Eur J Nutr (2004) 43 : 237–245
DOI 10.1007/s00394-004-0465-3

ORIGINAL CONTRIBUTION

Catherine Nicolle
Elyett Gueux
Claudine Lab
Lydia Jaffrelo
Edmond Rock
Andrzej Mazur
Pierre Amouroux
Christian Rémésy

Lyophilized carrot ingestion lowers lipemia and beneficially affects cholesterol metabolism in cholesterol-fed C57BL/6J mice

Summary *Background* Several lines of evidence indicate that diet rich in fruit and vegetable can protect against cardiovascular diseases by acting on cholesterol metabolism and on oxidative stress. *Aim of the study* The aim of this study was to assess whether daily carrot consumption (provided as lyophilized powder) could differentially influence the consequences of cholesterol supplementation on lipid metabolism and oxidative stress in C57BL/6J mice. *Methods* Fourteen mice were randomized in four groups. Mice were fed either control diets (without or with 0.25 % cholesterol added) or lyophilized carrot enriched diets (20 % wt/wt without or with 0.25 % cholesterol added) for 4 weeks. Cholesterol and triglycerides in plasma and in liver were measured at the end of the experimental period. Fecal excretion of sterols was evaluated. Vitamin E and carotenoid concentrations were also determined. Several biomarkers relative to oxidative stress such as FRAP (Ferric Reducing Ability of Plasma) and isoprostanes were investigated. *Results* Feeding the carrot diet resulted in a decrease of cholesterol (–41 %) and triglycerides (–49 %) in plasma and in the liver (–41 % and –39 %, respectively) in animals fed cholesterol-supplemented diets. Carrot diet induced an increase of total neutral sterols fecal excretion, which inhibits digestive cholesterol absorption. Carrot diet increased antioxidant status in cholesterol-fed mice as related by the 16 % higher FRAP values. Although vitamin E was not affected by carrot diet, vitamin E/TG ratio was significantly higher in animals fed carrot diets. The carrot diet induced an increase of vitamin E in the heart in both cholesterol-free and cholesterol-supplemented mice suggesting a higher protection of this tissue. *Conclusion* This study shows that carrot ingestion decreases lipemia and improves antioxidant status in mice. Such results suggest that carrot intake may exert a protective impact against CVD linked to atherosclerosis. It is likely that these effects could be due to the synergistic effect of fiber and associated antioxidants.

Key words carrot intake – bile acids – cholesterol – oxidative stress

Received: 4 June 2003
Accepted: 30 October 2003
Published online: 28 January 2004

C. Nicolle · P. Amouroux
Vilmorin, Clause & Cie – BP 1
Chappes, France

C. Nicolle Ph.D (✉) · E. Gueux · C. Lab · L. Jaffrelo · E. Rock · A. Mazur · C. Rémésy
Unité des Maladies Métaboliques & Micronutriments
INRA Clermont-Ferrand/Theix
63122 Saint Genès Champanelle, France
Tel.: +33-473/6246-76
Fax: +33-473/6246-38
E-Mail: cnicolle@clermont.inra.fr

Abbreviations

FRAP	Ferric Reducing Ability of Plasma
TG	triacylglycerol
LDL	low-density lipoprotein
VLDL	very low-density lipoprotein

Introduction

Consumption of fruit and vegetable is associated with a lowered risk of cancer and cardiovascular diseases common in the western countries [1–4]. Carrot is one of the major vegetables consumed in the western world, whatever the season. Its consumption has been increasing in western countries.

Carrot is one of the richest vegetables in fibers and carotenoids. Fibers represent 3–4 % of carrot with a

European Journal of Nutrition (2004) Vol. 43, Number 4
© Steinkopff Verlag 2004

large proportion of soluble fiber (more than 40 %). Carrot is also a valuable source of carotenoids (6 mg/100 g) mainly represented by α- and β-carotene (90 % of the total carotenoids, the remaining 10 % represent 9 *cis* and 13 *cis* isomers of β-carotene and a small amount of lutein). It also contains other antioxidants such as vitamin E (515 µg/100 g), vitamin C (7 mg/100 g) and phenolics such as *p*-coumaric, chlorogenic and caffeic acids [5].

Mainly dietary fiber and antioxydants, such as polyphenols, could be an important protective factor against hyperlipidemia and associated risk. Convincing evidence that fibers may also prevent the progression of atherosclerosis has been provided by animal and human studies [6–8]. Hypolipidemic or anti-atherogenic effects have been obtained with diverse fiber sources such as fruit and vegetable (apples, legumes or bran cereals). Among the fibers isolated, cellulose is generally without effect, whereas pectins or lignins appear to have a more consistent hypocholesterolemic potential by lowering plasma and liver cholesterol and increasing fecal bile acids or steroids output [9]. It was hypothesized that the lipid-lowering effect of fiber could contribute to improve the lipid protection by plant-antioxidants. Thus, the simultaneous supply of fiber and carotenoids in carrot diet could optimize the protective effect of this vegetable on condition that fiber does not avoid carotenoids' bioavailability.

Little is known about the lipid-lowering effect of carrot and its impact on antioxidant status. We determined the status of several antioxidant molecules (vitamin E, carotenoids) and several parameters related to oxidative stress, such as FRAP which represents total plasma antioxidant capacity, and isoprostane excretion which represents arachidonic end-product of lipid peroxidation. To investigate the cardiovascular protective effect of carrot consumption, we either supplemented or not the diet of C57BL/6J mice with 0.25 % of dietary cholesterol and then examined both lipid metabolism and antioxidant biomarkers. Under these conditions, we also investigated the impact of dietary cholesterol ingestion on oxidative stress.

