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DECREASE OF PLASMA CHOLESTEROL WITH THE MARINE MICROALGA *DUNALIELLA TERTIOLECTA* IN HYPERCHOLESTEROLEMIC RATS

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Female Sprague-Dawley rats, whose plasma cholesterol level had been previously increased, were fed on a diet including 22% biomass of the marine microalga *Dunaliella tertiolecta.* This diet was compared to two control diets, one of casein and the other of soy flour. After 14 days of feeding, the group fed the microalgal diet exhibited the largest decrease in the plasma cholesterol concentration. The plasma cholesterol level in the group fed the microalgal diet was 45.2% of that in the control group (casein diet) and 28.4% lower than the group fed the soy flour diet. These results suggest that the marine microalga *Dunaliella tertiolecta* has marked anti-hypercholesterolemic activity when incorporated into the diet.

High levels of cholesterol in the blood are a known risk factor for the development of atherosclerosis. High levels of cholesterol in plasma can accelerate the development of arterial disease by increasing the amount of circulating lipids available for incorporation into the arterial walls (*29*). Diets rich in polyunsaturated fatty acids reportedly reduce serum lipids by enhancing their excretion, increase membrane fluidity and reduce thrombosis by conversion of the lipids to eicosanoids (*9,14,15*).

Lipid-lowering diets have mainly been based on an increase of linoleic acid (18 : 2 n-6) content, but the metabolites of linoleic acid (*gamma*-linolenic acid, 18 : 3 n-6, and arachidonic acid, 20 : 4 n-6) have a much greater anti-hyper cholesterolemic activity than the parent molecule (*16,27*).

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On the other hand, polyunsaturated fatty acids of n-3 series, whose useful sources are fish and fish oils, may also have beneficial effects regarding ischemic heart disease and thrombosis (*17*); fish oils have been shown to reduce serum lipids levels in humans (*12,22*) and in the rat (*13*).

Plasma cholesterol can also be reduced with soy protein. Animal studies have shown that soy protein has an anti-hyper cholesterolemic effect in rats, rabbits, swine and primates, compared to casein diets (*8*). Significant decreases in plasma total cholesterol levels have been also observed in hypercholesterolemic subjects when soy protein is included in diet.

The studies made with microalgae to establish their anti-hyper cholesterolemic effect are rather limited. Lower cholesterol levels have been observed in rats fed with diets containing *Scenedesmus* (freshwater microalga) compared with control animals (*2,24*). Studies of marine microalgae have shown the effect of *Dunaliella tertiolecta* compared to a casein control diet. *D. tertiolecta* decreased significantly plasma cholesterol, triglycerides and creatine phosphokinase levels in rats (*7*). In this work, we analyzed the anti-hyper cholesterolemic effect of *D. tertiolecta* flour when introduced in diets of rats, whose level of plasma cholesterol has been increased.

MATERIALS AND METHODS

Culture. The marine microalga *Dunaliella tertiolecta* (Chlorophyceae) was grown in glass containers of 10 *l*, in sea water with 3.5% of salinity and nutrients, at a temperature of $19 \pm 1°C$ and an illumination of $115\,\mu mol\,photon\,m^{-2}\,s^{-1}$ produced by fluorescent lamps and applied with a light-darkness 12 h : 12 h regime (*6*). The cultures were kept aired with a flux of 15 *l*/min to maintain the pH below 8.0.

The cells were harvested by centrifugation at the end of the logarithmic phase, and immediately dehydrated by freeze-drying. The biomass obtained was triturated and homogenized, resulting in fine flour of microalgae, which was kept at $-20°C$ until use.

Biochemical analysis. Proteins were determined by the Kjeldahl method; the analysis of crude fat was made by the Soxlhet extraction and fiber was determined following a method of acid treatment of the sample (*1*). Amino acids analysis was made by acid hydrolysis and subsequent determination by HPLC (*20*).

Animals and diets. The animals used in the trials were female Sprague-Dawley rats with an initial average weight of 152 ± 4 g. They were housed individually, in a room at $22 \pm 2°C$ with alternating 12-h periods of light and darkness. The rats were allowed free access to experimental diets and water. Body weight, food intake and water consumption were recorded every day.

The experiments were carried out in two periods: hypercholesterolemic period and principal period, with a duration of 14 days each. During the first period, hypercholesterolemia was induced in the rats by means of a basal diet enriched

Table 1. Composition of the diets used in the feeding trials, expressed as grams of component per kilogram of diet.

