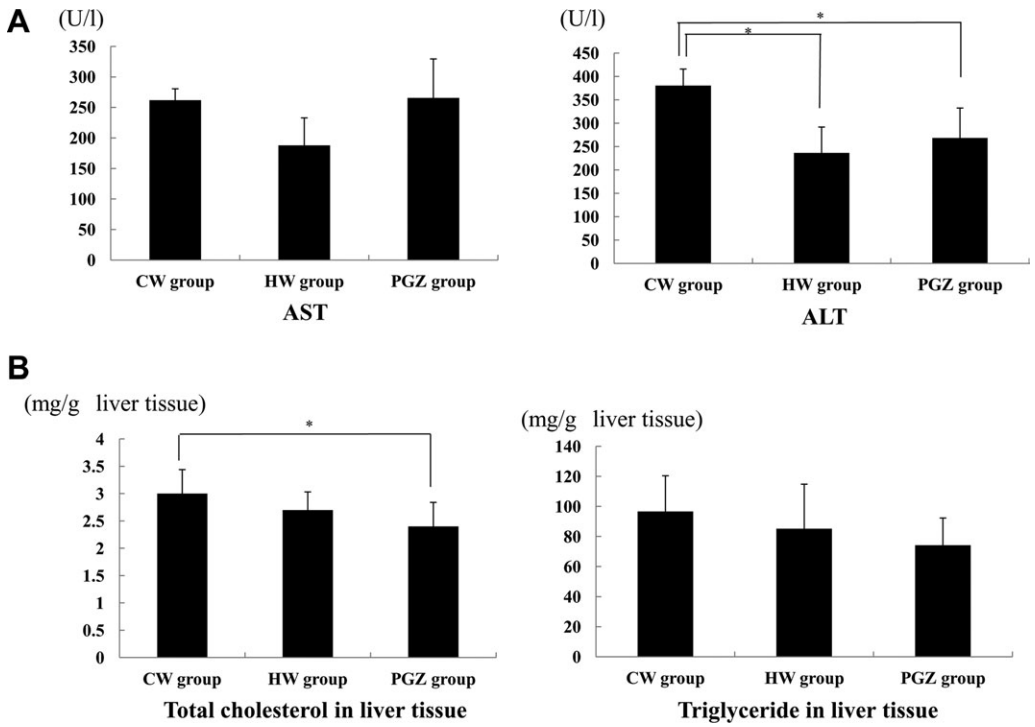

Fig. 1. Plasma biochemical findings and lipid concentrations in the liver. (A) Alanine aminotransferase levels were significantly lower in the HW and PGZ groups than in the CW group. (B) Total cholesterol levels in the liver were significantly lower in the PGZ group. Data are expressed as mean ± SD. *$P < 0.05$.

GCCCATCGAGG -3′ (forward), 5′-GTCCTTGTAG ATCTCCTGGAGCAG -3′ (reverse).

Hepatic 8-Hydroxydeoxyguanosine Concentration Analysis. 8-Hydroxydeoxyguanosine (8-OHdG), a modified DNA base product generated by free radicals, is considered to be a good biomarker of oxidative DNA damage.[14] DNA was extracted from livers using a DNA extractor kit (DNA Extractor TIS Kit; Wako, Osaka, Japan). Hepatic 8-OHdG concentration was measured using an enzyme-linked immunosorbent assay kit (Highly Sensitive 8-OHdG Check; Japan Institute for the Control of Aging, Shizuoka, Japan) after preparation with an exclusive kit (8-OHdG Assay Preparation Reagent Set; Wako, Osaka, Japan). Results are expressed as nanograms per milligrams DNA corrected with the amount of each sample DNA levels.

Measurement of Plasma Reactive Oxygen Metabolite Levels and Antioxidant Capacity. The reactive oxygen metabolite (ROM) blood levels were accepted as markers for circulating ROS. Measurement of ROM plasma levels was performed using a spectrophotometer (Diacron International, Grosseto, Italy) as described.[15] Measurements were made in terms of Carratelli units (CARR U); 1 CARR U corresponds to 0.08 mg/dL hydrogen peroxide.

To determine total plasma antioxidant capacity, an OXY-adsorbent test was performed using a spectrophotometer (Diacron International). This test evaluates the capacity of serum to oppose the massive oxidative action of a hypochlorous acid (HClO) solution and total antioxidant capacity was expressed in terms of HClO (μmol) consumed by 1 mL of sample (μmol HClO/mL). To compare parameters with different measurement units and variabilities, standardized values of the ROM and OXY-adsorbent tests were used to represent the oxidative index. The oxidative index was calculated as described.[15] Low oxidative index values indicated low oxidative stress in the blood.

