Effects of dietary supplementation with red-pigmented leafy lettuce (*Lactuca sativa*) on lipid profiles and antioxidant status in C57BL/6J mice fed a high-fat high-cholesterol diet

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The present study was undertaken to assess the beneficial effects of a daily consumption of 8 % freeze-dried red-pigmented leafy lettuce (*Lactuca sativa*) on CVD. C57BL/6J mice were fed a high-fat high-cholesterol diet supplemented with or without red-pigmented leafy lettuce for 4 weeks. The present results showed that the red-pigmented leafy lettuce-supplemented diet significantly decreased the level of total and LDL-cholesterol and TAG in the plasma of the mice. The atherosclerotic index was calculated to be 46 % lower in the mice fed with the lettuce diet compared with the control diet. Lipid peroxidation measured by 2-thiobarbituric acid-reactive substances was markedly reduced in the plasma, liver, heart and kidney of the mice fed the lettuce diet. The content of antioxidants (total glutathione and β-carotene) was significantly increased by lettuce supplementation. The antioxidant defence system by antioxidant enzymes including glutathione S-transferase, glutathione peroxidase, glutathione reductase, superoxide dismutase and paraoxanase in blood or liver tissues was also increased, and showed the improved oxidative stress in the mice fed the lettuce diet. The measurement of tail DNA (%), tail extent moment and olive tail moment indicated that the lettuce diet increased the resistance of hepatocyte and lymphocyte DNA to oxidative damage. The present study showed that the supplementation of a high-cholesterol high-fat diet with 8 % red-pigmented leafy lettuce resulted in an improvement of plasma cholesterol and lipid levels, prevention of lipid peroxidation and an increase of the antioxidant defence system and, therefore, could contribute to reduce the risk factors of CVD.

Antioxidants: Cholesterol: DNA: High fat diets: Lipid peroxidation: Mice: Red-pigmented leafy lettuce

CVD is one of the most prevalent diseases in Western developed countries, and its complications have been a leading cause of death and illness annually. Recent studies suggest that oxidative stress caused by reactive oxygen species (ROS) is involved in the pathogenesis of CVD including atherosclerosis[1]. ROS or so-called free radicals are highly reactive molecules due to the presence of unpaired valence shell electrons. They react with all classes of biological molecules, and the consequent molecular modification is known to be caused by oxidative stress[2–4]. Under the mechanism of vascular damage by oxidative stress in atherosclerosis, ROS drive the oxidative modification of LDL in arterial walls, and the oxidised LDL and oxidation products play a significant role in the initiation and progress of atherosclerosis[1].

Nature has provided animals and humans with antioxidant defence systems including enzymes such as superoxide dismutase (SOD) and glutathione peroxidase[5], and antioxidants including glutathione, tocopherols and carotenoids[6,7] which have a capacity to neutralise ROS. However, depleted antioxidant defences could lead to oxidative stress, that is, a disturbance in the equilibrium status of pro-oxidant–antioxidant systems in intact cells resulting in oxidative damage to lipids, proteins, carbohydrates and nucleic acids, and which contribute to pathological dysfunction in the organism[4]. Therefore, much attention has been focused on the activity of natural antioxidants such as ascorbic acid, tocopherols, carotenoids and polyphenols in fruits and vegetables because these compounds may potentially reduce the level of oxidative stress and account for the health beneficial effects[8]. The increased consumption of fruits and vegetables has been found to be associated with lowering the risks of degenerative diseases that come with ageing such as CVD, cancer, cataracts, and brain and immune dysfunction[9].

Lettuce is one of the most commonly consumed vegetables and a good source of antioxidant micronutrients such as carotenoids (β-carotene and lutein), vitamins E and C, phenolics (caffeic acid, quercetins, hydroxycinnamic acids and chlorogenic acid), as well as fibre. Red-pigmented leafy lettuce was found to be higher in 2,2-diphenyl-2-picrylhydrazyl hydrate free radical-scavenging activity and inhibition of human LDL oxidation compared with other leafy vegetables[10,11], and red-pigmented leafy lettuce contains relatively higher contents of phenolic compounds than green lettuce[11,12]. The effect of lettuce consumption on CVD has been previously reported in an animal model. Feeding rats with a 20 % lettuce diet for 3 weeks decreased the concentration of cholesterol in the plasma and liver, and increased cholesterol endproducts (faecal total steroid) excretion[13]. Compared with control, more antioxidants were retained in the rats' plasma, and lipid oxidation expressed as 2-thiobarbituric acid-reactive substances (TBARS) was significantly improved. However, more research is needed to make a conclusion for its impact on lipid and cholesterol metabolism with emphasis on the antioxidant status in animals fed with a high-fat high-cholesterol diet. Therefore, in present study, the *in vivo* effect of red-pigmented leafy lettuce (freeze-dried, 8 % of diet) on hyperlipidaemic mice was investigated against CVD. C57BL/6J mice were fed with a high-fat high-cholesterol diet (20 % of lipids plus 1 % of dietary cholesterol) for 4 weeks, and the changes of lipid profiles and antioxidant biomarkers in the blood and liver tissue were assessed. We determined the levels of TBARS induced by lipid peroxidation, and considered this level as an index of the suppressive effects of the lettuce diet on ROS-induced oxidative damage in mouse plasma and organs. In addition, the impact on oxidative stress of DNA was examined by tail DNA (%), tail extent moment and olive tail moment in hepatocytes and lymphocytes.

