glutathione [3–7]. Oxidative stress seems to play a critical role in cisplatin-induced nephrotoxicity [8–11]. So far, antioxidants that improve nephrotoxic side effects have been extensively explored; however, although some antioxidants exhibited protective effects in model animals, the effects were not satisfactory or the dosage of antioxidants was extremely high for clinical use [11–13]. In addition, concerns about possible interference with the anti-tumor activity of cisplatin limit its use to clinical trials [11].

We have reported that molecular hydrogen is a mild but efficient antioxidant by gaseous rapid diffusion into tissues and cells [14]. Moreover, we have recently shown that consumption of water dissolving molecular hydrogen at a saturated level (hydrogen water) prevents stress-induced cognitive declines in mice [15].

Here we show that inhalation of hydrogen gas and drinking hydrogen water ad libitum mitigate cisplatin-induced nephrotoxicity in mice. Drinking hydrogen water may be more convenient for consumption of hydrogen rather than hydrogen gas. Consuming hydrogen water ad libitum was efficacious for renal failure caused by cisplatin without compromising anti-tumor activity in mice. Thus, we propose that hydrogen consumption, whether hydrogen gas or hydrogen water, is applicable to alleviate nephrotoxic side effects induced by an anti-cancer drug.

Materials and methods

Animals

Female C57BL/6CrSlc mice (7 weeks old, 15–20 g) for the nephrotoxicity studies, male ddY mice (4 weeks old, 18–20 g) for the tumor studies, and male SD rats (7 weeks old, 210–230 g) for the measurement of hydrogen concentration in blood were purchased from Nippon SLC (Hamamatsu, Shizuoka, Japan). Mice were fed ad libitum and housed in a temperature-controlled room (22–24°C) under a 12-h light/dark cycle. The care and treatment of experimental animals were in accordance with institutional guidelines. This study was approved by the Animal Care and Use Committee of Nippon Medical School.

Cells

S-180 sarcoma (CFW sarcoma 180, mouse) and L-1210 (lymphocytic leukemia, mouse) cell lines were obtained from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). S-180 cells were maintained in MEME medium supplemented with 10% fetal calf serum, 1% NEAA and penicillin/streptomycin. L-1210 cells were maintained in RPMI1640 medium supplemented with 10% fetal calf serum and penicillin/streptomycin.

Reagents

Cisplatin (25 mg/50 mL) was purchased from Yakult Honsha Co., Ltd. (Tokyo, Japan). All other chemicals and reagents were of analytical grade.

Animal treatments for the nephrotoxicity studies

C57BL/6 mice were divided randomly into five groups. Group I (CTL) received physiological saline (0.9% NaCl) by intraperitoneal injection. Groups II–V received a single dose of CDDP (17 mg/kg) by intraperitoneal injection. Groups II [HG (+)] and III [HG (−)] inhaled air with or without hydrogen, respectively. Groups IV [HW (+)] and V [HW (−)] were allowed to freely drink water with or without hydrogen, respectively. Lee et al. [16] described renal injury was clearly seen with a dose of 20 mg/kg cisplatin at 72 h after the cisplatin treatment in C57BL/6 mice. However the lethality caused by a dose of 20 mg/kg cisplatin reached 67% in our preliminary experiment ($n = 10$; data not shown). To obtain almost 50% lethal dose of cisplatin, we used a dose of 17 mg/kg cisplatin in this experiment,

Hydrogen gas administration

Mice were housed in a standard cage with food and water available ad libitum and the cage was placed into a semi-closed box (55 × 35 × 30 cm; length × width × height), into which 1% H_2 in air was introduced at a rate of 10 L/min throughout the experiments. The box was placed in a temperature-controlled room (22–24°C) under a 12-h light/dark cycle. In the control group, air was administered at the same rate for the same time period. During each experiment, the concentration of hydrogen in the box was monitored using a gas analyzer (TGA-2000, Teramecs Co., Kyoto, Japan).

Hydrogen water administration

Molecular hydrogen (H_2) was dissolved in water under high pressure (0.4 MPa) to a supersaturated level using hydrogen water-producing apparatus (ver. 2) produced by Blue Mercury Inc. (Tokyo, Japan). The saturated hydrogen water was stored in an aluminum bag. Hydrogen water was freshly prepared every week, which ensured that a concentration of more than 0.6 mM was maintained. We confirmed the hydrogen content with a hydrogen electrode (ABLE). Each day, hydrogen water from the aluminum bag was placed into a closed glass vessel (70 mL) equipped with an outlet line containing two ball bearings, which kept the water from being degassed. This vessel ensured that the hydrogen concentration was more than 0.4 mM after 1 day. Hydrogen water degassed by gentle stirring was used for

control animals; the complete removal of hydrogen gas was confirmed with a hydrogen electrode.

Sample collection and biochemical assays

Three days after cisplatin injection, animals were killed under anesthesia, blood was collected from the heart, and the kidneys were obtained. The left kidney was used for measurement of the level of malondialdehyde (MDA) and the right kidney was used for H&E and TUNEL staining. Serum levels of creatinine and BUN were measured using a Creatinine Testwako kit and a Urea N B Testwako kit (Wako Pure Chemical Industries Ltd., Osaka, Japan), respectively. MDA levels in the kidney were determined using a BIOXYTHCH MDA-586 Assay kit (OxisResearch, Oregon, USA) as described previously [17].

Measurement of hydrogen concentration in blood

Rat received hydrogen water orally by stomach gavage at 15 mL/kg. Three minutes after administration, the rat was killed under anesthesia and blood was collected from the heart. Hydrogen concentration in blood was measured as described previously [14]. In brief, 5 mL of blood was kept in a closed aluminum bag with 25 mL air to transfer the hydrogen from blood to the air. The amount of hydrogen in the air was measured by gas chromatography.

H&E and TUNEL staining

The kidney was fixed with 4% paraformaldehyde in PBS. The tissues were dehydrated, embedded in paraffin, sectioned at 5-μm thickness, and stained by hematoxylin and eosin (H&E) for histopathological analysis. The degree of injury was scored according to the following scale: 0 no pathological findings, 1 mild, 2 moderate, 3 severe. Apoptosis was detected by DNA strand breaks using terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end-labeling (TUNEL) according to the procedure of the manufacturer (Chemicon International).

In vitro cytotoxicity assay

S-180 ($1 \times 10^4\,mL^{-1}$) or L-1210 ($5 \times 10^4\,mL^{-1}$) cells were seeded in 24-well plates. The cells were treated with various concentrations of cisplatin or PBS and cultured in medium with or without 0.6 mM hydrogen. After 72-h incubation, dead cells were assessed with 0.2% trypan blue staining [18] and scored viable cells. Under serum-free conditions, S-180 cells ($2 \times 10^4\,mL^{-1}$) were seeded in 24-well plates and trypan blue assay was performed after 120-h incubation with cisplatin. We repeated independent experiments using 3 wells for each concentration.

Cell culture in medium with or without hydrogen was performed as described previously [14]. In brief, we dissolved hydrogen into medium by bubbling hydrogen gas (75% H_2, 20% O_2 and 5% CO_2). We used medium bubbled with control gas (75% N_2, 20% O_2 and 5% CO_2) as a control. The cells were maintained at 37°C in a humidified box filled with gas with or without hydrogen gas.