Statistical Analysis. Results are expressed as the mean ± SD. All data were compared using the Tukey-Kramer method (Stat View, Cary, NC). Data were considered to be statistically significant at $P < 0.05$.

Results

Biochemical Analysis of Plasma and Liver. Aspartate aminotransferase levels were not lower in the HW and PGZ groups, but alanine aminotransferase levels were significantly lower in the HW and PGZ groups (Fig. 1A). In liver tissues, total cholesterol was lower in the PGZ group, whereas hepatic triglyceride levels were also slightly lower in the PGZ group, though they were not statistically significant (Fig. 1B).

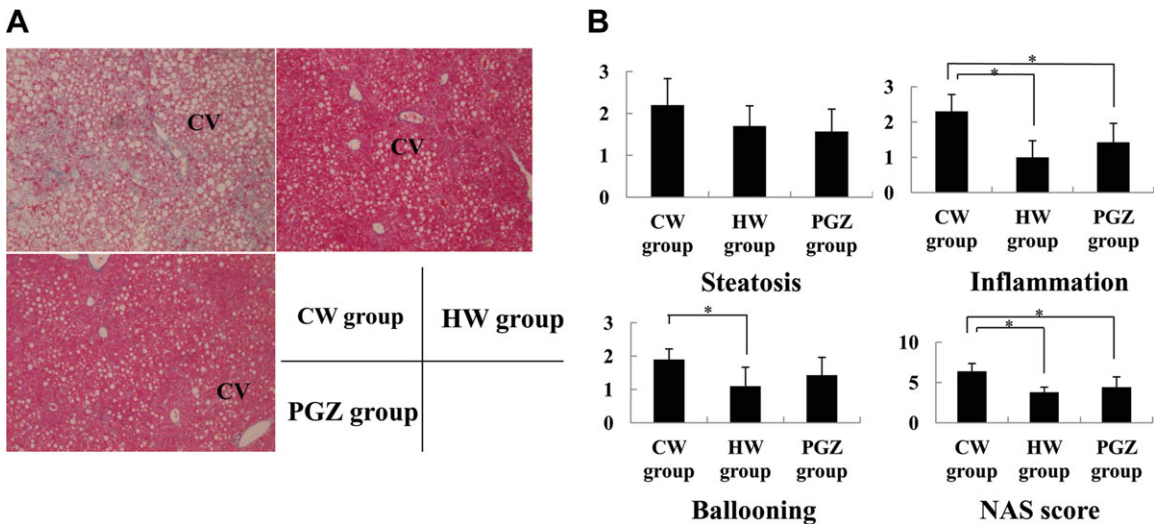

Fig. 2. Liver histological findings. (A) Representative Azan-stained liver sections are shown. The HW and PGZ group showed less inflammation and fibrosis (magnification ×100). (B) NAS for mouse liver specimens. The HW and PGZ groups had less inflammation and ballooning of hepatocytes, whereas steatosis did not differ significantly among the three groups. Data are expressed as mean ± SD. CV, central vein. *$P < 0.05$

Histological Findings in Liver. As shown in Fig. 2A, the MCD group developed hepatocyte steatosis, ballooning, and scattered inflammatory cell infiltration with fibrosis at 8 weeks. Necroinflammation, hepatocyte ballooning, and pericellular fibrosis were clearly reduced in the HW and PGZ groups at 8 weeks, while steatosis was not reduced (Fig. 2B). The NAS was significantly lower in the HW and PGZ groups.

Apoptosis, Inflammation, and Lipogenic Gene Expression in Liver. Hepatic messenger RNA (mRNA) expression of TNF-α was significantly down-regulated in the HW group. Expression of IL-6 was down-regulated in both the HW and PGZ groups (Fig. 3A). Lipid metabolism–related gene expression analysis revealed that free fatty acid uptake–related gene FAT and free fatty acid–induced β-oxidation–related gene AOX were down-regulated in the HW and PGZ groups (Fig. 3B). Whereas expression of PPARγ did not differ among the groups, PPARα was significantly down-regulated in the HW group compared with the other groups (Fig. 3C).

