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Antioxidant intervention as a route to cancer prevention

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Abstract

The notion of cancer prevention through antioxidant intervention arises from the fact that fruits and vegetables contain antioxidants and are linked to low cancer rates in those who consume them. Protection against DNA damage by plant food products can be demonstrated *in vitro*. However, particular care is needed when measuring the damage, since oxidation readily occurs during sample preparation, creating a serious artefact. In the case of DNA oxidation, estimates of background levels in human cells range over 3 orders of magnitude, depending on the method used. Using validated, reliable biomarker assays for DNA oxidation, it is possible to demonstrate a decrease in oxidative damage after supplementation with isolated antioxidants or whole plant foods in humans. In contrast, in several large-scale interventions with disease or death as the endpoint, supplementation with β-carotene resulted in no effect or an increase in cancer incidence. It is certainly true that we do not yet fully understand the role of phytochemicals as antioxidants, or as modulators of other processes related to carcinogenesis and its prevention.

Keywords: Antioxidants; Cancer prevention; DNA

1. Introduction

The title of this review should probably be followed by a question mark. There is a strong evidence from observational epidemiology that fruits and vegetables in the diet are associated with a lower incidence of various cancers. From this has developed the idea that it is the antioxidants in these foods that are the effective preventive agents. This is an attractive hypothesis; it is known that free radicals released during respiration can damage DNA, that oxidation damage to DNA can result in mutation, and that fruits and vegetables contain substantial amounts of various natural compounds with antioxidant properties. However, confirmation of this hypothesis remains an elusive goal of experimental scientists as well as conventional epidemiologists.

2. The antioxidants in plant foods

It was probably Bruce Ames who first drew general attention to the importance of oxidative damage in human cancer aetiology and the likely importance of antioxidant defences, both intrinsic (glutathione, uric acid, superoxide dismutase, *etc.*) and of dietary origin. In a paper published in 1983 [1] he surveyed the large number and variety of natural chemicals in plants used as human food that have been shown to be mutagenic (*i.e.*, capable of causing mutations in *in vitro* tests such as the Ames test) or carcinogenic (inducing cancer in animals), or both. Many of these may act through the generation of oxygen free radicals. Perhaps, as Ames implies, we are protected against deleterious effects of oxygen free radicals by the antioxidants and other anticarcinogens that are also present in plant-derived foods. These phytochemicals would also protect us against the effects of endogenous production of reactive oxygen, as a by-product of normal respiration, as part of

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the inflammatory response, or during xenobiotic metabolism.

The phytochemicals present in plant foods that have been shown to act as antioxidants are legion. Examples are shown in Fig. 1. Apart from vitamin C and the tocopherols, there are several hundred carotenoids, and an even greater number of flavonoids (of which quercetin is an example) and other polyphenols. Even if it were feasible to identify and measure all these compounds in a particular food, the information would not be very useful, since antioxidants act not in isolation but in combination, and synergistic and antagonistic effects are very hard to predict.

It is more realistic to attempt to measure the total antioxidant capacity of plant foods. Various methods are available, depending on ability to quench free radicals, simple reducing activity against ferric ion, or a more biological approach that looks at the protection of DNA or lipids against oxidation in an *in vitro* system.

Probably the most comprehensive survey of total antioxidant activities of plant foods was carried out by Halvorsen *et al.* [2], using a modification of the FRAP assay, originally developed for measuring the Ferric Reducing Ability of Plasma [3]. Hundreds of plant foods – including cereals, roots, vegetables, fruits, berries, pulses, nuts and seeds – were subjected to a standard water/methanol extraction, and the ability of the extract to reduce Fe^{3+} to Fe^{2+} determined with a simple colorimetric reaction. Table 1 shows a selection of the results, illustrating the wide range of antioxidant content determined with this method, and some of the surprises – notably, the low antioxidant capacity of carrots. Generally, fruits, berries, and some nuts and seeds come out high in this assay. Pellegrini *et al.* [4] employed three different assays to measure total antioxidant capacity in 34 vegetables, 30 fruits, 34 beverages and 6 vegetable oils – trolux equivalent antioxidant capacity (TEAC), total radical trapping antioxidant parameter (TRAP), and FRAP. In most cases there was broad agreement between the different tests, though there were some exceptions. When testing pure antioxidants (various flavonoids, vitamin C and trolux), Mitchell *et al.* [5] reported a high correlation between FRAP values and radical quenching assessed using electron spin resonance spectroscopy, but no correlation with TEAC determinations. Inhibition of lipid peroxidation in vitamin E-deficient liver microsomes, another measure of antioxidant activity, correlated with FRAP and ESR results. Serafini *et al.* [6] found that various phenolic-rich beverages (teas, wines) could protect LDL from oxidation.

