Effect of Chlorella and Its Fractions on Blood Pressure, Cerebral Stroke Lesions, and Life-Span in Stroke-Prone Spontaneously Hypertensive Rats

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Summary Effects of *Chlorella regularis* (dried cell powder)—cultured axenically under heterotrophic conditions, and provided as a dietary supplement—and its fractions on the blood pressure, cerebral stroke lesions, and life-span of stroke-prone spontaneously hypertensive rats (SHRSP/Izm) were investigated. When SHRSP were fed on diets with supplemented Chlorella to a commercial diet (Funabashi SP), elevation of blood pressure was significantly lower in the Chlorella groups than in the control group. At 21 wk of feeding, serum total cholesterol was significantly lower in the Chlorella groups than in the control group. Histopathological examination revealed cerebral vascular accidents in the brains of the control group, but those of Chlorella groups showed apparently low incidence compared to the control group. The average life-span of the Chlorella groups were significantly longer than that of the control group ($p < 0.001$). Chlorella powder was fractionated into three fractions, lipid-soluble, hot water-soluble, and residual fractions. The diets supplemented with lipid or residual fractions equivalent to 10% Chlorella significantly suppressed elevation of blood pressure in SHRSP, and then decreased the incidence rate of cerebral vessel lesions compared to the control group. Chemical analysis revealed that the lipid fraction contained large quantities of antioxidants, including carotenoids (especially lutein) and others, and phospholipids involved in aorta collagen and elastin metabolism; the residual fraction contained high concentrations of arginine, enhancing the function of blood vessels. The control diet contained only a little these substances. These experimental results suggest that the beneficial effect of Chlorella on SHRSP is caused by the synergistic action of several ingredients of Chlorella, which play a role in sustention of a vascular function of rats.

Key Words Chlorella, SHRSP, carotenoid, arginine, blood pressure

The stroke-prone spontaneously hypertensive rat (SHRSP/Izm) has a genetic predisposition to hypertension and cerebral stroke. Numerous nutritional and pathological studies using SHRSP have revealed that various food ingredients are protective against hypertension, and cerebral stroke, and these findings have contributed greatly to the prevention of hypertension and cerebral stroke in humans (1, 2). Okamoto et al. (1) and Murakami et al. (3) reported that Chlorella intake suppressed elevation of blood pressure and the incidence of cerebral stroke lesion in SHRSP, and the average life span was remarkably longer than that the control group. However, little is known about the active ingredients in Chlorella.

Chlorella produces protein and various physiologically active substances with high efficiency in utilizing light and carbon dioxide, or organic carbon sources. However, cellular ingredients of Chlorella fluctuate greatly with culture conditions. Therefore the mass cul-

ture and nutritional application of Chlorella have been studied for many years (4, 5). Recently, high-efficiency production of *Chlorella regularis*, which contains large quantities of arginine-rich protein and the phytochemicals typical of green plants, was accomplished under axenic heterotrophic conditions (6).

Several nutritional, physiological, and clinical studies have addressed whether Chlorella powder can help prevent arteriosclerosis (7–11). The purposes of the present study were to confirm the preventive effect of axenic heterotrophic Chlorella on hypertension and cerebral stroke in SHRSP, and to elucidate the components involved in this activity.

MATERIALS AND METHODS

Diets. The control diet used in the present study was an unrefined diet (Funabashi SP diet, Funabashi Farm, Chiba, Japan). The composition is shown in Table 1. Chlorella powder (Nihon Chlorella Co., Tokyo, Japan) was the dried powder of *C. regularis* cells that had been cultured axenically under heterotrophic conditions and

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Table 1. Composition of diets supplemented with Chlorella powder and Chlorella fraction.

(weight %)

	Dietary groups						
	Control ^a	5% Chl. ^b	10% Chl. ^c	20% Chl. ^d	Lipid F. ^e	Water-sol. F. ^f	Residual F. ^g
Chlorella powder	0.0	5.0	10.0	20.0	0.0	0.0	0.0
Control diet ^a	100.0	85.9	71.8	43.6	98.4	96.4	81.8
Lipid fraction	0.0	0.0	0.0	0.0	1.6	0.0	0.0
Water-sol. Fraction	0.0	0.0	0.0	0.0	0.0	1.2	0.0
Residual fraction	0.0	0.0	0.0	0.0	0.0	0.0	7.2
Corn starch	0.0	9.1	18.2	36.4	0.0	2.4	11.0
Ingredient							
Crude protein	20.8	20.8	20.8	20.8	20.8	20.8	20.8
Crude lipid	4.8	4.7	4.7	4.5	6.3	4.6	3.9
Crude fiber	3.2	3.1	3.1	2.9	3.1	3.5	3.2
Crude ash	5.0	4.6	4.3	3.5	4.9	5.1	4.5
Moisture	8.0	7.1	6.1	4.2	7.9	7.8	6.8
Carbohydrate	58.2	59.7	61.0	64.1	57.0	58.2	60.8

