

β -Carotene enhances the recovery of lymphocytes from oxidative DNA damage^{*^o}

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Epidemiological studies have revealed a strong correlation between high intake of fruit and vegetables and low incidence of certain cancers. Micronutrients present in these foods are thought to decrease free radical attack on DNA and hence protect against mutations that cause cancer, but the fine details of the causal mechanism have still to be elucidated. Whether dietary factors can modulate DNA repair – a crucial element in the avoidance of carcinogenesis – is an intriguing question that has not yet been satisfactorily answered. In order to investigate the effects of β -carotene on oxidative damage and its repair, volunteers were given a single 45 mg dose and lymphocytes taken before and after the supplement were treated *in vitro* with H₂O₂. DNA strand breaks and oxidised pyrimidines were measured at intervals, to monitor the removal of oxidative DNA damage. We found inter-individual variations in response. In cases where the baseline plasma β -carotene concentration was high, or where supplementation increased the plasma concentration, recovery from oxidative damage (i.e. removal of both oxidised pyrimidines and strand breaks) was relatively rapid. However, what seems to be an enhancement of repair might in fact represent an amelioration of the continuing oxidative stress encountered by the lymphocytes under *in vitro* culture conditions. We found that culture in a 5% oxygen atmosphere enhanced recovery of lymphocytes from H₂O₂ damage.

It has been recognised for many years that the common fatal cancers occur in large part as a result of life-style and other environ-

mental factors and are in principle preventable [1]. Among these environmental factors, dietary practices are the most promising area

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Abbreviations: SCGE, single cell gel electrophoresis; HPLC, high performance liquid chromatography; RPMI, Roswell Park Memorial Institute; DAPI, 4,6-diamidino-2-phenylindole; PBS, phosphate-buffered saline.

to explore. Low dietary intake of fruit and vegetables is associated with an increased risk of many types of cancer, and the apparent protective effect of fruit and vegetables is attributed at least in part to the presence of antioxidants such as vitamins C and E and carotenoids that prevent reactive oxygen species from damaging DNA [2-7]. In a molecular epidemiological study, we recently showed that supplementation of the diet for 20 weeks with vitamin C, vitamin E and β -carotene led to a significant decrease in endogenous oxidative base damage (a biomarker of cancer risk) in lymphocyte DNA, and also rendered lymphocytes more resistant to oxidative damage *in vitro* [8]. Thus there may be a major opportunity for influencing the incidence of cancer by altering diet or, more specifically, by increasing antioxidant uptake. However, direct intervention trials have tended to show, if anything, an increase in cancer incidence in groups receiving β -carotene in comparison with unsupplemented groups [9, 10].

Clearly, more work is required on the fundamental mechanisms of action of β -carotene (and other dietary antioxidants), and we focus here on *ex vivo* experiments in which lymphocytes, from blood collected before or after administration of a single dose of β -carotene, were challenged with H_2O_2 and incubated *in vitro* for up to 24 h; DNA strand breaks and oxidised pyrimidines were monitored using the comet assay, or single cell gel electrophoresis (SCGE). The comet assay has been used previously to assess oxidative damage in human lymphocytes, and the protective effects of dietary antioxidants [8]. We have also previously reported the rather slow kinetics of repair of H_2O_2 -induced damage in peripheral human lymphocytes in culture, compared with proliferating transformed cells [11]. We have now, for the first time, tried systematically to modulate the *in vitro* DNA repair/recovery process by *in vivo* supplementation.

MATERIALS AND METHODS

Supplementation and sampling

Four adult staff members of the laboratory (1 male, 3 female), gave their informed consent to participate in the study. Ages were between 20 and 50 (mean 29), and body mass index varied from 18.5 to 22.4 kg/m² (mean 20.3 kg/m²). All were non-smokers; none was on a particular diet (e.g. vegetarian), on medication, vitamins or mineral supplement or had an acute or chronic illness. Participants received 3 capsules of 15 mg β -carotene (Boots Company plc, Nottingham, U.K.) with water, with a 'wash-out' period of at least one week before repeating an experiment. The study was approved by the Joint Ethical Committee of the Grampian Health Board and the University of Aberdeen.