Materials and methods

Materials

Glutathione reductase (type III from baker's yeast), reduced glutathione (GSH), oxidised glutathione (GSSG), tetramethoxypropane, 5,5′-dithio-(2-nitrobenzoic acid), NADP, NADPH, thiobarbituric acid and bovine serum albumin were products of Sigma-Aldrich Chemical Co. (St Louis, MO, USA). All reagents used were of analytical grade.

Animals and diet

Male C57BL/6J mice (25–30 g), aged 6–8 weeks, were housed eight per polycarbonate cage (identified with the ear punch method) in an animal room with controlled temperature (23 ± 3°C) and humidity level (55 ± 10 %) under a 12 h light–dark cycle. Red-pigmented leafy lettuce (*Lactuca sativa*) was obtained from an organic farm (Gongju, Korea), and freeze-dried. After 1 week of acclimatisation to a pelleted commercial diet (Samyang Co., Seoul, Korea), the mice were randomly divided into two treatment groups: a high-fat control (HFC) group and a high-fat with freeze-dried red lettuce (HFL) group. The mice had *ad libitum* access to water and were assigned the diets for 4 weeks. For the experimental diets, HFC mice were fed a 20 % fat and 1 % cholesterol diet containing AIN-93 mineral and vitamin mixtures, and, for the HFL diet, freeze-dried red lettuce powder (8 %) was added into the HFC diet (Table 1). The fibre and antioxidant content of the red-pigmented leafy lettuce powder (in dry weight) is presented in Table 1. The body weight of mice and food intake were measured daily, and the feed efficiency ratio was calculated throughout the experiment. The animal experiment was conducted in compliance with the 'Guide for Care and Use of Laboratory Animals' of the National Institutes of Health Guidelines[14].

Sampling procedures

Fasted mice (12 h) were anaesthetised during the post-absorptive period between 08.00 and 10.00 hours. Blood was drawn from the vena cava into heparin tubes and centrifuged at 2500 rpm and 4°C. Plasma samples were stored in liquid N_2 for assays of lipid peroxidation, lipid profile and antioxidant markers. The fat pad was removed and weighed. Heart, kidneys and liver were rapidly washed in saline buffer, collected into cryovials, weighed and stored immediately in liquid N_2 for lipid peroxidation and antioxidant marker assays.

Table 1. Composition of experimental diets

Components (g/kg diet)	HFC	HFL
Casein	200·00	200·00
L-Cystine	3·00	3·00
Maize starch	388·00	370·32
Sucrose	100·00	49·20
Cellulose	50·00	42·08
Maize oil	20·00	20·00
Lard	180·00	180·00
Cholesterol	10·00	10·00
Mineral mixture*	35·00	32·20
Vitamin mixture†	10·00	9·20
Choline barbitrate	4·00	4·00
Red-pigmented leafy lettuce‡	0·00	80·00
Energy (kJ)	19 096	19 012
Diet composition (% energy)§		
Fat	39·4	39·6
Protein	17·8	17·9
Carbohydrate	42·8	42·5

HFC, high-fat control diet; HFL, high-fat diet with freeze-dried red lettuce (*Lactuca sativa*); USP, United States Pharmacopeia.

* AIN 93 mineral mixture (g/kg): calcium carbonate, 357·00; potassium phosphate (monobasic), 196·00; potassium citrate.H_2O, 70·78; sodium chloride, 74·00; potassium sulfate, 46·60; magnesium oxide, 24·00; ferric citrate USP, 6·06; zinc carbonate, 1·65; manganous carbonate, 0·63; cupric carbonate, 0·30; potassium iodate, 0·01; sodium selenate, 0·01025; ammonium paramolybdate.4H_2O, 0·00795; sodium metasilicate.9H_2O, 1·45; chromium potassium sulfate.12H_2O, 0·275; lithium chloride, 0·0174; boric acid, 0·0815; sodium fluoride, 0·0635; nickel carbonate, 0·0318; ammonium vanadate, 0·0066; finely powdered sucrose, 221·026.

† AIN 93 vitamin mixture (g/kg): niacin, 3·00; calcium pantothenate, 1·60; pyridoxine HCl, 0·70; thiamine HCl, 0·60; riboflavin, 0·60; folic acid, 0·20; biotin, 0·02; vitamin E acetate (500 mg/g), 15·00; vitamin B_{12} (0·1 %), 2·50; vitamin A palmitate (275 mg/g), 0·80; vitamin D_3 (10 mg/g), 0·25; vitamin K_1-dextrose mix (10 mg/g), 7·50; sucrose, 967·23.

