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ORIGINAL CONTRIBUTION

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Absorption and DNA protective effects of flavonoid glycosides from an onion meal

Summary *Background.* It is widely believed that antioxidant micronutrients obtained from fruit and vegetables afford significant protection against cancer and heart disease, as well as ageing. Flavonoids are potential antioxidants found in foods such as onions; information on their effectiveness *in vivo* is so far lacking. *Aims.* To determine uptake as well as *in vivo* antioxidant effects of flavonoids from foods. *Methods.* Six healthy non-obese normocholesterolaemic female volunteers in the age range 20–44 years participated in a randomised two-phase crossover supplementation trial to compare the antioxidant effects associated with (a) a meal of fried onions and (b) a meal of fried onions and fresh cherry tomatoes. Plasma flavonoids, lymphocyte DNA damage, plasma ascorbic acid, tocopherols and carotenoids, urinary malondialdehyde and 8-hydroxy–2'-deoxyguanosine were determined to assess flavonoid absorption and antioxidant efficacy. *Results.* Flavonoid glucosides (quercetin–3-glucoside and isorhamnetin–4-glucoside) were significantly elevated in plasma following ingestion of the onion meal and the increases were associated with an increased resistance of lymphocyte DNA to DNA strand breakage. A significant decrease in the level of urinary 8-hydroxy–2'-deoxyguanosine was evident at 4 h following ingestion of the onion meal. After the combined tomato and onion meal, only quercetin was detected in plasma. Endogenous base oxidation was decreased but resistance to strand breakage was unchanged. There was no significant change in the excretion of urinary malondialdehyde following either meal. *Conclusion.* Both meals – onions, and onions together with tomatoes – led to transient decreases in biomarkers of oxidative stress, although the particular biomarkers affected differ. It is possible that the differences in patterns of response reflect the different uptakes of flavonoids but the underlying mechanism is not understood.

Key words Flavonoids – lycopene – DNA damage – plasma lipid peroxidation

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Introduction

Oxidative stress and free radical production have been implicated in a variety of disease states including cancer and atherosclerosis and may also play a role in the ageing process. Epidemiological evidence suggests that a diet rich in fruit and vegetables is associated with a decreased incidence of cancer (1) and coronary heart disease (CHD) mortality (2).

The bioavailability and antioxidant properties of several plant-derived micronutrients including flavonoids, tocopherols, carotenoids and ascorbic acid have been studied. An inverse correlation between plasma levels of various carotenoids and incidence of coronary heart disease exists (3). Epidemiological studies indicate that intake of flavones and flavonols is associated with a reduced incidence of CHD (2). A number of mechanisms may be involved in this protection including antioxidant activity by free radical scavenging (4, 5, 6), metal ion chelating activities (7, 8) and modulation of the activities of phase I and phase II drug metabolising enzymes (9, 10). In addition

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flavonoids may regenerate other antioxidants such as α-tocopherol by donating a hydrogen atom to the tocopheryl radical (11).

Human feeding trials are necessary to obtain an accurate measure of the actual protective effect of dietary antioxidants. Whilst trials with single supplements provide useful pharmacokinetic data there are obvious limitations in extrapolating these data to complete diets where complex interactions including regeneration of chain breaking antioxidants may occur. Previous intervention trials examining the absorption and potential protective effects of nutritional antioxidants such as the carotenoids have reported competing effects at the level of intestinal absorption and have highlighted the need for caution in this area (12, 13). To date there have been no reports of additive or synergistic or indeed antagonistic effects of flavonoids with other dietary antioxidants such as carotenoids. Therefore, in this study we have examined flavonoid absorption in humans following ingestion of a flavonoid-rich meal and compared the associated antioxidant activity with that obtained following a combined flavonoid/carotenoid-rich meal.

Dietary sources of flavonoids include teas, fruits, vegetables, nuts and red wine (14, 15, 16). Dietary flavonoids are present predominantly as sugar conjugates, although a number of free flavonoids such as myricetin occur in red wine (17). Conjugation of flavonoids with glycosidic moieties impairs their antioxidant and metal chelating activities (18) and may affect their intestinal absorption and subsequent interaction with target tissues or partitioning into low-density lipoprotein (LDL). The intestinal absorption and transport of flavonoids is currently an area of intense research and until recently it was believed that only aglycones were absorbed to a limited extent in the large intestine. Absorption was blocked by an inhibitor of the Na^+/glucose transport and specificity of uptake suggested the involvement of intestinal sugar carrier (19). Hollman *et al*. (20, 21) subsequently demonstrated small intestinal absorption of intact quercetin glycosides in humans and postulated that the intestinal glucose transporter was the means of transport. In support of this, carrier-mediated efflux of labelled galactose from preloaded rat jejunum occurs after exposure to either quercetin monoglucosides (Q3-O-G, Q–4'-O-G) or quercetin diglucosides (Q–3, 4'-di-O-glucoside) (22). The glycosidic cleavage and glucuronidation of selected flavonol glycosides in isolated rat jejunum in the absence of gut microflora has since been demonstrated. Flavonoids containing a 3', 4'-orthodihydroxy B ring were mainly absorbed as glucuronides (88–100 %) but quercetin–3-glucoside and rutin were absorbed unmetabolised (23).

There is evidence of intestinal absorption of glycosylated flavonoids following dosing with flavonoid-rich meals (24, 20) or single flavonoids in humans (20). Inter-individual variation in the extent of absorption (20) has been reported and there is evidence of a preferential uptake of isorhamnetin–4'-glucoside compared with quercetin conjugates following the ingestion of onions (24). A similar differential uptake of carotenoids has been observed in humans; following supplementation with a carotenoid mixture chylomicrons accumulate proportionately more lutein and zeaxanthin than β-carotene. This selectivity of absorption may reflect differences in the physical properties of carotenoids but the involvement of membrane-bound proteins cannot be excluded (25). Factors such as these are particularly important when assessing the bioavailability of compounds from food sources.