Materials and methods

Animals and diet

Male C57BL/6J mice (obtained from IFFA CREDO, L'Arbresle, Lyon, France), 5 weeks old, were used. Animals weighing about 17 g each were housed five per cage in a room maintained at 22°C with a 12-h light-dark cycle (light from 8.00 a.m. to 8.00 p.m.) with access to food from 4.00 p.m. to 8.00 a.m. Mice were randomized in four groups of ten animals each and fed *ad libitum* for 4 weeks semipurified diets distributed as a powder (Table 1). Mice received diets containing either cellulose or lyophilized carrot in powder (200 g/kg of diet) with or without 2.5 g of cholesterol/kg.

The basal vitamin E supply was limited to one-third of the recommended value. During the last week of the experimental period, mice were housed two or three per cage in metabolic cages for urine and feces collection. Daily food consumption and body weight were recorded once a week.

Animals were maintained and handled according to the recommendations of the Institutional Ethic Committee (Institut National de la Recherche Agronomique), in accordance with decree n° 87–848. The experiment was also approved by the Institutional Ethic Committee.

Sampling procedures

Non-starved mice were anesthetized during the post-absorptive period (between 8.00 a.m. and 10.00 a.m.), when the cecal fermentation is still active, by sodium pentobarbital intraperitoneal injection (40 mg/kg of body weight). Blood was drawn from the vein cava into heparinized tubes and centrifuged at 12,000 g for 2 min. Plasma samples were immediately stored at –20°C for

Table 1 Composition of diets

Components (g/kg diet)	Control	Carrot	Control + cholesterol	Carrot + cholesterol
Casein	160	160	160	160
Wheat starch	610	542	607.5	539.5
Sucrose	30	/	30	/
Glucose	20	/	20	/
Fructose	20	/	20	/
Mineral mixture[1]	50	40	50	40
Vitamin mixture[2, 3]	10	8	10	8
Corn oil	50	50	50	50
Cellulose powder	50	/	50	/
Lyophilized carrot[4, 5]	/	200	/	200
Cholesterol	/	/	2.5	2.5

[1] Mineral mixture AIN-93M (per kg of diet) [36]: Ca HPO_4 15 g; K_2HPO_4 2.5 g; KCl 5 g; NaCl 5 g; MgCl 2.5 g; Fe_2O_3 2.5 mg; $MnSO_4$ 125 mg; $CuSO_4$ 125 mg; $ZnSO_4$, $7H_2O$ 100 mg; KI 0.4 mg

[2] Vitamin mixture (mg/kg of diet): thiamin (15), riboflavin (20), pyridoxine (10), nicotinamide (100), pantothenate (70), folic acid (5), biotin (0.3), cyanocobalamin (0.05), retinyl palmitate (1.5), cholecalciferol (0.15), menadion (1.5), ascorbic acid (50), myo-inositol (100), choline (1360). The mixture was prepared by mixing two AIN-76A [37] preparations purchased from ICN Biomedicals (Ohio), one with α-tocopherol and one without, which was further supplemented with choline

[3] Control and control + cholesterol diets provided α-tocopherol: 32.8 µg/g of diet

[4] Carrot and carrot + cholesterol diets provided α-tocopherol: 41.6 µg/g of diet

[5] Carrot composition (% of dry weight): sucrose 20 %; glucose 13.5 %, fructose 13.5 %, fiber 37 %, protein 7 %, mineral 3 %, lipid 2 %

Diet composition in carotenoids (µg/g): lutein (5.3); α-carotene (54.3); β-carotene (102.4)

lipid analysis or at –80°C for oxidative protection assays and antioxidant molecules quantification.

After blood sampling, the cecum (wall with contents) was removed and weighed. Samples of cecal contents were collected into 2 mL microfuge tubes, immediately frozen, and stored at –20°C. Supernatants from cecal content obtained by centrifuging one of the two microtubes at 20,000 g for 10 min at 4°C were used for short-chain fatty acid (SCFA) analysis. The cecal wall was flush-cleaned with water, dried and weighed (cecal wall weight).

The liver was freeze-clamped into liquid nitrogen and stored at –80°C for the measurement of lipid and carotenoid contents. The heart was rapidly washed in ice physiological buffer (9 g NaCl/L) to remove blood, and quickly frozen in liquid N_2 and stored at –80°C for lipid peroxidation assay.

Analyses on tissues were carried out within 3 weeks after sacrifice.

Analytical procedures

SCFA were measured on aliquots of cecal supernatants by gas-liquid chromatography as previously described [10].

Bile acids were quantified using the enzymatic method with 3 α-hydroxysteroid dehydrogenase (EC 1.1.1.50; Sigma Chemical Co., L'Isle d'Abeau Chesnes, France) from 20 µL of the extract [11]. Bile acids and neutral steroids were extracted twice from feces at 70°C for 2 h by 2 × 10 volume of alkaline ethanol (KOH 0.5 mol/L). Neutral steroids in alkaline ethanol solution (100 µL) were extracted three times with hexane (500 µL) after addition of 5 α-cholestane (internal standard, Sigma St Louis). The hexane extract was concentrated to 200 µL and 2 µL were injected into the gas chromatograph (Danieducational, Paris, France) fitted with a 12 m × 0.25 mm fused silica capillary column (BP 10) and a flame-ionization detector. Helium was used as a carrier gas, and an isocratic temperature (260°C) was used for the steroid separation. Sterol concentrations were calculated from the peak area relative to the area of the internal standard.

