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Green tea extract only affects markers of oxidative status postprandially: lasting antioxidant effect of flavonoid-free diet*

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Epidemiological studies suggest that foods rich in flavonoids might reduce the risk of cardiovascular disease and cancer. The objective of the present study was to investigate the effect of green tea extract (GTE) used as a food antioxidant on markers of oxidative status after dietary depletion of flavonoids and catechins. The study was designed as a 2×3 weeks blinded human cross-over intervention study (eight smokers, eight non-smokers) with GTE corresponding to a daily intake of 18·6 mg catechins/d. The GTE was incorporated into meat patties and consumed with a strictly controlled diet otherwise low in flavonoids. GTE intervention increased plasma antioxidant capacity from 1·35 to 1·56 ($P<0·02$) in postprandially collected plasma, most prominently in smokers. The intervention did not significantly affect markers in fasting blood samples, including plasma or haemoglobin protein oxidation, plasma oxidation lagtime, or activities of the erythrocyte superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase. Neither were fasting plasma triacylglycerol, cholesterol, α-tocopherol, retinol, β-carotene, or ascorbic acid affected by intervention. Urinary 8-oxo-deoxyguanosine excretion was also unaffected. Catechins from the extract were excreted into urine with a half-life of less than 2 h in accordance with the short-term effects on plasma antioxidant capacity. Since no long-term effects of GTE were observed, the study essentially served as a fruit and vegetables depletion study. The overall effect of the 10-week period without dietary fruits and vegetables was a decrease in oxidative damage to DNA, blood proteins, and plasma lipids, concomitantly with marked changes in antioxidative defence.

Green tea extract intervention: Dietary depletion of fruits and vegetables: Biomarkers of oxidative damage: Biomarkers of antioxidative defence: Protein oxidation

Consumption of fruit and vegetables is widely encouraged because of the epidemiological indications of a relationship between increased intake and reduced risk of cancer and cardiovascular diseases. A major group of antioxidative compounds in fruit and vegetables, the polyphenols, has been suggested to contribute to these beneficial effects (Hertog *et al.* 1993*a*, 1994, 1995). In a Dutch population, tea was found to be the main dietary source of five selected flavonoids (Hertog *et al.* 1993*b*). However, the beneficial effects of tea on cancer and cardiovascular diseases remain controversial (Goldbohm *et al.* 1996; Katan, 1997; Kohlmeier *et al.* 1997).

Abbreviations: AAS, 2-amino-adipic semialdehyde; EC, epicatechin; EGC, epigallocatechin; GTE, green tea extract; 8-oxo-dG, 8-oxo-deoxyguanosine.

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As much as 26–30 % of the DM of fresh tea leaves may consist of flavonoids (Lin *et al.* 1998), among which the catechins are the most abundant. During the manufacture of black tea (a fermentation process) catechins are progressively oxidised by polyphenol oxidase, to form polymerisation products (Lin *et al.* 1998). The catechins in tea have a potent antioxidative capacity in model systems, including radical scavenging (Yen & Chen, 1995; Gardner *et al.* 1998) and provide protection against oxidative changes in plasma (Ishikawa *et al.* 1997; Lotito & Fraga, 1998) and LDL (Ishikawa *et al.* 1997; Zhang *et al.* 1997). Some studies indicate that green tea is a more potent antioxidant than black tea in *in vitro* model systems (Yen & Chen, 1995; Gardner *et al.* 1998), probably due to its higher content of catechins and lack of polymerisation products (Lin *et al.* 1998).

If catechins can be incorporated into plasma they may be expected to affect the antioxidative status. Animal studies on pure catechins in rats have shown that these compounds are absorbed and transported to the tissues (Nakagawa & Miyazawa, 1997) and experiments with human subjects have shown that catechins are found in plasma (Nakagawa *et al.* 1997) and excreted into the urine (Das, 1971). Consumption of both green and black tea was found to increase plasma antioxidant capacity within 30–50 min (Serafini *et al.* 1996), but over a longer time scale (4 weeks) no effects on antioxidative parameters in blood were observed apart from a slight increase in antioxidative activity in plasma after green tea consumption (van het Hof *et al.* 1997).

If tea consumption has an effect on the antioxidative system an extract of tea could be expected to exhibit a similar effect. The present study investigated the effect of a green tea extract (GTE) on markers of oxidative status in blood and urine in a blinded double cross-over study with complete control of all dietary intakes. The GTE was incorporated into meat patties at a concentration of 1000 mg/kg, which has been demonstrated to protect against oxidative changes in dried chicken meat (Nissen *et al.* 2000).

In order to study individuals with different levels of oxidative stress the study included both smokers and non-smokers.

Methods

Subjects

Sixteen men volunteered for the study including eight non-smokers and eight smokers (10–15 cigarettes/d). Average age was 23 (range 20–31) years and average BMI was 22·7 (range 19·5–25·7) kg/m^2. None of the subjects had any chronic illness. Subjects received oral and written information about the study, and gave their written consent. The study was approved by the local Research Ethics Committee of Copenhagen and Frederiksberg (Journal number KF01-342/97).

Study design and diet

The study design is illustrated in Fig. 1. It was a double-blind randomised 2 × 3 week cross-over with 2 weeks wash-out before each intervention. Subjects were divided into groups A and B, with four smokers and four non-smokers in each group. A standardised diet with a low flavonoid content, including 200 g meat patties/10 MJ, was served for the 2 × 3 week study periods. For one of the 3-week periods the subjects consumed GTE incorporated into meat patties at a concentration of 1000 mg/kg. During the wash-out weeks the subjects excluded flavonoid-containing foods from their diet. Tea, wine, spices, chocolate, cocoa and all products containing fruits, berries and vegetables, except potatoes and carrots were excluded from their diet. A list of allowed dietary items was distributed to the participants, which included all meats, fish, shellfish, grain products (including whole grain) such as

