

intestinal absorption of redundant lipid from our daily intake and subsequently to prevent hyperlipidemia as well as atherosclerosis.

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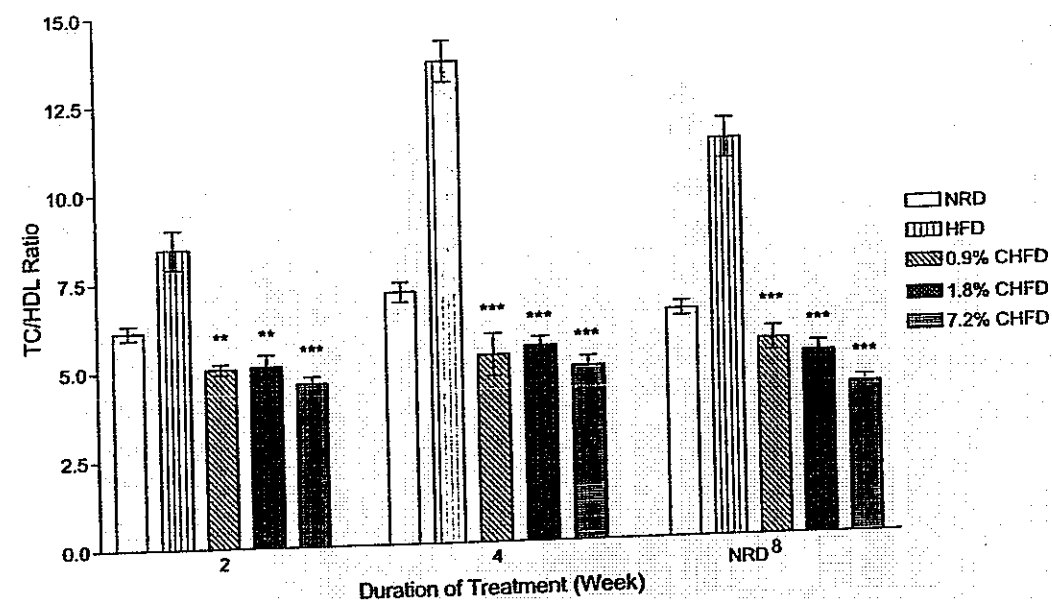


Fig. 10. Fasting serum total cholesterol/HDL ratio in chronic high fat with or without *Chlorella pyrenoidosa* treated hamster. Data expressed as means \pm SEM, $n = 8$ hamsters in each group. ** $p < 0.01$, *** $p < 0.005$ as compared to their relative HFD group.

produce a higher HDL cholesterol levels. There were some further increases of HDL levels in the 0.9%, 1.8%, and 7.2% CHFD groups. Importantly, total cholesterol/HDL ratios were increased in the HFD group (Fig. 10). However, these ratios were significantly suppressed in all CHFD hamsters and higher *Chlorella* content in diet or longer treatments further gradually decreased in total cholesterol/HDL ratios showing the benefit effect of *Chlorella* ($p < 0.005$, t-test).

Effects of *Chlorella pyrenoidosa* on body weight changes in high fat diet-treated rats and hamsters

The body weight of experimental rats and hamsters taking HFD and various CHFD were not statistically different to the NRD groups. Also no changes in food intake for HFD, CHFD groups comparing to NRD rats or hamsters (ANOVA test, data not shown).

Discussion

We showed the ameliorative effects on serum lipid profiles provided by the chronic *Chlorella pyrenoidosa* administration regarding to elevated fasting TG, total cholesterol, and LDL levels in HFD-treated rats and hamsters. These observations are consistent with early finding of others (Sano and Tanaka, 1987; Sano et al., 1988; Okudo et al., 1975). This is the first time to use hamster as an animal model in investigating the effects of *Chlorella pyrenoidosa* on serum lipid profiles since hamsters had similar lipoprotein and bile acid metabolism patterns as that in human (Spady et al., 1986a,b). Therefore, it is rational to use different animals to reconfirm the effects of chronic *Chlorella*

pyrenoidosa administration in lowering serum lipid levels. In two different species, we conclude chronic *Chlorella pyrenoidosa* intake is capable of preventing elevated blood lipids in high-fat diet intake.

