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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

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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

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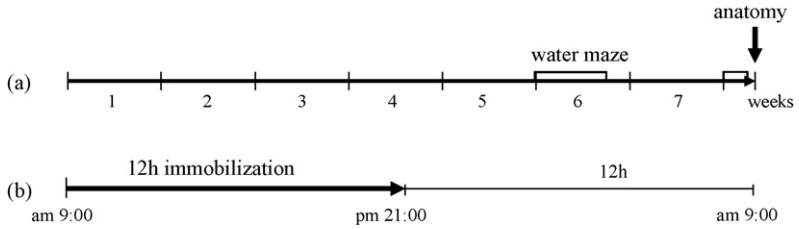

Fig. 1. Experiment protocol: (a) After 5 weeks of chronic immobilization, the spatial memory of all mice was evaluated with the MWM. (b) Chronic immobilization protocol for RA, VE, and GS mice. These mice were exposed to 12 h (9:00 a.m. to 21:00 p.m.) of immobilization in an immobilization cage (width 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

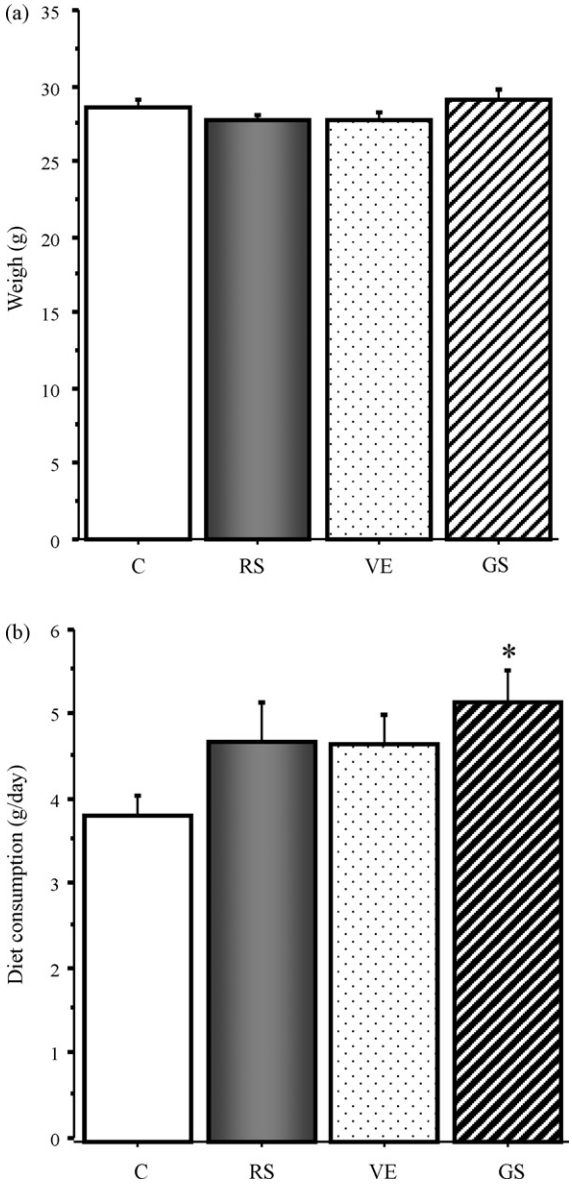

Fig. 2. Body weight and diet consumption: (A) The average weight of mice was not significantly different between conditions. (B) The average diet consumption of RS, VE, and GS mice tended to be greater than that of C mice.

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-

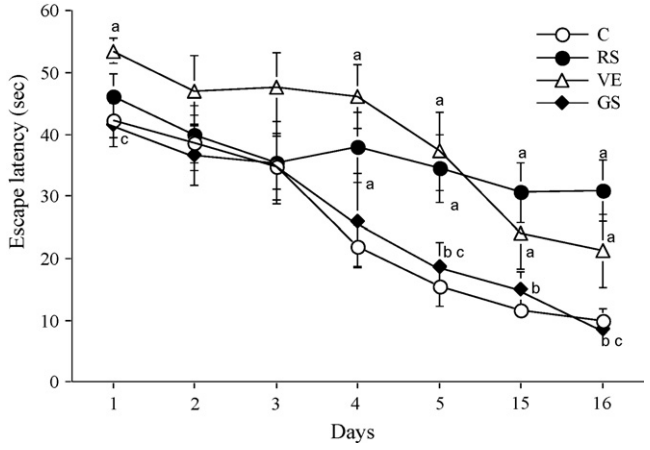

Fig. 3. Spatial learning and memory as measured by MWM RS mice showed significantly impairment of escape latency when compared to C mice. However, the escape latency of GS mice was significantly shorter than that of the RA group. Significant differences in escape latency between the RS and GS group were 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.

