Antioxidant and Anti-Cataract Effects of Chlorella on Rats with Streptozotocin-Induced Diabetes

Shinya SHIBATA1, Yu NATORI2, Terumi NISHIHARA2, Kazue TOMISAKA2, Keisuke MATSUMOTO1, Hiroshi SANSAWA1 and Van Chuyen NGUYEN2

1Yakult Central Institute for Microbiological Research, 1796 Yaho, Kunitachi, Tokyo 186-8650, Japan
2Department of Food and Nutrition, Japan Women's University, Mejirodai 2-8-1, Bunkyo-ku, Tokyo 112-8681, Japan

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Summary The antioxidant activities of Chlorella in vitro and in vivo were investigated. Chlorella showed a strong antioxidant effect compared to various vegetables in a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. To evaluate the antioxidant and anti-cataract effects in vivo, a 7.3% Chlorella powder was fed to rats with Streptozotocin-induced diabetes for 11 wk. At the end of the experiment, Chlorella had decreased the blood glycated hemoglobin (hemoglobin Alc) and serum cholesterol levels significantly, however, it had not affected the serum glucose concentration. The serum lipid peroxide value (TBARS value) in the rats fed Chlorella was lower than that of the control rats. In the liver and kidney, Chlorella also reduced chemiluminescent intensities. In addition, it delayed the development of lens opacities. The lens lipid peroxide content of the rats fed Chlorella was lower than that of the control rats, however the differences were not significant. These results indicate that Chlorella has antioxidant activity and may be beneficial for the prevention of diabetic complications such as cataracts.

Key Words Chlorella, antioxidant activities, DPPH, diabetes, cataract

Oxidative stress, induced by reactive oxygen species, is thought to be an important factor leading to chronic diseases in mammals. In diabetes mellitus, hyperglycemia causes the nonenzymatic glycosylation of proteins through the Maillard reaction, in which reactive oxygen species are produced and oxidative stress may arise (1–3). Enhanced oxidative stress has been proposed to cause harmful damage to cells and tissue proteins through cross-linking, fragmentation, and lipid peroxidation. Nonenzymatic glycation and lipid peroxidation have been estimated to play an important role in the progression of diabetic complications such as atherosclerosis, neuropathy, renal disease, retinopathy, and cataracts.

Dietary antioxidants such as ascorbic acid, α-tocopherol and carotenoids can act as inhibitors of free radicals and lipid peroxidation (4–6). The consumption of fruits and vegetables, which are rich in antioxidant components, has been reported to increase plasma antioxidant capacity in humans (7). Thus, the active intake of dietary antioxidants may decrease oxidative stress in diabetes mellitus and be effective for the prevention of diabetic complications.

Chlorella powder is used as a functional food in Japan and is reported to have antihypertensive and hypocholesterolemic effects in humans and animals (8–11). Chlorella powder contains many dietary antioxidants such as lutein (2500 ppm), α-carotene (500 ppm), β-carotene (500 ppm), ascorbic acid (250 ppm), and α-tocopherol (250 ppm). However, the antioxidant activity of Chlorella has not been fully clarified and there is no published study describing the antioxidant effect of Chlorella in rats with streptozotocin-induced diabetes (STZ rats).

The aim of this study was to determine the antioxidant activities of Chlorella both in vitro and in vivo. As antioxidant activity in vitro, the scavenging of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was measured. To determine the antioxidant effect of Chlorella on diabetes, we investigated the effect of Chlorella on the glycated hemoglobin (hemoglobin Alc) and lipid peroxide (TBARS) levels in the serum of STZ rats. Miyazawa and Kaneda have shown that the chemiluminescence of rat tissue homogenates was related to lipid peroxidation accompanied by the formation of singlet oxygen and free radicals (12). Thus, we measured the chemiluminescent intensities of kidney and liver homogenates to discuss the antioxidant activities of Chlorella. In addition, we also investigated the effect of Chlorella on the prevention of cataracts, a diabetic complication, in STZ rats.

MATERIALS AND METHODS

Determination of antioxidant activity by DPPH radical scavenging assay

Materials. Viable Chlorella (Chlorella regularis) cells were obtained from Nihon Chlorella Co., Ltd. (Tokyo, Japan). Vegetables, such as green sweet pepper, spinach, cabbage, and celery, were purchased in a local market in Tokyo, Japan. 1,1-Diphenyl-2-picrylhydrazyl (DPPH)
and 2-morpholinoethanesulfonic acid (MES) were obtained from Sigma Chemicals Co. (MO, USA). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Calbiochem (CA, USA).