High proportion of daily energy derived from fat component is a common situation in current living style in most of societies of the world. High prevalence of vascular diseases and other chronic diseases, e.g. hypertension, diabetes are likely related with abnormal blood lipid profiles that may be due long term of high fat intake. In this study, we showed the effects of lowering blood lipids of *Chlorella pyrenoidosa* could be due to reduce the absorption of fat in the intestinal tract as that shown in *Chlorella vulgaris* by Sano et al. (1988). Another possible mechanism of *Chlorella* was also shown by Shibata et al. (2001). They showed that *Chlorella* administration increased the total amount of fecal neutral steroids excreted and subsequently lowered liver cholesterol contents. As the result, peripheral cholesterol may be transferred back to the liver in response to the reduction. Kay (1991) proposed that the possible active ingredients of *Chlorella* in reducing blood lipid levels could be water-soluble fibers, vegetable protein, phospholipids, Vitamin C, Vitamin E and beta-carotene. Kritchevsky et al. (1982) also showed that protein from animal origin could be more cholesterolemic and atherogenic than plant protein. Since lysine content of a protein may be a determinant of its atherogenicity (Weigensberg et al., 1964). Moore et al. (1977) found a significant correlation between the lysine content of the diet and the trend towards increased severity of coronary heart disease CHD. These suggest that low lysine content of plant protein is advantageous to lower serum cholesterol. In fact, the arginine/lysine ratio in *Chlorella* proteins is approx. 1.08 comparing to that ratio in soy bean (approx 1.13) (Kritchevsky et al. (1982). This was supported by others, which replaced animal proteins by soy protein in patients' diet and showed a significant reduction of blood cholesterol levels (Carroll and Kurowska, 1995). Besides, patients with total cholesterol values in the desirable range (less than 200 mg/dL) may be at high risk of CHD if they have low HDL levels (below 35 mg/dL). Administration of *Chlorella pyrenoidosa* provides the "beneficial effect" in hamster (Fig. 9) regarding to the decrease in total cholesterol/HDL ratio. Although the effect of *Chlorella pyrenoidosa* on HDL showed marginal increase in 7.2% CHFD grouped rats (Fig. 4), the total cholesterol/HDL ratio decreased significantly (Fig. 5) in all CHFD treated rats. In human, a total cholesterol/HDL ratio of less than 3.5 indicates a patient at low CHD risk, while a ratio of 5 places a patient at average risk. Coronary heart disease risk continuously increases with higher total cholesterol/HDL ratios. This might explain that *Chlorella pyrenoidosa* was proven to show a reduction of aortic atheromatous area in high cholesterol fed rabbits (Sano and Tanaka, 1987).

In our data showed that high-fat diet did not induce any significant body weight change compared to NRD-fed animals. Several authors have recently shown that high fat diet do not always induce body weight gain in animals (Bullen et al., 2004; Jaber, 2004; Kovacs et al., 2003). Possible explanations for this phenomenon could be due to diet-induced thermogenesis, which plays an important role in energy homeostasis and maintain a constant body weight range. To support this, some authors found that high-fat diet induced uncoupling protein expression in adipose tissues (Jaber, 2004). Uncoupling proteins are known to play an important role in thermogenesis. Others found that high fat diet induces fatty acid oxidation in skeletal muscle by increasing the expression of the β -oxidation pathway enzyme β -hydroxyacyl-CoA dehydrogenase (β -HAD; EC 1.1.1.211) (Cameron-Smith et al., 2003; Schrauwen et al., 2001).

In conclusion, we studied the effect of *Chlorella pyrenoidosa* on blood lipids and showed it has remarkable action on lowering TG, total cholesterol, LDL but raising serum HDL level resulting a low total cholesterol/HDL ratio. Therefore, *Chlorella pyrenoidosa* could be potential in use to prevent

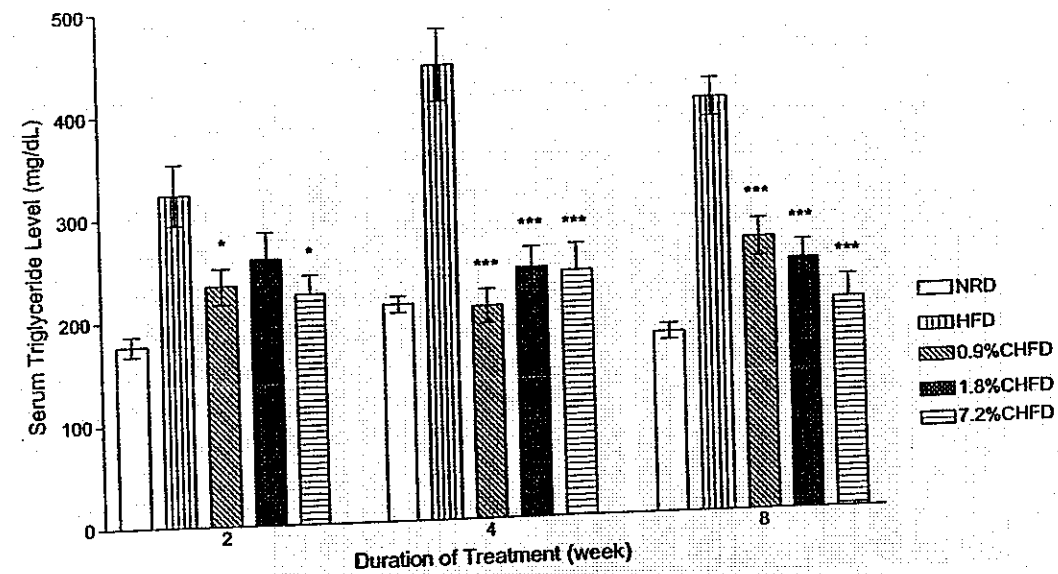


Fig. 6. Fasting serum triglyceride levels (mg/dL) in chronic high fat with or without *Chlorella pyrenoidosa* treated hamster. Data expressed as means \pm SEM, n = 8 hamsters in each group. * p < 0.05, *** p < 0.005 as compared to their relative HFD group.

