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Role of dietary antioxidants in the prevention of *in vivo* oxidative DNA damage

M. S. Cooke*, M. D. Evans, N. Mistry and J. Lunec

Oxidative Stress Group, Division of Chemical Pathology, University of Leicester, Robert Kilpatrick Clinical Sciences Building, Leicester Royal Infirmary, University Hospitals of Leicester NHS Trust, Leicester LE2 7LX, UK

Epidemiological evidence consistently shows that diets high in fresh fruit and vegetables significantly lower cancer risk. Given the postulated role of oxidative DNA damage in carcinogenesis, the assumption has been made that it is the antioxidant properties of food constituents, such as vitamin C, E and carotenoids, which confer protection. However, epidemiological studies with specific antioxidants, either singly or in combination, have not, on the whole, supported this hypothesis. In contrast, studies examining the *in vitro* effect of antioxidants upon oxidative DNA damage have generally been supportive, in terms of preventing damage induction. The same, however, cannot be said for the *in vivo* intervention studies where overall the results have been equivocal. Nevertheless, recent work has suggested that some dietary antioxidants may confer protective properties through a novel mechanism, unrelated to their conventional free-radical scavenging abilities. Upregulation of antioxidant defence, xenobiotic metabolism, or DNA-repair genes may all limit cellular damage and hence promote maintenance of cell integrity. However, until further work has clarified whether dietary supplementation with antioxidants confers a reduced risk of cancer and the mechanism by which this effect is exerted, the recommendation for a diet rich in fruit and vegetables remains valid empirically.

Antioxidants: Diet: DNA damage: Free radicals

Introduction

In the simplest terms, an antioxidant may be defined as a substance which, when present in small quantities compared with the substrate, prevents or delays the oxidation of that substrate (Halliwell & Gutteridge, 1989). Reactive oxygen species (ROS) comprise a group of potentially reactive O-containing molecules or free radical species, such as superoxide ($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$),

Abbreviations: 8-oxodA, 8-oxo-2′-deoxyadenosine; 8-oxodG, 8-oxo-2′-deoxyguanosine; dC-glyoxal, deoxycytidine-glyoxal adduct; EC, electrochemical detection; PBL, peripheral blood lymphocyte; PBMC, peripheral blood mononuclear cell; ROS, reactive oxygen species; T < > T, thymine dimer.

***Corresponding author:** Dr M. S. Cooke, fax +44 116 2525887, email msc5@le.ac.uk

hydrogen peroxide, singlet oxygen ($^1O_2$) and hypochlorite (^-OCl). Potential sources of cellular ROS include the generation of $O_2^{\bullet -}$ by 'electron leakage' from the mitochondrial, and other, electron transport chains, neutrophil or macrophage respiratory burst activities, oxidase activities and arachidonic acid metabolism (for reviews, see Janssen *et al*. 1993; Evans *et al*. 1997). Spontaneous or enzymically-catalysed dismutation of $O_2^{\bullet -}$ can lead to the production of hydrogen peroxide, which in turn, through the Haber-Weiss reaction, can yield the highly reactive $HO^{\bullet}$. Other important radicals and reactive species, such as lipid hydroperoxides, endoperoxides and reactive aldehydes may arise from autocatalytic lipid peroxidation, following the interaction of ROS with lipids, although such products may also derive from the synthesis of prostaglandins, leukotrienes and thromboxanes (Chow, 1988). It is accepted that whilst all these ROS are 'kept in check' by enzymic and non-enzymic defences, a little 'background' damage to cellular biomolecules is inevitable. However, oxidative stress, arising from the imbalance of pro-oxidative and anti-oxidative processes, in favour of the former, invariably leads to the significant modification of DNA (for reviews, see Marnett, 2000) generating a multitude of lesions such as 8-oxo-2′-deoxyguanosine (8-oxodG) and 8-oxo-2′-deoxyadenosine (8-oxodA; Dizdaroglu, 1991). The subject of much study has been 8-oxodG, given its ease of formation and mutagenic potential.

A broad spectrum of techniques are available for the analysis of oxidative DNA damage. HPLC with electrochemical detection (EC) was the first method used for the routine analysis of 8-oxodG (Floyd *et al*. 1986) and use of this technique remains in a large number of laboratories, in part, due to its versatility. HPLC–EC has been applied to the analysis of 8-oxodG in various biological matrices, most notably, DNA (Floyd *et al*. 1986) and urine (Shigenaga *et al*. 1989). Antibodies to oxidative DNA damage were first developed as part of a pre-purification step before HPLC–EC (Kasai & Nishimura, 1986), before becoming an optional technique for the measurement of DNA, serum and urinary 8-oxodG, in its own right (Cooke *et al*. 1998; Evans *et al*. 2000). Measurement, and identification, of oxidative DNA lesions other than 8-oxodG was provided by the application of GC–MS (Dizdaroglu, 1991). A technique frequently used in toxicological studies for measuring DNA strand breaks, and more recently applied to the field of oxidative stress, is single cell gel electrophoresis, or Comet assay (Angelis *et al*. 1999). However, to tailor the technique for oxidative DNA damage, lesion-specific endonucleases need to be incorporated into the assay.

Measurement of nucleic acid markers of oxidative stress is not without its problems. The very process of DNA extraction from isolated cells has been reported to artifactually elevate levels of oxidative damage (Halliwell, 1998), which will have an effect irrespective of the method used. As this issue is fundamental to so many of the techniques, an organisation known as the European Standards Committee on DNA Damage (ESCODD), and comprising many research groups throughout Europe, has set itself the task of addressing this problem. Clearly, methods which do not require DNA extraction before analysis, such as immunochemical or Comet assays, can circumvent this issue. However, accurate quantification of the number of modified deoxynucleosides, per native deoxynucleoside is largely impossible. Furthermore, to be specific for oxidative DNA damage the Comet assay, as mentioned earlier (p. 20), requires the introduction of repair enzymes. Whilst the specificity of the repair enzymes (primarily formamidopyrimadine glycosylase protein and endonuclease III) has been characterised, the effect of factors, such as DNA-related proteins, cofactors, effect of lesion clusters and DNA conformation, upon enzyme efficiency as applied to the Comet assay, has not been fully investigated.

Chromatographic methods, such as HPLC–EC and GC–MS are perhaps better suited to 'absolute quantification', although both have their associated 'weaknesses', largely associated with sample workup. HPLC–EC is regarded by some to be the simplest, reliable method for

measurement of a small number of lesions, principally 8-oxodG (Cadet *et al*. 1998), although the assay's limit of detection has, more recently, been brought into question (Lunec *et al*. 2000*b*). Criticism of the GC–MS approach has primarily been aimed towards the artifactual oxidation of native bases during the derivatisation step which, it has been suggested, may be overcome by HPLC-prepurification (Cadet *et al*. 1998). In stark contrast, a thorough review of the literature by Dizdaroglu (1998) illustrated that such criticisms of GC–MS may be unfounded, based upon comparisons of very different experimental procedures. Furthermore prepurification is said to be unnecessary when derivatisation is performed using a more refined protocol, in the absence of oxygen or at room temperature (Dizdaroglu, 1998). Whilst different research groups strongly advocate one technique, or the other, there does appear to be some agreement over an approach which, through liquid chromatography, coupled with tandem MS, avoids the need for sample derivatisation and combines this with the specificity and sensitivity of MS (Ravanat *et al*. 1998; Podmore *et al*. 2000).