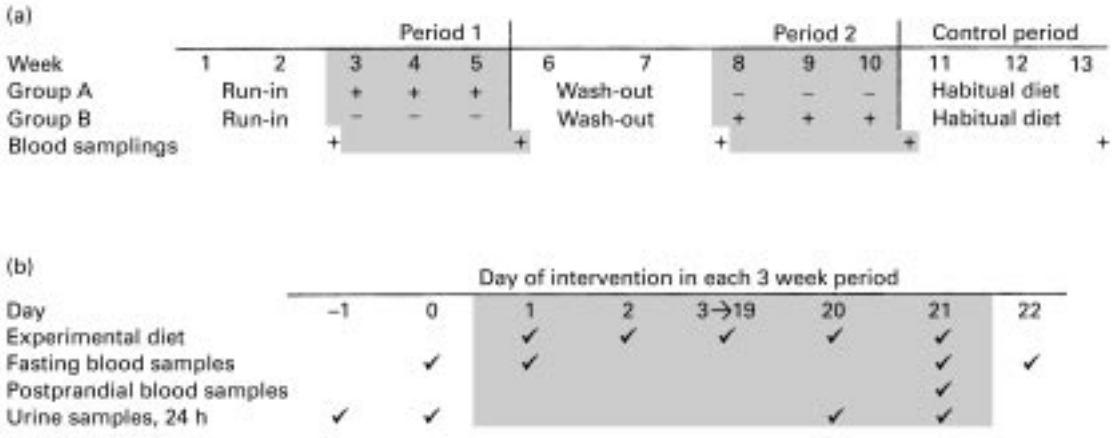

Fig. 1. Study design. (a) Depiction of the overall design over the entire 13-week period, including two sequential periods, each with an initial 2-weeks wash-out followed by a 3-weeks cross-over intervention, and a final control period. (b) Depiction of the exact sampling times for blood and urine during each of periods 1 and 2. (+) Intervention with green tea extract; (−) sample collection.

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Table 1. Composition of the experimental diet (g/d) at an energy intake of 10 MJ/d

	Component	Menu 1	Menu 2	Menu 3	Menu 4	Menu 5	Menu 6	Menu 7
Breakfast	Bread roll with carrots	77	77	77	77	77	77	77
	Cheese	25	25	25	25	25	25	25
	Sour milk	170	170	170	170	170	170	170
	Rye breadcrumbs	30	30	30	30	30	50	50
Lunch	Rye bread	100	100	100	100	100	100	100
	Butter	6	6	6	6	6	6	6
	Meat patties	50	50	50	50	50	50	50
	Tuna salad	34		34				
	Potatoes		50			50		
	Ham salad				44			
	Roast beef						30	30
	Carrot salad	100	100	100	100	100	100	100
Dinner	Meat patties	150	150	150	150	150	150	150
	Mashed potatoes	250					250	
	Pasta		70					
	Potatoes			200		200		
	Rice				70			70
	Stock	150					150	
	Brown sauce			153		153		
	Cheese sauce		160					
	Chicken sauce				180			180
Snack	Bread roll with carrots	77	77	77	77	77	77	77
	Butter	6	6	6	6	6		
	Sponge cake	100		100		100		
	Carrot cake		100		100		100	100
Milk		250	250	250	250	250	250	250

breads and pasta, eggs, potatoes, carrots, freeze dried coffee, mineral water and tap water. The freeze dried coffee was analysed by HPLC and found to be free from catechins. The diet during intervention consisted of seven menus consumed each week from Monday to Sunday (Table 1). The macronutrient and selected micronutrient contents were calculated using a computer program (Dankost) which is based on the Danish Veterinary and Food Administration composition database (Møller, 1989) (Table 2). Each subject's energy requirement was estimated from body weight and degree of physical activity. All meals were prepared at the department in individual portions according to energy requirement. One of the daily main meals was taken at the department while the remaining (cold) meals were provided daily for intake at home. Subjects were instructed to return any leftovers to the department for weighing and subtraction from planned intake and were asked daily about compliance. Freeze-dried coffee powder was provided for preparation of coffee. Coffee intake was recorded and varied between subjects but was held constant over the 6 weeks. No food or drink other than that provided from the department was allowed. Fasting blood samples were collected during each period on the morning of day 0, 1, 21 and 22, thus reflecting the preceding 24 h, i.e. day 0 and 1 samples reflect baseline concentrations before intervention (days −1 and 0, respectively) and days 21 and 22 samples reflect the last 2 d of each intervention period (days 20 and 21, respectively) (see Fig. 1(b)). In addition, one blood sample was taken 2.5 h after the hot meal at midday on day 21 of each period. The samples will be referred to in the text according to the 24 h they are reflecting within each period. A final blood sample was collected after week 13 when the subjects had been on their habitual diet for 3 weeks after the last intervention. This sample was collected to represent the participant's natural background. Twenty-four hour urine samples were collected at baseline, i.e. the 2 d before the intervention (days −1 and 0) and during the last 2 d of intervention (days 20 and 21). Body weight was determined before and after each intervention period.

Table 2. The macronutrient and selected micronutrient content of the experimental diet (per 10 MJ)*

	Content	
Nutrient	per 10 MJ	% Energy
Protein (g)	91	16
Fat (g)	99	37
Carbohydrate (g)	277	47
Vitamin E (tocopherol equivalents)	2·9	
Vitamin C (mg)†	12·6	
Vitamin A (retinol equivalents)	3180	
β-Carotene (mg)†	11·2	
α-Carotene (mg)†	3·6	
Lutein (mg)†	0·49	

* For details of diet and procedures, see Table 1 and p. 344.
† Analysed by HPLC; the remaining nutrients were calculated.

Green tea extract and pork meat patties

Extract of Chinese green tea (Licosa-P/Thé chinois) was produced using the process described in a patent (Aeschbach & Rossi, 1994). The extract was a hydrophilic mechanical extract obtained by pressing the tea on a hydraulic laboratory press using propylene glycol as the carrier. The total polyphenol content of the tea extract was

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Table 3. Daily intake of some polyphenols from green tea extract (1000 mg/kg in the 200 g meat patties served per 10 MJ)*

Compound	Daily intake (mg/10 MJ)
Catechin	Not detected
Epicatechin	1·68
Epicatechin gallate	2·78
Epigallocatechin	5·14
Epigallocatechin gallate	9·04
Total phenolics†	23·5

* For details of diet and procedures, see Table 1 and pp. 344–345.
† Total phenolic content was determined by the Folin-Ciocalteu method as mg gallic acid equivalent/g extract. Catechins were determined by HPLC.

117·3 mg gallic acid equivalent/g extract. Catechins content as determined by HPLC are shown in Table 3.

GTE was incorporated at 1000 mg/kg in pork meat patties, which were produced on a pilot scale using pork meat (25 % fat), water and salt as ingredients. Reference meat patties containing no added GTE were also produced. The process consisted in tempering of the meat (at −2°C), mixing all the ingredients, grinding (3 mm), patty forming (approximately 40 g each patty) and frying (belt fryer, no use of additional fat or oil). Meat patties were frozen at −25°C, packed under vacuum in plastic pouches and stored at −25°C until use. A microbiological control was performed on the finished products.