^aFunabashi SP diet. ^b5% Chlorella diet. ^c10% Chlorella diet. ^d20% Chlorella diet. ^eLipid fraction diet. ^fWater-soluble fraction diet. ^gResidual fraction diet.

dried using a spray dryer. The general composition of the Chlorella powder was 58.7% crude protein, 12.2% crude lipid, 7.7% crude fiber, 6.7% ash, 11.2% carbohydrate, and 3.5% moisture.

Experiment-1: To investigate the effect of Chlorella concentration on blood pressure and incidence of cerebral stroke in SHRSP, three test diets were prepared by adding Chlorella powder at a concentration (wt/wt) of 5, 10, or 20% to the control diet. The crude protein content of each diet was adjusted to 20.8% by adding cornstarch (Table 1). Experiment-2: In order to approach the active ingredients showing anti-hypertension, Chlorella powder was fractionated into three fractions, hot water-soluble, lipid-soluble, and residual fractions. First, the lipid fraction was extracted from Chlorella powder in accordance with the method of Folch et al. (12). The residual was suspended in water and water-soluble components were extracted by heating for 1 h at 100°C. The supernatant was used as the water-soluble fraction, and the insoluble portion was used as the residual fraction. The yields of each fraction from Chlorella powder were 16.0% lipid-soluble, 11.8% water-soluble and 72.2% residual fraction. The diets used in Experiment-2 were prepared by adding 1.2% water-soluble fraction, 1.6% lipid-fraction, or 7.2% residual fraction to the control diet. Each diet contained each fraction equivalent to the 10% Chlorella diets. The crude protein contents of each diet were adjusted to 20.8% by adding cornstarch (Table 1).

Animals. Male SHRSP were purchased from Funabashi Farm. For Experiment-1, eighty 11-wk-old SHRSP were divided into four groups ($n=20$ per group), which began receiving the experimental and control (i.e., 0% Chlorella powder added) diets at 12 wk of age. All rats were housed five animals per cage in a room at constant temperature ($24\pm2^\circ\text{C}$) and humidity ($55\pm10\%$) with a 12:12-h light:dark cycle. The rats had free access to the assigned diet and tap water

throughout the experimental period. Five rats in each group were euthanized at the end of the 21-wk experimental period, and blood biochemical tests and histopathological analyses of internal organs were completed on these animals. The remaining 15 rats in each group were used to study the incidence of cerebral stroke lesion after natural death and effects on life-span. For Experiment-2, forty-five 6-wk-old male SHRSP with systolic blood pressure of 202 ± 16 mmHg were divided into five groups ($n=9$ per group). The rats were individually housed in a room. Each group was fed on the diets for Experiment-2 supplemented water-soluble, lipid-soluble, residual fractions, or 10% Chlorella powder and control diet from 7 wk of age. Rats had free access to drinking water containing 1% NaCl. Other husbandry conditions were the same as for Experiment-1. The ethical committee for animal experiments of Yakult Central Institute approved this research project, and the animals were maintained in accordance with institutional guidelines for the care and use of laboratory animals.

Measurement of systolic blood pressure. The blood pressure of rats was measured once weekly for Experiment-1 for 13 wk and for Experiment-2 for 3 wk. After a rat was fixed for 5 min on a holder in the thermostat at 41°C , blood pressure of unanesthetized rat was measured by using a photoelectric oscillometric tail-cuff method (UR-5000, ELK Corp., Tokyo, Japan).

Blood biochemical analysis. At the end of the 21-wk experimental period, five rats in each group in Experiment-1 were deeply anesthetized with pentobarbital, and the animals were then exsanguinated through the abdominal aorta. Coagulation parameters (prothrombin time and activated partial thromboplastin time) were measured using a blood coagulation analyzer (Coagmaster II, Sankyo, Tokyo, Japan), and fibrin degradation products were measured using a spectrophotometer (UV-160A, Shimadzu, Kyoto, Japan). Serum biochemical parameters (total cholesterol, triglyceride,

phospholipid, sodium, chloride, and potassium concentrations) were measured using an automatic clinical analyzer (Type 7170, Hitachi, Tokyo, Japan).