Plasma total cholesterol concentration was enzymatically determined using a kit purchased from Bio-Merieux (Charbonnières-les-bains, France) and plasma triglyceride concentrations were determined using a kit from Biotrol (Paris, France). Liver lipids were extracted from approximately 200 mg of liver with chloroform/methanol (2:1, v/v) according to the method previously described [12]. Triglycerides in lipid residue were saponified by 0.5 mol/L KOH-ethanol at 70°C for 30 min, then 0.15 mol/L $MgSO_4$ was added to neutralize the mixture. Cholesterol in the lipid residue was measured with an enzymatic procedure as above. A polyvalent control serum (Biotrol-33-plus, Paris, France) was treated in parallel with samples and served as a control in tissue lipid analysis.

Ferric reducing ability of plasma (FRAP) was determined on 100 µL plasma samples diluted 1:2 with distilled water and the tripyridyltriazine complex formed immediately with the reduced ferrous ions were measured at 593 nm by spectrophotometer (Uvikon 941 plus series, Kontron Instruments, St Quentin en Yvelines, France) and the reaction was monitored up to 4.2 min [13]. Results were calculated from a standard scale of $FeSO_4$.

Free isoprostane (8-epiPGF2 alpha) were extracted from urine as described by Morrow et al. [14]. The urinary samples were assayed using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, and Oxford Biomedical research, Oxford, MI, respectively) [15].

Carotenoids and vitamin E in plasma were analyzed by HPLC-UV [16]. Briefly, carotenoids were extracted from plasma after addition of echinenone (internal standard) by 2 × 2 volumes of hexane. Tissues were previously homogenized with potter in three volumes of PBS 1X before hexane extraction. The HPLC equipment included a photodiode array detector on line with a Waters Millemium. The range of wavelength used was from 270 nm to 520 nm for vitamin E and carotenoids quantification. The separation was carried out on a Vydac TP54 (250 × 4.6 mm; Hesperia, CA) and a Nucleosil column (150 × 4.6 mm; Interchim, Montluçon, France) in series. Elution was performed with an isocratic mobile phase consisting of 70% acetonitrile, 15% methanol containing 50 mM ammonium-acetate, 10% dichloromethane and 5% water (v/v/v/v), at a constant flow of 2 mL/min. Carotenoids were analyzed in the diets as described previously [17].

Calculation

The neutral steroids balance was calculated as: [daily cholesterol intake – fecal excretion of neutral sterols excretion]. The total steroids balance was calculated as: [daily cholesterol intake – (fecal excretion of bile acids + fecal excretion of neutral steroids)].

Statistical analysis

The statistical comparisons were performed with STATVIEW software (version 5.0; SAS Institute Inc, Cary, NC). Values are given as the means ± SEM. A two-way ANOVA was employed with dietary cholesterol and carrot diet as main effects. ANOVA was followed by Fisher's least significant difference test to compare treatment means. Differences of $P < 0.05$ were considered significant.

© Steinkopff Verlag 2004

Results

C57BL/6J mice were used in the present study. Mice received 20 % of lyophilized carrot in the diet compared to 5 % cellulose to equilibrate fiber supply. Furthermore, they were fed on cholesterol-free diet or cholesterol-supplemented diet (2.5 g/kg).

Food intake, body and organ weight, and symbiotic fermentation

Neither dietary lyophilized carrot nor exogenous cholesterol affected food consumption and body weight (Table 2). Absolute and relative heart and liver weights were affected neither by the incorporation of 20 % of freeze-dried carrot nor by the incorporation of 0.25 % cholesterol.

The cecal short-chain fatty acids (SCFA) pool was markedly enhanced due to carrot diet consumption in both cholesterol-free and cholesterol fed mice ($P < 0.001$). Acetate was largely enhanced (+ 170 % between control and carrot); propionate concentration increased between cholesterol fed mice (+ 69 %), whereas there was no change for butyrate (Fig. 1).

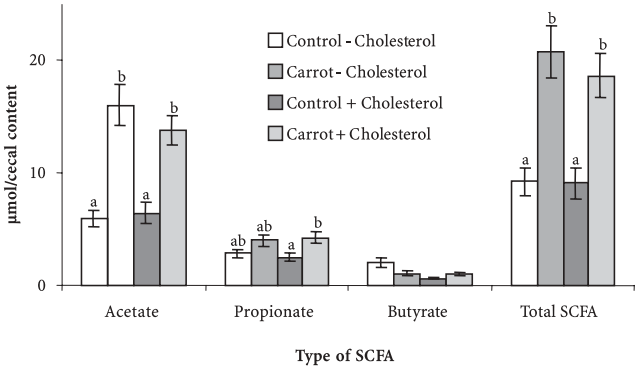

Fig. 1 Cecal short-chain fatty acids in mice fed control or 20 % lyophilized carrot diet for 4 weeks. Values are means ± SEM, n = 10 in each group

Effect of cholesterol metabolism

Plasma concentrations of cholesterol and triglycerides were 2.31 and 0.63 mmol/L, respectively, in control mice (Table 3). No difference was observed in plasma cholesterol concentrations of mice fed cellulose or lyophilized carrot in cholesterol-free diet. In contrast, cholesterolemia was substantially decreased by carrot diet in cholesterol fed mice (–41 %). Plasma triglyceride levels were significantly reduced by carrot diet under both conditions (–22 % with cholesterol-free diet and –49 % with diet containing cholesterol).

Liver cholesterol and triglyceride concentrations did not differ between control and carrot cholesterol-free fed mice. Hepatic cholesterol and triglyceride amounts were largely enhanced by cholesterol enriched diet leading to steatosis, although the accumulation of cholesterol and triglycerides in the liver was significantly lowered (–41 % and –39 %, respectively) by carrot diet.

