Original Article

A study on changes of biochemical and immunological markers by supplementations of *Spirulina* in elderly males in Tianjin

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Summary

The objective of this study was to assess the potential effects of daily supplementation of *Spirulina* on biochemical and immunological functions in elderly males. Two groups of male participants in Tianjin over 65 years old were recruited in this study. One group, consisting of 13 males, was diet-supplemented by tablets of *Spirulina* daily (4.2 g/day) for consecutive 12 weeks as the Sp group. The other group, consisting of 14 males, was kept intact without intervention as the Cont group. Changes of biochemical and immunological markers and results from questionnaires on life-style obtained before and after the experimental period were compared to clarify the effects of *Spirulina* supplementation. Levels of serum protein in the Sp group significantly increased after the experimental period, compared to those in the Cont group. Three subjects in the Cont group were suspected pneumonia infections by the increased levels of WBCs, however, data from these subjects were not excluded from the statistical analyses. No infected subject was observed in the Sp group. The levels of RBC, Ht, and Hb significantly decreased after the experimental period in both of the Sp and Cont groups, however, 2-factor ANOVA revealed no significance in interaction factors. Levels of NK cell activities in both of the Sp and Cont groups significantly increased after the experimental period also with no significance in interaction factors of ANOVA. Add-on of the *Spirulina* to ordinary diet in the hospitalized elderly may prevent protein loss. There was no effect observed on the *Spirulina* supplementation in the items of anemia caused by nutritional difficulties in elderly. Also, effects of *Spirulina* supplementation on the levels of NK cell activities and S-IgA productions were not certified in this study.

Keywords: *Spirulina*, male elderly, immunosenescence

Aging is an irreversible process, although public health efforts such as those for promotion of healthier diets have improved average lifespans of the citizens in developed countries[1)]. It has been recognized that aging is associated with the functional decline of the immune system, a process termed "immunosenescence", contributing to morbidity and mortality[2)]. Some studies have investigated the possibility that reduced bioavailability of key conditionally essential nutrients might limit immune response among the aging[3)]. Lifestyle factors such as good nutritional status, on the other hand, can sustain the ability of effective immune responses against infectious challenges[1, 4)].

It is generally accepted that the development of age-associated alterations occurs earlier in the intestinal mucosal immune system than in the systemic immune compartment[5)]. We previously reported that age-related changes in intestinal immune functions and functional preservation of intraepithelial lymphocytes were positively affected by ingestion of *Spirulina*[6)]. *Spirulina* (*Arthrospira*) *platensis*, a helicoidal filamentous blue-green alga (cyanobacterium), has been used as a food for more than a thousand years, and has been commercially produced for more than 30 years as a food supplement[7, 8)]. *Spirulina* is rich in high-quality proteins, vitamins and minerals, and is known

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to possess various therapeutic effects, as reviewed elsewhere, against hyperlipidemia, diabetes, and hypertension, as well as immune modulatory activity[9].

In the present study, male residents in a nursing home over 65 years old were recruited as subjects and divided into two groups. One group was supplemented by tablets of *Spirulina* daily for consecutive 12 weeks as the Sp group. The other group was kept intact without intervention as the Cont group. Changes of biochemical and immunological markers in the blood and saliva, together with scores of life-style questionnaire before and after the supplementation period, were assessed and compared between the two groups.

MATERIALS AND METHODS

Subjects

Participants of this study were recruited from residents of a private nursing home for the elderly in Tianjin, the People's Republic of China, administrated by the Hetong Elderly Welfare Association. Detailed information on this study was given in Chinese to the participants by nurses or dietitians who belonged to the nursing home, according to an experimental manual. Written informed consents were obtained from the participants directly or from protectors of participants who had some mental difficulties in responding to the agreements. Twenty-seven male residents between 65 and 92 years old agreed to join the study. One of the subjects was limited in his ability to recollect his families' names, day-long events, and/or feeding himself.

The 27 participants were randomly divided into two groups; one group, consisting of fourteen males (76.2±10.2 years), was kept intact without intervention as the Cont group having ordinary diets and the other group, consisting of thirteen males (78.6±7.3 years), was supplemented by tablets of *Spirulina* (4.2 g/day) daily added to ordinary served diets as the Sp group. One participant in the Cont group took medicine for heart disease and another participant answered as excessive fat and glucose in the blood but did not take any medicine. No supplement was taken by the participants in both groups. The diet served at the nursing home during the study was the ordinary weekly dietary menu planned and calculated by standard tables of food composition in China for one week by dietitians. No food from outside the nursing home was allowed, following the rules of the nursing home, and the subjects were asked not to intake foods other than the served diets during the investigation. The subjects of the Sp group were instructed to ingest 7 tablets per meal of *Spirulina* supplement (21 tablets per day; a total of 4.2 g of *Spirulina platensis* per day, DIC SPIRULINA 21 of Hainan DIC Microalgae Co. Ltd., Haikou, Hainan). It is generally recommended to take 2〜6 g of *Spirulina* as a supplement per day, and 4.2 g of *Spirulina* contain the same or enough amount of beta carotene (about 8 mg) as that of dark green