We have examined the potential antioxidant effects of flavonoids by feeding volunteers a meal of onions, a rich source of flavonols (14), predominantly quercetin–4'-O-β-glucoside and quercetin–3, 4'-diO-β-glucoside (26). Biomarkers of oxidative damage to DNA were measured in addition to individual antioxidant concentrations and overall plasma antioxidant capacity to assess any antioxidant or prooxidant activity associated with the meal. Subsequent ingestion of a combined onion/tomato meal allowed for an examination of any interaction of the flavonoids with carotenoids, principally lycopene. English cherry tomatoes were selected as they are rich in lycopene and have high concentrations of quercetin (17–77 μg of quercetin/g fresh weight) relative to other varieties of tomato (27). Lycopene, the predominant carotenoid in tomatoes, exhibits the highest antioxidant activity of all dietary carotenoids (28, 29) and contributes about one-third of all carotenoids in serum.

Materials and methods

Chemicals

Apigenin, isorhamnetin, luteolin, quercitrin, quercetin and quercetin–3-glucoside were purchased from Apin Chemicals Ltd., Oxon. European Pharmacopoeial ascorbic acid, United States Pharmacopoieal ascorbic acid and fat-soluble-vitamins (National Institute of Standards and Technology) were purchased from Promochem Ltd., Welwyn Garden City, UK. Echinone was kindly donated by F. Hoffmann-La Roche Ltd, Basel, Switzerland. Genesis C18 4μm (150 x 4.6 mm i. d.) and Nucleosil ODS 5μm (250 x 4.6 mm i. d.) columns were purchased from Jones Chromatography, Mid Glamorgan, UK. 5 ODS2 (C18) columns (250 mm x 4.6 mm i. d.) were from Hichrom Ltd, Reading Berks.,UK. All other chemicals were supplied by Sigma Chemical Co., Poole, Dorset, UK and were > 95 % pure.

Subjects and study design

Six healthy female non-smokers aged between 20 and 44 years were recruited. Subjects had no history of digestive disorders or smoking in the last ten years. Individuals taking nutritional supplements or medication (except oral con-

traceptive) were excluded from the trial. Ethical approval for the protocol was obtained from the Joint Ethical Committee of Grampian Health Board and the University of Aberdeen and details of the study were fully explained to the subjects who gave informed consent. Dietary restrictions: volunteers were given a list of fruits and vegetables containing more than 15 mg quercetin/kg and of beverages with more than 4 mg quercetin/L which they avoided from T–7 days until collection of the T+24 h sample (see protocol below). Individuals completed a validated diet questionnaire and a 7-day weighed intake record during their low flavonoid dietary regimen.

A two-phase crossover design was employed. Baseline determinations of each of the assays described were measured one week prior to T–48 h and one week after the T+48 h sampling points outlined below. Volunteers 1, 2, 4, 5 and 6 were common to both phases; volunteer 3 unavoidably left the study after Phase 1 and was replaced.

T–7d* T–48h* T0h* T+4h T+8h T+**24h*** T+48h* **T**+7d*
Maintenance on low flavonoid diet

Samples:
T_{-7d} T_{-48} T_0 T_{+4} T_{+8} T_{+24} T_{+48} T_{+7d}
MEAL

Blood (20 ml) and urine samples (from fasting subjects where indicated*) were collected at intervals as indicated. After collection of the 4 h sample volunteers had free access to food and drink but continued to consume the low flavonoid diet until T+24 h.

Phase 1:

Immediately following T_0 volunteers were fed 200 g of lightly fried onions (10 min with 5 g of extra virgin olive oil) with other low flavonoid foods and beverages. The breakfasts consisted of protein-free crisp bread, jams made from quercetin-free fruits, water or coffee without milk (thus minimising binding of flavonoids to proteins).

Phase 2:

Following a two-week washout period the volunteers participated in Phase 2 of the trial, which investigated the antioxidant effects, associated with the ingestion of a meal of fried onions and uncooked tomatoes. Dietary restrictions and blood and urine collection were as described for Phase 1. Immediately following T_0 volunteers were fed 200 g lightly fried onions + 100 g uncooked English cherry tomatoes with other low protein/low flavonoid food and beverages.

Collection of samples

Peripheral venous blood (20 ml) was collected into vacuum tubes containing EDTA. Aliquots (30 µl) were immediately taken for analysis of lymphocyte DNA damage. Plasma was separated from the remaining sample by centrifugation for 15 min at 3500 g at 4 °C, aliquoted, snap frozen in liquid N_2 and stored at –80 °C. Plasma for ascorbic acid determination was mixed with an equal volume of 10 % w/v metaphosphoric acid before freezing and storage at –80 °C. Urine samples were collected in 25 ml plastic universals and aliquots for malondialdehyde (MDA) and 8-hydroxy–2'-deoxyguanosine (8OHdG) stabilised with butylated hydroxytoluene (3.2 mM final concentration) or HCl (40µM final concentration) respectively before storage at –80 °C.

Analysis of plasma antioxidants

Levels of plasma flavonoid glycosides were determined following a liquid-liquid extraction with methanol: HCl (1:0.4 v/v) containing 20 mmol/L diethyldithiocarbamate and the extracts filtered (24). Acid hydrolysed plasma samples were used for the determination of total flavonoid concentration (24). Reverse phase HPLC using combined UV and fluorimetric detection was employed for flavonoid characterisation and quantitation (30).

Plasma ascorbic acid levels in metaphosphoric acid-treated samples were determined by reverse phase HPLC with UV detection at 263 nm (31). Determination of plasma retinol, α-tocopherol, γ-tocopherol and 6 carotenoids was by reverse phase HPLC with simultaneous UV and fluorimetric detection (32). Extraction efficiency was determined by use of an echinone internal standard. The total antioxidant activity of plasma was quantified as Trolox equivalents by the TRAP assay (33). Oxidised glutathione (GSSG) was quantified by measuring the total glutathione present after the derivatisation of reduced glutathione (GSH) with 2-vinyl pyridine (34). Plasma glutathione peroxidase activity was determined spectrophotometrically (35).