In vivo anti-tumor activity assay

S-180 cells (3×10^6 cells/mouse) were subcutaneously inoculated into the back of ddY mice. One week later, the tumors had grown to 70–130 mm^3, and the mice were randomly divided into three groups. The first group received physiological saline and the second and third groups received three consecutive daily injections of cisplatin (5 mg/kg). The second and third groups were given water with or without hydrogen throughout the experiment, as described above. Tumor volume was measured with LaTheta LCT-100, X-ray CT for experimental animals (Aloka Co., Ltd., Tokyo, Japan) after the administration of Omnipaque 300, a contrast medium (Daiichi Sankyo Co., Ltd., Tokyo, Japan).

Statistical analysis

We performed statistical analysis using StatView software (SAS Institute) by applying an unpaired two-tailed Student's *t* test and ANOVA followed by Fisher's exact test as described previously [14].

Results

Inhalation of hydrogen gas reduced mortality, body-weight loss and nephrotoxicity induced by cisplatin

To investigate the effect of hydrogen gas on cisplatin-induced toxicity, mice were intraperitoneally injected with a single dose of cisplatin (17 mg/kg) and housed in a box filled with 1% H_2 in air, as described in "Materials and methods". We monitored their survival rate daily (Fig. 1a). In the control air group, mice started to die on Day 2 and only 60% of mice survived to Day 6. In contrast, all mice survived to Day 5 and 80% of mice survived to Day 9 in the hydrogen gas group. No mice died after Day 9 in all groups. Body-weight loss in the control group on Day 3 was 9.7%, whereas inhalation of hydrogen gas significantly suppressed body-weight loss to only 3.5% on Day 3 (Fig. 1b).

Next we measured the levels of serum creatinine and blood urea nitrogen (BUN) to assess the functional effect of hydrogen on cisplatin-induced renal dysfunction (Fig. 1c, d). Cisplatin increased the levels of serum creatinine and

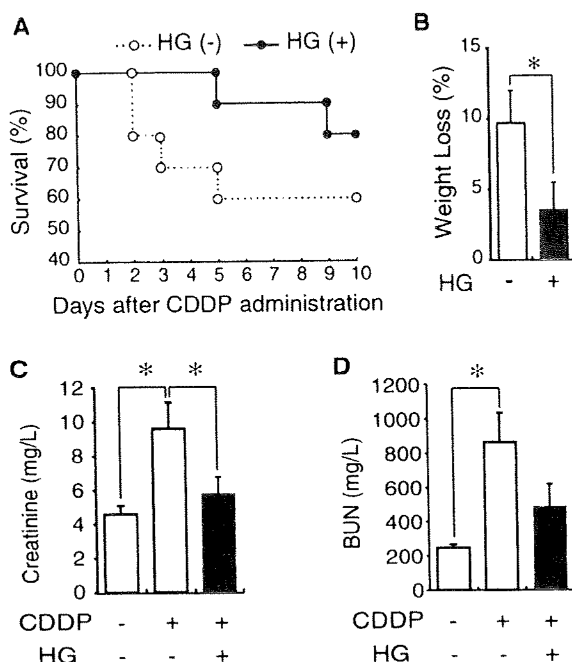

Fig. 1 Hydrogen gas (*HG*) reduces mortality, body-weight loss and nephrotoxicity induced by cisplatin (*CDDP*). Mice were injected intraperitoneally with a single dose of cisplatin (17 mg/kg) (Day 0). Hydrogen gas was administered by inhalation (1% H_2 in air) throughout the experiments (from Day 2 to Day 10). HG (+) and HG (−) were mice that inhaled air with or without hydrogen, respectively. **a** Survival rate was monitored daily ($n = 10$). **b** Body weight of each mouse was measured on Day 3 ($n = 12$). **c** Serum creatinine and **d** BUN levels were measured on Day 3 ($n = 5$). Data are the means ± SEM. Difference in body-weight loss was significant ($*P < 0.05$) by Student's *t* test. Differences in creatinine and BUN levels were significant ($*P < 0.05$) by one-way ANOVA

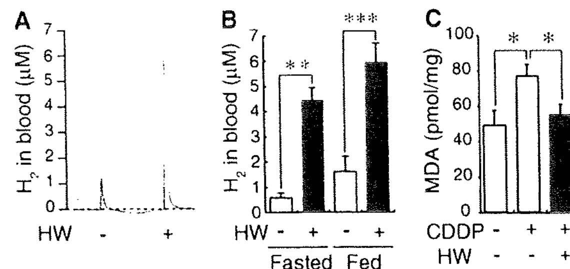

Fig. 2 Hydrogen is detected in blood after oral administration of hydrogen water and reduced oxidative stress in the kidney. **a** Rats (approximately 230 g) were administered 3.5 mL of hydrogen water (0.8 mM H_2 in water) into the stomach via a catheter. After 3 min, hydrogen concentration in blood was quantified using gas chromatography, as described in "Materials and methods". Representative profiles of gas chromatography for detecting molecular hydrogen are shown. **b** Hydrogen concentration in blood was quantified in fasted and fed state as described in **a** ($n = 5$ for fasted group and $n = 3$ for fed group). Data are the means ± SD. Differences in hydrogen concentration were significant ($**P < 0.01$, $***P < 0.001$) by Student's *t* test. **c** Mice were injected intraperitoneally with a single dose of cisplatin (17 mg/kg) (Day 0). Hydrogen water (0.8 mM H_2 in water) was available ad libitum throughout the experiments (from Day 2 to Day 3). HW (+) and HW (−) were mice given water with or without hydrogen, respectively. MDA was measured on Day 3 ($n = 15$). Data are the means ± SEM. Differences in the MDA level were significant ($*P < 0.05$) by one-way ANOVA

BUN by two- and fourfold, respectively, at 72 h after administration with cisplatin as compared with the non-treatment group. Inhalation of hydrogen gas decreased the levels of serum creatinine (9.6 ± 1.5 (SEM) vs. 5.7 ± 1.0 (SEM) mg/L) and BUN (863 ± 170 (SEM) vs. 477 ± 135 (SEM) mg/L) as compared with the control group with cisplatin and without hydrogen.

Hydrogen was detected in blood by oral administration of hydrogen water

Hydrogen gas may be inconvenient for daily intake; thus, we examined whether hydrogen can be administered as hydrogen water (water containing hydrogen) instead of hydrogen gas. Molecular hydrogen is dissolved in water at the saturated level of 0.8 mM [14]. Blood of several milliliters is necessary to measure the hydrogen concentrations in blood. Because it is difficult to obtain a sufficient volume of blood from mice, we used rats for the measurement of hydrogen concentration in the blood. We placed hydrogen water at 3.5 mL/230 g (15 mL/kg) in the stomach of a rat via a catheter in the fed and fasted state, and measured the concentration of hydrogen in blood after 3 min as described [14]. The concentration of hydrogen increased 3.7-fold and 7.6-fold in the fed and fasted state, respectively (Fig. 2a, b), suggesting that orally administered hydrogen can be incorporated into the body.

Next hydrogen water was given to mice ad libitum as described in "Materials and methods". We measured the consumed volume of hydrogen water and degassed control water in mice. Water intake was nearly the same (194 ± 12 (SD) vs. 188 ± 15 (SD) mL/(kg day)) between groups drinking hydrogen water and degassed control water. In addition, a 24-h water intake ad libitum (194 mL/kg) was almost 13-fold higher compared with a single water intake given by a catheter as mentioned above (15 mL/kg); thus we used the method in which hydrogen water was available ad libitum throughout the whole period.