Table 1 Nutritional value of commercially produced food-grade *Spirulina*

(per 100g dry weight)

General constituents		Vitamins	
calorific value	383 kcal	A (retinol eq..)	16.2 mg
protein	70.0 g	B1	3.78 mg
total lipid	8.1 g	B2	3.99 mg
carbohydrate	2.7 g	B6	0.84 mg
dietary fibers	9.5 g	niacin	38.5 mg
gamma linolenic acid	1.29 g	folic acid	0.17 mg
linoleic acid	1.22 g	pantothenic acid	1.3 mg
chlorophyll a	1320 mg	biotin	30.4 μg
phycocyanin	7400 mg	E	9.1 mg
zeaxanthin	98 mg	K1	1080 μg
inositol	105 mg	K2	80 μg
Minerals			
calcium	75.8 mg		
phosphorus	868 mg		
iron	63.1 mg		
sodium	296 mg		
potassium	1.58 g		
magnesium	242 mg		
zinc	0.98 mg		

Data from DIC LIFETEC Co., Ltd.;
http://www.dlt-spl.co.jp/spirulina/component.html

or yellow and other vegetables ingested per day. *Spirulina* tablets to be taken were directly handed to the subjects at every meal by nurses or dietitians who worked for the nursing home during the investigation period of 12 weeks. Twelve-week administration of *Spirulina* was used in relatively many reports of the studies on anti-hyperlipidemia, obesity, diabetic mellitus, and so on[10–13]. Nutritional value of commercially produced food-grade *Spirulina* was shown in Table 1.

The Institutional Review Board at Kagawa Nutrition University (Protocol Number 204) approved this study protocol, and the Board of Directors of Hetong Elderly Welfare Association allowed us to conduct the study at the nursing home. The study was carried out in compliance with the Helsinki Declaration.

Daily consumption of food in subjects

Daily consumption of a meal was assessed in food-stuffs categorized as rice and bread, potatoes, eggs, meat and processed meat, fish and fish products, bean and beans products, dairy products, vegetables, fruits, and seaweed. The categorized consumptions in the menus of the nursing home were scored with a three-item method[14], i. e. score 1; eat served food but leave some in every meal (about one third of food consumption in score 3), score 2; eat almost all served food but leave some in some meals (around average food consumption between scores 1 and 2), and score 3; eat almost all served food in every meal (basis food consumption). The consumption of food by each subject was checked every meal by nurses or dietitians who worked for the nursing home. Examination about statistical difference of the values scored by nurses and dietitians or accuracy was not carried out. Mean±standard deviations of data in each group were calculated before and after the intervention.

Questionnaire and description of physical and mental state in daily life

As physical characteristics, body weight (kg) with light garments and height (m) were measured and a body mass index (BMI kg/m^2) was calculated using these measurements. Systolic and diastolic pressures were measured using a sphygmomanometer.

All of the participants were interviewed by nurses or dietitians concerning their life styles, such as frequency of bowel movements, quality of sleep, consciousness of own health, state of stressfulness, and motivation of daily life, a week before and after the 12-week intervention period, and obtained answers from each item were scored with a four-item method[15] ; for examples, score 1; sleep well every day, score 2; occasionally sleep not well, score 3; often sleep not well, and score 4; sleep not well every night, as sleep categories.

The subjects were also asked about their health care, anamnesis and any diseases they had in the past one month before the examination.

Collecting blood and saliva specimens

Blood and saliva specimens were collected before and at the end of the experiment. In order to avoid the influences of meals and the circadian rhythms of secretory immunoglobulin A (S-IgA) and cortisol[16, 17], nurses collected the saliva first and then drew the blood before breakfast. Fasting at the previous night was not carried out because of presumable causation of hunger stress. Saliva was collected by using a cotton roll with Salivette (SARSTEDT Aktiengesellschaft & Co., Germany). Briefly, the sterilized cotton roll used in dentistry was gently chewed for 3 minutes without swallowing the saliva secreted. The cotton saturated with saliva was then returned to the suspended insert of the centrifuge tube and centrifuged at 1,000 g for 3 min. to recover the saliva. The obtained saliva specimens were frozen at −80 degree centigrade until assay.

