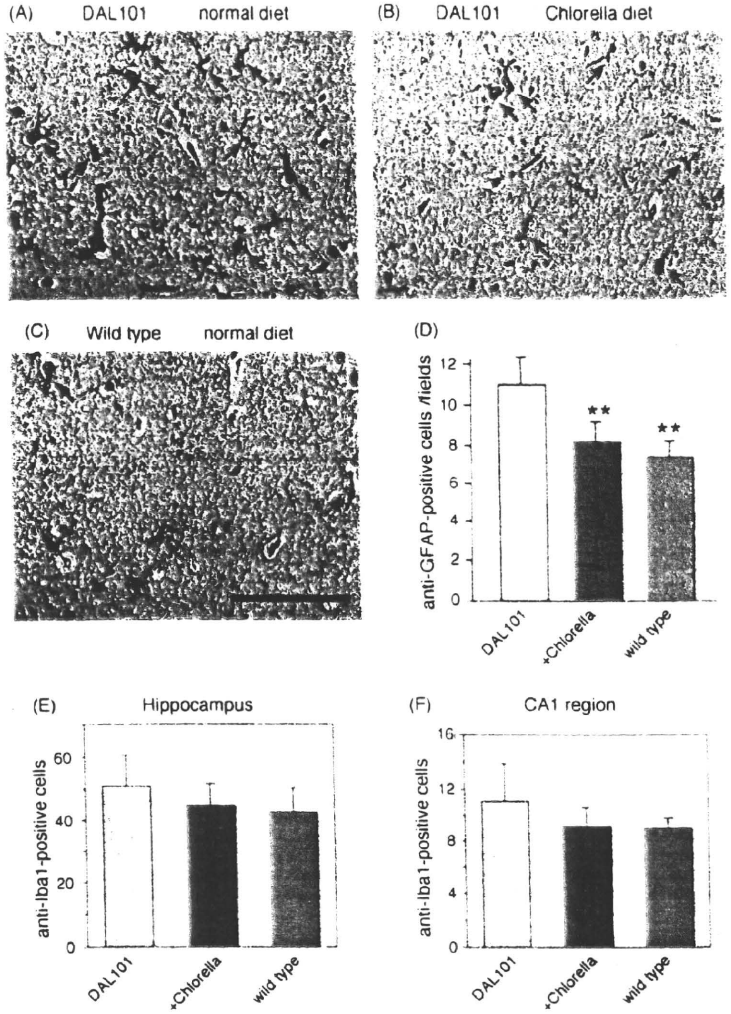

Fig. 4. Effects of *Chlorella* on activation of astrocytes. Activated astrocytes in the hippocampal CA1 region were immunostained using anti-GFAP antibody and anti-Iba1 antibody after 16-month *Chlorella* consumption. Representative pictures are shown (A, B and C). Arrows indicate activated astrocytes. Scale bar: 100 μm. (A) DAL101 (normal diet), (B) DAL101 (*Chlorella* diet), and (C) wild type (C57BL/6 with normal diet). (D) Positive cells in a single visual field in the hippocampal CA1 region were counted in a blinded fashion. (E) and (F) show anti-Iba 1-positive cells in hippocampus and the CA1 region, respectively. DAL101: DAL101 normal diet ($n = 8$), +Chlorella: DAL101 with *Chlorella* diet ($n = 7$), and wild type: C57BL/6 with normal diet ($n = 6$). $^{**}p < 0.01$: significant vs. DAL101 (normal diet).

deficiency affects the growth of animals (Supplementary Table 1). Prolonged consumption of *Chlorella* significantly improved the growth of DAL101, and reached the wild-type level (Supplementary Table 1), clearly indicating that *Chlorella* has a benefit to DAL101 growth.

First, the Morris water-maze test was performed to investigate the effects of a prolonged consumption of *Chlorella* on the decline in spatial memory and learning abilities of DAL101 mice. The physical strength of male DAL101 mice markedly decreases with aging, as reported previously [19]; however, female DAL101 can be subjected to the test because swimming speed of female DAL101 mice was not affected until 18 months old as reported previously [19]. A training trial to find a hidden platform was performed for 5 days, and then the time to reach the hidden platform was measured (Fig. 2). After *Chlorella* was fed for 10 months from 8 weeks of age, there was no difference among the DAL101 group without *Chlorella*, the DAL101 group with *Chlorella* and the wild-type group in the time required to reach the platform. As reported [16], DAL101 is still young at 12 months old (*Chlorella* diet for 10 months from 8 weeks of age) and no symptom of memory loss and cognitive decline appeared. After DAL101 mice had been fed for 16 months, the Morris water-maize test was performed. In this test, DAL 101 mice with the *Chlorella* diet reached the hidden platform significantly faster than DAL without *Chlorella* (Fig. 2B), suggesting that prolonged *Chlorella* consumption suppressed the decline in spatial memory and learning. In a probe trial performed the day after completing the 5-day training trial, DAL101 with *Chlorella* showed only a trend of a longer stay in the targeted quadrant (Fig. 2C).

Next, an alternative test was performed to confirm the effects of 16-month long-term *Chlorella* consumption. The object recognition test is not influenced by physical ability and the sniffing frequency was not significantly decreased even in old male DAL101 (data not shown). Thus, this test involved both male and female DAL101 mice. The animals explored 2 different objects for 10 min during the training period. As a result of the training trial, all control, *Chlorella*

treatment, and normal groups approached or sniffed the 2 objects with the same frequency (~50% of recognition index) (Fig. 3A), indicating comparable attention, motivation and visual perception. One day after training, one of the conditioned objects was replaced with a novel object. During 5-min testing, aged DAL101 with normal diet approached or sniffed the novel object at the same frequency as the original object (Fig. 3B). These results indicated that aged DAL101 showed a deficit in visual recognition memory. In contrast, DAL101 with the *Chlorella* diet showed an intact ability to detect the novel object at high frequency and reached the level of the wild type, indicating that this age-dependent deficit in recognition memory was significantly prevented by the *Chlorella* diet.

Increased astrocyte activation is a marker of inflammation and remodeling in brain impairment [2]. To investigate the effects of *Chlorella* on the central nervous system in DAL101, the number of reactive astrocytes in the hippocampal CA1 region was examined by immunohistological staining (Fig. 4A–C). The number of activated astrocytes immmunostained with anti-GFAP antibody in the hippocampal CA1 region was significantly greater in DAL101 than in wild-type mice (Fig. 4D). On the other hand, we could not find significant difference in anti-Iba 1 antibody staining, a marker of gliosis [3] (Fig. 4E and F). Since the number of activated astrocytes increases when the brain is injured, the brain of DAL101 mice may have been affected 16 months after experiment initiation, probably by accelerated aging. In contrast, the number of activated astrocytes in DAL101 with the *Chlorella* diet was similar to that in wild-type mice, suggesting that *Chlorella* prevented brain injury in DAL101 mice (Fig. 4D).

We investigated the effects of long-term *Chlorella* consumption on oxidative stress, body weight, age-dependent cognitive ability and the central nervous system, and found benefits of *Chlorella* from all points of view.

AD manifests with impaired memory, and finally leads to severe dementia through progressive memory/cognitive disorder. Since DAL101 exhibited a shortened life span, age-dependent neurodegeneration, hyperphosphorylation of tau and decline in cognitive ability, this model mouse is suitable to explore foods that prevent dementia by long-term feeding [16]. We investigated the effects of 16-month *Chlorella* consumption on spatial learning/memory (Morris water-maze test) and object recognition ability (novel object recognition test) using DAL101 mice and found that long-term *Chlorella* consumption prevented cognitive impairment in DAL101.

Increased activated astrocytes are a marker of inflammation and remodeling [2]. Prolonged *Chlorella* consumption significantly decreased activated astrocytes in the hippocampal CA1 region in aged DAL101, suggesting that *Chlorella* may suppress inflammation, perhaps owing to its antioxidative ability.

DAL101 mice cannot efficiently degrade aldehydes, such as 4-HNE, because their ALDH activity is suppressed [16,17]. An increase in the 4-HNE level in patients with mild cognitive disorder (MCI) and early stage AD, compared to that in healthy subjects, has been reported [32], which is consistent with the findings regarding oxidative stress markers in cerebrospinal fluid [20]. Moreover, elevation of the oxidative stress level has been observed in a mouse AD model with amyloid β-protein (Aβ) deposits [21]. Based on these findings, oxidative stress seems to play a crucial role before AD onset.