Consuming hydrogen water ad libitum reduces oxidative stress in the kidney

Cisplatin stimulates the generation of ROS such as hydroxyl radicals and renal lipid peroxidation [19]. We examined the effect of hydrogen on oxidative stress in the kidney as judged by the level of malondialdehyde (MDA), an oxidative stress marker derived from lipid peroxides [20]. Mice were given hydrogen water freely throughout

the experiment. Three days after cisplatin administration, the MDA level in the kidney fell to nearly the normal level in mice drinking hydrogen water (Fig. 2c), indicating that daily consumption of hydrogen water suppresses oxidative stress.

Consuming hydrogen water ad libitum reduced mortality, body-weight loss and nephrotoxicity induced by cisplatin

To reveal whether hydrogen water had similar effects to hydrogen gas, we next examined the survival rate, body-weight loss and nephrotoxicity induced by cisplatin. Taking hydrogen water ad libitum improved their survival rate (Fig. 3a), and significantly suppressed body-weight loss (Fig. 3b). We measured levels of serum creatinine and BUN at 72 h after administration with cisplatin as described above (Fig. 3c, d) to reveal the effect of hydrogen water on cisplatin-induced nephrotoxicity. Giving hydrogen water freely significantly decreased serum creatinine (9.6 ± 1.5 (SEM) vs. 5.7 ± 0.6 (SEM) mg/L) and BUN levels (863 ± 170 (SEM) vs. 452 ± 101 (SEM) mg/L) compared with cisplatin alone. Hydrogen gas appeared to be more protective than hydrogen water for the first 3 days in the survival curves; however, the inhalation of hydrogen gas showed no apparent difference with drinking hydrogen water on attenuating cisplatin-induced nephrotoxicity on Day 3. These data suggest that hydrogen water rescue mice less than hydrogen gas from severe damage, which caused death within 72 h after cisplatin administration, but could efficiently protect kidney of mice from moderate damage.

As observed by H&E staining, cisplatin caused histopathologically serious tubular damage as characterized by vacuolization, desquamation of epithelial cells, and many hyaline and protein casts in renal tubules (Fig. 4a). Daily consumption of hydrogen water markedly improved cisplatin-induced histopathological changes. Moreover, hydrogen water reduced the number of TUNEL-positive cells (Fig. 4c), suggesting that hydrogen suppressed apoptosis. Semi-quantitative analysis of metamorphosis is shown in Fig. 4b. Taken together, drinking hydrogen water ad libitum functionally and morphologically alleviates nephrotoxicity induced by cisplatin.

Hydrogen does not impair anti-tumor activity by cisplatin

We tested the possibility that hydrogen impairs anti-tumor activity of cisplatin using cultured cells. Hydrogen and oxygen concentrations were maintained in culture medium as described [14], where pH is not influenced by hydrogen. S-180 sarcoma and L-1210 leukemia cells were exposed to various concentrations of cisplatin to induce cell death and continued to culture in medium with or without 0.6 mM hydrogen (Fig. 5a–c). Cell death was assessed using trypan blue staining [18]. Hydrogen did not suppress cell death induced by cisplatin in vitro (Fig. 5a–c).

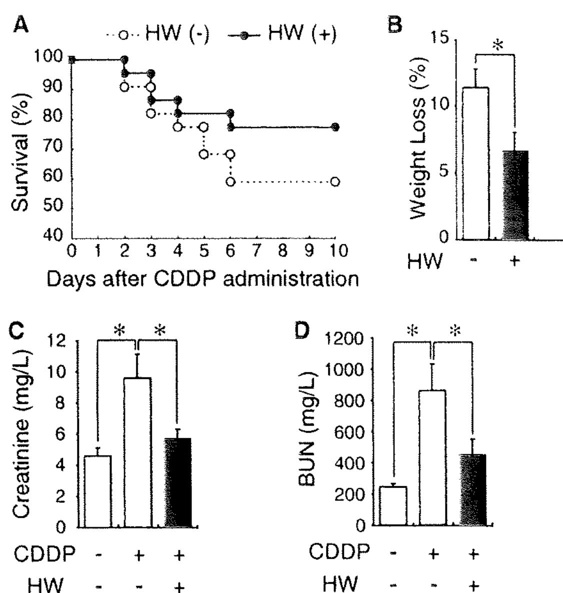

Fig. 3 Hydrogen water (*HW*) reduces mortality, body-weight loss and nephrotoxicity induced by cisplatin (*CDDP*). Mice were injected intraperitoneally with a single dose of cisplatin (17 mg/kg) (Day 0). Hydrogen water was administered by drinking ad libitum (0.8 mM H_2 in water) throughout the experiments (from Day 2 to Day 10). HW (+) and HW (−) were mice given water with or without hydrogen, respectively. **a** Survival rate was monitored daily ($n = 22$). **b** Body weight of each mouse was measured on Day 3 ($n = 25$). **c** Serum creatinine and **d** BUN levels were measured on Day 3 ($n = 15$). Data are the means ± SEM. Difference in body-weight loss was significant ($*P < 0.05$) by Student's *t* test. Differences in creatinine and BUN levels were significant ($*P < 0.05$) by one-way ANOVA

We next evaluated the effects of hydrogen on anti-tumor activity of cisplatin using tumor-bearing mice in vivo [21]. As the sublethal dose of cisplatin described above is not applicable for actual clinical uses, we examined anti-tumor activity of a safe dose of cisplatin using a transplantation model. To obtain an optimal dose and times, cisplatin was injected with different doses (5, 10, or 15 mg/kg) and times (once, twice or three times) ($n = 6$ in each experiment). Treatment of three consecutive daily injections of cisplatin (5 mg/kg) inhibited tumor growth and caused only a little weight loss. Higher doses of cisplatin (10 or 15 mg/kg, single injection) caused apparent weight loss (10–30%). Therefore, the regimen (5 mg/kg, three times) was used in this study. We transplanted S-180 sarcoma cells into ddY mice and monitored the tumor mass with a CT scan. When tumor-bearing mice received an injection of physiological saline instead of cisplatin, the tumor tissue increased in mass by twofold on Day 7 (Fig. 5d, e). Administration of three consecutive daily injections of cisplatin (5 mg/kg) inhibited tumor growth. Notably, cisplatin inhibited tumor growth in