‡ Red-pigmented leafy lettuce composition (in dry weight): energy, 1063·45 kJ/100 g (254 kcal/100 g); sugar (sucrose, glucose, and fructose), 6·88 %; fibre, 26·42 %; vitamin C, 434 mg/100 g; β-carotene, 53·02 mg/100 g; α-tocopherol, 3·42 mg/100 g; total phenols, 1140 mg/100 g; anthocyanins, 270·5 mg/100 g.

§ Energy content was calculated using 16·7 kJ/g (4 kcal/g) for protein and carbohydrate and 37·7 kJ/g (9 kcal/g) for fat.

Determination of plasma and faecal lipids

Plasma total cholesterol, HDL-cholesterol and TAG concentrations were enzymically determined using a kit purchased from Yeongdong Pharmaceutical (Seoul, Korea). Optical density was measured using a spectrophotometer (Pharmacia Biotech Co., Cambridge, UK). The concentration of LDL-cholesterol was calculated using the Friedewald equation[15]: LDL-cholesterol = total cholesterol − HDL-cholesterol − (TAG/5). Faecal lipid was extracted using a previously described method[16], and faecal TAG and cholesterol concentrations were determined as previously described in plasma TAG and cholesterol determination.

Lipid peroxidation determined by thiobarbituric acid-reactive substances

To determine lipid peroxidation of organs in the mice, liver, heart and kidney tissues were placed on ice, and homogenised with 50 mM-sodium phosphate buffer using a tissue homogeniser with a Teflon pestle. The tissue homogenates and plasma samples (each 1 ml) were mixed with 1 ml of 8·1 % SDS, 2 ml of 20 % acetic acid and 1 ml of 0·75 % thiobarbituric acid. The mixture was boiled for 30 min and centrifuged (14 000 rpm for 10 min). The absorbance of the malondialdehyde–thiobarbituric acid adduct formed in the supernatant fraction was measured colorimetrically at 533 nm as previously described[17]. The values of malondialdehyde were calculated from a standard curve which was prepared with tetramethoxypropane (Sigma Chemical Co.), and expressed as TBARS values.

Total glutathione content

Liver tissue (about 0·2 g wet weight) was pulverised in a cooled ceramic percussion motor with 6 % metaphosphoric acid, and the mixture was centrifuged (25 000 rpm for 20 min) at 4°C. Total glutathione level was determined enzymically according to a previous procedure[18] with a slight modification. The supernatant fraction (0·05 ml) was mixed with 100 mM-phosphate buffer (pH 7·4, 0·39 ml) containing 5 mM-EDTA, 10 mM-5,5′-dithiobis-(2-nitrobenzoic acid) (0·025 ml) and 5 mM-NADPH (0·08 ml). After 3 min of equilibration at 25°C, the reaction was started by adding 2 units of glutathione reductase (type III from baker's yeast; Sigma Chemical Co.). The formation of 5,5′-dithiobis-(2-nitrobenzoic acid) was continuously recorded at 412 nm with a UV/VIS spectrophotometer. The total amount of glutathione in the samples was determined from a standard curve obtained by plotting the known amount of glutathione *v.* the rate of change of absorbance at 412 nm.

Antioxidant enzyme activities

Liver tissue was homogenised in 9 volumes of 20 mM-phosphate buffer containing 0·1 M-KCl, 1 mM-EDTA and 0·5 % Triton X-100 (pH 7·4). The homogenate was centrifuged (25 000 rpm and 4°C) for 30 min, and the supernatant fraction was used for the following enzyme assays. Glutathione S-transferase (GST) activity was determined using 1-chloro-2,4-dinitrobenzene as a substrate in the presence of 0·1 mM-GSH according to the method of Habig *et al.* [19]. The formation of dinitrophenyl thioether by GST was monitored for 3 min at 37°C using a spectrophotometer with absorbance at 340 nm. The values were expressed as the mean values and standard deviations of quadruplicate sets. Glutathione reductase was determined using the method described by Pinto *et al.* [20]. The supernatant fraction was mixed with 1 M-glutathione disulfide (GSSG) and 5 mM-NADPH in 0·1 M-phosphate−0·5 mM-EDTA buffer (pH 7·0), and the formation of NADP + was monitored with a spectrophotometer at 340 nm[18]. To determine glutathione peroxidase activity, the supernatant fraction was mixed with 1 mM-EDTA, 100 mM-GSH, 5 mM-NADPH and 1 unit of glutathione reductase in 0·1 M-phosphate buffer (pH 7·0), and incubated for 3 min. Cumene hydroperoxide (10 mM) was added into the reaction mixture, and the oxidation of NADPH into NADP + was monitored spectrophotometrically by a decrease in absorbance at 340 nm. One unit of glutathione peroxidase causes the formation of 1 μmol NADP + per min[21]. For the SOD assay, the homogenate was mixed with 1 mM-xanthine, 0·2 mM-cytochrome and 0·05 M-potassium cyanide in 0·05 M-potassium phosphate−0·1 mM-EDTA buffer, and then xanthine oxidase was added into the reaction mixture. SOD activity was measured as the inhibition of the rate of reduction of cytochromes by superoxide radicals, observed spectrophotometrically at 550 nm[22]. The activities of glutathione reductase, glutathione peroxidase and SOD were expressed as international units/mg liver tissue.