Chlorella is rich in chloroplasts, where plastoquinone substitutes for ubiquinone as an electron carrier in the photosynthetic electron transport chain. Plastoquinone shows the better antioxidant properties, as shown in chemical experiments [7] and is not complicated by danger of prooxidant effect within a large range of concentrations [27]. It is possible that plastoquinone acts as better antioxidant to prevent the cognitive decline.

Elevated blood homocysteine has been reported to be a risk factor for cardiovascular disease, cerebral stroke, and AD [25]. It has also been reported that elevated blood homocysteine associated with folic acid deficiency increased the cytotoxicity of Aβ in a mouse AD model, in which Aβ accumulates [8]. Insufficient folic acid and vitamin B6 and B12 intake increases homocysteine [9]. *Chlorella* is rich in folic acid (1000–3000 μg/100 g) in addition to antioxidants [22,26]. Thus, it is possible to speculate that *Chlorella* improves cognitive ability in DAL101 mice through the interaction of folic acid and antioxidants. Even if a single dietary antioxidant exhibits no preventive effect, multi-compounds such as *Chlorella* may be efficient to prevent age-dependent disorders.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2009.08.044.

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Research report

Oral supplementation with melon superoxide dismutase extract promotes antioxidant defences in the brain and prevents stress-induced impairment of spatial memory

Sanae Nakajima[a], Ikuroh Ohsawa[a], Kazufumi Nagata[a], Shigeo Ohta[a], Makoto Ohno[b], Tetsuo Ijichi[c], Toshio Mikami[d,*]

[a] Department of Biochemistry and Cell Biology, Institute of Gerontology, Nippon Medical School, Kawasaki, Kanagawa 211-8533, Japan
[b] Department of Graduate School of Nippon Sport Science University, 7-1-1 Fukasawa, Setagaya-ku, Tokyo 158-8508, Japan
[c] Combi Corporation, 5-2-39 Nishibori, Sakura-ku, Saitama-shi, Saitama 338-0832, Japan
[d] Department of Health and Sports Science, Nippon Medical School, 2-297-2 Kosugi-cho, Nakahara-ku, Kawasaki, Kanagawa 211-0063, Japan

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ABSTRACT

The purpose of this study was to investigate the effect of antioxidant ingestion on stress-induced impairment of cognitive memory. Male C57BL/6 mice were divided into four groups as follows: (1) control mice (C mice) fed in a normal cage without immobilization; (2) restraint-stressed (RS mice) fed in a small cage; (3) vitamin E mice (VE mice), mice were fed in a small cage with a diet supplemented with vitamin E; (4) GliSODin mice (GS mice) fed in a small cage with a diet supplemented with GliSODin. RS, VE and GS mice were exposed to 12 h of immobilization daily. Five weeks later, spatial learning was measured using the Morris Water Maze (MWM) test. After water maze testing, we performed immunohistochemical analysis using 4-hydroxy-2-noneral (4-HNE) and an anti-Ki67 antibody. 4-HNE is a marker of lipid peroxidation. RS mice showed impaired spatial learning performance and an increased number of 4-HNE-positive cells in the granule cell layer (GCL) of the hippocampal dentate gyrus when compared to C mice. Moreover, RS mice showed a decreased number of Ki67-positive cells in the subgranular zone (SGZ). GS mice showed better spatial learning memory than RS mice. The number of 4-HNE-positive cells in the GCL of GS mice was significantly less than that of RS mice. The number of Ki67-positive cells in the SGZ of GS mice was significantly greater than that of RS mice. These finding suggests that GliSODin prevents stress-induced impairment of cognitive function and maintains neurogenesis in the hippocampus through antioxidant activity.

1. Introduction

Aging leads to suppression of brain functions such as learning and memory. This effect is accelerated by chronic stress, especially psychological stress. Chronic immobilization stress significantly impaired spatial performance in the MWM, elevated plasma corticosterone levels, and attenuated hippocampal long-term potentiation (LTP) [1]. Escape latencies in the MWM were longer in rats restrained for 21 days than in control rats [2].

Stress-induced impairment of learning and memory is closely related to suppression of hippocampal neurogenesis. Chronic restraint stress resulted in impaired performance in the MWM and a decreased number of BrdU-positive cells in the dentate gyrus [3]. Stress suppresses neurogenesis of dentate gyrus granule neurons, and repeated stress causes remodeling of dendrites in the CA3 region, which is particularly important for memory processing [4].

One of the reasons why stress suppresses hippocampal neurogenesis increased oxidative stress. Fontella et al. [5] reported that repeated restraint stress induced an increase in thiobarbituric acid reactive substance (TBARS) levels and in glutathione peroxidase activity in rats. A relationship between impairment of memory and oxidative stress has been reported. In addition, it has been reported that ingestion of the antioxidant flavanol improved spatial memory retention in adult mammals [6]. However, there have been no reports of protective effects of antioxidant on stress-induced impairment of learning and memory.

In the present study, we investigated whether ingestion of an antioxidant protected against stress-induced impairment of learning and memory. We used two types of antioxidants: GliSODin and α-tocopherol. GliSODin is superoxide dismutase (SOD) extracted from melons and combined with gliadin. SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide and

* Corresponding author. Tel.: +81 44 733 3719; fax: +81 44 733 3719.
E-mail address: mikami@nms.ac.jp (T. Mikami).

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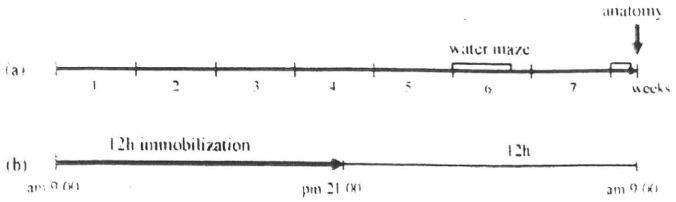

Fig. 1. Experiment protocol: (a) After 5 weeks of chronic immobilization, the spatial memory of all mice was evaluated with the MWM. (b) Chronic immobilization protocol for RA, VE, and GS mice. These mice were exposed to 12 h (9:00 a.m. to 21:00 p.m.) of immobilization in an immobilization cage (width 3 cm, length 3 cm, height 7.5 cm) at a frequency of 6 days per week for 5 weeks. After immobilization, RA, VE and GS mice were fed in six-divided cage. A six-divided cage was made by dividing a standard mouse cage into six sections with plastic boards to make the living space narrow. In this cage, the living space per mouse was 10 cm wide, 10 cm long, and 10.5 cm tall. C mice were fed in a standard mouse cage, where four mice were fed per cage.

is an important antioxidant in nearly all cells exposed to oxygen. In humans, three forms of SOD are present. SOD1 is located in the cytoplasm, SOD2 in the mitochondria, and SOD3 is extracellular. The physiological importance of SODs has been demonstrated by severe pathologies evident in mice genetically engineered to lack these enzymes [7,8]. Additionally, SOD administered as GliSODin led to an increased SOD activity in tissues and protection against oxidative stress. In previous studies, animals supplemented with GliSODin showed significant elevation of circulated antioxidant enzyme activity that was correlated with increased resistance of red blood cells to oxidative stress-induced hemolysis [9]. Supplementation with GliSODin was effective for controlling the thickness of carotid artery intimal and medial layers as measured by ultrasonography-B [10]. α-Tocopherol is also well known to have antioxidant activity. Therefore, we expected that supplementation of GliSODin or α-tocopherol would enhance the antioxidant capacity of the brain and protect against impairment of learning and memory by chronic stress.

Our findings demonstrate that administration of GliSODin prevented stress-induced impairment of spatial memory, increased the number of Ki67-positive cells, and decreased the number 4-HNE-positive cells. These findings suggest that GliSODin is a useful antioxidant for prevention of stress-induced impairment of cognitive function and neurogenesis in hippocampus.

2. Materials and methods

2.1. Animals and diet

All experimental procedures and animal treatments were performed in accordance with the guidelines of the laboratory animal manual of Nippon Medical School. Male C57/BL6 mice (Sankyo Lab Service, Tokyo, Japan) aged 7 weeks and weighing 22.1 ± 1.3 g, were used. Mice were randomly divided into four groups: control mice (C mice; $n = 12$), restraint-stressed mice (RS mice; $n = 10$), vitamin E mice (VE mice; $n = 10$), and GliSODin mice (GS mice; $n = 9$). C mice were fed in a standard mice cage (width 32 cm, length 21.5 cm, height 10.5 cm) with standard animal diet (Oriental Yeast Co., Tokyo, Japan). In this case, four mice were fed in a cage. RS mice were fed in a six-divided cage with standard animal diet. The six-divided cage was made from a standard mice cage divided into six partitions with plastic boards to make the living space narrow. In this cage, the living space per mouse was 10 cm wide, 10 cm long, and 10.5 cm tall.