Immunochemical methods are ideally suited to the detection of lesions in complex biological matrices, with their simplicity aiding automation and high throughput analyses (for comprehensive reviews of the benefits and drawbacks of immunochemical techniques, see Cooke & Lunec, 2002). However, comparison of antibody and HPLC–EC methods for the analysis of urinary 8-oxodG has revealed a discrepancy between the two techniques, although both techniques show good intra- and inter-laboratory agreement (Cooke *et al*. 2000). Explanation for this observation may derive from (1) possible cross-reactivity of the antibody with 8-oxoguanosine, present in the urine and also a perfectly acceptable marker of oxidative stress, and/or (2) urinary 8-oxodG being present in oligomers which are unavailable for detection by HPLC–EC. Evidence for the latter is increasing and suggests that these lesion-containing oligomers may derive from nucleotide excision repair (Cooke *et al*. 2001).

Many proponents of the hypothesis for the role of free radicals in disease acknowledge that oxidative DNA lesions are likely to participate in the pathogenesis of cancer, as well as many chronic and degenerative diseases. In particular, 8-oxo-guanine has been a focus of these studies, arising in part from the relative abundance of techniques available to measure this lesion and its potential biological importance (reviewed in Table 1; MS Cooke, MD Evans and J Lunec, unpublished results). The interest in dietary antioxidants, as a potentially simple means to modulate risk of disease development, is therefore obvious. Although many dietary compounds may be considered to have antioxidant properties, such as vitamin D (Chatterjee, 2001), unless they have been applied in human intervention studies of oxidative DNA damage, we have considered them outside the remit of this review.

Nutritional antioxidants: source and function

The human diet contains important micronutrients, such as vitamin C (ascorbate), vitamin E (tocopherols) and carotenoids, essential for the maintenance of human health. As such, the dietary route is a primary means of modulating endogenous antioxidant protection. Whilst these compounds are often referred to as antioxidants, as defined in the introduction, this may not be their primary function *in vivo* (Sies & Stahl, 1995). Indeed, as will become apparent, they may not have a solely antioxidant effect. Evidence *in vitro* suggests that many of these compounds can directly react with oxidants and free radicals (i.e. act as scavengers) and are sacrificial targets for more critical biomolecules such as DNA. Many of these molecules may alter cellular antioxidant defence in more subtle ways, for example through the induction of DNA repair processes or enzymic antioxidant defence at the gene level.

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Table 1. Experimental evidence for the role of 8-oxo-guanine in the pathogenesis of various diseases

Pathological condition	Oxidative DNA lesion levels
Cancer	
ALL	Lymphocyte DNA lesion levels significantly ($P < 0{\cdot}05$) elevated in ALL compared with control subjects (Stentürker *et al*. 1997)
Adult T cell leukaemia and lymphoma	Urinary lesion levels elevated ($P < 0{\cdot}05$) compared with controls (Honda *et al*. 2000)
Invasive ductal carcinoma	Levels of lesions significantly ($P \leq 0{\cdot}05$) increased compared with calf thymus DNA (Malins & Haimanot, 1991) Significantly elevated levels of lesion ($P < 0{\cdot}001$) in malignant breast tissue; also levels significantly greater ($P = 0{\cdot}007$) in oestrogen receptor positive (ORP) *v*. ORP negative malignant tissue (Mussarat *et al*. 1996)
Primary breast cancer	Significantly higher ($P < 0{\cdot}0001$) levels of lesion in tumour *v*. non-tumour tissue (Matsui *et al*. 2000)
Colorectal cancer	Significantly elevated levels of lesion ($P < 0{\cdot}005$) in tumour tissue compared with normal mucosa (Oliva *et al*. 1997)
	Significant correlation between lymphocyte lesion levels and colorectal cancer deaths in men (r 0·91, $P < 0{\cdot}05$; Collins *et al*. 1998*a*)
Gynaecological cancers	Levels significantly higher ($P \leq 0{\cdot}05$) in patients with gynaecological cancer compared with control subjects (Yamamoto *et al*. 1996)
Cervical cancer	Levels of lesion significantly increased ($P < 0{\cdot}001$) in low-grade and high-grade levels of dysplasia, compared with normal (Romano *et al*. 2000)
RCC	Levels of lesion significantly higher ($P < 0{\cdot}0005$) in RCC *v*. non-cancerous tissue (Okamoto *et al*. 1994)
SCC	Levels elevated in tumour tissue of all SCC patients *v*. controls (Jaruga *et al*. 1994)
Small cell carcinoma	Elevated lesion compared with controls ($P < 0{\cdot}05$) (Erhola *et al*. 1997)
Lung cancer	Lymphocyte DNA levels of lesion significantly elevated ($P < 0{\cdot}05$) compared with controls (Vulimiri *et al*. 2000)
	Elevated levels of lesions in lung cancer compared with normal lung tissue from control individuals (Inoue *et al*. 1998)
Arsenic-related skin neoplasms	Significantly elevated levels of lesions ($P < 0{\cdot}001$) in arsenic-related Bowen's disease, Bowen's carcinoma and actinic keratosis, compared with their corresponding non-arsenic related conditions (Matsui *et al*. 1999)
Breast, rectal and colon cancer	Women who develop breast, or colorectal cancer have elevated levels of serum auto-antibodies to lesions, compared with controls (Frenkel *et al*. 1998)
Assorted cancers	Elevated levels of urinary lesions noted in cancer patients before ($P < 0{\cdot}01$) and after anti-cancer therapy ($P < 0{\cdot}001$; Tagesson *et al*. 1995)
	Lesion significantly elevated ($P \leq 0{\cdot}05$) in assorted cancers: fibrillary astrocytoma (brain); lung cancer; mucinous carcinoma (stomach); ovarian cancer; colon cancer (Olinski *et al*. 1992)

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Table 1. *Continued*

Pathological condition	Oxidative DNA lesion levels
Cardiovascular disease	Strong association (r 0·95, $P < 0·01$) between premature CHD in men and lymphoctye lesion levels (Collins *et al.* 1998*a*)
Diabetes mellitus (insulin- and non-insulin-dependent)	Both groups had significantly higher levels of lesions ($P < 0·001$) in mononuclear cell DNA compared with controls (Dandona *et al.* 1996)
Diabetes mellitus (non-insulin-dependent)	Levels of urinary lesions significantly higher than controls ($P = 0·001$; Leinonen *et al.* 1997)
	PBMC levels of oxidised DNA base products significantly elevated in diabetes patients compared with controls (Rehman *et al.* 1999*b*)
Down's syndrome	Levels significantly increased ($P = 0·00011$) in Down's syndrome subjects compared with controls (Jovanovic *et al.* 1998)
Infection	
HCV	Leucocyte DNA lesion levels significantly higher than in HBV infection ($P < 0·04$), correlating with clinical diagnosis ($P < 0·025$) (Farinati *et al.* 1999)
Inflammatory disease	
Chronic hepatitis	Liver levels of lesion significantly elevated ($P < 0·05$) compared with controls (Shimoda *et al.* 1994)
Cystic fibrosis	Urinary levels of lesion significantly raised *v.* control subjects (Brown *et al.* 1995)
Atopic dermatitis	Urinary lesion levels significantly higher than in controls ($P < 0·0001$) and correlating with disease severity index (Tsuboi *et al.* 1998)
Rheumatoid arthritis	Levels of urinary lesions significantly elevated ($P < 0·001$) compared with control subjects (Lunec *et al.* 1994)
SLE	Titres of serum auto-antibodies to lesions significantly elevated in SLE (Frenkel *et al.* 1998)
	Levels of urinary lesions significantly reduced compared with control subjects; presence of 8-oxodG noted in circulating immune complexes (Lunec *et al.* 1994)
Neurological disease	
Parkinson's disease	DNA levels of lesion significantly elevated ($P = 0·0002$) in substantia nigra of Parkinson's disease brains (Alam *et al.* 1997)
	Levels of lesion in cytoplasmic DNA and RNA are elevated in substantia nigra neurons of Parkinson's disease patients and in multiple system atrophy Parkinsonian type and DLB (Zhang *et al.* 1999)
Alzheimer's disease	Higher levels of lesion in cortex and cerebellum of patients *v.* controls (Lezza *et al.* 1999)
	Ventricular cerebrospinal fluid DNA levels of lesion significantly ($P < 0·05$) elevated compared with controls (Lovell *et al.* 1999)
DLB	Increased levels of lesions in cortical region of brain in DLB patients compared with control tissue (Lyras *et al.* 1998)

ALL, acute lymphoblastic leukaemia; ORP, oestrogen receptor positive; RCC, renal cell carcinoma; SCC, squamous cell carcinoma; PBMC, peripheral blood mononuclear cell; HCV, hepatitis C virus; HBV, hepatitis B virus; SLE, systemic lupus erythematosus; 8-oxodG, 8-oxo-2′-deoxyguanosine; DLB, dementia with Lewy bodies.