Non-fasting blood was obtained by antecubital venepuncture before and 24 h after β -carotene supplementation. One ml of the 10 ml sample (with EDTA as anti-coagulant) was taken for the comet assay and the rest was used for the HPLC analysis of plasma antioxidants. In some experiments finger-prick samples of blood were taken for the comet assay.

Comet assay

Thirty μ l of whole blood from venepuncture or finger-prick was mixed with 1 ml of RPMI 1640 medium supplemented with 10% foetal calf serum and kept on ice for 30 min. Lymphocytes were separated by centrifugation over 100 μ l of Histopaque 1077 at 200 $\times g$ for 3 min at 4°C; the cells recovered from above the Histopaque layer were 95% mononuclear cells [11].

DNA breaks were detected using an adaptation of the single cell gel electrophoresis method of Singh *et al.* [12] as described previously [13]. In brief, cells were suspended in 85

μ l of 1% low melting point agarose (Gibco BRL, Paisley, U.K.), and pipetted onto a layer of 85 μ l of 1% normal melting point agarose (Gibco BRL) on a frosted microscope slide; the slides were immersed in lysis solution (2.5 M NaCl, 0.1 M Na₂EDTA, 10 mM Tris/HCl, pH 10, and 1% Triton X-100) at 4°C for 1 h to remove cellular proteins. Slides were then placed in 0.3 M NaOH, 1 mM Na₂EDTA for 40 min, before electrophoresis at 25 V for 30 min at an ambient temperature of 4°C. The slides were then immersed 3 times (5 min each) in 0.4 M Tris/HCl, pH 7.5, before staining with 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI).

As described, the comet assay detects strand breaks in DNA. The assay can be modified to expose oxidative base damage, by converting oxidised pyrimidines to strand breaks using the bacterial enzyme endonuclease III [14]; after lysis, the slides were washed 3 times for 5 min each in endonuclease buffer (40 mM HEPES/KOH, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin, pH 8.0); the agarose was covered with either buffer or endonuclease III in buffer, beneath a cover-slip, and incubated for 45 min at 37°C. Subsequent steps (NaOH treatment, electrophoresis and staining) were as described above.

DAPI-stained comets were examined with a Zeiss Axioskop fluorescence microscope. One hundred comets on each slide were scored visually as belonging to one of five classes according to tail intensity and given a value of 0, 1, 2, 3 or 4 (from undamaged 0 to maximally damaged 4). Thus, the total score for 100 comets could range from 0 (all undamaged) to 400 (all maximally damaged). This visual scoring was confirmed by computerised image analysis (Komet 2.2, from Kinetic Imaging Ltd., Liverpool, U.K.).

The comet assay was calibrated using X-rays [15] so that results could be expressed in terms of DNA breaks per 10⁹ Da of DNA.

H₂O₂ damage and repair in lymphocytes

Human lymphocytes recovered from the Histopaque gradient were washed with 1 ml of PBS, pH 7.4, before exposure to 100 μ M H₂O₂ for 5 min on ice. Treatment on ice minimises the possibility of cellular processing of damage during the exposure period. The cells were then centrifuged at 200 \times g for 3 min at 4°C. To examine repair of DNA damage, cells were incubated in petri dishes at 37°C in sterile RPMI growth medium, for various times up to 24 h. After incubation, cells were centrifuged, resuspended in low melting point agarose, set in gel on the slide and lysed. Strand breaks and oxidised pyrimidines were estimated by the comet assay.

Incubation under low O₂ atmosphere

After H₂O₂ treatment, lymphocytes were incubated at 37°C in an airtight moist box gassed with a low O₂ mixture (5% CO₂, 5% O₂, 90% N₂). At regular intervals, samples were removed for the comet assay and the box was regassed. For comparison, cells from the same finger prick were incubated in a 'normal' atmosphere (with 5% CO₂).