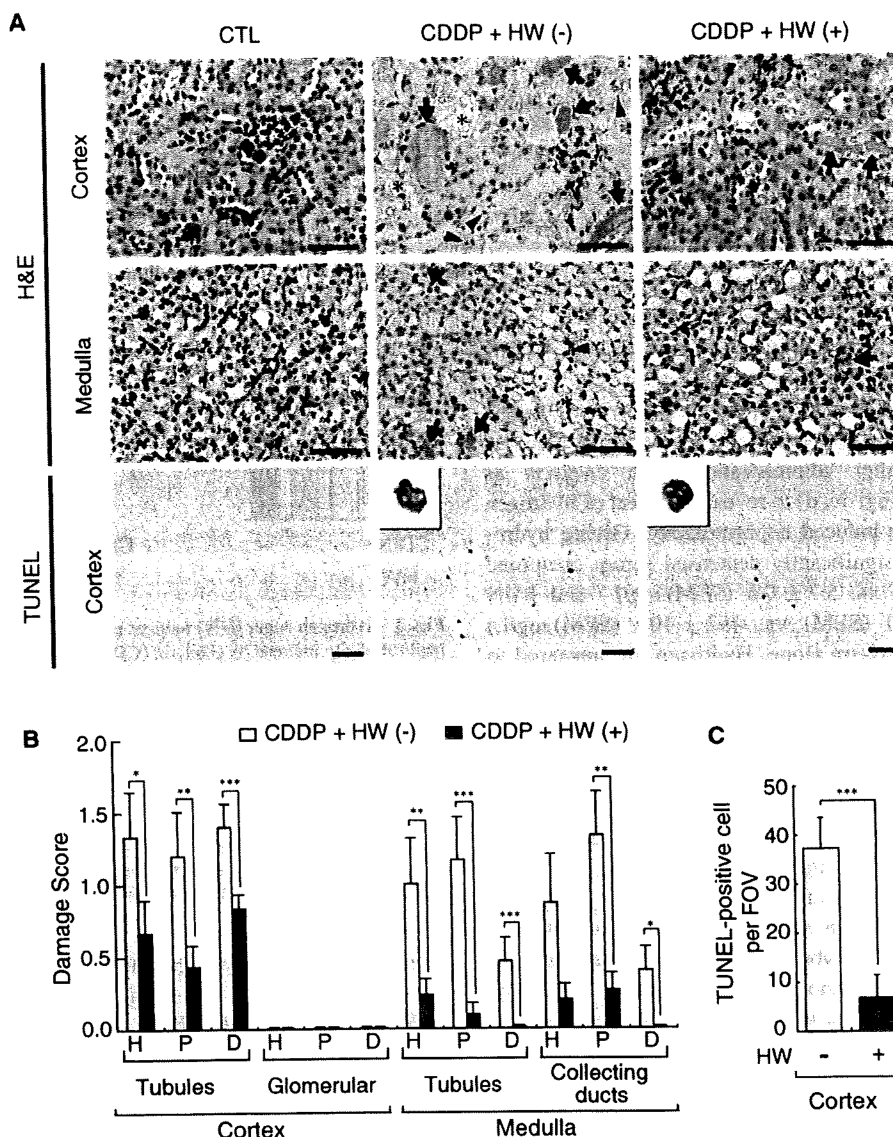

Fig. 4 Hydrogen prevents cisplatin-induced acute renal injury. **a** Mice were injected intraperitoneally with a single dose of cisplatin (17 mg/kg) (Day 0). Hydrogen water (0.8 mM H_2 in water) was available ad libitum throughout the experiments (from Day 2 to Day 3). HW (+) and HW (−) were mice given water with or without hydrogen, respectively. On Day 3, the kidney was fixed and stained with H&E and TUNEL as described in "Materials and methods". *Arrows* show hyaline cast, *arrowheads* show protein cast, and *asterisks* show degeneration of cell. Representative TUNEL staining of nucleus was enlarged in the *inset*. *Scale bar* 50 μm. **b** Semi-quantitative analysis of the metamorphosis. The degree of injury was scored on H&E stained sections and average scores in each group ($n = 15$) are shown. *H* hyaline cast formation, *P* protein cast formation, *D* degeneration of cell. Data are the means ± SEM. Difference in the score between groups drinking water with versus without hydrogen was significant ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$) by Student's *t* test. **c** The number of TUNEL-positive cells per field of view (*FOV*) were counted in five non-overlapping fields per slide ($n = 6$ mice). Data are the means ± SD. The difference in the number of TUNEL-positive cells between groups drinking water with versus without hydrogen was significant ($***P < 0.001$) by Student's *t* test

the group consuming hydrogen water ad libitum to the same level as in the group without hydrogen water. We measured levels of serum creatinine and BUN as described above (Fig. 1c, d) to assess nephrotoxicity. Giving hydrogen water freely decreased serum creatinine (6.4 ± 0.7 (SEM) vs. 4.1 ± 0.4 (SEM) mg/L) and BUN levels (302 ± 47 (SEM) vs. 217 ± 25 (SEM) mg/L) compared with cisplatin alone. These results clearly indicated that hydrogen does not interfere with the chemotherapeutic activity of cisplatin and attenuate cisplatin-induced nephrotoxicity.

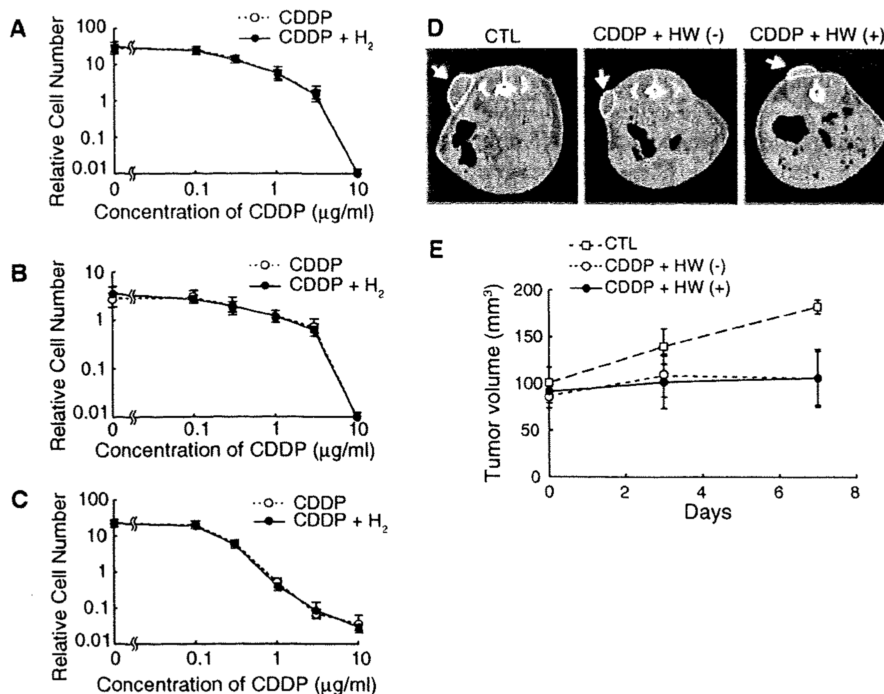

Fig. 5 Hydrogen does not impair cytotoxicity by cisplatin. **a–c** Hydrogen does not influence cytotoxicity of cisplatin against tumor cell lines in vitro. Relative cell number of (**a, b**) sarcoma 180 and (**c**) L-1210 cells were examined under (**a, c**) serum-containing or (**b**) serum-free medium. Cells were cultured in medium with or without 0.6 mM H_2 and treated with various concentrations of cisplatin for 72 (**a, c**) or 120 h (**b**). Cell number was measured by counting viable cells as described in "Materials and methods". Data show relative cell number at 72 (**a, c**) or 120 h (**b**) against the starting cell number. Data are the means ± SD. **d, e** Hydrogen does not inhibit anti-tumor activity of cisplatin in vivo. Sarcoma 180 cells were subcutaneously transplanted into ddY mice in the back. After their tumor volumes reached almost 100 mm^3 (Day 0), mice received three consecutive daily injections of cisplatin (5 mg/kg). Hydrogen water (0.8 mM H_2 in water) was available ad libitum throughout the experiments (from Day 2 to Day 7). On Days 0, 3, and 7, their tumor sizes were evaluated with a CT scan. **d** Representative images of CT scanning on Day 7 are shown. Tumor areas are indicated with *white dot lines* and *arrows*. **e** Tumor volumes were calculated by serial CT scan images, as described in "Materials and methods". Data are the means ± SEM. *CTL* were mice that received saline instead of cisplatin ($n = 4$). HW (+) and HW (−) were mice given water with or without hydrogen, respectively ($n = 4$ for each group)