Table 2 Food intake, body and organs weight of a 4-week period of feeding experimental diet. Values indicate mean ± SEM over the 4 weeks of the experiment (n = 10)

	Control	Carrot	Control + cholesterol	Carrot + cholesterol	ANOVA (P-value)		
					Cholesterol	Carrot	Cholesterol x carrot
Food intake (dry g/day)	2.7 ± 0.03	2.6 ± 0.03	2.7 ± 0.08	2.7 ± 0.03	ns	ns	ns
Body weight (g)	21.1 ± 0.4	20.0 ± 0.3	20.9 ± 0.5	20.8 ± 0.5	ns	ns	ns
Relative liver weight (g/100 g)	4.10 ± 0.15	4.07 ± 0.20	4.19 ± 0.22	4.17 ± 0.19	ns	ns	ns
Relative heart weight (g/100 g)	0.51 ± 0.02	0.58 ± 0.05	0.58 ± 0.05	0.51 ± 0.02	ns	ns	ns

Table 3 Plasma and liver lipids in mice fed control or 20 % lyophilized carrot diet for 4 weeks. Values are means ± SEM, n = 10 in each group

	Control	Carrot	Control + cholesterol	Carrot + cholesterol	ANOVA (P-value)		
					Cholesterol	Carrot	Cholesterol x carrot
Plasma mmol/L							
Cholesterol	$2.31 \pm 0.18^{a,b}$	$1.97 \pm 0.22^{a,c}$	2.76 ± 0.20^{b}	1.63 ± 0.13^{c}	ns	< 0.005	< 0.05
Triglyceride	0.63 ± 0.04^{a}	0.49 ± 0.03^{b}	0.84 ± 0.05^{c}	0.43 ± 0.04^{b}	ns	< 0.001	< 0.005
Liver mg/g							
Cholesterol	2.00 ± 0.08^{a}	1.90 ± 0.06^{a}	17.31 ± 0.94^{b}	10.23 ± 1.10^{c}	< 0.001	< 0.001	< 0.001
Triglyceride	9.47 ± 1.00^{a}	8.33 ± 0.42^{a}	24.27 ± 1.90^{b}	14.86 ± 1.24^{c}	< 0.001	< 0.005	< 0.005

[a, b, c] Values within a line not sharing the same superscript are significantly different ($P < 0.05$)

Cholesterol intake and digestive balance of bile acids

Since bile acids and neutral sterols are the major way by which cholesterol is eliminated from the body, we investigated the effect of carrot diet on the bile acids and neutral sterols excretions (Table 4; Fig. 2). In the absence of dietary cholesterol, fecal excretion of both bile acids and neutral sterols were of the same magnitude. Lyophilized carrot consumption induced a twofold higher excretion of both neutral sterols and bile acids. In diet supplemented with cholesterol, the fecal losses of cholesterol were highly increased and carrot consumption induced a significant 30 % increase in neutral sterol excretion. In spite of a relatively high level of cholesterol supplementation, the digestive neutral sterol balance was only slightly positive in the control mice; this balance remained negative in mice fed carrot diet. Dietary cholesterol significantly increased bile acids fecal excretion and carrot addition failed to enhance bile acids excretion by contrast to the cholesterol-free diets. The total steroid balance was negative not only in mice fed on cholesterol-free diet, but also in mice receiving cholesterol supplementation. This balance was twofold lower in both groups of mice fed carrot diet.

Effect on plasma and tissue antioxidants

Dietary cholesterol induced a decrease in plasma vitamin E concentrations (Table 5) ($P = 0.004$), whereas lyophilized carrot did not significantly affect vitamin E levels. Dietary carrot did not affect vitamin E/TG ratio in mice fed cholesterol-free diet, whereas, in mice fed cholesterol-enriched diet, carrot intake significantly improved the ratio meaning a higher lipid protection. Vitamin E was also determined in heart tissue (Table 6). We noticed an improvement of vitamin E content in heart due to the carrot powder supplementation ($P = 0.002$). Dietary cholesterol enhanced the pool of liver vitamin E in relation to the increase of triglyceride pool. The decrease of vitamin E in the liver of mice fed carrot-cholesterol diet could be explained by the decrease of triglyceride pool.

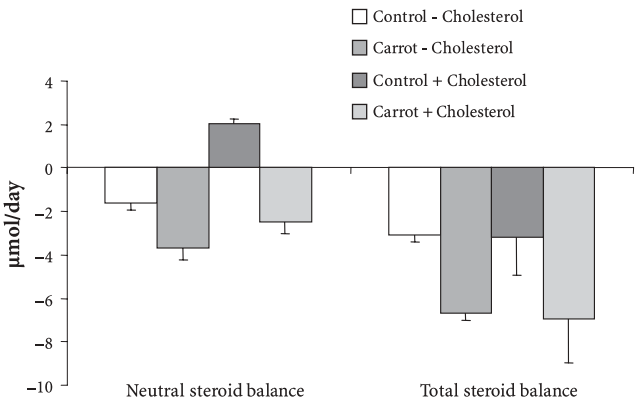

Fig. 2 Changes in fecal excretion of neutral steroids and in the total steroid balances. Values are means ± SEM, n = 10 in each group

In mice fed on carrot diet, carotenoids (α-carotene and β-carotene) were recovered in plasma (Table 5). Neither isomers nor carotenoid metabolites were detected. The level of these two carotenoids was lower in the plasma of mice fed cholesterol diet, but this decrease was not significant. Carotenoids were undetectable in heart in both groups. The levels of α-carotene and β-carotene in the liver were twofold higher in mice fed on cholesterol-rich diet (Table 6). This increase was explained by the higher levels of triglycerides in the liver of these mice (β-carotene/TG ratio was 0.03 ± 0.003 vs. 0.04 ± 0.002 in cholesterol-free and cholesterol-supplemented groups, respectively).