Interestingly, in contrast to rats, HDL levels in the HFD group were remarkable higher (Fig. 9) than that in the NRD group (22.9 ± 0.91 mg/dL, p < 0.005) throughout the experimental duration. This data is in an agreement with the findings of Glueck et al. (1982) showing that high cholesterol in diet tended to

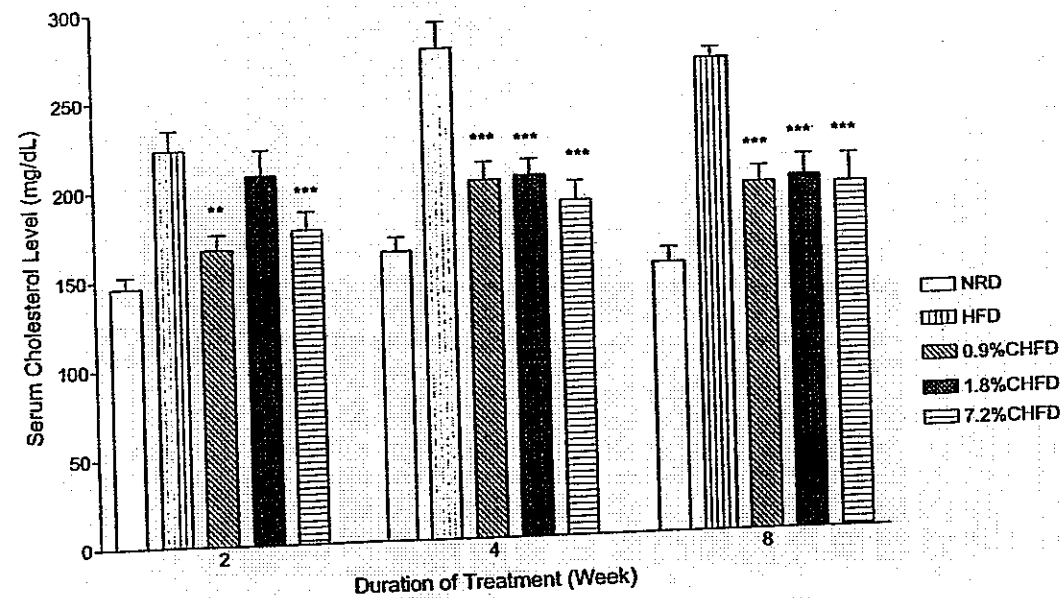


Fig. 7. Fasting serum total cholesterol levels (mg/dL) in chronic high fat with or without *Chlorella pyrenoidosa* treated hamster. Data expressed as means \pm SEM, n = 8 hamsters in each group. ** p < 0.01, *** p < 0.005 as compared to their relative HFD group.

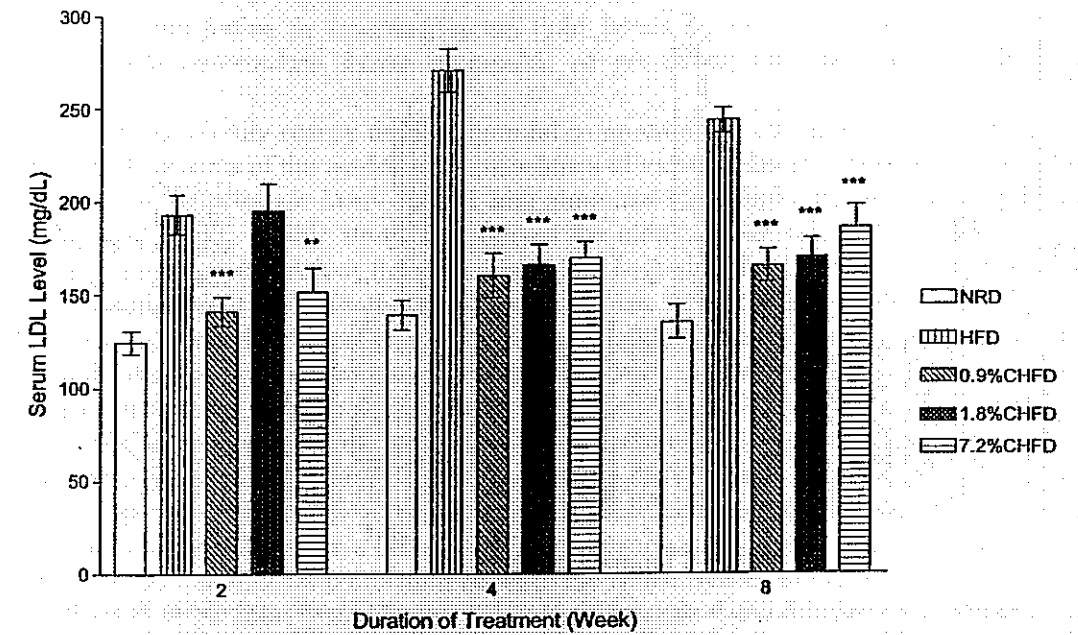


Fig. 8. Fasting serum LDL levels (mg/dL) in chronic high fat with or without *Chlorella pyrenoidosa* treated hamster. Data expressed as means \pm SEM, n = 8 hamsters in each group. ** p < 0.01, *** p < 0.005 as compared to their relative HFD group.