Serum biochemical indices

Total protein, albumin, globulin, albumin to globulin (A/G) ratio, glutamic pyruvic transaminase (GPT) or alanine transaminase (ALT), gamma-glutamyl transpeptidase (γ-GTP), uric acid, blood glucose, total- cholesterol (T-Ch), high- and low-density lipoprotein cholesterol (HDL- and LDL-C, respectively), triacylglycerol (TG), calcium, and iron were measured using an automatic analyzer in the Tianjin Wang Ding-Di Hospital.

Blood cell counts and natural killer (NK) cell activity

The numbers of white blood cells (WBC), red blood cells (RBC), hemoglobin (Hb), hematocrit (Ht), and the numbers of lymphocytes (LYM), monocytes (MON), and granular cell leukocytes (GRA) were measured at the laboratory in the Department of Pathology, Tianjin Medical University. Natural killer (NK) cell activity was also measured by lactate dehydrogenase (LDH) method as an index of innate immunity[18].

Cortisol, chromogranin A (CgA), and secretory immunoglobulin A (S-IgA) in saliva

The amounts of the cortisol and chromogranin A (CgA) were measured as indices of psychological stress.

Cortisol concentration in saliva was measured by Expanded-range High Sensitivity Salivary Cortisol

Enzyme Immunoassay Kit (Salimetrics LLC., USA). Optical density at 450 nm was detected by using a microplate leader, Multiskan MK3 Model 353 equipped with a data processing software Package Revision 4.5 lab system (Thermo Scientific, USA). CgA was measured by using the YK070 human Chromogranin A EIA kit (Yauchibara Research Institute, Japan), and optical density at 490 nm was detected by the same equipment as described above.

Secretory immunoglobulin A (S-IgA) in saliva was measured by the ELISA method described by Ishii et al.[19]. Purified human secretory IgA (Cappel laboratories Inc., USA) as a standard, goat IgG fraction to human secretory IgA (Cappel laboratories Inc., USA) as a primary capturing antibody, and peroxidase-conjugated IgG fraction to human secretory IgA as the secondary antibody (Cappel laboratories Inc., USA) were used for the indirect ELISA method. Optical density in 492 nm was detected by the same equipment as described above. Protein concentration in saliva was measured by using bovine serum albumin (#500-0007, BioRad Laboratories, USA) as a standard in the Bradford method, and optical density at 595 nm was detected. S-IgA concentration in saliva was adjusted as a ratio of mg protein in saliva according to the paper reported by Sakai et al.[20]

Statistical analysis

The data of daily consumption of foods and physical and mental state obtained from each subject were scored by a three-item and a four-item method, respectively, as described above. Serum biochemical indices, blood cell counts, and immunological indices were treated as numerical data in assumption of normal distribution. The scored data of daily consumption of foods were analyzed by Student's t-test if the data showed normal distribution and by Mann- Whitney U-test if the data did not show normal distribution. The other scored data of the Cont and Sp groups were categorized as "Before" and "After" according to data collected period in this experiment and analyzed by generalized linear mix model in two-way analysis of variance (AVOVA) at significant rates of 0.05 by using the statistical software SPSS Statistics ver. 23 for Windows.

Results

Daily consumption of food

Daily consumption of rice by the subjects of both Sp and Cont groups were not so large; that is, the subjects took rice but left some at some meals, 2.1±0.7 in Sp group and 2.2±0.4 in Cont group (Table 2). Similarly, ingestions of potato, eggs, milk and its products, and vegetables including dark green or yellow vegetables tended to be small. Daily consumptions of these foods in comparison between Cont and Sp groups were neither statistically different nor in comparison between before and after intervention. Meat and meat products, seafood and seafood products, and seaweed were relatively well ingested in both Sp and Cont group, but ingestions of the meat products and seafood by Sp group were relatively lower than those of the Cont. Especially, ingestion of meat products of Sp group was significantly lower than that of Cont group (2.6±0.7 in Sp group and 3.0±0.0 in Cont group) and main effect of Sp comparing Cont was observed in meat products. Bread was also well ingested in both Sp and Cont group (2.7±0.7 in Sp group and 3.0±0.0 in Cont group), and ingestion of bread by Sp group was statistically lower than that of the Cont. Other foods such as bean curd and its products, milk and its products, dark green or yellow vegetables, fruits, and seaweed were also well ingested in both groups but there was no statistically significance in the amount between the groups (data were not shown).

Physical characteristics

Weight and height involving BMI of both Sp and Cont groups did not change before and after intervention schedule (Table 3); that is, neither main effect nor interaction was observed. Both systolic and diastolic pressures of Sp group were almost the same as those of Cont. Systolic and diastolic pressures of both Sp and Cont groups were decreased after intervention period, and decrease of the systolic pressure observed in the Cont group was statistically significant.