Sample preparation. Chlorella (viable cells) and vegetables were lyophilized, and then each lyophilized sample (100 mg) was homogenized in 5–7.5 mL of 80% ethanol for 5 min with a glass homogenizer. After centrifugation, the supernatant was filtered through paper filter No. 7 (Kiriya-Seisakusho Ltd., Tokyo, Japan) to remove insoluble matter.

Measurement of antioxidant activity. The DPPH radical scavenging assay was performed according to the method described by Suda et al. (13). Briefly, 150 μL of 133 μM DPPH solution was added to 50 μL of test sample (diluted with 80% ethanol) in a well of a 96-well ELISA plate. Final concentrations in the reaction mixture were 100 μM DPPH, 50% ethanol, and 50 μM MES (pH 6.0). After the addition of DPPH solution, the plate was kept at room temperature for 20 min. If the test sample was colored, a blank test was conducted as described above without DPPH. The decrease in DPPH was measured using a microplate reader (Model 3550, Bio-Rad Laboratories, CA, USA) at 510 nm. From linear regression curves obtained using different concentrations of test samples and Trolox (positive standard), the ED_{50} values were calculated. The ED_{50} was the concentration of antioxidant required to decrease the initial level of the DPPH radicals by 50%. Based on each ED_{50} value, the results were expressed as micromole Trolox equivalents per gram of test sample on a dried basis.

Antioxidant and anti-cataract effects of Chlorella on rats with streptozotocin-induced diabetes

Animals. Male, 10-wk-old Wistar rats (Nihon Clea, Ltd., Tokyo, Japan), weighing about 270 g, were injected with streptozotocin (Sigma Chemicals Co., MO, USA) at a dose of 50 mg/kg body weight. Soon after the injection, rats were administered drinking water containing 10% glucose overnight to avoid hypoglycemic shock. After 1 wk, the diabetic rats (STZ rats) were separated into two groups having similar body weight. Each group was fed one of the experimental diets and water ad libitum for 11 wk. Food consumption was recorded every day and the body weights of the rats were recorded once a week. Cataract formation was checked every day after preliminary cataract formation was observed. During the experiment, the rats were housed in individual metabolic cages in a room with controlled temperature (23 ± 1°C) and a 12-h light-dark cycle. This experiment was conducted in accordance with the Guidelines for Animal Experimentation No. 6, established by the Prime Minister’s Office of Japan in 1980, and the guidelines of the Food and Nutrition Department, Japan Women’s University.

Diets. STZ rats were fed a control diet or Chlorella (CHL) 7.3% diet (7% without moisture). The Chlorella (Chlorella regularis) powder was manufactured by Nihon Chlorella Co., Ltd. (Tokyo, Japan). The compositions of the experimental diets are given in Table 1. The casein, corn oil, and vitamin mixtures were all vitamin E free.

All diets were designed to contain 20% crude protein (nitrogen × 6.25), 5% crude fat, and 5% total dietary fiber. Nitrogen levels were determined by conventional methods. Crude fat and total dietary fiber analyses were carried out according to the method of the Association of Official Analytical Chemists (14).

Sample collection. After 11 wk of feeding, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (Abbott Laboratories, IL, USA); 50 mg/kg body weight. Blood was obtained from the aorta ventrally. Various organs such as liver, kidney, and lens were removed, rinsed with 0.9% NaCl, and stored at −80°C until analysis.

