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Hypercholesterolemia Induced Oxidative Stress Is Reduced in Rats with Diets Enriched with Supplement from *Dunaliella salina* Algae

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Abstract

Dunaliella salina (D.salina), an alga is known as a potent free radical quencher and an antioxidant in biological systems. The present study examined the modulation of hypercholesterolemia and 5'-Deiodinase (5'-DI) activity on *D. salina* supplementation. Male SD rats were divided into three groups. Group-I rats acted as control and were fed standard pellet diet. Animals in group-II were supplemented with 2% cholesterol in the control diet. Further, the diet of group-III animals was supplemented with 2% cholesterol and 1.25g D.*salina*/Kg high cholesterol diet (HCD). After two months of diet feeding, significant increase in serum cholesterol and triglycerides in HCD fed animals was found as compared to the control counterpart. Significantly increased activities of antioxidant enzymes viz. catalase, superoxide dismutase (SOD) and oxidized glutathione (GSSG) in HCD fed group were observed as compared to control. Lipid peroxidation levels increased nearly 2 folds. Significant decrease in the T_3 (triiodothyronine) and increase in T_4 (thyroxine) levels on HCD feeding in comparison to the respective control was observed. *D. salina* supplementation caused restoration of catalase, SOD, MDA (Malondialdehyde) levels, as well as thyroid hormones compared to HCD group. The present study clearly indicated the protective role of D. salina on various events leading to atherogenicity in terms of maintaining basic lipid profile. The altered levels of 5'-DI obtained during high cholesterol diet feeding got normalized on *D. salina* supplementation. Hence, increased uptake of the *D. salina*, but within safe limits, in general population may be useful against development of hypercholesterolemia related disorders. Finally, on the basis of present observations, it could be concluded that *D. salina* protects against atherogenesis and hypothyroidism and could be exploited accordingly as a cheap diet supplement.

Keywords: *Dunaliella salina*; Hypercholesterolemia; Antioxidants; Hypothyroidism.

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1. Introduction

Prominent role of the antioxidants in food has gained importance in the recent years. Growing evidences indicate that chronic and acute overproduction of the reactive oxygen species (ROS) under pathophysiological conditions is integral in the development of cardiovascular diseases (CVD) [1]. Hence, there is exhorting for finding newer, stringent and potential sources of cardiovascular protective agents from naive sources.

Algae are unexploited sources due to their limited distribution in the natural habitat. However, their nutritional composition with various bioactive compounds produce accustom to the biodiversity of marine environment [2]. *Dunaliella salina* (*D. salina*) is a unicellular biflagellate green alga that belongs to the Chlorophyaceae family [3]. *D. salina* has the potential to accumulate maximum amount of the β-carotene under appropriate conditions which makes *dunaliella* alga much more effective as an antioxidant than synthetic β-carotene because *dunaliella* derived β-carotene consists of both 9-cis and all trans isomers, whereas synthetic β-carotene is rich in all the trans isomers [4]. β-carotene derived from *Dunaliella* alga is much more effective as antioxidant than synthetic β–carotene [5]. However, dunaliella is also a source of protein that has good utility value, rich in essential fatty acids and is safe to utilize directly in food formulations [6]. Dunaliella species have shown to exhibit various biological activities, like anti-hypertensive, bronchodilator, analgestic and muscle relaxant and anti-edima activity [7].

Strong evidences have been put forward by various investigators for the involvement of free radicals production and lipid peroxidation in the onset of atherosclerosis. Thus identification of the antioxidants, which can retard the process of lipid peroxidation by blocking the generation of free radical chain reaction, has gained importance in recent years. The antioxidants may act by raising the levels of endogenous defense by upregulating the expression of genes encoding the enzymes such as superoxide dismutase (SOD), Catalase, glutathione peroxidase or lipid peroxidase [8]. Thyroid hormone involvement has also been reported in context of atherosclerosis [9] as hypothyroidism is said to be associated with the disease process. Lipid abnormalities are reported to be more common in patients with overt hypothyroidism and contribute to the disproportionate increase in cardiovascular risk. In some studies, patients with subclinical hypothyroidism have also shown that they have elevations in their cholesterol levels [10]. Fall in T_3/T_4 levels causes qualitative changes in circulating lipoproteins that increase their atherogenicity, indicating the positive correlation between hypercholesterolemia and hypothyroidism. The present study was aimed to delineate the possible modulation of hypercholesterolemia induced oxidative stress and 5'-Deiodinase expression on *D. salina* supplementation.