Histopathological examination. In Experiment-1, the liver, spleen, kidneys, adrenal gland, testis, heart, thymus, lung, thoracic aorta, brain, and pituitary from rats euthanized at the end of the 21-wk test period and those that died during the experiment period were evaluated histopathologically. In Experiment-2, the brains of rats in each group were examined histopathologically at the end of 10 wk of the experiment period. Organs were fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for microscopic examination. A diplomate (coauthor, Takahashi) authenticated by the Japanese Society of Toxicologic Pathology judged the presence of a lesion. The results were expressed as number of rats showing symptoms of vascular lesion against number of rats examined.

Chemical analysis. Major components—protein, amino acids, lipids, vitamins, and carotenoids—and the general chemical composition of the control diet and Chlorella powder were analyzed according to the methods of the Association of Official Analytical Chemists (13) and as reported by Sansawa and Endo (6).

Statistical analysis. The results showing in Tables 2, 4 and Fig. 1, 2 are expressed as mean $\pm$ standard error (SE). Data were analyzed by repeated-measures analysis of variance (ANOVA) followed by Bonferroni's multiple-range test to estimate the significance of differences between the control and experimental groups. Survival data in Fig. 3 were analyzed by a log-rank test followed by Bonferroni's correction. Differences with $p < 0.05$ were considered statistically significant.

RESULTS

Experiment-1

Effect on body weight. During the first 3 wk of experimental feeding, intake of diet was significantly more in the Chlorella groups than in the control group, though during the first 11 wk of experimental feeding, when cerebral lesions first appeared in control animals, body weight did not differ significantly between the Chlorella groups and control group (Fig. 1). Thereafter, control animals began to show weight loss, and the number of rats that subsequently died from emaciation increased. In contrast, the body weights of Chlorella groups increased slowly throughout the 21 wk of the experiment. In particular, the body weights of the 10% and 20% Chlorella groups after 21 wk of supplementation were increased significantly compared with those of the control group.

Effect on blood pressure. Blood pressures in the 10% and 20% Chlorella groups were significantly lower than that of the control group, since 1 wk after the test diets were introduced (Fig. 2). During the experimental period, the blood pressure of the control group increased from 190 to >260 mmHg after 10 wk. In comparison, the blood pressures of the 10% and 20% Chlorella groups were maintained at <260 mmHg

Fig. 1. Change with time in body weight of SHRSP fed on Chlorella diets. SHRSP at 12 wk of age were fed on diets containing 0% (○), 5% (▲), 10% (●) or 20% (■) Chlorella. Rats were weighed once a week. Values are means $\pm$ SE ($n=20$). Significantly different from control (0% Chlorella), ** $p < 0.01$; *** $p < 0.001$.

Fig. 2. Change with time in blood pressure of SHRSP fed on Chlorella diets. SHRSP at 12 wk of age were fed on diets containing 0% (○), 5% (▲), 10% (●) or 20% (■) Chlorella. Blood pressure of the rats was measured once a week. Values are means $\pm$ SE ($n=20$). Significantly different from control (0% Chlorella), * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

until 34 wk, at which time the first deaths occurred in these groups. After 1 wk, the 5% Chlorella group showed a significantly lower blood pressure than did the control group; thereafter blood pressure remained lower in the 5% Chlorella group but not significantly different from that of controls.

Organ weight and blood biochemical analysis. Organ weights and blood biochemical values were measured after 21 wk of feeding the supplemented diets. Organ weights of the Chlorella groups did not differ significantly from those of the control group. The weights of the brain and kidney, corrected for body weight, were significantly lower for the 20% Chlorella group than for the control group, but no other body-weight-adjusted organ weights differed between any Chlorella group

Table 2. Serum biochemical values in SHPSP fed diet containing Chlorella for 21 wk.

