***Spirulina* Prevents Memory Dysfunction, Reduces Oxidative Stress Damage and Augments Antioxidant Activity in Senescence-Accelerated Mice**

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Summary *Spirulina* has proven to be effective in treating certain cancers, hyperlipidemia, immunodeficiency, and inflammatory processes. In this study, we aimed to investigate the effects of *Spirulina* on memory dysfunction, oxidative stress damage and antioxidant enzyme activity. Three-month-old male senescence-accelerated prone-8 (SAMP8) mice were randomly assigned to either a control group or to one of two experimental groups (one receiving daily dietary supplementation with 50 mg/kg BW and one with 200 mg/kg BW of *Spirulina platensis* water extract). Senescence-accelerated-resistant (SAMR1) mice were used as the external control. Results showed that the *Spirulina*-treated groups had better passive and avoidance scores than the control group. The amyloid β -protein ($A\beta$) deposition was significantly reduced at the hippocampus and whole brain in both *Spirulina* groups. The levels of lipid peroxidation were significantly reduced at the hippocampus, striatum, and cortex in both *Spirulina* groups, while catalase activity was significantly higher only in the 200 mg/kg BW *Spirulina* group than in the control group. Glutathione peroxidase activity was significantly higher only in the cortex of the 200 mg/kg group than in that of the SAMP8 control group. However, superoxide dismutase activity in all parts of the brain did not significantly differ among all groups. In conclusion, *Spirulina platensis* may prevent the loss of memory possibly by lessening $A\beta$ protein accumulation, reducing oxidative damage and mainly augmenting the catalase activity.

Key Words *Spirulina platensis*, memory dysfunction, amyloid β -protein, oxidative stress, antioxidant enzymes

Learning and memory dysfunctions are the main symptoms of Alzheimer's disease (AD), which is characterized by the deposition of amyloid β -protein ($A\beta$) in the brain. Alzheimer's patients also show an increased level of plasma thiobarbituric acid-reactive substances (TBARS), which indicates a higher oxidation of plasma unsaturated phospholipids, and an increased oxidation of red blood cell glutathione, which indicates oxidative stress in peripheral cells (1).

The senescence-accelerated prone mouse strain 8 (SAMP8) is often used as an animal model for AD (2, 3). The SAMP8 has a shorter life span and shows many typical signs of aging, such as deficits in learning and memory, emotional disorders (increased anxiety-like behavior and depressive behavior) and altered circadian rhythm associated with certain pathological, biochemical and pharmacological changes, at an earlier age

than control mice (senescence-accelerated resistant mouse, SAMR1). Changes in gene expression of neuroprotection, signal transduction, protein folding/degradation, cytoskeleton/transport, immune response and reactive oxygen species (ROS) production have been reported in SAMP8 (4, 5). In addition, mitochondrial dysfunction was also noted in SAMP8 (6). Oxidative stress is believed to have an important role in AD development (4–7), and it is reasonable to expect that supplementation of antioxidants might prevent memory dysfunction.

Spirulina is a microscopic blue-green algae in the shape of a perfect spiral coil, living both in sea and fresh water. The most common *Spirulina* product for human and animal food supplements contains primarily two species: *Spirulina platensis* and *Spirulina maxima*. It is a rich and valuable food source of macro- and micronutrients including high quality protein, iron, gamma-linolenic fatty acid, carotenoids, vitamins B₁ and B₂,

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minerals, C-phycocyanin (CPC), etc. (8). It has been reported that *Spirulina platensis* or its active ingredient (CPC) exerts anti-oxidative activity, anti-lipid peroxidation, anti-inflammatory effects, and inhibitory effects on prostaglandin and leukotriene biosynthesis (9, 10).

In vivo and in vitro studies have proven the effectiveness of *Spirulina* for certain cancers, hepatotoxicity, hyperglycemia, hyperlipidemia, immunodeficiency, and inflammatory processes (11). However, the effects of *Spirulina* on memory dysfunction and antioxidant enzyme activity have not been reported. As we know, the hippocampus, striatum, and cortex are key brain structures implicated in cognition and/or memory functions (12–16). Thus, in this study, we aimed to investigate these issues in SAMP8 mice.