VE and GS mice were fed in the six-divided cage with a standard animal diet supplied with α-tocopherol or GliSODin, respectively. The VE diet was the standard animal diet supplemented with α-tocopherol acetate at 88 mg per 100 g of diet to generate an α-tocopherol intake of 70 mg/(kg day) according to Li et al. [11]. The GliSODin diet was the standard animal diet supplemented with GliSODin at 125 mg per 100 g of diet to generate a GliSODin intake of 100 mg/(kg day) according to Vouldoukis [12]. All mice were fed with ad libitum access to food and water with a 12-h light/dark cycle (24 °C room temperature, 50% humidity).

2.2. Immobilization

All mice were acclimatized to the living conditions and diet for 5 days. RS, VE, and GS mice were then exposed to 12 h (9:00 a.m. to 9:00 p.m.) of immobilization in immobilization cage (width 3 cm, length 3 cm, height 7.5 cm) 6 days per week for 5 weeks (Fig. 1). During the daily immobilization period, the mice were only freely able to drink water.

2.3. Spatial learning and memory

After 5 weeks of chronic immobilization, the spatial memory of mice was evaluated using the MWM according to the method of Morris with some modifications [13]. Briefly, mice were trained with four trials/day for 5 days. A circular pool that had a diameter of 115 cm was filled with water 1.5 cm above the plastic platform to

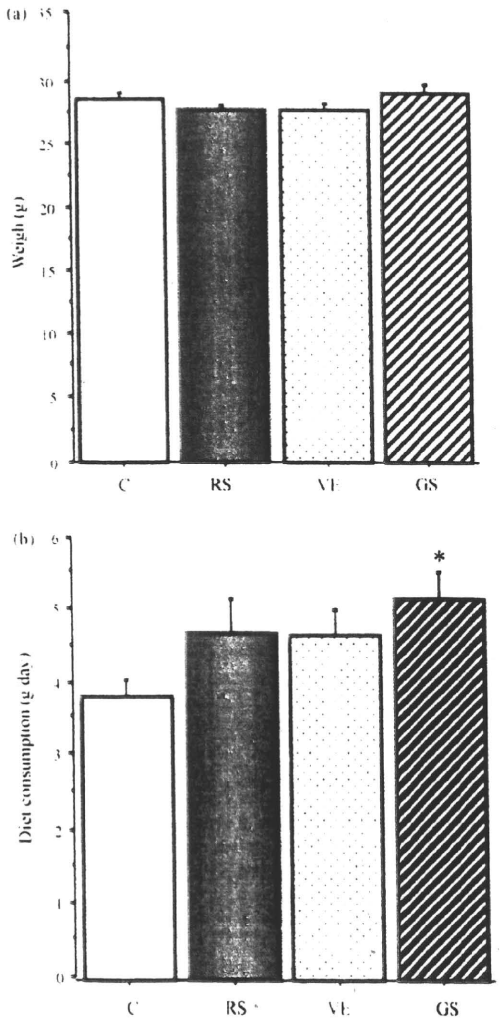

Fig. 2. Body weight and diet consumption: (A) The average weight of mice was not significantly different between conditions. (B) The average diet consumption of RS, VE, and GS mice tended to be greater than that of C mice.

hide it. The water was made opaque with white non-toxic paint and the water temperature was set at 24 °C. A mouse was released into the pool facing the pool wall from four different starting points that were varied randomly each day. The time to reach the platform (escape latency) was recorded for every trial. Each trial lasted either until the mouse had found the platform or for a maximum of 60 s. On each trial, mice were allowed to rest on the platform for 20 s at the end of each trail. To determine long-term retention (memory), the MWM was performed again on the 15th and 16th day after the first MWM.

2.4. Sample collection

The day after completion of MWM, mice were anesthetized with pentobarbital and transcardially perfused with 60 ml saline via the left ventricle. Brains were carefully removed and hemispheres were separated. The left hemisphere was fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; 137 mM NaCl, 8.10 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, pH 7.4) overnight at room temperature. After washing three times with PBS, the brain was cut rostrally at bregma −1.30 mm, caudally at bregma −5.80 mm, and ventrally at 4.5 mm. The areas were serially sectioned rostro-caudally with a Leica vibratome (VT 1000S, Leica Microsystems, Germany) at 50 μm and immersed free-floating in PBS. Ninety-six-well plates were used to keep the sections separate to preserve the order of the series in PBS at 4 °C. The right hemisphere was divided into hippocampus, cerebral cortex, hypothalamus and cerebellum. These samples were quickly frozen with liquid nitrogen and stored at −80 °C until analysis.

2.5. Ki67 immunohistochemistry

To investigate neurogenesis in hippocampus, Ki67-positive cells were identified immunohistochemically. A one-in-eight series of sections (400 μm apart) of every animal was used for stereology of cell counts. The sections were incubated with 3% hydrogen peroxide in methanol to block endogenous peroxidase activity and with normal goat serum to block non-specific staining. After washing with PBS, the sections were exposed to heat (100 °C) in 100 mM citric acid buffer (pH 6.0) for 5 min using a microwave for antigen retrieval. After washing with PBS, the sections were incubated with rabbit polyclonal anti-Ki67 antibody (Abcam, 1:500) for two nights at 4 °C with gentle shaking. After washing with PBS, the sections were incubated with goat anti-rabbit biotinylated IgG (Vector Laboratories, 1:100) for 1 h at room temperature. After washing with PBS, the sections were incubated with avidin–biotin–horseradish peroxidase complex (VECTASTAIN ABC reagent, Vector Laboratories) for 2 h at room temperature. Finally, the sections were washed in PBS and developed using 0.67 mg/ml 3′3-diaminobenzidine (DAB) for 5 min.

2.6. 4-HNE immunohistochemistry

To investigate lipid peroxidation in hippocampus, 4-HNE immunohistochemistry was performed using M.O.M. immunodetection kit (Vector laboratory, USA) according to the manufacturer's instructions. A one-in-eight series of sections (400 μm apart) of every animal was used for stereology of cell counts. Briefly, free-floating sections were washed in PBS and reacted with 3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. After washing with PBS, the sections were treated as described above for antigen retrieval. After washing with PBS, the sections were incubated with M.O.M. mouse IgG blocking solution for 1 h. After washing with PBS, the sections were incubated with 10 μg/ml of monoclonal anti-4-HNE antibody (Japan Institute for the Control of Aging, Japan) in M.O.M. diluent (0.1 M PBS; pH 7.4, 0.5% Triton X-100, 8% protein concentrate stock solution) for two nights at 4 °C with gentle shaking. After washing with PBS, the sections were incubated with biotinylated anti-mouse IgG in M.O.M. diluent (1:250) for 2 h at room temperature. After washing with PBS, the sections were incubated with avidin–biotin–horseradish peroxidase complex for 2 h at room temperature. Finally, the sections were then incubated with VECTASTAIN ABC reagent (Vector Laboratories) for 1 h and developed using DAB.

The sections reacted with Ki67 or 4-HNE antibodies were mounted, dehydrated, and coverslipped using Permount mounting medium For stereology, Ki67-positive cells and 4-HNE-positive cells were counted in subgranular zone (SGZ) or granule cell layer (GCL) using a light microscope (ECLIPSE E400 Nikon; Nikon, Japan) with a 40× objective (Nikon).

2.7. Analysis of SOD activity and α-tocopherol content

SOD activity was measured using the SOD Assay Kit-WST (Dojindo Molecular Technologies Co., Tokyo) as follows: 20 mg of hippocampus was homogenized in the dilution buffer included in the SOD assay kit and centrifuged at 18,000 × g for 10 min. The protein concentration of the supernatant was measured using Coomassie Plus Protein Assay Reagent Kit (Pierce Co., Ltd.). The supernatant (20–50 μg protein) was used for measurement of SOD activity in accordance with the manufacturer's instructions. SOD activity was expressed as SOD content per gram total protein (units/g protein). The level of α-tocopherol in hypothalamus was determined by high performance liquid chromatography (HPLC) according to the method of Milne and Botnen with some modifications [14]. We used the hypothalamus for α-tocopherol analysis because the hippocampus and cerebral cortex had already been used for other analyses.