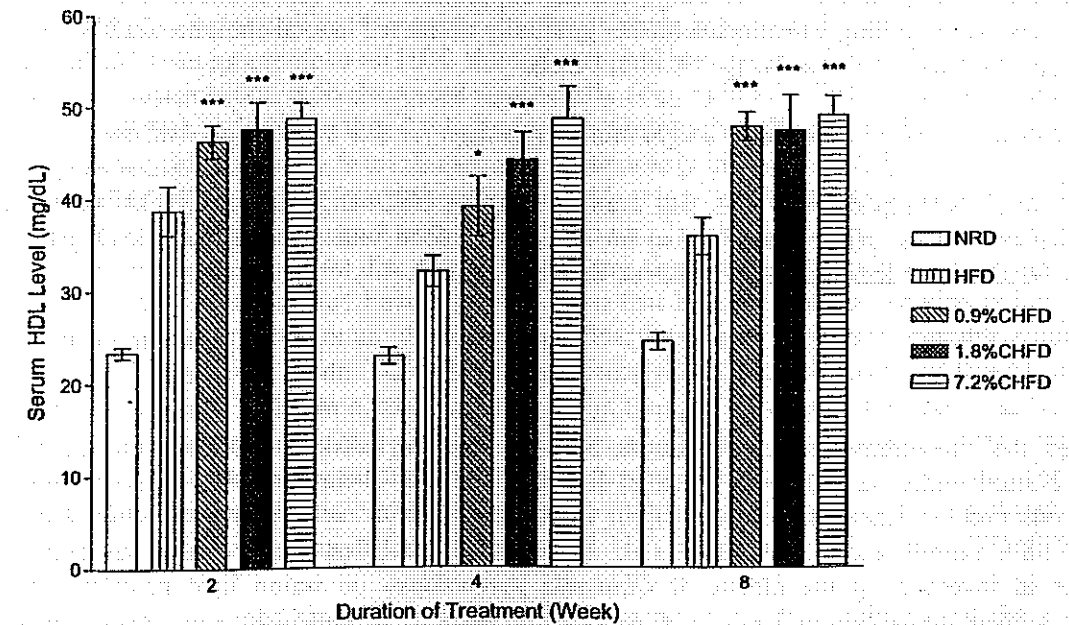


Fig. 9. Fasting serum HDL levels (mg/dL) in chronic high fat with or without *Chlorella pyrenoidosa* treated hamster. Data expressed as means \pm SEM, n = 8 hamsters in each group. * p < 0.05, *** p < 0.005 as compared to their relative HFD group.

1.8% CHFD) for longer treatment (8 week). High *Chlorella* content in diet (7.2% CHFD) gives earlier onset of the action on lowering total cholesterol level from 2 weeks. The change of LDL cholesterol level was similar to the changing pattern of total cholesterol level in rats (Fig. 3). *Chlorella pyrenoidosa* significantly prevented the increases of LDL level in 0.9%, 7.2% CHFD groups after 2, 4 and 8-week treatment schedules. After longer treatment (8 weeks), the LDL lowering effects of *Chlorella pyrenoidosa* in the 1.8% CHFD group was also observed ($P < 0.005$, t-test).

Neither HFD-treated nor all CHFD diets significantly affected HDL levels in rats throughout the whole period of treatments (Fig. 4). Measuring HDL cholesterol is important since the ratio of total cholesterol to HDL cholesterol (total cholesterol/HDL) is a predictive indication of coronary heart disease (CHD) instead of the total cholesterol value only (NECP, 1993). The higher ratio indicates higher risk of occurrence of coronary heart disease. Whereas the ratios were significantly increased in HFD-treated rats compared with NRD group, the elevated ratios returned to near basal levels in 0.9%, 1.8%, and 7.2% CHFD groups ($p < 0.005$, Fig. 5).

Effects of *Chlorella pyrenoidosa* on serum lipids profile in Syrian hamsters

In experimental hamsters, an increase of fasting serum TG levels (from 174.7 ± 7.93 mg/dL of NRD controls constantly, $p < 0.005$) after 2, 4, and 8-week chronic HFD treatment were also found as that observed in rats (Fig. 6). The TG levels were significantly lower in 0.9%, 1.8% and 7.2% CHFD groups. Total cholesterol and LDL levels in the HFD group were higher than that in NRD groups (151.6 ± 8.10 mg/dL and 124.7 ± 6.03 mg/dL, respectively) after 2, 4, and 8-week treatments. Again, the administration of *Chlorella* significantly suppressed the elevated total cholesterol and LDL levels in 0.9%, 1.8%, and 7.2% CHFD groups from 4 weeks thereafter (Figs. 7 and 8) ($p < 0.005$, t-test).