To determine the activity of plasma paraoxonase, the mouse plasma (5 μl) was transferred to the assay buffer containing 20 mM-2-amino-2-hydroxymethyl-propane-1,3-diol (Tris)−HCl and 1 mM-CaCl₂, and then 0·2 M-phenylacetate was added as a substrate. Paraoxonase activity was measured with the rate of formation of phenol by monitoring the increase spectrometrically at the absorbance at 270 nm. One unit of paraoxonase activity is equal to 1 μmol phenol per min[23].

β-Carotene content in plasma

The mouse plasma (100 μl) was mixed with ethanol (500 μl) and 91 mM-butylated hydroxytoluene in methanol (25 μl) in a test-tube in which β-apo-8′-carotenal as an internal standard was added into the mixture. Plasma was extracted by adding hexane (3 ml) and vortexing for 30 s. The organic phase containing β-carotene was separated by distilled water. After centrifugation at 1500 rpm for 2 min, the upper phase was collected, and hexane was evaporated under an N₂ stream. The residue was re-dissolved in 30 μl isopropanol−acetonitrile (50:50, v/v), and injected into an HPLC equipped with a UV detector set at 450 nm. A Nova-Pak C18 column (4 μm, 150 × 3·9 mm internal diameter; Waters, Milford, MA, USA) was used for the separation. Elution was performed with an isocratic mobile phase of acetonitrile−isopropanol−methanol (68:20:12, by vol.) with 0·02 % ammonium acetate, and the flow rate was 1·0 ml/min. The amount of β-carotene was determined with a calibration curve, and triplicate analysis was performed.

DNA damage of hepatocytes and lymphocytes

Liver tissue was homogenised using a Polytron homogeniser at 600 (SD 10) rpm with ten volumes of 50 mM-Hanks' balanced salt solution with 1 mM-EDTA (pH 7·4). Cells

were washed twice before the gel electrophoresis assay. The endogenous DNA damage in lymphocytes and hepatocytes was analysed by alkaline single-cell gel electrophoresis (comet assay) according to the method of Singh *et al.*[24] with a slight modification. Briefly, frosted slides were prepared with two basal layers of 0·5 % normal-melting agarose, one middle layer of lymphocytes (or hepatocytes) mixed with 75 µl of 0·7 % low-melting agarose, and a top layer of 75 µl of 0·7 % low-melting agarose, to make a microgel on each slide. Each layer was covered with a cover slip and kept in a refrigerator for at least 10 min before adding the next layer. After removal of the cover slips from the top layer, the slides were immersed in a jar containing cold lysing solution (pH 10) consisting of 2·5 M-NaCl, 100 mM-EDTA, 10 mM-Tris and 1 % sodium laurylsarcosine, in which 1 % Triton X-100 and 10 % dimethyl sulfoxide were freshly added to the solution upon immersion of the slides. The jars were then stored in the refrigerator for 1 h. After the lysis procedure, the slides were placed in a horizontal electrophoresis tank and submerged in fresh alkaline buffer (300 mM-NaOH and 10 mM-Na₂EDTA, at pH 13·0) for 40 min at 4°C. For electrophoresis of the DNA, an electric current of 25 V and 300 mA was applied for 20 min at 4°C. The slides were washed three times with neutralising buffer (0·4 M-Tris, pH 7·5) for 5 min at 4°C, and then washed with ethanol for another 5 min. All of these steps following the lysis treatment were processed in the dark to prevent additional DNA damage.

Image analysis

DNA was stained with 30 µl ethidium bromide. Observations were made using a 40 × objective lens on a fluorescent microscope equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. A total of 100 cells per animal (fifty cells on each of two replicate slides) were analysed for DNA migration. The tail length, tail DNA and tail moment of DNA were measured by a comet image analysing system[25].

Statistical analysis

Data are presented as the mean values and standard deviations, and statistical analyses were performed using SAS software (release 8.2; SAS Institute, Inc., Cary, NC, USA)[26]. The differences between means were assessed by the Student's *t* test, and statistical significance was defined at $P < 0.05$.