2. Materials and Methods

Animals: Male Sprauge-Dawley rats (100g body weight) were obtained from the Central Animal House, Panjab University, Chandigarh (India) and were kept in polypropylene cages under hygienic conditions in the animal rooms of the department. Before initiating the experiments, animals were adapted to the conditions of the animal rooms for a week. Necessary approvals were obtained from the Ministry of Social Justice and Empowerment for the use of experimental animals for this study.

Experimental Design: Animals were acclimatized to the departmental animal room for a week, divided into three groups and 6 rats were taken in each group: group-I (Control diet), group-II (High Cholesterol diet; HCD) and group-III (HCD + *D. salina*). Feed and water were given ad libitum. Treatment protocol was for 2 months.

Diet preparation: Standard rat pellet diet obtained from Ashirwad Industries (India) was given as control diet (Group-I). For Group-II animals, HCD was prepared by supplementing 2% cholesterol to the control diet and fat composition (%) was taken as per Kritchevsky [11]. Group–III animals were fed with HCD (as in group–II) but further supplemented with *D. salina* (1.25g/kg HCD diet).

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Dunaliella salina Culture: *D. salina* culture was obtained from Department of Botany, Panjab University, Chandigarh (India) and cultured in artificial seawater medium [12,13]. *D. salina* was grown in the medium comprising of NaCl 116 g/L, $NaHCO_3$ 4.2 g/L, $MgSO_4$ 1.21 g/L, KNO_3 75.82 mg/L, KH_2PO_4 27.20 mg/L, $CaCl_2$ 29.40 mg/L, $MnCl_2$ 1.38 mg/L, EDTA 1.86 mg/L, $FeCl_3$ 0.32 mg/L, $(NH_4)_6MO_7O_{24}$ 1.23 mg/L, $ZnCl_2$ 0.13 mg/L. $NaHCO_3$ was added after autoclaving and pH was adjusted to 8.0 with 1N KOH. Culture medium contained in Erlenmeyer flasks was autoclaved at a pressure of 15-lb/ $inch^2$ for 20 minutes and inoculated with *D. salina* culture. The culture flasks were incubated in culture room illuminated by 40W florescent light with intensity of 3,000 lux and 16:8 hr day/night cycle. The temperature was maintained at 28^0C. The algal biomass was collected by centrifugation and lyophilized to remove water. This lyophilized biomass was used to supplement the animal feed. After completion of diet feeding schedule, rats were kept on overnight fasting, anesthetized and exsanguinated. Serum from blood and tissue (liver) samples was collected from each animal. Tissues were snap frozen in the liquid nitrogen.

Total cholesterol and Triglycerides Level: Serum total cholesterol and triglycerides levels were estimated by enzymatic colorimetric kit obtained from E. MERCK diagnostic (Germany).

Lipid peroxidation (LPO): The level of MDA is used as an index for measuring the level of lipid peroxidation as according to the method of Wills [14]. For standard, 2-10nM range of 1, 1', 3, 3'-tetraethoxypropane (TEP) in 250µL volume was used and for control, distilled water was used instead of the sample. Results were expressed as nmoles MDA/mg protein.

Total and oxidized Glutathione: All biochemical analysis were done in liver PMF (Post mitochondrial fraction). Total and oxidized glutathione were quantitated by fluorimetric method of Hissin and Hilf [15]. Glutathione reacts specifically with O-phthaldehyde (OPT) at pH 8 resulting in the formation of highly fluorescent product that is activated at 350nm with the emission peak at 420nm.

For the estimation of the oxidized glutathione (GSSG), supernatant was mixed with N-ethylmaliemide and incubated at room temperature for 30 min to inhibit the reduced glutathione (GSH) at an alkaline pH 12. The fluorescence intensity produced by OPT-GSSG reaction at pH 12 was directly related to GSSG concentration.

