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Journal of Nutritional Biochemistry 15 (2004) 730–740

Journal of Nutritional Biochemistry

The influence of dietary vitamin E, fat, and methionine on blood cholesterol profile, homocysteine levels, and oxidizability of low density lipoprotein in the gerbil

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Received 4 February 2004; accepted 15 April 2004

Abstract

A 90-day feeding study with gerbils was conducted to evaluate the influence of dietary vitamin E levels (25 mg/kg diet, 75 mg/kg, 300 mg/kg, and 900 mg/kg), two levels of dietary methionione (casein or casein+L-methionine (1% w/w)) and two sources of lipid (soybean oil [20%] or soybean oil [4%]+coconut oil [16%, 1:4 w/w]) upon serum lipids (total cholesterol, HDL-cholesterol, LDL-cholesterol). In addition, this study examined the effects of diet-induced hyperhomocysteinemia and supplemental dietary vitamin E on the oxidation of low density lipoproteins. Tissue vitamin E (heart, liver, and plasma) demonstrated a dose response ($P \leq 0.001$) following the supplementation with increasing dietary vitamin E (25, 75, 300, and 900 mg/kg). In addition, tissue vitamin E levels were found to be higher ($P \leq 0.001$) in those animals receiving a combination of coconut oil+soybean oil as compared to the group receiving soybean oil solely. Blood cholesterol profiles indicated an increase ($P \leq 0.001$) in total cholesterol and LDL cholesterol by the influence of saturated fat and supplemental methionine. Low-density lipoprotein cholesterol profile demonstrated a reduction ($P \leq 0.001$) at the higher dietary vitamin E levels (300 and 900 mg/kg) as compared to the 25 mg/kg and 75 mg/kg dietary vitamin E. Plasma protein carbonyls were not influenced by dietary vitamin E nor by supplemental methionine intake. In vitro oxidation of LDL showed that vitamin E delayed the lag time of the oxidation phase ($P \leq 0.001$) and reduced total diene production ($P \leq 0.001$). On the contrary, supplemental methionine decreased ($P \leq 0.001$) the delay time of the lag phase, whereas total diene production was increased ($P \leq 0.001$). Plasma lipid hydroperoxides were significantly reduced ($P \leq 0.05$) with supplemental dietary vitamin E, whereas supplemental L-methionine (1%) resulted in a significant ($P \leq 0.05$) increase in lipid plasma hydroperoxide formation. Plasma homocysteine was elevated ($P \leq 0.001$) with supplemental dietary L-methionine (1%) as well as the inclusion of dietary saturated fat. The present data showed that 1) a combination of dietary lipids (saturated and unsaturated fatty acids) as well as vitamin E and methionine supplementation altered blood cholesterol lipoprotein profiles; 2) in vitro oxidation parameters including LDL (lag time and diene production) and plasma hydroperoxide formations were affected by vitamin E and methionine supplementation; and 3) plasma homocysteine concentrations were influenced by supplemental methionine and the inclusion of dietary saturated fat.

1. Introduction

Dietary fat composition is the primary determinant of serum cholesterol, LDL/HDL cholesterol ratio and lipoprotein [a], important risk factors for cardiovascular disease. Saturated fatty acids (especially lauric, myristic and palmitic) are known to elevate serum cholesterol, and conversely, dietary polyunsaturated fatty acids (especially linoleic acid) lower serum cholesterol [1]. The distribution of cholesterol between the various fractions of plasma lipoproteins is an important determinant in atherogenesis and is used as a biological marker for risk of atherosclerosis. Cholesterol in low-density lipoprotein (C-LDL) is considered to be atherogenic, while cholesterol in high density lipoprotein (C-HDL) is associated with a protective mechanism against the onset of atherosclerotic lesions [1].

Dietary protein is also known to influence cholesterol metabolism. Protein of animal origin such as casein is more

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doi:10.1016/j.jnutbio.2004.04.009

cholesterolemic and atherogenic than plant protein such as soybean protein in humans and a variety of animal models [2,3]. Casein may exert its hypercholesterolemic effects by mechanisms which may include increased absorption and decreased turnover of cholesterol [4]. Higher methionine and higher methionine/glycine and lysine/arginine ratios in casein compared to soybean protein were reported to be positively associated with the hypercholesterolemic effect of casein [5].

Protein sources were reported to have significant effects on plasma total homocysteine (tHcy, including both bound and free) in a pig model, and the increase in plasma tHcy was positively related to the methionine contents of protein sources, and to plasma methionine [6]. An excessive intake of methionine increases plasma homocysteine concentrations by enhancing the synthesis of homocysteine [7]. Elevated plasma total homocysteine (tHcy, both bound and free) is an independent risk factor for atherosclerotic vascular disease [8–10]. There is convincing evidence to suggest that high plasma concentration of this sulfur amino acid is an indicator of increased risk of cardiovascular morbidity and mortality [11].