Results

Food intake and body weight

After 4 weeks of 8 % freeze-dried red-pigmented leafy lettuce supplemented to the high-fat high-cholesterol diet, the weight gain, food intake and food efficiency ratio were not significantly different between the HFC and HFL C57BL/6J mice (Table 2). Visible fat deposition in major areas (retroperitoneal, mesenteric, epididymal, inguinal and splenic adipose tissue) was not significantly affected by the lettuce diet, and the weight of the total fat pad of mice fed the lettuce diet (4·67 g per 100 g body weight) was smaller than that of the

Table 2. Weight gain, food intake and food efficiency ratio (FER) of C57BL/6J mice after 4 weeks of experimental feeding†

(Mean values and standard deviations)

	Weight gain (g)		Food intake (g/d)		FER (%)	
	Mean	SD	Mean	SD	Mean	SD
HFC	3·73	1·2	2·98	0·3	4·47	2·2
HFL	2·63	0·5	3·29	0·3	2·86	1·6

HFC, high-fat control diet; HFL, high-fat diet with freeze-dried red lettuce (*Lactuca sativa*).
† There were no differences between the two groups (Student's *t* test; $P > 0.05$).

control mice (5·09 g) (Table 3). Organ weights (per 100 g body weight) of mice were compared between the HFC and HFL groups, and no significant weight differences were found in liver (4·06 g in HFC *v.* 4·10 g in HFL), heart (0·42 *v.* 0·41), and kidney (1·21 *v.* 1·20 g) and brain (0·98 *v.* 0·91 g).

Plasma and faecal lipid profiles

Plasma TAG level was significantly decreased by 23 % in the HFL mice compared with the HFC mice ($P < 0.01$) (Table 4). Total and LDL-cholesterol concentrations were significantly decreased by 8 and 56 %, respectively ($P < 0.05$). Therefore, the atherosclerotic index in the HFL mice was markedly changed, showing a significant 46 % reduction by the red-pigmented leafy lettuce diet ($P = 0.013$). HDL-cholesterol concentration was increased by 4 % in the HFL mice, but it was not significant ($P > 0.05$). Faecal weight was significantly increased by 53 % in the HFL mice compared with the HFC mice ($P = 0.018$) (Table 5). Faecal excretion of cholesterol and TAG per d (µmol/d) was increased by 29 and 13 %, respectively, in the HFL mice but the changes were not significant ($P > 0.05$), whereas the amounts of cholesterol and TAG excreted based on the faecal weight (mg per g faeces) were lower in the HFL mice than the HFC mice.

Antioxidant status

Fig. 1 shows that the malondialdehyde formations determined by the TBARS value in organs were significantly lowered in plasma (−39·75 %) and the liver (−19·86 %), heart (−25·10 %) and kidney (−17·19 %) tissues of mice fed with HFL diet compared with those of mice fed the HFC diet ($P < 0.05$). The concentration of plasma β-carotene was 0·073 nmol/ml in the HFL group; however, β-carotene was not detected in the plasma of the HFC mice (Table 6). The activities of antioxidant enzymes in liver and plasma are presented in Table 6. In liver tissue, a significantly higher concentration of total glutathione in the HFL mice was observed compared with the HFC mice ($P < 0.05$). The activities of liver GST, glutathione peroxidase and plasma paraoxonase were also significantly higher in the HFL group by 41, 18 and 19 %, respectively ($P < 0.05$) compared with the HFC group. Whereas no significantly different activities of glutathione reductase and superoxide dismutase in liver tissue were observed between the HFC and HFL mice

Table 3. Weight of visible fat (g/100 g body weight) of C57BL/6J mice after 4 weeks of experimental feeding†

(Mean values and standard deviations)

	Retroperitoneal		Mesenteric		Epididymal		Inguinal		Splenic		Total fat	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
HFC	0·70	0·11	1·05	0·61	1·70	0·62	1·08	0·12	0·57	0·13	5·09	0·81
HFL	0·68	0·18	0·72	0·12	1·78	0·26	0·95	0·17	0·54	0·08	4·67	0·57

HFC, high-fat control diet; HFL, high-fat diet with freeze-dried red lettuce (*Lactuca sativa*).
† There were no differences between the two groups (Student's *t* test; $P > 0.05$).

Table 4. Plasma lipid profile (mg/l) and atherogenic index of C57BL/6J mice after 4 weeks of experimental feeding

(Mean values and standard deviations)

	Total cholesterol		HDL-cholesterol		TAG		LDL-cholesterol		AI†	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
HFC	1804	110	1328	60	812	61	311	101	0·35	0·1
HFL	1657*	29	1392	62	623**	91	139*	76	0·19*	0·1

AI, atherogenic index; HFC, high-fat control diet; HFL, high-fat diet with freeze-dried red lettuce (*Lactuca sativa*).
Mean value was significantly different from that of the HFC group (Student's *t* test): * $P < 0.05$, ** $P < 0.01$.
† AI = (total cholesterol − HDL-cholesterol)/HDL-cholesterol.