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Vitamin C (ascorbic acid)

Vitamin C is widely distributed in many fruits and vegetables which serve as the primary source of dietary intake. This water-soluble vitamin may also be taken in as a food preservative. Because of its water solubility vitamin C readily leaches out of food during cooking. Structurally, vitamin C is derived from L-gulono-γ-lactone with the introduction of unsaturation as the last synthetic step (Fig. 1). Vitamin C can function as a radical scavenger in the aqueous phase, the initial product being the semidehydroascorbyl radical which can be resonance-stabilised to diminish reactivity relative to the scavenged species (Bielski & Richter, 1975). Further one-electron oxidation produces dehydroascorbate which may be reduced back to ascorbate via glutathione or NADH, as may the ascorbyl radical (Halliwell, 1999). Vitamin C may also be involved in the transfer of radical species from lipid to aqueous cellular compartments via regeneration of lipid-soluble membrane-bound antioxidants such as d-α-tocopherol. Although vitamin C has potential antioxidant properties, it has several other important physiological functions in Fe metabolism and as an essential cofactor for enzymic hydroxylation of lysine and proline during collagen synthesis.

Vitamin E (tocopherols and tocotrienols)

Vitamin E is a term encompassing a group of compounds which exhibit varying degrees of biological activity relative to d-α-tocopherol (Brigelius-Flohe and Traber, 1999). The naturally occurring tocopherols with antioxidant activity consist of a group of four tocopherols (α- to δ-) and four tocotrienols (α- to δ-). Structurally, they consist of a saturated phytyl chain, in the case of the tocopherols attached to a chroman ring, which contains varying numbers and positions of

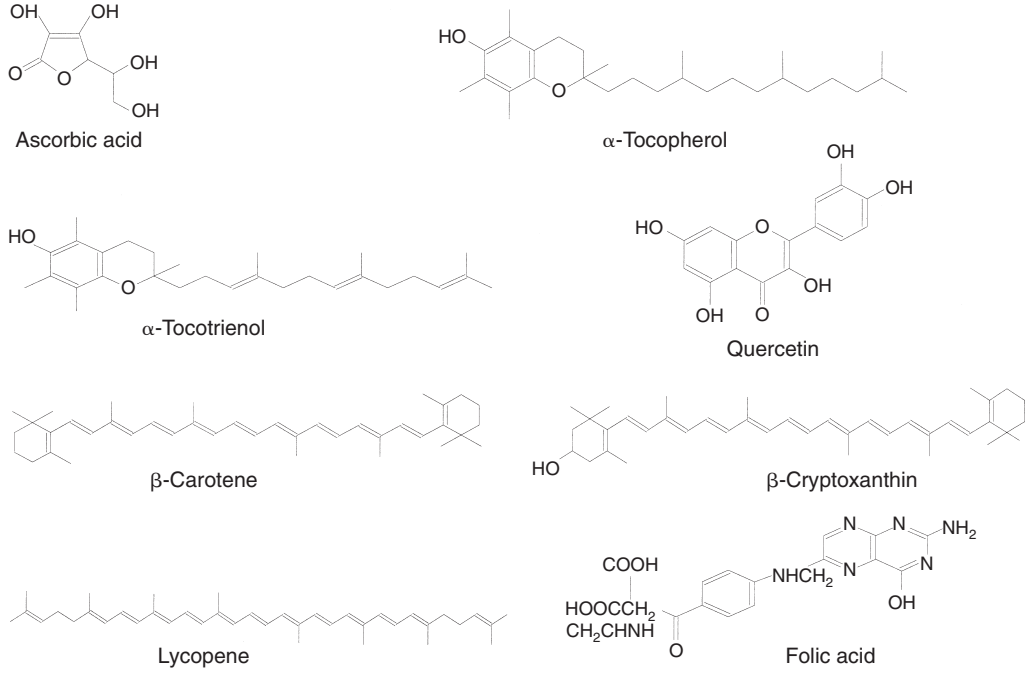

Fig. 1. Representative structures of antioxidant micronutrients.

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methyl substitution. Tocotrienols are similar apart from unsaturation in the phytyl side chain (Fig. 1). The biopotency of these groups of compounds is lower relative to the activity for d-α-tocopherol (set at 100 %) and this includes their antioxidant capacity. The richest, most common sources of vitamin E are vegetable oils (e.g. olive, sunflower, maize and soyabean), products made from them (e.g. margarine, mayonnaise) and whole grains, nuts and seeds. The exact composition of these sources in terms of the individual compounds and the relative intake by different human populations varies. For example, the relatively higher intake of soyabean and maize oils in the American diet accounts for a higher intake of γ-tocopherol in this population. Intestinal absorption is equivalent for all forms of vitamin E; however passage through the liver results in a distribution such that at least for the tocopherols, d-α-tocopherol is preferentially taken up into the bloodstream whereas the other three tocopherols are metabolised and excreted in bile. This selective uptake into the circulation is the result of the hepatic α-tocopherol transfer protein (Hosomi *et al.* 1997). However, γ-tocopherol is also taken up into the bloodstream but is often present at about 10-fold lower level than α-tocopherol, and is rapidly replaced by α-tocopherol if this is increased in the diet. Tocopherols would be expected to partition equally into lipid-rich cellular compartments, although studies on preferential sub-cellular distribution patterns are lacking. The antioxidant activity of the tocopherols is expressed in the lipid membrane compartments of cells where it can act as a chain-breaking antioxidant to prevent the propagation of free radical reactions, such as those arising from lipid peroxidation (Kamal-Eldin & Appelqvist, 1996). The ability of the tocopherols to function effectively as antioxidants is a result of the phenol group. H abstraction by a peroxyl or C-centred radical, for example, gives a phenoxyl radical, which can be resonance-stabilised and is much less reactive than the lipid-derived radicals and thus poorly available for propagation reactions. Regeneration of tocopherol may occur by redox reaction with ascorbate (vitamin C) or reduced glutathione, thus transferring the radical to the aqueous phase. Although the physiological function of vitamin E is primarily regarded as an antioxidant, studies are emerging that it may have functions unrelated to its antioxidant activity.