HPLC Analysis of plasma carotenoids

Heparinised whole blood was centrifuged at 1700 \times g, 4°C for 15 min and aliquots were immediately snap frozen and stored at -80°C. All plasma was prepared within half an hour of blood collection.

Carotenoids were analysed by a method adapted from Hart & Scott [16]. An aliquot of freshly thawed plasma was added to aqueous sodium dodecyl sulphate and ethanol, containing an internal standard (tocopherol acetate). This solution was extracted with hexane, the hexane removed and dried down. The dried extract was redissolved. Retinol, α -tocopherol, α - and β -carotene, lycopene, β -crypto-

xanthin, lutein and zeaxanthin were simultaneously separated by reversed phase HPLC. A mixed carotenoid solution was run as external standard. Carotenoids were measured at 450 nm.

RESULTS

Recovery of lymphocytes from damage *in vitro*

After a 5 min treatment with 100 μ M H₂O₂ on ice, to introduce oxidative damage to DNA, lymphocytes were incubated for up to 24 h. The comet assay, applied to samples at intervals, allowed us to follow the occurrence of strand breaks and oxidised pyrimidines and their repair (Fig. 1). Strand breaks were rejoined slowly but by 24 h the process was complete. However, the difference between strand breaks, and strand breaks plus endonuclease III-sensitive sites (i.e. oxidised pyrimidines) was maintained throughout this period, indicating that essentially no base repair had taken place. In addition, it is noticeable that in the case of control cells, not treated with H₂O₂, there is a slight but significant increase in strand breaks and oxidised bases after 6 h of *in vitro* culture ($P < 0.02$ by the two-tailed paired sample *t*-test).

β -Carotene supplementation

Recovery from H₂O₂ treatment *in vitro* was followed in samples of lymphocytes taken just before and 24 h after supplementation with a single dose of 45 mg of β -carotene. Incubation *in vitro* continued for 24 h after H₂O₂ treatment. β -Carotene plasma concentrations were not at first available, but on the basis of the DNA damage recovery experiments the samples were divided into two groups; a set of four experiments in which rejoining of strand breaks over the first 6 h of incubation was significantly faster in lymphocytes taken after supplementation than in those isolated before ($P < 0.01$, one-tailed paired sample *t*-test); and a pair of samples which showed no such effect (Fig. 2). β -Carotene levels were subsequently determined and are shown in Fig. 3. It is striking that the four samples that respond to supplement with an enhanced removal of DNA damage also show an increase in plasma β -carotene. The other two samples, from two separate individuals, show no significant effect of β -carotene supplement on plasma concentration (which is already high in each case), and no effect on rate of recovery from damage. The extent of removal of damage is, however, already noticeably higher in these two samples (Fig. 2). Thus, rapid recovery from oxidative damage correlates with an elevated plasma β -carotene concentration.

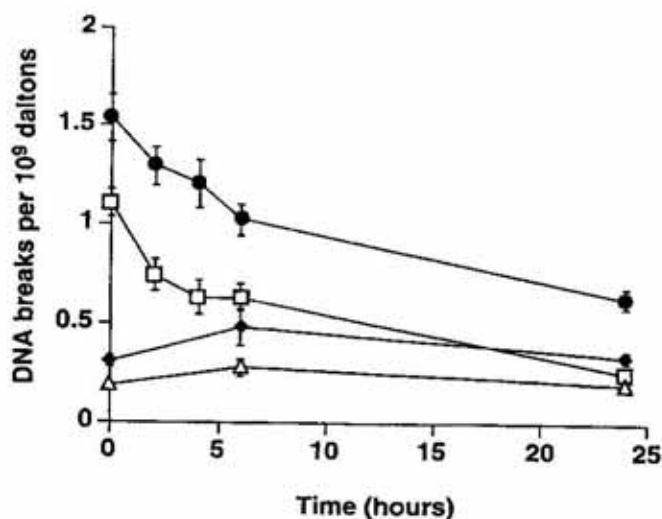


Figure 1. Recovery from oxidative DNA damage in human lymphocytes.