MATERIALS AND METHODS

Animals and food intake. SAMP8 and SAMR1 mice were procured from Kyoto University, Japan, and were maintained by inbred breeding in the standard animal room of Providence University. Male SAMP8 mice (3 mo old) were randomly divided into three groups ($n=8$ each): one control and two *Spirulina* (50 and 200 mg/kg BW per day) groups. Male SAMR1 mice, which showed normal characteristics, were used as the external control. The mice were housed about 5 per cage under controlled environmental conditions ($22\pm 2^\circ\text{C}$, $65\pm 5\%$ relative humidity, 0700–1900 h lighting period). Animals were allowed free access to drinking water and the AIN 93-M basal diet for 12 wk.

For the two *Spirulina* groups, we used the water extracts of *Spirulina platensis* supplied by Far East Bio-Tec Co., Ltd. as experimental materials. The water extract of *Spirulina platensis* contained $15\pm 5\%$ CPC and $35\pm 5\%$ polysaccharide after dialysis to remove molecules less than 3 kDa and lyophilization. As in previous studies (11, 17), the final concentration of *Spirulina platensis* extract was administered orally with food at a concentration of 50 or 200 mg/kg BW per day. To assure the potency of *Spirulina*, we checked the concentration of phycocyanin, a biliprotein pigment found in *Spirulina platensis*, in the whole diet 1 d after exposure to light. The results revealed the water extract of *Spirulina platensis* remained stable under controlled environmental conditions ($22\pm 2^\circ\text{C}$, $65\pm 5\%$ relative humidity, 0700–1900 h lighting period). Therefore, the diet containing *Spirulina* water extract at the prescribed concentrations was replaced every other day. The food intake of the mice was recorded every day, and the mice were weighed weekly. The study protocol was approved by the animal research ethics committee at Providence University, Taichung, Taiwan.

Memory evaluation. One week before the memory function evaluation, the mice were individually placed in cubic boxes (each side, 25 cm). Ambulatory activity of all mice was measured for 10 min using a video activity monitor (model E61-21, Coulbourn Instruments, Philadelphia, PA). Mice with poor activity were excluded from subsequent tests.

Behavioral studies were performed in a shuttle box

($35\times 17\times 20$ cm, model E10-15, Coulbourn Instruments) that consisted of two equal compartments connected by a small opening (7.5×6.5 cm, Guillotine door, model E10-15GD, Coulbourn Instruments). One compartment was lit, while the other was darkened by a black semi-transparent plastic cover. The floor of the box was a platform of steel rods.

The single-trial passive avoidance test used a shuttle box ($35\times 17\times 20$ cm: width $\times$ length $\times$ height, model E10-15, Coulbourn Instruments) that consisted of two equal compartments connected by a small opening (7.5×6.5 cm, Guillotine door, model E10-15, GD, Coulbourn Instruments). One of the compartments was lighted while the other compartment was darkened by covering the top with a black semi-transparent plastic. The floor of the box was a platform of steel rods. In the acquisition trial, each mouse exposed to this test was placed in the lighted compartment, and after a brief orientation period (10 s), the partition of the opening was raised to allow the mouse to explore the apparatus freely. The mice would suffer an electric foot shock (3 mA) for 3 s through the stainless steel grid floor when it entered the dark compartment. After 24 h, the retention trial was performed in the same manner; the mice were put into the bright compartment again, and the time when they entered the dark compartment was recorded. The time of the retention trial was 180 s.

Active (shuttle) avoidance test examined the successful avoidance response which was conducted if a tested mouse moved itself from one compartment to another compartment within the shuttle box after receiving a conditional stimulus (CS), 10 s of tone and red, yellow and green light. If the tested mouse did not perform a successful avoidance response, an unconditional stimulus (UCS), a 0.3 mA, 5 s scrambled foot shock, would be given during the CS presentation. The test was conducted for 2 d including one acquisition trial and one testing trial. Each mouse received four daily sessions of a combination of 5 CS/UCS trials, a total of 20 trials. Between the sessions the tested mouse was allowed to rest for 15–20 min. The avoidance responses of tested mice were recorded automatically (18).