Superoxide Dismutase: Superoxide dismutase assay was performed according to the method of Kono [16]. The method is based on the principle of inhibitory action of SOD on the reduction of NBT by superoxide anions, which generate by the photo-oxidation of hydroxylamine hydrochloride. One unit of enzyme is expressed as inverse of the amount of protein required inhibiting the reduction rate of NBT by 50%.

Catalase: Catalase was estimated by the method described by Luck [17] using H_2O_2 as substrate. Decrease in the absorbance at 240 nm was measured and the amount of H_2O_2 decomposed was calculated using the molar extinction coefficient of H_2O_2 (0.71 M^{-1} cm^{-1}) and results were expressed as µmol of H_2O_2 decomposed/min/mg protein.

Protein: Protein assay was done by the method of Lowry et al [18].

T_3 and T_4 levels: Serum T_3 and T_4 estimation were done by radioimmunoassay (RIA) kits procured from Babha Atomic Res Centre, Mumbai, India.

Type-I 5'-iodothyronine deiodinase activity: Type-I iodothyronine deiodinase (5'-DI) activity in liver was estimated by following the method of Behne [19]. The protein containing 5'-DI enzyme was incubated with T_4 to convert it into T_3. The activity of 5'-DI can be estimated in terms of T_3 produced in the reaction mixture.

5'DI mRNA expression using RT-PCR: Total RNA from liver was extracted using TRI REAGENT (Molecular Research Centre, Inc. Ohio). 3µg of the total isolated RNA from each group was used in the total RT-PCR reaction mixture containing 10µL of 5X QIAGEN One Step RT-PCR buffer (2.5mM $MgCl_2$ as final concentration), 2µL of dNTP mix (10mM of each dNTP), 5µL of each forward and reverse gene specific primers (from 10µM stock) and 2µL QIAGEN One Step RT-PCR enzyme Mix, 1µL RNase inhibitor (1U/µL) made to 50µL total volume with PCR grade RNase free water

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(provided in the kit). Mixed the contents gently and the PCR reaction was performed in the thermal cycler (Techne Ltd., England) using following conditions: RT reaction at 50^0C for 50 minutes, initial PCR activation was done at 95^0C for 15 min, followed by 35 cycles of 94^0C (denaturation) for 45s, 58^0C (annealing) for 45s, 72^0C (extension) for 1min. Finally incubated at 72^0C for 10 min to extend any incomplete strands. Optimal oligonucleotide primer pairs for RT-PCR were designed and selected with the aid of the software Gene Runner. The primer sequences (5'-3') for rat 5'DI coding (+), noncoding (-) strands were TCTGGGATTTCATTCAAGGC and TAGAGCCTCTCAGGCAGAGC respectively. Final PCR products formed were analyzed on 1.5% agarose gel electrophoresis and densitometric analysis of the bands was done by Image J software (NIH, USA). For the comparison of each analysis the densities of the three independent densitometric analyses of PCR product bands were normalized with respect to the PCR bands of β-actin, which act as internal control.

Statistical analysis: Statistical analysis of the data was preformed by analysis of variances (one way ANOVA) following one-way ANOVA post-Hoc test using least significance difference (LSD).

3. Results

Rats fed on HCD for two months (Group–II) showed a progressively significant increase ($P<0.05$) in the concentration of total cholesterol and triglyceride levels in the serum when compared to control (Table I). However, when *D. salina* was supplemented along with HCD (Group-III), serum total cholesterol and triglycerides levels were significantly decreased ($P<0.05$) when compared to group-II animals (Table–1).

Table 1: Lipid profile and lipid peroxidation in rat serum after 2 months of diet feeding

	Control Group-I	HCD Group-II	HCD + *D. salina* Group-III
Cholesterol estimation (mg/dL)	87.86 ± 5.82	175.94 ± 12.40*	128.20 ± 6.82*
Triglyceride estimation *(mg/dL)*	83.57 ± 7.01	159.80 ± 10.10*	123.72 ± 7.21*
Lipid peroxidation (nmoles of MDA/mg of protein)	0.08 ± 0.01	0.96 ± 0.45*	0.20 ± 0.06*

The values are mean ± SD of six independent observations.
*, **, *** represent $p<0.05$, $P<0.01$, $P<0.001$, respectively
HCD represents high cholesterol diet