While the mechanism of homocysteine's involvement in the development of premature cardiovascular disease is not understood, homocysteine has been shown to cause damage to endothelial cells, probably by the generation of reactive oxygen species such as hydrogen peroxide with the formation of oxidized low density lipoprotein [12]. Endothelial lesions induced by oxidized LDL may contribute to atherogenesis. Oxidation of plasma proteins (including apolipoproteins) may be discernible as increased content of carbonyl (aldehyde or ketone) adducts on the proteins. Protein oxidation, measured as an increase in dinitrophenylhydrazine-reactive carbonyl groups on the proteins, has been shown to be an early event in oxidative stress in vitro [13]. Protein oxidation products may be more persistent than direct products of lipid peroxidation. Vitamin E, a chain breaking antioxidant, may play an important function in protecting low density lipoproteins against oxidation under the conditions of oxidative stress [14] that appear to be present with mild/moderate hyperhomocysteinemia. Evidence supports the hypothesis that antioxidant dietary factors like vitamin E may inhibit LDL oxidation and thereby possibly protect against the development of atherosclerosis. Vitamin E status has also been shown to potentially modulate serum lipoprotein and cholesterol profiles. Some studies including animal and human clinical trials have shown a beneficial role of vitamin E supplementation in redistributing cholesterol among the lipoprotein fractions (from LDL-C to HDL-C) as well as lowering plasma total cholesterol levels. In studies with rats, supplementation with various dietary levels of vitamin E decreased cholesterol levels in serum and liver [15], whereas Kritchevsky et al. [16] reported no effect on serum cholesterol levels.

The present study was conducted to clarify whether vitamin E supplementation can influence the distribution of cholesterol among various lipoprotein fractions as well as serum lipid profiles induced by the type of dietary fat and methionine level. Moreover, information on the potential inhibitory effect of vitamin E supplementation on the methionine/homocysteine-induced LDL oxidation was obtained. Two types of dietary fat (soybean oil and soybean oil+coconut oil), two levels of dietary methionine (casein and casein+methionine) and four levels of supplemental vitamin E (25, 75, 300, and 900 mg/kg) were tested in gerbils. The gerbil model is more similar to humans than other rodent species in terms of plasma lipoprotein distribution and responses to dietary changes, which are limited to plasma LDL analogous to the human situation.

2. Methods and materials

2.1. Diets

The composition of the 16 experimental diets is shown in Table 1. The experimental diets included casein as the protein source ± L-Met (10 g/kg diet). The fat source (20% w/w) was either soybean oil (SBO) or a 4:1 (20% w/w) mixture of coconut oil (CO) and SBO (Mix). Diets containing each of the protein and fat type were supplemented with four levels of vitamin E (25, 75, 300, and 900 mg/kg diet). Diets were prepared weekly and stored at 4°C.

2.2. Animal protocol

Male Mongolian gerbils (*Meriones unguiculatus*), weighing 30 (SD 3g), were purchased from Tumblebrook Farms (West Brookfield, MA) and housed individually in regular plastic bins in a room with controlled temperature, 23°C (SD 2°C), relative humidity, 55% (SD 2%) and light (lights on 0600 to 1800 h). The experiment and animal handling procedures were approved by the Animal Care Committee of Health Canada and were performed in accordance with the guidelines of the Canadian Council of Animal Care (1993).

Upon reception at Health Canada's Animal Resource Division, the gerbils were weighed and randomly assigned to the 16 dietary treatments ($n=10$ animals/treatment). Animals were given free access to water and experimental diets for a period of 90 days. Weekly food consumption and body weights were recorded. At the end of the 90 day period, gerbils were sacrificed while anaesthetized with 3% isoflurane (in O_2) with blood and tissues collected. Blood from the dorsal aorta was collected into EDTA-tubes and spun at 1500×*g* for 15 minutes at 4°C immediately after sampling to obtain plasma. Collected plasma was then partitioned into aliquots for the various assays.

2.3. Analytical methods

Cholesterol distribution among lipoproteins was determined on fresh plasma according to the high performance gel filtration chromatographic method of Kieft et al. [17]. Plasma homocysteine (tHcy) was determined by a reverse

Table 1
Composition of experimental diets

Diet	Casein (g/kg)	L-methionine (g/kg)	Coconut oil (g/kg)	Soybean oil (g/kg)	Corn starch (g/kg)	Vitamin E (mg/kg)	Cellulose (g/kg)
C-SBO (E1)	250	—	—	200	222	25	150
C-SBO (E2)	250	—	—	200	222	75	150
C-SBO (E3)	250	—	—	200	222	300	150
C-SBO (E4)	250	—	—	200	222	900	150
C-SBO (E1)	250	10	—	200	222	25	150
C-SBO (E2)	250	10	—	200	222	75	150
C-SBO (E3)	250	10	—	200	222	300	150
C-SBO (E4)	250	10	—	200	222	900	150
C-(SBO+CO) (E1)	250	—	160	40	222	25	150
C-(SBO+CO) (E2)	250	—	160	40	222	75	150
C-(SBO+CO) (E3)	250	—	160	40	222	300	150
C-(SBO+CO) (E4)	250	—	160	40	222	900	150
C-(SBO+CO) (E1)	250	10	160	40	222	25	150
C-(SBO+CO) (E2)	250	10	160	40	222	75	150
C-(SBO+CO) (E3)	250	10	160	40	222	300	150
C-(SBO+CO) (E4)	250	10	160	40	222	900	150

Minerals (AIN-93), vitamins (AIN-93, excluding α-tocopherol), choline chloride, and cholesterol added at 3.5%, 1.0%, 0.3%, 0.1% respectively. Glucose was added to all diets at 13.0%.
C=vitamin free casein; SBO=soybean oil, tocopherol stripped; CO=coconut oil; E1–E4 four levels of added α-tocopheryl acetate added to respective oil combinations.

phase HPLC method (Fiskerstrand et al. [18]) using a Waters NOVA-PAK-C_{18} 15 cm long column at 25°C.