Effect on biomarkers of oxidative capacity and oxidative stress

FRAP values, which reflect the total antioxidative capacity of plasma protection, increased by about 15 % in mice fed 20 % lyophilized carrot diet; however, this increase was only significant in cholesterol-fed groups (Table 5). Indeed, lyophilized carrot improved the antioxidant protection in both the cholesterol-free and enriched groups, concurrently with a marginal increase when cholesterol was added to the diet.

Table 4 Digestive balance of total steroids in mice after a 4-week period of feeding experimental diet. Values are means ± SEM, n = 10 in each group

	Control	Carrot	Control + cholesterol	Carrot + cholesterol	ANOVA (P-value)		
					Cholesterol	Carrot	Cholesterol x carrot
Cholesterol intake (µmol/day)	nd	nd	17.5 ± 0.5	17.6 ± 0.2	< 0.001	ns	ns
Fecal cholesterol (µmol/day)	1.6 ± 0.4[a]	3.3 ± 0.5[a]	15.0 ± 2.6[b]	21.3 ± 3.3[c]	< 0.001	ns	ns
Fecal coprostanol (µmol/day)	nd	0.4 ± 0.1	0.4 ± 0.1	1.0 ± 0.1	< 0.001	< 0.005	ns
Total neutral sterols	1.6 ± 0.4[a]	3.7 ± 0.3[a]	15.4 ± 1.7[b]	20.1 ± 2.1[c]	< 0.001	< 0.001	< 0.001
Bile acids fecal excretion (µmol/day)	1.4 ± 0.3[a]	3.0 ± 0.5[c]	5.3 ± 0.2[b]	4.5 ± 0.5[b]	< 0.0001	ns	< 0.05

[a, b, c] Values within a line not sharing the same superscript are significantly different ($P < 0.05$)

© Steinkopff Verlag 2004

Table 5 Plasma FRAP level and plasma concentrations of antioxidant compounds in mice fed control or 20 % lyophilized carrot diet for 4 weeks. Values are means ± SEM, n = 10 in each group

	Control	Carrot	Control + cholesterol	Carrot + cholesterol	ANOVA (P-value)		
					Cholesterol	Carrot	Cholesterol x carrot
FRAP (µmol Fe/L)	240 ± 7[a]	274 ± 10[a]	267 ± 10[a]	309 ± 12[b]	< 0.005	< 0.0005	ns
Vitamin E (µmol/L)	9.9 ± 2.5[a]	9.0 ± 1.6[a, b]	6.6 ± 1.4[b]	7.3 ± 1.0[a, b]	< 0.005	ns	ns
Vitamin E/TG ratio	16.6 ± 0.6[a]	19.9 ± 1.0[a]	8.3 ± 0.6[b]	1.0 ± 0.1	< 0.05	< 0.01	ns
Carotenoids (nmol/L)							
α-carotene	nd	123.6 ± 26.1	nd	100.8 ± 11.2			
β-carotene	nd	158.1 ± 37.4	nd	113.3 ± 16.3			

Detection limit is 2 ng for α- and β-carotene and 20 ng for α-tocopherol
[a, b] Values within a line not sharing the same superscript are significantly different (P < 0.05)

Table 6 Liver and heart carotenoids and vitamin E levels in mice fed control or 20 % lyophilized carrot diet for 4 weeks. Values are means ± SEM, n = 10 in each group

	Liver			Heart		
	α-carotene	β-carotene (µg/organ)	Vitamin E	α-carotene	β-carotene (µg/organ)	Vitamin E
Control	nd	nd	18.85 ± 1.44[a]	nd	nd	0.09 ± 0.01a
Carrot	0.14 ± 0.01	0.22 ± 0.02	14.19 ± 1.01[a]	nd	nd	0.15 ± 0.03[b]
Control + cholesterol	nd	nd	27.93 ± 1.95[b]	nd	nd	0.07 ± 0.01[a]
Carrot + cholesterol	0.25 ± 0.02***	0.48 ± 0.05***	18.51 ± 2.22[a]	nd	nd	0.13 ± 0.02[a, b]

*** P < 0,0005
[a, b] Values within a row not sharing the same superscript are significantly different (P < 0.05)

Urinary excretion of isoprostanes was markedly increased by dietary cholesterol ($P = 0.015$), indicating an increase of oxidative injury *via* free radical catalyzed lipid peroxidation. Carrot addition remained with a moderate effect; a non-significant 18 % decrease of urinary excretion of isoprostane was only observed between mice fed carrot-cholesterol and control-cholesterol diets (Fig. 3).

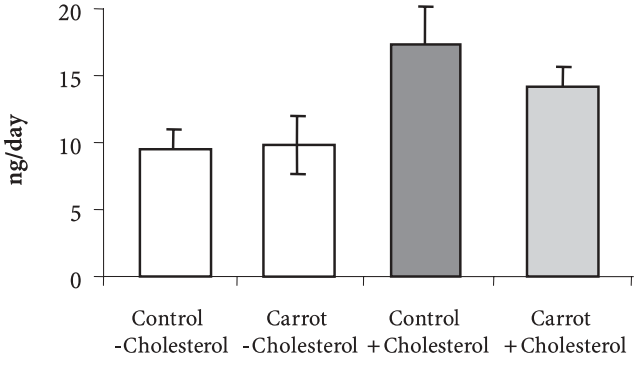

Fig. 3 Changes in isoprostane urinary excretion in mice fed control or 20 % lyophilized carrot diet for 4 weeks. Values are means ± SEM, n = 10 in each group

Discussion

Effect on cholesterol metabolism

Both animal and clinical studies have previously suggested that fruit and vegetable fibers can potentially exert an hypocholesterolemic effect [7, 18, 19] and it is interesting to describe the main cardiovascular protective effects of a vegetable largely consumed in human nutrition.