2.8. Statistical analysis

Data are presented as mean ± S.E. Statistical analysis was performed using Fisher's PLSD post hoc test. $p < 0.05$ was accepted as significant.

3. Results

3.1. Weight and diet consumption

Chronic immobilization and feeding in the six-divided cage did not result in any differences in body weight between groups (Fig. 2A). However, the diet consumption of RS, VE, and GS mice tended to be greater than that of C mice (Fig. 2B).

3.2. Learning and memory

To examine whether stressful conditions (12 h immobilization and feeding in a narrow space) would influence cognitive performance, we tested both learning and memory (Fig. 3). In learning, control mice showed a reduced latency for finding the hidden platform during 5 days of training, whereas RS mice had a significantly longer latency than C mice on days 4 and 5 during the training period ($p < 0.05$). This finding confirms that the experimental conditions used in the present study were stressful enough to impair the learning of RS mice. On the other hand, GS mice showed a latency reduction equal to that of C mice and had a significantly shorter latency than RS mice on day 5 ($p < 0.05$), whereas VE mice did not show a latency reduction equal to that of C mice.

To test memory, mice were exposed to the MWM again on days 15 and 16 (Fig. 3). Both C and GS mice remembered the position of the platform well and showed a latency reduction between days 15 and 16, whereas RS mice had a significantly longer latency than C and GS mice and did not improve. VE mice showed reduced latency but still showed a significantly longer latency than C and GS mice.

3.3. 4-HNE-positive cells in the GCL of the dentate gyrus

Previous studies have shown that psychological stress leads to increased lipid peroxidation in the brain [15]. However, these findings were based on analyses of brain homogenate; no study has actually shown the localization of lipid peroxide in the hippocam-

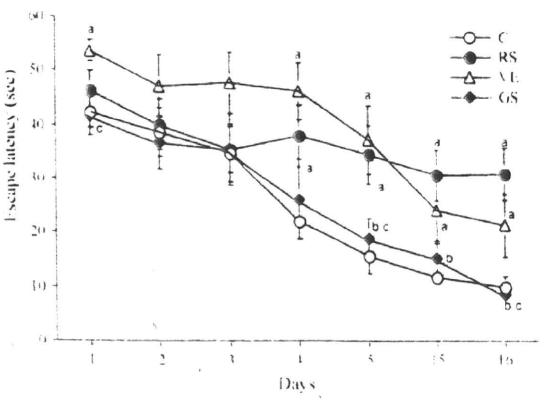

Fig. 3. Spatial learning and memory as measured by MWM RS mice showed significantly impairment of escape latency when compared to C mice. However, the escape latency of GS mice was significantly shorter than that of the RA group. Significant differences in escape latency between the RS and GS group were also maintained on days 15 and 16. The data are shown as the mean ± S.E. (a) $p < 0.05$ vs. C mice, (b) $p < 0.05$ vs. RS mice. (c) $p < 0.05$ vs. VE mice.

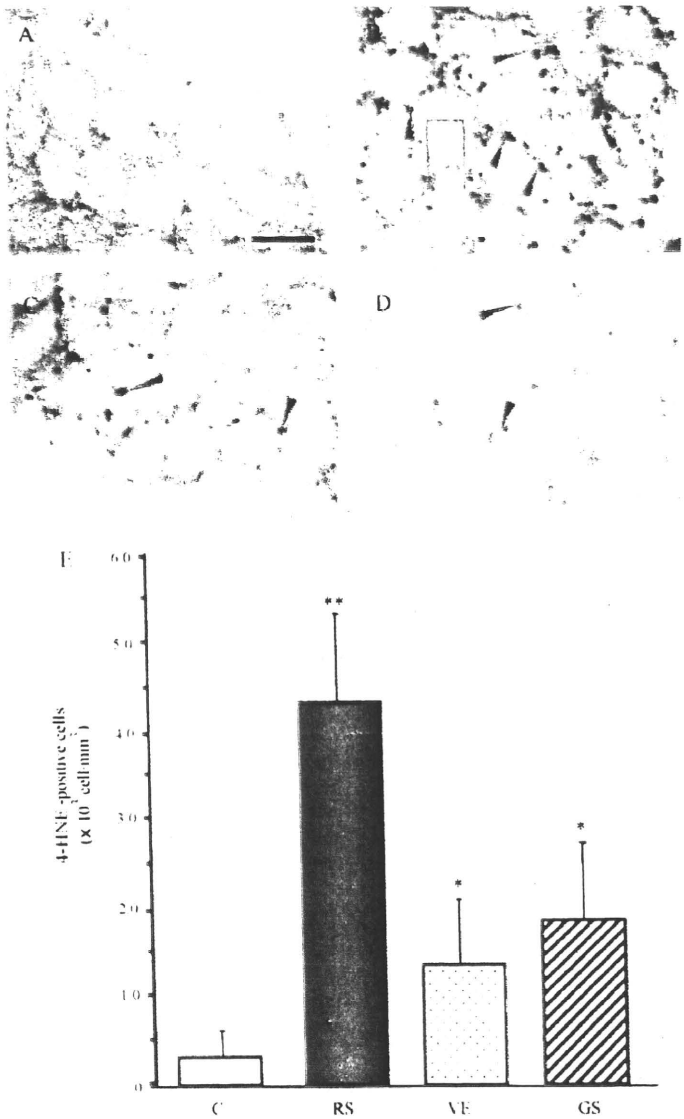

Fig. 4. Number of 4-HNE-positive cells in the GCL Representative images of 4-HNE-positive cells in GCL are shown. (A) C mice, (B) RS mice, (C) VE mice, and (D) GS mice. Bar: 50 μm. (E) Total number of 4-HNE-positive cells in the SGZ. RS mice had significantly more 4-HNE-positive cells in the GCL of the dentate gyrus than C mice. GS and VE mice had significantly less 4-HNE-positive cells in the GCL of the dentate gyrus than RS mice. The data are shown as the mean ± S.E. (a) $p < 0.05$ vs. C mice and (b) $p < 0.05$ vs. RS mice.

pus. Therefore, we used immunohistochemistry to determine whether lipid peroxide was produced in hippocampal neurons under our experimental conditions. As shown in Fig. 4, RS mice had significantly more 4-HNE-positive cells in the dentate gyrus than C mice. GS and VE mice had significantly fewer 4-HNE-positive cells in the dentate gyrus than RS mice. These findings suggest that the stress condition used in the present study caused increased lipid peroxidation in the dentate gyrus and that supplementation with of antioxidants prevented lipid peroxidation induced by chronic stress.

3.4. Neurogenesis in the dentate gyrus

To examine whether the beneficial effects of GliSODin on learning and memory could be mediated by increased neurogenesis in the hippocampus, we measured the number of Ki67-positive cells in the hippocampal dentate gyrus. The number of Ki67-positive cells in the SGZ of the dentate gyrus was significantly lower in RS mice than in control mice (Fig. 5). However, the number of Ki67-positive cells in GS mice was equal to that in C mice and significantly higher than that of RA mice. VE mice did not have an equal number of Ki67-positive to C mice.

3.5. SOD activity and α-tocopherol content

We examined the effects of GilSODin and α-tocopherol administration on SOD activity and α-tocopherol content in the brain. The mouse hippocampus was too small to measure α-tocopherol content. Therefore, we used the hypothalamus to measure α-tocopherol content in the brain.