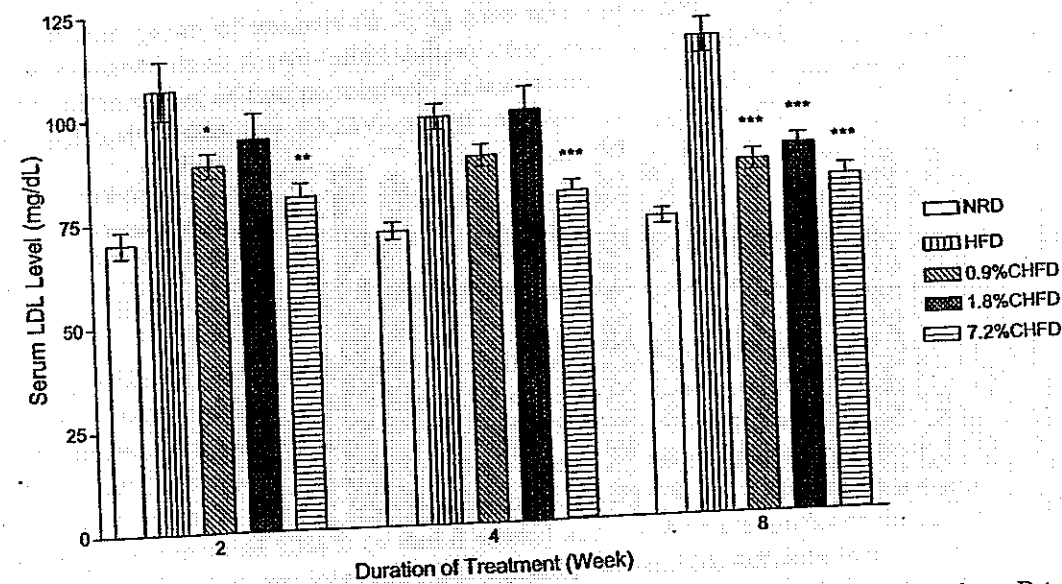


Fig. 3. Fasting serum LDL levels (mg/dL) in chronic high fat with or without *Chlorella pyrenoidosa* treated rats. Data expressed as means \pm SEM, $n = 8$ rats in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ as compared to their relative HFD group.

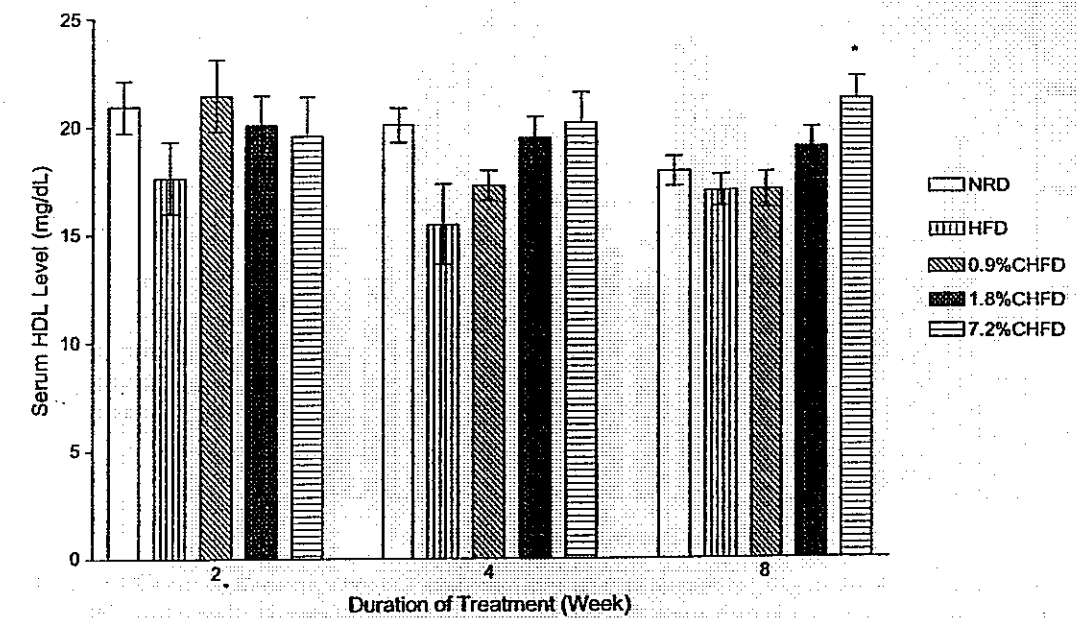


Fig. 4. Fasting serum HDL levels (mg/dL) in chronic high fat with or without *Chlorella pyrenoidosa* treated rats. The values in NRD groups were not statistically different to HFD rats. Data expressed as means \pm SEM, $n = 8$ rats in each group. * $p < 0.05$ as compared to the HFD group.

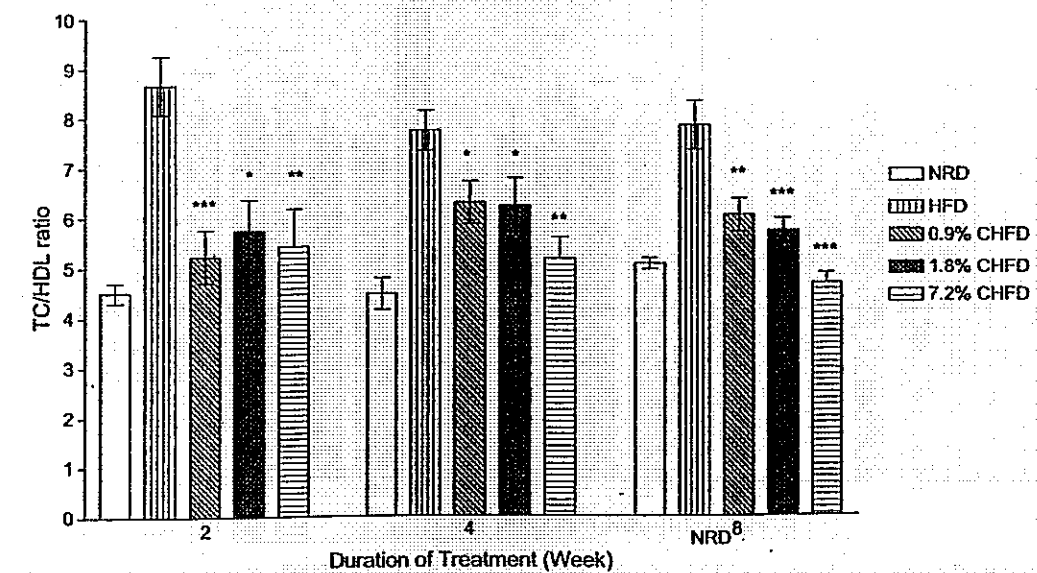


Fig. 5. Fasting serum total cholesterol/HDL ratio in chronic high fat with or without *Chlorella pyrenoidosa* treated rats. Data expressed as means \pm SEM, $n = 8$ rats in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ as compared to their relative HFD group.

