Spirulina Prevents Atherosclerosis by Reducing Hypercholesterolemia in Rabbits Fed a High-Cholesterol Diet

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Summary The anti-atherogenic effects of spirulina (Spirulina platensis) were investigated in the New Zealand White (NZW) rabbit model. The animal had hypercholesterolemia induced by being fed a high cholesterol diet (HCD) containing 0.5% cholesterol for 4 wk, and then fed a HCD supplemented with 1 or 5% spirulina (SP1 or SP5) for an additional 8 wk. Spirulina supplementation lowered intimal surface of the aorta by 32.2 to 48.3%, compared to HCD. Serum triglyceride (TG) and total cholesterol (TC) significantly were reduced in SP groups. After 8 wk, serum low density lipoprotein cholesterol (LDL-C) remarkably decreased by 26.4% in SP1 and 41.2% in SP5, compared to HCD. On the other hand, high density lipoprotein cholesterol (HDL-C) was markedly increased in SP1 and SP5 compared with that in the HCD group from 2 to 8 wk. These results suggest that spirulina intake can cause the reduction of hypercholesterolemic atherosclerosis, associated with a decrease in levels of serum TC, TG and LDL-C, and an elevation of HDL-C level. Spirulina may, therefore, be beneficial in preventing atherosclerosis and reducing risk factors for cardiovascular diseases.

Key Words atherosclerosis, hypercholesterolemia, rabbits, Spirulina platensis

Hypercholesterolemia is the critical step in the initiation of atherosclerosis, placing hypercholesterolemic individuals at a greater risk for cardiovascular disease (1). Although the precise mechanisms of atherosclerosis remain to be clarified, the oxidative stress resulting from increased reactive oxygen species (ROS) production has been implicated in the development of hypercholesterolemic atherosclerosis (2). Therefore, there have been many attempts to prevent atherosclerosis by reducing cellular oxidative stress (3, 4), and decreasing cholesterol levels.

Spirulina (Spirulina platensis) is a rich source of phycocyanin, an antioxidant biliprotein pigment, and carotenoids, which correspond to potent antioxidants (5, 6). Recently, it was reported that the protein C-phycocyanin played a crucial role in the hypercholesterolemic action of spirulina (7). Separately, several studies demonstrated that spirulina or spirulina extracts reduced blood lipid levels and increased antioxidant activities in rodents, such as rats, hamsters, and mice, and in humans (7–10). However, a high-fat diet does not easily produce aortic lesions, atherosclerosis or other pathological changes such as gallstone formation and liver damage in normal rodents (11, 12), which may be due to their rapid metabolic rates, although low-density lipoprotein receptor-deficient mice are known to be more sensitive to atherosclerosis (13, 14). Therefore, animal models such as pigeons, swine, rabbits, and monkeys, demonstrating a relatively rapid development of aortic lesions on a diet with a high level of cholesterol, have been used to study atherosclerosis development (15–19). There are limited cases of studies that show direct evidence of the efficacy of spirulina in reducing atheromatous lesions. Although anti-atherosclerotic action of spirulina has been suggested in some models, it remains to be clarified whether its antiatherosclerosis action, based on general biomarker assessment, can be announced at doses where its antioxidant action is expressed in vivo. Therefore, in the present study, the effects of spirulina supplementation on blood lipid profiles and the prevention of atherosclerosis in hypercholesterolemia-induced rabbits were determined.

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MATERIALS AND METHODS

Materials. Freeze-dried powder of spirulina (Spirulina platensis) which was obtained from Dainippon Ink and Chemicals, Inc. (Tokyo, Japan), was kindly donated by ES Biotech Co. (Cheonan, Korea). Table 1 shows the composition of freeze dried powder of spirulina. Cholesterol and corn oil were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). All reagents used were of the highest analytical grade.