Discussion

In this study, we demonstrated that hydrogen functionally and morphologically protects the kidney against cisplatin-induced toxicity without impairing its anti-tumor activity. Cisplatin is a platinum-based drug that possesses clinical activity against a wide variety of tumors. Its primary target is DNA and platinum–DNA adducts activate various cellular processes, including the signaling of DNA damage, cell-cycle checkpoints and arrest, DNA repair and cell death [22–24]. Hydrogen does not interfere with the activity of cisplatin, possibly because hydrogen does not interact with platinum–DNA adducts and its downstream pathways. On the other hand, hydrogen significantly alleviated nephrotoxicity, the major dose-limiting side effect. In addition to the main target of cisplatin of DNA, cisplatin has high affinity to SH (sulph-hydryl) groups [19]. The interaction of cisplatin with SH groups leads to GSH depletion, resulting in reduction of the cellular antioxidant system and accumulation of ROS or its products [3, 4, 19]. Cisplatin accumulates predominantly in the kidney than other tissues because the major route of its excretion is via the kidney [11]. The accumulation of cisplatin and the generation of ROS in the kidney may be attributed to cisplatin-induced nephrotoxicity. DNA-damaging agents usually have less toxicity in non-dividing cells, whereas ROS has severe toxicity in quiescent cells. In this study, we administrated a high dose of cisplatin into mice by a single shot to exhibit apparent side effects although the drug is consecutively administrated into patients at lower doses.

A wide variety of antioxidants have been reported to exhibit a protective effect on cisplatin nephrotoxicity. The administration of a wide variety of antioxidants, such as vitamin E [12, 25, 26], vitamin C [12, 25, 27, 28], selenium [26, 29], carotenoids [30, 31], melatonin [32], allopurinol [33], erdosteine [34, 35], edaravone [36] and *N*-acetylcysteine [36, 37] have been reported to ameliorate cisplatin-induced nephrotoxicity in various rodent models; however,

in animal experiments, high doses of antioxidants were required to obtain a significant effect; for example, the effect at 250 mg/kg dose of vitamin C or vitamin E was shown to protect against oxidative renal damage induced by cisplatin in mice [12]. If the same dose is given to humans (15 g for 60 kg body weight), the amount would be much higher than the tolerable upper intake concentration of vitamin C (2 g/day) or vitamin E (1 g/day), as recommended by the Food and Nutrition Board of the U.S. Institute of Medicine [38]. Moreover, it is known that excess vitamin C functions as a pro-oxidant [39]. Compared to these antioxidants, hydrogen has an advantage to protect cells within a safe dosage. Notably, hydrogen water was ad libitum provided to mice in this study. Moreover, even when too much hydrogen is taken in, the excess would be expired via the lungs. Thus, hydrogen gas or hydrogen water should be applicable for patients with cancer to reach efficient amounts.

Low concentrations of ROS, such as superoxide anion and hydrogen peroxide, function as signaling molecules and regulate apoptosis, cell proliferation, and differentiation [40, 41]. In fact, recent studies have suggested that excessive antioxidant increased mortality and rates of cancer, because it may interfere with essential defensive mechanisms [42–44]. Hydrogen selectively reduces hydroxyl radicals but not superoxides and hydrogen peroxides having physiological roles [14]; thus, we suggest that the side effects of hydrogen must be small, different from other antioxidants. Inhalation of hydrogen gas does not influence physiological parameters such as body temperature, blood pressure, pH and pO_2 in the blood, as shown previously [14]. Hydrogen has already been used for human in the prevention of decompression sickness in divers at the level of 2 MPa partial pressure of hydrogen, suggesting that 16 mM hydrogen in blood could be safe [45].

This study showed that inhalation of hydrogen gas has effective protection against cisplatin. For acute and strong oxidative stress induced by ischemia/reperfusion, 1% of hydrogen gas is sufficient protection, as shown previously [14, 17, 46–48]. Inhalation of 1 or 2% hydrogen gas may be applicable for short-term treatments. Such a low concentration of hydrogen gas is safe because hydrogen cannot burn or explode under 4.7% of hydrogen gas. In addition to hydrogen gas, this study demonstrated that drinking hydrogen water ad libitum was sufficient to obtain a significant effect. We showed that hydrogen from the stomach delivered to blood in 3 min and that it reduced the level of oxidative stress (Fig. 3). Even with no administration of hydrogen water, a small amount of hydrogen was detected in blood (Fig. 3). This hydrogen is probably derived from hydrogen produced by large intestinal bacteria.

The brain, heart and liver were protected from oxidative stress by inhalation of 1% hydrogen gas, whose concentration in blood was expected to be 8 μM because the saturated level of hydrogen in water reaches 800 μM under atmosphere pressure [14, 17, 46]. It is possible that continuous consumption of hydrogen protects the kidney from chronic oxidative stress even at much lower concentrations than 8 μM. In this study, we presented that the incorporation of hydrogen from the stomach into blood reaches the level of several μM orders. The water volume that we placed in the stomach corresponds to almost one tenth of consumption volume for 24 h. Frequency of drinking episodes was 11.13 ± 1.28 (mean ± SE) per day in mice [49]. Thus, these data suggest that mice having free access to hydrogen water would take several μM hydrogen into blood 11 times a day. Continuous exposure to hydrogen may change blood components towards the reductive state, and indirectly influence the oxidative state in the kidney. In fact, a randomized clinical test has recently shown that drinking water dissolving hydrogen reduced an oxidative stress marker of patients with diabetes [50]. It is very convenient to drink hydrogen water to take hydrogen during chemotherapeutic treatments; thus, hydrogen has potential to improve quality of life during chemotherapy. Furthermore, we expect that hydrogen would allow higher doses of cisplatin to patients by efficiently mitigating the side effects.

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Conflict of interest statement Dr. Ohta is a director of Mitos Co. Ltd. (Kawasaki, Japan), and a scientific adviser to Blue Mercury Inc. (Tokyo, Japan). Blue Mercury Inc. supplied the fresh hydrogen water used in this study and has donated a research division to our institute.

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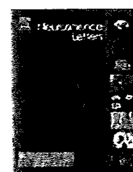

Molecular hydrogen is protective against 6-hydroxydopamine-induced nigrostriatal degeneration in a rat model of Parkinson's disease

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ABSTRACT

Molecular hydrogen serves as an antioxidant that reduces hydroxyl radicals, but not the other reactive oxygen and nitrogen species. In the past year, molecular hydrogen has been reported to prevent or ameliorate eight diseases in rodents and one in human associated with oxidative stress. In Parkinson's disease, mitochondrial dysfunction and the associated oxidative stress are major causes of dopaminergic cell loss in the substantia nigra. We examined effects of ~50%-saturated molecular hydrogen in drinking water before or after the stereotactic surgery on 6-hydroxydopamine-induced nigrostrital degeneration in a rat model of Parkinson's disease. Methamphetamine-induced behavioral analysis showed that molecular hydrogen prevented both the development and progression of the nigrostrital degeneration. Tyrosine hydroxylase staining of the substantia nigra and striatum also demonstrated that pre- and post-treatment with hydrogen prevented the dopaminergic cell loss. Our studies suggest that hydrogen water is likely able to retard the development and progression of Parkinson's disease.
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Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease, affecting ~0.3% of the population over age 50. PD is characterized by resting tremor, bradykinesia, and rigidity. PD is caused by loss of dopaminergic neurons in the substantia nigra pars compacta. Although the pathomechanims of PD remain mostly unknown, oxidative stress to dopaminergic neurons is one of the major causes leading to dopaminergic neuronal cell loss [33]. Mitochondrial dysfunction and the associated oxidative stress in PD are directly or indirectly supported by studies of genetic forms of PD including *PINK1* [2], *DJ1* [19], and *HTRA2* [38], as well as by mitochondrial toxins including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [15] and rotenone [3].

