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ORIGINAL CONTRIBUTION

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Recovery of human lymphocytes from oxidative DNA damage; the apparent enhancement of DNA repair by carotenoids is probably simply an antioxidant effect

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Summary *Background*: Many epidemiological studies have identified a protection against cancer associated with consumption of fruit and vegetables. One factor in this protection may be the enhancement of cellular DNA repair activity by micronutrients, such as carotenoids, found in these foods.

Aims of the study: To measure the capacity of lymphocytes isolated from volunteers supplemented with β-carotene, lutein or lycopene to recover from DNA damage induced *in vitro* by treatment with H_2O_2.

Methods: Healthy volunteers were given supplements of lutein (15 mg/day), lycopene (15 mg/day) and β-carotene (15 mg/day), each for 1 week, the supplementation periods being separated by 3-week wash-out periods. Blood samples were taken at the beginning and end of each supplementation, and at 1 week and 3 weeks during the wash-out period. Carotenoid levels were measured in plasma. Lymphocytes were isolated and frozen. Subsequently, they were treated with 100 μM H_2O_2 and incubated for up to 24 h; DNA damage was measured with the comet assay (single cell gel electrophoresis) after 0, 2, 4, 8 and 24 h.

Results: Increases of 2- to 3-fold in mean plasma lutein and β-carotene concentrations were seen at the end of the respective supplementation periods; they returned virtually to basal levels after wash-out. Lycopene concentrations were less affected by supplementation, and were more variable. H_2O_2-induced DNA strand breaks were apparently only slowly rejoined by the lymphocytes. The rejoining of breaks in the first few hours appeared substantially faster in lymphocytes following supplementation with β-carotene, but no such effect was seen with lutein. In those individuals who showed increases in lycopene concentrations, the recovery was significantly faster. Lymphocytes that were not treated with H_2O_2 showed a transient increase in DNA breakage to about double the background level in 2 h, presumably as a result of exposure to atmospheric oxygen; this effect, too, was relieved by supplementation with lycopene or β-carotene.

Conclusions: While certain carotenoids appear to enhance recovery from oxidative damage, this is probably in fact an antioxidant protective effect against additional damage induced by atmospheric oxygen, rather than a stimulation of DNA repair.

Key words Carotenoids – oxidative DNA damage – DNA repair – comet assay

Introduction

Oxidative damage to DNA has been suggested as a likely contributor to cancer aetiology [1]. Oxidised bases have been detected in significant amounts in the DNA of normal human cells [2], arising probably from attack by ·OH radicals present as a by-product of normal respiration. The amount of damage occurring is limited by the intrinsic antioxidant defences, which include thiol reagents such as glutathione, and the enzymes catalase, superoxide dismutase, glutathione peroxidase etc. This protection may be augmented with antioxidants of dietary origin. There are now various studies which show that supplementation with vitamin C, vitamin E, carotenoids or flavonoids, or with foods rich in these substances leads to a decrease in the steady-state level of DNA oxidation in lymphocytes [3–5]. The damage that does occur is mostly removed by enzymes of the cellular base excision repair pathway [6].

It is clear that individual variations in intrinsic DNA repair capacity would have a bearing on cancer risk, and there are extreme cases of rare diseases where a specific defect in a DNA repair pathway is associated with a greatly elevated risk of cancer. The best known of these is xeroderma pigmentosum; defective nucleotide excision repair results in very high incidence of skin cancer caused by deficient repair of cyclobutane pyrimidine dimers induced by UV light [7]. In the normal population, polymorphism of repair genes may give rise to more subtle loss of repair capacity, revealed in inter-individual variations in the cellular response to DNA damage. In addition, there may be significant intra-individual variation in DNA repair reflecting metabolic effects or envionmental modulation. Little is known of the extent of such variation, largely because of the lack of a suitable assay for repair in lymphocytes, the only cell type readily available for investigating this biomarker. We have used the comet assay (single cell gel electrophoresis) to study the ability of lymphocytes, incubated *in vitro*, to remove the oxidative DNA damage induced by H_2O_2. Compared with cultured cells such as HeLa, lymphocytes seem to be very slow to repair both DNA strand breaks and oxidised bases [8].