Tissue and plasma vitamin E were extracted according to the method of Ingold et al. [19] and quantified by high pressure liquid chromatography [20].

Plasma lipid oxidation was performed on EDTA fresh plasma by the inclusion of Cu^{2+} as previously described by Spranger et al. [21] with minor modifications. In brief, 10 μl of plasma were diluted with 490 μl of Dulbecco's phosphate-buffered saline (DPBS, GIBCO/BRL, Burlington, Ont. Canada). The oxidation was started by adding 5 μl of 5 mM $CuSO_4$ solution into the plasma samples, followed by incubation at 37°C for different time periods (1 h, 2 h, 3 h, 4 h or 5 h). The oxidation of the plasma was determined by a lipid hydroperoxide (LPO) assay kit (Cayman Chemical Co., Ann Arbor, MI). Briefly, 500 μL of each sample were mixed with an equal volume of extract R (saturated methanol) and vortexed, then 1 mL of cold chloroform was added followed by vortexing. The sample mixture was then centrifuged at 1500×*g* for 5 minutes at 0°C. The bottom chloroform layer was carefully transferred to another test tube using a pasteur pipette. A 500–μL aliquot of the chloroform extract was mixed with 450 μL of chloroform-methanol (v/v 2:1) solvent mixture and 50 μL of chromogen. The assay tubes were kept at room temperature for 5 minutes and then the absorbance at 500 nm was measured spectrophotometrically. The amount of plasma lipid oxidation was calculated according to the hydroperoxide standard curve and converted to a concentration as nmol/ml plasma.

For the assessment of protein carbonyl content, plasma proteins were derivatized with dinitrophenylhydrazine (DNP) before SDS-polyacrylamide gel electrophoresis on precast 4–12% gradient gels (NOVEX, San Diego, CA), followed by Western transfer to nitrocellulose filters (Shacter et al. 1994). DNP-derivatized plasma protein carbonyl groups were sequentially reacted with rabbit anti-DNP and goat anti-rabbit IgG antibodies (OxyBlot Protein Oxidation Detection Kit, Intergen Company, Purchase, NY) followed by chemiluminescence detection with ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech, Inc., Baie d'Urfé, PQ, Canada. Oxidized protein standards included with the Oxy Blot kit were used to assess the size (in kDa) of protein bands on the Western blots. Total protein staining with Amido Black was done on a duplicate nitrocellulose filter prepared at the same time from the same DNP-derivatized sample. Quantitation of densitometry scans from chemiluminescence detection and from total protein staining was done using a Howtek Scanmaster 3 scanning densitometer and Quantity One software for analysis of 1D gels (pdi, Huntington Station, NY).

Protein concentration in plasma samples was assayed using the bicinchoninic acid kit for protein determination (Sigma-Aldrich Canada, Oakville, ON).

Low density lipoproteins (LDL) were isolated from pooled plasma (2 samples/ each corresponding dietary treatment) using a NVT-90 rotor on a Beckman S1-80 centrifuge as described by Ordovas [22]. The oxidation of isolated LDL was carried out by incubation with copper sulphate with the measurement of conjugated diene formation continuously monitored as the change in absorbance at 234 nm [23].

2.4. Statistical analysis

All data are reported as means and standard errors. Each response variable (except plasma and low density lipoprotein oxidation parameters and plasma carbonyls) was analysed by a three-factor ANOVA test using a statistical package

Table 2
Cholesterol profile (mmol) among lipoproteins and plasma homocysteine (μmol) in gerbils after 90 days with a dietary combination of oils, proteins, and vitamin E

Dietary treatment	Total Chol (mmol/L)		C-LDL (mmol/L)		C-HDL(mmol/L)		Homocysteine (μmol)	
	LSM	SE	LSM	SE	LSM	SE	LSM	SE
Proteins								
Main effects								
Casein (unsuppl)	4.06[a]	0.01	2.07[a]	0.008	1.96[a]	0.005	5.89[a]	0.08
Casein+L-Met (1%)	5.52[b]	0.01	2.58[b]	0.008	0.67[b]	0.005	10.98[b]	0.08
Vitamin E								
Main effects								
25 mg/kg	4.98[a]	0.02	2.42[a]	0.01	1.36[a]	0.01	8.01[a]	0.11
75 mg/kg	4.83[b]	0.02	2.40[a]	0.01	1.30[a]	0.01	8.12[a]	0.11
300 mg/kg	4.62[c]	0.02	2.26[b]	0.01	1.26[a]	0.01	7.86[a]	0.11
900 mg/kg	4.69[d]	0.02	2.16[b]	0.01	1.32[a]	0.01	8.36[a]	0.11
Oils								
Main effects								
Soybean Oil	4.49[a]	0.01	2.13[a]	0.01	1.44[a]	0.005	7.25[a]	0.08
Soybean Oil+Coconut oil (1:4)	5.10[b]	0.01	2.52[b]	0.01	1.19[b]	0.005	8.91[b]	0.08
Interactions								
Oils *vit E	NS		NS		$p=0.034$		NS	
Oils* protein	NS		NS		$p=0.045$		NS	
Vit E * protein	NS		NS		NS		NS	
Oi1s*vit E*protein	NS		NS		NS		NS	

Least square means within a column (within a category (protein, vitamin E, and oils)) for each variable not sharing a common superscript letter were significantly different ($P \leq 0.001$).
Chol=cholesterol; HDL=high density lipoprotein; LDL=low density lipoprotein; LSM=least square mean; SE=standard error; Vit=vitamin.