Three subjects of the Cont group suffered from infections such as pneumonia or had a medical examination during the last one month of the intervention schedule. On the other hand, there was no subject of the Sp group who suffered from infection during the intervention schedule.

Physical and mental state

States of daily mood and sleep as well as physical state involving bowel condition in the subjects of both Cont and Sp groups were generally good or not bad. Consciousness of own health of both Cont and Sp groups, however, tended to be not so good and scores of stressfulness of both groups were relatively high (2.6±0.7 in Sp group and 2.6±0.8 in Cont group). Daily lives also tended to be dependent on family members and some of the staff members of the nursing home. Two-way ANOVA of these items involving awakening, however, showed neither statistically significant interaction nor main effect.

Table 2 Daily dietary ingestion of foods in each group before and after *Spirulina* intervention

Food items	Group	Before intervention	After intervention
	Sp	n = 13	
	Cont.	n = 14	
Rice	Sp	2.1 ± 0.7	2.1 ± 0.7
	Cont.	2.2 ± 0.4	2.2 ± 0.4
Bread	Sp	2.7 ± 0.7 b	2.7 ± 0.7 b
	Cont.	3.0 ± 0.0	3.0 ± 0.0
Potato	Sp	2.3 ± 0.8	2.3 ± 0.8
	Cont.	2.5 ± 0.5	2.5 ± 0.5
Eggs	Sp	2.5 ± 0.7	2.5 ± 0.77
	Cont.	2.3 ± 0.5	2.6 ± 0.5
Meat	Sp	2.6 ± 0.7	2.6 ± 0.7
	Cont.	2.9 ± 0.4	2.6 ± 0.7
Meat products*	Sp	2.6 ± 0.7 b	2.6 ± 0.7 b
	Cont.	3.0 ± 0.0	3.0 ± 0.0
Seafood	Sp	2.6 ± 0.7	2.7 ± 0.6
	Cont.	2.9 ± 0.3	2.9 ± 0.3
Seafood products	Sp	2.7 ± 0.6	2.8 ± 0.6
	Cont.	2.8 ± 0.4	2.8 ± 0.4

Values; mean ± SD of scores answered by the subjects in each group.
score 1; eat served food but leave some in every meal
score 2; eat almost all served food but leave some in some meal
score 3; eat almost all served food in every meal
b; $p < 0.05$ in comparison to corresponding value of Control
Two-way ANOVA showed main effects of Sp diet (Sp group vs Cont.)
* Meat products include the following items in the weekly menu of meals; meat-filled steamed bun, thin-sliced meat and potato julienne stir-fries, seasoned rice porridge with ground meat and egg, meat and vegetable dumplings (Chiaotzu), minced pig meat and cauliflower stir-fried, minced pig meat and egg noodle soup, etc.

Table 3 Physical characteristics

		Group	Before intervention	After intervention
		Sp	n = 13	
		Cont.	n = 14	
Age	years	Sp	78.6 ± 7.3	
		Cont	76.2 ± 10.2	
Height	cm	Sp	169.5 ± 5.7	169.5 ± 5.7
		Cont	170.9 ± 4.1	170.9 ± 4.1
Weight	kg	Sp	63.2 ± 9.9	64.3 ± 9.6
		Cont	62.9 ± 7.0	63.2 ± 7.2
BMI	kg/m^2	Sp	22.0 ± 2.9	22.3 ± 2.8
		Cont	21.5 ± 1.7	21.6 ± 1.8
Systolic pressure	mmHg	Sp	144.2 ± 11.9	136.5 ± 8.0
		Cont	141.5 ± 11.0	136.5 ± 10.0 b
Diastolic pressure	mmHg	Sp	75.2 ± 6.2	74.8 ± 6.3
		Cont	77.0 ± 4.8	76.0 ± 5.2
Heart rate	BPM	Sp	78.8 ± 3.2	79.1 ± 3.1
		Cont	78.4 ± 5.0	76.2 ± 5.1

Values mean average ± SD of the subjects in each group.
b; $p < 0.05$ in main effect between after and before intervention

Table 4 Nutritional and biochemical variables in the serum of each group before and after *Spirulina* intervention