	Dietary groups (Chlorella %)			
	0	5	10	20
PT (s)	11.8±0.3	11.5±0.2	11.9±0.2	11.9±0.1
APTT (s)	18.9±0.6	18.3±0.6	19.5±0.6	18.8±0.5
FDP (μg/mL)	1.82±0.72	1.72±0.31	2.49±0.83	1.84±0.91
T. cho. (mg/dL)	84.9±5.2	74.3±2.3*	74.6±2.9*	67.5±1.9**
TG (mg/dL)	39.7±9.5	39.8±0.8	36.3±3.8	33.6±1.8
PL (mg/dL)	125.2±13.6	120.3±2.4	113.4±4.1	111.4±3.2
Na (mEq/L)	148.1±1.2	143.8±0.2**	144.1±0.7*	144.5±0.5*
K (mEq/L)	4.9±0.3	4.8±0.2	4.7±0.3	4.2±0.2
Cl (mEq/L)	108.3±1.1	101.1±0.4***	101.1±0.4***	101.5±0.6***

Values are means±SE (n=5). PT, prothrombin time; APTT, activated partial thromboplastin time; FDP, fibrin degradation product; T. cho., total cholesterol; TG, triglyceride; PL, phospholipid; Na, sodium; K, potassium; Cl, chlorine. Significantly different from control (0% Chlorella), * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Table 3. Incidence rates of brain lesions and kidney lesions in SHRSP fed diet containing Chlorella for 21 wk.

Dietary groups (Chlorella %)	0	0	5	10	20
Cases	survived	died	survived	survived	survived
Brain					
Focal degeneration of nerve cells	5/5	8/8	4/5	1/5	1/5
Edema	1/5	8/8	0/5	0/5	0/5
Fibrinoid degeneration of arteriola	2/5	6/8	1/5	1/5	0/5
Encephalomalacia	2/5	3/8	1/5	1/5	0/5
Hemorrhage	0/5	6/8	0/5	0/5	0/5
Kidneys					
Cronic nephropathy	5/5	8/8	4/5	4/5	2/5
Fibrinoid degeneration of arteriola	4/5	8/8	1/5	1/5	0/5

Results are expressed as No. of rats with lesions/No. of rats examined.

and the control group (data not shown). Serum levels of sodium, chloride, and total cholesterol were significantly lower in the Chlorella groups than in the control group (Table 2).

Histopathological examination. At 21 wk of experimental period (age 33 wk, the point at which half of rats in the control group had died), the brains of surviving rats in the control group showed encephalomalacia, focal degeneration of nerve cells, edema, and fibrinoid degeneration of arterioles. In addition, the kidneys of these animals had high incidence rates of chronic nephropathy and fibrinoid degeneration of arterioles. In comparison, the incidence rates of these lesions in the Chlorella groups were suppressed in accordance with the concentration of Chlorella. At this time point, all groups lacked lesions in the liver and spleen. The control rats that died during the first 21 wk had higher incidence rates of fibrinoid degeneration of arterioles, encephalomalacia, and hemorrhage in the brain than the surviving rats. In addition, the kidneys of these animals had chronic nephropathy and fibrinoid degeneration of arterioles (Table 3). The ratio of rats with brain lesions in Chlorella-supplemented groups who died during the experiment period was suppressed, and

Fig. 3. Survival time of SHRSP fed on Chlorella diets. SHRSP at 12 wk of age were fed on diets containing 0% (○), 5% (▲), 10% (●) or 20% (■) Chlorella. The survival time of SHRSP was monitored.

inversely correlated with concentration of Chlorella. Rats dying in the 20% Chlorella group (during days 300 to 370 shown in Fig. 3) had few symptoms of cerebral stroke. The incidence rates of lesions in kidneys and lungs of these rats increased, consistent with the ani-

Table 4. Change of blood pressure in SHRSP fed on diets containing Chlorella fractions.

(mmHg)

	Dietary groups				
	Control	10% Chlorella	Water-sol. F. ^a	Lipid F. ^b	Residual F. ^c
0 wk	237±12	235±22	236±14	231±16	234±16
1 wk	253±17	245±18	247±16	237±16*	236±16*
2 wk	276±20	256±19**	261±12	253±12***	255±18**

Diets were prepared so as to contain each fraction equivalent to the addition of 10% Chlorella.

Rats were given 1% NaCl water for drinking.

^a Water-soluble fraction. ^b Lipid fraction. ^c Residual fraction.Values are means±SE (n=9). Significantly different from control, **p*<0.05, ***p*<0.01, ****p*<0.001.

Table 5. Microscopical findings of brain in SHRSP fed on diet containing Chlorella fractions.

Dietary groups	Control	10% Chlorella	Water-sol. F. ^a	Lipid F. ^b	Residual F. ^c
Brain					
Focal degeneration of nerve cells	6/6	3/6	3/6	3/6	2/6
Edema	5/6	1/6	4/6	3/6	1/6
Fibrinoid degeneration of arteriola	1/6	1/6	1/6	1/6	1/6
Encephalomalacia	4/6	0/6	3/6	3/6	2/6
Hemorrhage	6/6	1/6	3/6	3/6	1/6

Diets were prepared containing each fraction equivalent to the addition of 10% Chlorella and administered for 10 wk.