(Statistical Systems for Personal Computers, SAS Institute, Cary, NC). The factors of interest were levels of methionine (casein and casein+methionine, 10 g/kg diet), two types of fat (SBO and coconut oil/SBO mix, 4:1) and four levels of vitamin E supplementation (25, 75, 300, and 900 mg/kg diet). Plasma and low density lipoprotein oxidation measurements and plasma carbonyls were analyzed by a two-way factor ANOVA test using a statistical package (Statistical Systems for Personal Computers, SAS Institute, Cary, NC) that included :levels of methionine (casein and casein+methionine, 10 g/kg diet) and four levels of vitamin E supplementation (25, 75, 300, and 900 mg/kg diet).

3. Results

3.1. Body weights and food consumption

At the beginning of the experimental period, mean body weights (37 ± 3.0 g) for the 16 groups were not different ($P > 0.05$). After 90 days of dietary treatments, no differences ($P > 0.05$) in mean final body weights (93.6 ± 8.12 g) were observed among the sixteen groups. Food consumption patterns over the course of the 90 day period did not reveal significant differences ($P > 0.05$) for the sixteen dietary treatments. Mean daily feed consumption was 5.77 ± 0.66 g.

3.2. Plasma cholesterol profile among lipoproteins

The effects of fat type, levels of methionine and amounts of supplemental vitamin E on plasma cholesterol profile are shown in Table 2. Methionine content, fat source had significant effects ($P < 0.001$) on the levels of plasma total cholesterol, LDL-cholesterol, and HDL-cholesterol, whereas amounts of supplemental vitamin E had significant influence ($P < 0.006$) on the levels of plasma total and LDL-cholesterol.

On average, the eight casein+methionine groups had higher levels of plasma total cholesterol (5.52 [SE 0.01] vs. 4.06 [SE 0.001] mmol/L), LDL-cholesterol (2.58 [SE 0.008] vs. 2.07 [SE 0.008] mmol/L) and lower HDL-cholesterol (0.67 [SE 0.005] vs. 1.96 [SE 0.005] mmol/l) than the eight unsupplemented casein groups ($P < 0.01$, ANOVA analysis of pooled groups, $n = 80$).

On average, the eight coconut oil–SBO mixture groups had higher levels of plasma total cholesterol (5.10 [SE 0.01] vs. 4.49 (SE 0.01) mmol/L) and LDL-cholesterol (2.52 [SE 0.01] vs. 2.13 [SE 0.01) mmol/L but had lower levels of HDL-cholesterol (1.19 [SE 0.005] vs. 1.44 [SE 0.005] mmol/L) compared to the SBO groups ($P < 0.001$, ANOVA analysis of pooled groups, $n = 80$).

On average, the 25 and 75 mg/kg supplemental vitamin E groups had higher levels of plasma LDL (2.40–42 [SE 0.01] than the 300 and 900 mg/kg vitamin E groups (2.16–2.26 [SE 0.01] mmol/L) ($P < 0.006$, ANOVA analysis of pooled groups, $n = 40$)).

3.3. Homocysteine status

Methionine level and fat source had significant effects ($P < 0.001$) on plasma tHcy, whereas the amounts of

Table 3
Plasma and tissue vitamin E response in gerbils after 90 days with a dietary combination of oils, proteins, and vitamin E

Dietary treatment	Plasma (μg/mL)		Liver (μg/g, fresh)		Heart (μg/g, fresh)	
	LSM	SE	LSM	SE	LSM	SE
Proteins						
Main effects						
casein (unsupplemented)	18.13[a]	0.133	715.45[a]	6.41	72.64[a]	0.24
casein+L-Met (1%)	18.51[a]	0.133	533.91[b]	6.41	63.23[b]	0.24
Vitamin E						
Main effects						
25 mg/kg	10.91[a]		221.21[a]	9.07	40.76[a]	0.34
75mg/kg	13.29[b]		309.79[b]	9.07	51.14[b]	0.34
300 mg/kg	19.24[c]		576.82[c]	9.07	69.74[c]	0.34
900mg/kg	29.85[d]		1390.91[d]	9.07	110.11[d]	0.34
Oils						
Main effects						
Soybean Oil	15.53[a]	0.13	903.34[a]	6.41	75.01[a]	0.24
Soybean Oil+coconut oil (1:4)	21.11[b]	0.13	346.03[b]	6.41	60.87[b]	0.24
Interactions						
Oils *Vit E	NS		NS		$P=0.012$	
Oils* Protein	NS		$P=0.007$		NS	
Vit E *Protein	NS		NS		NS	
Oils* Vit E*Protein	NS		NS		NS	

Least square means within a column (within a category (protein, vitamin E, and oils)) for each variable not sharing a common superscript letter were significantly different ($P \leq 0.001$).
Chol=cholesterol; HDL=high density lipoprotein; LDL=low density lipoprotein; LSM=least square mean; SE=standard error; Vit=vitamin.

supplemental vitamin E had no effect ($P>0.05$) on plasma tHcy (Table 2).