Nutritional and biochemical variables		Group	Before intervention		After intervention	
		Sp	n = 13			
		Cont.	n = 14			
Total protein	60–80 g/L	Sp	70.6 ± 6.9		74.5 ± 2.8	b, c
		Cont	74.1 ± 3.6		72.5 ± 3.7	
Albumin (A)	35–55 g/L	Sp	41.8 ± 3.9		43.3 ± 2.9	b
		Cont	45.9 ± 6.9		43.4 ± 3.0	
Globulin (G)	15–30 g/L	Sp	29.6 ± 4.1		30.2 ± 3.1	
		Cont	29.7 ± 2.4		29.1 ± 3.1	
A/G	1.2–2.0	Sp	1.4 ± 0.3		1.5 ± 0.2	
		Cont	1.6 ± 0.3		1.5 ± 0.2	
GPT (ALT)	0–40 U/L	Sp	30.8 ± 12.4		39.9 ± 9.1	b
		Cont	34.9 ± 12.0		43.3 ± 8.5	b
GOT (AST)	0–40 U/L	Sp	38.1 ± 12.1		36.5 ± 8.3	
		Cont	35.1 ± 6.6		39.9 ± 8.6	
γ-GTP	0–40 U/L	Sp	18.8 ± 10.6		15.1 ± 10.0	
		Cont	16.6 ± 6.1		11.9 ± 7.3	
Urea (BUN)	2.8–8.2 mmol/L	Sp	6.3 ± 1.5		6.1 ± 1.4	
		Cont	6.2 ± 1.4		6.5 ± 1.8	
Uric acid	148–416 μmol/L	Sp	226.9 ± 72.7	a	242.1 ± 68.0	c
		Cont	320.5 ± 123.7		261.4 ± 76.5	b
Creatinine	65–133 μmol/L	Sp	98.9 ± 23.2		98.6 ± 17.4	
		Cont	98.7 ± 25.3		104.5 ± 33.7	
Glucose	3.6–6.1 mmol/L	Sp	4.6 ± 1.4		5.9 ± 2.9	
		Cont	4.7 ± 0.8		5.2 ± 0.8	b
T-Ch	2.09–6.31 mmol/L	Sp	4.3 ± 1.0		4.60 ± 0.84	
		Cont	4.06 ± 0.68		4.25 ± 0.73	
HDL-C	1.2–1.55 mmol/L	Sp	1.36 ± 0.18		1.42 ± 0.20	
		Cont	1.28 ± 0.17		1.30 ± 0.19	
LDL-C	2.7–3.36 mmol/L	Sp	2.3 ± 0.8		2.4 ± 0.7	
		Cont	2.2 ± 0.7		2.5 ± 0.6	b
Triacylglycerol (TG)	0.32–3.04 mmol/L	Sp	1.21 ± 0.26		1.41 ± 0.42	
		Cont	1.28 ± 0.40		1.28 ± 0.36	
Ca	2.15–2.55 mmol/L	Sp	2.16 ± 0.12		2.35 ± 0.24	b
		Cont	2.16 ± 0.29		2.34 ± 0.33	b
Fe	10.7–26.9 μmol/L	Sp	17.7 ± 4.0		17.5 ± 4.4	
		Cont	19.8 ± 4.4		19.7 ± 4.3	
Transferrin	2.0–4.0 g/L	Sp	3.1 ± 0.3		3.2 ± 0.4	
		Cont	3.2 ± 0.5		3.3 ± 0.5	

Values mean average ± SD of specimens from the subjects in each group.
a; $p < 0.05$ in main effect between Cont and Sp group
b; $p < 0.05$ in main effect between after and before intervention
c; $p < 0.05$ in interaction of Cont and Sp groups before and after intervention
Standard values were based on the data from the Clinical laboratory of Tianjin Medical University.

Serum biochemistry index

Relatively low total protein and albumin (A) of Sp group before *Spirulina* supplementation increased significantly after 12-week Sp supplementation and significant interaction of total protein in the Cont and Sp groups before and after intervention was observed (Table 4). Neither main effect nor interaction of the serum globulin (G) and A/G ratio was observed in two-way ANOVA.

GPT (ALT) levels of both Sp and Cont groups significantly increased after intervention schedule, but significant interaction of Cont and Sp groups before and after intervention was not observed. Gamma-GTP of both Cont and Sp groups, however, tended to decrease after Sp intervention schedule.

The levels of urea (BUN) in both Sp and Cont groups did not change before and after intervention. Although uric acid of Cont group as an index of gout, which was significantly higher than that of Sp group, decreased significantly after intervention, that of Sp group tended to increase after Sp supplementation. Significant interaction of uric acid levels in Cont and Sp groups before and after intervention was observed. Neither main effect nor interaction of creatinine was observed in two-way ANOVA.

The levels of glucose and LDL- C in Cont group were significantly increased after intervention schedule, although changes of the levels in Sp group were not significant. Neither main effect nor interaction of the, T-Ch and HDL-, and TG was observed.

Serum calcium of Sp and Cont groups significantly increased after Sp intervention. Main effects of calcium before and after intervention period were observed. There was no characteristic change in Fe and transferrin levels of both Cont and Sp groups.