Erythrocyte vitamin E

Erythrocyte vitamin E was determined fluorimetrically. To 1 ml of red cells was added 1 ml of 25 % w/v ascorbic acid and 2 ml of ethanol. Samples were incubated at 70 °C for 10 min, saponified with 2 ml of 10M potassium hydroxide and incubated for 30 min at 70 °C. Vitamin E was extracted with 4 ml of hexane. Three milliliters of the hexane extract was neutralised with 0.6 ml of 60 % v/v sulphuric acid. Following centrifugation (5 min at 1500 g) the aqueous layer was aspirated off and the fluorescence intensity of the hexane extract determined (λexc 292 nm; λem 322 nm).

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Quantitation of urinary 8-hydroxy–2'-deoxyguanosine

A competitive *in vitro* enzyme-linked immunoassay kit produced by "Japan Institute for The Control of Ageing", Fukori City, Japan 437–0122 was used for the quantitation of urinary 8-hydroxy–2'-deoxyguanosine (8OHdG) in morning void samples as well as samples collected at 4 h and 8 h after feeding.

Analysis of DNA damage (comet assay)

Endogenous oxidative DNA damage was measured in lymphocytes using a modified comet assay (alkaline single cell gel electrophoresis) which estimates both strand breakage and oxidised pyrimidines (36). In brief, cells were embedded in a thin layer of agarose on a microscope slide, lysed for 1 h in 2.5 M NaCl, 0.1M Na_2EDTA, 10 mM Tris-HCl, pH 10, 1 % TritonX–100, washed with 0.1 M KCl, 0.5M Na_2EDTA, 40 mM HEPES-KOH, 0.2 mg/ml bovine serum albumin, pH8 and incubated with endonuclease III in this buffer at 37 °C to convert oxidised bases to strand breaks. Control slides were incubated with buffer but no enzyme. Under alkaline electrophoresis, DNA with breaks extends towards the anode, forming a "comet tail" when viewed by fluorescence microscopy; the percentage of total fluorescence in the tail is linearly related to DNA break frequency up to about 2 per 10^9 daltons. Resistance of lymphocyte DNA to oxidative damage was also determined using the comet assay to measure DNA strand breaks induced by exposure to hydrogen peroxide (H_2O_2; 50μM and 200μM) *ex vivo* (37). Comet scoring was by visual classification – a method which has been validated by comparison with computerised image analysis (Komet 3.0, Kinetic Imaging Ltd., Liverpool, UK) (38) applied to selected comets.

Indices of lipid peroxidation

The malondialdehyde (MDA) content of urine and plasma was determined by resolution of the TBA-MDA adduct using isocratic reverse phase HPLC with fluorimetric detection. A standard curve of malondialdehyde was prepared using 1,1,3,3-tetraethoxypropane (39). The kinetics of copper-induced oxidation of plasma lipids was measured in diluted plasma by monitoring the absorbance at 245 nm (40). Following a 100-fold dilution of the plasma with 0.01 M phosphate buffered saline (PBS, pH 7.4) the reaction mixture was brought to 37 °C and the oxidation initiated by the addition of 50 μM $CuCl_2$. The kinetics of oxidation were followed by continuously monitoring the change in absorbance in a thermostat-controlled ATI Unicam UV4 spectrophotometer equipped with an 8-position automatic sample changer. The oxidisability of the plasmas was assessed in terms of the lag time preceding oxidation (41) and these lag times calculated using Slidewrite 4.0 software purchased from Advanced Graphics Software Inc., California, USA.

Determination of malondialdehyde content of red blood cells

The susceptibility of washed erythrocytes to oxidation by hydrogen peroxide was assessed as malondialdehyde production following a 60 min incubation (42). To 100 μl of washed red cells (triplicate determinations) was added 900 μl of PBS containing sodium azide and 1 ml of 1.5 % v/v H_2O_2. Samples were incubated for 1 h at 37 °C and the reaction terminated by the addition of 1 ml of 20 % w/v trichloroacetic acid. Samples were vortexed and centrifuged for 5 min at 3500 rpm. Aliquots of the supernatant were snap frozen and stored at –80 °C. One milliliter of 0.67 % w/v 2-thiobarbituric acid (TBA) was added to 1 ml of thawed supernatant and the samples boiled for 30 min. The TBA-reactive materials were recovered by butanol extraction (5 ml) and the fluorescence intensity (λexc 515 nm; λem 546 nm) measured.

Spectrophotometric assays

Metal chelation studies used the method of Belinky *et al.* (43). Plasma cholesterol and high density lipoprotein (HDL) were determined spectrophotometrically at 510 nm (44). Urinary creatinine was reacted with picrate under alkali conditions to form a chromogen and the absorbance at 510 nm measured (45). Whole blood haemoglobin was determined spectrophotometrically at 540 nm with reference to a standard.

Results

During Phase 1 of the trial plasma flavonoid levels were determined in non-hydrolysed samples. Levels of plasma aglycones, quercetin and isorhamnetin, in non-hydrolysed plasma samples were low prior to dosing (in the range of 0–5 ng/ml) and there was no significant change as a result of the onion meal (Fig. 1). Flavonoid glycosides were not detected in any of the baseline samples. Following supplementation with onions the predominant flavonoid present in plasma was quercetin–3-glucoside (Q3G), with slightly lower levels of isorhamnetin–4'-glucoside (I4G). There was evidence of inter-individual variation in the extent of absorption of Q3G into plasma and also the time at which the highest plasma concentration was observed (Fig. 2a). Volunteers 1, 2 and 3 had highest plasma Q3G concentration at 4 h. In contrast, plasma Q3G concentrations in volunteers 4, 5 and 6 were highest at 8 h (Fig. 2b); mean values of plasma Q3G levels are shown in Fig. 1. A similar

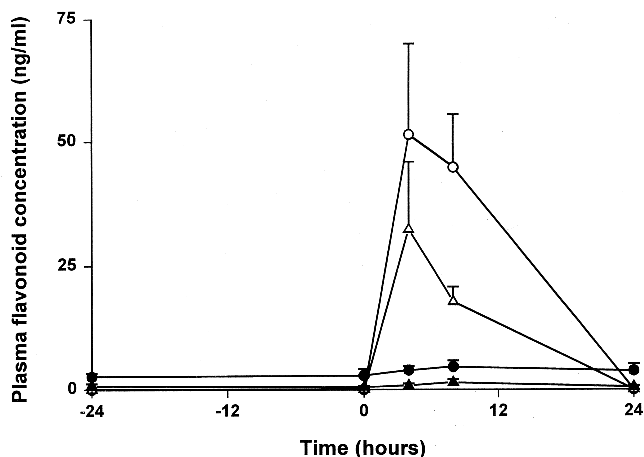

Fig. 1 Changes in plasma aglycone [quercetin (●) and isorhamnetin (▲)] and plasma glucoside [I4G (Δ) and Q3G (o)] concentrations during Phase 1 of trial. Plasma flavonoid levels were measured at three intervals prior to feeding and then at intervals following the onion meal. Data show the mean ± SEM for six volunteers.