Results are expressed as No. of rats with lesions/No. of rats examined.

^a Water-soluble fraction. ^b Lipid fraction. ^c Residual fraction.

mals' advanced age (data not shown).

Effect on life span. The average life span of each group was as follows: 367±13 d 5% Chlorella group, 384±8 d 10% Chlorella group, and 420±5 d 20% Chlorella group, as compared with 232±8 d of the control group (Fig. 3). Average life span was significantly longer in the Chlorella groups than in the control group (*p*<0.001). Significant differences were also shown between Chlorella groups, except relation between the 5% and 10% Chlorella group. The 20% Chlorella group was significantly different from the 5% Chlorella group (*p*<0.001) and from the 10% Chlorella group (*p*<0.01).

Experiment-2

Effects of Chlorella fractions. In Experiment-2, in order to identify the active ingredients involving in prevention of hypertension and cerebral stroke in SHRSP, Chlorella powder was fractionated into three fractions: water-soluble, lipid-soluble, and residual. The effects of diet supplemented with each fraction equivalent to 10% Chlorella, on blood pressure and the incidence of cerebral lesions in SHRSP were investigated. As shown in Table 4, blood pressure was significantly lower in the rats fed the diet containing the lipid- or residual-fraction than in the control group. Prolonged feeding of these Chlorella fractions decreased the incidence rates of cerebral lesion of rats in both groups, as compared with the control group. The blood pressure of the water-soluble fraction group showed no significant difference from those in the control diet. The incidence rate of cerebral lesion showed a low tendency (Table 5).

Chemical composition of Chlorella powder, each fraction

and control diet. The control diet and the experimental diets supplemented with Chlorella powder or its fraction contained nearly equal amount of crude protein, crude lipid, crude fiber crude ash and carbohydrate (Table 1).

Chemical analysis revealed evident differences in ingredients between Chlorella powder and control diet (Table 6). One hundred grams of Chlorella powder contained 720 mg carotenoids (including 350 mg lutein, 60 mg α -carotene, 50 mg β -carotene, 139 mg violaxanthin, 104 mg neoxanthin), 3.5 g chlorophyll, and 6.3 g arginine, whereas the control diet had little carotenoids (<10 mg) or chlorophyll (<10 mg), and 1.34 g arginine. Furthermore, as active ingredients which have been proven to be involved in vascular function, Chlorella powder contained 42% indigestible fraction (CIF, 10) and 4.4% phospholipids. Quantities of vitamin B₂, vitamin E, folic acid, pantothenic acid, biotin, inositol, lysine, methionine, and fatty acids C₁₆₌₀, C₁₆₌₁ and C₁₈₌₃ were over threefold those of the control diet. The main components in each fraction of Chlorella powder were analyzed. One hundred grams of the residual fraction contained 72.9 g crude protein, 19.4 g dietary fiber, 5.6 g hemicellulose, 3.9 g ash, and 7.2 g arginine as well as 38.6 g CIF. One hundred grams of the lipid fraction contained 20.3 g neutral lipid, 27.4 g phospholipid, and 27.9 g glycolipid. Regarding components involved in protecting against arteriosclerosis, the lipid fraction contained 4.2 g total carotenoids, 2.1 g lutein, 0.38 g α -carotene, 0.28 g β -carotene, 0.78 g violaxanthin, 0.59 g neoxanthin, 0.17 g vitamin E, 0.31 g ubiquinones, 20 g chlorophyll, fatty acids (1.13 g C_{16:1},

Table 6. Chemical composition of Chlorella powder and control diet.

