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ORIGINAL ARTICLE

Long-term walnut supplementation without dietary advice induces favorable serum lipid changes in free-living individuals

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Background/Objectives: Walnuts have been shown to reduce serum lipids in short-term well-controlled feeding trials. Little information exists on the effect and sustainability of walnut consumption for longer duration in a free-living situation.
Subjects/Methods: A randomized crossover design in which 87 subjects with normal to moderate high plasma total cholesterol were initially assigned to a walnut-supplemented diet or habitual (control) diet for a 6-month period, then switched to the alternate dietary intervention for a second 6-month period. Each subject attended seven clinics 2 months apart. At each clinic, body weight was measured, and in five clinics (months 0, 4, 6, 10 and 12), a blood sample was collected.
Results: Our study showed that supplementing a habitual diet with walnuts (12% of total daily energy intake equivalent) improves the plasma lipid profile. This beneficial effect was more significant in subjects with high plasma total cholesterol at baseline. Significant changes in serum concentrations of total cholesterol ($P=0.02$) and triglycerides ($P=0.03$) were seen and nearly significant changes in low-density lipoprotein cholesterol (LDL-C) ($P=0.06$) were found. No significant change was detected in either high-density lipoprotein (HDL) cholesterol LDL to HDL ratio.
Conclusions: Including walnuts as part of a habitual diet favorably altered the plasma lipid profile. The lipid-lowering effects of walnuts were more evident among subjects with higher lipid baseline values, precisely those people with greater need of reducing plasma total and LDL-C.
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Keywords: walnuts; total cholesterol; triglyceride; LDL-C

Introduction

It is widely recognized that diet is one of the important factors in lowering the risk of cardiovascular disease. Dietary guidelines for cardiovascular disease prevention emphasize the reduction of fat intake or replacement of saturated fatty acids by unsaturated fatty acids (US Department of Health and Human Services, 2005). Another approach that might invigorate current knowledge is the addition of a whole food to the existing diet for the same beneficial effect. Nuts, as whole food, have received particular attention because of the epidemiological association of frequent intake with protection from coronary heart disease (Fraser *et al.*, 1992; Sabaté and Fraser, 1994; Kushi *et al.*, 1996; Hu *et al.*, 1998; Spiller *et al.*, 1998; Hu and Stampfer, 1999). Walnuts have a high fat content, as do other nuts, but are low in saturated fatty acids. Walnuts have consistently reduced serum cholesterol and improved the lipoprotein profile in several well-controlled short-term clinical trails (Sabaté *et al.*, 1993; Almario *et al.*, 2001; Iwamoto *et al.*, 2002; Morgan *et al.*, 2002; Zibaeenezhad *et al.*, 2005) and recently reviewed by Banel and Hu (2009). The Food and Drug Administration issued a qualified health claim that says, 'Supportive but not conclusive research shows that eating 1.5 ounces per day of walnuts, as part of a low saturated fat and low cholesterol diet may reduce the risk of coronary heart disease' (Food and Drug Administration, 2003).

Considering previous research on walnuts, there are questions that remain unanswered: (1) Can the lipid-lowering

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effect of walnut consumption in the short term be maintained and extended to a long-term period in a free-living situation in which no dietary advice is given to participants? (2) Can people sustain a diet with daily walnut consumption for a long duration such as 6 months or more? Most of the previous studies were conducted either over a short period of time or with a modified diet plan (Banel and Hu, 2009). We designed a long-term study in which free-living healthy adults participated to compare the effects on serum lipid and lipoprotein levels of a walnut-supplemented diet without any dietary advice, with those of their habitual diet. This report on serum lipids is a sub study of a study on the long-term effect of walnut on body weight (Sabaté *et al*., 2005).

Subjects and methods

Subjects

In all, 113 nonsmoking men and women aged 30–72 years were recruited from Southern California communities using advertisement flyers. They all underwent a selection process, which included two telephone interviews, an informational meeting and a person-to-person interview. Subjects were eligible as they met the following inclusion criteria: (i) apparently healthy as indicated by a general medical questionnaire; (ii) body mass index $\leqslant 30\,kg/m^2$; (iii) not allergic or sensitive to nuts; (iv) not pregnant or lactating; (v) eager to keep a habitual diet similar to a typical American diet and willing to not consume any other nuts during the study; (vi) not follow a weight-reducing diet; and (vii) not taking any lipid-lowering medication and/or dietary supplements. After the selection process, 94 people were enrolled in the study. Two of the ninety-four participants dropped out because of compliance difficulty, two were dropped when diagnosed with a metabolic disorder at the time of the study, and three were excluded from the statistical analysis because they were found to be taking lipid-lowering medications. A total of 87 people, 49 females and 38 males aged 30–72 years comprised the analytic study population. Table 1 presents descriptive characteristics of study participants at baseline. The average age was 54 years, and mean body mass index, weight and height were $26.5\,kg/m^2$, 75.6 kg and 169.5 cm, respectively. The study protocol was approved by the University institutional review board before the study. All volunteers signed a written informed consent before beginning the study and were free to withdraw from the study at any time without obligation.