Molecular hydrogen (H_2) can reduce only hydroxyl radicals ($^{\bullet}OH$), but not superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), or nitric oxide ($NO^{\bullet}$) [5,25]. To date, H_2 has no known side effects in rodents or humans. Being prompted by these unique features of H_2, studies of molecular hydrogen for oxidative stress-associated disorders have flourished this past year. Molecular hydrogen in the form of gas reduces the cerebral infarction volume in rats [25], suppresses hepatic ischemia/reperfusion injury in mice [12], reduces the infarct size of myocardial ischemia/reperfusion injury in rats [16], reduces apoptosis in neonatal hypoxic brain injury in rats [8], and mitigates small intestinal transplantation-induced inflammation in rats [4]. Effects on the neonatal hypoxic brain injury are also shown in peritoneal injection of H_2-saturated saline [7]. Molecular hydrogen dissolved in drinking water similarly prevents stress-induced learning impairment in mice [23], improves lipid and glucose metabolism in type 2 diabetes and impaired glucose tolerance in humans [18], reduces atherosclerotic lesions in mice [26], and prevents cisplatin-induced nephrotoxicity [24].

Intrastriatal injection of the catecholaminergic neurotoxin 6-hydroxydopamine (6-OHDA) exerts its toxic effect by increasing oxidative stress in dopaminergic neurons [30]. The technique has been widely used to evaluate neuroprotective effects of therapeutic modalities. For example, the rat PD model has been treated with vitamin E [6], serofendic acid [17], estrogen [28], and insulin-like growth factor-1 (IGF-1) [27]. Compared to MPTP, 6-OHDA triggers

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more prominent dopaminergic cell loss that cannot be readily prevented by neuroprotective methodologies [1].

In an effort to examine a neuroprotective effect of molecular hydrogen for PD, 6-OHDA-induced PD rats were given free access to ~50%-saturated hydrogen water starting either before or after the stereotactic surgery. Behavioral and pathological analyses demonstrated that molecular hydrogen efficiently prevents both the development and progression of the nigrostriatal degeneration in rats.

Seven-week-old male Sprague–Dawley rats, ranging from 245 to 255 g, were purchased from Charles River Laboratories (Osaka, Japan). Rats were housed in a room temperature-controlled environment at 25 °C under a 12-h light/dark cycle with *ad libitum* access to food and water. The animal studies were approved by the Animal Care and Use Committee of the Nagoya University Graduate School of Medicine.

Fifteen rats were randomly divided into three groups: a control group (Ctr, $n = 5$) with unlimited access to dehydrogenized water, a pretreatment group (PreH, $n = 5$) with unlimited access to hydrogen water starting from 7 days before surgery, and a posttreatment group (PostH, $n = 5$) with unlimited access to hydrogen water starting from 3 days after surgery. Rats were supplied with fresh hydrogen water 7 days a week. We also confirmed before surgery that the weight gains and the amounts of drinking water were not statistically different between the Ctr and PreH groups.

As the immunostaining of 8-hydroxy-dG (8-OHdG) and 4-hydroxy-2-nonenal (4-HNE) failed to stain any cells in the substantia nigra and striatum 4 weeks after surgery (data not shown), we examined the rat brains 48 h after surgery. We randomly divided six additional rats into three groups. The Ctr and PreH groups were essentially the same as above. The third group was a sham operation group that was infused with saline into the striatum and supplied with dehydrogenized water.

Hydrogen water was provided by Blue Mercury (Tokyo, Japan) or TYK (Tokyo, Japan). For both suppliers, we measured that the hydrogen concentration was more than 0.4 mM (equivalent to 50% saturation at room temperature) immediately before transferring to a 50-ml closed glass vessel equipped with an outlet line having two ball bearings. With the glass vessel, the hydrogen concentration remained more than 0.2 mM after 24 h. We measured the hydrogen concentrations with a hydrogen electrode (ABLE, Tokyo, Japan). To make dehydrogenized water, we left hydrogen water uncovered overnight at room temperature and confirmed the complete removal of hydrogen gas.

We administered 6-OHDA (Sigma–Aldrich Japan, Tokyo, Japan) steretotactically into the right striatum as previously described [22]. Briefly, each rat was anesthetized by an intraperitoneal injection of chloral hydrate (400 mg/kg) and was placed in a stereotactic frame. We infused 20 μg of 6-OHDA in 2 μl of saline containing 0.02% ascorbic acid each into two sites of the right striatum that corresponded to the following coordinates: AP 1.6 mm, ML 2.4, and DV 4.2; and AP 0.2, ML 2.6, and DV 7.0.

On days 3, 7, 14, 21, and 28 after surgery, rats were intraperitoneally injected with 5.0 mg/kg of methamphetamine (Dainippon Sumitomo Pharma, Osaka, Japan) to provoke dopamine release from the dopaminergic nerve terminals. We started counting turns at 10 min and counted the total number of turns in the following 30 min. We only counted turns with a diameter of 20 cm or less in order not to include counts when the rats walked along the edge of a 40-cm round platform. The investigator was blinded whether the rats were taking dehydrogenized or hydrogen water.

The rats were deeply anesthetized with chloral hydrate and transcardially perfused with 4% paraformaldehyde. The brains were frozen at −80 °C, and cut into coronal sections. After blocking the sections with horse serum, we incubated the sections either with an anti-TH rabbit polyclonal antibody (Biomol International, Plymouth Meeting, PA) at a dilution of 1:200, an anti-8-OHdG monoclonal antibody (JaICA, Nikken SEIL, Shizuoka, Japan) at 1:100, or an anti-4-HNE monoclonal antibody (JaICA, Nikken SEIL) at 1:40 at 4 °C overnight. We next incubated the sections with the biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) at a dilution of 1:200, followed by incubation with the avidin–biotin–horseradish peroxidase complex (the Vectastain ABC kit, Vector Laboratories) and the ImmPACT DAB Substrate (Vector Laboratories). The images of the striatum were reconstructed by the BZ-8000 microscope (Keyence, Woodcliff Lake, NJ). Two blinded investigators counted the numbers of TH-positive cells.

We counted the number of clockwise turns in 30 min in response to an intraperitoneal injection of methamphetamine (Fig. 1). In two rats before surgery, we confirmed that methamphetamine induced no turn with a diameter of 20 cm or less. The Ctr group exhibited gradual development of hemiparkinsonism over the course of 4 weeks. On the contrary, the PreH group demonstrated a gradual and significant decrease of the number of turns over the course (Fig. 1A). In the PostH group, the number of turns was similar to that of the Ctr group on day 3. After the rats started drinking hydrogen water on day 3, however, the number of turns gradually decreased in 2 weeks, and the improved state persisted up to the end of our observation period of 4 weeks (Fig. 1B).