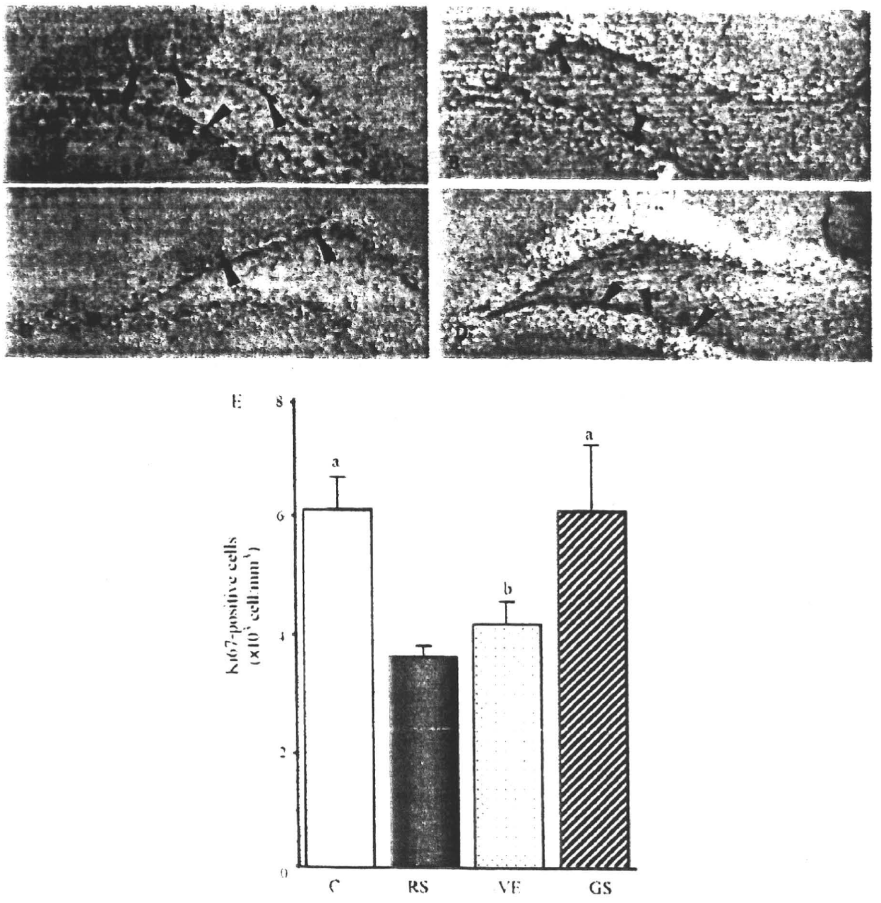

Fig. 5. Number of Ki67-positive cells in the SGZ Representative pictures of Ki67-positive cells in the SGZ are shown. (A) C mice, (B) RS mice, (C) VE mice, (D) GS mice, and (E) Total number of Ki67-positive cells in the SGZ. The number of Ki67-positive cells in the SGZ was significantly lower in RS mice than in C and GS mice. (a) $p < 0.05$ vs. RA mice and (b) $p < 0.05$ vs. C mice.

The hippocampal SOD activity of GS mice was significantly greater than that of the other three groups of mice ($p < 0.05$) (Fig. 6A). There was no significant difference in hippocampal SOD activity among C, RS, and VE mice.

RS mice showed a significantly lower content of α-tocopherol in the hypothalamus compared with control mice (Fig. 6B). In spite of α-tocopherol administration, α-tocopherol content in VE mice did not significantly increase when compared to C mice. On the other

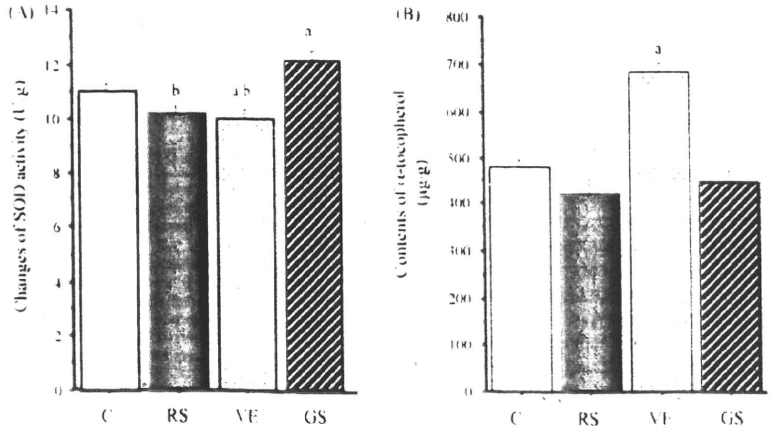

Fig. 6. SOD activity and α-tocopherol content in the brain: (A) SOD activity in hippocampus; SOD activity of GS mice was significantly increased when compared to other mice. (a) $p < 0.05$ vs. C mice and (b) $p < 0.05$ vs. GS mice. (B) α-tocopherol content of the hypothalamus. α-Tocopherol content of VE mice was significantly increased when compared to other mice. (a) $p < 0.05$ vs. C mice.

hand, although GS mice were fed a diet with the same amount of α-tocopherol as the standard diet, they showed a significant higher content of α-tocopherol than C mice.

4. Discussion

To investigate whether administration of antioxidants improves stress-induced cognitive memory impairment, spatial memory in mice exposed to chronic immobilization and feeding in narrow cages was determined using the MWM after administration of the antioxidants GliSODin and vitamin E. Chronic immobilization and feeding in narrow cages resulted in suppression of spatial memory. In addition, the stressful environment resulted in increased and decreased numbers of 4-HNE-positive and Ki67-positive cells in the dentate gyrus, respectively. Administration of GliSODin prevented the impairment of spatial memory, the reduced number of Ki67-positive cells, and the increased number of 4-HNE-positive cells, whereas administration of vitamin E did not prevent the impairment of spatial memory or the loss of Ki67-positive cells in spite of preventing the increase in 4-HNE-positive cells. These findings suggest that GliSODin prevents stress-induced impairment of cognitive function by suppressing of oxidative stress and maintaining neurogenesis in the hippocampus.

In the present study, mice were fed in narrow cages with 12 h of immobilization to generate psychological stress. Unpredictably, the body weight of mice exposed to stress was not significantly different from C mice. On the other hand, the daily food intake of stressed mice tended to be greater than that of C mice. Chronic stress increases daily food intake of animals [16]. In other experiments, we have investigated plasma corticosterone concentrations in mice exposed to same stressful conditions as in the present study. On the third day after initiation of stressful conditions, the plasma corticosterone concentration was significantly higher than at the pre-stress state (data not shown). Therefore, it is likely that the conditions used in the present study resulted in a stress response.

In the present study, restraint stress impaired spatial memory as measured by the Morris MWM, which corresponded to previous findings [2]. However, mice exposed to restraint stress who received GliSODin did not show impairment of spatial memory (Fig. 3). Decreased hippocampal neurogenesis can impair spatial memory [17]. Chronic restraint stress impaired performance in the MWM and decreased the number of BrdU-positive cells in the dentate gyrus of the hippocampus [3]. In addition, impairment of spatial memory is negatively correlated with hippocampal neurogenesis [18]. Some factors such as environmental enrichment or habitual exercise can increase the number of BrdU-positive cells in the dentate gyrus of hippocampus and in turn enhance spatial memory [19]. In the present study, GliSODin treatment prevented impairment of spatial memory and loss of Ki67-positive cells in the dentate gyrus of hippocampus (Figs. 3 and 5). An increased number of Ki67-positive cells in the dentate gyrus reflects increased hippocampal neurogenesis [20]. Therefore, our findings suggest that GliSODin prevents stress-induced suppression of spatial memory by maintaining hippocampal neurogenesis.

Increase of oxidative stress in the hippocampus also suppresses hippocampal neurogenesis during chronic restraint stress [2,5,21–23]. Repeated restraint stress induced an increase in TBARS levels and glutathione peroxidase activity in rats [5]. Chronic restraint stress also significantly elevated the levels of nitrites and TBARS in the frontal cortex and hippocampus [2]. In the present study, we showed that chronic restraint stress increased the number of 4-HNE-positive cells in the GCL of the dentate gyrus. In addition, our findings show that GliSODin treatment reduced the number of 4-HNE-positive cells (Fig. 4). 4-HNE is a representative oxidative stress marker that specifically labels lipid peroxidation in cellular membranes [24]. GliSODin treatment simultaneously increased SOD activity in the hippocampus and decreased the number of 4-HNE-positive cells (Fig. 6A). Therefore, GliSODin might prevent lipid peroxidation in hippocampus by increasing hippocampal SOD activity.

In the present study, we investigated the effects of GliSODin and α-tocopherol on stress-induced lipid peroxidation and impairment of spatial memory. Both GliSODin and α-tocopherol protected against lipid peroxidation; however GliSODin also prevented impairment of spatial memory. The reason for this discrepancy is unclear; however we speculate that GliSODin treatment may have upregulated neurotrophic factors such as insulin-like growth factor 1 (IGF-1), or nerve growth factor (NGF) in the brain or other tissues. IGF-1 enhances hippocampal neurogenesis and protects against stress-induced impairment of spatial memory [25]. In the intestine, macrophages regard GliSODin as non-self and attacked it by releasing reactive oxygen, resulting in the release of NO into the blood. This NO is transferred to the tissues and stimulates induction of several proteins such as SOD and catalase [12]. In addition, NO induces stimulates induction of IGF-1 [26]. In the present study, GliSODin induced SOD activity in hippocampus (Fig. 6). However, whether GliSODin actually induces expression of neurotrophic factors was not determined in the present study. Further investigations are necessary to elucidate this point. Collectively, our findings suggest that GliSODin prevents stress-induced impairment of cognitive function by preventing lipid peroxidation and maintaining neurogenesis in hippocampus.