On average, the eight methionine-supplemented casein groups had higher levels of plasma tHcy (10.98 [SE 0.079] vs. 5.89 [SE 0.079] μmol/L) than the eight unsupplemented casein groups ($P<0.001$, ANOVA analysis of pooled groups, $n=80$).

On average, the eight coconut oil–SBO mixture groups had higher levels of plasma tHcy (8.91 [SE 0.079] vs. 7.25 [SE 0.079] μmol/L ($P<0.001$, ANOVA analysis of pooled groups, $n=80$).

3.4. Vitamin E status

The influence of methionine level, fat type and amounts of supplemental vitamin E on plasma, liver and heart vitamin E status is reported in Table 3. Methionine level had significant effects ($P<0.001$) on liver and heart vitamin E levels; fat type had significant effects ($P<0.001$) on plasma, liver and heart vitamin E levels; and amounts of supplemental vitamin E had significant effects ($P<0.001$) on plasma, liver and heart vitamin E concentrations.

On average, the eight methionine-supplemented casein groups had lower levels of liver vitamin E (533.91 [SE 6.41) vs. 715.45 [SE 6.41] μg/g) and heart vitamin E (63.23 [SE 0.24] vs. 72.64 [SE 0.24] μg/g) compared to the eight unsupplemented casein groups ($P<0.001$, ANOVA analysis of pooled groups, $n=80$).

On average, the eight coconut oil–SBO mixture groups had higher levels of plasma vitamin E (21.11 [SE 0.13] vs. 15.53 [SE 0.13] μg/mL) compared to the eight SBO groups ($P<0.001$, ANOVA analysis of pooled groups, $n=80$).

On average, the eight coconut oil–SBO mixture groups had lower levels of liver vitamin E (346.03 [SE 6.41] vs. 903.34 [SE 6.41] μg/g) and heart vitamin E (60.87 [SE 0.24] vs. 75.01 [SE 0.24] μg/g) than the eight SBO groups ($P<0.001$, ANOVA analysis of pooled groups, $n=80$).

Supplemental vitamin E caused dose-related increases in the levels of plasma, liver and heart vitamin E. On average, the four 900 mg/kg supplemental vitamin E groups had the highest levels of plasma vitamin E (29.8 μg/mL), liver vitamin E (1390 [SE 9.07] μg/g) and heart vitamin E (110.13 [SE 0.343] μg/g compared to the corresponding values for the four 25 mg/kg supplemental vitamin E groups (10.91 μg/ml, 221.21 [SE 9.07] μg/g, 40.76 [0.34] μg/g), the four 75 mg/kg supplemental vitamin E groups (13.29 μg/ml, 309.79 [SE 9.07] μg/g, 51.14 [SE 0.34] μg/g), and the four 300 mg/kg supplemental vitamin E groups (19.24 μg/mL, 576.82 [SE 9.07] μg/g, 69.74 [SE 0.34] μg/g) ($P<0.001$, ANOVA analysis of pooled groups, $n=40$).

3.5. Lipid oxidation

No lipid oxidation in plasma for all the treatments was detected without copper induction. Thus, lipid oxidation was induced by adding $CuSO_4$ (5 μmol/L) for different time periods (1, 2, 3, 4, and 5 hours) and assayed by a commercially available lipid hydroperoxide assay kit (Cayman Chemical, Ann Arbor, MI). There were significant diet effects at all time points ($P \leq 0.05$) (Table 4). For the 1-hour

Table 4
Plasma induced lipid oxidation (lipid hydroperoxides, via copper sulphate addition) in gerbils with different experimental diets

Time (h)	Diet 1*		Diet 2*		Diet 3*		Diet 4*		Diet 5†		Diet 6†		Diet 7†		Diet 8†		*P* value		
	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM	Diet	Vit. E	D*V
1	58.8^{bc}	12.8	57.9^{bc}	13.7	41.1^{b}	12.1	47.7^{bc}	12.1	99.4^{a}	12.8	82.0^{ac}	12.1	76.8^{ac}	12.1	68.2^{abc}	14.8	0.0016	0.3120	0.848
2	128.1^{b}	25.7	114.0^{b}	27.1	99.4^{b}	28.8	79.1^{b}	28.8	267.1^{a}	28.8	159.8^{b}	28.8	106.2^{b}	28.8	73.5^{b}	46.9	0.0392	0.0015	0.071
3	289.9^{d}	41.9	129.9^{cf}	44.4	66.3^{cg}	51.3	34.6^{efg}	44.4	436.5^{a}	39.7	273.5^{bd}	39.7	163.9^{bc}	39.7	114.3^{ce}	51.3	0.0004	0.0001	0.839
4	382.2^{b}	46.0	274.1^{bc}	48.4	52.9^{d}	51.4	40.7^{d}	65.0	546.7^{a}	46.0	384.5^{b}	46.0	202.1^{c}	46.0	150.5^{cd}	59.4	0.0005	0.0001	0.926
5	478.7^{ab}	56.2	343.5^{bd}	56.2	109.6^{e}	56.2	60.0^{e}	62.1	583.0^{a}	70.5	382.9^{b}	70.5	301.4^{bc}	70.5	166.5^{cde}	76.1	0.0197	0.0001	0.697

Values in the same row with different superscript letters are significantly different ($P < 0.05$).
D*V = diet × vitamin E; LSM = least square mean; SE = standard error.