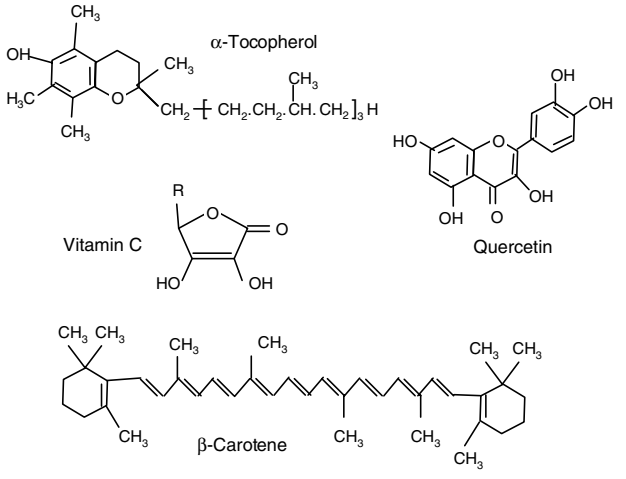

Fig. 1. Examples of common antioxidants from different chemical groups.

This *in vitro* approach is attractively simple, and certainly provides impressive comparative information on different plant foods. It examines the antioxidant properties of mixtures of compounds, rather than micronutrients in isolation, and it is mixtures that are presented to the human body after all. However, it has several serious limitations. The actual levels of antioxidants in plants depend greatly on the variety of plant analysed, the way in which it was cultivated (since synthesis of antioxidants can be a response to stress, plants

Table 1
Some examples of the ferric reducing activity of common plant foods

Cereals	
Oats	0.59
Wholemeal flour	0.33
White flour	0.13
Roots	
Ginger	3.76
Blue potato	0.80
Potato	0.09
Carrot	0.04
Vegetables	
Capsicum	2.46
Spinach	0.98
Onion	0.67
Tomato	0.31
Cabbage	0.09
Fruits	
Pomegranate	11.33
Grape	1.45
Kiwifruit	0.91
Apple	0.29
Berries	
Dog rose	39.46
Bilberry	8.23
Elderberry	4.31
Gooseberry	1.45
Pulses	
Broad bean	1.86
Garden pea	0.12
Nuts and seeds	
Walnut	20.97
Hazelnut	0.49

Activity is expressed as millimolar (mM), for 100 g of the food prepared as a water/methanol extract. Data are from [2].

grown under more natural, or 'organic' conditions may contain higher levels – an idea that should be tested), and how it was harvested and stored. Most importantly, this is an *in vitro* approach, and tells us nothing about bioavailability and metabolism, *i.e.*, how efficiently the compounds are taken up from the gut, how they are transported to tissues and into cells, and how quickly they are excreted.

The next step rationally would be to incubate cells in culture with a plant food extract, and to measure an endpoint related to oxidative stress (DNA oxidation, or lipid peroxides). Eberhardt *et al.* [7] treated human cancer cell lines Caco-2 and HepG2 with extracts of apples and found an inhibition of cell proliferation, though at very high concentrations. There are rather more reports on the effects of individual antioxidant compounds on cultured cells. For example, Fig. 2 shows the effect on DNA integrity of incubating cells with quercetin, a common and abundant flavonoid in many fruits, vegetables, wines and other plant foods. Quercetin actually induces DNA damage when present alone, though the effect is significant only at concentrations above those that would be present in the body [8]. But when administered in combination with H_2O_2, quercetin significantly decreases the amount of damage done. This rather paradoxical result is perhaps what might be expected from the observations by Ames [1] on the presence of both mutagenic and protective activities in common foods.