Measurements of brain $\text{A}\beta$ deposition. After the memory evaluation, the mice were sacrificed. Each brain was quickly dissected from the skull and individually fixed in a 10% buffered neutral formalin solution for 1 wk. The brain was sectioned ($5\text{-}\mu\text{m}$ thickness) using a microtome (RM 2145, Leica, Nussloch, Germany) based on the method described by Popesko et al. (19).

β -Amyloid in the B-section of brains was stained by the immunohistochemical method of Popesko et al. (19) using an UltraTech H (DAB) Streptavidin-Biotin Universal Detection System (Immunotech, Cedex, France). To identify β -amyloid plaque, sections were immersed in 10 mmol citrate buffer and microwaved three times (each for 2 min), incubated with primary anti- β amyloid antibody (1 : 300; MAb to β -amyloid a.a. 17–24, Biodesign International, Kennebunk, ME, USA) for 2 h at room temperature, incubated in polyvalent-biotinylated antibody (goat anti-rabbit antibodies, 1 : 1,000)

for 45 min, incubated in DAB substrate solution for 30 min, and stained with Mayer's hematoxylin (Sigma, St. Louis, MO, USA) for 3 min. Areas of β -amyloid appeared brownish in color.

The A β positive areas in sections of the hippocampus and whole brain were measured according to Lim et al. (20) with slight modification. Briefly, A β positive areas were measured in sections from the middle part of the brain under 100 $\times$ magnification, and more than 20 fields were assessed using an image analyzer (Q500MC, Leica).

Redox status analysis. After sacrificing the mice, the hippocampus, striatum, and cortex were separated, immediately placed in sodium phosphate buffer (100 mM/L; pH 7.4), homogenized, and centrifuged at 3,000 $\times g$ for 10 min in a refrigerated centrifuge (Hettich Universal 16 R, Tuttlingen, Germany). The supernatants were collected and assayed for lipid peroxide, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) activity.

The level of lipid peroxidation in each tissue was determined according to the method described by Ohkawa et al. (21). The supernatants were mixed with 2'-thiobarbituric acid (4 g/kg in 0.2 M HCl) and butylated hydroxytoluene (2 g/kg in 95% ethanol) at a ratio of 1 : 2 : 0.3, and then the mixture was heated at 90°C for 45 min, cooled down, and mixed with 5 mL of *n*-butanol. The *n*-butanol layer was separated by centrifugation (1,000 $\times g$ for 10 min) and assayed for TBARS spectrophotometrically at 532 nm. The results were expressed as μ mol equivalents of malondialdehyde per g of tissue, using malondialdehyde from tetramethoxypropane as the standard and double-distilled water as the control.

CAT activity was measured using the method of Aebi (22). Changes in absorbance of a mixture consisting of 1.0 mL of H₂O₂ (30 mM) and 2 mL of supernatant were recorded at 240 nm in a spectrophotometer at 25°C. CAT activity was calculated in terms of moles of H₂O₂ consumed/min/g of tissue protein.

GSH-Px activity was measured using the method of Paglia and Valentine (23). Briefly, the production of formazan in a mixture consisting of 200 μ L of glutathione reductase (5 U/mL; GSSG-R), 50 μ L of glutathione (40 mM; GSH), 620 μ L of phosphate buffer (0.25 M; pH 7.4), 100 μ L of supernatant, 10 μ L of β -nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (20 mM; NADPH), and 20 μ L of cumene hydroperoxide (15 mM) was determined at 340 nm in a spectrophotometer at 25°C. One unit of GSH-Px was defined as the number of μ mol of NADPH oxidized per minute at 25°C.

The SOD activity was measured using the method of Marklund and Marklund (24). The production of formazan in a mixture containing 10 μ L of supernatant, 50 mM Tris-HCl (pH 8.2), and 50 mM pyrogallol in a final volume of 3.017 mL was determined at 325 nm in a spectrophotometer at 25°C. One unit of SOD was defined as the amount of protein that inhibits the rate of pyrogallol auto-oxidation by 50%. Total SOD activity

Fig. 1. Memory evaluation in 6-month-old male SAMP8 mice fed different diets. The passive avoidance time (A) and active avoidance time (B) were longer or higher in both *Spirulina* groups than in the SAMP8 control group. Both avoidances in the 200 mg/kg group were similar to that in the SAMR1 group. Each value is the mean $\pm$ SE of $n=8$ /group. *Indicates significant difference from the SAMP8 control group.

but not the activities of SOD isoforms were measured.