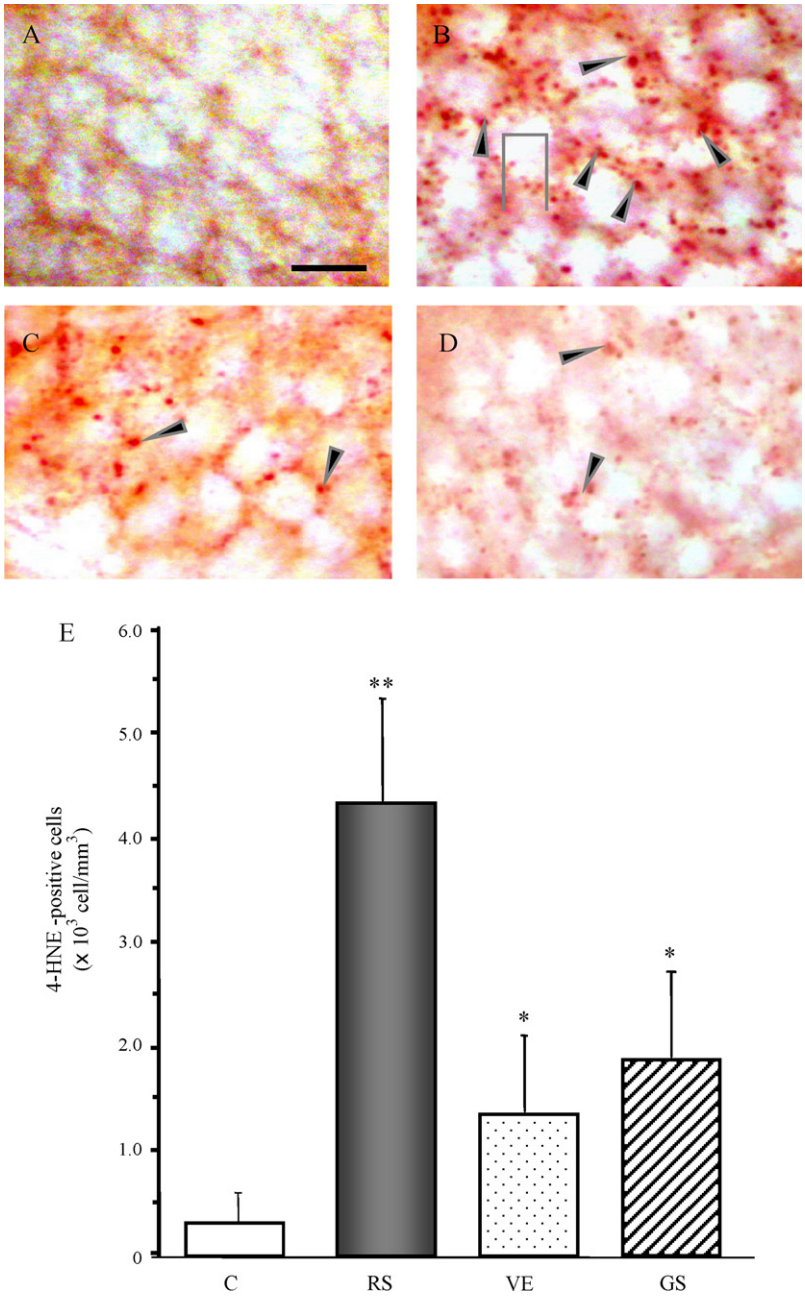

Fig. 4. Number of 4-HNE-positive cells in the GCL Representative images of 4-HNE-positive cells in GCL are shown. (A) C mice, (B) RS mice, (C) VE mice, and (D) GS mice. Bar: 50 μm. (E) Total number of 4-HNE-positive cells in the SGZ. RS mice had significantly more 4-HNE-positive cells in the GCL of the 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.

