Free Rad. Res., Vol. 27(5), pp. 533–537
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Ex vivo Assessment of Lymphocyte Antioxidant Status Using the Comet Assay

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Accepted by Prof. B. Halliwell

(Received 10 Dec 1996; In revised form 19 February 1997)

Lymphocytes were isolated from volunteers before and after receiving a single supplement of vitamin C, vitamin E or β-carotene. The lymphocytes were treated with H_2O_2, and DNA strand breaks were measured by single cell gel electrophoresis (the comet assay). Significant protection against oxidative DNA damage was evident 2–4 h after vitamin C intake, and 18–24 h after consumption of the other antioxidants. Lymphocytes from smokers were more sensitive to DNA damage than those from non-smokers, and they showed at least as great a protective effect with antioxidants.

Keywords: DNA damage, human lymphocytes, comet assay, dietary antioxidants

INTRODUCTION

Epidemiological evidence that dietary antioxidants can protect against certain cancers[1] has recently been substantiated by the results of intervention studies based on the use of a biomarker for oxidative DNA damage. Antioxidants such as vitamin C, vitamin E, carotenoids etc. are thought to protect by quenching endogenous or exogenous reactive oxygen species before they are able to damage the DNA. Using single cell gel electrophoresis (modified to permit the quantitation of oxidised pyrimidines),[2] we found a very significant decrease in endogenous oxidative damage in the lymphocytes of men who had received a daily supplement of vitamin C, vitamin E and β-carotene when compared with the damage in lymphocytes from a placebo group.[3] In that study, we also investigated the resistance of lymphocytes to DNA damage (strand breaks) induced *in vitro* by incubation with H_2O_2. Samples taken at the end of the 20-week trial were subjected to this test, and supplementation was associated with a very significant decrease in damage. This *ex vivo* test for resistance was based on experiments reported by Green *et al.*,[4] in which resistance to ionising radiation damage was found to be greater in lymphocytes collected shortly after a single large dose of vitamin C than in lymphocytes from the same subjects taken just before the vitamin C was administered. A short-

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term bioassay for resistance to oxidative damage would be a valuable tool for investigating putative dietary antioxidants, and so we have now looked in detail at the extent and time course of the protective effect that follows single doses of vitamin C, vitamin E and β-carotene. In addition, we have compared the response of lymphocytes from smokers and non-smokers.

MATERIALS AND METHODS

Subject Selection

Volunteers were aged between 20 and 32, free of acute or chronic illness and not taking medication, vitamins or other supplements; none was vegetarian. All lived in the city of Aberdeen and were of similar socio-economic backgrounds. There were six non-smokers (3 male, 3 female) and six smokers (4 male, 2 female).

The study was approved by the Joint Ethical Committee of the Grampian Health Board and the University of Aberdeen.

Supplementation and Sampling

Volunteers received a single dose of 1 g of vitamin C (from Boots, Nottingham, UK), followed by a 1-week "wash-out" period, then a 1 g dose of "Natural source vitamin E" (Dα-tocopherol) (Boots) with a further 1-week interval before the final supplement of 45 mg of natural β-carotene (from *Dunaliella)* (Boots). Finger-prick blood samples were taken before and at prescribed times after supplementation. Lymphocytes were isolated by sedimentation in a density gradient as described previously.[3]

Comet Assay (Single Cell Gel Electrophoresis)

The comet assay was based on the method of Singh *et al.*[5] A layer of 85 μl 1% standard agarose (Gibco BRL) in PBS (phosphate-buffered saline) was set on a fully frosted microscope slide, and overlaid with 85 μl of 1% low melting point agarose (Gibco BRL) in PBS, in which approximately 2×10^4 cells were suspended at 37°C. The cells were lysed by immersing the slides in 2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris-HCl pH 10, 1% Triton X-100 at 4°C for 1 h; this treatment leaves residual nuclei, or nucleoids, embedded in the gel. They were then placed in an electrophoresis tank in 0.3 M NaOH, 1 mM Na_2EDTA for 40 min. Electro-phoresis was carried out at 25 V and approximately 300 mA, for 30 min, at an ambient temperature of 4°C. After staining with DAPI (4,6-diamidino-2-phenylindole), each slide was viewed by fluorescence microscopy and the degree of damage in the nucleoids was assessed visually. Each of 100 nucleoids, or comets, was assigned a score, from 0 to 4, depending on the fraction of DNA pulled out into the "tail" under the influence of the electric field. The overall score for each slide was therefore between 0 (undamaged) and 400 (maximally damaged). The arbitrary units assigned by visual scoring are closely related to the tail intensity as measured by computer image analysis.[6]

Statistical Analysis

A paired t-test (two tails) was used to assess the significance of effects of supplementation at each concentration of H_2O_2 within the different groups. When comparing effects between smokers and non-smokers, an unpaired t-test with unequal variances was applied. Intraindividual variability was investigated by analysis of variance (F-test). The statistical package of Excel 5.0 (Microsoft Corporation) was used for these tests.