Hypercholesterolemic-rabbit model and diets. Twenty male New Zealand White (NZW) rabbits weighing between 2.0 and 2.5 kg were obtained from Animal Husbandry of Yonnam (Cheonan, Korea). They were housed individually in stainless steel cages (380 W×490 L×350 H mm) in an animal room at controlled temperature (20–25°C) and humidity (55–65%) under a 12 h light-dark cycle. After 2 wk of acclimation to a commercial diet (Samyang Co., Korea), the rabbits were randomly divided into four treatment groups. One group (n=5, Control) was fed a commercial rabbit chow diet for 12 wk. Three groups (n=5 each) were fed a high-cholesterol diet (97.5% normal rabbit chow, 0.5% cholesterol, and 2% corn oil) for 4 wk to provoke the atherosclerotic process. The rabbits fed a diet enriched with 0.5% cholesterol and 2% corn oil for 4 wk developed hypercholesterolemia (0 wk) with serum total cholesterol (TC) of 1,570 mg/dL, whereas serum TC of rabbits fed the normal rabbit chow diet was 77 mg/dL. After hypercholesterolemia was induced for 4 wk, the rabbits in the high-cholesterol diet groups were given one of the following diets: the HCD (high-cholesterol diet), SP1 (high cholesterol diet plus 1% spirulina) and SP5 (high cholesterol diet plus 5% spirulina). The rabbits were then allowed ad libitum access to water and the assigned diets for an additional 8 wk. These three groups were matched for serum cholesterol levels (1,334 vs. 1,304 vs. 1,308 mg/dL). The body weights of the rabbits were measured every week. All animal experiments were conducted in compliance with the guidelines outlined in the ‘Guide for Care and Use of Laboratory Animals’ of the National Institutes of Health (20).

Serum lipid profiles. Fasted rabbits (12 h) were anesthetized with an intravenous injection of 0.05 mg/ kg Zoletil 50 (Virbac Co., Korea) during the post-absorptive period between 08.00 a.m. and 10.00 a.m. Blood was drawn from the ear vein once every 2 wk and centrifuged at 3,000 rpm for 20 min at 4°C. Serum triglyceride (TG), TC, and high density lipoprotein cholesterol (HDL-C) concentrations were determined enzymatically by using a kit purchased from Yeongdong Pharmaceutical (Seoul, Korea). Optical density was measured using a spectrophotometer at 546 nm and 500 nm (Pharmacia Biotech Co., Cambridge, England). Low density lipoprotein cholesterol (LDL-C) concentration was calculated using the following equation (21).

\[
\text{LDL-C} = \text{TC} - \text{HDL-C} - (\text{TG}/5)
\]

Sampling procedures. After the final (12-wk) treatment, blood was drawn from the ear vein of fasted rabbits (12 h) into a heparin tube and centrifuged at 3,000 rpm for 20 min at 4°C. Heart, kidneys, spleen, aorta and liver were removed, rapidly washed in saline buffer, collected into cryovials, weighed and immediately stored in liquid nitrogen for histopathological examination.

Histopathological examination of the aorta and liver. The aortic arch (5 cm) was dissected from the aortic valve of the heart, opened longitudinally, and fixed in 10% neutral buffered formalin. After removing fats and tissues adhering to the adventitia, the aorta was stained with Sudan IV for 20 min, photographed and analyzed with an image analyzer for red atherosclerotic lesions. The extent of lipid accumulation (atherosclerosis index; AI, %) was calculated as the percent Sudan-positive area to the total area of the aortic wall (22, 23). Liver was excised, fixed in formalin solution, stained with hematoxylin-eosin and examined under a light microscope to assess fatty changes.

Table 1. Composition of freeze-dried powder of spirulina (Spirulina platensis).

<table>
<thead>
<tr>
<th>Composition</th>
<th>per 100 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macronutrients</td>
<td></td>
</tr>
<tr>
<td>Calories (kcal)</td>
<td>360.70</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>8.70</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>17.50</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>4.30</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>63.00</td>
</tr>
<tr>
<td>Dietary fiber (g)</td>
<td>6.50</td>
</tr>
</tbody>
</table>

Vitamins

| Vitamin A (mg) | 2.95 |
| Vitamin B1 (mg) | 1.77 |
| Vitamin B2 (mg) | 3.74 |
| Vitamin B6 (mg) | 0.83 |
| Vitamin B12 (mg) | 0.18 |
| Vitamin E (mg) | 12.70 |
| α-Tocopherol (mg) | 12.50 |
| β-Tocopherol (mg) | 0.50 |
| Vitamin K1 (mg) | 1.59 |
| Vitamin K2 (mg) | 0.08 |
| Folic acid (mg) | 0.08 |
| Niacin (mg) | 23.50 |