Study design

This study used a randomized crossover design in which subjects were initially assigned to a walnut-supplemented diet or habitual (control) diet for 6 months. The subjects were then crossed over to the alternate diet and continued for another 6 months. A crossover design was used to eliminate ordering effects. Each subject participated in seven clinic visits 2 months apart. At each clinic visit body weight was measured, and in five of these visits (months 0, 4, 6, 10 and 12) blood samples were collected.

Table 1 Descriptive characteristics of study participants at the baseline[a]

Characteristics	*Values*
Age (years)	54 ± 10.2
Gender (% female)	56
Weight (kg)	75.6 ± 13.23
Height (cm)	169.5 ± 9.65
BMI (kg/m^2)	26.5 ± 3.3
Fat %	31 ± 7.09
Lipid values at baseline	
Total cholesterol (mmol/l)	5.72 ± 0.83
LDL cholesterol (mmol/l)	3.35 ± 0.88
HDL cholesterol (mmol/l)	1.53 ± 0.39
LDL: HDL	2.37 ± 0.91
Triglycerides (mmol/l)	1.39 ± 0.77

Abbreviations: BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
[a]All results except gender are expressed in means ± s.d.

Diet

While assigned to the walnut-supplemented diet, participants were expected to consume the provided amount of walnuts (12% of total energy intake), which was the equivalent of 28–64 g/day based on individual characteristics, such as gender, height, weight, age and level of physical activity. The total energy need for each participant was estimated with the Harris-Benedict equation before the study. For subsequent clinical visits, the prescribed walnut amount was adjusted based on the daily energy intake as assessed by 24-h dietary recalls. Conversely, while on the control diet, participants were advised to continue their habitual diet and refrain from eating walnuts and substantial amounts of any other nuts during this period. Daily amounts of walnuts were provided in individual packages and subjects were instructed to consume the walnuts everyday. Subjects were advised to avoid additional walnut consumption outside of that provided by the study and to maintain their regular diets. To prevent confounding, we instructed the participants not to change their physical activity and dietary intake, and not to attempt to lose weight while in the study. No other dietary guidance was given to keep the study as free-living as possible.

Compliance

Dietary compliance was defined as non-intake of walnuts (intake <2 g) during the control period and intake of the allotted amounts of walnuts (intake $\geqslant 28$ g) during the walnut-supplemented period. To ensure whether the subjects had eaten all the supplied walnuts and whether they had consumed nuts other than those that were provided, 14

dietary recalls were collected during the 12-month study. Seven recalls were collected during each 6 months. Recalls included 2 weekend days and 5 nonconsecutive weekdays, in order to capture daily variations in intake. Dietary recalls were unannounced and administered nonconsecutively to reduce the possibility of subjects changing their intake. Nutrient intake was evaluated using Nutritional Data Systems software (NDS-R) Version 5.0, developed by the Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN, USA, Food and Nutrient Database 29. Compliance was also assessed at the end of each dietary period by measuring the concentration of two essential fatty acids, linolenic and α-linolenic, in the erythrocyte membrane.

Blood sample collection and body weight measurement

The subjects attended each morning clinic after fasting 12 h. Whole blood was collected into tubes containing EDTA to determine plasma lipids. Plasma was separated by centrifugation at 1500 *g* for 20 min at room temperature within 1 h of extraction. The resulting plasma was frozen at −80 °C and shipped in batches to the Laboratory for Clinical Studies (University of Connecticut, Department of Nutritional Sciences). Plasma total cholesterol, high-density lipoprotein cholesterol (HDL-C) and Triacylglycerol (TAG) were determined using methods previously reported, and low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald formula. Crude Values (CVs) assessed by the Standardization Program during a recent study were 0.76–1.42% for total cholesterol, 1.71–2.72% for HDL-C, and 1.64–2.47% for triglycerides.