There are some hints that antioxidant micronutrients might influence the recovery of cells from oxidative DNA damage. Among small groups of volunteers receiving a single 1 g dose of vitamin C, or 45 mg of β-carotene, those who responded with a substantial increase in plasma concentration of vitamin C or β-carotene also showed a significantly faster removal of H_2O_2-induced strand breaks [9, 10]. We report here further studies, with larger numbers of subjects, supplemented for longer periods, with β-carotene, lutein or lycopene; responses of lymphocytes taken just before supplementation, at the end of the week of supplementation, and after 1 and 3 weeks of 'washout' (no supplementation) were compared.

Table 1 Supplementation regime

lutein				lycopene				β-carotene				
weeks:												
0												12
samples:												
*	*	*		*	*	*		*	*	*		*

Materials and methods

Subject selection, sampling, and supplementation regime

Eight healthy volunteers took part in this trial – 5 male, 3 female (1 smoker in each group), average age 28 y (range 24–34), average body mass index 24.5 kg/m^2 (range 22–27). The supplementation schedule was: 15 mg/day of lutein for one week; 3-week wash-out period; 15 mg/day of 'lycopene' for one week; 3-week wash-out period; 15 mg/day of β-carotene; 3-week wash-out period (Table 1).

Capsules containing lutein (80% *trans*-lutein, 20% 13-/15-*cis*-lutein) or lycopene (with 10% β-carotene) were prepared by R. P. Scherer International Corp. with lutein from Quest International, Cork, Ireland and lycopene from Makhtesheim Chemical Works Ltd., Beer-Sheva, Israel. Capsules of 'natural β-carotene' capsules (prepared from Dunaliella, a sea plant rich in β-carotene) were obtained from Boots, Nottingham, England. Early morning (but not fasted) samples of about 10 ml of blood were collected by venepuncture into Vacutainers (Greiner Labortechnik, Stonehouse, UK) containing EDTA, on the day before the supplementation trial began, at the end of each week of supplementation, and 1 week and 3 weeks into each wash-out period. The 3-week wash-out sample following the lutein supplementation was also the pre-trial sample for lycopene; the 3-week wash-out sample following the lutein supplementation was also the pre-trial sample for β-carotene. A preliminary trial of lycopene supplementation in 8 volunteers is referred to as the pilot study in the Results section. Plasma samples were snap-frozen in liquid nitrogen and stored at –80 °C. Lymphocytes were isolated from the buffy coat by centrifugation on a Ficoll-based density gradient, suspended in 90% foetal calf serum/10% DMSO (freezing medium) at a density of 3×10^6/ml, and aliquots frozen slowly overnight to –80 °C before transferring to liquid nitrogen. Storage times (average 64 days) were similar for baseline and supplemented samples within each phase of the trial.

The trial was approved by Grampian Research Ethics Committee.

Analysis of carotenoid content of plasma

Carotenoids (α- and β-carotenes, β-cryptoxanthin, lycopene, lutein/zeaxanthin, and phytofluene) as well as α- and γ-tocopherol were measured by reverse phase HPLC [11].

Comet assay for DNA damage

Plain glass microscope slides were coated with 1 % aqueous solution of electrophoresis grade agarose (Gibco-BRL, Paisley, Scotland) and dried. 100 μl of 1 % agarose in PBS was placed on the slide, covered with a glass cover-slip, and left at 4 °C to set.