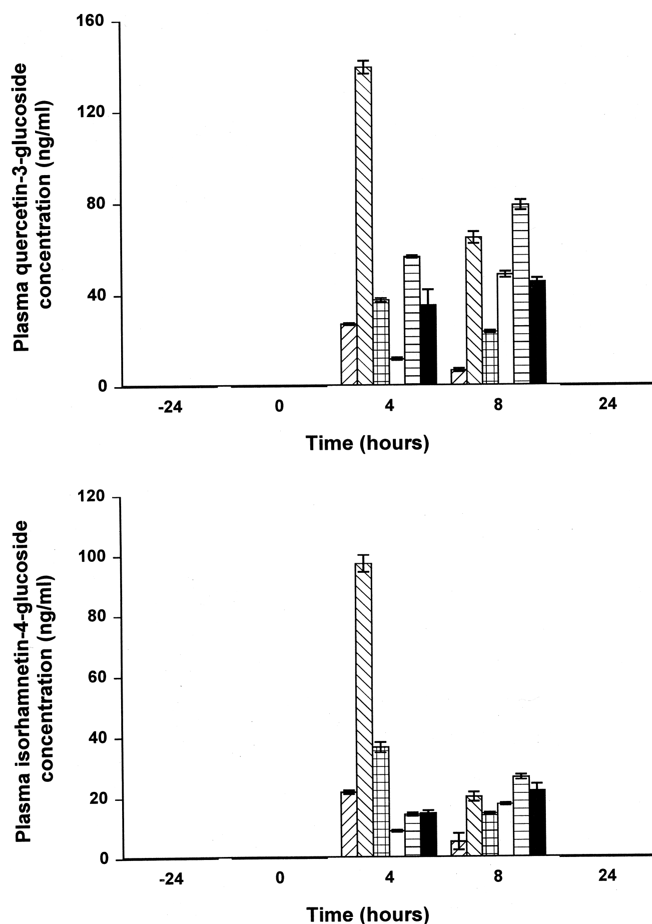

Fig. 2 Inter-individual variation in plasma concentration of (a) Q3G (b) I4G after consumption of the onion meal. Data show the mean ± SEM for triplicate determinations; different bar shading indicates each volunteer. Levels at –24 h, 0h and 24 h were not detectable.

inter-individual variation in both the extent and rate of absorption of I4G was observed (Fig. 2b). Mean values of plasma I4G levels are shown in Fig. 1.

During Phase 2 of the study plasma flavonoid determinations were measured in acid hydrolysed samples enabling an estimation of total aglycone content; i. e. the distribution between aglycones and glycosides was not studied (Fig. 3). At all timepoints the principal flavonoid in acid hydrolysed samples was quercetin (Fig. 3). The combined concentration of kaempferol and isorhamnetin in plasma did not exceed 5 ng/ml at any of the timepoints and so these data have not been shown. Following the tomato and onion meal, levels of plasma quercetin increased dramatically from the baseline level of 16.5 ± 2.7 ng/ml (mean ± sem; n=6) to 104.9 ± 10.42 ng/ml (mean ± sem) at 4 h ($p < 0.0005$) and remained significantly elevated at 8 h ($p < 0.005$). Plasma quercetin levels at T+24 h were significantly higher ($p < 0.005$) than those at T_o suggesting a slow clearance of the dietary flavonoids from the body. Estimates of total plasma quercetin during Phase 1 were made at each timepoint assuming a 100 % conversion of Q3G to quercetin and these results are summarised in Fig. 3. Concentrations of plasma quercetin were greater following the combined tomato and onion meal relative to those estimated for the onions only.

Plasma tocopherol/carotenoid levels did not change significantly following feeding with onions or tomato and onions (Table 1). There was no significant change in the concentration of any other plasma antioxidants measured (Table 1). Wide variation in the plasma concentration of lycopene was seen between individuals but for each subject the concentration remained remarkably constant (Figs.

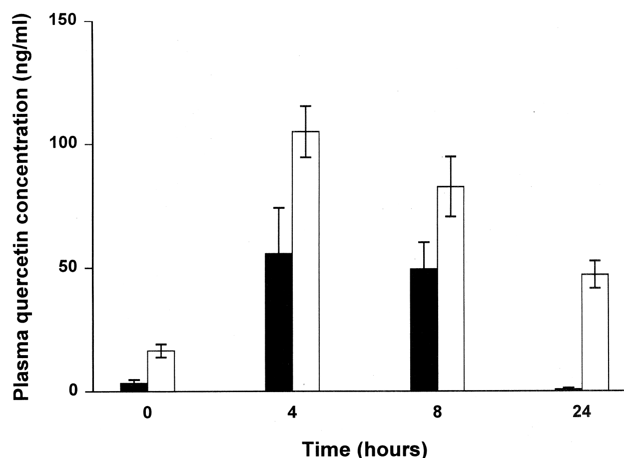

Fig. 3 Mean levels of plasma quercetin determined in acid hydrolysed samples from the onion and tomato phase of the trial (open bars). For comparison, corresponding total quercetin levels are shown (solid bars) for the onion phase (estimated from separately determined levels of quercetin and quercetin glucosides as shown in Fig. 1). Data are the mean of six individuals (duplicate determinations) ± SEM.