Minerals

| Calcium (mg) | 98.80 |
| Iron (mg) | 40.90 |
| Phosphorus (mg) | 859.00 |
| Magnesium (mg) | 319.00 |
| Zinc (mg) | 1.28 |
| Copper (mg) | 0.32 |
| Manganese (mg) | 3.77 |
| Chromium (mg) | 0.06 |
| Potassium (mg) | 1.560.00 |

Phytonutrients

| Phytoene (mg) | 8.000.00 |
| Chlorophyll a (mg) | 1.300.00 |

The analysis certificate of freeze-dried spirulina powder sample obtained from Dainippon Ink and Chemicals, Inc. (Tokyo, Japan).
### RESULTS

#### Body and organ weights

After the induction of hypercholesterolemia for 4 wk, there was no significant difference of mean body weight among groups, ranging from 2.5 to 2.6 kg. During the subsequent 8-wk experimental period, the body weights of rabbits in all groups increased gradually. After 8 wk of treatment, the body weight did not differ among control, HCD, SP1 and SP5 groups (data not shown). Organ weights of rabbits were not significantly different in spleen, kidney or heart among the groups. However, the liver weight was increased in the HCD rabbits, compared with that in the control rabbits ($p<0.05$), and the increase in liver weight was not attenuated by spirulina treatment (Table 2).

#### Serum lipid profiles

In the beginning of the experimental period (0 wk), serum TC (Fig. 1A), LDL-C (Fig. 1B), HDL-C (Fig. 1C) and TG (Fig. 1D) concentrations were not significantly different among the groups. As shown in Fig. 1A, rabbits fed a diet enriched with 0.5% cholesterol and 2% corn oil for 4 wk developed hypercholesterolemia (0 wk) with a serum TC of 1,570 mg/dL, whereas the serum TC of rabbits fed the normal diet was 77 mg/dL. Most of this increase was observed with the atherogenic particle LDL-C (510 mg/dL for hypercholesterolemic vs. 58 mg/dL for normal rabbits) (Fig. 1B). In particular, spirulina 5% supplementation significantly reduced serum TC and LDL-C level from the second week of the experimental period, compared with HCD rabbits ($p<0.05$). After 8 wk, the serum TC level decreased by 29.4% in the SP5 group (from 1.185 mg/dL in hyper-

### Table 2. Effect of spirulina on the organ weight (g) of hypercholesterolemic rabbits after 8 wk of treatment.

<table>
<thead>
<tr>
<th>Organ weight</th>
<th>Liver</th>
<th>Spleen</th>
<th>Kidney</th>
<th>Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>85.4±30.4a</td>
<td>1.6±0.8aNS</td>
<td>6.8±1.7aNS</td>
<td>6.3±1.8aNS</td>
</tr>
<tr>
<td>HCD</td>
<td>97.0±5.0a</td>
<td>1.4±0.2</td>
<td>6.9±1.3</td>
<td>5.7±0.6</td>
</tr>
<tr>
<td>SP1</td>
<td>95.4±13.1a</td>
<td>1.0±0.5</td>
<td>5.9±1.5</td>
<td>6.0±2.0</td>
</tr>
<tr>
<td>SP5</td>
<td>95.8±9.8a</td>
<td>1.4±0.4</td>
<td>7.0±0.7</td>
<td>5.5±0.3</td>
</tr>
</tbody>
</table>

Control: Control group ($n=5$), HCD: High cholesterol diet group ($n=5$), SP1: High cholesterol diet plus 1% spirulina supplementation group ($n=5$), SP5: High cholesterol diet plus 5% spirulina supplementation group ($n=5$).

Values are means±SD.

Means in the same column with different superscripts differ ($p<0.05$).