Collection of urine and determination of 8-oxo-deoxyguanosine

Twenty-four hour urine samples were collected in 2·5 l plastic bottles. Fifty ml of 1 mol/l HCl and 10 ml of 10 % ascorbic acid were added to each 2·5 l bottle to increase stability during collection. Urines were weighed, density determined, pH adjusted to 3–4 with 1 mol/l HCl and stored at −80 °C until analysis. Urinary concentrations of 8-oxo-deoxyguanosine (8-oxo-dG), a marker of total oxidatively damaged DNA in the body, were determined by column-switching HPLC with electrochemical detection as described previously (Loft & Poulsen, 1999).

Collection of blood samples and separation of erythrocytes and plasma

Venous blood samples were taken in the morning after at least 12 h of fasting. An additional postprandial sample was drawn on day 21 of each intervention, 2.5 h after consumption of the cooked meal. Blood samples were taken in EDTA-coated tubes after supine resting for 10 min. All subjects abstained from alcohol for at least 24 h before blood sampling, and the smokers abstained from smoking for at least 8 h before fasting blood samples, and 4.5 h before sampling of non-fasting blood samples. Subjects were instructed to avoid heavy physical activity for 36 h before blood sampling. Plasma samples for 2-amino-adipic semialdehyde (AAS) determination were centrifuged at 1500 ***g*** for 10 min at room temperature. Erythrocytes were washed three times with 3 vol. 0·9 % NaCl, resuspended in 1 vol. ultrapure (resistance >18 Mohm) water for lysis, and then stored at −80 °C until analysis. Samples for α-tocopherol, retinol, β-carotene, antioxidant capacity and vitamin C determination were protected from light exposure and kept on ice. Samples were centrifuged at 3000 ***g*** for 15 min at 4°C, and plasma for vitamin C analyses were added to1 volume of 10 % meta-phosphoric acid before freezing. Plasma was saturated with N_2 gas and stored at −80 °C until analysis for antioxidant capacity (maximum 7 d), retinol, α-tocopherol and β-carotene (maximum 7 months), cholesterol and triacylglycerol (20 months).

Ascorbate, plasma protein oxidation and antioxidant enzymes

Plasma and dietary samples were analysed for ascorbate (Kall & Andersen, 1999) and plasma proteins were analysed for oxidised lysine residues (Daneshvar *et al.* 1997) by HPLC as described previously. Erythrocyte lysates were also analysed for protein oxidation products and for the activities of catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase by methods detailed previously (Nielsen *et al.* 1999). Inter-series CV was less than 10 % for all of these analyses.

Plasma oxidation lagtime and plasma antioxidant capacity

Oxidation of lipoproteins in unfractionated plasma was continuously measured by a fluorescence method as described previously (Hofer *et al.* 1995).

The postprandial samples collected on day 21 of each period were used immediately for determination of plasma antioxidant activity. Fifty μl of plasma (or controls: 10 μl 2 mmol/l Trolox +40 μl buffer or 50 μl buffer) were added to 2·45 ml 1·33 mmol/l methyl linoleate emulsion in 5·0 mmol/l oxygen-saturated PBS (pH 7·4) containing 0·1 % Tween 20 and the oxygen consumption was determined as described previously (Jørgensen & Skibsted, 1993). Antioxidative capacity was estimated by calculating the area under oxygen consumption curves and the influence of plasma was expressed as an antioxidative index (I) relative to samples including Trolox:

$$I_{\text{oxygen consumption relative to reference including Trolox}} = \text{area (plasma)}/\text{area (reference including Trolox)}.$$

Determination of plasma retinol, α-tocopherol, β-carotene, triacylglycerol and cholesterol

Plasma retinol, α-tocopherol and β-carotene were determined by a contract laboratory (Medi–Lab, Copenhagen, Denmark) using a standard HPLC–procedure (Thurnham *et al.* 1988). Intra–series CV was 4 %, 4 % and 7 % and inter–series variation was 5 %, 5 % and 10 %, respectively, for retinol, α-tocopherol and β-carotene. Plasma triacylglycerol (kit no. 07 36791 Unimate5, Roche) and cholesterol (kit no. CHOD–PAP 2016630; Boehringer Mannheim, Mannheim, Germany) were determined on a Cobas Mira + (Roche, Basel, Switzerland). Carotenoids in the

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experimental diet were determined as described by Hart & Scott (1995).

Follow–up studies

Two small follow–up studies were performed in order to assess catechin uptake and whether catechins from meat patties containing GTE were absorbed to the same extent as catechins from GTE given in a water–alcohol mixture.

In the first study five volunteers consumed an experimental diet with a low content of flavonoids as described in the main study for 4 d. On the second day in the morning they voided a spot urine sample and subsequently ingested 2 g GTE in 25 ml water–ethanol (2:1). Total urines were collected 4, 24 and 48 h after GTE intake.

The second study was a 3 × 1 d cross-over, where three volunteers consumed a low-catechin diet as described in the main study for 2 d (day 1–2). On days 3–5, they were each day given either 500 mg GTE in 10 ml water–ethanol (2:1), 500 mg GTE in meat patties stored at −25°C for 12 months (from the main study), or 500 mg GTE mixed into freshly prepared meat patties. Urine samples (24 h) were collected from each volunteer during days 2–5. The volunteers in these studies were different from those in the main study.

The containers for urine collection in both follow-up studies were prepared as described for the main study and were additionally filled with Ar. Immediately at the end of each collection period the urine samples were saturated with Ar and analysed for catechins.

HPLC analyses of urine samples, green tea extract and instant coffee for content of epicatechin and epigallocatechin

The content of epicatechin (EC) and epigallocatechin (EGC) was determined in the GTE, in the freeze dried coffee, and in fresh urine samples from the follow-up studies. The assay used for analysis was similar to those previously described for other flavonoids (Nielsen & Dragsted, 1998*a*,*b*) with respect to enzymic hydrolysis and solid phase extraction.