Hepatocyte Apoptosis Assay. As shown in Fig. 4A, the MCD diet induced TUNEL-positive cells in the

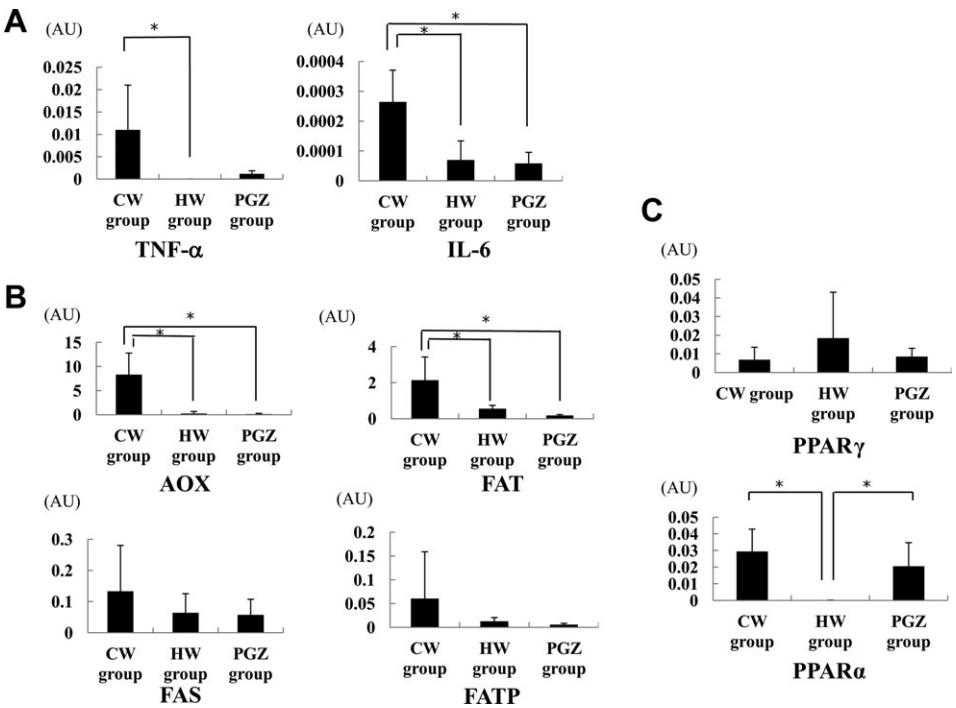

Fig. 3. Quantitative real-time polymerase chain reaction findings of the liver. (A) Hepatic mRNA expression levels of TNF-α and IL-6 were lower in the HW group compared with the CW group. In the PGZ group, only IL-6 was lower. (B) Hepatic mRNA expression levels of fatty acid metabolism-related genes. AOX and FAT were lower in the HW and PGZ groups. (C) Hepatic mRNA expression levels of PPARs. PPARα was significantly down-regulated in the HW group. Data are expressed as the mean ± SD. *$P < 0.05$