Carotenoids

Carotenoids comprise a group of compounds closely related in structure that are abundant as plant pigments. Out of 500–600 carotenoids that have been identified, only about six are found in relative abundance in human blood; α-carotene (carrots), β-carotene (orange vegetables and fruits and dark green leafy vegetables), xanthophylls (zeaxanthin, cryptoxanthin and lutein, found predominantly in spinach and kale) and lycopene (tomatoes and tomato products). Structurally, the carotenoids consist of variations on a basic C_{40} skeleton composed of conjugated double-bonds (Fig. 1); the carotenoid hydrocarbons are composed of the bicyclic carotenes and the acyclic lycopene (Rice-Evans *et al*. 1997). Xanthophylls are distinguished as O-containing carotenoids (Fig. 1). β-Carotene is the major precursor of retinol (vitamin A); retinol is found only in foods of animal origin such as liver, dairy products, egg yolks and fish liver oils, as a product of β-carotene metabolism. These lipid-soluble compounds are absorbed by passive absorption in the small intestine in association with lipid micelles (Parker, 1996). Their lipid solubility means that carotenoids are reduced to basal levels relatively slowly following supplementation, for example. The electron-rich polyene chain of the carotenoids renders them susceptible to electrophilic attack, thereby making them effective free radical scavengers. Carotenoids, because of their lipophilicity are involved in the antioxidant protection of lipid membranes and can act synergistically with tocopherols. They function to inhibit free radical propagation reac-

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tions, such as lipid peroxidation, in a similar manner to tocopherols. The resultant carotenoid-free radical is stabilised and therefore rendered less reactive by delocalisation of the free electron over the conjugated double bond system. The extensive conjugated double bond system is also responsible for the effective $^1O_2$ scavenging abilities of carotenoids.

Phytochemicals

Among phytochemicals we include a range of compounds that have more recently become thought of as important in modulating oxidative stress. As the name implies these are compounds derived from plant material and they are not classified amongst the groupings outlined above. However, an appreciation of their potential importance is growing as many representatives of this group of compounds have demonstrated antioxidant function and are being studied in human subjects. There are multiple dietary sources of these compounds as they are present in virtually all plant material. Flavonoids are ubiquitously distributed in vascular plants and comprise a group of over 2000 compounds. The richest dietary sources for these compounds are fruits, vegetables, coffee, tea, cocoa and alcoholic beverages. These compounds are also termed polyphenols and are grouped under flavones (e.g. chrysin; fruit skin), flavanols (e.g. catechin, epicatechin, epigallocatechin; green and black teas, wine), flavonones (e.g. naringin and taxifolin; citrus fruits), flavonols (e.g. quercetin; many fruits and vegetables), methylated flavones and *O*-glycoside derivates of the flavonoids which are the most abundant form of these compounds (Hollman & Katan, 1999). Formation of the free polyphenol by glycosylase activity in the gastrointestinal tract, for example, is necessary to confer antioxidant function. Structurally the flavonoids all have the basic structure of the diphenylpropane skeleton ($C_6C_3C_6$) substituted with differing numbers and arrangements of hydroxyl groups to produce the individual members (Rice-Evans *et al.* 1996). The phenolic groups serve as a source of readily available H atoms such that the subsequent radical produced can be delocalised over the flavonoid structure. More simple phenolic compounds, as with the flavonoids, are found mostly as the *O*-glycoside derivatives and are widely distributed in plants; examples include caffeic and ferulic acids. Again it is the phenolic moieties that confer the ability of these compounds to act as effective radical scavengers, in conjunction with the ability of the electron to become delocalised across the structure. Phyto-oestrogens, including isoflavonoids are also polyphenolic compounds but possess weak oestrogenic activity exerted through the oestrogen receptor. Their protective effects in cells lacking oestrogen receptors suggests they possess other functions, such as antioxidant activity imparted by their polyphenolic nature. Allium compounds derived from garlic, onions and chives are S-containing compounds that are in part responsible for the distinctive taste and odour properties of these vegetables. There are several unique water- and lipid-soluble organosulfur compounds with antioxidant properties in these vegetables, e.g. allixin and allicin (from garlic, this is converted to more bioavailable water-soluble organosulfur compounds, S-allylcysteine and S-allylmercaptocysteine upon ageing of crushed garlic). Allium compounds can exert antioxidant function by direct scavenging of ROS in addition to enhancing antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase and glutathione (Borek, 2001).

Folic acid

Although folic acid is not traditionally recognised as an antioxidant nutrient, recent evidence indicates that this compound is an effective free radical scavenger and thus its role may extend beyond that of its critical function in one-C metabolism, specifically methylation processes (Joshi

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et al. 2001). The major dietary sources of folic acid are dark leafy vegetables and wholegrain cereals. The parent molecule, folic acid (pteroyl glutamic acid), forms the structural basis for multiple derivatives involved in one-C metabolism, dihydrofolic acid, tetrahydrofolate, 5-methyltetrahdrofolate and 10-formyltetrahydrofolate. These compounds are then involved in the synthesis of thymidine for example, and it is this function which impacts directly on DNA stability.

Epidemiological evidence for the possible protective effects of dietary antioxidants

Given the postulated significant role for free radical species in disease, many epidemiological studies have examined the relationship between serum and plasma antioxidant levels and cancer incidence (results summarised in Table 2). Whilst these results support the protective effect of a diet rich in fruit and vegetables, the role of individual antioxidants would appear to be very much more under question (for reviews, see Byers & Perry, 1992). Nevertheless, the explanation for how antioxidant micronutrients may have a protective effect against cancer is plausible, although failure of epidemiological studies to support this has led to focus upon the effects of supplementation on *in vivo* oxidative DNA damage.

Experimental evidence for the protective effects of antioxidants

The data relating to the possible protective effects of dietary antioxidants can be simply divided into two groups; the first group of studies examines diets rich in antioxidant-containing vegetables; the second relates to supplementation with specific antioxidants. The latter group contains by far the larger number of studies.

Table 2. Potential for high dietary antioxidant levels to affect the risk of cancer development at specific sites*

	Nutritional antioxidant			
Cancer site	Vitamin C	Vitamin E	Carotenoids	Vegetables and fruit
Bladder	Insufficient		Insufficient	Probable
Breast	Insufficient		Possible	Probable
Cervix	Possible	Possible	Possible	Possible
Colon, rectum	Insufficient	Insufficient	Possible	Convincing (for vegetables)
Endometrium			Insufficient	Possible
Kidney				Possible
Larynx	Insufficient		Insufficient	Probable
Liver				Possible (for vegetables)
Lung	Possible	Possible	Probable	Convincing
Mouth and pharynx	Possible			Convincing
Oesophagus	Possible		Possible	Convincing
Ovary			Insufficient	Possible
Pancreas	Possible			Probable
Prostate				Possible
Stomach	Probable		Possible	Convincing
Thyroid				Possible

* Data derived from World Cancer Research Fund/American Institute for Cancer Research (1997). Judgements are graded according to the strength of the evidence derived from case–control (≥ 200 cases) and cohort (≥ 100 cases) studies. Definitions: convincing, conclusive evidence for a causal relationship; probable, evidence strong enough to conclude a likely causal relationship; possible, a causal relationship may exist; insufficient, suggestive evidence exists but there is too little or it is imbalanced.

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Vitamin E (tocopherols and tocotrienols)