* Diets 1–4 = casein and soybean oil with varying amounts of vitamin E (25, 75, 300, and 900 mg/kg); see Table 1 for details.

† Diets 5–8 = casein + L-Met (1%) and soybean oil with varying amounts of vitamin E (25, 75, 300, and 900 mg/kg); see Table 1 for details.

time period, vitamin E treatment did not have a significant effect on copper-induced lipid oxidation within each diet group. At the 2-hour period, the group with low dietary vitamin E (group A, 25 mg/kg) had a significantly higher ($P \leq 0.05$) lipid oxidation induced by copper than other groups in animals fed with diet 1. For the gerbils in the other treatment groups, there were no significant differences regarding lipid oxidation. Starting from the 3-hour induction time period there was a dose-dependent effect of vitamin E supplementation on the lipid oxidation in both diet groups. The higher the amount of vitamin E supplemented, the lower the oxidation was induced by copper. Furthermore, the copper induced oxidation was higher ($P < 0.05$) when gerbils were fed the dietary treatment of soybean oil plus L-methionine (1%) versus those receiving solely the soybean oil diet treatment.

3.6. Plasma protein oxidation

Assessment of plasma protein concentrations and evaluation of Amido Black staining on duplicate Western transfers demonstrated consistency of protein loading and transfer to nitrocellulose. Several plasma proteins contained sufficient carbonyl groups to be detectable by this method. No significant differences were noted in the carbonyl content of these plasma proteins between casein/soybean oil treatment groups with and without added methionine at any of the levels of vitamin E addition examined in this experiment (data not shown).

3.7. Oxidizability of LDL

The results obtained from this study showed that various dietary vitamin E levels had a marked impact ($P \leq 0.001$) on the lag time (min) of the oxidation phase as well on the total diene formation (Table 5). A significant vitamin E dose response ($P \leq 0.001$) was observed for the lag time (min) as well as for total diene production (Table 5). The percent change in lag time (min) over the lowest dose vitamin E administered (25 mg/kg) were as follows: 75 mg/kg, Δ 31.93%; 300 mg/kg, Δ 54.49%; and 900 mg/kg, Δ 60.67%. On the contrary, additional dietary L-methionine resulted in a significantly ($P \leq 0.001$) decreased lag time (min) of the oxidation phase, whereas total diene production was elevated ($P \leq 0.001$) as compared to the unsupplemented casein group.

A positive correlation coefficient (+0.86, $P \leq 0.001$) was found between plasma vitamin E levels and the lag time in the oxidation phase. In addition, a negative correlation coefficient (−0.84, $P \leq 0.001$) was observed with plasma vitamin E and total diene production. No significant correlation coefficients ($P \geq 0.05$) were present between plasma homocysteine and lag time in the oxidation phase or total diene production. No interactions ($P \geq 0.05$) were found between vitamin E and L-methionine in either lag times or total diene production.

Table 5
Low density lipoprotein oxidation (copper induced) parameters in gerbils after 90 days with a dietary source of (SBO), proteins (Casein or Casein plus L-Met), and vitamin E

Dietary treatment	Lag time (mins)		Total diene (μmolar)	
	LSM*	SE**	LSM	SE
Proteins				
Main effects				
Casein (unsuppl.)	161.5[a]	4.3	4.37[a]	0.06
Casein (unsuppl) + L-Met (1%)	131.1[b]	4.3	4.64[b]	0.06
Vitamin E				
Main effects				
25mg/kg	81[a]	6.02	5.63[a]	0.09
75mg/kg	119[b]	6.02	4.79[b]	0.09
300mg/kg	178[c]	6.02	4.04[c]	0.09
900mg/kg	206[d]	6.02	3.56[d]	0.09
Interactions				
Proteins times vitamin E	NS		NS	

Least square means within a column for each variable (dietary methionine levels, vitamin E) not sharing a common superscript letter were significantly different ($P \leq 0.001$).
LSM = least square mean; SE = standard error.

4. Discussion

4.1. Influence of methionine and fat sources on cholesterol profile and homocysteine status

The methionine-induced increase in serum total cholesterol obtained in our study is in agreement with the data of Sugiyama and Muramatsu [24], who found that the addition of methionine to a 25% casein diet resulted in significant ($P < 0.05$) increase in plasma cholesterol in rats. This increase was prevented by the addition of glycine to the casein diet. Similarly, in rats fed cholesterol-free diets containing different animal and vegetable proteins, significant ($P < 0.05$) positive correlations between serum cholesterol and dietary methionine and dietary methionine/glycine ratios have been reported [5,25]. Glycine is required in disproportionately large amounts for the formation of bile salts, heme, creatine, collagen and nucleic acids [26]. Addition of methionine to casein, which is a poor source of glycine, results in higher methionine/glycine ratio, which in turn may be responsible for the hypercholesterolemic effect of supplemental methionine, as noted in this investigation. However, a mechanism by which the methionine/glycine ratio could regulate serum cholesterol is still unclear. Similarly, the mechanisms for the cholesterol modulating effects of sulfur amino acids are poorly understood. Some of the hypolipidemic effect of cysteine has been attributed to enhanced taurine production, as taurine participates in bile acid conjugation; however, administration of taurine has not been shown to be as effective as cystine in reducing serum cholesterol [27]. Methionine is also metabolized to yield cystine and taurine. In the absence of low glycine content as found in casein, however, the plasma cholesterol elevating effect of methionine overcomes the cholesterol-lowering effect due to its nonmethyl portion [28]. It has been shown