Total red cell fatty acids were extracted with isopropanol and chloroform according to the method of Rose and Oklander (1965). This was followed by preparative chromatography to separate individual lipid classes from each extract (Watkins *et al.*, 2001). Lipid fractions were transesterified in 3 N methanolic HCl in a sealed vial under N_2 atmosphere at 100 °C for 45 min. The resulting fatty acid methyl esters were extracted with hexane containing 0.05% butylated hydroxytoluene and prepared for gas chromatography by sealing the extracts under N_2. Fatty acid methyl esters were separated and quantified on an HP-88 column and a 6800 capillary gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame-ionization detector. Body weight was also measured at baseline and at each clinic visit to account for the potential influence of this factor on plasma lipid levels. Weight measurements were recorded to the 0.1 of a kilogram. Subjects were weighed twice and the mean was used for data analysis (Lohman *et al.*, 1991).

Statistical analysis

Our primary interest was the contrast between the walnut-supplemented and control diet in the main outcomes of total cholesterol, triglycerides, LDL and HDL-C, and the ratio between LDL and HDL-C. For each outcome the between-diet differences in end-of-period measurements was considered. To reduce intraindividual variability in blood lipid determination, the results of all lipid measurements made at the last two clinics of each diet period (months 4 and 6, and months 10 and 12) were averaged. Subsequent analysis was on the basis of each subject's average value for that period. SAS 9.1 (SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses (PROC MIXED procedure). Data are given as means ± s.d. Changes from baseline were used as the dependent variables. If the original data were of approximate normal distribution, then changes from baseline were calculated on the original scale; if a log transformation was deemed appropriate, then changes from baseline were calculated on the log scale, and these changes then corresponded to a multiplicative change from the baseline original scale. Statistical significance was defined as $P<0.05$ (two-sided) with and without adjustment for body weight.

Results

Across the treatment sequence groups and the diet periods, degree of compliance, as assessed in the dietary recalls, was excellent at about 95%. In addition, of the 87 subjects, 67.5% showed an increase in the red blood cell of linoleic acid and a 90% increase was found in α-linolenic acid. The sum of both fatty acids was 68% higher during the walnut-supplemented diet compared with the values during control diet. Table 2 presents the mean of red blood cell fatty acids, linoleic and α-linolenic acid in both walnut-supplemented and control diet during the study period.

Table 2 Mean changes of key red blood cell fatty acids, linoleic and α-linolenic acids (mole %) during consumption of walnut-supplemented and control diets for 6 months by healthy, free-living men and women[a,b]

Fatty acids	*Diet*		*Difference between diets*	P-*value*
	Walnut-supplemented	*Control*		
Linoleic acid (18:2ω6)	12.61 ± 0.23	11.41 ± 0.23	1.2 ± 0.26	<0.0001
α-Linolenic acid (18:3ω3)	0.257 ± 0.006	0.185 ± 0.006	0.072 ± 0.007	<0.0001

[a]All values are least square (geometric) mean ± s.e. in (mole%); $n=87$ (38 men and 49 women).
[b]Analysis was performed on log-transformed values.

After evaluating the 24-h dietary recalls, we found that subjects while on the walnut-supplemented diet had a higher total energy consumption, 8171 kJ (1952 kcal), than during the control diet, 7614 kJ (1819 kcal). The mean difference between daily total energy intake during the two diet periods (mean (s.d.) = 557 (142) kJ (133 (34) kcal)) is less than the actual energy intake from walnuts (967 kJ (231 kcal)).

The effects of the two diets on serum lipid and lipoprotein concentrations are given in Table 3. Significant changes in serum concentrations of total cholesterol ($P = 0.02$ for diet effects) and triglycerides ($P = 0.03$ for diet effects) were found. Effects on LDL ($P = 0.1$) and HDL-C ($P = 0.47$) and ratio between LDL and HDL ($P = 0.85$) concentrations did not differ between the two diets. After adjusting for body weight, the effects of the walnut-supplemented and control diets on serum lipid and lipoprotein concentration showed a significant change in serum concentrations of total cholesterol ($P = 0.01$), triglycerides ($P = 0.01$) and borderline significance for LDL-C ($P = 0.06$). No significant change was detected in either HDL-C or LDL to HDL ratio.