Feeding of HCD to group–II animals showed a significant decrease in total glutathione levels ($P<0.05$) in liver when compared to group–I rats (Table-2). These levels significantly decreased ($p<0.05$) on combined feeding of HCD and *D. Salina* to the group-III animals in comparison to the Group-II animals (Table 2). Feeding of HCD to rats showed highly significant increase in the GSSG in liver as compared to the control group (Table 2). On simultaneous feeding of HCD and *D. Salina* (Group-III) rats showed significant decrease ($P<0.05$) in oxidized glutathione levels in liver as compared to groups-II animals. A significant decrease ($p<0.05$) in the GSH/GSSG ratio was observed in the liver in high cholesterol fed group II when compared to group-I (Table-2).

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Co feeding of *D. salina* with HCD did not show any significant change in the GSH/GSSG ratio in liver in comparison to the group-II rats.

Superoxide dismutase (SOD) and Catalase levels were seen to increase significantly in liver in HCD fed group in comparison to the control rats (Table-2). Results indicated that when *D. salina* was supplemented in group-III rats along with HCD, levels of SOD and Catalase decreased significantly ($p<0.05$).

Table 2. Antioxidant enzymes status in the rat after two months of diet feeding schedule

	Control Group-I	HCD Group-II	HCD + *D. salina* Group-III
Total glutathione (ng/mg)	62.20 ± 0.48	51.92 ± 1.11	52.48 ± 2.77
Oxidized glutathione (ng/mg)	13.32 ± 0.47	22.40 ± 0.38*	20.12 ± 0.65*
Redox ratio (GSH/GSSG)	4.67 ± 0.20	2.49 ± 0.33*	2.61±0.27
Superoxide Dismutase (U/mg protein)	36.25 ± 5.30	46.68 ± 0.91*	42.79 ± 1.85*
Catalase (µmoles H_2o_2 decomposed/ mg protein	0.78 ± 0.01	1.64 ± 2.20*	1.36 ± 0.07*

The values are mean ± SD of six independent observations.
*, **, *** Represent $p<0.05$, $P<0.01$, $P<0.001$, respectively
HCD represent high cholesterol diet

In the present study, MDA levels (as a marker of lipid peroxidation) were assayed in liver. Significant increased production of MDA was observed in liver of HCD fed (Group–II) animals (Table-1). Levels of MDA decreased in liver ($p<0.05$) when *D. salina* supplemented along with HCD (Group- III) in comparison to the Group–II animals.

Levels of serum T_3 decreased significantly ($P<0.05$) on HCD feeding in comparison to the control group (Table-3). Further, these levels seemed to be significantly higher on the simultaneous feeding of HCD and *D. salina* in comparison to the Group–II rats. Results of serum T_4 levels followed opposite trends as that of serum T_3. HCD feeding resulted in a significant increase ($p<0.05$) in circulating T_4 levels in Group–II rats (Table-3) in comparison to the control. However, on feeding of *D.salina* along with HCD (Group–III), a marked decrease ($p<0.05$) in circulating T_4 values in comparison to Group–II values were seen.

The 5'–DI activity decreased significantly ($p<0.05$) in liver of HCD fed animals in comparison to control. Supplementation with *D. salina* along with HCD significantly increased deiodinase activity in liver ($p<0.05$) in comparison to Group–II animals (Table-3). 5'-DI mRNA expression in liver was analyzed after 2 months of experimental diet feeding. In liver, mRNA expression decreased significantly ($p<0.05$) in HCD fed Group-III in comparison to the control. However in Group-III, expression increased ($p<0.05$) significantly in comparison to the HCD fed Group–II (Fig. 1).