RESULTS

Preliminary experiments were carried out to establish suitable doses of antioxidant and to optimise the sampling times. A volunteer was given a single dose of 1 g of vitamin C. Blood samples were taken just before and at intervals up to 6 h after the administration. Lymphocytes were isolated, suspended in PBS, and incubated on ice for 5 min with

150 μM H_2O_2. Figure 1 indicates that significantly less damage was induced in lymphocytes taken after vitamin C administration, a minimum being reached after 2–4 h. Similar experiments were carried out on the same individual given a supplement of either 1 g of vitamin E or 45 mg of β-carotene. Since neither of these antioxidants is water-soluble, uptake was expected to be slower than that of vitamin C, and so samples were taken over a longer period, up to 24 h. A significant protective effect, in each case, was seen at the later times of sampling (Fig. 1).

Since it is possible that, in the period of the vitamin C supplementation trial, breakfast itself is protective, a control experiment was carried out, without any supplements, taking samples during the first part of the day with breakfast (cereal, glass of orange, bread rolls, omelette) taken as normal or omitted. Results indicate a small, significant effect of breakfast, but no significant variation with time of sampling (Fig. 1). The magnitude of the effect of breakfast is clearly much less than that of vitamin C.

Having established optimal sampling times (2 h after vitamin C, 24 h after vitamin E or β-carotine), we proceeded with the main study, in which the responses of lymphocytes to different doses of H_2O_2 were compared before and after supplementation, and between smokers and non-smokers.

Six non-smokers and six smokers were given, in sequence, 1 g of vitamin C, 1 g of vitamin E, and 45 mg of β-carotene, with intervening wash-out periods of 1 week. Before and after each supplement, blood samples were taken, lymphocytes isolated and treated *in vitro* with H_2O_2 (72 μM and 240μM). In both groups, there is a significant protective effect of vitamin C, at both concentrations of H_2O_2 (Table I). This is true also for vitamin E (Table II) and for β-carotene (Table III).

The levels of DNA damage induced (in cells isolated before supplementation) by 72μM and 240 μM H_2O_2 were significantly less in non-smokers than in smokers ($P < 0.05$ in each case). Endogenous damage (i.e. strand breakage in the absence of H_2O_2) was not significantly different.

DISCUSSION

The ability of vitamin C, given as a single large dose, to protect lymphocytes against DNA dam-

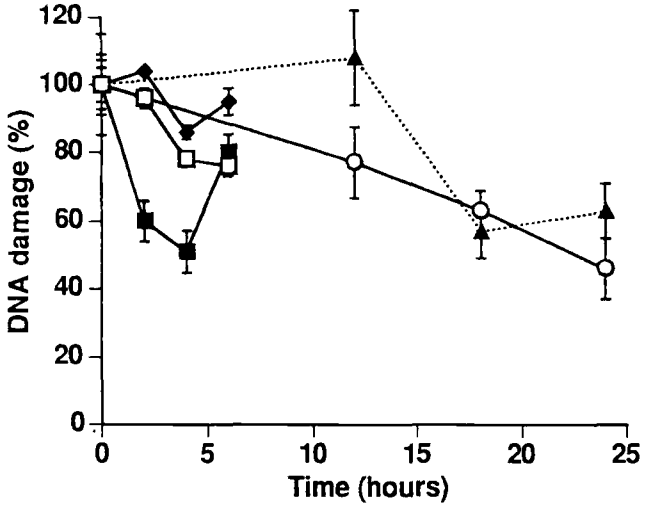

FIGURE 1 DNA damage (strand breaks) measured by SCGE in lymphocytes isolated at intervals after a single dose of 1 g of vitamin C (■), 1 g of vitamin E (○), or 45 mg of β-carotene (▲), or at corresponding times of day with (□) or without (♦) breakfast. Mean values are shown from two experiments, expressed as the % of the damage seen in samples taken at t_0. Bars indicate the range of duplicates.

TABLE I Effects of a single dose of 1 g of vitamin C on *ex vivo* resistance of lymphocytes to treatment with H_2O_2. Blood samples were taken before and 2 h after vitamin C administration. Damage to DNA is shown in arbitrary units obtained by visual estimation of 100 comets. S.E. of means are indicated, as well as the % decrease in induced damage after supplementation. These decreases are significant at $P < 0.05$.

H_2O_2 concentration (μM)	DNA damage (arbitrary units)			
	Non-smokers		Smokers	
	Before	After	Before	After
0	35 ± 1.7	34 ± 1.6	36 ± 1.6	34 ± 0.9
72	101 ± 3.5	75 ± 6.6 (26%)	130 ± 2.4	103 ± 6.4 (21%)
240	138 ± 6.4	117 ± 7.3 (15%)	198 ± 3.0	156 ± 5.2 (21%)

age induced by ionising radiation, was demonstrated by Green *et al.*[6] Since radiation and H_2O_2 both act via free radical attack on DNA, it is not surprising that vitamin C is also effective at decreasing the damage induced in lymphocytes by H_2O_2. A decrease in DNA strand breakage of around 20% is consistently seen in the different volunteer groups and at different concentrations of H_2O_2. Increasing the dose of vitamin C to 3 g increased the degree of protection (results not shown). The effect of vitamin C is seen very soon after treatment, and it is possible that breakfast might be interfering and having a protectant effect in addition to that of the supplement. As we show, however, the effect is only a small one. In fact, this breakfast test serves as a negative control for the supplementation experiments.