Lipid peroxide in serum and lens. The lipid peroxide level in the serum was determined using the thiobarbituric acid test (15) with 1,1,3,3-tetraethoxypropane (Sigma Chemicals Co.) as the standard. The level in the lens was also measured as the amount of thiobarbituric acid reactive substances (TBARS) using the method of Ohkawa et al. (16) with a slight modification. Briefly, the lens was homogenized in 0.5 mL of phosphate buffer (0.01 M, pH 7.4 containing 1 mM EDTA), the homogenate was centrifuged, and then the supernatant was used for the TBARS determination. One-hundred microliters of sample was added to 0.1 mL of 8.1% SDS, 0.75 mL of 20% acetic acid (pH 3.5), 0.8 mL of 0.01 M phosphate buffer (containing 1 mM EDTA), and 0.1 mL of 0.8% thiobarbituric acid prior to being heated at 95°C for 1 h and then cooled in tap water. The standard was also prepared as above, using 0.1 mL of 1,1,3,3-tetraethoxypropane of different concentrations. Samples were again added to 1 mL of distilled water and 5 mL of 15:1 (v/v) n-butanol: pyridine before centrifugation. The organic layer was measured at excitation 515 nm, emission 553 nm with a spectrofluorophotometer (RF-1500, Shimadzu Co., Kyoto, Japan).

Chemiluminescence of liver and kidney homogenates. Wet liver or kidney (0.5 g) was homogenized in 9.5 mL of 0.1 M phosphate buffer (pH 7.4) to achieve a 10% concentration prior to centrifugation at 4°C, 10,000 × g for 5 min. Supernatant was measured with a CLD-110 Chemiluminescence Analyzer System (Tohoku Electric Co., Sendai, Japan) at 40°C. The chemiluminescent

<table>
<thead>
<tr>
<th>Table 1. Composition of experimental diets (g/kg diet).</th>
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<tr>
<td>Ingredients</td>
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<tr>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Chlorella</td>
</tr>
<tr>
<td>Casein†</td>
</tr>
<tr>
<td>Stripped corn oil‡,§</td>
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<tr>
<td>Mineral mixture§</td>
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<tr>
<td>Vitamin mixture†,§</td>
</tr>
<tr>
<td>Cellulose</td>
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<tr>
<td>Corn starch</td>
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<td>Sucrose</td>
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† Vitamin E free.
‡ Stripped corn oil was obtained from Eizai Co. (Tokyo, Japan).
§ Harper mixture (Oriental Yeast Industry, Tokyo, Japan).
intensities were expressed as average counts per minute of 5-min measurements.

Biochemical analysis of blood. Serum triglyceride, cholesterol, and glucose concentrations were measured enzymatically using an Olympus AU5200 automatic analyzer (Olympus, Tokyo, Japan) at the clinical laboratory facility in Mitsubishi Kagaku BCL (Yokohama, Japan). Hemoglobin A1C (HbA1C) was measured with a commercially available kit (Glyc-Affin-GHb, Seikagaku Co., Tokyo, Japan).

Cataract grading. Grading of cataracts began at 8 wk and continued twice every week until dissection. The light was focused close to both eyes of the rats while grading. Scores ranged from 0 to 9, depending on the progression of opacity and cloudiness.

Statistical analysis. Differences between the two groups were calculated using Student’s t test except for cataract grading comparisons. Cataract grading comparisons were made using the Wilcoxon rank sum test with SAS system version 6.12 (SAS Institute Inc., NC, USA). The level of statistical significance was set at \( p<0.05 \).

RESULTS

Antioxidant activity determined by DPPH radical scavenging assay

The dose-response curves of Chlorella and vegetables for DPPH radical scavenging activities are shown in Fig. 1(A). All these samples had scavenging activities and their ED50 values varied. Based on the data, the results are expressed in Fig. 1(B) as Trolox equivalents to compare antioxidant activities. In this system, the ED50 of Trolox was 92.5 \( \mu \)M. The rank order of antioxidant activities was green sweet pepper>Chlorella>spinach>celery>cabbage.

Antioxidant and anti-cataract effects of Chlorella on STZ rats

Throughout the experiment, food intake, body weight, and urine volume did not differ significantly between groups.

Table 2. Serum glucose, total cholesterol, triglycerides and hemoglobin A1c (HbA1c) values of diabetic rats fed experimental diets for 11 wk.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Chlorella</th>
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<tr>
<td>Glucose (mg/dL)</td>
<td>625.2±106.8</td>
<td>664.2±42.3</td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td>24.0±2.9</td>
<td>19.9±0.6**</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>75.8±8.5</td>
<td>63.8±9.4*</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>105.8±40.4</td>
<td>97.0±27.1</td>
</tr>
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</table>

Values are means±SD (Control \( n=6 \), Chlorella \( n=5 \)).

* Significantly different from the control group at \( p<0.05 \).