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Table 3. Serum T_3, T4 and tissue type-I 5'-deiodinase activity in rats after two month of diet schedule

	Control Group I	HCD Group II	HCD + *D. salina* Group III
T_3 levels (nmoles/L)	54.66 ± 3.99	30.76 ± 3.18*	33.80 ± 3.23
T_4 levels (nmoles/L)	4.54 ± 0.58	7.66 ± 0.64*	4.90 ± 0.63
Tissue DI activity (ng T_3 liberated /mg Protein/hr)	9.20 ± 0.18	5.39 ± 0.36*	8.30 ± 0.52*

The values are mean ± SD of six independent observations.
*, **, *** Represent p<0.05, P<0.01, P<0.001, respectively
HCD represent high cholesterol diet

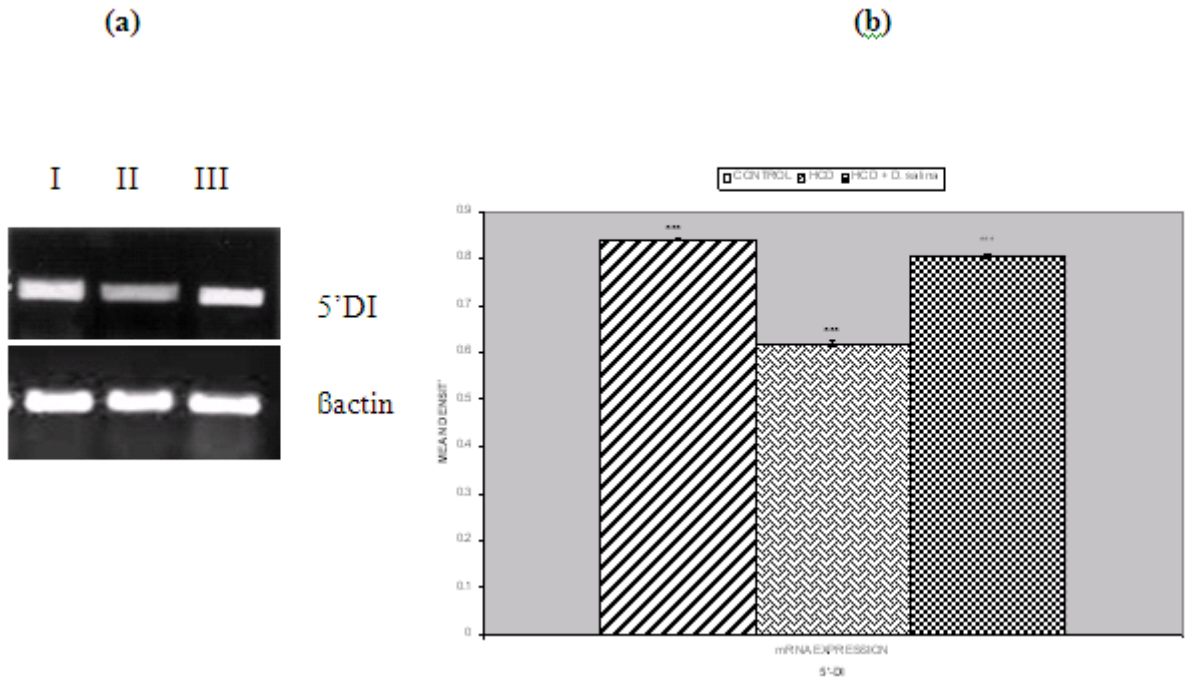

Figure 1: 5'-DI mRNA expression in liver by RT-PCR after 2 months of diet feeding schedule (a) and its densitometric analysis (b). Lane I- Control, Lane II- HCD Fed, Lane III- HCD + D. Salina Fed. The values are mean ± SD of three independent observations. *, **, *** Represent p<0.05, P<0.01, P<0.001, respectively

4. Discussion

High plasma cholesterol represents a major risk factor for ischemic heart diseases [20]. Total serum cholesterol is really an indicator of the amount of the free radical damage in the body. Higher the free radical level, higher the body needs to produce cholesterol internally from liver to act as an antioxidant and free radical scavenger. The significant decrease in the cholesterol and the triglycerides levels on *Dunaliella* supplementation can be explained on the basis that presence of *D.*

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salina triggers: Intestinal binding of cholesterol, an enzymatic shift decreasing the liver cholesterol production, increased production of fat degrading enzymes. Basically β-carotene of the *D. salina* decreases absorption by blocking the fat molecule absorption gates in the intestine [21].

MDA concentration detected in the present study showed that an increase in lipid peroxidation occur in animals fed on HCD when compared to the controls. Significant decrease of MDA levels in *D. salina* group established the efficacy of *D. salina* in quenching singlet oxygen and intercepting deleterious free radicals and ROS. Indeed *in vitro* experiments have demonstrated that components of *D. salina* i.e. lycopene, alpha carotene, zeaxanthin, lutein and cryptoxanthin quench singlet oxygen and inhibit lipid peroxidation [22].