In the present study, we choose to evaluate the effect of carrot in the C57BL/6J mice in which cholesterol metabolism is well documented. However, mice are a less suitable model for carotenoid research because their absorption of carotenoids is lower than that of humans. Despite this inconveniency, the use of mice allows investigation of the effects of carotenoid-free and enriched diet. Although the C57BL/6J mice are frequently used for lipid metabolism study [20], we did not observe any effect of cholesterol addition in the diet as was observed in a previous study despite cholesterol accumulation in the liver. However, the carrot diet showed a plasma cholesterol-lowering effect in mice fed on cholesterol-enriched diets (–41 %). In cholesterol-free diet, the carrot effect was lower (–15 %). Similar phenomena were found with a diet containing 5 g cholesterol/kg plus 5 %

of rhubarb fiber [21]. These results confirm that numerous legumes or vegetables have a cholesterol-lowering effect, but they may not all be equally potent. For example, apple was found to be more potent than carrot in rat model [22, 23].

Unlike cholesterolemia, carrot diet decreased markedly triglyceride levels in both cholesterol-free and cholesterol-fed mice. In mice fed the cholesterol diet, the effect of carrot on triglyceridemia was particularly potent (–49 %).

Although dietary cholesterol has small effect on plasma cholesterol, it induced a large increase of liver cholesterol. Mice fed on the cholesterol diet did not have heavier livers, but this tissue was very pale in color. These observations were in agreement with previous ones reporting fatty liver in rats and in mice fed on high-fat, high-cholesterol diets [24, 25]. There was a massive triglycerides and cholesterol accumulation in the liver, which was effectively reduced by carrot diet.

The main mechanism involved in the lipid-lowering effect of many vegetables was certainly linked to their content of dietary fiber and associated compounds. In the present study, we observed a marked increase of neutral sterol fecal excretion, especially of cholesterol. In this case, 5 % carrot dietary fiber could completely inhibit digestive cholesterol absorption. Carrot fibers, rich in pectin or in soluble fermentable hemicellulose, were, thus, very efficient in reducing cholesterol absorption in contrast to cellulose. This result was previously observed by Elliott et al. [26] who compared the respective effect of pectin, cellulose and tomato pomace. Other compounds such as plant-sterols, carotenoids or tocotrienols could also be involved in the cholesterol-lowering effect of carrot, but the respective role of fiber and associated compounds was not identified. It is possible that minor compounds could enhance the fiber effect. Moreover, without fiber, the isolated micronutrients may have a marginal effect on cholesterol absorption and metabolism.

Dietary fibers also act on the fecal excretion of bile acids. Degradation of cholesterol to bile acids is one of the major pathways by which cholesterol is eliminated from the body. The sequestrants (useful drug therapy for patients with hypercholesterolemia) work by binding the bile salts in the gut lumen, thus inhibiting their re-absorption by the intestinal cells and facilitating their fecal excretion. It has been demonstrated *in vitro* that rhubarb fiber (mainly insoluble fraction, 66 %) has the ability to bind bile salts [27], which could then be responsible for its consistent lipid-lowering effect observed in humans [28]. The effect of carrot on bile entero-hepatic cycling is unlikely to be due to a unique component, but rather results from the effect of a combination of several types of fiber (hemicellulose, pectin) as well as lignin. As carrot contains about 1.5 % of dry weight of lignin, this polyphenol may possibly be involved in the improvement of bile acids fecal excretion.

When cholesterol is included in the diet, enzymes in the supply pathway are suppressed and cholesterol 7 α-hydroxylase is induced (the first enzyme involved in the bile acids biosynthesis). This induction in turn leads to an increase in bile acid production and to an increase of their excretion [29], which could explain the difference between diets without cholesterol and 0.25 % supplemented diets. In mice fed cholesterol diet, carrot apparently failed to enhance bile acids fecal excretion. This result may be explained by the fact that in mice fed carrot diet, the liver received a lower amount of digestive cholesterol.

Besides, the down-regulation of the cholesterol 7 α-hydroxylase leads to a decrease of bile acids and then to an increase of the hepatic cholesterol. This could also explain the fatty livers we observed. Carrot fibers largely prevent the down-regulation leading to a lower accumulation of cholesterol and triglycerides.

In conclusion, lipid-lowering effect seems mainly due to the presence of carrot fibers, and associated compounds are only an indicator of exposure. Nevertheless, it is likely that other nutrients could also affect lipid status. Thus, it should be interesting to investigate clearly the impact of fiber or various lipophilic micronutrients on lipid absorption and transport.

Effect on plasma and tissue antioxidants

Besides effects on cholesterol metabolism, carrot may also be involved in other beneficial health effects due to a large supply of antioxidants. The mouse as model provides many advantages for experimental atherosclerosis research in which antioxidants are largely involved [30].