Table 5. Faecal weight and faecal cholesterol and TAG levels of C57BL/6J mice after 4 weeks of experimental feeding

(Mean values and standard deviations)

	Faecal weight (g/d)		Cholesterol				TAG			
			μmol/d		mg/g		μmol/d		mg/g	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
HFC	0·15	0·06	44·9	14·9	115·7	13·8	2·4	0·4	13·9	2·9
HFL	0·23*	0·02	57·8	9·5	97·6	11·4	2·7	0·9	10·4	3·1

HFC, high-fat control diet; HFL, high-fat diet with freeze-dried red lettuce (*Lactuca sativa*).
* Mean value was significantly different from that of the HFC group (Student's *t* test; $P < 0.05$).
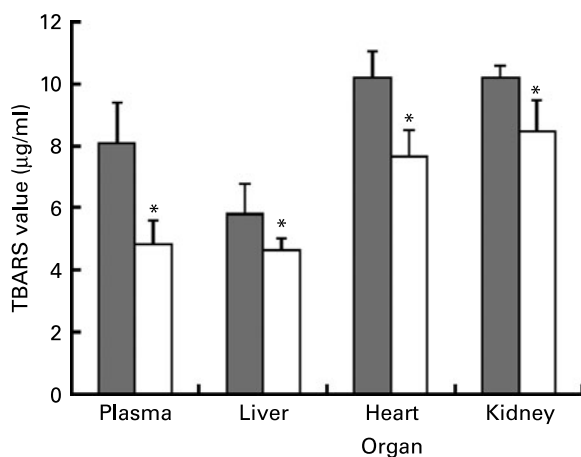
Fig. 1. Thiobarbituric acid-reactive substances (TBARS) values (μg/ml) in plasma and different organs of C57BL/6J mice after 4 weeks of experimental feeding of a high-fat control diet (■) or a high-fat diet with freeze-dried red lettuce (*Lactuca sativa*) (□). Values are means, with standard deviations represented by vertical bars. * Mean value was significantly different from that of the group fed the high-fat control diet (Student's *t* test; $P < 0.05$).

(Table 6), an increasing trend of their activities, however, was shown in the HFL mice.

Oxidative damage of hepatocyte and lymphocyte DNA

After 4 weeks of red-pigmented leafy lettuce intake, the extent of DNA oxidative damage caused by the high-fat high-cholesterol diet was determined by comet assay. Results are presented in Table 7 and Fig. 2. In liver, the comet parameters such as tail DNA (%), tail extent moment, olive tail moment and tail length were significantly lowered by 28·16, 63·52, 49·67 and 47·39 %, respectively, in the HFL mice compared with the HFC mice ($P < 0.05$), indicating that the red-pigmented leafy lettuce diet improved the hepatocyte DNA damage in the HFL mice. The lymphocyte DNA was also influenced by the dietary lettuce since the tail DNA (%), tail extent moment and olive tail moment were found to be significantly decreased by 29·89, 45·70 and 36·32 %, respectively, in the HFL mice ($P < 0.05$) (Table 7 and Fig. 2). Therefore, the consumption of 8 % freeze-dried red-pigmented leafy lettuce with the high-fat high-cholesterol diet reduced the susceptibility of hepatocyte and lymphocyte DNA to oxidative damage.

Table 6. Antioxidants and antioxidant enzymes in liver and plasma of C57BL/6J mice after 4 weeks of experimental feeding

(Mean values and standard deviations)

Biomarkers		HFC Mean	SD	HFL Mean	SD
Total glutathione	Liver (mg/ml)	1·07	0·11	1·48*	0·14
GST	Liver (nmol/min per mg protein)	1·31	0·43	1·84*	0·11
GPx	Liver (units/mg protein)	1·13	0·11	1·33***	0·07
GR	Liver (units/mg protein)	0·45	0·05	0·54	0·09
SOD	Liver (units/mg protein)	0·52	0·06	0·59	0·08
Paraoxonase	Plasma (units)	43·6	6·50	52·1*	8·2
β-Carotene	Plasma (nmol/ml)	ND	–	0·073	0·057

HFC, high-fat control diet; HFL, high-fat diet with freeze-dried red lettuce (*Lactuca sativa*); GST, glutathione S-transferase; GPx, glutathione peroxidase; GR, glutathione reductase; SOD, superoxide dismutase; ND, not detected.
Mean value was significantly different from that of the HFC group (Student's t test): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

Table 7. Hepatocyte and lymphocyte DNA oxidative damage of C57BL/6J mice after 4 weeks of experimental feeding

(Mean values and standard deviations)

	Hepatocytes HFC Mean	SD	HFL Mean	SD	Lymphocytes HFC Mean	SD	HFL Mean	SD
Tail DNA (%)	13·39	4·12	9·62*	2·96	12·31	2·11	8·63***	1·73
Tail extent moment	8·06	4·68	2·94*	1·77	3·26	1·91	1·77*	0·71
Olive tail moment (arbitrary units)	4·51	1·16	2·27***	0·86	2·12	0·72	1·35*	0·34
Tail length (μm)	49·53	11·02	26·06***	10·75	19·43	11·21	16·13	4·63

HFC, high-fat control diet; HFL, high-fat diet with freeze-dried red lettuce (*Lactuca sativa*).
Mean value was significantly different from that of the HFC group (Student's t test): * $P < 0.05$, *** $P < 0.005$.