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Table 1 Plasma Antioxidant Content

	Phase 1 (time of sampling)			
	T_0	T+4	T+8	T+24
Vitamin C (μM)	51.9 ± 8.2	57.6 ± 6.04	55.7 ± 6.4	55.7 ± 7.63
Retinol (μg/ml)	0.41 ± 0.03	0.40 ± 0.03	0.41 ± 0.03	0.41 ± 0.04
γ-tocopherol (μg/ml)	0.78 ± 0.10	0.73 ± 0.09	0.78 ± 0.06	0.90 ± 0.12
α-tocopherol (μg/ml)	9.53 ± 0.65	9.53 ± 0.47	9.62 ± 0.38	9.27 ± 0.57
Lutein/Zeaxanthin (μg/ml)	0.15 ± 0.04	0.15 ± 0.04	0.15 ± 0.03	0.14 ± 0.04
β-cryptoxanthin (μg/ml)	0.046 ± 0.016	0.046 ± 0.015	0.047 ± 0.015	0.04 ± 0.016
Lycopene (μg/ml)	0.189 ± 0.031	0.189 ± 0.030	0.188 ± 0.030	0.172 ± 0.028
α-carotene (μg/ml)	0.073 ± 0.016	0.072 ± 0.016	0.074 ± 0.016	0.071 ± 0.016
β-carotene (μg/ml)	0.35 ± 0.09	0.35 ± 0.08	0.35 ± 0.08	0.46 ± 0.09

	Phase 2 (time of sampling)			
	T_0	T+4	T+8	T+24
Vitamin C (μM)	60.8 ± 8.4	64.9 ± 6.1	69.3 ± 7.5	50.5 ± 4.2
Retinol (μg/ml)	0.37 ± 0.02	0.38 ± 0.03	0.37 ± 0.02	0.34 ± 0.03
γ-tocopherol (μg/ml)	0.71 ± 0.09	0.64 ± 0.08	0.62 ± 0.09	0.63 ± 0.08
α-tocopherol (μg/ml)	8.68 ± 0.48	8.71 ± 0.51	8.55 ± 0.55	8.96 ± 0.73
Lutein/Zeaxanthin (μg/ml)	0.14 ± 0.04	0.14 ± 0.04	0.14 ± 0.04	0.23 ± 0.06
β-cryptoxanthin (μg/ml)	0.59 ± 0.02	0.06 ± 0.02	0.13 ± 0.06	0.15 ± 0.05
Lycopene (μg/ml)	0.185 ± 0.036	0.194 ± 0.043	0.186 ± 0.04	0.183 ± 0.04
α-carotene (μg/ml)	0.095 ± 0.008	0.096 ± 0.008	0.092 ± 0.008	0.097 ± 0.012
β-carotene (μg/ml)	0.40 ± 0.061	0.41 ± 0.08	0.40 ± 0.07	0.46 ± 0.09

Triplicate analyses at each timepoint; results show mean ± SEM for six volunteers in each phase of the trial

4a , 4b) and there was no evidence of a significant change in the level of plasma lycopene as a result of the tomato feed.

The comet assay was used in two distinct ways to measure DNA damage in lymphocytes. In its simplest form, the assay detects strand breaks, i. e. the low 'background' level

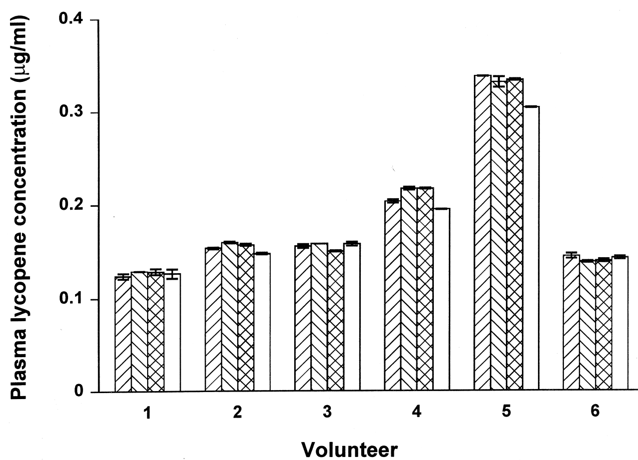

Fig. 4a Inter-individual variation in plasma lycopene concentration before and after consumption of the onion meal. Individual data for all volunteers 1–6 are shown (mean ± SEM for triplicate determinations) for the four timepoints (in the order of T_0, T+4, T+8, T+24) as indicated by individual shading on the graph.

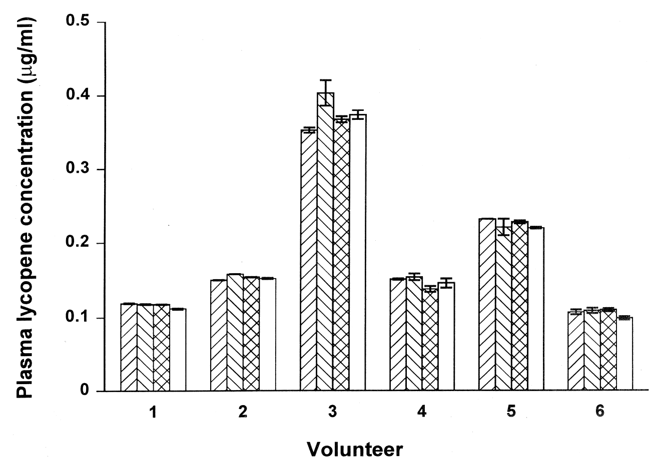

Fig. 4b Inter-individual variation in plasma lycopene concentration before and after consumption of the onion and tomato meal. Individual data for all volunteers are shown (mean ± SEM for triplicate determinations) for the four timepoints (in the order of T_0, T+4, T+8, T+24) as indicated by individual shading on the graph.