Component	Chlorella	Control diet ^a	Component	Chlorella	Control diet ^a
Vitamins (mg/100 g dry weight)			Amino acids (mg/100 g dry weight)		
Vit. A (IU)	61,100	1,000	Arginine	6,350	1,340
Vit. B ₁	1.7	1.0	Lysine	4,510	990
Vit. B ₂	5.1	1.1	Methionine	1,230	300
Vit. B ₆	2.2	1.2	Cystine	780	290
Vit. B ₁₂ (μg)	0	1.7	Tryptophane	950	230
Vit. D ₃ (IU)	nd ^b	200.0	Glycine	2,830	850
Vit. E (mg)	53.5	5.0	Isoleucine	2,110	880
Vit. K	1.4	0.5	Leucine	4,460	1,460
Niacin	30.7	10.0	Phenylalanine	2,420	870
Panto. acid ^c	22.1	1.5	Threonine	2,180	680
Folic acid	1.2	0.1	Valine	2,880	970
Biotin	0.3	<0.001	Histidine	1,110	500
Choline	305.3	230.0	Tyrosine	1,810	620
Inositol	530.1	90.0	Alanine	4,060	nd
Ubiquinons	49.2	nd	Aspartic acid	4,530	nd
Carotenoids (mg/100 g dry weight)			Glutamic acid	6,830	nd
T-Carotenoids ^d	720.0	<1.0	Proline	2,450	nd
α-Carotene	60.0	nd	Serine	1,980	nd
β-Carotene	50.0	nd	Lipids (g/100 g dry weight)		
Lutein	368.0	nd	Neutral lipid	4.3	4.8
Violaxanthin	139.0	nd	Phospholipid	4.4	nd
Neoxanthin	104.0	nd	Glycolipid	4.5	nd
Chlorophyll	3,600.0	<10.0	Fatty acids (mg/100 g dry weight)		
Minerals (mg/100 g dry weight)			14:0	17	24
Ca	128.0	1,200.0	16:0	1,550	716
P	1,900.0	960.0	16:1	190	24
Mg	380.0	260.0	18:0	190	221
K	1,420.0	520.0	18:1	1,390	938
Na	52.0	390.0	18:2	2,150	2,524
Fe	73.0	20.0	18:3	1,620	226
Mn	18.0	10.0	20:0	0	10
Cu	0.6	0.5	20:1	0	53
Zn	2.0	4.4	22:0	0	43
I (μg)	0	40.0	22:1	0	19
Co	0	0.2			

^aFunabashi Farm data. ^bNot determined. ^cPantothenic acid. ^dTotal-carotenoids.

7.88 g C_{18:1}, 11.79 g C_{18:2}, and 9.0 g C_{18:3}), and 27.4 g phospholipids. One hundred grams of the water-soluble fraction contained 59 g peptide, (11.6 g arginine, as almost a peptide bond), 28 g inorganic salts (10.2 g K, 3.6 g Mg, and others), and some amount of nucleic acid-related substances, and was rich in water-soluble vitamins (vitamin B₂, pantothenic acid, folic acid, biotin, inositol, and others).

DISCUSSION

Nutritional and pathological studies on hypertension and cerebral stroke in SHRSP have contributed greatly to the prevention of these diseases in humans. These studies have revealed that increased or decreased levels of the following dietary components have an influence on blood pressure and incidence of cerebral stroke when SHRSP were fed a generalized commercial diet (Funabashi SP diet, etc): quality and quantity of protein (1, 2), methionine, taurine, tyrosine (2), arginine (14, 15), cholesterol (2, 16), Na, K (2, 17), Ca (18, 19), fatty

acids (20), vitamin E (21–25), and dietary fiber (26). The present study showed that the diets supplemented with Chlorella suppressed elevation of blood pressure and incidence of cerebral stroke in SHRSP, and then noticeably prolonged the average life span compared with the control diet. The effect of Chlorella on SHRSP was basically similar to the effect of Chlorella on SHRSP previously reported by Okamoto et al. (1) and Murakami et al. (3). This experiment showed further that the protective effect of Chlorella on SHRSP was brought about by the lipid-portion and residual-portion fractionated from Chlorella powder. Firstly, deductions about the active ingredient contained in Chlorella could be drawn from the evident difference in chemical components between Chlorella and the control diet. Chemical analyses suggest that K, methionine, lysine, tyrosine, and fatty acid C_{16:1} richly contained in Chlorella must have had no effect in this experimental system, because these substances were contained in larger amounts in the control diet than in 10% Chlorella diet.

Chlorella contained a higher concentration of antioxidants, carotenoids and others, phospholipids, and arginine, which are proven to be involved in the function of blood vessels. Each diet supplemented with the fractions including these ingredients shown to suppress elevation of blood pressure and incidence of cerebral lesion, compared with the control diet. Meanwhile, the control diet contained only a little of these substance.

In recent years, related studies using cell powder obtained from a pure culture of *C. regularis* have proven that this organism is rich in many physiologically active substances that protect against atherosclerosis and life-style-related diseases (7–11). We will discuss the components and activities of each fraction in light of these related studies on the basis of SHRSP studies.