Blood cell counts

WBC of Cont group before intervention, 6.1±1.7 x 10^6 cells/mL, significantly increased to 7.4±1.8 x 10^6 cells/mL after 12-week intervention schedule, although that of the Sp group slightly increased after 12-week Sp supplementation (Table 5). There was no significant interaction between groups and intervention period in two-way ANOVA of WBC.

RBCs of both Sp and Cont groups were significantly decreased after Sp intervention. Ht and Hb of Cont groups as well as Ht of Sp group also significantly decreased after 12-week intervention schedule.

As for lymphocyte (LYM), monocyte (MON), and granulocyte (GRA) counts, neither main effect nor interaction was observed.

Immunological indices

As shown in Fig. 1, NK cell activity of Sp group before intervention, 62.8±13.8%, significantly increased to 73.7±9.5% after Sp supplementation. Since amounts of the blood specimen from one participant of the Sp group and two participants of Cont were not enough for detecting NK cell activity, the number of specimens finally forced to be reduced. That of Cont group, 60.0±13.8% also signifi-

Table 5 Blood cell counts

Blood cells		Group	Before intervention	After intervention	
		Sp	n = 13		
		Cont.	n = 14		
WBC	4.0–10.0	Sp	5.6 ± 1.5	6.5 ± 1.4	
	10^9/L	Cont	6.1 ± 1.7	7.4 ± 1.8	b
RBC	3.50–5.80	Sp	4.95 ± 0.84	4.45 ± 0.47	b
	10^{12}/L	Cont	5.33 ± 0.69	4.75 ± 0.46	b
Ht	0.35–0.50	Sp	0.46 ± 0.07	0.40 ± 0.04	a b
	L/L	Cont	0.50 ± 0.06	0.45 ± 0.05	b
Hb	110–165	Sp	148 ± 20	138 ± 8.7	a
	g/L	Cont	160 ± 17	152 ± 14	b
LYM	17.0–48.0	Sp	29.8 ± 12.0	26.3 ± 7.4	
	%	Cont	25.2 ± 7.2	28.6 ± 2.1	
MON	4.0–10.0	Sp	6.4 ± 1.5	5.4 ± 1.5	
	%	Cont	5.9 ± 1.5	6.0 ± 1.6	
GRA	43.0–76.0	Sp	63.8 ± 12.0	68.4 ± 8.1	
	%	Cont	68.9 ± 8.4	65.4 ± 13.1	

Values mean average ± SD of specimens from the subjects in each group.
a; $p < 0.05$ in main effect between Cont and Sp group
b; $p < 0.05$ in main effect between after and before intervention
c; $p < 0.05$ in interaction of Cont and Sp groups before and after intervention
Standard values were based on the data from the Clinical laboratory of Tianjin Medical University.

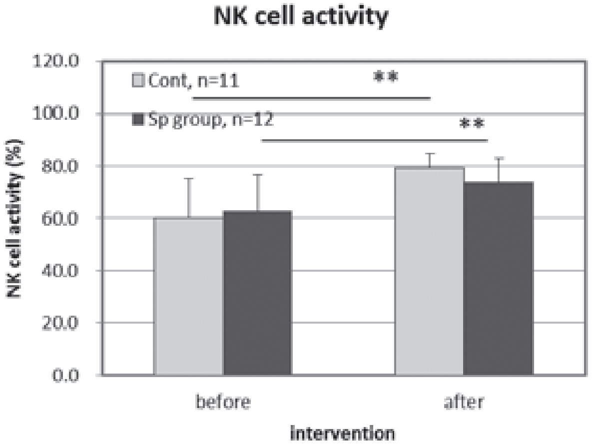

Fig. 1 Change of the NK cell activity before and after *Spirulina* intervention
Two-way ANOVA showed main effects of both period (before and after intervention) and diet (Cont and Sp) groups (**; p<0.01), but did not show interaction of period and diet groups.

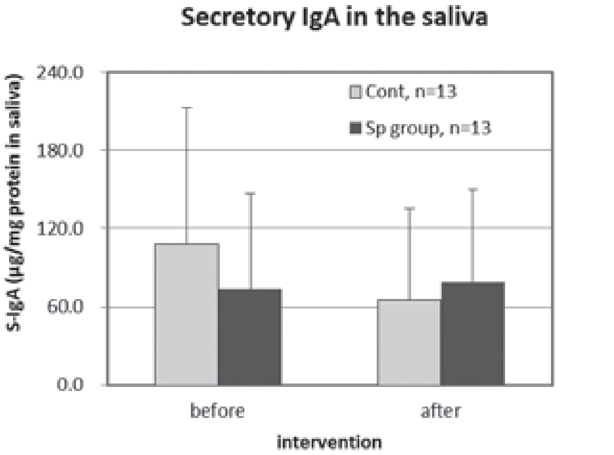

Fig. 2 Change of secretory IgA in the saliva before and after *Spirulina* intervention
Two-way ANOVA showed main effect of neither period (before and after intervention) nor diet (Cont and Sp) groups. There was no interaction of period and diet groups in the two-way ANOVA.

cantly increased to 79.3±5.9% after intervention schedule. Main effects of both period (before and after intervention) and diet (Cont and Sp) groups were observed in two-way ANOVA, but significant interaction of period and diet groups was not observed.