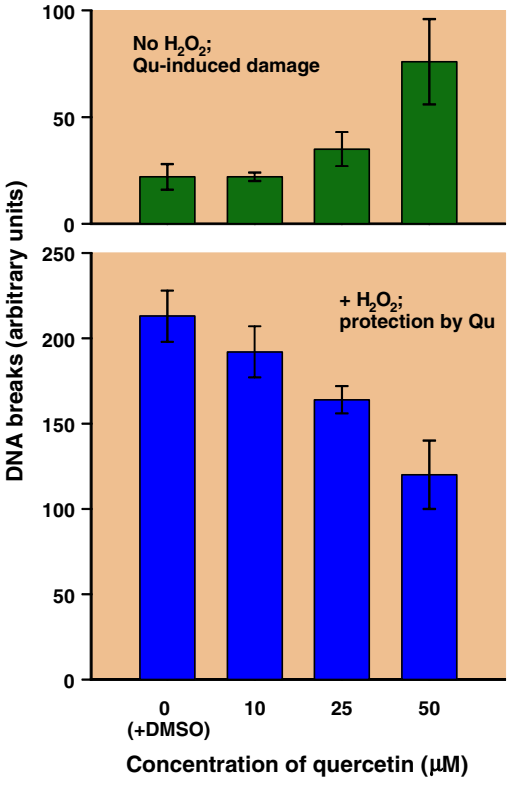

Fig. 2. DNA-damaging and protective effects of quercetin. Human lymphocytes were incubated for 30 min with quercetin at different concentrations before measuring DNA breaks with the comet assay. In the lower panel, the cells were treated with 200 μM H_2O_2 for 5 min at the end of the incubation with quercetin. DNA breaks were measured with the comet assay. Data are redrawn from [8], copyright 1997, with permission from Elsevier.

3. Testing for effects of antioxidants in humans: use of biomarkers

In view of the issue of bioavailability, the best way to examine the effectiveness of antioxidants is to carry out *in vivo* experiments, with antioxidant supplements or foods rich in putative antioxidants, preferably in humans. The design of the trial is crucially important, and should include a placebo group, with samples taken before, during and after supplementation. If a crossover design is adopted (*e.g.*, for different supplements, or different doses, or placebo and supplement in the same subjects), washout periods between the different treatments are essential, and randomly selected subgroups should receive the treatments in different orders.

The so-called molecular epidemiological approach is based on the use of biomarkers, indicating some aspect of disease risk, rather than disease itself. It must be possible to measure the biomarkers on samples that are readily obtained, which generally means blood or urine. The markers can indicate levels of exposure to disease-causing or preventive agents, or individual susceptibility factors such as polymorphisms in relevant genes, or early stages in the disease process, such as mutations in the case of cancer. Biomarkers are useful only if they are validated.

A biomarker assay should be reliable and reproducible, giving the same answers on identical samples in different laboratories. It should have a clear and quantitative relationship to whichever facet of the disease process it is supposed to represent, *i.e.*, exposure, susceptibility, or pathology. Unfortunately, biomarker assays are too often used without paying attention to the need for validation.

Possible biomarkers for use in investigations of antioxidant effects include:

- Total antioxidant capacity of plasma.
- Lipid peroxidation products, of which malondialdehyde (MDA) in blood is most commonly measured.
- Endogenous DNA oxidation, measured in white blood cells.
- *Ex vivo* resistance of leukocyte DNA to oxidation by H_2O_2.
- *Ex vivo* resistance of blood lipids to oxidation.

Total antioxidant capacity (TAC). TAC tends to be a rather insensitive indicator of change in antioxidant levels, since it is a composite measure, made up of many

individual antioxidant components (including endogenous antioxidants such as uric acid and albumin), and an increase in one (or a few) exogenous antioxidants is hard to detect above this background [9]. On the other hand, seasonal changes in diet can influence TAC – an effect that should be detected in the placebo group [10]. Serafini *et al.* [6] found increases in TRAP values for serum from volunteers given green or black tea, or alcohol-free red wine.