Enzymically hydrolysed urine samples, GTE or coffee were suspended in 5 % acetonitrile in water before addition of internal standard (10 mg catechin/l) and subjected to solid phase extraction. The HPLC–MS system consisted of a Hewlett Packard (Waldbronn, Germany) 1090 system with a a MSD 1100 mass spectrometric detector. The columns used were a Zorbax SB-C8 (4·6 × 75 mm, 3·5 μm) column as column 1 and a Purospher RP-18 (4 × 125 mm, 5 μm) column with guard cartridge (4 × 4 mm, 5 μm) as column 2 (Hewlett Packard). Column temperature was maintained constant at 37 °C using a thermostatically controlled column compartment. U.v. detection was carried out at 280 nm, with peak scanning between 190 and 600 nm (2 nm step). The MS-detection was performed by single-ion monitoring using atmospheric pressure ionisation with electrospray interface in positive mode. The drying gas (N_2) was heated to 350 °C and introduced at a flow-rate of 10·0 l/min. Nebuliser pressure was set to 30 psig and capillary voltage to 4000 V. The mobile phases used for HPLC were (flow of 0·4 ml/min): A, 1 % aqueous formic acid; B, 100 % acetonitrile; C, 100 % methanol. With the automatic six-port column-switching valve in position 1, the sample was injected onto column 1 and eluted through the diodearray detector, bypassing column 2 and the MS-detector, with a following linear gradient of mobile phase B in A (v/v): 5 % to 15 % from 0 to 5 min; 15 % to 25 % from 5 to 14 min. Between 14·1–15 min 100 % B was applied resulting in a short wash of column 1. EGC was eluted from column 1 at 7·5 min. By switching the column switching valve from position 1 to 2 at 7·2 min and back to position 1 at 7·8 min, this compound was eluted onto the second column. This procedure was repeated between 8·3–8·8 min and 9·5–10·0 min for catechin (internal standard) and EC, respectively. From 15·1–23 min column 1 was preconditioned with pure mobile phase A, and after shifting to column 2 at 21·5 min, elution of the tea catechins through the MS-detector was initiated using the following gradient of mobile phase C in A (v/v): 24 min, 25 % C; 28–37 min, 32 % C; 37·5–39 min, 100 % C; 39·1 min, 25 % C. A freshly prepared standard solution containing EC, EGC and catechin was analysed before and after each run to ensure a correct column-switching schedule. The EC and EGC detected were corrected for the internal standard (catechin) recovery in each urine sample.

Statistics

The statistical analysis of biomarkers in the cross-over study was done by paired *t* tests according to Pocock (1998). Significant period effects were observed for plasma ascorbate, 8-oxo-dG in urine and plasma antioxidant capacity, and corrections were made according to Pocock (1998). Plasma oxidation lagtime, β-carotene and AAS in erythrocyte lysates were analysed by comparing groups A and B after the first period using a two-sample *t* test due to period–effect interactions. The effect of smoking was analysed by two-sample *t* tests, comparing smokers and non-smokers using the baseline samples collected after run-in. The effect of intervention on smokers and non-smokers was analysed separately using the same procedure as outlined above for the whole study. Multivariate ANOVA with a repeated statement for the day on intervention was performed for all biomarkers to additionally test for time trends during the whole intervention trial using the SAS statistical package (SAS 6.12; SAS Institute Inc., Cary, NC). A *P*-value of $<0{\cdot}05$ was considered statistically significant.

Results

Subjects, baseline comparisons and influence of smoking status

All subjects were weight-stable, i.e. body weight changed less than 1 kg during the total experimental period for each subject. Group A and B differed with respect to plasma oxidation lagtime at week 2. There were no other differences between the groups after run-in. Smoking did not influence any of the markers after the 2 week run-in

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Table 4. Comparison of oxidative status markers in blood or urine at the end of 3 weeks intake of the experimental diet without and with green tea extract (1000 mg/kg) in meat patties (200 g/10 MJ per d) in sixteen subjects (average of blood samples from 2 successive days)*

(Mean values with their standard deviations)

	Basal diet		Tea extract		Paired difference	
Biomarker	Mean	SD	Mean	SD	Mean	95 % CI
Change in oxidation lagtime (min)†	32·6†‡	26·0	42·0†‡	30·0	†‡	
8-Oxo-deoxyguanosine (pmol/24 h per kg body wt)	257	79	261	81	4·3§	−31·4, 39·9
Plasma antioxidant capacity ($I_{ref}^{\|\|}$)	1·35	0·25	1·56	0·34	0·21§¶	0·021, 0·41
AASpl (nmol/mg protein)	18·8	3·8	19·5	3·7	0·75	−1·78, 3·28
AAShb (nmol/mg protein)	36·2‡	4·9	36·4‡	4·5	‡	
GGShb (nmol/mg protein)	21·9	4·0	21·5	3·7	−0·39	−2·25, 1·46
Gpx (U/g Hb)	37·9	9·8	37·4	10·8	−0·48	−5·58, 4·61
Glutathione reductase (U/g Hb)	7·05	1·2	7·06	1·2	0·01	−0·13, 0·16
Superoxide dismutase (U/g Hb)	1009	149	1035	139	25·9	−3·7, 55·5
Catalase (U/g Hb)	12·0	2·1	11·4	1·2	−0·65	−1·54, 0·24

AASpl, Plasma 2-amino-adipic semialdehyde; AAShb, 2-amino-adipic semialdehyde in erythrocyte lysates; GGShb, γ-glutamyl semialdehyde in erythrocyte lysates; Gpx, glutathione peroxidase, Hb, haemoglobin.

* For details of diet and procedures, see Table 1 and pp. 344–345.

† Due to a significant difference between the groups at baseline, the change in plasma oxidation lagtime from start to end of period I is shown.

‡ There was significant period–effect interaction for this marker, so statistical analysis was performed between groups in period I only.

§ There was a significant period effect for this marker, and statistical analysis was performed according to Pocock (1998).

|| I_{ref}, antioxidative capacity relative to reference including Trolox (see p. 346).

¶ There was a significant effect of green tea extract intervention ($P<0·02$).

period, but in the sample representing habitual diet (13 weeks), plasma ascorbate was significantly lower in smokers.

Biomarkers of oxidative damage and antioxidant defence

Effects on blood and urine parameters after 3 weeks of experimental diet with or without addition of tea extract to a low-flavonoid diet are shown in Tables 4 and 5. There was no significant effect of intervention with tea extract on plasma protein oxidation (plasma AAS) or plasma oxidation lagtime analysed as delta change for each individual from beginning to end of period 1.

In erythrocytes no effect of GTE intervention was observed on markers of protein oxidation (AAS and γ-glutamyl semialdehyde in erythrocytelysates), and the excretion of 8-oxo-dG in urine was not affected either.