Various carrot constituents are potentially protective against peroxide attack, chiefly lipid-soluble compounds such as carotenoids (β-carotene) or vitamin E. After 4 weeks of carrot feedings, the level of α-tocopherol in plasma was marginally affected, whereas α-tocopherol/TG ratio was increased by carrot diet, suggesting a higher protection of lipid against radical attack. On the other hand, we observed a significant direct correlation between β-carotene and α-tocopherol in plasma, promoting greater defenses. Indeed, β-carotene has been shown to act synergistically with α-tocopherol as radical-trapping antioxidant in membrane models [31]. Heart was also protected by carrot diet as suggested by the α-tocopherol levels, which were 1.8 times higher than in control mice. Carotenoids were undetectable in heart, but were recovered in liver. The large differences of vitamin contents in the tissues could be explained by the lipid metabolism. In contrast to the heart, the liver has the capacity to accumulate vitamin E in a large pool of triglycerides and to release this vitamin by lipoprotein secretion. The development of steatosis enhances the capacity of the liver to sequestrate vitamin E and

© Steinkopff Verlag 2004

carotenoids. Liver carotenoid levels were twofold higher in mice fed cholesterol-enriched diet due to the eight-fold and 2.5 fold increases of cholesterol and TG, respectively.

Effect on biomarkers of antioxidative capacity and oxidative stress

The results of the present study show that supplementation with 20% of lyophilized carrot, with or without 0.25% cholesterol, enhances plasma antioxidant capacity, especially with cholesterol-enriched diets. Surprisingly, FRAP also increased with cholesterol supplementation, whereas the higher isoprostane excretion shows an increase of lipid peroxydation. At present, we have no explanation for such effect: it could be discussed that oxidative damage caused by dietary cholesterol may be occulted in plasma by the involvement of endogenous antioxidants such as bilirubine or acid uric.

Among all the biomarkers of oxidative stress, the LDL capacity to protect against exogenous stress is one of the most investigated. Previous investigators have suggested that β-carotene may protect mice tissues against lipid peroxidation [32] or should inhibit atherosclerosis in cholesterol-fed rabbits by a pathway independent of making LDL resistant to oxidation [33]. However, no inhibition of atherogenesis was found with the antioxidant combination of β-carotene (0.05%) and α-tocopherol (0.05%) in the diet fed to apoE-deficient mice [34], although this supplementation corresponded to a very large dose (threefold higher in carotenoids and seventeen-fold higher in vitamin E).

Isoprostane urinary excretion, another biomarker of oxidative stress, showed that cholesterol addition in the diet induces isoprostane excretion and that carrot ingestion decreases non-significantly the F2-isoprostane excretion (–18%). We have to keep in mind nutritional conditions, as the amount of antioxidant contained in 20% carrot is lower than in pharmacological studies, as demonstrated for vitamin E by Pratico et al. [35].

In conclusion, we have found that a carrot ingestion can reduce plasma and hepatic lipids or improve antioxidant status and several biological parameters considered as a factor of risk for cardio-vascular diseases and especially in nutrition-induced stress conditions. The lipid-lowering effect of carrot mainly mediated by fibers may contribute to enhance the effect of antioxidant by increasing lipid-soluble antioxidants/TG ratio. The question arises whether the impact of micronutrients antioxidant could be enhanced by fibers or other minor compounds in a complex vegetable.

Acknowledgement We gratefully acknowledge B. Lyan, P. Lamby and C. Cubizolles for their respective advice, technical assistance and their contribution in animal handling.

References

1. Steinmetz KA, Potter JD (1991) Vegetables, fruit, and cancer. I. Epidemiology. Cancer Causes Control 2:325–357
2. Block G, Patterson B, Subar A (1992) Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. Nutr Cancer 18:1–29
3. Ness AR, Powles JW (1997) Fruit and vegetables, and cardiovascular disease: a review. Int J Epidemiol 26:1–13
4. Gillman MW, Cupples LA, Gagnon D, Posner BM, Ellison RC, Castelli WP, Wolf PA (1995) Protective effect of fruits and vegetables on development of stroke in men. JAMA 273:1113–1117
5. Alasalvar C, Grigor JM, Zhang D, Quantick PC, Shahidi F (2001) Comparison of volatiles, phenolics, sugars, antioxidant vitamins, and sensory quality of different colored carrot varieties. J Agric Food Chem 49:1410–1416
6. Robertson J, Brydon WG, Tadesse K, Wenham P, Walls A, Eastwood MA (1979) The effect of raw carrot on serum lipids and colon function. Am J Clin Nutr 32:1889–1892
7. Jenkins DJ, Kendall CW, Popovich DG, Vidgen E, Mehling CC, Vuksan V, Ransom TP, Rao AV, Rosenberg-Zand R, Tariq N, Corey P, Jones PJ, Raeini M, Story JA, Furumoto EJ, Illingworth DR, Pappu AS, Connelly PW (2001) Effect of a very-high-fiber vegetable, fruit, and nut diet on serum lipids and colonic function. Metabolism 50:494–503
8. Brown L, Rosner B, Willett WW, Sacks FM (1999) Cholesterol-lowering effects of dietary fiber: a meta-analysis. Am J Clin Nutr 69:30–42
9. Vahouny GV (1982) Dietary fiber, lipid metabolism, and atherosclerosis. Fed Proc 41:2801–2806
10. Rémésy C, Demigné C (1974) Determination of volatile fatty acids in plasma after ethanolic extraction. Biochem J 141:85–91
11. Turley SD, Dietschy JM (1978) Re-evaluation of the 3 alpha-hydroxysteroid dehydrogenase assay for total bile acids in bile. J Lipid Res 19:924–928
12. Mazur A, Rémésy C, Gueux E, Levrat MA, Demigné C (1990) Effects of diets rich in fermentable carbohydrates on plasma lipoprotein levels and on lipoprotein catabolism in rats. J Nutr 120:1037–1045
13. Benzie IF, Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem 239:70–76
14. Morrow JD, Awad JA, Boss HJ, Blair IA, Roberts LJ 2nd (1992) Non-cyclooxygenase-derived prostanoids (F2-isoprostanes) are formed in situ on phospholipids. Proc Natl Acad Sci USA 89: 10721–10725
15. Feillet-Coudray C, Tourtauchaux R, Niculescu M, Rock E, Tauveron I, Alexandre-Gouabau MC, Rayssiguier Y, Jalenques I, Mazur A (1999) Plasma levels of 8-epiPGF2alpha, an in vivo marker of oxidative stress, are not affected by aging or Alzheimer's disease. Free Radic Biol Med 27:463–469
16. Lyan B, Azais-Braesco V, Cardinault N, Tyssandier V, Borel P, Alexandre-Gouabau MC, Grolier P (2001) Simple method for clinical determination of 13 carotenoids in human plasma using an isocratic high-performance liquid chromatographic method. J Chromatogr B Biomed Sci Appl 751:297–303