** Significantly different from the control group at \( p<0.01 \).
Antioxidant and Anti-Cataract Effects of Chlorella

Table 3. Lipid peroxide contents of serum and lens in diabetic rats fed experimental diets for 11 wk.

<table>
<thead>
<tr>
<th>Lipid peroxide</th>
<th>Control</th>
<th>Chlorella</th>
</tr>
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<tbody>
<tr>
<td>Serum (nmol MDA/mL)</td>
<td>7.9±2.4</td>
<td>5.4±0.4**</td>
</tr>
<tr>
<td>Lens (nmol MDA/g lens)</td>
<td>83.4±10.7</td>
<td>65.3±13.2</td>
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Lipid peroxide was measured as TBARS (described in Materials and Methods).

Values are means±S.D. (Control n=6, Chlorella n=5).

** Significantly different from the control group at p<0.01.

Table 4. Chemiluminescent (CL) intensities of the liver and kidney of diabetic rats fed experimental diets for 11 wk.

<table>
<thead>
<tr>
<th>CL intensity (counts/min)</th>
<th>Control</th>
<th>Chlorella</th>
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<tbody>
<tr>
<td>Liver</td>
<td>43.090±9.160</td>
<td>35.890±5.610*</td>
</tr>
<tr>
<td>Kidney</td>
<td>26.450±1.220</td>
<td>19.830±1.740**</td>
</tr>
</tbody>
</table>

Values are means±SD (Control n=6, Chlorella n=5).

* Significantly different from the control group at p<0.05.

** Significantly different from the control group at p<0.01.

DISCUSSION

In the present study, we evaluated the antioxidant activity of Chlorella using the DPPH radical scavenging assay, focusing on comparisons with selected vegetables known to have antioxidant potency. In Japan, Chlorella is used as a supplement to improve the low intake of vegetables and fruits, because the components of Chlorella are similar to those of green-yellow vegetables. Cao et al. (17) investigated the antioxidant activity of common vegetables, including spinach, cabbage, and celery, based on the oxygen radical absorbance capacity (ORAC). In their study, the rank order of activity was spinach>cabbage>celery (17). Ou et al. (18) reported that green sweet pepper had very strong activity in common vegetables based on ORAC and ferric-reducing antioxidant activity and that the rank order of activity was green sweet pepper>spinach>cabbage. An extract of green sweet pepper obtained with 80% ethanol inhibited the autooxidation of linoleic acid better than green cabbage (19). As a general trend, our results were consistent with those reported in other literature (17-19), except for the ranking order of celery and cabbage. The present study showed that the antioxidant activity of Chlorella is similar to that of green sweet pepper or spinach, thus it is estimated that Chlorella has high antioxidant activity compared to common vegetables.

The daily intake of Chlorella is generally 3–9 g (as tablets of dried powder) in Japan. The vegetables shown in Fig. 2 contain over 90% moisture. Consequently, the daily intake of Chlorella is equal to each wet weight of the vegetables, such as 67–201 g of cabbage, 50–150 g of celery, 39–117 g of spinach, and 26–78 g of green sweet pepper on the basis of DPPH radical scavenging activity.

Limited information is available on the bioavailability of nutrients and micronutrients contained in Chlorella. Yamada reported that the true digestibility of protein in Chlorella was approximately 80% in rats (20). In humans, the ingestion of Chlorella tablet (6 g/d) increased serum ß-carotene and lutein concentrations (unpublished observation). Thus, Chlorella is thought to be a beneficial source for protein and carotenoids. However, the bioavailability of other antioxidant substances in Chlorella was not determined in this study because they were not fully identified. This should be investigated in future research.

The present study demonstrated that the ingestion of Chlorella significantly decreased blood HbA1c levels and serum lipid peroxide levels without a hypoglycemic effect in STZ rats. However, in this study, we did not measure serum glucose levels until 11 wk, thus it was not clear whether the decrease in HbA1c levels reflected a specific period of change in the serum glucose levels of between the two groups (data not shown). Serum glucose, total cholesterol (TC), triglyceride (TG), and blood glycated hemoglobin (HbAlc) levels of the rats at the end of the study are shown in Table 2. Serum glucose concentrations did not differ significantly between the groups. However, HbA1c levels were significantly lower in the Chlorella group than the control group. Chlorella also decreased the serum TC level, but did not affect the serum TG concentration.