The cancer preventative ability of vitamin E would appear to be least investigated (Table 2), perhaps because most of the studies examining the protective effects of vitamin E have focused upon cardiovascular disease. Indeed, only limited studies have examined the effects of vitamin E upon oxidative DNA damage *in vivo* (Claycombe & Meydani, 2001). Of these studies, three, supplementing with 200, 400, 500, or 800 IU/d failed to show any effect upon urinary 8-oxodG (Priemé *et al*. 1997), lymphocyte single strand breaks (Astley *et al*. 1999) or lymphocyte micronuclei frequency (Fenech *et al*. 1997), respectively. Equally, a liquid formula diet, described as nutritionally complete and fortified with high vitamin E and polyunsaturated fatty acids did not lower leucocyte 8-oxodG levels, although a significant ($P = 0{\cdot}04$) downward trend was noted (Chen *et al*. 1999). In contrast, only two studies reported a significant decrease in DNA strand breaks; 80 IU/d in conjunction with a 5 % polyunsaturated fatty acid diet; $P = 0{\cdot}01$ (Jenkinson *et al*. 1999) and 280 IU/d; $P < 0{\cdot}002$ (Duthie *et al.* 1996). In the case of the latter study, however, a cocktail of antioxidant nutrients was used (100 mg vitamin C/d and 25 mg β-carotene/d in addition to vitamin E) and thus a DNA protective effect cannot be ascribed to vitamin E alone. A third study, supplementing volunteers with 400 IU/d, showed a decrease in peripheral blood mononuclear cell (PBMC) levels of 8-oxodG, as measured by HPLC–EC, although no change in 8-oxodG or 8-oxodA was seen, in the same cells, measured by GC–MS (Lunec *et al*. 1999). The authors attributed this lack of agreement between the two methods to superior sensitivity and reproducibility of the HPLC–EC (Lunec *et al*. 1999). Testing the hypothesis for a relationship between dietary antioxidants in a typically Mediterranean diet and oxidative DNA damage did not show any correlation between vitamin E levels and lymphocyte levels of 8-oxodG, as measured by HPLC–EC (Bianchini *et al*. 2000). Nor were plasma vitamin E levels significantly higher in the Mediterranean diet compared with a population in Sweden (Bianchini *et al*. 2000). Indeed, contrary to the expected findings, lymphocyte 8-oxodG levels were significantly higher ($P < 0{\cdot}01$) in the Mediterranean group compared with the Swedish group (see also for carotenoids, below).

Carotenoids

Studies suggest that the DNA protective effect of carotenoids is concentration-dependent and protection has been noted *in vitro* using physiological concentrations, i.e. those detected in plasma of individuals consuming healthy diets without supplementation (Lowe *et al*. 1999). At levels that may be achieved when using supplements, carotenoids may increase DNA damage; this phenomenon has been put forward as one explanation for the increased incidence of lung cancer in smokers seen on recent carotenoid supplementation studies (Alpha tocopherol, β-carotene Cancer Prevention Study Group, 1994; Omenn *et al*. 1996). Although a common feature of these two supplementation studies was that smokers were used, it has also been suggested that carotenoids may increase xenobiotic metabolism through cytochrome P-450 induction, perhaps facilitating metabolic activation of smoke-borne carcinogens (Murata & Kawanishi, 2000). Factors such as the O tension and the identity of the oxidising species may also determine whether carotenoids are protective or not under certain conditions (Woods *et al*. 1999; Yeh & Hu, 2000; Zhang & Omaye, 2001).

Studies examining the relationship between plasma carotenoid levels, oxidative DNA damage in peripheral blood lymphocytes (PBL), and the resistance to exogenous oxidative insult of PBL, have been reported. Many of these studies have involved either supplementation regimens with single carotenoids or the use of carotenoid-rich, ‘functional’, foods. In the case of the lat-

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ter, to ascribe any protective effect to carotenoids alone some measurement of blood levels of carotenoids and correlations with levels of DNA damage need to be performed, since several other plant-derived antioxidants such as flavonoids could be responsible for any observed protective effect. The various supplementation or dietary manipulation studies performed in human subjects to address the antioxidative effects of carotenoids on oxidative DNA damage show a predominant protective effect. Significant negative correlations were detected between total serum carotenoid levels and oxidised pyrimidine levels in PBL DNA, from subjects supplemented with α- or β-carotene, lutein or lycopene, measured using a modified Comet assay (Collins *et al*. 1998*b*). In a similar study subjects given lycopene, lutein or β-carotene (15 mg/d for 1 week) showed 2- to 3-fold increases in plasma carotenoid concentration, which reverted to basal levels after a 3-week wash-out period. An increased resistance of PBL isolated from the supplemented subjects to hydrogen peroxide-induced DNA strand breakage as measured by the Comet assay was observed (Torbergsen & Collins, 2000). A lowering of baseline levels of 24-h urinary 8-oxodG was detected in eight healthy non-smoking male subjects supplemented with 30 mg β-carotene/d for 1 month, which increased plasma β-carotene levels 17-fold compared with a placebo group (Sumida *et al*. 1997). In terms of dietary manipulation studies or intervention with functional foods, the antioxidant effects of carotenoids have been similarly encouraging. Twenty-eight healthy female subjects participated in a 14-d recipe-defined diet rich in fruit and vegetables (Thompson *et al*. 1999). Post-intervention plasma carotenoid levels increased relative to pre-intervention levels but to differing extents, with α-carotene levels increasing most markedly out of those measured. Overall, fruit and vegetable consumption showed a trend towards reduction in levels of PBL and urinary 8-oxodG, although this was not statistically significant. Plasma α-carotene levels did correlate significantly with a reduction in PBL 8-oxodG, at least for individuals with inherently low pre-supplementation levels of α-carotene (< 98 ng/ml). Those subjects with higher pre-supplementation levels of α-carotene showed a reduction in PBL 8-oxodG, although this was not statistically significant. Whilst there was a trend toward reduction in urinary levels of 8-oxodG in both groups this was also not statistically significant. These data would suggest that individuals with higher levels of carotenoids may maintain inherently lower levels of oxidative DNA damage. Strong inverse correlations between plasma carotenoids (lutein, β-cryptoxanthin) and markers of oxidative DNA damage (urinary and PBL DNA levels of 8-oxodG measured by ELISA and HPLC–EC respectively) were detected in thirty-seven female subjects participating in a 14-d dietary intervention study equivalent to twelve servings of fruit and vegetables/d (Haegele *et al*. 2000). Damage to DNA, measured by the Comet assay, as strand breaks or oxidised pyrimidines, was examined in PBL from twenty-three healthy, non-smoking male subjects who initially abstained from a carotenoid-rich diet for 2 weeks before receiving either tomato juice (lycopene), carrot juice (α- and β-carotene) or dried spinach powder (lutein). All three dietary regimens induced a decrease in DNA strand breaks with carrot juice specifically decreasing oxidised pyrimidine levels (Pool-Zobel *et al*. 1997). Again, using the Comet assay, a highly significant decrease in PBL DNA damage was detected in twenty-eight healthy elderly volunteers (mean age 68 years) given a fruit and vegetable extract juice twice daily for 80 d (Smith *et al*. 1999).

Processed tomato products, such as tomato juice or tomato puree, are a significant source of lycopene and in some cases this carotenoid in particular has been the focus of attention. Significant increases in plasma and/or PBL lycopene levels were noted in two studies where adult females consumed tomato puree (25 g/d for 14 d) as part of a dietary manipulation study (Riso *et al*. 1999; Porrini & Riso, 2000). Resistance of PBL to DNA damage following hydrogen peroxide challenge in these subjects was increased, as assessed by the Comet assay, with an inverse relationship between lycopene concentration and DNA damage. Using GC–MS to mon-

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itor oxidised base lesions in PBL, Rehman *et al.* (1999*a*) showed that a single serving of tomatoes in healthy human subjects was sufficient to decrease the level of some oxidative DNA lesions (8-oxo-guanine), yet the levels of other lesions (8-oxo-adenine) were increased. This trend is the same as that described by Podmore *et al.* (1998*a*) in a widely reported vitamin C supplementation study. Subsequent studies have implied that this divergent trend for two oxidative lesions may be explained by a modulation of DNA repair activities for one lesion over another. Although evidence for this sort of function from vitamin C is emerging, this apparent effect for carotenoids has been questioned (Torbergsen & Collins, 2000). Since all oxidative DNA lesions do not behave in the same way, but change in a divergent manner in such supplementation studies, this would question the idea that carotenoids merely act as antioxidants rather than modulating DNA repair in PBL. As discussed in the next section (pp. 30–31), vitamin C may modulate DNA repair by providing a priming oxidative stimulus. Such an oxidative priming phenomenon for carotenoids would not be an unreasonable route to modulate DNA repair, since pro-oxidant properties for carotenoids have been noted in some studies.