Component	Cholesterol-enriched diet	Casein diet	Soy flour diet	*D. tertiolecta* diet
Casein	151.6	151.6	—	—
Soy flour	—	—	290.2	82.8
Dunaliella tertiolecta	—	—	—	220.8
Methionine	2.0	2.0	2.0	3.5
Olive oil	97.6	97.6	94.7	72.6
α-Cellulose	50.0	50.0	15.2	38.0
Mineral mixture[a]	35.0	35.0	35.0	—
Vitamin mixture[a]	10.0	10.0	10.0	—
Choline hydrochloride	2.0	2.0	2.0	2.0
Cholesterol	10.0	—	—	—
Sodium cholate	2.5	—	—	—
Starch	150.0	150.0	150.0	150.0
Sucrose	489.3	501.8	400.9	430.3

[a] Minerals and vitamins were incorporated according to prepared mixtures (*7*).
Protein, fiber and fat contents were 14, 5 and 10%, respectively.

with cholesterol and a bile salt (Table 1). Then the rats were divided into three groups of 10 animals each and fed on the experimental diets free of cholesterol and the bile salt. Casein (92.3% of protein), soy flour (48.3% of protein) and the freeze-dryed microalgal biomass (47.15% of protein) were the sole sources of protein in each of these three diets, at a protein level of 14%. Diets were formulated using a linear calculation program operated on a Hewlett Packard HP 9817 computer, taking into account the complete biochemical composition of each of the sources used. All diets needed a supplement of methionine. All diets were adjusted to 5% of crude fiber and 10% of oil by appropriate additions of cellulose and olive oil. Minerals and vitamins were incorporated into the diet according to prepared mixtures (*7*), except in the *D. tertiolecta* diet, since microalgal biomass for the protein supply was sufficient for the vitamin and mineral requirements of rats (*4,5*). The composition of all diets is shown in Table 1.

Lipids analysis. The diets prepared were analyzed for their composition of fatty acids by GC-MS; lipids were previously extracted in methanol and dichloromethane-methanol (*28*).

At the end of the feeding periods, blood was extracted from the rats by cardiac puncture using lithioheparinate as an anticoagulant. They were previously anaesthetized with sodium pentobarbital (40 mg/kg body weight). Plasma was separated by centrifugation at $2{,}600 \times g$ for 25 min. Plasma lipids were analyzed for cholesterol, triglycerides and phospholipids with an automatic analyzer HITACHI 747.

Statistical analysis. Data were analyzed by the non-parametric test of Mann-Whitney-Wilcoxon to inspect all differences between pairs of means.

RESULTS

The amino acid profile of microalgal protein, casein and soy flour and the fatty acid composition of the experimental diets are shown in Tables 2 and 3.

Feeding with the diet rich in cholesterol for 14 days produced significant

Table 2. Amino acid profile casein, soy flour and *Dunaliella tertiolecta*, expressed as grams per 16 grams of nitrogen.

	Casein (*10*)	Soy flour (*21*)	*D. tertiolecta*
Ile	6.10	4.48	3.12
Leu	9.20	7.84	9.82
Lys	8.20	6.40	5.20
Met	2.80	1.28	2.37
Phe	5.00	4.96	5.40
Thr	4.90	3.84	5.82
Val	7.20	4.80	4.90
Trp	—	1.28	—
Tyr	6.30	3.20	3.25
Ala	3.20	4.32	9.87
Arg	4.10	7.20	7.25
Asp	7.10	11.68	9.95
Cys	0.30	1.60	—
Glu	22.40	18.72	12.95
Gly	2.00	4.16	7.30
His	3.10	2.56	1.40
Pro	10.60	5.44	—
Ser	6.30	5.12	6.45
Asn	—	—	0.67

Table 3. Fatty acid composition of the diets used in the nutrition trials, expressed as a percentage of total fatty acids.

Fatty acid	Casein diet	Soy flour diet	*D. tertiolecta* diet
Saturated			
16:0	11.6	11.7	16.8
18:0	—	—	—
20:0	0.2	0.2	—
Monounsaturated			
16:1			
18:1	81.7	80.9	66.3
20:1	0.2		
Polyunsaturated			
16:3 (n-3)	—	—	1.8
18:2 (n-6)	5.6	6.3	8.2
α18:3 (n-3)	0.5	0.6	6.8
P/S ratio	0.5	0.6	1.0
n-3/n-6 ratio	0.09	0.10	1.05

Table 4. Values (mean $\pm$SE) of plasma cholesterol, phospholipids and triglycerides in rats fed the different experimental diets.