There was a significant decrease in plasma AAS and in γ-glutamyl semialdehyde in erythrocyte lysates when the samples representing the participants' habitual diet (week 13) were compared with the samples collected at the end of the periods with restricted diet. The downward trends in plasma AAS (Fig. 2(a)) and in AAS in erythrocyte lysates during the period with restricted diets were also significant as determined by repeated measures ANOVA. Plasma oxidation lagtimes increased significantly in each strictly controlled period, and also overall during the 10 weeks with a restricted diet as compared with habitual diet (Table 6 and Fig. 2(c)). The urinary excretion of 8-oxo-dG decreased significantly during each of the two

Table 5. Comparison of plasma lipid status markers and plasma antioxidant vitamins at the end of 3 weeks intake of experimental diet without or with green tea extract (1000 mg/kg) in meat patties (200 g/10 MJ per d) in sixteen subjects (each represented by the average of blood samples from 2 successive days)*

(Mean values with their standard deviations)

	Basal diet		Tea extract		Paired difference	
Biomarker	Mean	SD	Mean	SD	Mean	95 % CI
Triacylglycerol (mmol/l)	1·02	0·23	1·04	0·22	0·019	−0·036, 0·074
Cholesterol (mmol/l)	4·25	1·20	4·21	1·03	−0·047	−0·20, 0·11
Retinol (μmol/l)	1·73	0·31	1·75	0·25	0·019	−0·082, 0·121
Vitamin C (mg/l)	2·36	0·88	2·50	1·06	0·14†	−0·52, 0·80
β-Carotene (μmol/l)	1·43‡	0·58	2·07‡	0·60	‡§	
α-Tocopherol (μmol/l)	17·6	4·2	17·4	4·2	−0·19	−0·76, 0·38

* For details of diet and procedures, see Table 1 and p. 344.

† There was a significant period effect for this marker, and corrected statistical analysis was performed according to Pocock (1998).

‡ There was significant period–effect interaction for this marker, so statistical analysis was performed between groups in period I.

§ There was a significant effect of green tea extract intervention ($P<0·05$).

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Table 6. Overall changes in biomarkers of oxidative status and in blood lipids from start to end of periods with controlled diets*
(Mean values with their standard deviations)

	Restricted diet period I				Restricted diet period I				Habitual diet	
	Start (week 2)		End (week 5)		Start (week 7)		End (week 10)		End (week 13)	
Biomarker	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Glutathione peroxidase (U/g Hb)	40·3	9·0	38·7	13·8	40·8	14·8	41·9	9·4	35·6‡	12·1
Glutathione reductase (U/g Hb)	7·20	1·11	7·05	1·12	7·01	1·08	7·06	1·21	7·50‡‡	0·83
Superoxide dismutase (U/g Hb)	1035	177	1011	158	1019	154	1034	128	1098‡	105
Catalase (U/g Hb)	11·8	1·8	11·7	2·3	12·1	2·6	11·4	1·4	9·92‡‡	1·16
AASpl (nmol/mg protein)	21·6	3·4	20·0	4·2	18·6	4·4	18·3	3·0	29·0‡‡‡	8·3
AAShb (nmol/mg protein)	37·4	5·1	35·9	4·9	36·1	5·3	36·6	4·5	38·9	4·9
GGShb (nmol/mg protein)	23·3	3·7	22·0	3·6	20·8	3·3	21·5	4·0	23·4‡	3·2
Plasma lagtime for lipid marker (min)	373	46	457†††	56	430	59	468†††	65	203‡‡‡	31
Plasma antioxidant capacity (I_{ref})	ND		1·35	0·21	ND		1·57§	0·22	ND	
8-Oxo-dG (pmol/24 h per kg body wt)	337	131	277†††	86	262	90	235	69	ND	
Plasma cholesterol (mmol/l)	4·13	0·98	4·24	1·04	4·39	1·08	4·22	1·20	4·15	1·03
Plasma triacylglycerol (mmol/l)	1·14	0·24	1·03	0·25	1·31	1·41	1·03††	0·19	1·30‡‡‡	0·40
Retinol (μmol/l)	1·88	0·36	1·75	0·26	2·06	0·29	1·73†††	0·30	2·01‡‡‡	0·32
Vitamin C (μmol/l)	43·5	18·4	16·7†††	5·2	14·1	6·5	10·0	3·46	78·4‡‡‡	25·6
β-Carotene (μmol/l)	0·18	0·10	1·75†††	0·56	0·55	0·26	1·76†††	0·61	0·45‡‡‡	0·17
α-Tocopherol (μmol/l)	18·5	4·1	17·7	4·1	19·6	4·5	17·4†††	4·3	19·5‡‡‡	5·2

Hb, haemoglobin; AASpL, plasma 2-amino-adipic semialdehyde; AAShb, 2-amino-adipic semialdehyde in erythrocyte lysates; GGShb, γ-glutamyl semialdehyde in erythrocyte lysates; I_{ref}, antioxidative capacity relative to reference including Trolox in postprandial plasma samples; 8-oxo-dG, 8-oxo-deoxyguanosine; ND, not determined.
* For details of diet and procedures, see Table 1 and p. 344.
Mean values were significantly different from those at the start of the period by paired *t* test: †† $P<0·01$, ††† $P<0·001$.
Mean values were significantly different from those at the end of the restricted diet period by paired *t* test (week 10): ‡ $P<0·05$, ‡‡ $P<0·01$, ‡‡‡ $P<0·001$.
Mean value was significantly different from that obtained in week 5: § $P<0·05$.