Increase in the SOD and the catalase levels in the high cholesterol fed animals is again attributed to increased oxidative stress on cholesterol feeding in these animals and is supported by the literature [23]. H_2O_2 is the end product of SOD dismutation while degradation of H_2O_2 and O_2 is catalyzed by GSH-P_x and Catalase. Since the end product of SOD is substrate for catalase catalyzation and owing to the fact that H_2O_2 is known to inactivate SOD [24]. It is possible that increase in oxyradicals during hypercholesterolemia could increase catalase activity, which in turn would protect SOD inactivation by H_2O_2 and lead to an increase in SOD levels. Supplementation of *D. Salina* along with HCD decreases the free radicals by quenching and lowering oxidative stress.

Intracellular reduction oxidation (redox) state is regulated in part by the presence of GSH [25]. The GSH/GSSG ratio may be sensitive indicator of oxidative stress. Significant decrease in the levels of total glutathione observed on HCD feeding might be due to impaired GSH biosynthesis and constant on slaught of $ONOO^-$ formed by reactions of O_2^- and NO, both of which increased in hypercholesterolemia. GSH plays critical role in the detoxification process against reactive nitrogen species e.g. NO, NO_2 & $ONOO^-$ [26]. Oxidized glutathione (GSSG) is formed by the linking of two tripeptides by disulfide bridge. The generation of GSSG takes place during the oxidation of GSH by glutathione peroxides in the following reaction to maintain the sufficient level of GSH. The increased oxidized glutathione levels in hypercholesterolemic rats can be attributed to spontaneous non-enzymatic GSH oxidation as has been suggested by Meister and Anderson [27]. Decreased levels of GSSG in *Dunaliella* supplemented group is due to the interaction of *D. salina* with free radicals, as carotenoids are most effective singlet oxygen quenchers in which singlet oxygen is restored to its ground state with no oxygen consumption or product formation.

Studies have shown that patients with subnormal plasma T_4 concentrations have elevated plasma LDL cholesterol. Present studies showed a significant decrease in the levels of T_3 and increase in T_4 on HCD feeding in comparison to respective control groups. So this study indicates that hypercholesterolemia induces hypothyroidism [28]. This could be due to the decreased activity as well as mRNA expression of 5'-DI enzyme observed in the present study on high cholesterol feeding. Basically 5'-DI converts T_4 to T_3 and therefore lesser the expression of this enzyme lesser will be T_3 production. Sudaram et al [29] have shown that LDL is more susceptible to oxidation in patients with hypothyroidism and this oxidized LDL is not taken up by LDL receptors. It accumulates in the body and in turn leads to hypercholesterolemia so vice versa i.e. hypothyroidism leads to hypercholesterolemia is also true.

Thyroid hormone depletion leads to the decreased LDL receptors expression and elevated serum cholesterol levels. LDL receptors levels are subject to negative feedback regulation by cellular cholesterol through sterol regulatory binding protein 2 (SREBP–2). Agents that directly increase SREBP-2 expression can reverse hypercholesterolemia associated with hypothyroidism. These findings suggest that hypercholesterolemic is directly related to low level of 5'-DI. Increased level of T_3 and decreased level of the T_4 was observed on *Dunaliella* supplementation. This could be due to increased expression of 5'-DI on *Dunaliella* supplementation. Curran-celentano et al [30] reported that thyroid hormones T_4 and T_3 were significantly depressed in hypercarotenemic group. In conclusion, the present studies showed

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the altered level of 5'-DI, during atherogenic condition. 5'-DI is involved in lipid metabolism through T_3 which in turn regulates the LDL-R expression at molecular level. The altered levels of 5'-DI obtained during high cholesterol feeding got somewhat normalized by *D. salina*. Due to the high accumulation of β-carotene, even up to 10% of the dry weight, the green alga *D.salina* may be one of the rich biological sources of β-carotene. Beneficial effects of *D. salina* intake in terms of its protective role require further investigation on a long-term study. Therefore, interaction of 5'-DI and dunaliella may represent a new therapeutic approach in atherosclerosis.

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