of damage (including alkali-labile sites and baseless sugars), and also breaks introduced by treatment with H_2O_2 as shown in Figs. 5a and 6a. Fig. 5a shows that, after the onion meal, lymphocytes become more resistant to H_2O_2-induced damage. In the modified form of the assay (Figs. 5b, 6b), lymphocytes were incubated in the gel with endonuclease III, which cuts DNA at sites of oxidised pyrimidines; as a control, lymphocytes were incubated with enzyme buffer alone. The background strand breaks detected after this incubation is slightly higher than without incubation. After the onion meal, lymphocyte DNA incubated with endonuclease III shows a significant decrease in damage (Fig. 5b), though the decrease is mostly in background breaks rather than enzyme-sensitive sites (the same trend is seen in the level of background strand breaks in Fig. 5a). The minimum level of DNA damage was seen at 8 h – lagging somewhat behind the peak plasma concentrations of flavonoid glycosides (see Fig. 1). Fig. 6a indicates that there is no decrease in H_2O_2-induced DNA strand breakage following the meal of onions with tomatoes, nor in the background level of strand breaks (with or without buffer incubation). There was no correlation between the levels of individual carotenoids or tocopherols and the resistance of volunteers' lymphocytes to oxidative damage resulting from an H_2O_2-challenge. However, the level of endogenous base oxidation, measured as endonuclease III sensitive sites, is significantly decreased at 4 h and 8 h after the meal (Fig. 6b).

In addition to determining lymphocyte DNA damage, two further markers of oxidative stress were measured, namely urinary MDA and 8OHdG. No significant change in the level of urinary MDA was observed in either phase of the trial (Table 2). There was, however, a significant decrease (42 %, $p < 0.05$) in the excretion of urinary 8-hydroxy–2'-deoxyguanosine at 4 h following the ingestion of the onion meal (Table 2) which corresponds to the point of maximal plasma flavonoid glycoside levels following the onion meal (Fig. 1). There was no change in the excretion of urinary MDA or 8OHdG following the combined onion and tomato meal (Table 2). Plasma antioxidant capacity showed a significant decrease ($p < 0.05$) in samples throughout Phase 1 of the trial (Fig. 7) possibly reflecting compliance with the low flavonoid diet but was not obviously reflected by changes in either individual or total di-

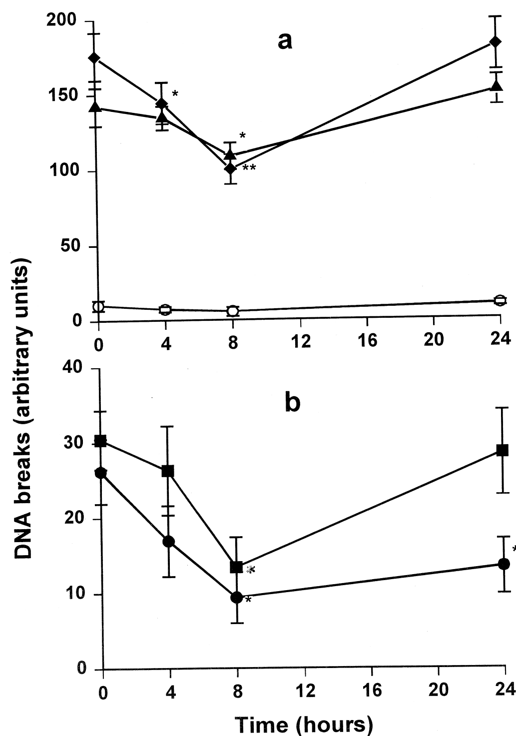

Fig. 5 DNA damage measured with the comet assay in lymphocytes isolated at different times during the onion phase of the trial: (a) background strand breaks (o) and breaks induced by endogenous treatment of cells with H_2O_2 at 50 µM (▲) and 200 µM (◆); (b) endogenous pyrimidine oxidation detected on incubation with endonuclease III (■); strand breaks revealed on incubation with buffer alone are also shown (●). Data are the mean ± SEM for six volunteers with duplicate gels per treatment.

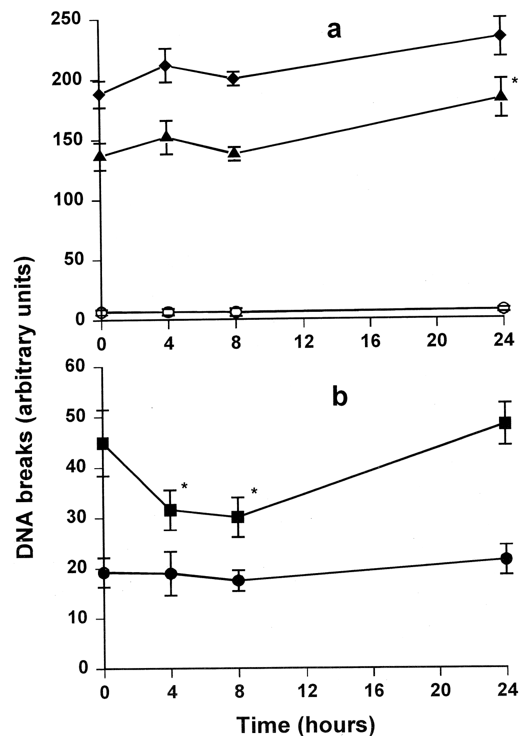

Fig. 6 DNA measured with the comet assay in lymphocytes isolated at different times during the onion and tomato phase of the trial: (a) background strand breaks (o) and breaks induced by endogenous treatment of cells with H_2O_2 at 50 µM (▲) and 200 µM (◆); (b) endogenous pyrimidine oxidation detected on incubation with endonuclease III (■); strand breaks revealed on incubation with buffer alone are also shown (●). Data are the mean ± SEM for six volunteers with duplicate gels per treatment.

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Table 2 Urinary markers of oxidative stress.