Frozen lymphocytes were thawed, mixed with PBS, centrifuged at 200×g for 3 min at 4 °C, and resuspended in PBS at 2×10^6/ml. For H_2O_2 treatment, 100 μl of 1 mM H_2O_2 was added to 0.9 ml of cells in PBS; after 5 min on ice, the cells were collected by centrifugation as before. Treated cells and untreated controls were suspended in RPMI medium with 10 % foetal calf serum, and incubated in open microcentrifuge tubes at 37 °C, in a 5 % CO_2 atmosphere. After 0, 2, 4, 8 and 24 h of incubation, cells were centrifuged, suspended in 1 % low melting point agarose (Gibco-BRL) at 6×10^5 per ml, and 100 μl applied to the gel already on the microscope slide, covered with a cover-slip and allowed to set at 4 °C. Slides were placed for 1 h at 4 °C in lysis solution (2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris/HCl pH 10, 1 % Triton X–100) to remove membranes, cytoplasm and nuclear proteins, leaving DNA as nucleoids. Slides were then placed in 0.3 M NaOH, 1 mM Na_2EDTA for 40 min, before electrophoresis at 25 V for 30 min at an ambient temperature of 4 °C. After neutralising the gels with 0.4 M Tris/HCl, pH 7.5 (3 changes), gels were stained with 4',6-diamidine–2'-phenylindole dihydrochloride (DAPI). Slides were viewed by fluorescence microscopy. Nucleoid DNA extends under electrophoresis to form 'comet tails', and the relative intensity of DNA in the tail reflects the frequency of DNA breaks [12]. In these experiments, tail intensity was assessed with a visual scoring method, the identity of slides being concealed before scoring; 100 comets selected at random were graded according to degree of damage into 5 classes (0 to 4) and the overall score expressed as between 0 and 400 arbitrary units.

Statistical analysis

Data representing the various time points were compared using the t-test; paired two sample for means, or (when data were lost for one of the two time points) two sample assuming equal variance.

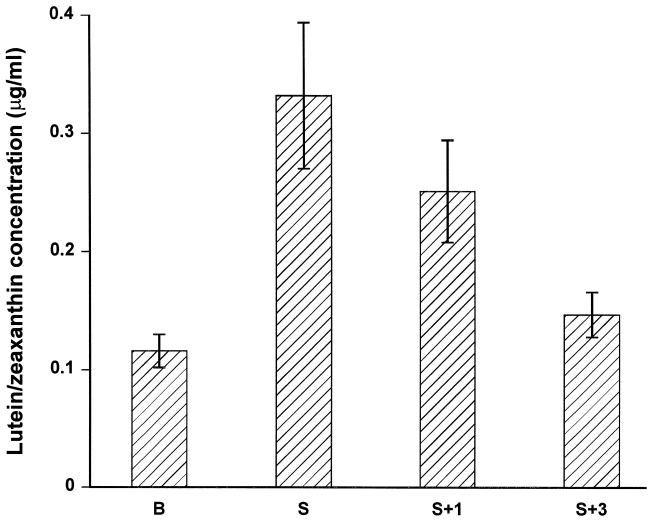

Fig. 1 Plasma concentrations of lutein/zeaxanthin during lutein supplementation phase of study. Mean values from 8 subjects are shown, ±SE. B, baseline; S, after 1 week of supplementation; S+1, after 1 week of wash-out; S+3, after 3 weeks of wash-out.

Results

Lutein supplementation

Figure 1 shows mean values for plasma lutein/zeaxanthin concentration, before and after lutein supplementation. An almost 3-fold increase occurred as a result of supplementation, and after 3 weeks of wash-out the plasma concentration was still slightly (and significantly) above the baseline level. The mean values conceal a high degree of inter-individual variation; one volunteer showed only a 10 % increase after one week (curiously increased to 50 % after one week of wash-out), while others showed increases of up to 7-fold. Measurements of DNA damage in lymphocytes treated with H_2O_2 and incubated for up to 24 h are shown in Figure 2. Repair of strand breaks appears to be very slow and incomplete – at whatever time during the trial the samples were taken. Thus there is no indication of an effect of lutein supplementation on DNA repair. Note that the control cells, not treated with H_2O_2, show a pronounced increase in DNA breakage within a few hours of being placed in *in vitro* culture.