The majority of dietary intervention studies indicate that a carotenoid-rich diet reduces the levels of oxidative DNA damage, although there are studies that show a positive correlation of plasma carotenoid levels with markers such as PBL 8-oxodG level. Such a study was reported recently by Bianchini *et al.* (2000) using subjects from Northern and Southern Europe. While this study may indicate that high plasma carotenoids are inducing or leading to a persistence of oxidative DNA damage, the authors admit that there may be other dietary components of the population being studied that are promoting oxidative DNA damage. A 12-week carotene supplementation regimen (palm oil carotenes (α- and β-carotene), lycopene and lutein) along with a placebo group was conducted in five European countries. Not all samples were analysed for the same parameters, but in those instances where pre- and post-supplementation assessments of serum carotenoids and PBL DNA levels of 8-oxodG were assessed, levels of carotenoids increased significantly on supplementation but 8-oxodG levels were unaffected. One feature of this supplementation study was that there was a 4-week vitamin E supplementation phase immediately preceding the carotenoid supplementation (Collins *et al.* 1998*a*). What may be apparent is a complex interplay between individual carotenoids and probably other food components to determine any overall *in vivo* antioxidative effect. An intake of these species in a complex food matrix may be more useful to study and of more benefit to the population than the promotion of individual nutrient supplements.

Phytochemicals

Studies of the protective effects of flavonoids, phyto-oestrogens and the other phytochemicals detailed earlier (see p. 26) on oxidative DNA damage *in vivo* in human subjects are relatively limited at present. Most of the studies to date have focused on *in vitro* experiments using cells pretreated with the compound of interest before exposure to an oxidative stress and also studies using rodents fed phytochemical-rich diets (Duthie *et al.* 1997; Noroozi *et al.* 1998; Casalini *et al.* 1999; Aherne & O'Brien, 2000; Johnson & Loo, 2000). Studies of the DNA protective effects of dietary flavonoids *in vivo* are required since extrapolation of *in vitro* data is probably an oversimplification. Since these compounds are ubiquitous in plant species there could be any number of compounds involved in DNA protective effects. As with the carotenoid studies, measurements of blood levels of the compounds of interest should be made and correlations drawn between these levels and any modulation of DNA damage. Only in recent years have studies moved beyond the *in vitro* and rodent studies and on to dietary supplementation with flavonoid rich foods. Findings from these

studies, when taken together are still equivocal, in part because insufficient studies have been performed addressing one compound or a group of related compounds. A recent study in thirty-six healthy human subjects assessed the impact of a high-flavonol diet (onion cake and black tea; primarily quercetin) on a number of factors in particular oxidative DNA damage in PBL (Beatty *et al*. 2000). The high-flavonol dietary regimen significantly increased plasma quercetin levels, but no significant decrease in oxidative DNA damage products in PBL was detected by GC–MS. Significant elevation of plasma flavonoids (quercetin, kaempferol and isorhamnetin) was observed in a group of eighteen healthy female volunteers receiving rutin (quercetin-3-O-beta-rutinoside) supplements (500 mg/d for 6 weeks). No significant change in urinary 8-oxodG levels was noted in either the rutin- or placebo-supplemented groups whilst both groups showed significant decreases in oxidised pyrimidine levels as measured by the Comet assay using endonuclease III (Boyle *et al*. 2000*a*). Although the nature of the compounds investigated was slightly different, a study using volunteers fed rye crispbread (76·5 g/d; 42 μg secoisolariciresinol and 33 μg matairesinol/100 g dry weight) found little evidence to indicate that isoflavonoids or lignin would exert a significant antioxidative effect systemically. In this case oxidised purine and pyrimidine lesions were assessed in PBL DNA using a modified Comet assay (Pool-Zobel *et al*. 2000). This lack of DNA protection could in part be due to the fact that adequate concentrations of these compounds are not achieved physiologically. Whereas these compounds may have a significant protective effect in an *in vitro* study, these findings again emphasise the need for controlled *in vivo* studies in human subjects.

There are some studies that show an apparent DNA protective effect of dietary flavonoids. A dietary regimen consisting of fried onions or fried onions plus fresh cherry tomatoes was used to study the effects of flavonoids on markers of oxidative damage to biomolecules, including DNA (Boyle *et al*. 2000*b*). Plasma flavonoid glycosides were significantly increased following the fried onion meal and this increase was associated with an increased resistance of PBL to oxidant-induced DNA strand breakage and a decrease in urinary 8-oxodG, 4 h after the meal. The decrease in urinary 8-oxodG is taken to reflect a decrease in oxidative DNA damage. Quercetin alone was increased in plasma following the onion and tomato meal, with a reduction in endogenous oxidative DNA damage but an unchanged resistance to strand breakage. The dietary manipulation using onion meals does not apparently take into account the protective roles of allium compounds, for example. Ten patients with stable type II diabetes were treated for 2 weeks on a low flavonol and then for 2 weeks on a flavonol-supplemented diet (approximately 100 mg flavonols, predominantly quercetin, provided as 400 g onion and tomato sauce and six cups of tea). Plasma flavonol concentrations increased 12-fold on the flavonol-supplemented diet. DNA damage in PBL from the patients was assessed using the Comet assay and was significantly decreased when taking the flavonol-supplemented diet (Lean *et al*. 1999).

Finally, a supplementation study using soya milk (a rich source of phyto-oestrogens) in ten healthy male volunteers gave significant increases in plasma levels of the phyto-oestrogens genistein and daidzein, and to a significant reduction in oxidative DNA damage in PBL using the Comet assay (Michell & Collins, 1999). The antioxidant effects of phyto-oestrogens may go beyond their inherent potential to scavenge free radical species, since they could also modulate the activity of antioxidant enzymes (Cai & Wei, 1996); this aspect of DNA protection by antioxidants is considered in more detail in the next section.

Vitamin C

From the epidemiological studies summarised in Table 2, it would appear that the cancer-preventing ability of vitamin C remains questionable. The contentious nature of this subject

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perhaps derives, in part, from the *in vitro* studies, summarised in a recent review (Carr & Frei, 1999) which appeared to show that there is as much evidence for a pro-oxidant effect upon DNA damage, as antioxidant. This would also seem to hold true for some of the *in vivo* studies.

Perhaps the first evidence for a protective effect *in vivo* derived from the seminal work by the group of Bruce Ames in which the vitamin C levels of ten volunteers were depleted and repleted (Fraga *et al*. 1991). This work demonstrated that decreasing vitamin C intake from 250 mg/d to 5 mg/d, led to a doubling of sperm DNA levels of 8-oxodG (34·0 (SD 2·4) fmol/μg DNA to 66·90 (SD 8·5 fmol/μg DNA; $P < 0{\cdot}01$), although continued depletion resulted in a 248 % increase (Fraga *et al*. 1991). Repletion at 250 mg/d for 28 d led only to a 36 % decrease in 8-oxodG levels. An identical study, by the same group, also showed an increase in sperm levels of 8-oxodG, although depletion had no effect on either urinary 8-oxodG or the 8-oxodG content of PBMC (Jacob *et al*. 1991).