	Phase 1: onion meal				
Marker	T–24	T_0	T+4	T+8	T+24
8OHdG (ng/mg creat)	27.2 ± 9.8	24.0 ± 7.9	*13.8 ± 6.5	21.3 ± 8.0	37.1 ± 12.0
MDA (nmol/μmol creat)	0.29 ± 0.18	0.12 ± 0.05	0.20 ± 0.05	0.23 ± 0.07	0.16 ± 0.03

	Phase 2: tomato and onion meal				
Marker	T–24	T_0	T+4	T+8	T+24
8OHdG (ng/mg creat)	11.04 ± 2.2	15.5 ± 1.8	15.3 ± 5.5	15.3 ± 5.5	11.9 ± 1.5
MDA (nmol/μmol creat)	0.11 ± 0.01	0.18 ± 0.07	0.09 ± 0.04	0.23 ± 0.03	0.15 ± 0.02

Triplicate analyses at each timepoint; results show mean ± SEM for six volunteers in each phase of the trial. * $p < 0.05$, where p refers to significant difference from T_0

etary antioxidant concentrations (Table 1). As a further measure of the antioxidant and metal chelating capacity of plasma, we followed the appearance of conjugated dienes at 245 nm following a copper-mediated initiation of peroxidation of plasma lipids. A lag in the formation of conjugated dienes would indicate an enhanced resistance of the plasma to peroxidation and could be due to either chelation of the copper ions and/or an increased ability of the plasma to quench free radicals. In most volunteers' plasma there was no evidence of an inhibition of peroxidation of plasma lipids following ingestion of the onion meal; typical volunteer data are depicted in Fig. 8a. In only one case (volunteer 2) is there an apparent protective effect following the onion meal; at T+4 h, peroxidation is significantly delayed (Fig. 8b). This subject had a two-fold higher absorption of Q3G and I4G than any other subject at T+4 h (Figs. 2a, 2b). Thus, there is some evidence to suggest an inhibition of metal-mediated peroxidation of plasma lipids associated with high concentrations of flavonoids.

There was no significant change in the susceptibility of erythrocytes to peroxidation at any of the timepoints measured during Phase 1 of the trial. Following the tomato and onion meal, the level of lipid peroxidation in erythrocytes collected at T+24 h (147.4 nmole TBARs/gHb) was significantly lower ($p < 0.05$) than that at T_0 (165.9 nmole

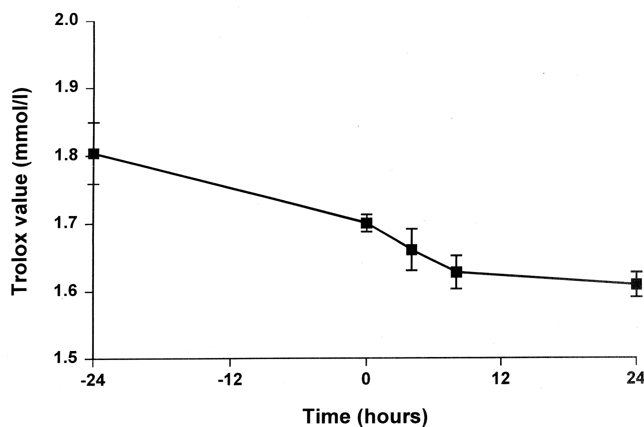

Fig. 7 Changes in mean plasma antioxidant capacity during Phase 1 of trial. Volunteers maintained a low flavonoid diet from T–24 h to T+24 h and consumed a high flavonoid onion meal at T_0. Data show the mean plasma trolox (mmol/l) ± SEM for six volunteers.

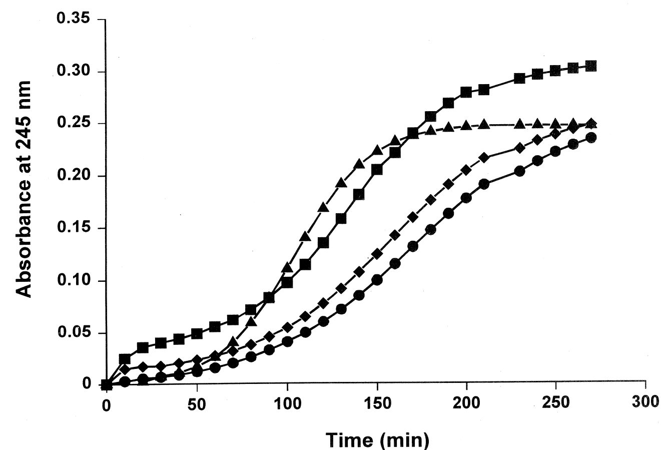

Fig. 8a Typical kinetics of copper-induced oxidation of plasma lipids collected from volunteers during the onion feed at various timepoints (●T_0, ■T4, ▲T8, ◆T24). Preparations were diluted 100-fold in PBS, Cu^{2+} (50 μM final) was added to EDTA-plasma and the absorbance at 245 nm monitored.

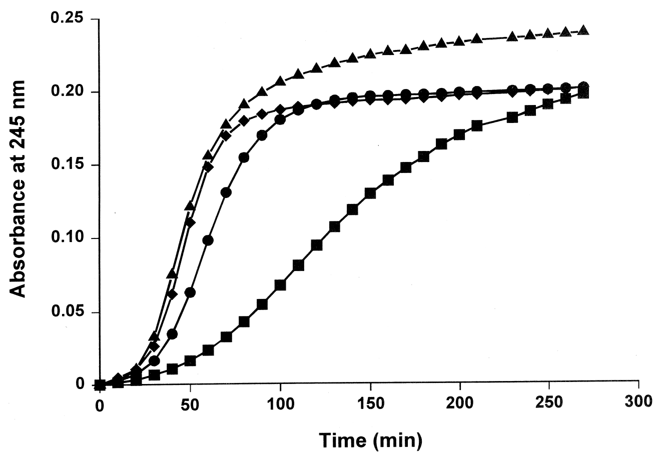

Fig. 8b Kinetics of copper-induced oxidation of plasma lipids collected from volunteer 2 during the onion feed at various timepoints (●T_0, ■T4, ▲T8, ◆T24). Preparations were diluted 100-fold in PBS, Cu^{2+} (50 µM final) was added to EDTA-plasma and the absorbance at 245 nm monitored.

TBARs/gHb) suggesting an increased resistance to the oxidant challenge. There was no significant change in erythrocyte vitamin E concentrations in any of the samples measured during either phase of the trial (data not shown).