Lipid peroxidation products. In the TBARS assay, MDA is complexed with thiobarbituric acid, and the resulting thiobarbituric acid-reacting substances (TBARS) are measured spectrometrically. However, there are many compounds apart from MDA that can react with thiobarbituric acid. A greater level of specificity is achieved if the MDA–TBARS compounds are analysed by HPLC. MDA was not affected by supplementation with vitamins C, E, β-carotene and selenium [10] or with vitamins C and E [9]. However, in a seasonal study we found higher levels of plasma MDA in winter/spring compared with summer/autumn [11].

Endogenous DNA oxidation. As it is well characterised and easy to measure, the base oxidation product 8-oxoguanine has become the most commonly measured biomarker of oxidative damage in human cells (generally leukocytes/lymphocytes). It is measured using gas chromatography–mass spectrometry (GC–MS), or high performance liquid chromatography (HPLC) with electrochemical detection (ECD), or liquid chromatography linked to (tandem) mass spectrometry (LC–MS/MS). Alternatively, an enzymic approach can be followed, in which the lymphocyte DNA is digested with a bacterial DNA repair enzyme, formamidopyrimidine DNA glycosylase (FPG), which creates strand breaks at sites of 8-oxoguanine (as well as ring-opened purines, or formamidopyrimidines, which are also products of oxidation). The lack of complete specificity is a disadvantage of this approach. An analogous enzyme, endonuclease III, makes breaks at oxidised pyrimidines. The breaks are measured using the comet assay (single cell gel electrophoresis), alkaline unwinding or alkaline elution. It became clear in the mid-1990s that there were enormous discrepancies (up to several orders of magnitude) between estimates of background levels of 8-oxoguanine in normal human cells by different methods. GC–MS tended to give the highest values, while estimates with the enzymic approach were invariably much lower [12–14]. The European Standards Committee on Oxidative DNA Damage (ESCODD) was set up to try to resolve the discrepancies, to decide on standard protocols for measuring 8-oxoguanine, and to reach a consensus on the true basal level of DNA oxidation. In a series of papers [15–19], we reported the results of trials in which identical samples of 8-oxodGuo, oligonucleotides with defined 8-oxodGuo content, calf thymus DNA, pig liver, and cultured human cells (HeLa) were sent to the participating laboratories for analysis. It was already recognised that substantial oxidation of DNA was occurring during the derivatisation step in preparation of samples for GC–MS analysis, and protocols were revised to try to eliminate this [13]. Even so, in our trials GC–MS was unable to detect a dose-response in the samples of HeLa cells in which we had induced additional oxidation, and it is not to be recommended for biomarker studies (though it remains exceptionally useful for characterising and identifying DNA oxidation products). HPLC was very good at detecting the dose response, and 7 of 8 laboratories obtained identical slopes [19]. However, estimates of the background level of damage by HPLC varied enormously (75-fold, as judged by *y*-axis intercepts of regression lines), indicating that substantial spurious oxidation of DNA occurs during sample preparation. In contrast, the enzymic approach showed a closer agreement over the background level, and gave a median value about 7 times less that that with HPLC. In the final phase of ESCODD, we analysed oxidation levels in lymphocytes from healthy young adult male volunteers in different countries in Europe [20]. There were differences between countries, but they were not consistent between the two experimental approaches. Once again, the enzymic method gave lower estimates, by a factor of about 12. We concluded that the actual level of damage lies between 4.2 and 0.38 8-oxoguanines per 10^6 guanines (the median values of the means from different laboratories using HPLC and the comet assay, respectively). We also suggest that reports of values of 8-oxoguanine much above 4 per 10^6 should be treated with caution.

I have described the ESCODD project in detail because it probably typifies the situation with biomarkers generally. Few, if any, have been examined in such a rigorous way, but it is generally accepted that there are large discrepancies and unresolved problems of reproducibility, sensitivity, specificity and other methodological issues (for a comprehensive review, see [21]).

Ex vivo resistance of DNA to oxidative attack. If an enhanced antioxidant status in lymphocytes is maintained when they are isolated, it should result in a higher resistance to oxidative attack *in vitro*. Oxidative damage is most simply measured as strand breaks induced by treatment with H_2O_2 and the comet assay is normally used to estimate the breaks. This approach can show up antioxidant effects after a single dose [22], whereas to detect an effect on endogenous damage generally requires a longer-term supplementation of the diet.