N.S.: Not significant.
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cholesterolemic rabbits to 837 mg/dL in 5% spirulina-treated rabbits), whereas 1% spirulina supplement decreased TC by 19.6% (from 1,185 mg/dL in hypercholesterolemic rabbits to 953 mg/dL in 1% spirulina-treated rabbits). In addition, the serum LDL-C level was significantly reduced by 41.2% in the 5% spirulina supplement group (from 599 mg/dL in hypercholesterolemic rabbits to 352 mg/dL in 5% spirulina-treated rabbits), whereas 1% spirulina supplement decreased LDL-C by 26.4% (from 599 mg/dL in hypercholesterolemic rabbits to 441 mg/dL in 1% spirulina-treated rabbits) after 8 wk. On the other hand, serum HDL-C was remarkably increased after feeding on 1% and 5% spirulina compared with that in the HCD group from 2 wk to 8 wk (Fig. 1C). In particular, the ratio of TC to HDL-C in serum was reduced by 40% (from 59.3 in hypercholesterolemic rabbits to 43.3 in 1% spirulina-treated rabbits) and 39% (from 59.3 in hypercholesterolemic rabbits to 36.4 in 5% spirulina-treated rabbits) in the SP1 and SP5 groups, respectively, compared with the HCD group after 8 wk (data not shown). In particular, spirulina 5% supplementation significantly decreased serum TC and LDL-C, but increased HDL-C level after 2 wk of the experimental periods. Eight weeks following the intake of the high-cholesterol diet, the concentration of serum TG was decreased by 31.3% (from 48 mg/dL in hypercholesterolemic rabbits to 33 mg/dL in 1% spirulina-treated rabbits) and 52.1% (from 48 mg/dL in hypercholesterolemic rabbits to 23 mg/dL in 5% spirulina-treated rabbits) compared to the HCD group (p<0.05) (Fig. 1D).

**DISCUSSION**

Atherosclerosis is a chronic inflammatory disease that is initiated by a series of highly specific cellular and molecular events. The pathogenesis of atherosclerosis involves the interaction of multiple factors, including inflammation, lipid accumulation, and endothelial dysfunction. In this study, we investigated the effects of spirulina supplementation on atherosclerosis in hypercholesterolemic rabbits. Our results show that spirulina supplementation significantly reduced the levels of TC, LDL-C, and LDL-C, while increasing HDL-C levels. These findings are consistent with previous studies that have demonstrated the anti-atherosclerotic effects of spirulina in animal models.

Histopathological appearance of the aorta has been used as one criterion to determine the degree of atherosclerosis. Figure 2A shows a representative appearance of the aorta from the normal control group based on the Sudan IV staining. As illustrated in Fig. 2B, all rabbits that received the high-cholesterol diet for 8 wk developed atherosclerotic lesions that appeared as marked alterations in the aortic wall. Meanwhile, 1% and 5% spirulina supplementation of the hypercholesterolemic diet for 8 wk remarkably improved the atheromatous lesions induced by hypercholesterolemia (Fig. 2C, 2D). The atherosclerotic lesion area (Sudan IV-positive area) in the SP1 and the SP5 group were significantly lower than that in the HCD group (38.8% and 29.5% vs. 57.2%) (p<0.05). These results indicate that the aortic lipid accumulation area (atherosclerotic plaque) was smaller in spirulina-fed rabbits than in HCD-fed rabbits (Fig. 3). In order to obtain more information on lipid accumulation in the liver as a result of the induction of hypercholesterolemia and the beneficial effect of dietary spirulina on lipid accumulation, a histological examination of the liver tissue was carried out (Fig. 4). Liver sections from normal control rabbits showed normal liver architecture, i.e., well-preserved hepatic cords, cytoplasm and prominent nucleus (Fig. 4A). In contrast, liver sections from most rabbits fed the cholesterol-enriched diet showed micro- and macro-cellular fatty changes (Fig. 4B). It is noteworthy that 1% and 5% spirulina supplementation significantly prevented the fatty change of the liver tissue induced by the high-cholesterol diet (Fig. 4C, 4D).

**Fig. 2.** Representative examination of atheroma in rabbits fed a high-cholesterol diet alone or with spirulina. (A) Control group (n=5), (B) HCD: High cholesterol diet group (n=5), (C) SP1: High cholesterol diet plus 1% spirulina supplementation group (n=5), (D) SP5: High cholesterol diet plus 5% spirulina supplementation group (n=5).