Discussion

To date, there have been no reports of investigations into competing effects of the absorption of flavonoids and carotenoids when presented in a food matrix. Such studies are important given the coexistence of these potential dietary antioxidants in many foods. This study has examined the pharmacokinetics and effects of flavonoid and carotenoid absorption in human volunteers following dosing with a flavonoid-rich meal and then a combined flavonoid/carotenoid rich meal.

Following supplementation with onions there was evidence of protection against DNA damage. DNA strand breaks decreased following onion consumption to a minimum at 8 h while a significantly increased resistance of the lymphocytes to an *ex vivo* H_2O_2 challenge was observed at 4 and 8 h. A simultaneous increase in the plasma level of flavonol glucosides, quercetin–3-glucoside and isorhamnetin–4'-glucoside, was observed in all volunteers following the onion meal but there was no significant change in the level of any other plasma antioxidants measured. These results suggest that the absorption of flavonol glucosides may have resulted in an increased resistance of lymphocyte DNA to oxidant stress. Whilst an earlier study has reported an increase in the level of flavonol glucosides, quercetin–4'-glucosides and isorhamnetin–4'-glucoside, following onion dosing (24) there was no investigation of concomitant antioxidant activity of these flavonoids *in vivo*. The mechanism of flavonoid protection against DNA damage is unknown but may involve a free radical scavenging activity (4, 5) or chelation of metal ions (7, 8). Hydrogen peroxide challenge of lymphocytes results in the generation of a highly reactive hydroxyl radical generated by the Fenton reaction and is, thus, dependent on concentrations of iron. Any flavonoid capable of chelating metal ions may modulate the damaging effects of H_2O_2 by preventing the metal-mediated formation of hydroxyl radicals. Of the two flavonol glucosides which showed elevated levels following onion dosing, only isorhamnetin–4'-glucoside has any metal chelating activity (24). Flavonoids having free 3-hydroxyl and 4-keto oxygen and a 5-hydroxyl and 4-keto oxygen are able to bind metal ions (46). Thus, the 3-O-glucoside on quercetin blocks this activity and no bathochromic shift of the spectra was found in the presence of metal ions (data not shown). Further evidence in support of this was obtained during the analysis of volunteers' plasmas.

There was no significant change in the total *in vitro* plasma antioxidant capacity following onion consumption. This is not surprising since serum proteins and uric acid can account for the bulk of the antioxidant capacity of plasma (47). However, plasma from one volunteer exhibited an increased resistance to a copper-mediated peroxidation at T+4 h and had significantly higher plasma glucoside levels than each of the other five volunteers. This inhibition could reflect the ability of the plasma to chelate metal ions such as copper, thus preventing the initiation of peroxidation, and/or it could be due to an increased ability of the plasma to quench free radicals produced in the chain reaction of lipid peroxidation. The lack of a significant effect using the TRAP assay suggests that the former is more likely.

Plasma quercetin levels following feeding with tomatoes and onions were significantly higher than those occurring as a result of the onion meal. While there was no protection against DNA strand breakage (whether endogenous background or H_2O_2-induced), a decrease in endogenous DNA base oxidation was seen after the meal of onions with tomatoes. Lean *et al.* (1999) (48) reported a similar phenomenon in a comparison of fried onions alone and onions fried with tomato ketchup and herbs; onions alone caused a significant decrease in H_2O_2-induced DNA damage, whereas no significant effect was seen when ketchup and herbs were included. It seems that onions alone (which increase plasma Q3G and I4G) have a protective effect against DNA strand breakage, while onions and tomatoes (which increase the plasma content of quercetin) protect against endogenous base oxidation without increasing resistance to exogenous breakage. If this difference is real, as it seems to be, it may reflect a subtle interaction of tomato- and onion-derived micronutrients but the detailed mechanism remains obscure.

In contrast to the profile of plasma flavonoids (Q3G and I4G) apparent after the feeding of onions, there was no significant absorption of isorhamnetin (and/or its glucosides)

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following the combined tomato and onion meal. Thus, the presence of tomatoes appears to have altered the specificity of flavonoid absorption favouring quercetin or quercetin glycoside uptake. The actual form of quercetin absorbed is not known owing to the hydrolysis of these samples but the lack of a protective effect following the hydrogen peroxide challenge suggests an inability to chelate metal ions indicating that little or no quercetin aglycone exists in the plasma prior to hydrolysis. The DNA protective effect at the level of oxidised bases is consistent with high levels of lycopene, which exhibits the highest antioxidant activity of all dietary carotenoids (28, 29). An inverse correlation between dietary lycopene intake and lycopene serum levels and the risk of cervical intraepithelial neoplasia (49) and bladder cancer (50) has been reported. There was no significant change in the level of plasma antioxidants, in particular plasma lycopene, as a result of the combined tomato and onion meal. Increased concentrations of lycopene in chylomicrons but not serum have previously been reported for volunteers fed a variety of experimental meals, including a fresh tomato meal (lycopene dose = 23 mg) (51). Following single carotenoid doses, peak concentrations in plasma are reached 24–48 h post-dose. In chylomicrons they occur between 4 and 6 h and decline to near basal levels within 12 h (52, 53, 54, 55). There was no evidence in our study of an increase in plasma lycopene at T+24 h or T+48 h following the tomato and onion meal. Nor was there any protective effect on lymphocyte DNA at the level of either endogenous strand breaks or resistance to H_2O_2 challenge in direct contrast to the protective effect observed with the meal of onions alone. The possibility that these results reflect a competing effect between flavonoids, in particular isorhamnetin glycosides, and other nutrients cannot be excluded. The influence of one micronutrient on the uptake of another is potentially very important since fruits and vegetables are complex mixtures. Recently Sheng *et al.* (1998) have examined the effects of a combined supplement of carotenoids, nicotinamide and zinc on DNA repair in human volunteers. A significantly increased resistance to DNA single strand breakage by H_2O_2 was observed following 7 weeks combined supplementation of zinc, nicotinamide and carotenoids (Nicoplex). When the same subjects were supplemented with Nicoplex and 17 additional nutrients or minerals there were no changes in DNA damage suggesting that the multinutrient supplementation may have compromised the effects of Nicoplex possibly by inhibitory actions at the level of uptake or absorption of unidentified components.

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