Lymphocytes were incubated for 24 h at 37°C after H₂O₂ treatment (100 μ M for 5 min on ice; □, ●). Control cultures (△, ◆) were not treated with H₂O₂. The comet assay was performed without or with endonuclease III to estimate strand breaks (open symbols) or strand breaks plus oxidised pyrimidines (solid symbols), respectively. Mean values from 20 experiments are shown, with S.E.

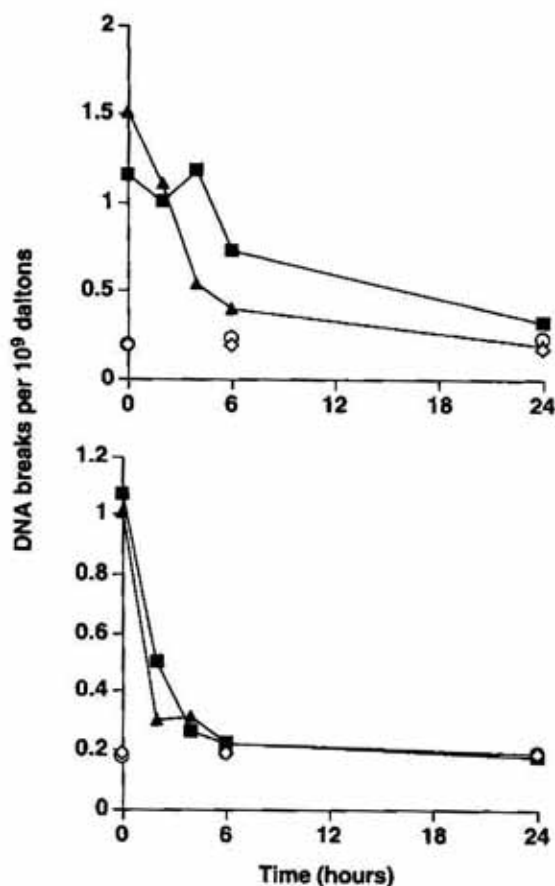


Figure 2. Effect of β -carotene *in vivo* on recovery from oxidative DNA damage *in vitro*.

Lymphocytes from blood taken before (■) or 24 h after (▲) β -carotene supplementation were treated with H₂O₂ and incubated for 24 h. Controls (○, before and ◇, after β -carotene) were incubated without H₂O₂ treatment. Strand breaks and strand breaks plus oxidised pyrimidines were measured by the comet assay. Upper graph: mean values from four experiments in which enhancement of recovery is seen (individuals F and S in Fig. 3). Lower graph: mean values from two experiments in which no enhancement was seen (individuals A and E in Fig. 3).

Effect of oxygen concentration

In view of the observed increase in oxidative damage in the DNA of untreated lymphocytes on incubation (Fig. 1), we investigated the possibility of significant oxidant stress under *in vitro* culture conditions by incubating lymphocytes in an atmosphere of only 5% oxygen (with 5% CO₂). For comparison, lymphocytes isolated from the same sample of blood were

incubated in the normal atmosphere (air plus 5% CO₂). Figure 4 shows that recovery from H₂O₂-induced strand breaks is faster in the cells exposed to a lower oxygen concentration.

DISCUSSION

In spite of antioxidant protection against the action of free radicals within cells, DNA damage still occurs and most of it is dealt with by cellular repair processes. However, if damage remains unrepaired when cells come to replicate their DNA, it may be fixed as a permanent sequence change, i.e. mutation. Mutations in appropriate genes can ultimately produce cancer, and cellular repair of DNA damage before it becomes fixed is therefore of crucial importance in controlling carcinogenesis. The extent of inter-individual variations in normal human repair capacity is not yet known; nor is it clear which, if any, nutritional factors may influence DNA repair. We have investigated these questions by giving human volunteers a single large dose of β -carotene, and examining lymphocytes collected before and a suitable time after supplementation for their ability to recover from *in vitro* H₂O₂ treatment.