Urinary 8-oxodG in 24-h urine collections was the sole DNA damage marker measured by ELISA during a supplementation study with 500 mg vitamin C/d for 2 months (Huang *et al*. 2000). At baseline, dietary intakes of vitamin C were inversely associated, although not significantly, with urinary 8-oxodG (17·6 (SD 10·4) ng/mg creatinine); upon supplementation no decrease in urinary 8-oxodG was noted, either with vitamin C alone (19·3 (SD 9·3) ng/mg creatinine), or in combination with vitamin E (17·7 (SD 9·5) ng/mg creatinine). The authors suggested that the lack of significance may be due to the 2-month study period being too short, and this issue is discussed later (p. 35). Similarly, urinary levels of 8-oxoguanosine (the damaged RNA product) did not alter from baseline (about 400 (SD 50) pmole 8-oxoguanosine/kg per d) following supplementation with 1 g vitamin C/d for 1 month (Witt *et al*. 1992). In contrast to measuring lesion levels in PBMC following *in vivo* supplementation, PBMC may be challenged *ex vivo* by hydrogen peroxide before assessment, by cytokinesis-block micronucleus assay, Comet assay (DNA strand break measurement) and chomosomal aberrations, for example. Using the former approach, Crott & Fenech (1999) reported no apparent DNA damage following a single 2 g bolus of vitamin C. Although equally, there was no protection of the PBMC against hydrogen peroxide toxicity. Even doses as large as 6 g vitamin C/d failed to show any beneficial effects, as assessed by DNA strand break measurement using the Comet assay, following hydrogen peroxide challenge in non-smoking individuals with 'low' and 'high' cholesterol (Anderson *et al*. 1997*)*.

A number of studies have shown that larger increases in plasma vitamin C levels are seen following supplementation of individuals with low initial levels (Calzada *et al*. 1997; Rehman *et al*. 1998). Indeed, initial ascorbate status may also affect 8-oxodG levels (Rehman *et al*. 1998) and a number of studies have compared intrinsic vitamin C levels with oxidative DNA damage. A negative correlation was demonstrated between intracellular ascorbate and lymphocyte DNA levels of 8-oxodG ($r -0{\cdot}28$; $P < 0{\cdot}01$) and 5-OH-deoxycytidine ($r -0{\cdot}07$; $P < 0{\cdot}01$), from a study of 105 healthy volunteers (Lenton *et al*. 1999), although no such correlation was seen with urinary 8-oxodG, in a similar study by Poulsen *et al*. (1998).

Vitamin C is commonly used to enhance peroxidative processes involving transition metal ions and has been suggested to generate radicals from the decomposition of lipid hydroperoxides (Baysal *et al*. 1989). However, whilst the point has been made that such a pro-oxidant activity does not occur in healthy human plasma, containing low levels of lipid hydroperoxides and no detectable metal ions (Stocker & Frei, 1991), this argument may not be simply applied to the environment surrounding DNA. Furthermore, pathological conditions do exist where lipid hydroperoxide or metal ions may conceivably be elevated and therefore vitamin supplementation may very well exert a pro-oxidant effect. Indeed, a number of supplementation stud-

ies in healthy volunteers with vitamin C alone or in combination with Fe have shown an increase in some oxidative DNA lesions, although with a concomitant antioxidant effect (Podmore *et al*. 1998*a*; Rehman *et al*. 1998). However, upon repeating the study of Rehman *et al*. (1998; 260 mg vitamin C/d and 14 mg Fe/d), no overall change in the 'total' oxidative DNA damage was noted, despite some lesion levels increasing and some decreasing (Proteggente *et al*. 2000).

Vitamin C supplemented at a level of 500 mg/d for 6 weeks significantly increased plasma vitamin C levels in healthy volunteers and was shown to increase levels of 8-oxodA, whilst at the same time lowering levels of 8-oxodG in PBMC DNA (Podmore *et al*. 1998*a*). As the lesions acted in a completely divergent manner in response to vitamin C, this phenomenon, contrary to many criticisms levelled at the work, could not be due to artifact and demonstrated an apparent pro-oxidant effect (Podmore *et al*. 1998*b*). A study extending supplementation to doses of 500, 1000 and 5000 mg vitamin C/d failed to demonstrate a pro-oxidant effect at any dose (Vojdani *et al*. 2000). Explanation for this finding may derive from the authors measuring 8-oxodG in leucocyte DNA which decreased in the study of Podmore *et al*. (1998*a*), whereas 8-oxodA increased. Equally, no decrease in 8-oxodG and hence antioxidant activity was noted (Vojdani *et al*. 2000).

A compelling hypothesis explaining these results derived from the analysis of serum and urinary levels of 8-oxodG in the same subjects (Cooke *et al*. 1998). The sequential decrease in PBMC DNA levels of 8-oxodG and increase in serum and urinary 8-oxodG levels suggested that, rather than a purely radical-scavenging antioxidant effect, vitamin C was influencing DNA repair enzymes (Cooke *et al*. 1998). Similar, albeit non-significant, increases in urinary 8-oxodG were noted during vitamin C supplementation (Priemé *et al*. 1997) or high fruit and vegetable diet (Hertog *et al*. 1997). This lack of significance during supplementation is consistent with the findings of Cooke *et al*. (1998) and led to the suggestion of a post-supplementation effect of vitamin C. This effect is exemplified by a recent result with 400 mg/d supplementation for 10–20 weeks (Carty *et al*. (2000); Fig. 2). A pro-oxidant effect was seen initially, with PBMC DNA levels of 8-oxodG, as measured by HPLC–EC, rising significantly ($P = 0{\cdot}05$) 28 % before a highly significant ($P = 0{\cdot}001$) reduction of 60 % which was maintained even after the volunteers were split into two groups, with one taking placebo (Fig. 2). The same study also demonstrated a dose effect upon urinary oxidative DNA damage markers, with a transient, significant increase in urinary 8-oxodG levels ($P = 0{\cdot}05$) and only when compared with baseline, rather than placebo (MS Cooke, N Mistry, J Ahmad and J Lunec, unpublished results). Furthermore, the initial pro-oxidant phase seen is consistent with the 500 mg/d study of Podmore *et al*. (1998*a*), despite only being seen in 8-oxodA, but nevertheless agrees with the suggestion that this effect primes DNA repair (Lunec *et al*. 2000*a*).

The potential role of vitamin C in DNA repair was further elucidated in a study of deoxycytidine-glyoxal adduct (dC-glyoxal), an oxidative DNA lesion, and thymine dimer (T<>T), a non-oxidative, u.v.-derived lesion, removal from PBMC DNA, following supplementation with 400 mg/d (N Mistry, ID Podmore, MS Cooke, J Ahmad, L Dickinson, Jones GDD, HR Griffiths, KE Herbert and J Lunec, unpublished results). The authors noted an initial increase in dC-glyoxal levels, which was not seen in the T<>T, proposed to derive from an initial pro-oxidant effect, followed by a subsequent reduction in both dC-glyoxal and T<>T, implying their removal by the same, or similar repair processes. Given that the removal of T<>T, which is exclusively repaired, in man, by nucleotide excision repair, coincides with the removal of dC-glyoxal, the authors propose that cytosine-glyoxal adducts are repaired by nucleotide excision repair pathways and it is these which are influenced by vitamin C supplementation.