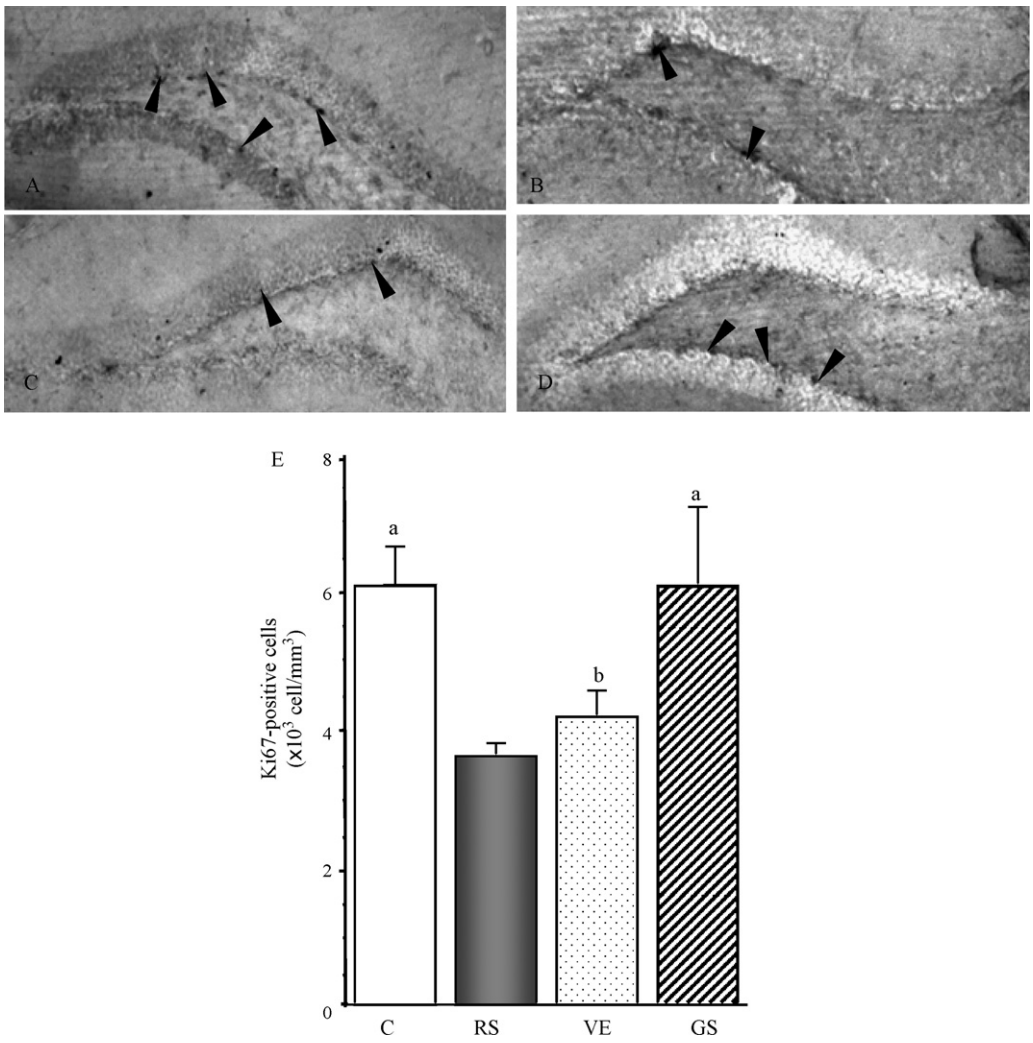

Fig. 5. Number of Ki67-positive cells in the SGZ Representative pictures of Ki67-positive cells in the SGZ are shown. (A) C mice, (B) RS mice, (C) VE mice, (D) GS mice, and (E) Total number of Ki67-positive cells in the SGZ. The number of Ki67-positive cells in the SGZ was significantly lower in RS mice 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

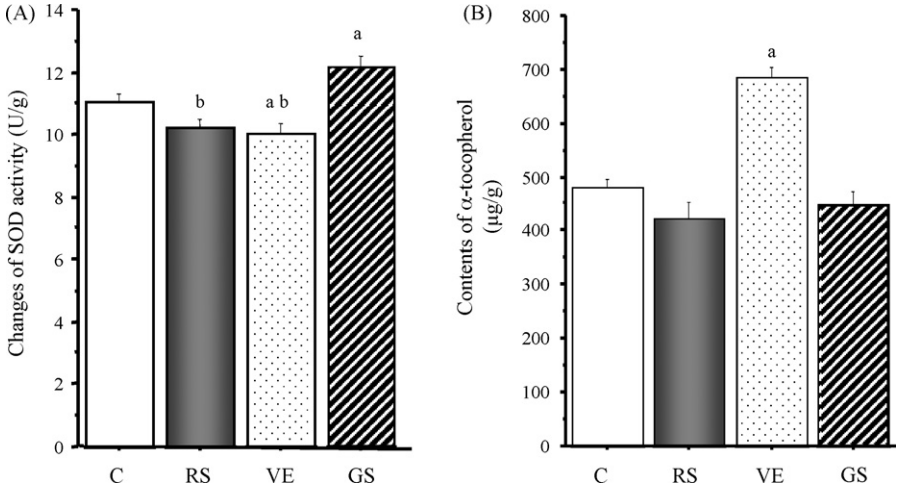

Fig. 6. SOD activity and α-tocopherol content in the brain: (A) SOD activity in hippocampus; SOD activity of GS mice was significantly increased when compared to other mice. (a) $p < 0.05$ vs. C mice and (b) $p < 0.05$ vs. GS mice. (B) α-tocopherol content of the hypothalamus. α-Tocopherol content of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