Lycopene supplementation

Of the common dietary carotenoids, lycopene tends to be the most poorly assimilated (B. Olmedilla, personal communication). In a pilot study, one week of lycopene supplementation resulted in a small (25 %) but significant increase in mean plasma lycopene concentration. In the lycopene supplementation stage of this present study, the mean plasma

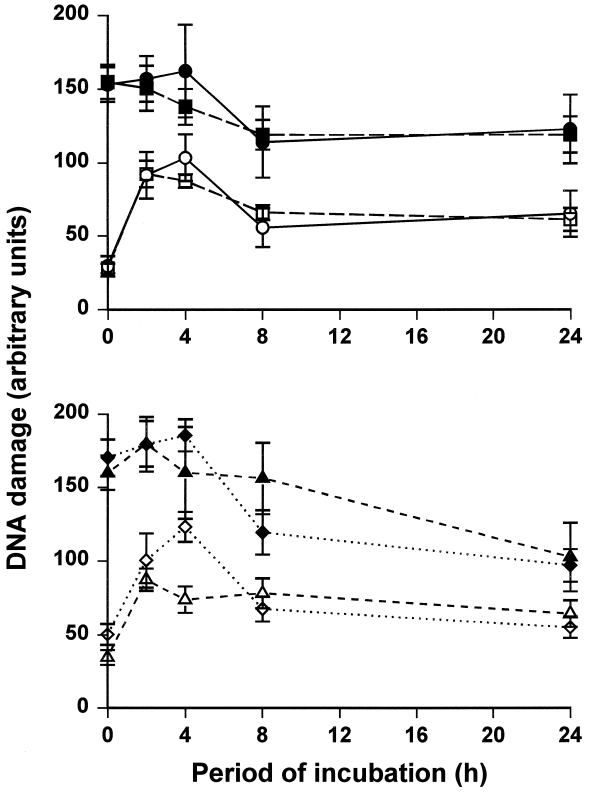

Fig. 2 Recovery of lymphocytes from DNA damage inflicted by H_2O_2: lutein supplementation phase. Solid symbols (upper two curves in each panel) represent lymphocytes from the lutein phase of the trial, incubated for up to 24 h after treatment with 100 μM H_2O_2. Open symbols (lower two curves in each panel) represent control cells, not treated with H_2O_2. The upper panel shows lymphocytes from baseline samples (squares) and after 1 week of supplementation (circles); the lower panel shows lymphocytes after wash-out for 1 week (triangles) and 3 weeks (diamonds). Bars indicate SE; n=8.

lycopene concentration increased by about 17 % during the supplementation (Fig. 3), but this was not statistically significant. (Although this supplement contains some β-carotene, no change in plasma β-carotene concentrations was seen.) There were no significant changes in DNA strand break rejoining over the period of the trial when data from all volunteers were pooled. It can be seen from Figure 3 that individual responses to supplementation, indicated by plasma lycopene concentrations, are very variable; the washout period is not equally effective in bringing plasma lycopene down in all subjects, and in some the maximum plasma concentration does not occur until after the supplementation period. The variability of plasma lycopene levels may reflect varying intake of carotenoids from dietary sources during the trial. We decided to examine separately those volunteers who did show an increase in plasma lycopene, defined as a 20 % rise above the baseline value. We looked at the kinetics of recovery from DNA damage in baseline lymphocyte samples, compared with lymphocytes from those sampling periods when – for that individual – the