that the plasma cholesterol-elevating effect of methionine could be abolished by eritadenine supplementation, a potent inhibitor of phosphatidylethanolamine (PE) N-methylation [29]. This suggested that the stimulation of PE N-methylation and a resultant increase in the phosphatidylcholine (PC)/PE ratio of liver microsomes might participate in the enhancement of plasma cholesterol by methionine. This was supported by a significant positive correlation between the PC/PE ratio and the plasma total cholesterol concentration in rats fed different types of dietary proteins varying in methionine content.

We found that even moderate methionine supplementation (10 g/kg) of the adequate casein diet (increasing dietary methionine to about two times the requirement) caused a significant ($P<0.001$) increase in plasma tHcy. This is in agreement with our previous findings in rats [30], and would suggest a detrimental effect of excess dietary methionine under conditions of adequate vitamin nutrition. Methionine-rich meals have been reported to cause slight increases in plasma tHcy [31]. Intake of excess dietary methionine (a precursor of homocysteine) may raise the requirements for folate, vitamin B_{12} and/or vitamin B_6, which are involved in the metabolism of homocysteine.

We also found that compared to SBO, the feeding of coconut oil–SBO mixture resulted in a considerable increase (about 60%) in plasma tHcy. Similar observations about the influence of the type of fat on serum tHcy have been made [32,33]. Fish oil was reported to lower serum homocysteine levels in 14 of 17 subjects [33], whereas olive oil supplements had no effect on serum homocysteine levels. It was suggested that the high levels of n-3 fatty acids in fish oil may be responsible for its homocysteine lowering effect. Feeding of a synthetic diet containing corn oil decreased the level of plasma homocysteine in rabbits compared with the same diet containing butter [32]. Corn oil is a rich source of linoleic acid, an n-6 polyunsaturated fatty acid precursor of arachidonic acid; olive oil contains an abundance of oleic acid, a monounsaturated fatty acid; butter contains predominantly saturated and monounsaturated fatty acids. Therefore, the lowering of serum homocysteine by polyunsaturated n-3 fatty acids of fish oil in humans and by the polyunsaturated n-6 fatty acids of corn oil in rabbits may provide an explanation for the antiatherogenic effect of dietary polyunsaturated fatty acids [33].

The coconut oil–SBO (4:1) mixture used in our study contains predominantly saturated fatty acids, whereas SBO contains abundance linoleic acid (a n-6 polyunsaturated fatty acid precursor of arachidonic acid) and some α-linolenic acid (a n-3 polyunsaturated fatty acid precursor of docosahexaenoic acid). Therefore, based on the present study and on the observations of McCully et al. [32] and Olszewski and McCully [33], it is suggested that homocysteine accumulation in blood may be suppressed by dietary polyunsaturated fatty acids. The mechanism for the influence of type of fat on serum homocysteine is, however, unknown at the present time.

4.2. Influence of dietary vitamin E on cholesterol profile among lipoproteins

The various dietary vitamin E levels administered to the gerbils over the 12-week period resulted in an alteration in the cholesterol profile among the lipoproteins, specifically low density lipoprotein. There have been numerous conflicting reports on the effect of vitamin E on cholesterol lipoprotein patterns among various animal species including man. In humans, Hermann et al. [34]) reported that vitamin E administered daily at 600 IU for 30–45 days contributed to a significant rise in plasma C–HDL concentration, with decreases in C-VLDL and total triglycerides, whereas no changes were found in total serum cholesterol. In a double blind study with hypercholesterolemic patients, Cloarec et al. [35] observed that supplementation with DL–alpha-tocopheryl acetate at 500 IU/day over 3 months resulted in significantly increased C-HDL with a concomitant decrease in the ratio of total cholesterol/C-HDL compared to the placebo group. However, other human studies [36–38] have reported that serum cholesterol or C-HDL is unrelated to vitamin E supplementation.

In animal models, conflicting reports have also been observed. Kritchevesky et al. [16] observed that vitamin E supplementation in rabbits had no significant effect on serum cholesterol profile. However, Komaratat et al. [39] demonstrated that the administration of vitamin E to rabbits over a 9-week period with a basal diet containing stripped corn oil and varying amounts of vitamin E depressed plasma cholesterol with the effect being proportional to the amount of dietary supplemental vitamin E. The hypocholesterolemic effect of vitamin E was accompanied by a redistribution of plasma lipoprotein cholesterol profile. Vitamin E supplementation at a level of 2100 IU/week over an 8-week period to rabbits receiving added dietary cholesterol (0.5%), also resulted in a 50% reduction in serum cholesterol levels [40]. In a sheep study, no observed influence on plasma cholesterol, triglycerides or total lipids following the provision of varying doses of vitamin E for 3 months [41]. Similar findings with regards to lipid and cholesterol plasma profile were reported by Hidiroglou et al. [42] in lambs ingesting various forms of vitamin E during a 60-day period. Discrepancies on the effect of vitamin E on plasma lipid profile may be associated to the extent of dosing time with the antioxidant [43] and these investigators [43] suggest that changes in serum lipoprotein profile may occur after receiving the antioxidant nutrient for more than 3 months. Other possible explanations for the discrepancies observed in the literature could be related to analytical methodologic differences or to selections of patients and subjects [36].