Therefore, 0.9%, 1.8%, and 7.2% of supplemental amounts of *Chlorella* were chosen in this study. Duration of various diet treatments lasted for 2, 4, or 8 weeks. All animals were provided ad libitum access to tap water. Their housing was maintained at a temperature of 20–22 °C, relative humidity of 50–80%, and a cycle of 12 hours light/dark (7.00hr to 19.00 hr with no twilight). All animals were anaesthetized briefly prior to be killed following the Guide for the Care and Use of Laboratory Animals.

Serum lipid measurements

After 2, 4 or 8 weeks of chronic administration of respected diet, animals ($n = 4$ from each group) were fasted 12–15 hours prior to experiments. Serum samples were collected from cervical veins for following serum lipid assays (in duplicate): (a) *Total triglycerides* were determined after enzymatic hydrolysis with lipases. The formed quinoneimine as indicator was produced from hydrogen peroxide, 4-aminophenazone, and 4-chlorophenol under the catalytic influence of peroxidase. (b) *Total cholesterol* was determined after enzymatic hydrolysis and oxidation. Measuring the indicator quinoneimine was formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase. (c) *Low density lipoprotein (LDL) cholesterol level*: LDL was precipitated by the heparin at its isoelectric point (pH 5.04). After centrifugation, the HDL and very low density lipoproteins remained in the supernatant. The cholesterol contents in the supernatant were measured as total cholesterol and subtracted by total cholesterol to obtain LDL cholesterol levels. (d) *High density lipoprotein (HDL) cholesterol level*: LDL and very low density lipoprotein and chylomicron fractions were precipitated by the addition of phosphotungstic acid in the presence of Mg^{++} . After centrifugation, the HDL cholesterol remained in the supernatant and the cholesterol content was measured.

Analysis

Data from each group of animals ($n \geq 8$) were taken into account for analysis from at least two different experimental days. A two-tailed student's unpaired test (GraphPad Prism software) was used to compare the mean values of two populations of continuous data. Body weight changes and food intake data were analyzed by one-way ANOVA test (GraphPad Prism software).

Results

Effects of *Chlorella pyrenoidosa* on serum lipids profile in Wistar rats

Total triglyceride (TG) levels in high-fat diet (HFD) treated rats were significantly higher than that in normal diet-fed controls (129.4 ± 9.24 mg/dL constantly, $p < 0.005$) after 2, 4, and 8-week treatments (Fig. 1). In contrast, the elevated TG levels were significantly suppressed in rats fed with 0.9% and 7.2% *Chlorella*-contained HFD (CHFD). Although the TG level of the grouped rats fed with 1.8% CHFD was not statistically different to control grouped rats fed with normal rodent diet (NRD) after 2 weeks, however, longer treatment of CHFD (4 and 8 weeks) again shows a significant decrease in TG level.

Total cholesterol level in rats received HFD was elevated and higher than that in rats received NRD (85.9 ± 2.045 mg/dL constantly, $p < 0.005$) after 2, 4, and 8-week period of treatments (Fig. 2). It is clear that total cholesterol level was decreased in rats fed with low *Chlorella*-content diet (0.9% and

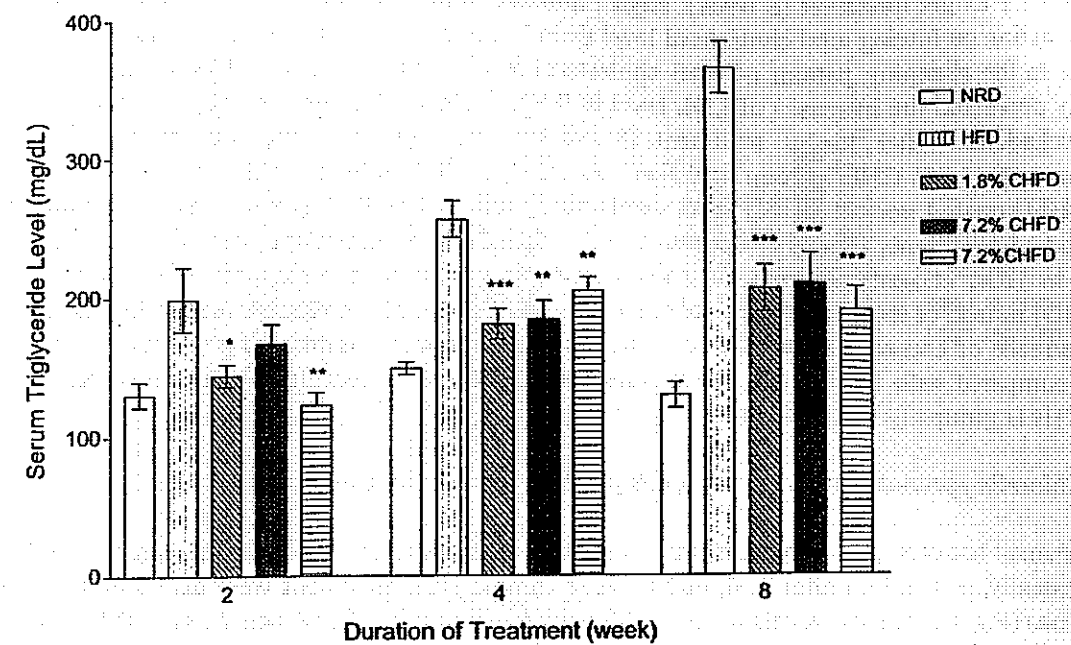


Fig. 1. Fasting serum triglyceride levels (mg/dL) in chronic high fat with or without *Chlorella pyrenoidosa* treated rats. Data expressed as means \pm SEM, $n = 8$ rats in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ as compared to their relative HFD group.