CAT, GSH-Px, and SOD activities are expressed as units/g protein. Soluble protein concentration in the samples was determined using the Pierce Micro BCA protein assay kit.

Statistical analysis. All data are expressed as mean $\pm$ standard error of mean and analyzed using the SPSS 10.0 software package (SPSS Inc., Chicago, IL, USA). Data were evaluated by independent samples *t*-test and one-way ANOVA. $p<0.05$ was considered statistically significant.

RESULTS

Spirulina does not affect body weight, food intake, or ambulatory activity

Body weight, food intake, and ambulatory activity were not significantly different among the three groups (one-way ANOVA, $p>0.05$) (data not shown).

Spirulina improved memory function

Compared to the SAMP8 control group, both *Spirulina* groups had significantly prolonged passive avoidance time (independent samples *t*-test, $p<0.05$ for both groups; Fig. 1A) and active avoidance time (independent samples *t*-test, $p<0.05$, for both groups; Fig. 1B) on the next day. Moreover, the passive or active avoidance time of both *Spirulina* groups was similar to that of the SAMR1 control group (independent samples *t*-test, $p>0.05$ for both groups in each test). These results indicated that the addition of *Spirulina platensis* to the diet could improve the emotional memory of SAMP8 mice.

Fig. 2. β -Amyloid ($A\beta$) deposition in the brains of 6-month-old SAMP8 mice fed different diets. $A\beta$ accumulation in the hippocampus (A) and whole brain (B) is lower in the 50 mg/kg BW *Spirulina* group, 200 mg/kg BW *Spirulina* group, and the SAMR1 group than in the SAMP8 control group. Each count is the mean $\pm$ SE of $n=8$ /group. $A\beta$ accumulation was assessed under $100\times$ magnification by photomicroscopy. *Indicates significant difference from the SAMP8 control group.

Spirulina reduced the accumulation of $A\beta$ deposition in the hippocampus and whole brain

$A\beta$ accumulation in the hippocampus (Fig. 2A) and whole brain (Fig. 2B) was markedly lower in both *Spirulina* groups than in the SAMP8 control group (independent samples t -test, $p<0.05$, for both groups), indicating that the *Spirulina* supplements can reduce accumulation of $A\beta$ in the hippocampus and whole brain.

Spirulina enhanced the redox status

Compared to the SAMP8 control group, both *Spirulina* groups had significantly lower levels of lipid peroxides in the hippocampus (0.67 ± 0.10 , 0.64 ± 0.06 and 1.13 ± 0.05 , respectively, for the 50 mg/kg, 200 mg/kg, and SAMP8 control groups), striatum (0.91 ± 0.09 , 0.72 ± 0.054 , and 1.15 ± 0.053 , respectively, for the 50 mg/kg, 200 mg/kg, and SAMP8 control groups), and cortex (0.67 ± 0.050 , 0.66 ± 0.053 , and 1.13 ± 0.15 , respectively, for the 50 mg/kg, 200 mg/kg, and SAMP8 control groups; all means compared by the independent samples t -test, $p<0.05$ for both groups; Fig. 3) and had significantly higher CAT activity in the hippocampus (3.16 ± 0.38 for the 200 mg/kg group versus 2.21 ± 0.088 for the SAMP8 control group), striatum (2.95 ± 0.30 versus 1.89 ± 0.14), and cortex (3.25 ± 0.33 versus 2.05 ± 0.05 ; all means compared by the independent samples t -test, $p<0.05$ for both groups; Fig. 4). GSH-Px activity was significantly higher

Fig. 3. The level of lipid peroxidation in 6-month-old male SAMP8 mice fed different diets. Lipid peroxide level was reduced in the brain hippocampus, striatum, and cortex of mice in the 50, 200 mg/kg BW *Spirulina* groups and SAMR1 control group (when compared to that of the SAMP8 control group). Each value is the mean $\pm$ SE of $n=8$ /group. *Indicates significant difference from the SAMP8 control group.