4.3. Influence of supplemental dietary methionine and vitamin E on plasma carbonyls

The presence of a basal level of carbonyl content in gerbil plasma proteins in this study was not unexpected.

Measurable basal levels of plasma protein carbonyl have been documented in rats [44] and in human nonsmokers, with higher levels found in smokers [45]. No change in circulating plasma protein carbonyl content was found in the present study, although this does not rule out the possibility that plasma proteins undergoing oxidative changes may have been cleared from circulation. Free radical–damaged bovine serum albumen (BSA) has been shown to be more rapidly endocytosed and degraded than native BSA by murine peritoneal macrophages, although a portion of the endocytosed free radical–damaged BSA was resistant to degradation and consequently tended to accumulate intracellularly [46]. Accumulation of oxidized protein has been suggested to play a role in atherosclerosis [47], so it may be very relevant to investigate the possibility that protein oxidation products may accumulate in blood vessel walls under similar experimental conditions to those reported here. Plasma total protein carbonyl content measured by HPLC was increased after 1 week of feeding of a very high protein (60% casein) diet to rats [48], this increase was not evident after 14 weeks of feeding the same diets. Thus it is possible that measurement of plasma protein carbonyls after 12 weeks in the present study may have missed an earlier, transient increase.

4.4. Effect of supplementation with vitamin E and methionine on LDL oxidation

Accumulating evidence from epidemiological and experimental studies supports the involvement and role of LDL oxidation in the early development and progression of coronary heart disease [49–53], although the mechanism by which LDL is oxidized in vivo is presently unknown [54]. Over the last few years, there has been great interest from both the scientific and public communities in the possibility that antioxidant vitamins (in particular, tocopherols, ascorbate, and carotenoids) might safely prevent lipoprotein oxidation and either prevent or slow down the atherosclerotic process [55].

In the present study it was found that varying dietary vitamin E levels resulted in marked changes in LDL oxidation parameters, specifically the lag time and total diene production. Our vitamin E data are in agreement with previous animal and human studies [56–59] that have shown that vitamin E supplementation is associated with the prolongation of lag time, decreased maximal rate of LDL oxidation and reduced total diene production.

Furthermore, in accordance with previous findings [58,60–62], our study demonstrated a significant dose-response increase in the prolongation of the lag phase before the onset of LDL oxidation following incremental vitamin E supplementation. In addition, previous reports [61–64] have shown significant positive correlations between the period of lag time before oxidation and vitamin E content in LDL. Unfortunately, in our study, we were not able to measure vitamin E levels in LDL due to the lack of sufficient amount of LDL aliquots.

To our knowledge, there is very scant information on the influence of methionine on lipoprotein oxidation, in particular LDL oxidation. In a rat study conducted by Moundras et al. [65], they showed that a diet containing a moderate protein level (soy protein, 13%) which was methionine deficient, resulted in a significantly increased susceptibility of VLDL+LDL fractions to oxidation as determined by the rate of conjugated diene formation. However, according to Moundras et al. [65], the addition of 0.4% methionine to the unsupplemented soy protein (13%), resulted in a prolonged lag phase (min) during the oxidation of LDL+VLDL. Thus, according to these investigators, imbalances in amino acids including sulphur amino acids might influence the effectiveness of systems involved in resistence against oxidative damage including damage to plasma lipoproteins. Radiolabeled studies in rat liver have previously shown that a high dietary ratio of methionine to cystine such as occurs in casein and especially in methionine-supplemented casein will suppress incorporation of cystine to glutathione [66]. A suppression of glutathione synthesis could, in turn, increase the susceptibility of tissues to oxidative stress. Methionine feeding has been reported to enhance hepatic [67] and plasma [68] lipid peroxidation as well as to decrease antioxidant activity [68].

Our vitamin E and methionine (1% w/w) data obtained from the LDL oxidation parameters were further substantiated from the plasma oxidation results. Further studies are needed to clarify the role of imbalances in amino acids and oxidative stress conditions.

In summary, the results of this study showed that a dietary combination of saturated and unsaturated oils (16%:4% w/w) elevated plasma total cholesterol and LDL cholesterol. In addition, L-methionine independently raised both total cholesterol and LDL cholesterol. Vitamin E supplementation at the higher dose levels (300 and 900 mg/kg) resulted in a lowering of plasma LDL cholesterol. Both LDL and plasma oxidation parameters demonstrated that Vitamin E had a protective effect, whereas conversely L-methionine had a depressing effect. Plasma homocysteine were elevated by additional dietary L-methionine and as well by the inclusion of saturated fat. The results obtained suggest potential dietary manipulations for modifying risk factors for cardiovascular disease such as blood cholesterol profiles and plasma total homocysteine, and against in vitro indicators of lipid oxidation parameters.

Acknowledgments

The authors extend their appreciation to the staff of the Animal Resource Division, Health Canada, for the care and feeding of the animals.

Dedication

The author (Nick Hidiroglou) dedicates this paper to his late father (Dr. Mike Hidiroglou) a highly recognized research scientist.

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