Lipid fraction

The lipid fraction of Chlorella contained large quantities of fat-soluble antioxidants and phytochemicals typically found in green plants, such as carotenoids, particularly lutein, vitamin E, ubiquinones, phospholipids, and others. One hundred grams of diet supplemented with 1.6% lipid fraction contained 68 mg total carotenoids, 32 mg lutein, 5 mg α -carotene, 5 mg β -carotene, 3 mg vitamin E, and 4.5 mg ubiquinone, as ingredients having antioxidant activity (27, 28). The control diet (100 g) contained only 5 mg vitamin E as an antioxidant. Furthermore, the lipid fraction diet (100 g) contained 396 mg phospholipids, which are involved in the aortic collagen and elastin synthesis (7, 8). Shibata et al. (10) reported that Chlorella powder or its extract show potent antioxidant activity in *in vitro* and *in vivo* systems. Wistar rats fed a vitamin E-free basal diet containing 7.3% Chlorella powder showed significantly reduced serum lipid peroxidation (TBARS value) and vascular release of super-oxide anion in the liver and kidney. Tomita et al. (21) and Yamori et al. (22) reported that a vitamin E-enriched diet (20 mg per 100 g diet) diminished serum lipid peroxidation in SHRSP and prolonged their life span. However, when SHRSP were fed with the supplement loaded in 1% NaCl drinking water, the effect of vitamin E had a tendency to be impaired. Noguchi et al. (23) also reported that in SHRSP fed the diet containing an excessive quantity of vitamin E (100 mg per 100 g diet), vascular oxygen stress, production of serum lipid peroxide, elevation of blood pressure, and thrombosis were suppressed to levels below those of the control group. Several experimental results also suggest that antioxidant treatment to reduce oxidative stress prevents the age-related development of blood pressure and cerebral vascular disease in an animal model of genetic hypertension (24, 25). Experiment-2 was conducted by a short-term feeding test under the 1% NaCl loaded condition, which causes a rapid increase in blood pressure (26, 29) and represses some anti-hypertensive effects. In spite of such conditions, the Chlorella lipid fraction suppressed the elevation of blood pressure in SHRSP, and decreased the incidence rate of cerebral stroke. The potent preventive effect of the Chlorella lipid fraction may have been caused by synergistic action due to the high content of

multiple antioxidants, carotenoids and others. Recently, Iribarren et al. (30), and Dwyer et al. (31, 32) have presented papers in which epidemiological study and mouse model findings support the hypothesis that increased dietary intake of lutein suppresses the progression of intima-media thickness of the common carotid arteries, and is protective against the development of early atherosclerosis. Extensive epidemiological studies also have shown that increased intake of carotenoids, especially lutein, is correlated significantly with decreased risk of cerebral stroke and coronary heart disease (33–35). Currently, numerous studies have revealed that a reduction of oxygen stress in blood vessels is effective to suppress the development of vascular disease. It seems reasonable to suppose that the antioxidants contained in Chlorella are involved in preventing the development of hypertension and cerebral stroke.

Hashimoto et al. (7, 8) reported that the diet containing 1% Chlorella phospholipids (CPL) increased significantly the prolyl hydroxylase activity in the thoracic aorta of rats, and degradation of aortic collagen was stimulated. Content of collagen and elastin, and ratio of collagen/elastin in the thoracic aorta fell in the CPL group. The experimental results suggested that change in the crosslinking of collagen and elastin may induce qualitative or structural changes leading to a reduction in aortic tension and strength. Many studies have shown that the synthesis of aortic collagen and elastin is increased in experimental arteriosclerosis. Yamori (2) and Murakami et al. (17) have reported that collagen and elastin in aorta of SHRSP increased with age compared with SHR and WK rats, and that these composition ratios in the aorta were improved by feeding a fish-meal diet. In the present study, the diet supplemented with 10% Chlorella powder contained 440 mg CPL per 100 g diet. CPL also may function synergistically with other ingredients for the prevention of cerebral stroke.

Hashimoto et al. (7, 8) also showed that CPL decreased the serum cholesterol of rats. Serum levels of total cholesterol at 21 wk in the present experiment were significantly lower in the rats fed on Chlorella diets than in those fed on the control diet. However, it has been proven that cerebral vascular lesion in SHRSP is caused by hypertensive arterionecrosis without involving cholesterol. It is not clear that whether the cholesterol-lowering effect of Chlorella involves preventing cerebral vascular lesion. It is necessary to research further. Chlorella and its lipid fraction was rich in chlorophyll. Recently, it has been found that chlorophyllin (a water-soluble analogue of chlorophyll) shows potent antioxidant ability in various experimental systems (36, 37). However, chlorophyll is absorbed little from the digestive tract, and there is no positive evidence showing the antioxidant activity of chlorophyll, unlike water-soluble chlorophyllin. Chlorophyll may contribute little to antioxidant *in vivo*. As a whole, the lipid fraction among the three fractions of Chlorella powder was shown to most effectively suppress the progress of hypertension and cerebral stroke in unit of activity per intake.