The rats were sacrificed 4 weeks after surgery. We then immunostained the substantia nigra and striatum for TH (Fig. 2). TH is a rate-limiting enzyme in catecholamine synthesis, and is a marker for dopaminergic neurons in the central nervous system [9]. We counted the number of TH-positive cells at the nigra and found that the 6-OHDA-treatment reduced the number of TH-positive

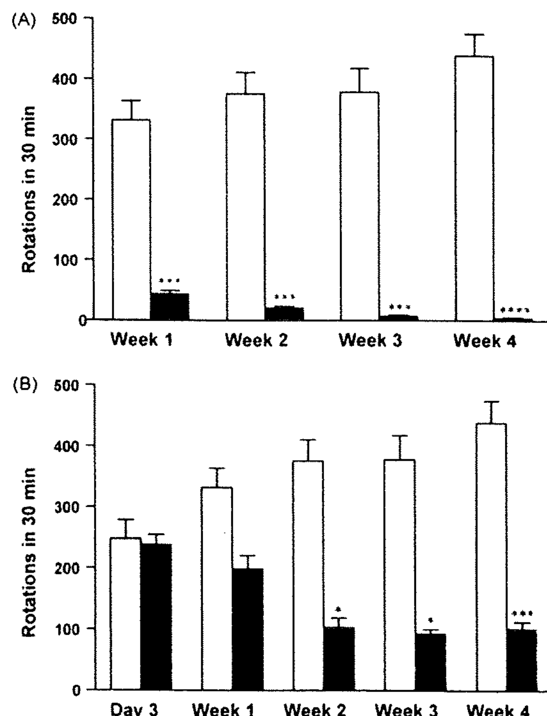

Fig. 1. Behavioral assays of 6-OHDA-treated rats. (A) Temporal profiles of the Ctr (open bars) and PreH (closed bars) rats. (B) Temporal profiles of the Ctr (open bars) and PostH (closed bars) rats. Vertical bars represent means ± S.E.M. of the number of turns in 30 min after intraperitoneal injection of methamphetamine in five rats. Student's *t*-test results are indicated by asterisks: *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$; ****$p < 0.001$.

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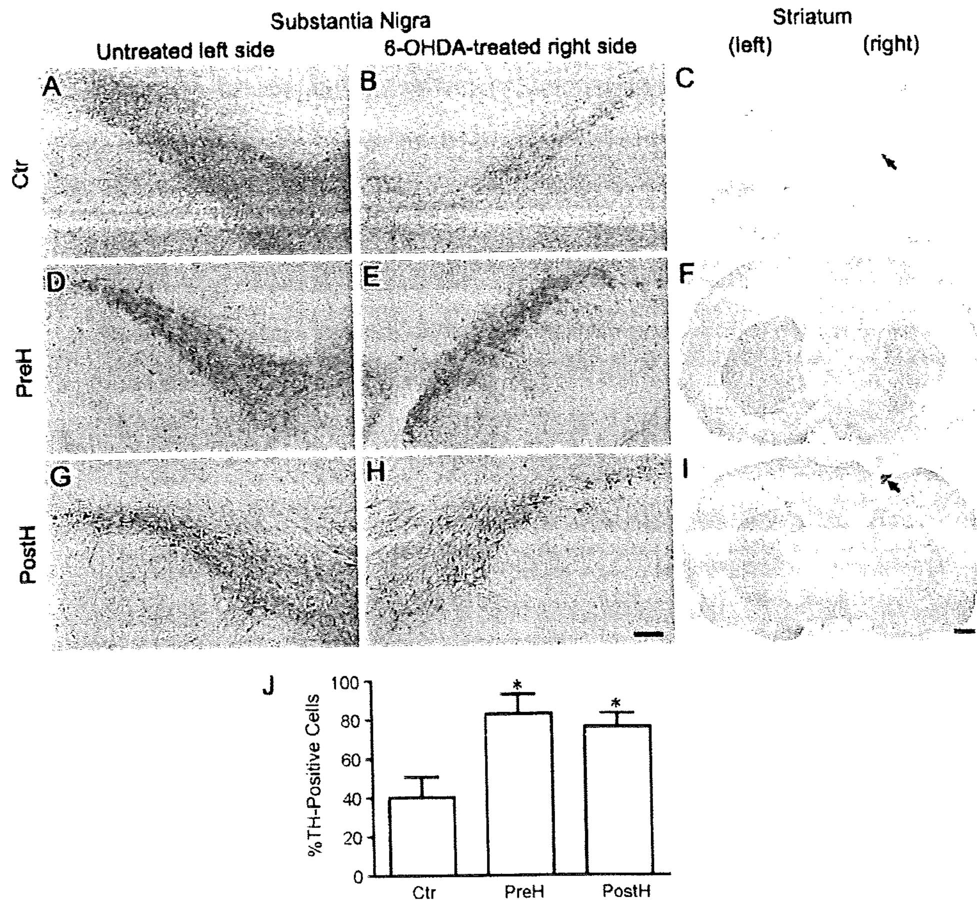

Fig. 2. Representative TH staining of the substantia nigra and striatum of 6-OHDA-treated rats. (A–C) Ctr; (D–F) PreH; (G–I) PostH. (A, D, and G) Untreated left side of the nigra; (B, E, and H) 6-OHDA-treated right side of the nigra; (C, F, and I) the striatum. Arrows point to bleeding scars by the stereotactic needle. (J) The number of TH-positive cells at the substantia nigra on the treated side is divided by that of the untreated side to calculate the ratio of the remaining dopaminergic cells (mean ± S.D., $n = 5$ for each group). Asterisks indicate $p < 0.001$ compared to Ctr (Student's t-test). Bar = 200 μm for A, B, D, E, G, and H; bar = 1 mm for C, F, and I.

cells to 40.2 ± 10.6% (mean ± S.D., $n = 5$) in the Ctr group, whereas the pre- and post-treatment with hydrogen water increased the ratios to 83.0 ± 10.2% and 76.3 ± 7.0%, respectively (Fig. 2J). We did not observe TH-positive nerve terminals in the striatum in the Ctr group, whereas in the PreH and PostH groups we observed patchy sparing of TH-positive nerve terminals (Fig. 2C, F, and I).

The rats were sacrificed 48 h after surgery, and the substantia nigra and striatum were immunostained for TH, 8-OHdG, and 4-HNE (Fig. 3). We observed patchy loss of TH staining (Fig. 3A and B), as well as appearance of 8-OHdG-positive (Fig. 3D and E) and 4-HNE-positive cells (Fig. 3G and H) in the ipsilateral striatum in the Ctr and PreH groups. We observed no effects on the substantia nigra in 48 h after surgery. Smith and Cass similarly report that 4-HNE-positive cells and reduced dopamine concentrations are observed in the striatum but not in the substantia nigra in day 1 after surgery [36]. Forty-eight hours were likely to be too short for 6-OHDA to exert its toxic effect on the substantia nigra.

In the substantia nigra, the oxidative damage and the loss of TH-staining were similar between the Ctr and PreH groups, and H_2 showed no discernible protection against oxidative stress in 48 h.

Our current studies demonstrate that molecular hydrogen in drinking water before the stereotactic surgery efficiently prevents development of PD in a 6-OHDA rat model. In humans, parkinsonian symptoms appear after 80% reduction of striatal dopamine concentration [29] or 50–70% dopaminergic cell loss at the substantia nigra [11]. We observed a loss of 17.0% TH-positive cells in the substantia nigra in the PreH group, which was likely to be below the threshold of the development of PD.