Discussion

C57BL/6J mice were a fed high-fat high-cholesterol diet (20 % fat and 1 % cholesterol) for 4 weeks to induce atherogenic profiles. Since there was no control group fed a standard diet in the present study, we compared the outcome with our previous study[27], in which the same mice fed a standard diet (5·6 % fat) had much lower concentrations of plasma cholesterol (984 mg/l) and TAG (663 mg/l) after the same experimental period. A regular chow (4 % fat) diet for 12 weeks resulted in a plasma cholesterol concentration of about 700 mg/l with C57BL/6J mice[28]. Further, in the report of Nicolle *et al.*[29], the mice fed a 5 % fat standard diet had a plasma cholesterol concentration of 2·31 mmol/l (893 mg/l) and a plasma TAG concentration of 0·63 mmol/l (543 mg/l), the levels of which increased by 102 and 50 %, respectively in the present study.

The red-pigmented leafy lettuce diet elicited a significant TAG- and cholesterol-lowering effect in the plasma of mice ($P < 0.05$), and could be considered as beneficial for the

Fig. 2. Comet image of lymphocyte DNA of C57BL/6J mice fed a high-fat control diet (a) and a high-fat diet with freeze-dried red lettuce (*Lactuca sativa*) (b).

prevention of CVD. This effect is in keeping with a previous investigation on animals supplemented with vegetables, showing that 20 % lyophilised lettuce was able to lower plasma cholesterol as well as increase faecal cholesterol and bile acid secretion in rats[13]. Similarly, the same author[29] reported that the diet supplemented with 20 % carrot plus 0·25 % cholesterol showed a remarkable reduction of cholesterol by 41 % in mice after 4 weeks of feeding.

This cholesterol-lowering effect in mouse plasma could be explained by an appreciable amount of fibre contained in the lettuce[13,30]. During lipid digestion, bile acids emulsify and solubilise lipids and cholesterol by forming micelles to promote hydrolysis by lipase, and facilitate their absorption by the small intestine. Soluble fibres could exert a destabilising effect on lipid emulsions, and impair the action of lipase on the emulsion[31]. As a result, these fibres inhibit digestible cholesterol absorption, and increase the faecal excretion of neutral sterols, especially cholesterol[12,13]. Consequently, the increase in faecal excretion leads to an increase of cholesterol metabolism in the liver, which results in lowered cholesterol levels in the plasma.

It is also conceivable that there could be synergistic effects with other constituents of red-pigmented leafy lettuce such as antioxidants (β-carotene, anthocyanin or α-tocopherol) or polyphenolic compounds on the cholesterol-lowering effect in mouse plasma. In the present study, the HFL diet contained 4·26 mg β-carotene, 0·274 mg α-tocopherol and 21·64 mg anthocyanin per 100 g diet. Similar results to the present study have been reported; β-carotene reduced the plasma cholesterol content in rabbits fed an atherogenic diet (0·5 % cholesterol), and
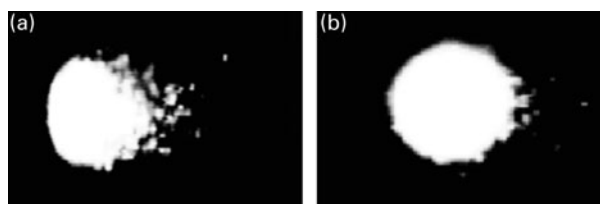
the more significant lowering effect was observed in a combined β-carotene and α-tocopherol diet[32]. Beneficial effects of anthocyanin have been reported; the administration of anthocyanin-rich juice such as blackcurrant juice (58 mg per 100 ml) and *Aronia melanocrpa* fruit juice (106·8 mg per 100 ml) lowered plasma cholesterol concentrations in rabbits and rats fed a standard diet and a high-cholesterol diet (4 %), respectively[33,34]. However, an adverse effect of purified anthocyanin (100 mg/ 100 g standard diet) from blackcurrants was observed on total and LDL-cholesterol in the plasma of rabbits[34]. This effect suggests that the other components contained in the juices and red lettuce reduce the adverse effect of anthocyanins on cholesterol metabolism.

Most clinical trials have been failed to show beneficial effect of an individual antioxidant or antioxidant cocktails on CVD for the past 10 years[35]. Although some studies with a small population (i.e. Antioxidant Supplementation in Atherosclerosis Prevention study in 2000) reported beneficial effects, most trials such as the Alpha-Tocopherol Beta-Carotene Cancer Prevention Study in 1994 and 1998, and the Heart Protection Study in 2002 showed no effect or adverse effect of antioxidants by long-term consumption (about 6 years). However, according to the meta-analysis of cohort studies, the intake of various antioxidants by fruit and vegetable consumption is inversely associated with the risk of CHD[36].