Ex vivo resistance of blood lipids to oxidative attack. There are some reports of *in vitro* treatment of LDL-particles isolated from blood with a source of reactive oxygen (macrophages, or $CuSO_4$), resulting in increased resistance to oxidation following supplementation of the

volunteers with antioxidant-rich food, including liquorice, red wine and olive oil [23–25].

4. Human intervention trials with DNA oxidation as endpoint

In the past few years, there has been an explosion of studies in which antioxidant effects have been investigated using human subjects, and the most informative studies have been those based on oxidative DNA damage as the endpoint. Møller and Loft [26,27] have recently reviewed 41 investigations with antioxidants and antioxidant-rich diets (generally lasting two weeks or more) in which 8-oxoguanine, FPG-sensitive or endonuclease III-sensitive sites were assessed in white blood cells. Roughly half the studies reported protective effects, while the rest showed no effect. They found that study design played a significant role in whether or not a protective effect was found. The better the study design, the more likely it was to show a null effect. These reviews did not attempt to assess seriously the issue of biomarker reliability. In view of the conclusions of ESCODD, this should, I believe, be a major consideration.

Endogenous damage in the form of oxidised pyrimidines was measured by us in lymphocytes from male smokers and non-smokers taking part in a placebo-controlled 20-week intervention study with vitamin C (100 mg/day), vitamin E (α-tocopherol) (280 mg/day) and β-carotene (25 mg/day) [28]. Damage was significantly decreased in the supplemented groups. We subsequently showed a very substantial decrease in oxidised pyrimidines in subjects taking one litre of soya milk (rich in isoflavones) per day for 4 weeks, compared with control groups taking cow's milk or a rice-based drink [29]. In a staggered multiple crossover study with kiwifruit (1, 2 or 3 per day for 3 week periods, with intervening washout periods), we found significant decreases in oxidised pyrimidines and, to a lesser extent, oxidised purines (Fig. 3) [30]. Pool-Zobel and colleagues [31] in a trial with consecutive 2-week periods of supplementation with tomato, carrot and spinach (a less-than-optimal design, lacking washout periods and with no placebo group), found a decrease in oxidised pyrimidines during the carrot phase. On the other hand, there was no significant effect of a 6-week supplementation of female volunteers with the flavonoid rutin in comparison with a placebo group [32]. In a recent Danish trial, subjects (healthy young people) were randomised into three groups receiving an antioxidant-free diet with the addition of 600 g/day of fruit and vegetables, or a supplement containing equivalent micronutrients and minerals, or a placebo, for 24 days [33]. Here, too, there was no significant decrease in endogenous base damage in those taking the antioxidant-rich diet.