17. Tyssandier V, Reboul E, Dumas JF, Bouteloup-Demange C, Armand M, Marcand J, Sallas M, Borel P (2003) Processing of vegetable-borne carotenoids in the human stomach and duodenum. Am J Physiol Gastrointest Liver Physiol 284:G913–923
18. Mee KA, Gee DL (1997) Apple fiber and gum arabic lowers total and low-density lipoprotein cholesterol levels in men with mild hypercholesterolemia. J Am Diet Assoc 97:422–424
19. Anderson JW, Hanna TJ (1999) Impact of nondigestible carbohydrates on serum lipoproteins and risk for cardiovascular disease. J Nutr 129: 1457S–1466S
20. Paigen B, Holmes PA, Mitchell D, Albee D (1987) Comparison of atherosclerotic lesions and HDL-lipid levels in male, female, and testosterone-treated female mice from strains C57BL/6, BALB/c, and C3H. Atherosclerosis 64:215–221
21. Goel V, Cheema SK, Agellon LB, Ooraikul B, Basu TK (1999) Dietary rhubarb (Rheum rhaponticum) stalk fibre stimulates cholesterol 7 alpha-hydroxylase gene expression and bile acid excretion in cholesterol-fed C57BL/6J mice. Br J Nutr 81:65–71
22. Aprikian O, Levrat-Verny M-A, Besson C, Busserolles J, Rémésy C, Demigné C (2001) Apple favourably affects parameters of cholesterol metabolism and anti-oxidative protection in cholesterol-fed rats. Food Chemistry 75: 445–452
23. Nicolle C, Cardinault N, Aprikian O, Busserolles J, Grolier P, Rock E, Demigné C, Mazur A, Scalbert A, Amouroux P, Rémésy C (2003) Effect of carrot intake on cholesterol metabolism and on antioxidant status in cholesterol-fed rat. Eur J Nutrition 42: 254–261
24. Beynen AC, Klaasen HL, Koopman JP, Fielmich-Bouman AM, Lemmens AG (1989) Liver cholesterol concentrations in mice fed diets containing various sources of fat, carbohydrates or fiber. Int J Vitam Nutr Res 59:401–405
25. Beynen AC, Meijer GW, Van der Meer R (1988) Comparison of rats with mice concerning the response of lipid metabolism to dietary fats. Z Ernahrungswiss 27:143–149
26. Elliott J, Mulvihill E, Duncan C, Forsythe R, Kritchevsky D (1981) Effects of tomato pomace and mixed-vegetable pomace on serum and liver cholesterol in rats. J Nutr 111:2203–2211
27. Goel V, Cheema SK, Agellon LB, Ooraikul B, McBurney MI, Basu TK (1998) In vitro binding of bile salt to rhubarb stalk powder. Nutrition Res 18:893–903
28. Goel V, Ooraikul B, Basu TK (1997) Cholesterol lowering effects of rhubarb stalk fiber in hypercholesterolemic men. J Am Coll Nutr 16:600–604
29. Russell DW, Setchell KD (1992) Bile acid biosynthesis. Biochemistry 31: 4737–4749
30. Smith JD (1998) Mouse models of atherosclerosis. Lab Anim Sci 48:573–579
31. Palozza P, Krinsky NI (1992) Beta-carotene and alpha-tocopherol are synergistic antioxidants. Arch Biochem Biophys 297:184–187
32. Iyama T, Takasuga A, Azuma M (1996) Beta-carotene accumulation in mouse tissues and a protective role against lipid peroxidation. Int J Vitam Nutr Res 66:301–305
33. Shaish A, Daugherty A, O'Sullivan F, Schonfeld G, Heinecke JW (1995) Beta-carotene inhibits atherosclerosis in hypercholesterolemic rabbits. J Clin Invest 96:2075–2082
34. Shaish A, George J, Gilburd B, Keren P, Levkovitz H, Harats D (1999) Dietary beta-carotene and alpha-tocopherol combination does not inhibit atherogenesis in an ApoE-deficient mouse model. Arterioscler Thromb Vasc Biol 19:1470–1475
35. Pratico D, Tangirala RK, Rader DJ, Rokach J, FitzGerald GA (1998) Vitamin E suppresses isoprostane generation in vivo and reduces atherosclerosis in ApoE-deficient mice. Nat Med 4: 1189–1192
36. Reeves PG, Nielsen FH, Fahey GC Jr (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 123: 1939–1951
37. AIN-76A (1977) Report of the American Institute of Nutrition ad hoc Committee on Standards for Nutritional Studies. J Nutr 107:1340–1348