As a part of the normal metabolic process, cells continuously produce ROS. In biological systems, cells are normally able to defend themselves against ROS through the use of numerous antioxidants (i.e. vitamins A, E and C, glutathione and flavonoids), and antioxidant enzymes (i.e. SOD, glutathione peroxidase, paraoxonase and GST)[5]. However, oxidative stress is caused by an imbalance between the production of ROS and neutralisation by an elaborate antioxidant defence system[4]. Such disturbances in this normal redox state result in deleterious effects that cause damage to biomolecules (i.e. lipids, amino acids, protein and DNA). A high-fat diet is believed to induce oxidative stress that plays an active part in developing CVD linked to atherosclerosis.

For assessing oxidative stress in mice, a measurement of the products of oxidised macromolecules (i.e. lipid peroxidation) and a determination of endogenous antioxidant capacity were approached in the present study. Malondialdehyde is used as a reliable biomarker of oxidative stress in blood and organ tissues, and the malondialdehyde content was measured by the TBARS value. The supplementation of red-pigmented leafy lettuce considerably decreased TBARS values in the blood and organs of mice, and showed a positive antioxidant effect in the mice. The lower TBARS value could be related to a marked increase of antioxidant nutrients (β-carotene and glutathione) and distinctly increased activities of antioxidant enzymes such as GST, glutathione peroxidase, glutathione reductase and SOD in liver, and paraoxanase in plasma. Aprikain *et al.* [37] also reported similar results on improved lipid peroxidation status in rats after feeding with 15 % lyophilised apple in a cholesterol diet.

The glutathione (γ-glutamyl-cysteinyl-glycine, GSH) system plays an important role in antioxidant defence by scavenging free radicals and ROS, removing lipid peroxides and preventing the oxidation of biomolecules[38]; therefore, GSH ultimately prevents the build-up of oxidised fats that may contribute to atherosclerosis. GSH is oxidised by ROS to form glutathione disulfide (GSSG) and, hence, facilitates

the removal of ROS. In addition, the endogenously produced H_2O_2 or other peroxides are reduced by GSH in the presence of glutathione peroxidase to form GSSG. Then the GSSG is readily converted to GSH by the NADPH-dependent glutathione reductase[39]. GST also facilitates the detoxification reaction by conjugating electrophilic compounds with GSH, and these conjugates formed are excreted from the cell (i.e. hepatocytes) into bile acid[40]. SOD is one of the primary enzymic antioxidant defences against superoxide radicals, and the increased activity has been reported to enhance the resistance to oxidative stress in the cell[41]. Paraoxanase, which is located on HDL in serum, is highly effective in preventing the lipid peroxidation of LDL. An animal study with transgenic paraoxonase knockout mice indicated that paraoxonase was a potent inhibitor of LDL oxidation, and could protect against CHD[42]. In an *in vitro* experiment, red-pigmented leafy lettuce efficiently inhibited Cu ion-induced oxidation of human LDL[10]. Therefore, it appears that red-pigmented leafy lettuce supplementation contributes to reduce oxidative stress by an improved antioxidant capacity through activation of the GSH system, and by the suppressed formation of malondialdehyde in mice.

Since the liver serves as the major detoxifying organ in the body, and lymphocytes play a number of roles in the immune system, we measured the oxidative damage of endogenous DNA of hepatocytes and lymphocytes as biomarkers of genetic change. The present results demonstrated that red lettuce supplementation influenced the level of DNA damage of high-fat high-cholesterol-fed mice, and resulted in a positive correlation between antioxidant levels (i.e. antioxidants and antioxidant enzymes) and oxidative DNA damage. This indicates that DNA was protected by antioxidants in the lettuce diet against the attack by endogenously produced ROS. The results are comparable with the study of Riso *et al.* [43] regarding the lymphocyte DNA status of females who consumed a tomato-rich diet. The consumption of tomato for 3 weeks significantly reduced DNA tail moment by about 33 to about 42 %, and increased the resistance of lymphocyte DNA to oxidative damage. In addition, a high intake of fruits and vegetables containing a high concentration of vitamins C and E and β-carotene induced a significant decrease in oxidative damage to DNA, giving support to the protective effect of fruits and vegetables against oxidative damage to DNA[44].

In the present study, the dietary supplementation with 8 % freeze-dried red-pigmented leafy lettuce into a high-fat high-cholesterol diet in C57BL/6J mice was observed to decrease levels of plasma total and LDL-cholesterol and TAG, lower lipid peroxidation in the blood and tissues, improve antioxidant status and provide a resistance of DNA to oxidative damage. These effects might be contributed by fibres, antioxidant components and other various micronutrients contained in the red-pigmented leafy lettuce. Therefore, the present results suggest that a regular intake of red-pigmented leafy lettuce is a good way to reduce oxidative damage caused by ROS, and also to prevent the development of CVD.

Acknowledgements

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