Any effect of breakfast can safely be ignored when assessing the other agents, vitamin E and β-carotene, since they act over a longer time course, and both samples (pre- and post-supplement) were taken at the same time of day. Their effect, at the doses used, is as great as that of 1 g of vitamin C. A higher dose of vitamin E (3 g) or of β-carotene (60 mg) did not further enhance resistance to damage (results not shown).

The H_2O_2 responses of different individuals, sampled before supplementation on two or three occasions during the whole trial, were examined statistically. No significant intra-individual variation was seen, indicating that sufficient time was allowed in the wash-out period.

Strand breaks are a major species of oxidative DNA damage, and so are a valid indicator of oxidative damage incurred by H_2O_2 *in vitro*. However, the interpretation of the low level of strand breaks detected (by the comet assay) in control lymphocytes that have not been treated with H_2O_2 is impossible. DNA breaks may reflect endogenous oxidative damage (by reactive oxygen released during respiration); but such breaks are short-lived, as repair is rapid. Apurinic and apyrimidinic (or AP) sites occur spontaneously[7] and will be seen as strand breaks in the comet assay as they are alkali-labile. Both AP sites and strand breaks occur as intermediates in the cellu-

TABLE II Effects of a single dose of 1 g of vitamin E on *ex vivo* resistance of lymphocytes to treatment with H_2O_2. Blood samples were taken before and 24 h after vitamin E administration. Damage to DNA is shown in arbitrary units obtained by visual estimation of 100 comets. S.E. of means are indicated, as well as the % decrease in induced damage after supplementation. These decreases are significant at $P < 0.05$.

H_2O_2 concentration (μM)	DNA damage (arbitrary units)			
	Non-smokers		Smokers	
	Before	After	Before	After
0	37 ± 1.4	35 ± 1.5	37 ± 2.0	35 ± 2.0
72	99 ± 3.5	80 ± 3.1 (19%)	131 ± 4.2	74 ± 8.1 (44%)
240	138 ± 6.6	119 ± 7.0 (14%)	197 ± 8.1	142 ± 7.3 (28%)

TABLE III Effects of a single dose of 45 mg of β-carotene on *ex vivo* resistance of lymphocytes to treatment with H_2O_2. Blood samples were taken before and 24 h after β-carotene administration. Damage to DNA is shown in arbitrary units obtained by visual estimation of 100 comets. S.E. of means are indicated, as well as the % decrease in induced damage after supplementation. These decreases are significant at $P < 0.05$.

H_2O_2 concentration (μM)	DNA damage (arbitrary units)			
	Non-smokers		Smokers	
	Before	After	Before	After
0	36 ± 1.9	35 ± 1.9	37 ± 1.3	36 ± 1.6
72	97 ± 5.2	75 ± 5.6 (23%)	135 ± 4.5	79 ± 4.0 (44%)
240	136 ± 5.6	113 ± 2.2 (17%)	204 ± 7.4	150 ± 3.9 (26%)

lar processing of a variety of DNA lesions, via either base excision repair or nucleotide excision repair. So it is uncertain whether DNA breaks in untreated cells represent DNA damage or DNA repair.

In several studies, plasma antioxidant levels have been shown to be relatively low in smokers compared with non-smokers, reflecting either lower dietary intake, or higher turnover as a result of smoking-induced oxidative stress.[8,9] It is therefore not surprising that their lymphocytes show more DNA damage when challenged with H_2O_2 *in vitro*. The relative protective effects of the supplements of vitamin E and β-carotene are more substantial in smokers than in non-smokers (see Tables), and at low H_2O_2 doses this brings the amount of induced damage in smokers and non-smokers down to the same level. However, with a more severe challenge (from the higher H_2O_2 dose), smokers still incur more damage. Vitamin C, on the other hand, seems to be equally effective in smokers and non-smokers at high and low H_2O_2 concentrations.

The main conclusion of this investigation is that the three major dietary antioxidants, vitamin C, vitamin E and β-carotene, have a demonstrable, short-term protective effect against oxidative DNA damage, which is widely regarded as an important element in the aetiology of cancer. The comet assay, used to assess *ex vivo* resistance to DNA-damaging agents, is a powerful experimental tool which can be applied in investigating different possible cancer-protective components of the diet.

Acknowledgments

We are grateful for the personal support to MP from Nutricia Foundation, and to the Scottish Office Agriculture, Environment and Fisheries Department. We thank Catherine Gedik for her valuable advice.

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