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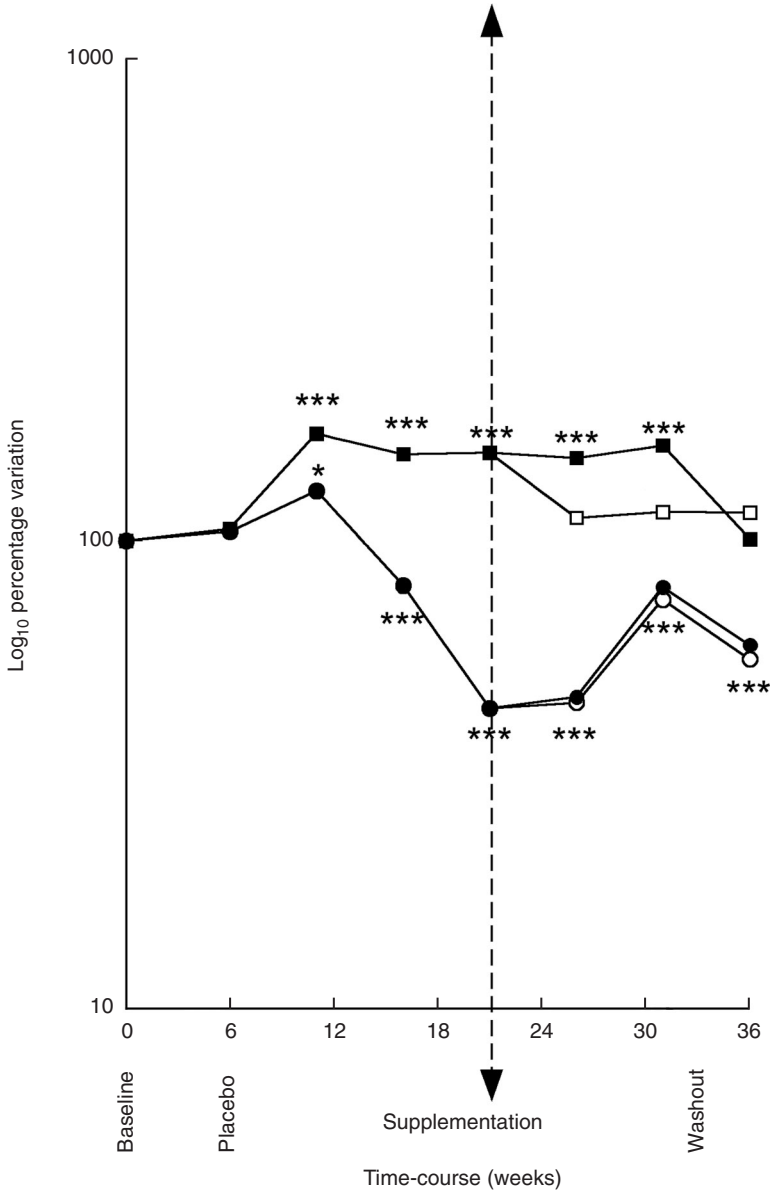

Fig. 2. Representation of 8-oxo-2′-deoxyguanosine levels in peripheral blood mononuclear cell DNA, assessed by HPLC–electrochemical detection, in healthy volunteers following supplementation with 400 mg vitamin C/d (●) or placebo (○) and comparison with plasma vitamin C levels in the supplemented (■) and placebo (□) groups. At the point indicated (↕) the group was split and double-blind, randomly-assigned to either remain on vitamin C or change to placebo (calcium carbonate). The trial format is described fully in Carty *et al.* (2000). Data are expressed in terms of mean percentage variation from baseline (100 %). Variation was significantly different from baseline and placebo measurements: $^{*}P = 0{\cdot}05$, $^{***}P = 0{\cdot}001$.

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Folic acid

A role for a direct antioxidant function of folic acid *in vivo* and indeed *in vitro* is currently extremely limited and presents novel avenues for study. However, the function of folic acid and derivates in methylation reactions indicates that a deficiency of this vitamin can adversely affect DNA synthesis and repair, by impacting on thymidine synthesis. Folate deficiency induces an increase in uracil mis-incorporation into DNA, and an overall decrease in DNA stability, by increasing the frequency of DNA strand breaks (Duthie *et al*. 2000). Some studies, although not in human subjects, indicate that base excision repair is specifically affected by folate deficiency (Choi *et al*. 1998; Duthie & Hawdon, 1998). Because folate deficiency can diminish the repair of DNA damage, including oxidative DNA damage, then its deficiency would increase the impact of such damage on DNA integrity by uracil mis-incorporation into DNA during post-excision DNA synthesis. Folic acid would not therefore appear to impact directly on the formation or removal of oxidative DNA damage, but may enhance the genotoxicity of oxidised DNA by an impairment of DNA repair processes. Significant uracil incorporation into DNA would result in removal by uracil DNA glycosylase and the introduction of nicks. Should nicks also arise on opposite strands, this could lead to double-strand break formation.

Intervention study considerations

Whilst the following points relate to vitamin C supplementation specifically, there is no reason why these considerations may not be applied to the study of other antioxidants.

Demonstrating evidence for an anti- or even a pro-oxidant effect for vitamin C *in vivo* would seem to depend on a number of factors:

Dose. There is evidence for a dose effect; urinary levels of 8-oxodG following 400 mg vitamin C/d (see p. 33) did not rise to the same level of significance as the previous study (Cooke *et al*. 1998). A dosing study, comprising the measurement of more than one oxidative lesion and in a number of biological matrices, would seem to be essential.

Duration of supplementation. Supplementation studies comprising a few weeks to a couple of months may not fully represent the effects seen with long-term antioxidant intake. Even so, supplementation with 500 mg slow release vitamin C/d (and/or 128 mg vitamin E) over a period of 36 months failed to show any effects upon urinary 8-oxodG, when sampled at 12 and 36 months (Porkkala-Sarataho *et al*. 2000), although this might be explained by when sampling is taken.

When sampling is taken. Any effect may be transient and the correct timepoint might be missed, or sampling may not continue for long enough and miss any post-cessation effect (see vitamin C, p. 33).

Lesion(s) measured. Certainly the work of Rehman *et al*. (1998) and Proteggente *et al*. (2000), due to the large number of lesions measured, illustrates how supplementation may have a pro-oxidant effect on some lesions and an antioxidant effect on others. However, the problem with defining effect based on 'total' oxidative DNA damage changes is that every single oxidative DNA lesion, and presumably this would include ribose damage and backbone modification, would need to be measured, not just the lesions that have been identified and can be measured.

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Which tissue to sample. In which tissues or biological matrices, such as serum and urine, is it important to assay for oxidative DNA damage? Clearly this can affect the results; for example, Jacob *et al*. (1991) showed that upon depletion of plasma vitamin C in volunteers, sperm levels of 8-oxodG went up, but no effect was seen in PBMC 8-oxodG or urinary 8-oxodG. PBMC are often used as surrogate cells, to provide information relating to tissues less easily accessible for analysis. However, a study of 8-oxodG levels in PBMC, tumour and normal human tissue, failed to show any correlation between the tissues and PBMC (Fokinski *et al*. 2000), warranting further investigation of this issue.

Study format. Double-blind cross-over studies, normally reserved for pharmaceutical drug intervention trials, are rarely applied to the examination of antioxidant function *in vivo*. Invariably, antioxidant studies are of a longitudinal design, using subjects as their own controls, effectively accounting for physiological differences. This has clearly not prevented the acquisition of meaningful data, and in some instances has facilitated novel findings such as the post-supplementation effect, described earlier. Clearly it is important to use the most appropriate trial format which will vary depending on the hypothesis to be tested (Jones & Payne, 1997).

Conclusion

The ability of fruit and vegetables to reduce the risk of cancer development does appear to have increasing epidemiological, if not experimental, support (Lee, 1999). The role of micronutrients in this effect, although not specifically antioxidants, has been extensively argued (Ames, 2001). However, it is not easy to agree with conclusions such as those by Duthie *et al*. (1996) that fruit and vegetables exert a cancer-protective effect via a decrease in oxidative DNA damage. Firstly, supplementation studies have failed to show conclusively that antioxidants alone can modulate cancer risk and second, the role of oxidative DNA damage in carcinogenesis has likewise not been conclusively demonstrated. Whilst the antioxidant effect of fruit and vegetables may be under question, evidence is growing that their constituents may affect gene expression and hence modulate DNA damage, either via DNA repair pathways (Lunec *et al*. 2000*a*) or enzymes associated with xenobiotic metabolism (Verhagen *et al*. 1995; Deng *et al*. 1998). Furthermore, it is very likely that antioxidants act jointly with other components in fruit and vegetables, and that it is this concerted effort which has a protective effect (Block, 1991). Overall, it would seem that a diet high in fruit and vegetables, along with the avoidance of known carcinogens, remains the current choice for maintenance of health.

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