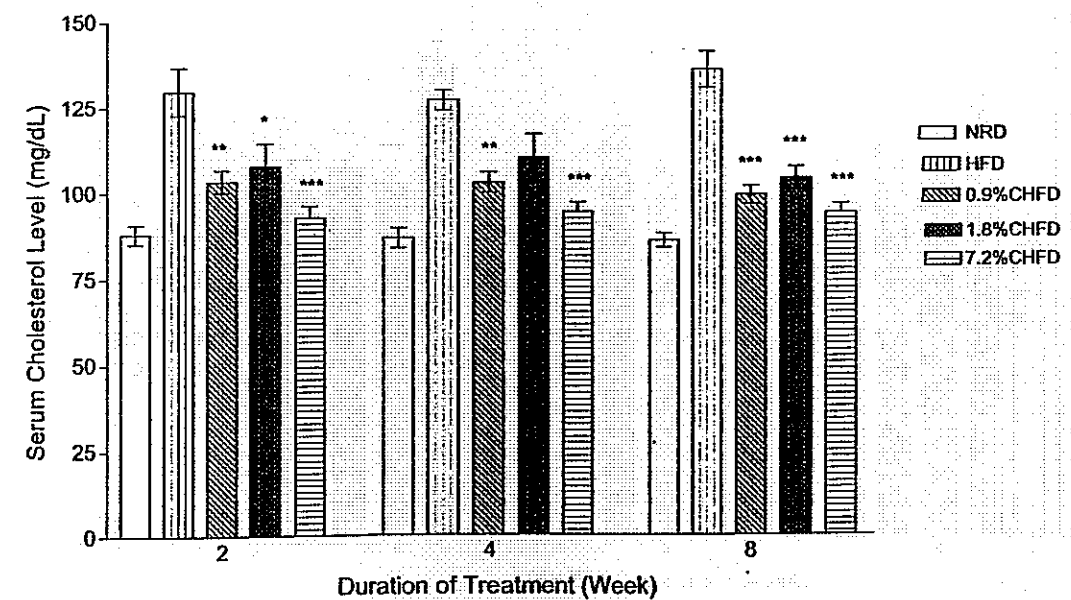


Fig. 2. Fasting serum total cholesterol levels (mg/dL) in chronic high fat with or without *Chlorella pyrenoidosa* treated rats. Data expressed as means \pm SEM, $n = 8$ rats in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ as compared to their relative HFD group.

Introduction

Chlorella, a type of unicellular green algae, has been a popular foodstuff worldwide especially in Japan and Taiwan. It contains essential amino acids, minerals, and fibers (Borowitzka, 1988; Schubert, 1988). Administration of *Chlorella* has been shown to play some biochemical functions, such as antihypertensive effects from its peptides inhibiting angiotensin I converting enzyme (Suetsuna and Chen, 2001; Merchant et al., 2002); antioxidant and anti-cataract effects in streptozotocin-induced diabetes rats (Shibata et al., 2003); lowering blood glucose in diabetic animals (Rodriguez-Lopez and Lopez-Quijada, 1971); anti-inflammatory and immunomodulatory activities from its polysaccharides (Guzman et al., 2003); production of cytokine and boosting immune function (Queiroz et al., 2002; Konishi et al., 1996; Singh et al., 1998; Tanaka et al., 1998); ameliorating oxidative stress in mice and preventing stress-induced ulcer (Lee et al., 2003; Tanaka et al., 1997) as well as anti-tumor activities from its glycoproteins (Hasegawa et al., 2002). *Chlorella vulgaris*, one strain of *Chlorella*, has also been shown to influence rats' lipid contents in the liver and serum (Shibata et al., 2001). For cholesterol-fed rabbits, *Chlorella vulgaris* has anti-lipidemic and anti-atherosclerotic actions (Sano and Tanaka, 1987). In human study, Okudo et al. (1975) showed that *Chlorella* intake reduced cholesterol levels in patients with hypercholesterolemia. *Chlorella pyrenoidosa* is the main strain of *Chlorella* produced in Taiwan industries. However, the effects on blood lipids profile have not yet been shown by *Chlorella pyrenoidosa* in normal rats and hamsters but only in ovariectomized rats (Hidaka et al., 2004). Also in our study, hamster was additional included as one of the research animal models, since hamsters had similar lipoprotein and bile acid metabolism patterns as that in human (Spady et al., 1986a,b). The aims of this study are to investigate the effects of *Chlorella pyrenoidosa* powder on the blood lipid profiles in rats and hamsters after chronic fed with high-fat diet containing cholesterol. This study might shed a light on the potential use of *Chlorella pyrenoidosa* for a treatment of hyperlipidemia or hypercholesterolemia in human.