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Hypothesis

Are the effects of α-glucosidase inhibitors on cardiovascular events related to elevated levels of hydrogen gas in the gastrointestinal tract?

Yoshihiko Suzuki [a], Motoaki Sano [c,*], Kentaro Hayashida [c], Ikuroh Ohsawa [a,b], Shigeo Ohta [a], Keiichi Fukuda [c]

[a] Department of Biochemistry and Cell Biology, Institute of Development and Aging Science, Graduate School of Medicine, Nippon Medical School, Kawasaki City 211-8533, Japan
[b] The Center of Molecular Hydrogen Medicine, Institute of Development and Aging Science, Graduate School of Medicine, Nippon Medical School, Kawasaki City 211-8533, Japan
[c] Department of Regenerative Medicine and Advanced Cardiac Therapeutics, Keio University School of Medicine, Tokyo 160-8582, Japan

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ABSTRACT

The major side-effect of treatment with α-glucosidase inhibitors, flatulence, occurs when undigested carbohydrates are fermented by colonic bacteria, resulting in gas formation. We propose that the cardiovascular benefits of α-glucosidase inhibitors are partly attributable to their ability to neutralise oxidative stress via increased production of H_2 in the gastrointestinal tract. Acarbose, which is an α-glucosidase inhibitor, markedly increased H_2 production, with a weaker effect on methane production. Our hypothesis is based on our recent discovery that H_2 acts as a unique antioxidant, and that when inhaled or taken orally as H_2-dissolved water it ameliorates ischaemia–reperfusion injury and atherosclerosis development.

1. Introduction

A growing body of evidence supports the notion that postprandial hyperglycaemia plays an important role in the development of cardiovascular disease. Large epidemiological studies have shown that the serum glucose concentration 2 h after an oral glucose challenge is a powerful predictor of cardiovascular risk [1,2].

α-Glucosidase inhibitors are pharmacological agents that specifically reduce postprandial hyperglycaemia through retardation of disaccharide digestion, thereby reducing glucose absorption by the small intestine. The STOP-NIDDM trial demonstrated that the treatment of patients who had impaired glucose tolerance with the α-glucosidase inhibitor acarbose was associated with a 25% reduction in the risk of progression to diabetes, a 34% reduction in the risk of developing *de novo* hypertension, and a 49% risk reduction for cardiovascular events [3]. Furthermore, a meta-analysis of seven long-term studies suggested that acarbose reduced the risk of myocardial infarction for patients with type 2 diabetes [4]. Such risk reduction for coronary heart disease events in patients with type 2 diabetes was not observed by the improved glycaemic control achieved with intensified treatment with insulin and glibenclamid [5]. Inhibition of postprandial hyperglycaemia by α-glucosidase inhibitors alleviates cardiac ischaemia–reperfusion injury in mice [6]. These findings suggest that α-glucosidase inhibitors interfere with the development of macrovascular diseases through additional mechanisms distinct from the expected modulation of postprandial hyperglycaemia.

2. Molecular hydrogen (H_2) acts as a novel antioxidant

Clinical evidence and experimental results strongly implicate reactive oxygen species (ROS) as the leading etiologic agent of cardiovascular diseases, including hypertension, atherosclerosis, angina pectoris, myocardial infarction, and heart failure [7,8]. The mechanisms for ROS production are diverse, and include increases in the activities of NAD(P)H-oxidase, xanthione oxidase, cyclooxygenase, and lipoxygenase, as well as uncoupling of nitric oxide synthase, dysfunction of the mitochondrial respiratory chain, and decreased bioavailability of antioxidants, all of which contribute to increased oxidative stress. An increase in ROS production reduces the bioavailability of nitric oxide (NO), synergistically advancing the pathogenesis of cardiovascular disease, since NO plays important roles in the regulation of vascular tone, inhibition of platelet aggregation, and suppression of smooth muscle cell (SMC) proliferation. Increases in the renal levels of ROS raise the blood pressure by influencing afferent arteriolar tone, tubulo-glomerular feedback response, and sodium reabsorption [9]. Increases in vascular ROS promote endothelial dysfunction, increased

* Corresponding author. Address: Department of Regenerative Medicine and Advanced Cardiac Therapeutics, Keio University School of Medicine, 35 Shinanomachi Shinjuku-ku, Tokyo 160-8582, Japan. Fax: +81 3 5363 3875.
E-mail address: msano@sc.itc.keio.ac.jp (M. Sano).

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contractility, monocyte invasion, VSMC proliferation, and increased deposition of extracellular matrix proteins, all of which contribute to the pathogenesis of hypertension, atherosclerosis, and plaque rupture. In the brain, increased production of ROS mediates hypertension by increasing sympathetic outflow. Various antioxidants have been tested for their abilities to reduce the risk of cardiovascular disease. However, these trials have not verified the importance of antioxidants in the prevention of cardiovascular disease [10]. These outcomes can be partially explained by the dual roles of ROS. Most of the detrimental effects of ROS are attributed to ·OH, which is the most reactive oxygen species. In comparison, O_2^{-}· and H_2O_2 have lower oxidative energies and, paradoxically, are implicated as crucial signalling components in the establishment of favourable tolerance to oxidative stress. Consequently, the inhibition of both these pathways (e.g., by antioxidants) can have a deleterious outcome.

Recently, we discovered that molecular hydrogen (H_2) acts as an antioxidant with the following interesting properties: (i) H_2 permeates cell membranes and can target the cellular organelles, including the mitochondria and nuclei; and (ii) H_2 specifically quenches detrimental ROS, such as ·OH and peroxynitrite ($ONOO^-$), while maintaining the metabolic oxidation–reduction reaction and other less-potent ROS, such as O_2^{-}·, H_2O_2 and nitric oxide (NO·) [11]. We showed that inhalation of H_2 gas, given at an incombustible level, limited the extent of myocardial infarction resulting from myocardial ischaemia–reperfusion injury, thereby preventing deleterious left ventricular remodelling in the rat [12]. Importantly, the inhaled H_2 gas was transported rapidly in the circulation and reached the 'at-risk' ischaemic myocardium before the coronary blood flow of the occluded infarct-related artery was re-established.

H_2 can also be administered orally in the form of H_2-dissolved water. Kajiyama et al. reported that supplementation with 900 ml/day (300 ml given three times a day) of H_2-dissolved water for 8 weeks reduced the levels of several biomarkers of oxidative stress, such as plasma oxidized low-density lipoprotein (LDL) cholesterol and urinary 8-isoprostanes, and improved glucose metabolism in patients with type 2 diabetes or impaired glucose tolerance [13]. Furthermore, supplementation with H_2-dissolved water normalized the oral glucose tolerance test in four out of six patients with impaired glucose tolerance. The reduction in the expression of biomarkers associated with systemic oxidative stress can be ascribed to the reductive property of H_2 gas. The formation of 4-hydroxynonenal (HNE) through lipoprotein oxidation plays an etiologic role in atherosclerotic lesion progression [14,15]. Oxidized LDL is taken up by macrophages through scavenger receptors, to form foam cells. Foam cells secrete growth factors that induce SMC migration from the media into the neointima. We demonstrate that the ingestion of H_2-dissolved water *ad libitum* for 6 months prevents the development of atherosclerosis in apolipoprotein E-knockout mice, which represent a model of spontaneously developing atherosclerosis [16]. This anti-atherogenic effect of H_2-dissolved water is associated with a reduction of HNE immunoreactivity in the aorta. These results suggest that persistent intake of H_2 has the potential to reduce oxidative stress and may prevent cardiovascular disease.