S-IgA antibody of Sp group did not change so much or slightly increased from 73.4±73.3 μg/mg protein of saliva before intervention to 79.1±71.2 μg/mg protein after Sp supplementation (Fig. 2). S-IgA of Cont group, however, decreased from 108.6±104.3 μg/mg protein to 64.8±70.7 μg/mg protein after the 12-week intervention schedule, although the decrease was not statistically significant.

Cortisol in saliva of both Cont and Sp groups tended to decrease after intervention schedule, and neither main effect nor interaction was observed. Chromogranin A (CgA) of both Cont and Sp groups slightly increased after intervention schedule, and neither main effect nor interaction was observed.

DISCUSSION

Recently, the number of intervention studies of *Spirulina* has been increasing, along with the recognition of the safety[21], for example, in Human Immunodeficiency Virus (HIV)-infected children[22] and in patients such as those having nephropathy[23], rhinitis[10], hypercholesterolemia[11], and others. At first we planned to interview and collect experimental specimens from a total of 39 aged subjects (27 men and 12 women) in this *Spirulina* intervention study. But, practically, it was hard to collect the blood and/or saliva specimens from some women because of the thin vein or involuntarily swallowing the secreted saliva. Therefore, we deleted the data obtained from the women, and used only those from the men in the study.

Infections, cancer, and autoimmune diseases occur more frequently in the elderly. Although many factors contribute to these difficulties, age-related remodeling of the immune system, termed immunosenescence, plays a major role[24]. Total serum protein of Sp group increased significantly after 12-week Sp intervention (Table 4), in spite of lower daily consumption of meat products and seafood by the Sp group in comparison with those by the Cont group. Serum protein of Cont group, on the other hand, was relatively high before intervention and slightly decreased during intervention period (Table 4). Mani et al.[23] designed a study on the efficacy of *Spirulina* supplementation for a period of 4 months in nephrotic patients who were matched for age, gender, and severity of disease to control subjects, and reported that a clinical manifestation of nephrotic syndrome, hypoproteinemia, was improved by supplementation with *Spirulina*, accompanied by raised levels of albumin and albumin: globulin (A:G) ratio. Since *Spirulina* has a rich content of protein, 60–70% protein by weight, and has a balanced amino acid profile, as well as vitamins, trace elements, and essential fatty acids[25], it has been widely used as an edible protein source for many years. Although protein intake of both Sp and Cont group was not calculated in this study, ingestions and scores of daily consumption of foods except bread and meat products differed neither in comparison between Sp and Cont group nor in comparison between before and after intervention. *Spirulina* supplementation for 3 months may have worked effectively to some degree to increase serum protein in the subjects of Sp group who ingested relatively low levels of meat products and seafood. GPT (ALT) levels significantly increased in the both groups after intervention schedule. Degree of increase was appeared to be controlled in Sp group. Cause of the increase of GPT over the normal range in Cont group was not clear. Statistically significant increase of calcium in the blood was observed in both groups. Certain nutritional factors from dietary ingestion of foods might contribute to the increase but details were not known yet.

WBC of Cont group significantly increased 12 weeks after intervention schedule, while those of the Sp group did not change or increased only slightly after 12-week Sp supplementation (Table 5). Three subjects with bacterial infections such as pneumonia were observed in Cont group during the experiment period. Significant increase in WBC of the Cont group may have been caused by infected subjects in the Cont group. No infected subjects were observed in the Sp treatment group. Immunomodulation properties of *Spirulina* have been reported in various models of humans and animals[9]. Increased indole amine 2,3-dioxygenase (IDO) enzyme activity as a sign of immune function was shown in the subjects aged 50 years or older at 6 and 12 weeks of *Spirulina* intervention, suggesting that *Spirulina* might ameliorate immunosenescence in older subjects[26]. It has been reported that ingestion of a hot-water extract of *Spirulina* may contribute to the functional preservation of the intestinal epithelium as a first line of mucosal barrier through retaining the number of intra-epithelial lymphocytes (IELs), as shown in aged mice[6]. As shown in Fig. 1, NK cell activities of Sp group as well as those of Cont groups significantly increased after intervention schedule. It is generally accepted that cytotoxicity and interferon gamma (IFN-γ) production as NK cell activity declines with age, especially in frail elderly, but the absolute number of NK cells adversely increases in elderly[1, 27]. It has been reported that IFN-γ productions in response to IL-12/IL-18 as NK cell activity of healthy male volunteers of 40–65 years old were much higher 2 months after *Spirulina* administration than those before the administration[28]. Although increase of NK cell activity can be anticipated after Sp supplementation, the increases of NK cell activity observed in both Sp and Cont group in the present study might have been caused by certain technical errors in the assay performed on the separate days before and after intervention.