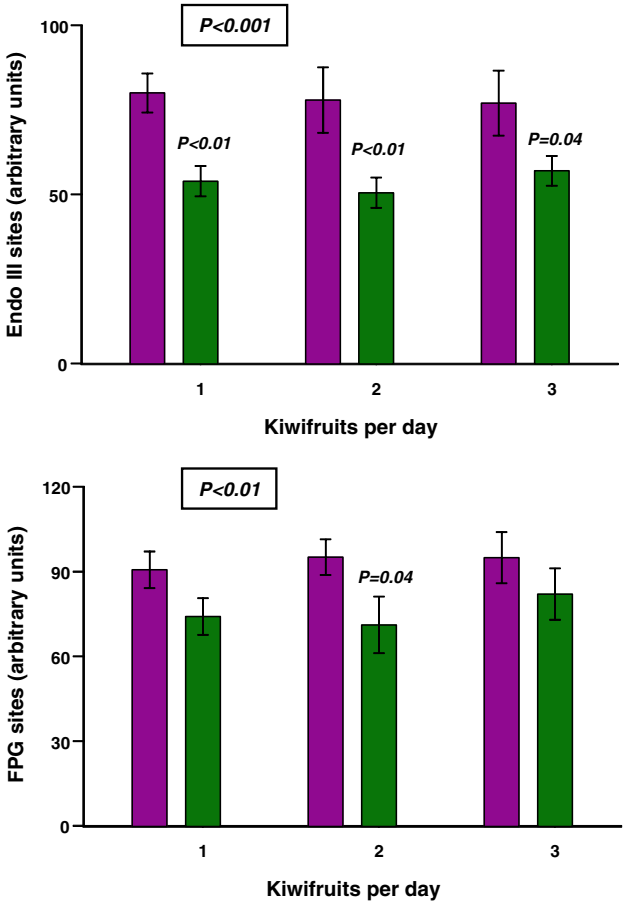

Fig. 3. Protection by kiwi fruit supplementation against endogenous DNA oxidation. Volunteers were given supplements of 1, 2 or 3 kiwi fruits per day for 3-week periods separated by 2-week washout periods. Lymphocytes were isolated before and after each supplementation period, and endogenous DNA damage – oxidised pyrimidines and oxidised purines – was measured with the use of endonuclease III and FPG, respectively, in the comet assay. From [30, pp. 513–514], by permission of Oxford University Press.

The other approach to assessing antioxidant effectiveness is to challenge lymphocytes from supplemented subjects *in vitro* with an agent that induces oxidative damage and to measure the damage that results. The first such use of the comet assay was in the early 1990s. Green *et al.* [34] showed that ionising radiation-induced damage was lower in leukocytes taken shortly after a single large dose of vitamin C than in leukocytes taken before the vitamin C was given. Using a similar protocol, we used H_2O_2 to challenge lymphocytes, isolated before and after giving subjects single doses of vitamin C (1 g), vitamin E (1 g) or β-carotene (45 mg), and found significant decreases in DNA strand breakage [22]. The greatest effects were seen at 24 h after the lipid-soluble supplements, and at shorter times (up to 4 h) after vitamin C, consistent with the known rapid uptake and clearance of vitamin C. At about this time, we were carrying out our placebo-controlled intervention trial with vitamin C, vitamin E and β-carotene in combination.

The lymphocytes isolated after 20 or more weeks of supplementation, and treated with H_2O_2, suffered significantly less damage (by about 25%) than did lymphocytes from placebo-treated subjects [28]. Porrini and Riso [35] fed female volunteers 25 g/day of tomato puree, equivalent to 7 mg/day of lycopene, for 14 days. There was a highly significant decrease in H_2O_2-induced damage to lymphocyte DNA, and lycopene, measured in plasma and also within the cells, showed an inverse correlation with DNA oxidation. A single meal of fried onions (rich in flavonoids) also resulted in a decrease in DNA breaks induced by H_2O_2 in lymphocytes [36], but a 6-week supplementation with rutin, while significantly increasing flavonoid concentrations in plasma, did not alter the resistance of lymphocytes to H_2O_2 [32]. We were able to demonstrate a protective effect of supplementing the diet with 1 or 2 kiwi fruit per day for 3 weeks [30]. However, in the recent Danish trial [33], there was no increase in resistance of lymphocyte DNA to H_2O_2 damage *in vitro*. This was also the case in a multiple crossover placebo-controlled intervention trial with single large doses of vitamin C and E (alone and in combination) [9].

To summarise these studies, and the other studies reported in the comprehensive reviews of Møller and Loft [26,27], it is hard to explain why in some intervention studies a protective effect is seen (against endogenous or *in vitro* induced damage) whereas in others there is no effect; but it is surely noteworthy that in no studies have deleterious effects been described. Populations studied inevitably differ in many respects, such as age, sex, state of health and especially basal diet. Although there is no clear indication that these factors determine whether antioxidant effects are seen or not, it has been suggested [9,27] that the best chance of success is likely when an 'at risk' population is selected – for instance, smokers, with elevated oxidative stress, or people with poor diet and marginal antioxidant status.

5. Human intervention trials with cancer as the endpoint

A couple of years before the paper by Ames appeared, Peto and colleagues [37], had examined the epidemiological evidence, from many studies, of an inverse association of cancer risk with blood retinol or with blood or dietary β-carotene, in the light of our biochemical understanding of how these compounds operate in the human body. The evidence was apparently so strong that the recommended next step was to carry out randomised, placebo-controlled intervention studies with high doses of β-carotene to determine whether cancer incidence could really be decreased by this micronutrient.