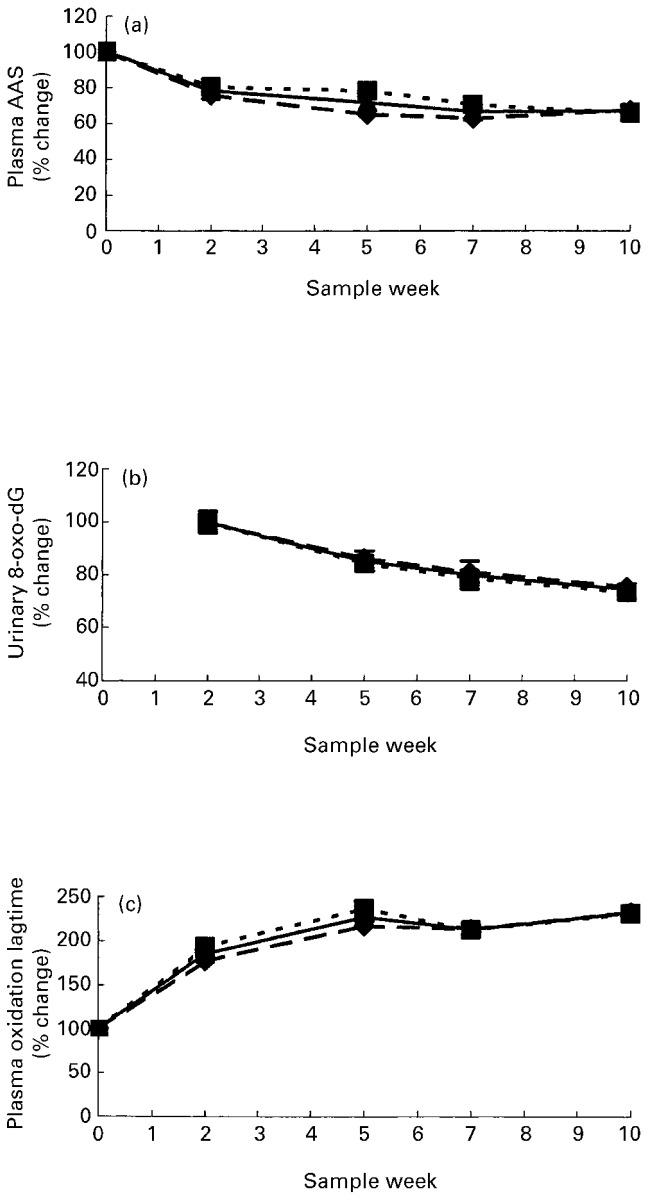

Fig. 2. Biomarkers of oxidative stress (mean values and standard deviations): (a) plasma protein oxidation (2-amino-adipic semialdehyde; AAS); (b) urinary excretion of 8-oxo-deoxyguanosine (8-oxo-dG); (c) plasma total lipoprotein lagtime in sixteen subjects throughout the study period with restricted diets expressed as percentage of the value obtained 3 weeks after its completion (reflecting habitual diet, depicted as day 0). Values for smokers (--■--), non-smokers (--◆--) and both (—▲—) are shown.

intervention periods, and also exhibited a significant overall decrease of 29 (95 % CI 14, 44) % over time during the 8 weeks on restricted diet (Fig. 2(b)). At baseline (week 2) there were no significant differences between smokers and non-smokers for any of the biomarkers of oxidative stress although the urinary excretion of 8-oxo-dG was 17 (95 % CI −4, 48) % higher in smokers than in non-smokers. There were no significant differences between smokers and non-smokers with respect to the changes observed over time for the oxidative damage markers (Figs. 2 and 3).

Enzymes in antioxidant defence

No changes were observed in activities of the antioxidative enzymes superoxide dismutase, glutathione peroxidase, glutathione reductase and catal in erythrocytes after intervention and none of these markers were affected by smoking status at baseline.

All the enzyme activities were fairly stable during the periods with restricted diets, but changed markedly as the volunteers returned to their habitual diets. Superoxide dismutase and glutathione reductase increased when the restricted diet was substituted for the habitual diet as determined by paired comparisons. Glutathione peroxidase and catalase decreased (Table 6).

Total antioxidative capacity in postprandially collected plasma

There was a significant period effect for plasma antioxidative capacity. The capacity was higher in the second period. Plasma antioxidative capacity increased significantly overall as determined by paired *t* test and the increase remained after correction for the period effect (Table 4). The effect was most pronounced in group B which had GTE in the second period. The effect was not significant overall in the non-smokers but was significantly increased among the smokers after intervention with tea extract.

Plasma cholesterol and triacylglycerol

Cholesterol and triacylglycerol in plasma were not affected by tea extract intervention (Table 5). Tea extract did not affect these markers in either smokers or non-smokers.

Plasma cholesterol and triacylglycerol did not differ between the groups A and B or between smokers and non-smokers at baseline (week 2). Triacylglycerols in plasma decreased significantly in the last intervention period, and also increased when the volunteers returned to their habitual diets.

Vitamin C in diet and plasma

The calculated content of vitamin C in the diet is listed in Table 2. Plasma ascorbate was significantly lower in smokers than in non-smokers on their habitual diets. The actual ascorbic acid content of the intervention diet was below the detection limit, i.e. <1 mg/100 g diet, but the dehydroascorbic acid content was 12·6 mg/10 MJ per d. There was no effect of the GTE intervention on plasma ascorbate, neither on total plasma vitamin C (Table 5), nor when smokers and non-smokers were analysed separately.

Compared with the reference blood sample reflecting habitual diet (week 13), the plasma vitamin C concentration decreased steeply within the first 2 weeks of the study by 36 % resulting in a total decrease by 85 % at the end of the 10th week (Table 6), similar for smokers and non-smokers (Fig. 3(a)).

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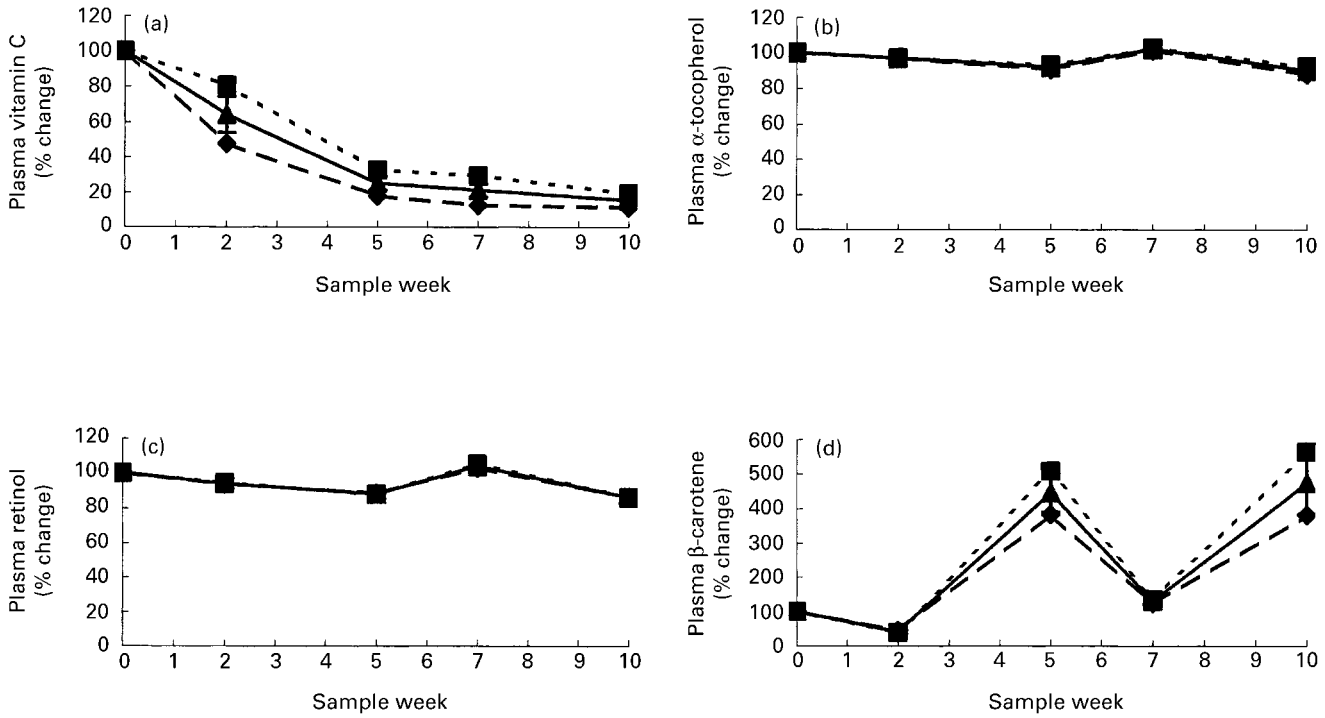

Fig. 3. Plasma concentrations of antioxidant vitamins (mean values and standard deviations): (a) vitaminC; (b) α-tocopherol; (c) retinol; (d) β-carotene in sixteen subjects throughout the study period with restricted diets expressed as percentage of the value obtained 3 weeks after its completion (reflecting habitual diet, depicted as day 0). Values for smokers (--■--), non-smokers (– –◆– –) and both (—▲—) are shown.