Residual fraction

Chlorella powder contained the large quantity of 6.4% arginine per dry weight, compared to the control diet containing 1.3% arginine. The diets supplemented with 5, 10, or 20% Chlorella contained 1.5, 1.6, and 1.9% arginine, respectively. The diet supplemented with a 7.2% residual fraction contained 1.6% arginine. Noguchi et al. (15) reported that supplementation with L-arginine significantly suppressed blood pressure, thrombosis, and stroke in SHRSP. Arginine is a physiologic substrate for production of endothelial nitric oxide (NO). Studies on arginine in various sorts of animal models have shown that orally provided arginine yields systemic NO, improves endothelium-dependent vasodilation, suppresses elevation of blood pressure, decreases platelet aggregation, and inhibits monocyte adhesion to endothelial cells, all of which processes are key early events in arteriosclerosis (38–40). Kohli et al. (41) showed that endothelial NO production in rats rose significantly after the increase of the daily intake from 0.35 to 1.78 g arginine/kg body weight of rats. In our study, the daily intake of arginine of SHRSP fed the diets supplemented with 0 to 20% Chlorella ranged from 0.86 to 1.85 g arginine/kg body weight of rat. These arginine amounts are within the range reported as having significant effects on blood pressure and endothelial NO production. Therefore, it is reasoned that arginine is one of active factors contained in the residual fraction of Chlorella powder. Okamoto et al. (1) presented a paper in which a potent antihypertensive glycoprotein was obtained by alkali-treatment (pH 9.3 with 0.1 N NaOH, 60°C, 15 h) of defatted Chlorella powder or by hot water-treatment, and that the activity was reduced by treatment with pepsin or trypsin. The present study showed that the diet supplemented with the hot water-soluble fraction of Chlorella didn't have the effect of suppressing elevation of blood pressure (Table 4), though a large portion of water-soluble peptide contained in Chlorella powder was extracted into the water-soluble fraction. We didn't examine in this study whether an antihypertensive peptide from the alkali-treatment of Chlorella is produced or not, because it seems unlikely that Chlorella is exposed to such alkali conditions during animal oral ingestion of Chlorella as a diet supplement. Further investigation into details on production of antihypertensive substances is required.

Chlorella powder did not show a Na excreting effect in the intestine. Although the serum level of Na at 21 wk of the test period was significantly lower in the Chlorella groups than in the control group, there was no significant difference in Na levels in response to the amount of Chlorella ingested. Serum level of Na in the Chlorella groups maintained its initial level, but Na in the control group was significantly higher than that. The control rats at 21 wk of feeding had increased the incidence rate of lesions in the kidneys as well as the brain (Table 3), and were on the verge of death (Fig. 3). Therefore it is presumed that the control rats couldn't maintain normal serum levels of Na owing to disorder of homeostasis.

Water-soluble fraction

The diet supplemented with the 1.2% water-soluble fraction didn't significantly suppress the elevation of blood pressure in SHRSP, but the incidence rate of cerebral lesions showed a low tendency (Tables 4 and 5). Although the activity of the water-soluble fraction was low, we are unable to disregard the activity shown by the addition of a small quantity. The water-soluble fraction was rich in several water-soluble vitamins typical in green plants, or unknown factors. Further research is needed.

In conclusion, the diet supplemented with Chlorella suppressed the elevation of blood pressure and decreased the incidence rate of cerebral stroke in SHRSP, compared with the control diet, and significantly prolonged the average life span. Additional experiments revealed that the lipid portion and residual portion fractionated from Chlorella powder suppressed the elevation of blood pressure and decreased the incidence rate of cerebral stroke in SHRSP. The lipid fraction contained high levels of carotenoids (particularly lutein) and other substances enhancing the antioxidant activity in serum and organs, and phospholipids involved in arteriosclerosis prevention. The residual fraction was rich in arginine, increasing the generation of endothelium-derived relaxing factor. The control diet contained only a little of these ingredients. These experimental results suggest that the protective effects of Chlorella on SHRSP were caused by the synergistic action of several ingredients involved in enhancement of vascular function in rats. Further research is needed to definitively identify the components of Chlorella that participate in suppressing hypertension and cerebral stroke in SHRSP.

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