**Fig. 3.** Effect of spirulina on the atheroma area (%) of hypercholesterolemic rabbits (n=5, per group). Values are means±SD in bars. Means with different superscripts differ (p<0.05).
molecular responses of the vascular endothelium to atherogenic factors (24, 25). In particular, in the initial event in the process of atherogenesis, high levels of lipid peroxides are supposed to injure blood vessels, causing increased adherence and aggregation of platelets at the injured sites (25, 26). Several studies in both animals and humans have demonstrated that prolonged high cholesterol concentrations in the circulating blood are positively correlated with atherosclerosis development. In general, supplementation of the diet with cholesterol rapidly results in a marked increase in the production by the liver and the intestine of \( \beta \)-migrating very low density lipoproteins (\( \beta \)-VLDL), rich in cholesteryl ester. In turn, this change in plasma lipoproteins lead to the development of advanced atherosclerotic lesions (27, 28). Currently the high cost of medicines is a limitation for pharmacological therapy adhesion. Therefore, alternative strategies for atherosclerosis prevention, such as dietary therapy, have received considerable attention in the scientific community.

In this study, we have demonstrated for the first time the atherosclerosis protective effect of dietary spirulina supplementation as a whole in a hypercholesterolemic rabbit model. In general, the rabbit model used in the present study has been used extensively for the study of atherosclerosis as it is sensitive to induction of atheromatous lesions and hypercholesterolemia. Rabbits do not naturally develop atherosclerosis. Moreover, the aortic lesions in rabbits do not develop into complicated plaques, so they are very similar to human fatty streaks, which makes it a useful model for antiatherogenic studies (29).

Our present study demonstrated that in rabbits fed a high-cholesterol diet for 4 wk, serum TC and LDL-C levels increased by 20.6 and 8.83 times, respectively, compared to those in normal rabbits. As shown in Table 1, our spirulina powder in the high cholesterol diet significantly reduced the levels of TC, TG, and LDL-C in blood as well as the area of atherosclerotic lesions in high cholesterol diet-fed rabbits. The mechanism by which the spirulina reduces hypercholesterolemia and atheromatous lesion formation has not been examined in detail. However, this might be consistent with previous findings that supplementation with spirulina decreased LDL-C and increased HDL-C with a probable beneficial effect on atherothrombotic indices in humans (30).

In addition, serum and liver cholesterol levels were significantly lower in rats fed spirulina with a high-cholesterol diet, compared to those fed casein, and these hypocholesterolemic actions of spirulina were suggested to involve the inhibition of both jejunal cholesterol absorption and ileal bile acid resorption, thus attenuating hypercholesterolemia (7). Nagaoka et al. (7) reported that a novel protein C-phycocyanin containing a large amount of cystine derived from spirulina can powerfully influence serum cholesterol concentrations and impart a stronger hypocholesterolemic activity. Sugiyama et al. (31) reported significantly negative correlation between the blood cholesterol concentrations and the level of cystine in dietary protein. Moreover a few previous studies suggested that the amounts of carbohydrates and dietary fiber in spirulina induce a hypocholesterolemic action mediated by increases in the size of the pool of bile acids and fecal steroid excretion (32, 33).

On the other hand, several previous studies indicated that spirulina contained antioxidants such as \( \beta \)-carotene, vitamin C, vitamin E, SOD, and selenium, as well
as polypeptide pigment phycocyanin (34, 35), some of which might contribute to antiatherosclerosis action together with various other micronutrients in spirulina. Phycocyanin, an antioxidant biliprotein pigment, is similar in chemical structure to bilirubin, acts as a potent free radical scavenger (hydroxyl and peroxyl radicals) and inhibits microsomal lipid peroxidation (36, 37). Moreover, phycocyanin in spirulina was reported to prevent early development of atherosclerosis, characterized by lipid deposition on the aorta and formation of foam cells (38), which might be engendered by accumulation of oxidized LDL in monocyte-derived macrophages. These previous studies could be supported by the effect of spirulina mediating several steps in the inflammation process that finally reduces atherothrombotic plaque formation.

Our present findings with rabbits may also provide further support for antiatherosclerosis action of spirulina, which at doses used here expressed a remarkable anti-atherosclerotic action by decreasing the levels of serum TG, LDL-C, and TC, while increasing serum HDL-C level, and by reducing the area of atherosclerosis lesions.

Although the mechanism of the antihypercholesterolemic and anti-atherosclerotic effect is not clear, the anti-atherosclerotic effect as evidenced by a decrease in lipid biomarkers of atherosclerosis may also contribute to the protective effect of spirulina, which is a rich source of vitamins, minerals, carotenoids, dietary fiber and phycocyanins. These results thus suggest that spirulina may be regarded as a functional food able to reduce blood cholesterol levels and to prevent the progression of atherosclerosis.

Acknowledgments

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