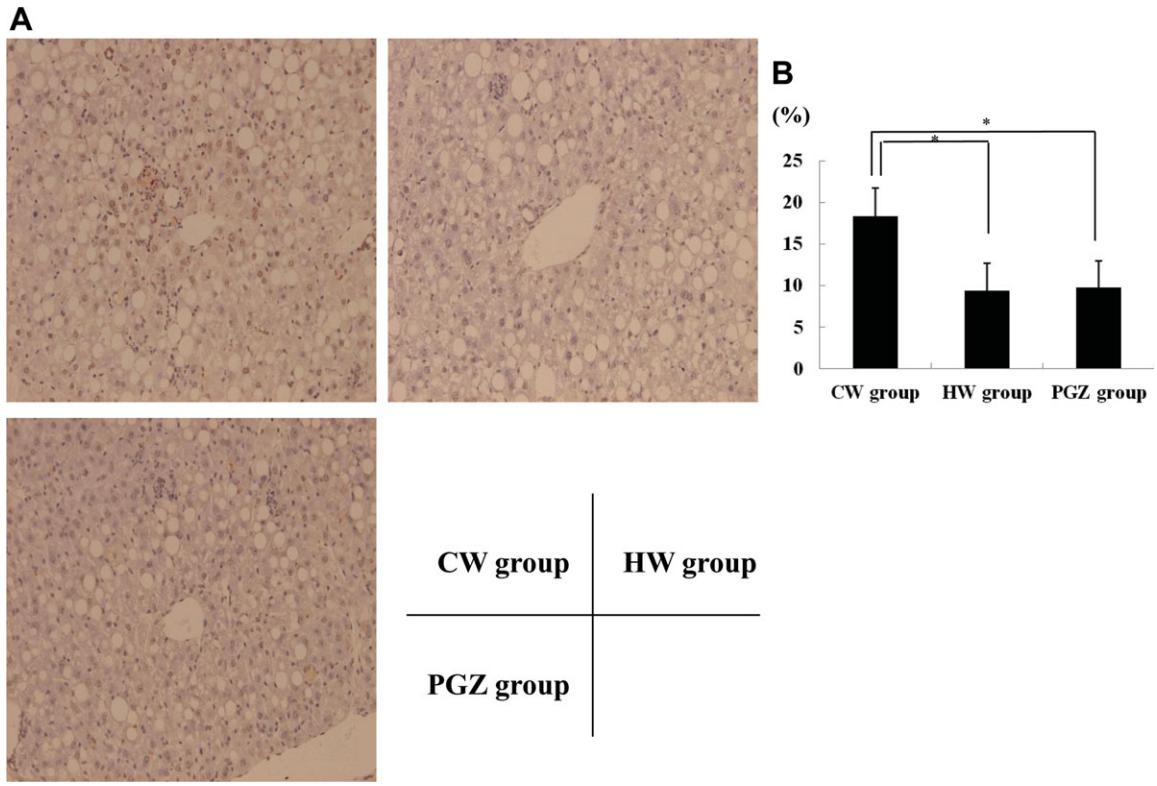

Fig. 4. Assessment of apoptosis by TUNEL assay. (A) Apoptotic hepatocytes were reduced in the HW and PGZ groups (magnification ×200). (B) The apoptotic cell number was significantly lower in the HW and PGZ groups. Data are expressed as the mean ± SD. *$P < 0.05$.

liver. The numbers of TUNEL-positive apoptotic cells were significantly reduced in the HW and PGZ groups (Fig. 4A,B).

Oxidative Stress Status in Liver and Circulating Blood. The concentration of 8-OHdG in the liver was significantly reduced in the HW and PGZ groups (Fig. 5A). Data on the oxidative stress marker ROM, antioxidative stress marker OXY-adsorbent test, and the oxidative index, which is the balance between oxidative and antioxidative markers, are shown in Fig. 5B. The HW group showed the lowest ROM value and the highest OXY-adsorbent test value, resulting in an oxidative index below zero.

Morphological Changes in Mitochondria. As shown in Fig. 6, the MCD diet resulted in morphological changes in mitochondria, with dissection occurring between the outer and inner membranes. This change was not observed in the HW and PGZ groups.

Hepatic Tumorigenesis. We assessed the background liver histology of the STAM NASH model using the NAS score (Fig. 7A). Hepatocyte ballooning was reduced in the HW-STAM and PGZ-STAM groups at 16 weeks, whereas steatosis was not reduced. The NAS was significantly lower in the HW-STAM group when compared with the other groups. Plasma glucose level was very high and insulin level was very low, because pancreatic beta cells were destroyed by the streptozotocin treatment in this model. Insulin resistance index did not differ between the groups (Fig. 7B). Liver tumors were observed in CW-STAM mice at 16 weeks. The PGZ-STAM group exhibited fewer tumors. The HW-STAM group exhibited fewer and smaller tumors, even smaller than those in the PGZ-STAM group (Fig. 7C,D). Histological findings revealed that the tumors were HCC (Fig. 7E). The number of PCNA-positive nuclei in noncancerous tissue was significantly lower in the HW-STAM group compared with the other groups (Fig. 8A,B).

Discussion

Our results confirm that drinking HW improves NASH and NASH-related hepatocarcinogenesis in mouse models. Hepatic and general oxidative stress markers were all improved and free fatty acid uptake–related enzymes, inflammatory cytokines, and PPARα were suppressed in the liver. The HW group showed a smaller hepatic cholesterol decrease than the PGZ group but exhibited a greater antioxidative effect and a stronger anti-hepatocarcinogenesis effect. These results indicate that drinking HW represents a simple and novel therapeutic strategy for NASH and NAFLD.