Figure 1 presents the estimated difference of effect between the walnut-supplemented and control diets on plasma total and LDL-C, and the LDL-C to HDL-C ratio according to the plasma total cholesterol values at baseline. After adjusting for body weight, a statistically significant diet–baseline value interaction effect was observed for overall total cholesterol (*P*-value for interaction = 0.018), LDL-C (*P*-value for interaction = 0.014), LDL-C and HDL-C ratio (*P*-value for interaction = 0.007). Compared with the control diet, lipid-lowering effect of walnuts was magnified in subjects with higher baseline total cholesterol levels. All blood lipid components showed a significant reduction at the 80th percentile after walnut ingestion. For total cholesterol, results were significant at the 60th (TC > 5.87 mmol/l) ($P = 0.005$) and 80th percentile (TC > 6.38 mmol/l) ($P = < 0.001$), whereas in the 20th percentile (TC < 4.94 mmol/l) total serum cholesterol concentrations did not differ between the two diets ($P = 0.88$). In both serum LDL-C and LDL to HDL-C ratio, significant diet–baseline interaction effect was observed at the 80th percentile. No significant change was observed for either triglycerides or HDL-C in either of the two diet groups.

Discussion

In this 12-month crossover dietary intervention trial on 87 free-living adults with normal to moderate high plasma total cholesterol, we found that supplementing walnuts for ~12% of total daily energy intake without changing habitual diet decreased total cholesterol and triglyceride levels. The beneficial effects of walnuts were more evident in subjects with higher baseline total cholesterol values. As the main purpose of this crossover feeding trial was to investigate the long-term effect of walnut consumption on body weight change (Sabaté *et al*., 2005), no intentional dietary modifications were applied during the study. Thus, adjusting for changes in body weight (−0.5 to 0.4 kg) provides the best estimate of the dietary effects on blood lipid.

In previous studies, nuts were used to replace other forms of fat such as animal fat and saturated fat (Dreaon *et al*., 1990; Ginsberg *et al*., 1990; Abbey *et al*., 1994; Hodson *et al*., 2001). Some other studies such as those conducted by Tapsell *et al*. (2004, 2009) three dietary advice groups of type II diabetics were given daily walnuts. Our study was designed to add walnuts to an existing diet and use a 'free-living' situation for a long duration of 6 months with no dietary advice. Our findings showed that without adjusting for body weight, total cholesterol and triglyceride levels were both significantly reduced in free-living subjects on a walnut-supplemented diet compared to habitual diet.

After adjusting for body weight, in addition to total cholesterol and triglyceride, LDL-C showed a significant reduction of 3% between the walnut-supplemented and control diets. Furthermore, according to baseline values of

Table 3 Fasting serum lipid and lipoprotein concentrations and the ratio of LDL to HDL cholesterol during consumption of walnut-supplemented and control diets for 6 months by healthy, free-living men and women[a]

Blood Lipids	*Diet*		*Crude values*		*Adjusted values*[b]	
	Walnut	*Control*	*Estimated effect*[c]	P-*value*	*Estimated effect*[c]	P-*value*
Total cholesterol	5.41 ± 0.09	5.54 ± 0.09	−0.13 ± 0.08	0.02[d]	−0.18 ± 0.07	0.01[d]
LDL cholesterol	3.29 ± 0.09	3.38 ± 0.09	−0.09 ± 0.05	0.10	−0.16 ± 0.09	0.06[d]
HDL cholesterol	1.50 ± 0.04	1.51 ± 0.04	−0.01 ± 0.01	0.47	−0.02 ± 0.05	0.72
LDL: HDL[e]	2.38 ± 0.10	2.44 ± 0.10	−0.06 ± 0.03	0.85	−0.07 ± 0.01	0.58
Triglycerides[e]	1.35 ± 0.05	1.44 ± 0.07	−0.09 ± 0.04	0.03[d]	−0.16 ± 0.06	0.01[d]

Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein.
[a]All values are mean ± s.d. in (mmol/l); $n = 87$ (38 men and 49 women).
[b]The values are adjusted for body weight.
[c]Control-diet values are subtracted from walnut-supplemented diet values.
[d]Statistically significant in walnut-supplemented diet compared with control diet.
[e]Analysis was performed on log-transformed values. Results are shown in original unit for ease of interpretation.