	Initial value	Cholesterol-enriched diet	Casein diet	Soy flour diet	*D. teriolecta* diet
Cholesterol (mg/dl)	77 ± 6^{e}	539 ± 42^{a}	190 ± 15^{b}	145 ± 11^{c}	104 ± 4^{d}
Phospholipids (mg/dl)	$138\pm10^{b,c}$	287 ± 28^{a}	165 ± 4^{b}	141 ± 8^{c}	138 ± 6^{c}
Triglycerides (mg/dl)	74 ± 4^{a}	97 ± 11^{a}	55 ± 4^{b}	49 ± 3^{b}	99 ± 10^{a}

For each parameter, the same letter indicates no significant differences, $p<0.05$.

increases in plasma cholesterol and phospholipid levels (Table 4). The level of triglycerides did not show significant variations.

The average weight of the rats at the end of the hypercholesterolemic period was 194 ± 8 g. During the 14 days of the principal period, body weight gain was significantly higher in rats fed on soy flour diet (37 ± 8 g) than in those fed on casein and microalgal diets (28 ± 7 and 25 ± 9 g, respectively).

After other 14 days of feeding with casein, soy flour and microalgal diets, significant decreases were observed in the plasma cholesterol level in the three groups of rats with regard to the hypercholesterolemic level; but the values continued to be higher than the initial ones (Table 4). The final concentration of plasma cholesterol was significantly lower in the *D. tertiolecta* group (104 ± 14 mg/dl) than in the control groups of casein (190 ± 47 mg/dl) and soy flour (145 ± 35 mg/dl).

Phospholipids also decreased significantly with the three diets during the principal period, returning to the initial values in all groups (Table 4). Even so, the final level of phospholipids in the plasma was significantly higher in the rats fed on casein (165 ± 14 mg/dl) than in those fed on soy flour (141 ± 24 mg/dl) or microalgal biomass (138 ± 20 mg/dl).

The final concentration of plasma triglycerides was similar in the rats fed with casein and soy flour diets; but these values were significantly lower than the initial ones (Table 4). The group fed on the *D. tertiolecta* diet had a triglyceride level significantly higher than the other two groups, but similar to the initial value.

DISCUSSION

The plasma cholesterol level of hypercholesterolemic rats decreased in a 80.7% when the biomass of the marine microalga *D. tertiolecta* was incorporated into their nutrition, and this decrease was higher than that of control diets of casein (64.8%) and soy flour (73.1%).

The anti-hyper cholesterolemic effect of the soy flour diet, with respect to the casein control diet, was 23.5%. When the biomass of *D. tertiolecta* was included in the diet, the decrease in plasma cholesterol was 45.2% with regard to casein control and 28.4% with respect to soy flour control. These differences could be due to two reasons: the different contents of amino acids of dietary proteins, and the composition of fatty acids.

Different dietary proteins exert different effects on plasma cholesterol levels. Animal proteins, mainly casein, increase plasma total cholesterol levels compared to vegetable proteins such as soy (*3,8*).

With regard to this anti-hyper cholesterolemic effect of vegetable proteins, the relation between lysine and arginine is important. This ratio has a significant effect on cholesterol metabolism in rats (*18,23*). A lysine/arginine ratio of 1.0 decreases serum, liver and aorta cholesterol significantly, and increases the degradation of cholesterol to bile acids compared to a lysine/arginine ratio of 2.0 (*23*).

The protein of *D. tertiolecta* presented a lysine/arginine ratio of 0.72; the soy protein value was 0.98 and that of casein was 2.0. The smaller the lysine/arginine ratio was, the greater the reduction in plasma cholesterol.

With respect to the fatty acid composition of the diets, the microalgal diet had a higher percentage of polyunsaturated fatty acids than the casein and soy flour diets, with a very high content of α-linolenic acid. The polyunsaturated-saturated (P/S) fatty acid ratio was 1.0 in the microalgal diet, while the values in casein and soy flour diets were 0.5 and 0.6, respectively.

The P/S ratio of a diet is important regarding plasma lipids metabolism. Diets with low fat content and high P/S ratios ($\geq$1.0) produce significant decreases in plasma cholesterol, in contrast to what happens with P/S ratios lower than 1.0 (*19, 26*).

Moreover, the microalgal diet had a greater proportion of polyunsaturated fatty acids of the n-3 series compared to casein and soy control diets, in which these fatty acids were practically non existent. The presence of n-3 polyunsaturated fatty acids also reduces plasma lipids (*11,25*).

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