Fig. 4. CAT activity in 6-month-old male SAMP8 mice fed different diets. Activity was higher in the brain hippocampus, striatum, and cortex of mice in the 200 mg/kg BW *Spirulina* group and SAMR1 control group, but not the 50 mg/kg BW *Spirulina* group (when compared to that of the SAMP8 control group). Each value is the mean $\pm$ SE of $n=8$ /group. *Indicates significant difference from the SAMP8 control group.

only in the cortex of the 200 mg/kg group than in that of the SAMP8 control group (18.12 ± 2.17 versus 10.00 ± 2.63 ; independent samples t -test, $p<0.05$). However, SOD activity in all parts of the brain did not significantly differ between the 200 mg/kg group and SAMP8 control group (data not shown; independent samples t -test, $p<0.05$). In addition, anti-oxidant enzyme activities of the 50 mg/kg group were similar to those of the SAMP8 control group in all parts of the brain (data not shown; independent samples t -test, $p>0.05$). These results indicated that high-dose *Spirulina* supplements could reduce oxidative stress damage and augment CAT activity mainly.

DISCUSSION

This experimental study showed that dietary supplementation with 200 mg/kg BW *Spirulina platensis* water extracts significantly slowed loss of memory by lessening $A\beta$ protein accumulation, reducing oxidative damage, and augmenting the catalase activity in SAMP8,

an animal model for AD.

Experimentally and clinically it is well known that a primary source of ROS generation is through oxidative phosphorylation, ischemia/reperfusion or prolonged hypoperfusion, such as that seen in myocardial infarction, cerebrovascular accidents, aging, and presbycusis. Although the exact mechanisms of AD are still unclear, it has been reported that A β and A β -related reactive oxidative species play a critical role in the development of AD (25). In addition, reduced activities of endogenous enzymes, such as superoxide dismutase (SOD), catalase and glutathione peroxidase, and/or low intake of a variety of small molecules with anti-oxidative activity in the human diet, might lead to the development of AD. Liu and Mori (7) showed that cognitive dysfunction in senescence-accelerated mice correlates well with elevation in lipid peroxidation level and decrease in glutathione (GSH) level in the brain.

On the other hand, Massaad et al. (15) demonstrated that over-expression of SOD could prevent AD-related learning and memory deficits by decreasing hippocampal superoxide, and reducing A β plaques in the Tg2576 mouse. In this study, *Spirulina* water extracts reduced A β accumulation in the hippocampus and whole brain, and decreased lipid peroxidation in the hippocampus, striatum, and cortex. Besides, *Spirulina* had differential effects on the activity of antioxidant enzymes. Notably, the high dose of *Spirulina platensis* extract enhanced CAT activity in the hippocampus, striatum, and cortex, and enhanced GSH-Px activity in the cortex, but had no effect on SOD activity anywhere in the brain.

In addition to antioxidant capability, the detailed neuroprotective mechanisms of *Spirulina* water extracts were not very clear. Wang et al. (26) reported that *Spirulina* can reduce the ischemia/reperfusion-induced apoptosis and cerebral infarction in mice with focal ischemia. *Spirulina* can also enhance striatal dopamine recovery and induce rapid, transient microglia activation after injury of the rat nigrostriatal dopamine system (27). CPC attenuated doxorubicin-induced reactive oxygen species formation, Bax protein activity, mitochondrial cytochrome *c* release, caspase-3 activity, DNA fragmentation, and apoptosis via suppression of p38 MAPK and NF- κ B signal pathway in cardiomyocytes (28). CPC also reduced apoptosis of R-HepG2 cells through up-regulation of the Bcl-2 protein and down-regulation of the Bax protein (29). However, more study will be needed to link CPC-induced improvement in emotional memory function to changes in signaling pathways.

CONCLUSIONS

Dietary supplementation with *Spirulina plantensis* ameliorates deterioration of memory in SAMP8 mice via reducing A β accumulation, lowering the level of lipid peroxidation, and augmenting the antioxidant system, especially CAT activity, all of which might have an important role in the prevention of AD.

Competing interests

The authors declare that they have no competing interest.

Author's contribution

YCC conceived of and designed the study. SMW performed most of the experiments. JHH and KCJ analyzed the experimental results. JHH and YCC drafted the manuscript. ITL, MFW, RCWH, and YCC supervised the study. All authors participated in the critical revision of the manuscript; and all authors have given final approval of the version to be published.

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