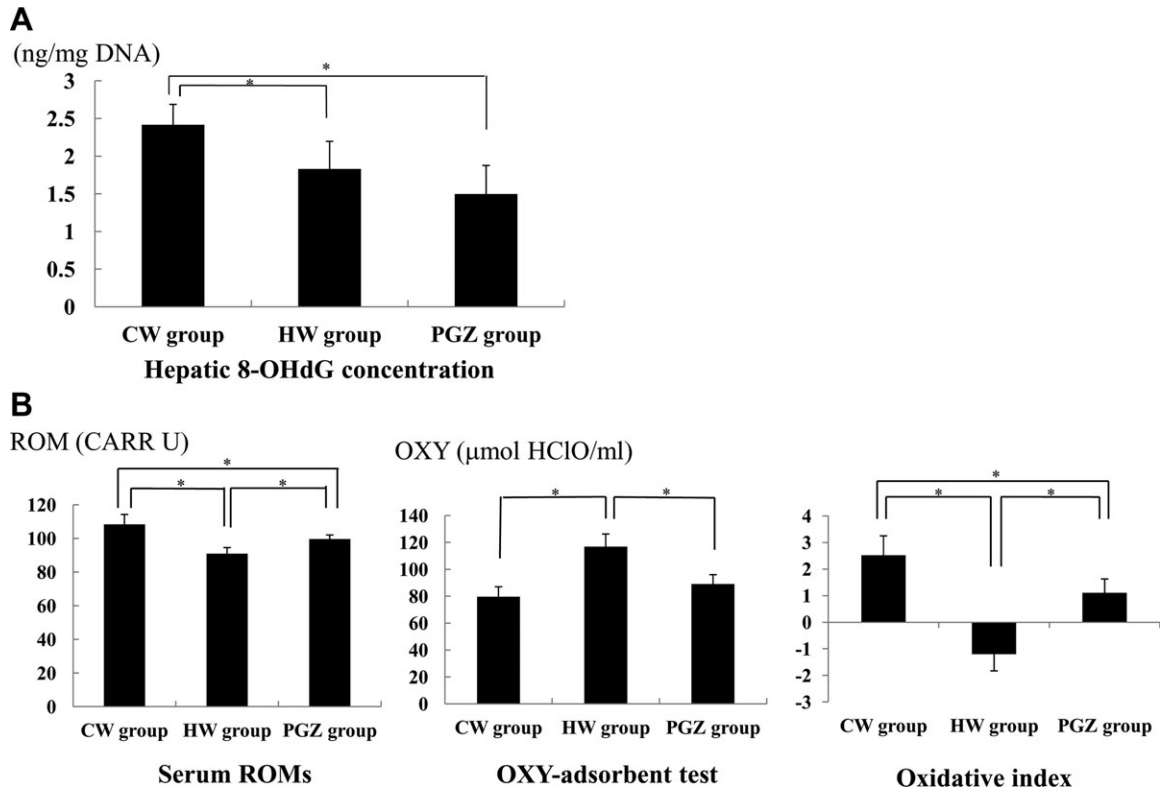

Fig. 5. Assessment of oxidative stress. (A) Concentrations of 8-OHdG in the liver were lower in the HW and PGZ groups. (B) Oxidative stress marker ROMs, antioxidative stress marker OXY-adsorption test, and oxidative index results are shown. The HW group showed the lowest ROM data and the highest OXY-adsorption test data, resulting in an oxidative index below zero. Data are expressed as the mean ± SD. *$P < 0.05$.

Ohsawa et al.[9] reported that molecular hydrogen selectively reduced hydroxyl radicals, the most cytotoxic ROS, but did not react with other ROS, which play physiological roles and effectively protect cells. Drinking HW or inhaling hydrogen gas has been accepted to have favorable effects on several disease models, such as chronic allograft nephropathy[11] and focal cerebral and myocardial ischemia-reperfusion injuries.[9] The effects of hydrogen on liver damage have also been reported in diabetic and CCl_4-induced acute liver failure models.[16,17] However, the effects of hydrogen administration on NASH remain unknown.

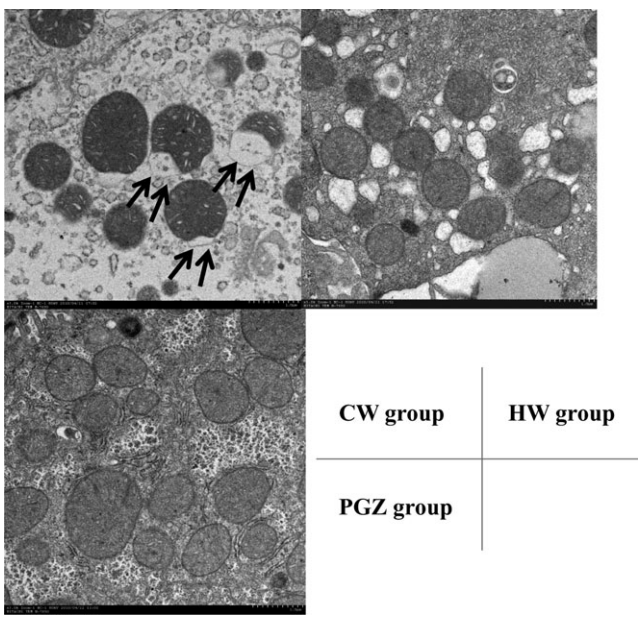

Fig. 6. Electron microscopic features of hepatic mitochondria. In the CW group, some mitochondria exhibited dissection between the outer and inner membranes (arrows), whereas the HW and PGZ groups exhibited no such mitochondrial damage (magnification ×10000).