3. Unexpected benefit of flatulence caused by α-glucosidase inhibitors

Is there any other way to supply H_2 to the body? H_2 is not produced endogenously in mammalian cells, since the hydrogenase activity responsible for the formation of H_2 gas may not be present [17]. Instead, spontaneous production of H_2 gas in the human body occurs *via* the fermentation of undigested carbohydrates by the resident enterobacterial flora. H_2 is transferred to the portal circulation and excreted through the breath in significant amounts [18]. Flatulence is regarded as the major side-effect of treatment with α-glucosidase inhibitors [19]. Therefore, we examined whether the administration of α-glucosidase inhibitors increases the levels of H_2 production in the gastrointestinal tract. Eleven healthy volunteers (10 males and 1 female) were administered acarbose at a dosage of 300 mg/day (100 mg three times a day) for 4 days under free-feeding conditions (Table 1). On Day 4 of the experiment, the levels of exhaled H_2 and methane (CH_4) were measured using the Breath Gas Analyzer Model TGA-2000 (TERAMECS, Kyoto, Japan). Acarbose treatment significantly increased the amount of exhaled H_2 at every time-point examined ($n = 11$, $P < 0.05$, paired t-test, as compared to before treatment with acarbose), whereas it had modest effects on CH_4 production (Fig. 1). Acarbose treatment had no effect on H_2 or CH_4 production in 2/11 volunteers.

Kajiyama treated patients with type 2 diabetes or impaired glucose tolerance with 900 ml/day (300 ml three times a day) H_2-dissolved water. After drinking 300 ml of H_2-dissolved water, the exhaled H_2 gas concentration reached a maximum of 56 ± 27.8 ppm at 15 min, and returned to the baseline level at 150 min. This peak level of H_2 gas reduced the levels of oxidative stress biomarkers and improved glucose metabolism in patients with type 2 diabetes or impaired glucose tolerance [13]. In the present study, we show that oral administration of acarbose at a dosage of 300 mg/day (100 mg given three times a day) can reach

Table 1
Eleven healthy volunteers (10 males and 1 female) were administered acarbose at a dosage of 300 mg/day (100 mg three times a day) for 4 days under free-feeding conditions. Exhaled gas was collected in an aluminium bag at the point of mid-expiration at the indicated time-points (i.e., morning, before lunch, 2 h after lunch, before retiring), both before and after acarbose treatment. The exhaled gas samples were injected into the Breath Gas Analyzer to measure the H_2 and CH_4 concentrations.

Sex	Hydrogen								Methane							
	Before				After				Before				After			
	Morning	Before lunch	After lunch	Before retiring	Morning	Before lunch	After lunch	Before retiring	Morning	Before lunch	After lunch	Before retiring	Morning	before lunch	After lunch	Before retiring
M	1	2	11	10	34	21	74	90	0	3	2	2	8	3	9	9
M	8	6	3	1	17	25	48	19	4	4	2	2	4	4	6	3
M	46	14	20	20	76	32	52	56	5	2	2	3	7	4	5	6
M	3	6	3	8	85	44	58	91	23	34	20	19	11	9	8	12
M	43	31	25	10	64	62	62	45	4	32	2	2	8	6	6	5
M	8	3	9	13	20	24	40	41	1	1	3	5	4	4	5	7
M	37	15	17	11	30	53	46	38	5	2	1	1	5	5	3	5
F	10	30	32	29	54	15	14	30	2	8	7	8	11	7	6	7
M	15	2	5	6	26	20	33	11	5	1	1	2	7	6	8	4
M	52	44	56	42	38	54	31	49	18	15	18	17	10	16	12	16
M	5	5	1	21	3	5	21	70	11	22	14	44	29	27	38	50

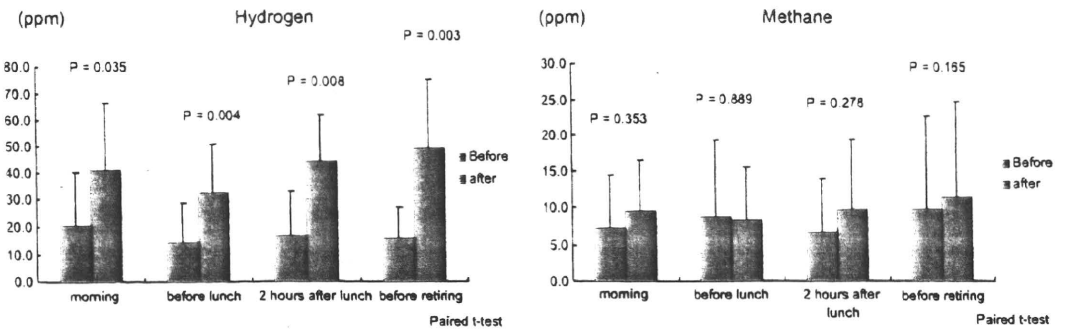

Fig. 1. Effects of acarbose on the levels of exhaled H_2 and CH_4. The values shown in the bar graphs are means ± S.D.

the same maximum levels of exhaled H_2 gas as compared to the consumption of 300 ml of H_2-dissolved water. Moreover, acarbose maintained this peak level continuously. It is noteworthy that the breath concentration of H_2 on a fasting morning remains high in people who take acarbose. These observations clearly indicate that the amounts of H_2 gas generated by acarbose in our current experiments are sufficient to reduce systemic oxidative stress. Oral administration of acarbose may be superior to drinking H_2-rich water in terms of maintenance of the appropriate H_2 gas levels in the body.

4. Conclusion

Based on these observations and experimental results, we propose that α-glucosidase inhibitors reduce the risk of cardiovascular disease in patients with impaired glucose tolerance or type 2 diabetes, and that these benefits can be attributed at least in part to the abilities of these drugs to neutralise oxidative stress by increasing the production of H_2 in the gastrointestinal tract. To investigate the relationship between the cardiovascular benefits of α-glucosidase inhibitors and H gas production by the gut microbiota, we should examine whether the cardiovascular benefits afforded by these drugs are diminished by scavenging H_2 gas in the gastrointestinal tract before absorption into the blood stream.

Conflict of interest statement

None declared.

Acknowledgement

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ORIGINAL ARTICLE

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

Naomi Nakashima-Kamimura · Takashi Mori · Ikuroh Ohsawa · Sadamitsu Asoh · Shigeo Ohta

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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 (H_2) 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% H_2 in air). Furthermore, instead of inhaling hydrogen gas, we examined whether drinking water containing hydrogen (hydrogen water; 0.8 mM H_2 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

N. Nakashima-Kamimura · I. Ohsawa · S. Asoh · S. Ohta (✉)
Department of Biochemistry and Cell Biology,
Institute of Development and Aging Sciences,
Nippon Medical School, Kawasaki,
Kanagawa 211-8533, Japan
e-mail: ohta@nms.ac.jp

I. Ohsawa
The Center of Molecular Hydrogen Medicine,
Institute of Development and Aging Sciences,
Nippon Medical School, Kawasaki,
Kanagawa 211-8533, Japan

T. Mori
Institute of Medical Science,
Saitama Medical Center/University,
Kawagoe, Saitama 350-8550, Japan

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*-diamminedichloroplatinum 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

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 (1×10^4 mL^{-1}) or L-1210 (5×10^4 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 (2×10^4 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

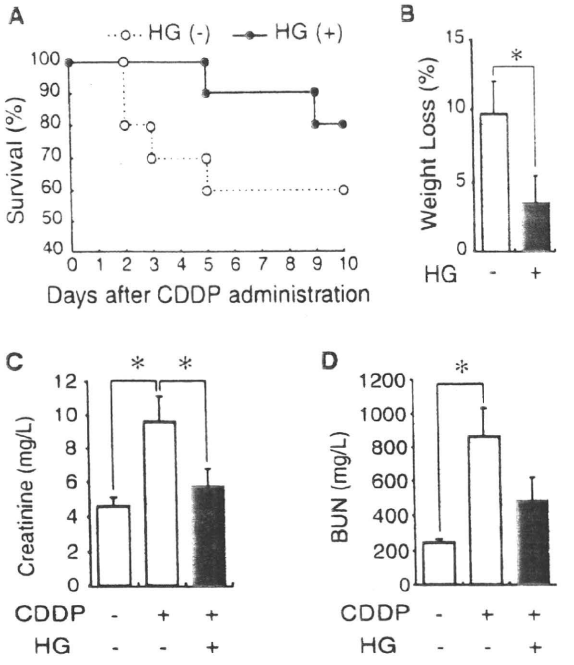

Fig. 1 Hydrogen gas (*HG*) reduces mortality, body-weight loss and nephrotoxicity induced by cisplatin (*CDDP*). Mice were injected intraperitoneally with a single dose of cisplatin (17 mg/kg) (Day 0). Hydrogen gas was administered by inhalation (1% H_2 in air) throughout the experiments (from Day 2 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