In any human nutritional trials, the problem of individual variability has to be confronted. Antioxidant levels in plasma vary according to dietary intake; the effect of a supplement of antioxidant depends on individual uptake and absorption characteristics. Therefore, in this investigation of possible modulating effects of an antioxidant supplement on the behaviour of lymphocytes *ex vivo*, it was essential to monitor plasma antioxidant levels. Baseline plasma concentrations of β -carotene in this group of volunteers, ranging from approximately 100 to 700 ng/ml, compare with the local average of 290 ng/ml [8]. Those volunteers who took part in the experiment more than once showed very similar concentrations on the different occasions. The two individuals with already high β -carotene levels showed no

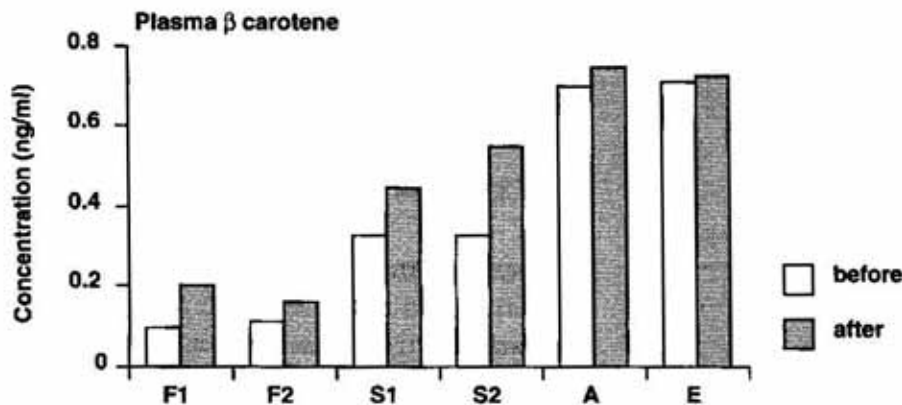


Figure 3. Plasma concentrations of β -carotene before (open bars) and after (shaded bars) supplementation.

Letters represent individuals; '1' and '2' indicate experiments on different occasions.

effect of supplementation, while in other samples the concentration was between 36% and 105% higher after supplementation.

We have previously reported [8] that a supplement of vitamin C, β -carotene and vitamin E (given over 20 weeks) decreases endogenous oxidative damage (oxidised pyrimidines) in lymphocyte DNA and increases resistance to oxidative damage induced *in vitro* by H_2O_2 .

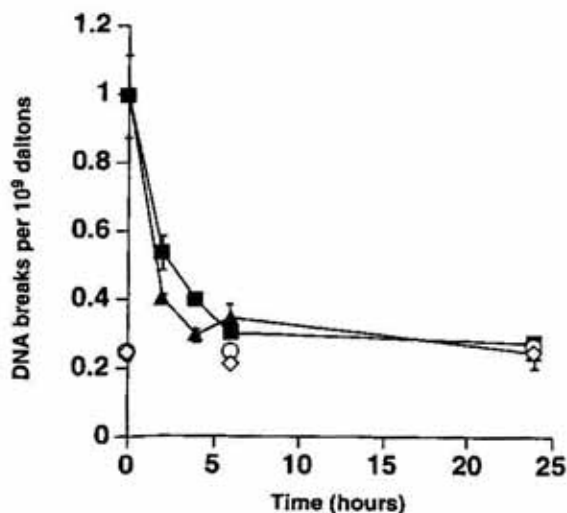


Figure 4. Effect of decreased oxygen concentration on recovery from oxidative damage.