Hydrogen is produced continuously under normal physiological conditions, during the fermentation of nondigestible carbohydrates by intestinal bacteria in the large intestine.[11] As molecules in the intestines flow into the portal vein and reach the liver, molecular hydrogen may reach the liver via this route. Monitoring of hepatic hydrogen revealed that hydrogen accumulates in the liver after oral administration of HW. Hydrogen monitoring in an ischemic myocardium revealed that hydrogen is distributed into ischemic areas, indicating that its distribution characteristics allow it to penetrate biomembranes and diffuse into the cytosol, in contrast to other antioxidants.[10] Microarray analysis of the liver revealed that drinking HW can induce up-regulation of numerous genes that encode the oxidoreductase proteins involved in steroid

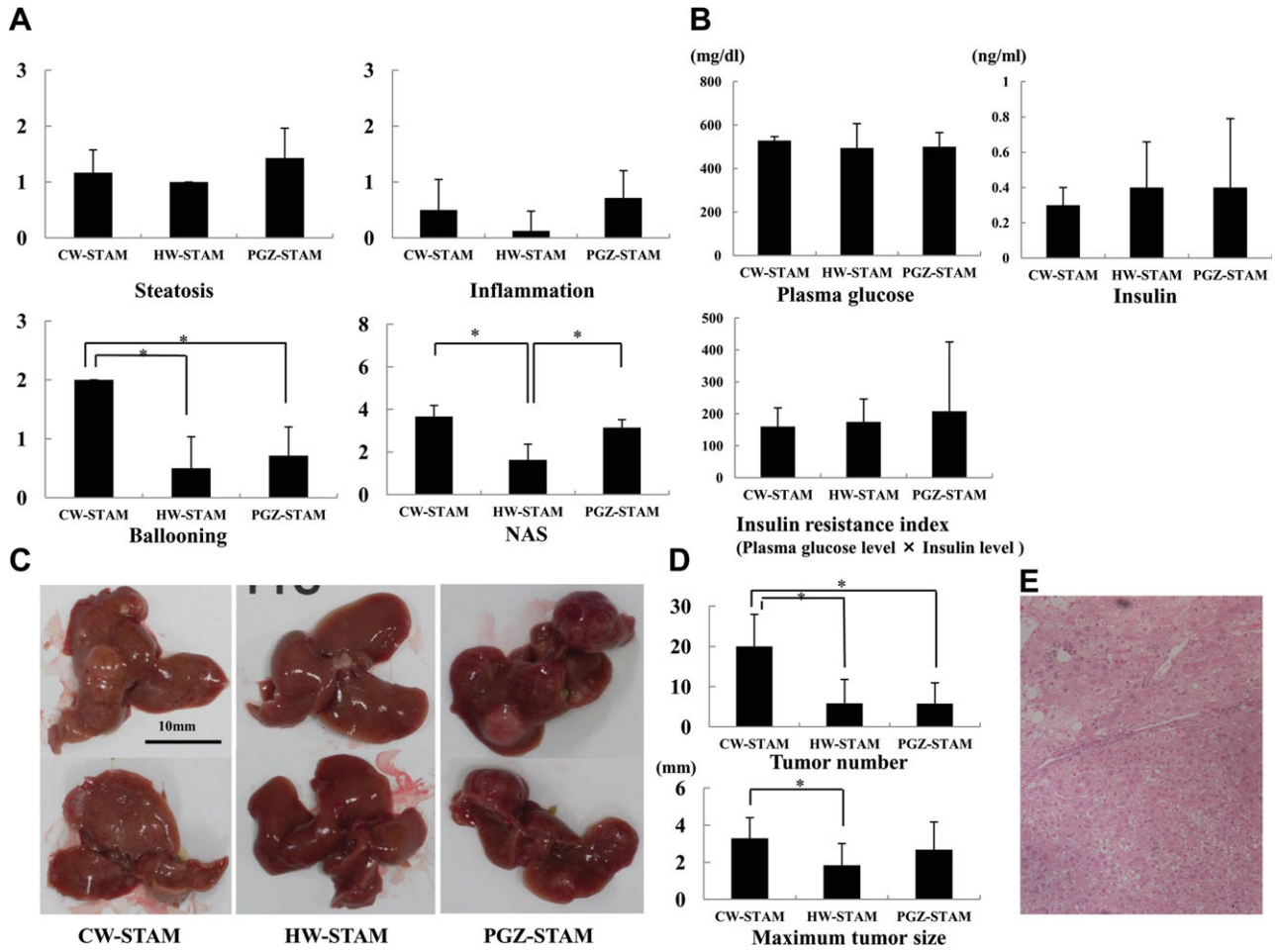

Fig. 7. Characteristics of the NASH model STAM mice. (A) STAM NASH mice developed relatively weak steatosis and inflammation. Hepatocyte ballooning was clearly reduced in the HW-STAM and PGZ-STAM groups at 16 weeks. NAS was significantly lower in the HW-STAM group. (B) Plasma glucose level and insulin level were not different between groups. (C) The CW-STAM group exhibited numerous tumors on the liver surface, whereas the HW-STAM group exhibited fewer and smaller liver tumors. (D) The size and number of the tumors were significantly lower in the HW-STAM group. In the PGZ-STAM group, the average tumor size was not reduced. (E) Histological findings revealed that the tumors were HCC. Data are expressed as the mean ± SD. *$P < 0.05$ (magnification ×100).

metabolism, amino acid metabolism, sterol biosynthesis process, glycogen metabolic process, and coenzyme metabolic processes.[18]