In Linxian County, in China, an area with a very high incidence of oesophageal/gastric cancer, almost 30 000 subjects aged 40–69 were randomly assigned to intervention groups to receive various combinations of retinol, zinc, riboflavin, niacin, vitamin C, molybdenum, β-carotene, vitamin E and selenium [38]. At the end of the 5-year supplementation period, significantly lower cancer mortality – resulting mainly from lower cancer rates – among those receiving supplementation with β-carotene, vitamin E and selenium was seen.

The first large-scale European trial to be completed was the Alpha-Tocopherol, Beta Carotene Cancer Prevention Study, in Finland [39]. Twenty nine thousand male smokers aged 50–69 took part in a randomised placebo-controlled trial with α-tocopherol (50 mg/day) alone, β-carotene (20 mg/day) alone, α-tocopherol and β-carotene together, or a placebo. Supplementation continued for up to 8 years, at the end of which it was found that the incidence of lung cancer was significantly higher (by 18%) in those who had received β-carotene.

In the USA, the CARET trial (β-Carotene and Retinol Efficacy Trial) looked at over 18 thousand smokers, former smokers and asbestos workers [40]. They were given 30 mg/day of β-carotene and 25 000 IU of retinol, or a placebo, for an average of 4 years. Lung cancer incidence was, again, significantly elevated (by 28%) in the supplemented group.

The Physicians' Health Study, also in the USA, tested β-carotene (50 mg on alternate days against placebo) in healthy male physicians (11 000 in each group) [41]. Almost 90% were non-smokers at the start of the study, which lasted for 12 years. Incidence of lung cancer and number of deaths from cancer did not vary significantly between the groups.

More recently, the MRC/BHF Heart Protection Study reported the results of a 5-year supplementation trial with antioxidants (600 mg vitamin E, 250 mg vitamin C, 20 mg β-carotene per day, or placebo) in over 20 000 UK adults with coronary disease, other occlusive arterial disease, or diabetes [42]. There were no significant effects of antioxidant supplementation on cancer incidence or on any other health outcome measured.

In the Suvimax study [43], 13 000 French men and women received modest daily supplementation with vitamin C (120 mg), vitamin E (30 mg), β-carotene (6 mg), selenium (100 μg) and zinc (20 mg), or a placebo, for about 7.5 years. A significant protective effect against cancer incidence was seen in men (relative risk 0.69 in the supplemented group) but not in women. The sex difference may be explained by the lower baseline antioxidant status in men.

Finally, disappointing results were found in a randomised trial with α-tocopherol as a supplement given to patients with head and neck cancer during and after radiation therapy with the aim of decreasing the incidence of second primary cancers [44]. The incidence of such cancers was higher in patients receiving α-tocopherol during the three year supplementation period (compared with the placebo group) but lower after

supplementation ceased, and overall cancer-free survival was similar in both groups after 8 years of follow-up. This and other attempts at chemoprevention were reviewed in an editorial [45].

The disappointing results of these studies have caused much heart-searching and head-scratching. It is hard to justify recommending a supplementation of the diet with antioxidants on health grounds. It is also difficult to secure ethical approval for further trials with β-carotene, which might elucidate the reasons for the paradox of the demonstrable efficacy of carotenoids (and other antioxidants) in suppressing oxidative damage, together with their lack of protection against cancer itself (and apparent enhancement of carcinogenesis, at least in groups such as smokers who are already at risk).

6. Conclusion

In view of the mixed results that the intervention trials produced, it was certainly prescient of the editor of the review by Peto *et al.* [37] to add this footnote to: 'Unwary readers (if such there are) should not take the accompanying article as a sign that the consumption of large quantities of carrots (or other major dietary sources of β-carotene) is necessarily protective against cancer, and the correlation between blood retinol and cancer avoidance is, for the time being, *sub judice*'. A quarter century later, this remains an apt comment; we still do not know how fruits and vegetables protect against cancer, but it seems increasingly unlikely that it is simply because they contain high concentrations of antioxidants. We need also to consider the effects of phytochemicals (which may or may not be antioxidants) on many other cellular functions, including cell-signalling, apoptosis, antioxidant enzymes, the phase I and II xenobiotic-metabolising enzymes, DNA repair, plus of course, the enormous potential for effects on gene expression that might have an impact on the carcinogenic process.

Conflict of interest statement

None declared.

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