Lymphocytes were treated with $100 \mu M H_2O_2$ and incubated either in air (with 5% CO_2), ■, or in a mixture of 5% oxygen, 5% CO_2 and nitrogen (▲). Corresponding controls (not treated with H_2O_2) are, respectively, ○ and ◇. Mean values are shown from two experiments; other experiments have shown qualitatively similar results.

These effects are consistent with the action of the micronutrients as antioxidants.

Turning to the kinetics of recovery of lymphocytes from oxidative damage *in vitro*, the combined results from numerous incubations of H_2O_2 -treated lymphocytes isolated before supplement administration show, as expected, only a slow decrease in the frequency of DNA breaks (detected with the comet assay) and apparently no repair at all of oxidised pyrimidines. In a series of experiments, shown in full elsewhere [17], recovery from H_2O_2 treatment *in vitro* was followed in samples of lymphocytes taken just before and 2 h after ingestion of a 1 g dose of vitamin C. Plasma concentrations of vitamin C were measured in the samples used to obtain the lymphocytes. In general, no significant difference was seen in the rate of removal of strand breaks or of oxidised pyrimidines between lymphocytes taken before and after vitamin C supplementation, and the supplementation had a modest effect on plasma concentration. However, in the two samples which showed the greatest rise in plasma vitamin C, the lymphocytes showed a markedly enhanced rate of removal of strand breaks, from 8% removal in 6 h before supplementation to 81% removal in 6 h after supplementation (mean values). The improvement in removal rate was statistically significant ($P < 0.01$).

With the individual data on β -carotene levels and responses to supplementation reported

here, we can see a striking influence of β -carotene on the recovery from H_2O_2 damage. Pre-existing high levels of β -carotene in the plasma of two subjects are associated with relatively rapid removal of strand breaks and oxidised pyrimidines, while the other subjects show slower removal rates but an enhanced recovery on supplementation.

There are at least two ways to interpret the effects of vitamin C and β -carotene on recovery from H_2O_2 damage. It might be that repair enzyme activity is stimulated by these two unrelated xenobiotics. Perhaps more likely is an explanation in terms of antioxidants. As has been remarked, untreated lymphocytes seem to accumulate oxidative damage over the first few hours in culture. Oxidative stress resulting from the sudden exposure to high oxygen tension can account for this, and also for the apparent slow removal of H_2O_2 -induced damage, which may in fact be the result of an input of 'new' damage (from atmospheric oxygen) rather than slow repair of the H_2O_2 -induced damage. The ability of vitamin C and β -carotene to promote recovery, then, is simply a protection against further oxidation.

The experiment done under 5% oxygen supports this interpretation; recovery is faster than under standard atmospheric conditions, suggesting, again, that a decreased amount of 'new' damage gives the impression of faster repair. Exposure to a hyperoxic environment is known to lead to toxic effects in the organism, probably caused by reactive oxygen species, and it is recognised that standard *in vitro* culture conditions with atmospheric oxygen represent a non-physiological, hyperoxic environment for most cell-types (the exception being cells from lung tissue) [18]. It is therefore not surprising that freshly isolated lymphocytes appear to suffer oxidative damage during *in vitro* incubation.

It may be meaningless to try to estimate the real repair ability of lymphocytes by incubating them *in vitro*, since it is probably impossible to recreate the conditions pertaining *in vivo*. An alternative approach is needed, and

we have developed a method, also based on the comet assay, which allows an assessment of repair capacity in a simple cell extract, provided with a DNA substrate (gel-embedded nucleoids) carrying specific kinds of damage (A.R. Collins, M. Somorovská, M. Dušinská and R. Štětina, in preparation). This is independent of antioxidant status or oxidative stress and should permit an answer to the questions of whether repair is modulated by vitamin C, β -carotene or other micro- or non-nutrient compounds. Meanwhile, we can at least say that, by whatever mechanism, vitamin C and β -carotene given *in vivo* can afford protection against effects of active oxygen *in vitro* and presumably also *in vivo*.

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