In an MCD diet model, loss of body weight is the converse of obesity-related NASH. However, the liver pathology recapitulates the major characteristics of human NASH, including steatosis, ballooning degeneration, inflammation and fibrosis.[19] With this model, the hepatic lipogenic gene expression profile and the oxidative stress marker attenuation by PGZ showed convincing results for estimating the effect of the drug in our study. We believe that the MCD diet model is worthy of further investigation, even though it has not proven to be a very good model so far. Hepatic fatty acid uptake is thought to occur by several mechanisms, including a transporter-mediated mechanism. In patients with NAFLD, hepatic expression of fatty acid synthesis genes and fatty acid oxidation-related genes is up-regulated. AOX is thought to be a rate-limiting enzyme in the peroxisomal β-oxidation pathway.[20] FAT and FATP are thought to be important regulators of fatty acid uptake, and FAS is thought to be a key gene in *de novo* lipogenesis.[21]

In this study, we examined the expression of these key genes of lipid metabolism and found that lipid toxicity–accumulating genes such as AOX and FAT were significantly down-regulated in the HW and PGZ groups, while FAS and FATP were not. Although AOX, FAT, and FATP are regulated by PPARs, which are central regulator of triglyceride homeostasis, FAS is not. We suggest that the effects of HW might be involved in PPAR pathway. PPARα was down-regulated only in the HW group. The activation of PPARα, which is the dominant form in the liver, induces the expression of mitochondrial oxidase and increases fatty acid oxidation. PPARα may play a key