The effects of Chlorella on the lipid peroxide content of serum and lens are indicated in Table 3. The lipid peroxide level in the serum was significantly decreased in Chlorella-treated rats. The level in the lens was also lower than that of the control rats, but not significantly.

The chemiluminescent intensities of liver and kidney homogenates are shown in Table 4. Chlorella decreased the chemiluminescent intensities of both the liver and kidney significantly.

The development of cataracts is shown in Fig. 2. The grading of cataracts began at 8 wk. The onset did not differ between the groups in 8 wk. However, Chlorella lowered the grading score at 10 and 11 wk significantly.

Fig. 2. Degree of cataract formation in diabetic rats fed experimental diets. (●): Control, (■): Chlorella. Degree of cataract formation was graded from 0 to 9. Values are means±SD (Control n=6, Chlorella n=5).

* Significantly different from the control group at p<0.05.

** Significantly different from the control group at p<0.01.
Chlorella-fed rats. There have been some studies on the effects of dietary antioxidants in experimental diabetic rats, but their results are not consistent with respect to hypoglycemic effect (21–23). Similarly, it was not clear whether micronutrient antioxidants reduced protein glycosylation in diabetic animals and humans (22–25). Some dietary fibers have been reported to inhibit the absorption of glucose (26). As speculation, the dietary fiber of Chlorella might inhibit the absorption of glucose, leading to a decrease in the level of HbA1C.

Serum lipid peroxide levels measured as TBARS in diabetic rats and mice were decreased by treatment with ascorbic acid and α-tocopherol (21–23). *Spirulina maxima*, a carotenoids-rich cyanobacterium containing β-carotene and zeaxanthin, has been reported to lower serum and liver TBARS values in mice with alloxan-induced diabetes (27). These antioxidants in Chlorella may inhibit lipid peroxidation, but the antioxidative substances contained in Chlorella have not been fully elucidated.

We also found that Chlorella reduced the chemiluminescent intensities of liver and kidney homogenates. Chemiluminescence is generated via free-radical reactions and is related to lipid peroxidation (12, 28). In α-tocopherol-deficient rats, liver and kidney chemiluminescent intensities were significantly higher than in normal rats (12). These results suggest that Chlorella exhibits antioxidant activity and reduces lipid peroxidation and oxidative stress in the liver and kidney of STZ rats.

Furthermore, in this study, serum cholesterol levels were lower in the rats fed Chlorella than the control rats. The *Chlorella* indigestible fraction had good bile acid binding capacity and improved the lipid metabolism in the cholesterol-fed rats (11). Kamata et al. (29) has reported that the serum cholesterol level in mice with STZ-induced diabetes was reduced by the administration of cholestyramine, a hypocholesterolemic drug that enhances fecal bile acid excretion by an anion exchange reaction. Regarding this result, the *Chlorella* indigestible fraction may exert a hypocholesterolemic effect in STZ rats.

Cataracts are an age-related disease as well as diabetic complication. The mechanism of cataract formation is still not fully understood. However, it is thought to be related to three biochemical phenomena: (1) the accumulation of sorbitol in the lens, (2) oxidative stress affecting the crystalline proteins, and (3) non-enzymatic glycation of the crystalline proteins (30). From a nutritional point of view, several vitamins such as ascorbic acid and α-tocopherol, which can act as antioxidants, may prevent the formation of cataracts by decreasing the oxidative stress in the lens. The anti-cataract effect of ascorbic acid or α-tocopherol in experimental diabetic animals has been reported (31, 32). Epidemiological studies suggest that dietary antioxidants, especially lutein and zeaxanthin which are xanthophylls contained in vegetables and fruits, reduce the risk of developing cataracts (33–35). However, the anti-cataract effect of lutein has not been determined in animal models. The anti-cataract effect of Chlorella described in this paper will need further study, especially regarding the contribution of lutein.

In conclusion, Chlorella had DPPH radical scavenging activity and inhibited lipid peroxidation and cataract formation in STZ rats. Accordingly, *Chlorella* would be a good source of dietary antioxidant and may serve as a functional food with beneficial effects on diabetic complications.

REFERENCES

17) Cao G, Sofic G, Prior RL. 1996. Antioxidant capacity of...