Plasma α-tocopherol, retinol and β-carotene

Plasma α-tocopherol and retinol were not affected by intervention with tea extract in either smokers or non-smokers (Table 5).

There was a small decrease by 16 % and 11 %, respectively, from start to end of the second intervention period in plasma retinol and α-tocopherol (Table 6). Similar decreases were observed in smokers and non-smokers between habitual and intervention diet (Fig. 3).

The basal intervention diet had a relatively high content of β-carotene, 11·2 mg/10 MJ per d, which meant that mean daily intake was 16·2 mg/d, similar in group A and B (range 13·4–20·2 mg/d) and that plasma β-carotene increased significantly during both intervention periods. There was a significant decrease in β-carotene after the last intervention when compared with the level after 3 weeks with habitual diet. The β-carotene plasma levels did not reach baseline levels within the wash-out period giving rise to carry-over from the first to the second intervention period (Fig. 3). When tested at the end of the first intervention period tea extract had an increasing effect upon the plasma β-carotene level (Table 5). Group A had a uniformly larger increase in β-carotene than group B in both periods, indicating that this was not due to the tea extract. The response was not influenced by smoking status.

Catechins in urine

The analysis for catechins was very sensitive with a detection limit of 0·5 ng/ml urine and linearity in the range of 0·5–50 ng/ml (r^2 0·998). The standards were stable in the HPLC vials for up to 1 week at room temperature, and at −20°C for 3 months.

In the first follow-up study we observed that 80 % of the excreted EC and EGC were detected in urine within 4 h, whereas 20 % were excreted between 4 and 24 h. At 48 h no catechins were detectable (see Fig. 4). This points to an excretion half-life of about 1.6 h for both substances. There was good concordance between double determinations for each individual (CV was 6·1 % for EC and 5·3 % for EGC), but relatively large inter-individual variations in total excretion (CV was 27 % for EC and 19 % for EGC). A mean of 3·2 % of EC and 1·3 % of EGC was excreted in the first follow-up study.

In the second follow-up study, EC and EGC were observed in 24-h urine samples from each of the three volunteers on the days where GTE was ingested, whereas only a trace was found in urine from day 2 where the volunteers had abstained from catechin-containing foods for 24 h, (see Table 7). After correcting for the recovery of catechin in each sample, 3·7 % of the EC and 2·8 % of the EGC from the ingested GTE were found in the urine. EC and EGC were excreted in urine after ingestion of the same GTE-containing meat patties which were used in the main study, indicating that EC and EGC are absorbable from enriched meat patties.

Discussion

It is still controversial whether green tea can protect against cancer and heart disease (Goldbohm *et al.* 1996; Katan,

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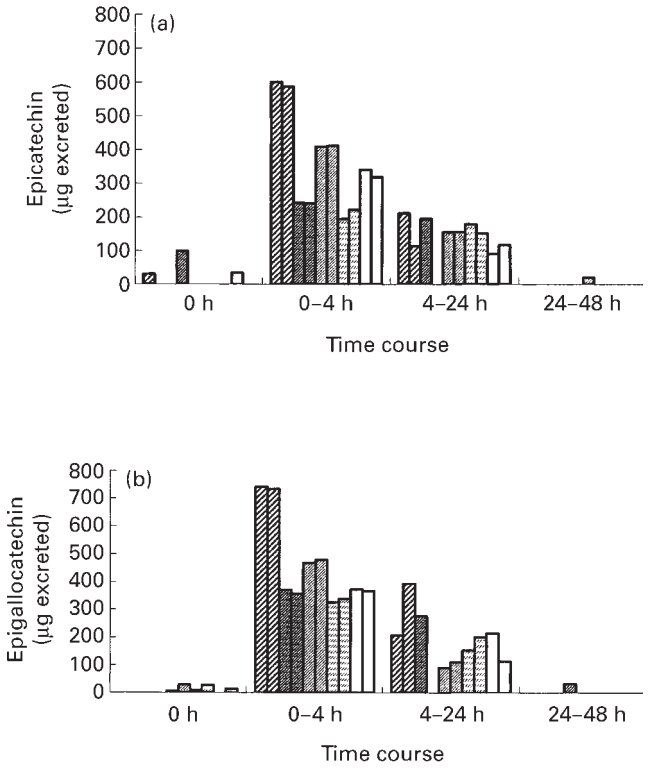

Fig. 4. Urinary excretion of (a) epicatechin and (b) epigallocatechin in five volunteers (▨, ▨, ▨, ▨, □) at various intervals up to 48 h after ingestion of 2 g green tea extract containing 16·3 mg epicatechin and 51·4 mg epigallocatechin. Each sample was analysed in duplicate and the total amounts excreted in each interval are shown as bars.

1997; Kohlmeier *et al.* 1997), but the high content of antioxidants in green tea, catechins in particular, has been suggested to cause such effects through a protective mechanism against radical-mediated damage (Imai & Nakachi, 1995; Benzie *et al.* 1999; Klaunig *et al.* 1999). Although green or black tea, tea extracts or isolated catechins have shown potent protective effects against oxidative stress in various model systems *in vitro* and in animal experiments (Loft & Poulsen, 1999), recent studies with human subjects have recorded limited effects in accordance with our present results. Van het Hof *et al.* (1997) did not observe any effects on resistance to LDL oxidation, plasma lipid oxidation or antioxidative enzyme activities in volunteers after intake of tea for 2 weeks. Similarly, black tea polyphenols increased the resistance of human plasma to lipid peroxidation *in vitro* but not *ex vivo* after ingestion of black tea (Cherubini *et al.* 1999).

Table 7. Excretion of epigallocatechin (EGC) and epicatechin (EC) after intake of green tea extract in three different regimens*

	EGC†		EC†	
Diet	Mean	SD	Mean	SD
Enriched meat patties‡	2·02	1·00	2·73	1·26
Meat patties with added extract§	2·12	0·06	2·62	1·75
Extract only	2·68	0·83	3·20	0·57

* For details of procedures, see p. 344.
† Expressed as percentage of ingested amount.
‡ Enriched during production and stored for 12 months at −25°C.
§ Green tea extract added right before ingestion.