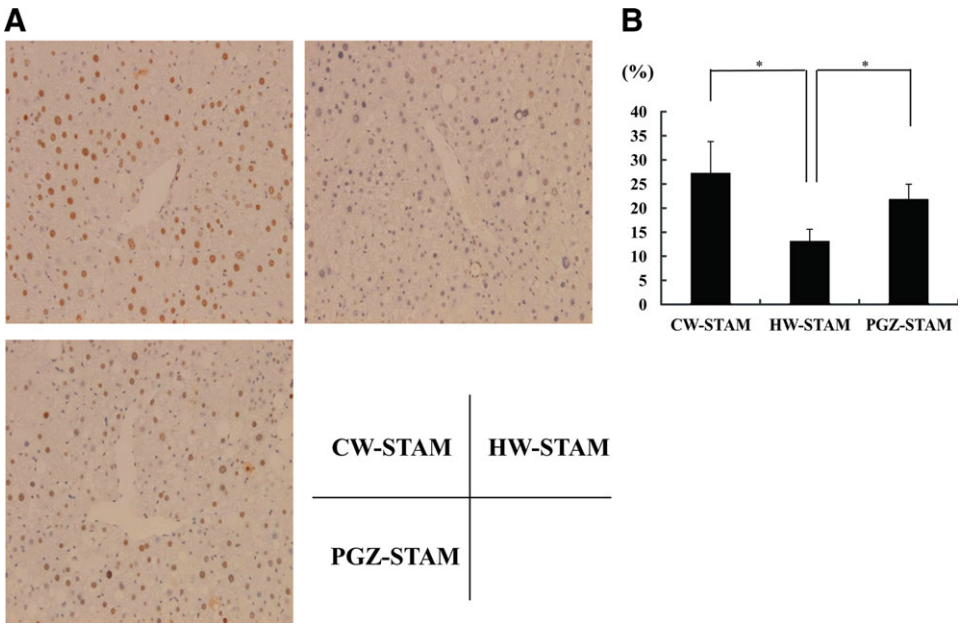

Fig. 8. (A) Representative immunostaining for PCNA of STAM mouse noncancerous liver tissue (magnification ×400). (B) The mean number of PCNA-positive nuclei of each group was significantly lower in the HW-STAM group. Data are expressed as the mean ± SD. *$P < 0.05$.

role in the "second hit," which involves oxidative stress in the liver, by controlling fatty acid oxidation in all potential sources.[22] When cytosolic fatty acids accumulate due to impairment of the oxidative capacity in mitochondria, alternative pathways in the peroxisomes are activated. In peroxisomal β-oxidation, AOX is responsible for the initial oxidation of fatty acyl-CoAs. In NAFLD, the expression of AOX was increased compared with that in healthy liver.[20] PPARα up-regulates the expression of a suite of genes that includes peroxisomal and mitochondrial β-oxidation enzymes as well as AOX. On the other hand, previous reports suggest that sustained activation of PPARα increases the risk of liver cancer development and is related in part to excess energy combustion.[23] In this study, the expression of AOX was significantly lower in the HW and PGZ groups. In the HW group, PPARα expression was significantly lower regardless of AOX suppression. PPARα activation is regulated by other nuclear receptor families such as liver X receptors and retinoid X receptors.[24] In the HW group, the expression of PPARα might be down-regulated by these other nuclear receptors. In addition, suppression of PPARα may enhance the anticarcinogenetic effects of PGZ.

Oxidative DNA damage is involved in the mechanisms of aging, carcinogenesis, and the progression of atherosclerosis. Several peripheral blood markers such as thioredoxins, ferritin, and ROMs are also reported to be hepatic oxidative markers.[25] Recently, antioxidant status was found to be measurable by peripheral blood serum spectrophotometric estimation using the OXY-adsorbent test.[15] Furthermore, we assessed global oxidative stress index (oxidative index), which reflects both oxidative and antioxidant components.[15] The present results of hepatic 8-OHdG and peripheral plasma ROMs, the OXY-adsorbent test, and the oxidative index data demonstrated that molecular hydrogen plays an important role in protecting DNA from oxidative stress in NASH.

Among cytokines related to the progression of NASH, TNF-α plays a pivotal role in hepatocyte apoptosis and inflammation.[26] In addition, the inflammatory cytokine IL-6 is involved in the inflammatory pathogenesis of NASH.[27] Our data revealed that molecular hydrogen prevents inflammation and apoptosis in the NASH model liver.

NASH is an accepted risk factor for hepatic carcinogenesis.[1] In rodent models, it is difficult to induce HCC without genotoxic carcinogens such as nitrosamines. NASH mouse models, including an MCD diet or a choline-deficient diet, may develop HCC, but the process requires long periods.[28] Recently, a combination of high-fat diet and streptozotocin-induced diabetes was reported as a mouse model that resembles human NASH.[29] In this study, we used STAM mice given a high-fat diet and streptozotocin treatment and the mice developed hepatic tumors at 16 weeks of age. We assessed the background liver histology of the STAM NASH model with the NAS score. In this model, relatively weak steatosis and inflammation developed. However, "hepatocyte ballooning," a hallmark finding of NASH, was severe and was clearly reduced in the HW-STAM and PGZ-STAM groups at 16 weeks. Hepatic tumor number was reduced in the HW-STAM and PGZ-STAM groups, whereas the tumor size was reduced only in the HW-STAM group. The proliferative activity of nontumorous hepatocytes was estimated by

immunostaining for PCNA. The expression of PCNA-positive proliferative cells in noncancerous tissue was significantly lower only in the HW-STAM group, which suggests stronger antiproliferative effects from drinking HW.

Although the hepatic triglyceride level and insulin resistance (STAM model is not a good model to estimate insulin resistance because the pancreatic beta cells were destroyed) were not significantly improved in the HW group, oxidative stress and inflammation were improved. Because molecular hydrogen has been reported to be an antioxidative molecule, these effects may be possible even in the absence of significant improvements in triglyceride and insulin resistance.

In conclusion, drinking HW reduces oxidative stress and fatty acid synthesis resulting in improvement of NASH and accompanying HCC.

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