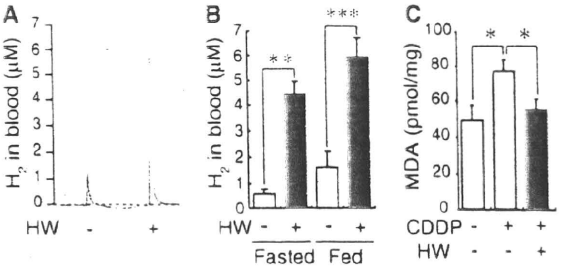

Fig. 2 Hydrogen is detected in blood after oral administration of hydrogen water and reduced oxidative stress in the kidney. **a** Rats (approximately 230 g) were administered 3.5 mL of hydrogen water (0.8 mM H_2 in water) into the stomach via a catheter. After 3 min, hydrogen concentration in blood 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

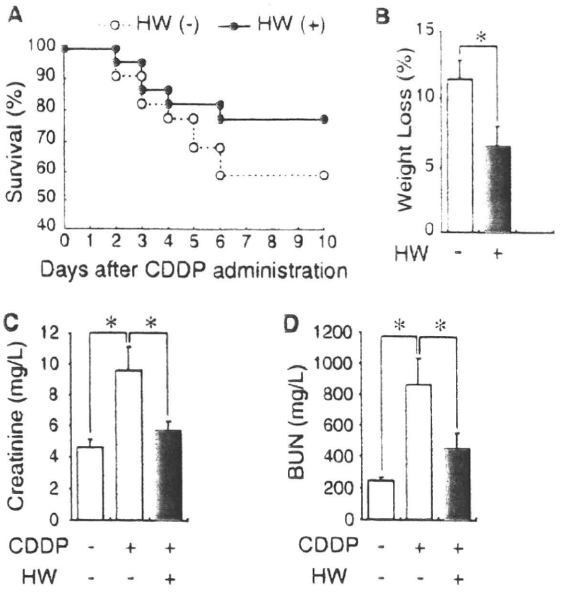

Fig. 3 Hydrogen water (*HW*) reduces mortality, body-weight loss and nephrotoxicity induced by cisplatin (*CDDP*). Mice were injected intraperitoneally with a single dose of cisplatin (17 mg/kg) (Day 0). Hydrogen water was administered by drinking ad libitum (0.8 mM H_2 in water) throughout the experiments (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

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) ($n = 6$ 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

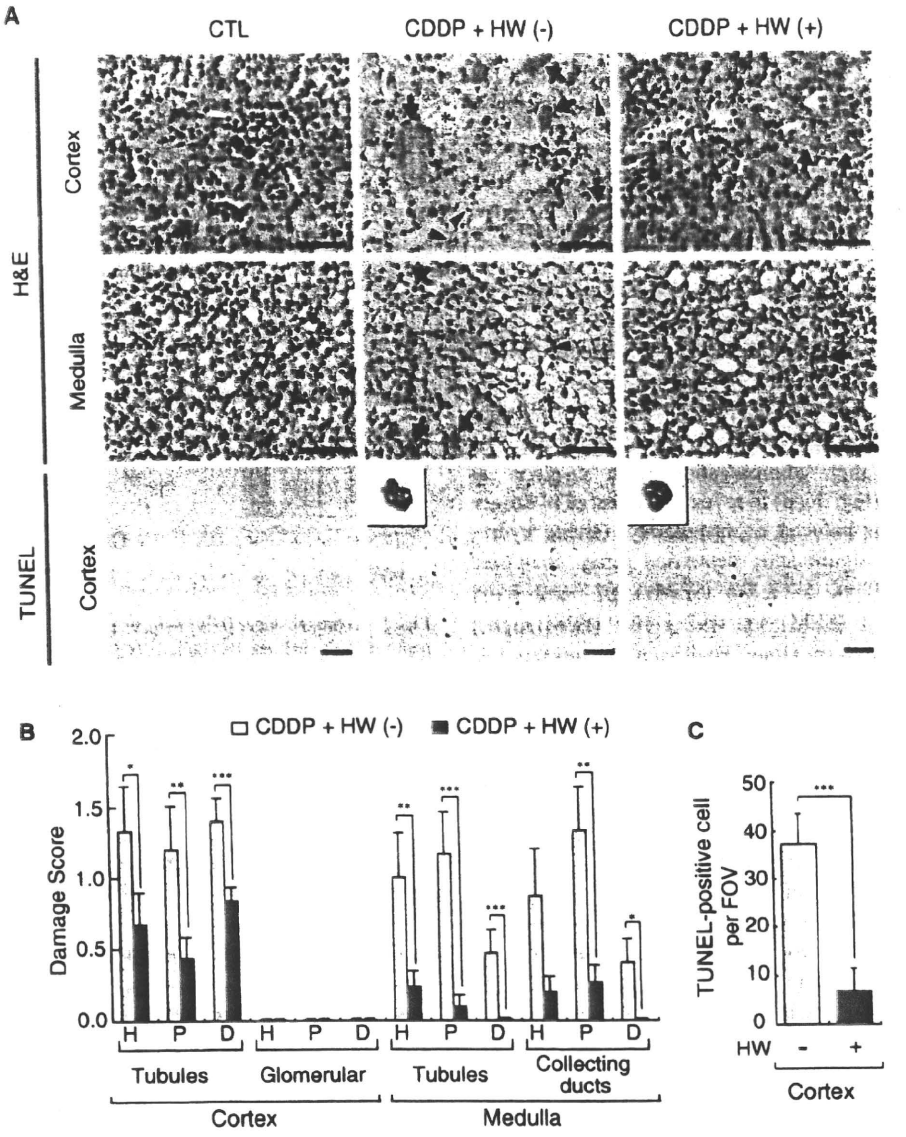

Fig. 4 Hydrogen prevents cisplatin-induced acute renal injury. **a** Mice were injected intraperitoneally with a single dose of cisplatin (17 mg/kg) (Day 0). Hydrogen water (0.8 mM H_2 in water) was available ad libitum throughout the experiments (from Day 2 to Day 3). HW (+) and HW (−) were mice given 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.

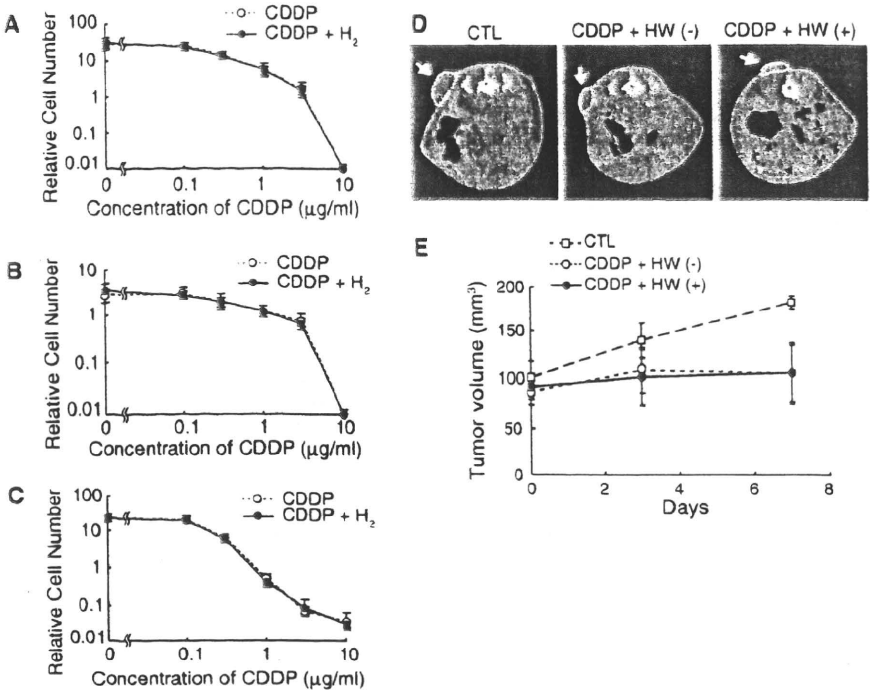

Fig. 5 Hydrogen does not impair cytotoxicity by cisplatin. **a–c** Hydrogen does not influence cytotoxicity of cisplatin against tumor cell lines in vitro. Relative cell number of (**a**, **b**) sarcoma 180 and (**c**) L-1210 cells were examined under (**a**, **c**) serum-containing or (**b**) serum-free 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,