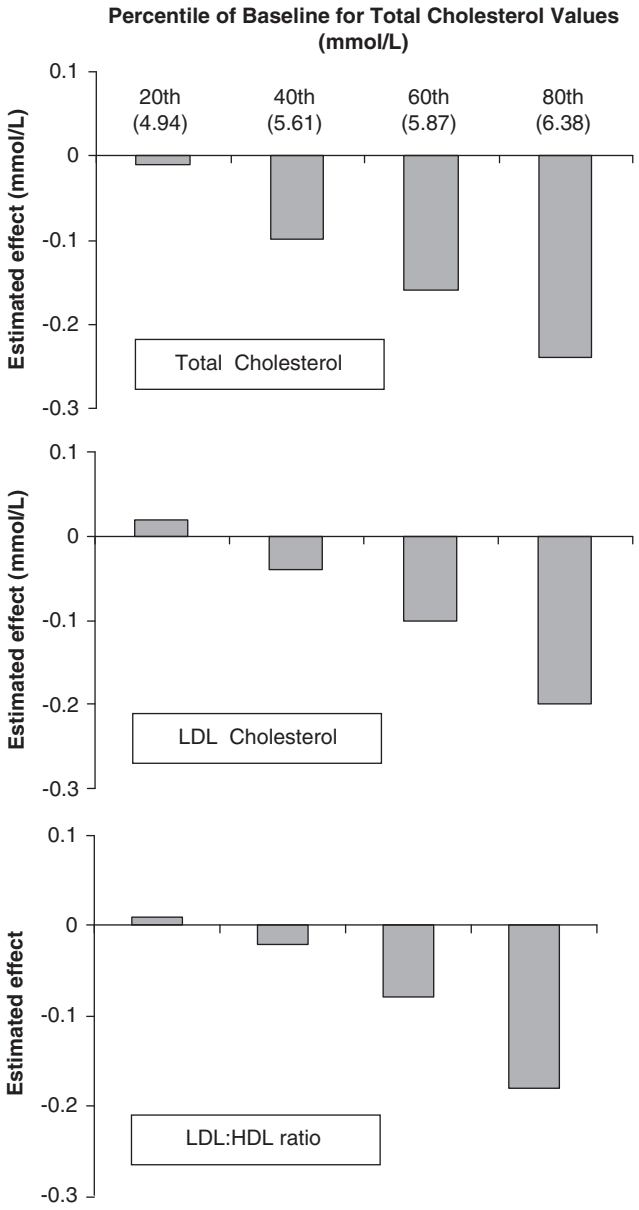

Figure 1 Estimated difference of effect between walnut and control diets in plasma total and LDL-C, and LDL to HDL ratio according to plasma total cholesterol baseline values.

plasma total cholesterol, the highest reduction was seen in the 80th percentile for total cholesterol, LDL-C and the ratio between LDL and HDL-C compared with other percentiles. This outcome showed the most cholesterol-lowering effect of walnuts in people who started the study with higher baseline values of total cholesterol compared with those with low or moderate plasma total cholesterol levels. This means hyperlipidemic people may benefit the most by incorporating walnuts into their diets.

After evaluating the 24-h dietary recalls and compliance method, we found that subjects while on the walnut-supplemented diet had a higher total energy consumption, than during the control diet. The mean difference between daily total energy intake during the two diet periods is less than the actual energy intake from walnuts, which suggests partial substitution of other foods in the walnut-supplemented diet. We also found an excellent (95%) degree of dietary compliance, which indicates that people can sustain a diet with walnuts for 6 months.

The lipid-lowering effects of walnuts that we found in our study were consistent with previous reports, and with a recent meta-analysis (Banel and Hu, 2009), showing that a walnut diet decreases total cholesterol levels, in particular, in subjects with higher baseline plasma total cholesterol levels (Zambón *et al*., 2000). No significant change was observed in HDL-C between the two diets, which is in broad agreement with previous study (Sabaté *et al*., 1993; Abbey *et al*., 1994). However, findings of Tapsell *et al*. (2004, 2009) on type II diabetic patients were different from ours. In one study they found significant increase in HDL-C and no change in total cholesterol was observed, whereas in the other study there was no change in HDL and an increase in LDL-C. These differences might be due to a study population who were type II diabetics and/or the modified diet plan that study subjects were following during that study.

Walnuts have many beneficial attributes. Foremost is the cardioprotective effect of walnuts because of a high amount of polyunsaturated fatty acids. There may be a number of other bioactive compounds in nuts, such as vitamin E, fiber, phytochemicals or polyphenols, which potentiate cholesterol-lowering effects and independently affect risk factors for various chronic diseases. However, the data on this topic are limited and much research is needed to define the roles that these compounds may have in reducing the risk of chronic diseases, as well as the biological mechanisms involved. Sufficient data are available to justify the recommendation to include nuts in the diet and they can be readily incorporated. Efforts are needed to educate the public about the health benefits of nuts and ways to use them in planning a healthy diet.

In summary, the inclusion of walnuts as part of a habitual diet favorably altered the plasma lipid profile. The lipid-lowering effects of walnuts were more evident among subjects with higher lipid baseline values, precisely those people with greater need of reducing plasma total and LDL-C. The take-home message is that walnuts work to lower your cholesterol on the long term.

Conflict of interest

The authors declare no conflict of interest.

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