Molecular hydrogen alleviates nephrotoxicity induced by an anti-cancer drug cisplatin without compromising anti-tumor activity in mice

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Abstract

Purpose Cisplatin is a widely used anti-cancer drug in the treatment of a wide range of tumors; however, its application is limited by nephrotoxicity, which is affected by oxidative stress. We have reported that molecular hydrogen (H₂) acts as an efficient antioxidant (Ohsawa et al. in Nat Med 13:688–694, 2007). Here we show that hydrogen efficiently mitigates the side effects of cisplatin by reducing oxidative stress.

Methods Mice were administered cisplatin followed by inhaling hydrogen gas (1% H₂ in air). Furthermore, instead of inhaling hydrogen gas, we examined whether drinking water containing hydrogen (hydrogen water; 0.8 mM H₂ in water) is applicable by examining oxidative stress, mortality, and body-weight loss. Nephrotoxicity was assessed by morphological changes, serum creatinine and blood urea nitrogen (BUN) levels.

Results Inhalation of hydrogen gas improved mortality and body-weight loss caused by cisplatin, and alleviated nephrotoxicity. Hydrogen was detected in blood when hydrogen water was placed in the stomach of a rat. Consuming hydrogen water ad libitum also reduced oxidative stress, mortality, and body-weight loss induced by cisplatin in mice. Hydrogen water improved metamorphosis accompanying decreased apoptosis in the kidney, and nephrotoxicity as assessed by serum creatinine and BUN levels. Despite its protective effects against cisplatin-induced toxicity, hydrogen did not impair anti-tumor activity of cisplatin against cancer cell lines in vitro and tumor-bearing mice in vivo.

Conclusion Hydrogen has potential for improving the quality of life of patients during chemotherapy by efficiently mitigating the side effects of cisplatin.

Keywords Antioxidant · Cisplatin · Dihydrogen · Oxidative stress · Side effect

Introduction

The development of chemotherapeutic drugs exhibiting weak side effects is desired; at the same time, overcoming side effects is essential for the clinical use of anti-cancer drugs. Cisplatin (cis-diaminedichloroplatinum II) is currently one of the most effective chemotherapeutic agents in the treatment of a variety of tumors, including those of the head, neck, testis, ovary and breast [1]. Higher doses of cisplatin are more efficacious; however, high-dose therapy is limited by nephrotoxic side effects [2]. Cisplatin causes the accumulation of reactive oxygen species (ROS), such as superoxide anions and hydroxyl radicals, by suppressing antioxidant activity through decreasing the reduced form of
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. In the control group, air was administered 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. 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% H2 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 administrated 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 (H2) 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 × 10⁴ mL⁻¹) or L-1210 (5 × 10⁴ 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 × 10⁴ 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% H₂, 20% O₂ and 5% CO₂). We used medium bubbled with control gas (75% N₂, 20% O₂ and 5% CO₂) 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 × 10⁶ cells/mouse) were subcutaneously inoculated into the back of ddY mice. One week later, the tumors had grown to 70–130 mm³, 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 (Daichi 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% H₂ 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% H₂ 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.

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) mg/L) vs. 5.7 ± 1.0 (SEM) mg/L) and BUN (863 ± 170 (SEM) mg/L) 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. Given 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).

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 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 120% (Fig. 4c), indicating that hydrogen suppresses apoptosis. We next evaluated the effects of hydrogen on anti-tumor activity of cisplatin using tumor-bearing mice in vivo [21].
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 ($6.4 \pm 0.7$ (SEM) mg/L) vs. $4.1 \pm 0.4$ (SEM) mg/L) and BUN levels ($302 \pm 47$ (SEM) mg/L) vs. $217 \pm 25$ (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. 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.
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 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 pO2 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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