Ishizu et al.[29] reported in a study of the elderly living on Kudakajima Island of Okinawa that S-IgA per volume unit of saliva continued to show relatively high levels without reduction in the subjects aged 65–75 years and over 75 years old. In another study in Okayama[30], S-IgA in saliva significantly declined with age in elderly in their 70s and 80s, whereas S-IgA levels were almost the same in middle-aged people in their 40s to 60s. Although aging appears to have a detrimental effect on IgA response with regard to the magnitude of S-IgA response, conflicting results have been reported in animal and human studies[31]. Several studies have demonstrated that chronic stress down-regulated the level of S-IgA, while an acute psychological challenge induced fluid change; that is, down- or up-regulation[32]. It has been reported that total and ovalbumin (OVA)-specific IgA antibody levels in the intestinal mucosa were elevated in mice administered with phycocyanin, a photosynthetic biliprotein of *Spirulina*, for 7 weeks and orally immunized with either OVA-microparticles[33]. Ishii et al.[34] demonstrated that total S-IgA level in the saliva of subjects who habitually used *Spirulina* was correlated to the total amount of *Spirulina* ingested, and the level in the subjects who consecutively ingested it over one year significantly increased, at a higher level than that in the subjects who ingested it less than 0.5 year. In the present study, although S-IgA of Cont group decreased after the 12-week intervention schedule, that of the Sp-supplemented group did not change so much, or slightly increased without statistical significant interaction (Fig. 2).

Selmi et al.[26] reported that there was a steady increase in average values of mean corpuscular hemoglobin in the subjects of both sexes with an age of 50 years or older following a *Spirulina* intervention for 12 weeks, suggesting that *Spirulina* might ameliorate anemia in older subjects. RBC levels, as well as hematocrit and hemoglobin of both Cont and SP groups after intervention schedule, significantly decreased within normal ranges (Table 5). Hematopoietic effect of supplementation with *Spirulina* could not surpass the decline of RBCs or force up the generation of the cells in the present aged subjects. Uric acid in the serum of Sp group slightly increased after intervention without significant main effect (Table 4) although that of Cont significantly decreased after intervention schedule and significant interaction of Cont and Sp groups before and after intervention. All of the uric acid levels of both groups before and after intervention were within normal range (148–416 μmol/L). Other biochemical variables in both groups were also within normal range except only one or two data slightly deviated. It has been reported that *Spirulina* normalized the elevated levels of AST, ALT, uric acid, and creatinine detected as hepatonephrotoxicity induced by deltamethrin, a synthetic pyrethroid insecticide in rats[21], and increased levels of creatinine, urea, and uric acid by anti-tuberculosis drugs such as isoniazid and rifampicin were ameliorated by *Spirulina* in a dose-dependent manner[35]. As general feature, it may be possible to say that *Spirulina* tends to ameliorate the vulnerable conditions in elderlies or in individuals who are necessitated to use medicine such as antibiotics or anticancer drug nevertheless it operates not so much in ordinary individuals

Other assessments, including physical conditions such as BMI and blood pressure and mental state such as daily mood and sleep, were generally good or not bad at least during the Sp supplementation period. In a randomized double-blind placebo-controlled trial enrolling 40 Caucasian patients aged 40 to 60 years with hypertension but not cardiovascular disease, *Spirulina* not only improved BMI and weight but also resulted in improvements in blood pressure and endothelial function[12]. Suliburska et al.[13] reported that treatment with *Spirulina maxima* for 3 months resulted in significantly decreased serum iron levels in hypertensive obese adults, aged 25 to 60 years, suggesting lowered blood pressure and improvement in markers of cardiovascular risk.

In summary, a daily supplementation with *Spirulina* to an ordinary balanced diet is possibly effective to some extent in improvement of nutritional difficulties such as protein loss in elderly. Ameliorating effect on immunosenescence such as in NK cells and S-IgA production needs further investigation.

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Competing financial interests:

The authors declare no competing financial interests.

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