Materials and methods

Chlorella pyrenoidosa material

Spray-dried algae materials from *Chlorella pyrenoidosa* cultured in outdoors cultivation pools were prepared by GONG BIH Enterprise Co., Ltd (Doo-Liu City, Taiwan). The *Chlorella* compositions of the product were analyzed and shown in Table 1.

Assay reagents

All the assay reagents were provided as assay kits purchased from Randox Laboratories Ltd. (USA).

Animals

Male Wistar rats (5 weeks old, 180 g) and male Syrian hamsters (100 g) were purchased from National Science Council Animal Center, Taiwan. Experiments on animals were approved by a local animal ethics committee and followed recommendations of the Committee for Research and Ethical

Table 1
Compositions of *Chlorella pyrenoidosa* dry powder (g/kg)

Compositions	g/kg
Protein	580
Lipids	130
Carbohydrates	107
Dietary Fiber	96
Chlorophyll	28
Iron	0.843
Calcium	2.44
Carotenoids	0.608
Vitamin A	338000 IU
Vitamin B1	0.0156
Vitamin B2	0.0497
Vitamin B6	0.0183
Vitamin B12	0.002
Vitamin C	0.4
Vitamin E	0.089

Data are provided from GONG BIH Enterprise Co., Ltd.

Issues as well as the Guidelines on Ethical Standards for animal studies, Republic of China. The animals were housed for a week prior to administration of powder diet. Subsequently they were randomly divided into five groups (n = 4/cage); Group 1: animals received normal powdered rodent diet (NRD) (purchased from LabDiet, Nutrition International Inc. USA); Group 2: animals received 0% of *Chlorella*-contained high fat diet mixed with 5% (w/w) cholesterol for rats or 1% for hamster (HFD); Group 3, 4 and 5: 0.9%, 1.8% and 7.2%, animals received high fat diet mixed with 0.9%, 1.8% and 7.2% (w/w) *Chlorella pyrenoidosa* powder (CHFD), respectively. High-fat diet (cat. # 960241, with total 20% saturated fatty acid) was purchased from ICN pharmaceutical Inc., (USA). The compositions of the five diets are shown in Table 2. Our previous study found that the effective dosage of *Chlorella pyrenoidosa* was 100mg/kg per day equivalent to approx. 1.2% of solid food intake in human (Shih, 2002).

Table 2
Compositions (g/kg) of the experimental diets used in the chronic high fat diet study

Composition	NRD	HFD	0.9% CHFD	1.8% CHFD	7.2% CHFD
Casein purified high nitrogen	200	195	189.9	184.6	153.2
DL-methionine	3	3	3	3	3
Sucrose	500	305.8	305.2	303.6	293.9
Corn starch	150	200	199.1	198.1	192.3
Coconut Oil Hydrogenated ²	50	200	198.8	197.7	190.6
Alphacel, Non-Nutritive bulk	50	50	50	50	50
DL-a-tocopherol powder (250IU/g)	—	120	120	120	120
AIN-76 Mineral Mixture (below in g/kg of mixture)	35	40	40	40	40
Cholesterol in rats	0	5	5	5	5
In hamsters	0	1	1	1	1
<i>Chlorella pyrenoidosa</i> powder	0	0	9	18	72

1. Abbreviations: NRD, normal rodent diet; HFD, high fat diet; CHFD, *Chlorella* contained-high fat diet.

2. Coconut Oil Hydrogenated is replaced by corn oil in NRD.



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Preventing dyslipidemia by *Chlorella pyrenoidosa* in rats and hamsters after chronic high fat diet treatment

Jong-Yuh Cherng, Mei-Fen Shih*

Department of Pharmacy, Chia-Nan University of Pharmacy and Science, 60 Erh-Jen Road, Sec.1, Tainan, 717, Taiwan R.O.C.

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Abstract

The effects of *Chlorella pyrenoidosa* on serum lipid profiles, after concomitant long-term treatment of high-fat diet (HFD) in rats and hamsters was studied. Wistar rats and Syrian hamsters were fed with or without various concentrations of *Chlorella pyrenoidosa* contained high-fat diet (CHFD) for 2, 4 and 8 weeks prior to assay of serum lipids. Fasting triglycerides, total cholesterol, and LDL cholesterol as well as HDL cholesterol levels in high-fat diet treated rats and hamster were determined. Results showed that triglycerides, total cholesterol and LDL cholesterol levels in HFD treated rats and hamsters were increased from the normal rodent diet (NRD) treated controls after 2, 4, and 8-week treatments. However, the presence of *Chlorella pyrenoidosa* in high-fat diets significantly decreased the levels of triglycerides, total cholesterol and LDL cholesterol with comparison to HFD group in rats and hamsters. The total cholesterol/HDL ratios, an indication of occurrence of coronary heart disease, were decreased in all CHFD treated grouped rats and hamsters which suggests administration of *Chlorella pyrenoidosa* could lower the occurring risk of heart diseases. In conclusion, *Chlorella pyrenoidosa* has the ability to prevent dyslipidemia in chronic high-fat fed animals and could be potential in use to prevent intestinal absorption of redundant lipid from our daily intake and subsequently to prevent hyperlipidemia as well as atherosclerosis.

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Keywords: *Chlorella pyrenoidosa*; High-fat diet; Serum lipids; Rats; Hamsters

* Corresponding author. Tel.: +886 6 2492707; fax: +886 6 2666411.
E-mail address: meifenshih@mail.chna.edu.tw (M.-F. Shih).