The present study included a group of smokers with a potentially higher oxidative stress. In a cross-sectional study Imai & Nakachi (1995) observed a decrease in lipid peroxides in fasting blood samples from heavy smokers who took ten cups of green tea/d or more, but Princen *et al.* (1998) did not observe any effect on LDL-oxidation, cholesterol levels, or plasma triacylglycerols in a randomised controlled study on smokers receving six cups of black or green tea a day for 4 weeks. In the present study we did not observe differences between smokers and non-smokers in fasting blood samples using any of the selected markers of oxidative status or lipid status either before or after intervention with catechin-rich tea extract after what corresponds to a more ordinary intake of about 1–2 cups of tea/d which is within the estimated median intake of 20–50 mg daily of catechins in Denmark (Dragsted *et al.* 1997). As expected, the smokers in the present study had lower ascorbate levels in plasma samples representing habitual diet. In the postprandial samples we observed a significant effect of GTE intervention on plasma antioxidant capacity in smokers only, when smokers and non-smokers were analysed separately, indicating that GTE has a more prominent effect in oxidatively stressed individuals. Thus, our observations in smokers is in fair agreement with the results of others although the dose of catechins in the present study may not have been sufficiently large for an effect on lipid oxidation.

The unexpectedly high bioavailability of β-carotene caused significant period–effect interactions and when analysed at the end of the first intervention period, tea extract seemed to increase plasma β-carotene. A sparing effect of GTE catechins on plasma β-carotene can therefore not be excluded to be present also *in vivo*. However, there is a large inter-individual variation in response to β-carotene, and extreme subjects are sometimes characterised as 'non-responders' and 'high-responders', respectively (Bowen *et al.* 1993; Parker, 1996). In the present study the larger response in group A, observed in both intervention periods, seemed to be due to a larger number of 'high-responders' in this group and we therefore conclude that GTE probably had no true sparing effect on β-carotene in the present study. This is in line with the observation that plasma β-carotene did not have a time-course similar to any markers of free-radical mediated damage in the present study.

Results of our follow-up studies indicate that as much as 2·7 % of the dose of EC and 2·0 % of EGC may have been absorbed from the meat patties fortified with catechins, but that excretion is very fast with urinary excretion half-lives of less than 2 h. The protein-rich matrix did not seem to greatly influence catechin uptake in accordance with results concerning other polyphenols (Hollman *et al.* 1997). Nakagawa *et al.* (1997) have shown that up to 2 % epigallocatechin gallate and 1.3 % EGC are incorporated into plasma 90 min after ingestion of GTE in capsules in fair agreement with our results. Our results on antioxidant vitamins in plasma also confirm the results of others, who did not

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see any changes in tocopherols, β-carotene, ascorbic acid or uric acid after consumption of teas or tea extracts at 5–20 times higher doses than ours but with less control over dietary intakes (Nakagawa *et al.* 1997; van het Hof *et al.* 1997).

We observed that the antioxidant capacity increased, as measured 2 h after a tea extract-containing meal. This effect on the antioxidative capacity in plasma confirms the results of several others (van het Hof *et al.* 1997; Pietta *et al.* 1998; Benzie *et al.* 1999; Sung *et al.* 2000) who also observed an increased total antioxidant activity in plasma shortly after consumption of a single or multiple doses of green or black tea. The presence of only a short-term effect may readily be explained by the short half-life of catechins from GTE as seen in Fig. 4.

The lack of long-term effects from the GTE intervention allows for a *post-hoc* analysis of the longitudinal effects of a flavonoid-free diet on the biomarkers of oxidative damage and defence in the present study. We have previously observed a significant increase in plasma AAS after high intakes of juices rich in ascorbate and plant phenols (Young *et al.* 1999) and Castenmiller *et al.* (1999) observed a significant correlation between plasma ascorbate and plasma AAS among sixty-four volunteers in a spinach intervention study (Castenmiller *et al.* 1999). We have also observed a decrease in plasma ascorbate and in plasma protein oxidation in a recent study over a period of 2 weeks with a fruit- and vegetable-free diet similar to the one used in the present study (Young *et al.* 2000). It is possible that dietary changes induced a change in protein turnover or degradation. However, a similar decrease in protein oxidation, in 8-oxo-dG excretion and in the increased resistance of plasma lipoproteins to oxidation in the present study points to a more general relief of oxidative stress after depletion of flavonoid- and ascorbate-rich fruits and vegetables from the diet, contrary to common beliefs.

The decrease in the urinary excretion of 8-oxo-dG might also have resulted from a decrease in the efficiency of DNA-repair. Other studies on DNA damage in relation to fruit and vegetable intakes show an equivocal picture (Velthuis-te Wierik *et al.* 1995; Hertog *et al.* 1997; Djuric *et al.* 1998), whereas no significant effect of vitamin C, vitamin E or β-carotene on the excretion of 8-oxo-dG has been observed in placebo-controlled intervention studies and in meta-analyses (van Poppel *et al.* 1995; Priemé *et al.* 1997; Poulsen *et al.* 1998; Loft & Poulsen, 2000). Studies on plasma lipid oxidation, plasma oxidation lagtimes and plasma malondialdehyde in relation to diet generally point to an increase in oxidative damage with diets low in fruits, vegetables or vitamin C, in disagreement with our findings (Fuller *et al.* 1996; Omaye *et al.* 1996; Anderson *et al.* 1997; Miller *et al.* 1998; Maskarinec *et al.* 1999). Complex pro- and antioxidant effects could together with large differences in study material and protocols possibly explain much of the apparent extensive variation and discrepancies regarding the effects of antioxidants, fruits and vegetables on DNA, lipid and protein oxidation.

In conclusion, GTE incorporated into meat patties in doses realistic for addition to processed foods only leads to a short-term change in plasma antioxidant capacity and has no long-term effects on oxidation parameters within the blood or urine compartments in smokers or in non-smokers. The study can therefore be seen as a 10 weeks controlled study with dietary depletion of all food antioxidants derived from fruits and vegetables, except for carrots and potatoes. During the depletion we observed a decrease in oxidative damage to proteins, DNA, and lipids, concomitantly with a major reduction in plasma ascorbate and minor changes in other vitamins and in antioxidant enzyme activities. We speculate that these seemingly positive effects on oxidative status are partly due to depletion of some pro-oxidant compounds co-existing with vitamin C in fruits and vegetables and this underlines the general lack of solid knowledge of the mechanisms by which a diet rich in fruits and vegetables cause a decrease in the risk of chronic diseases.

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