The formation of hydroxyl radical and the subsequent lipid peroxidation and protein oxidation maximize 48 h after 6-OHDA administration and persist for 7 days [30]. We thus examined the oxidative stress in 48 h after surgery, and observed appearance of 8-OHdG-positive and 4-HNE-positive cells, as well as loss of TH-staining, in the ipsilateral striatum. Pretreatment with H_2, however, exhibited no apparent protective effect. This is likely because the acute toxicity of 6-OHDA has exceeded the capacity that molecular hydrogen in drinking water can scavenge. Alternatively, as the treated rats were scarcely capable of drinking water 24 h after surgery, our experimental protocol of *ad libitum* administration of hydrogen water might not be suitable for protecting the acute oxidative stress posed by 6-OHDA. The protective effect against the dopaminergic cell loss in the substantia nigra is thus not likely due to immediate extinction of 6-OHDA in the striatum, but to prevention of the delayed toxic effect of 6-OHDA on the dopaminergic neurons in the substantia nigra. This can also explain why we observed the similar but less efficient effect with the PostH group.

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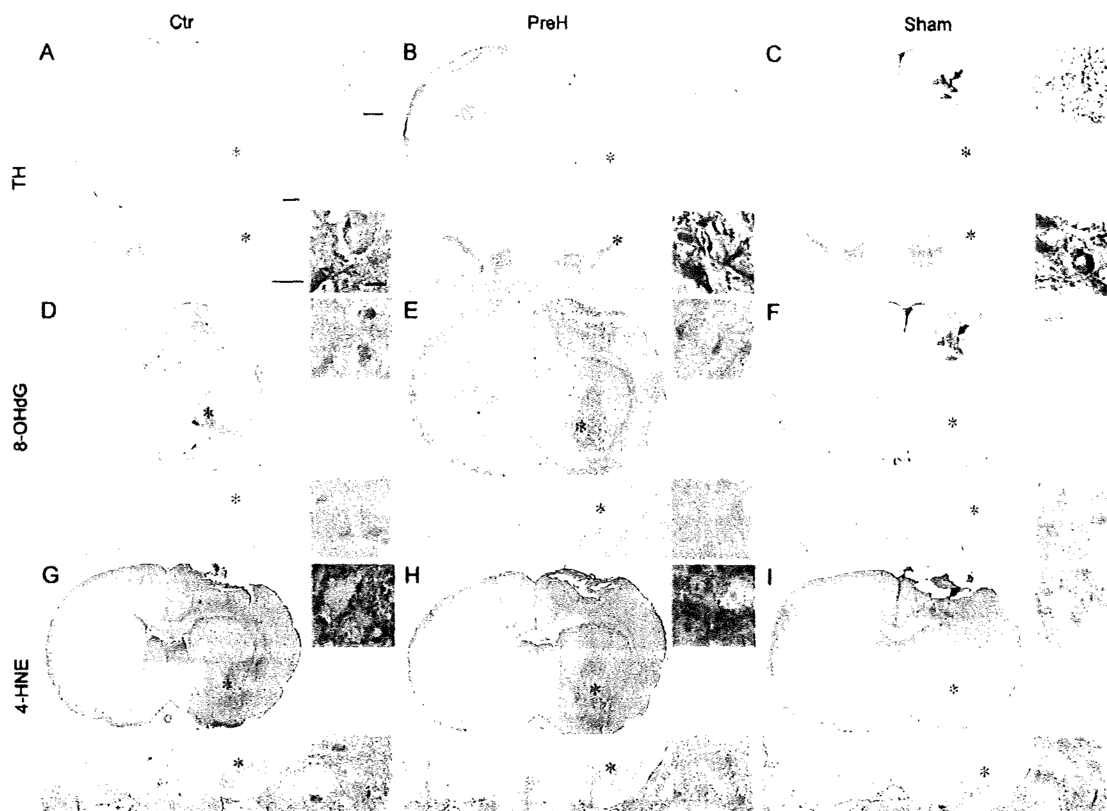

Fig. 3. Representative TH, 8-OHdG, and 4-HNE staining of the substantia nigra and striatum of 6-OHDA-treated and sham-operated rats at 48 h after surgery. (A–C) TH staining; (D–F) 8-OHdG staining; (G, H, and L) 4-HNE staining and nuclear staining with hematoxylin. (A, D and G) Ctr; (B, E, and H) PreH; (C, F, and I) sham-operation. For each panel, the upper part shows the striatum and the lower part shows the substantia nigra. High magnification images are attached on the right side of each panel, and the stars point to the magnified positions. The ipsilateral side is shown on the right side. Arrows point to bleeding scars by the stereotactic needle, where cells are positive for 8-OHdG and 4-HNE even in the sham-operated brain. Bars = 1 mm for low magnification images on the left; 10 μm for high magnification images on the right.

When we administered 3 ml of 0.4 mM hydrogen water directly into the rat stomach, the H_2 concentration in the carotid artery increased by 0.011 mM in 10 min (unpublished data), which was similar to an increase by 0.009 mM when rats were placed in 2% H_2 gas [25]. Hydrogen in drinking water, however, should not stay in the body as long as the inhaled hydrogen. In addition, the scavenging activity of hydroxyl radicals is observed at 0.2 mM or higher concentrations of H_2 in cell-free systems, and is demonstrated at 0.6 mM H_2 in culture cells [25]. The amount of H_2 taken by the rats was likely to be too low to reduce a large amount of hydroxyl radicals generated by 6-OHDA. One possible explanation would be that H_2 activates yet unidentified pathways that culminate in activation of the antioxidant activities. Indeed, H_2 prevents formation of $O_2^{\bullet-}$ in brain slices [31]. As H_2 cannot directly reduce $O_2^{\bullet-}$ [5,25], a subtle reduction of hydroxyl radicals might have exerted beneficial effects on the integrity of mitochondria and led to reduced production of $O_2^{\bullet-}$, but the exact molecular mechanisms remain to be elucidated.

α-Tocopherol demonstrates a neuroprotective effect for PD in rats [6], but not in humans in a double-blind placebo-controlled study [14,34]. Coenzyme Q_{10} similarly exerts a neuroprotective effect in cultured cells and in rodents [20]. Coenzyme Q_{10}, however, may [35] or may not [37] be beneficial in humans in double-blind placebo-controlled trials. Neuroprotective effects of vitamin C and β-carotene are not observed in patients with PD even in open trials [10]. In contrast to the other radical scavengers, molecular hydrogen can reduce only hydroxyl radicals ($^{\bullet}OH$), but not the other reactive oxygen and nitrogen species including superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and nitric oxide ($NO^{\bullet}$) [5,25]. Reactive oxygen and nitrogen species other than the hydroxyl radical play essential roles in biological processes including cell proliferation [32], defense against bacterial infection [39], neurotransmission [13], and vasodilation [21]. Lack of therapeutic effects of α-tocopherol, coenzyme Q_{10}, vitamin C, and β-carotene in PD may be owing to aberrations of the biological processes mediated by the reactive oxygen species. On the other hand, hydroxyl radical has no known biological activities. The ability of molecular hydrogen to exclusively reduce hydroxyl radical may hold the greatest promise for a dependable and efficient neuroprotective modality for PD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2009.02.016.

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