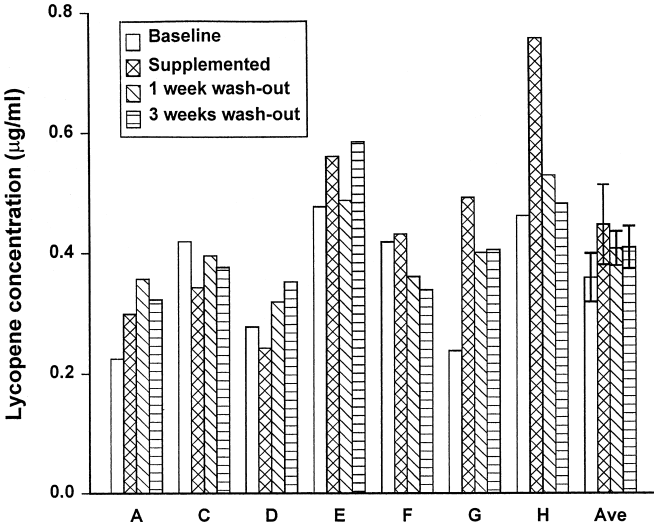

Fig. 3 Plasma concentrations of lycopene during lycopene supplementation phase of study. Individual data for the four sampling times are shown for 7 subjects (one volunteer not taking part in this phase of the study), together with average values ±SE.

increase in lycopene concentration was greater than 20 %. Figure 4 shows that there does, indeed, appear to be an association between elevated plasma lycopene levels and faster recovery from H_2O_2-induced DNA damage.

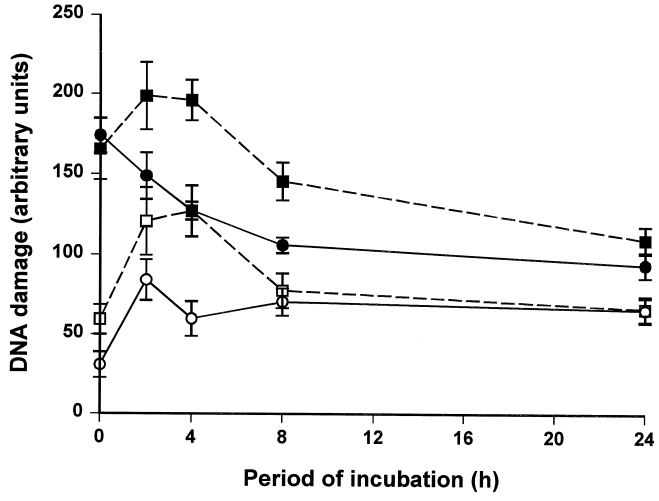

Fig. 4 Recovery of lymphocytes from DNA damage inflicted by H_2O_2: lycopene supplementation phase. Solid symbols (upper two curves) represent lymphocytes from the lycopene phase of the trial, incubated for up to 24 h after treatment with 100 μM H_2O_2. Open symbols (lower two curves) represent control cells, not treated with H_2O_2. This analysis is limited to those volunteers (5 of 7) showing, at some point during the trial, a greater than 20 % increase in plasma lycopene level (circles) compared with baseline samples (squares). Bars indicate SE.

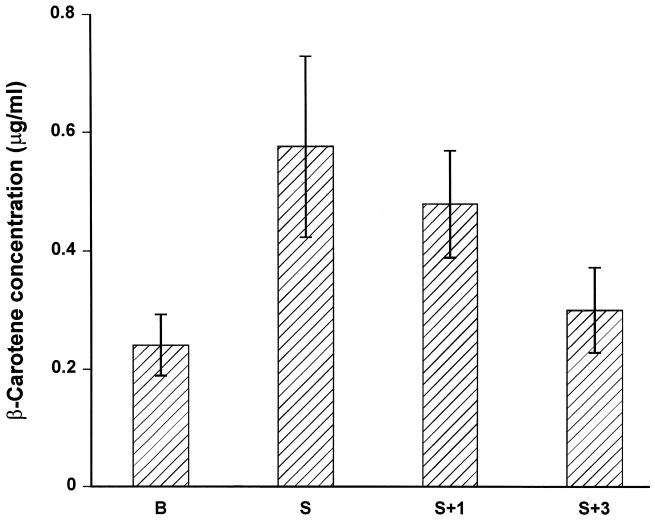

Fig. 5 Plasma concentrations of β-carotene during β-carotene supplementation phase of study. Mean values from 8 subjects, ±SE. B, baseline; S, after 1 week of supplementation; S+1, after 1 week of wash-out; S+3, after 3 weeks of wash-out.

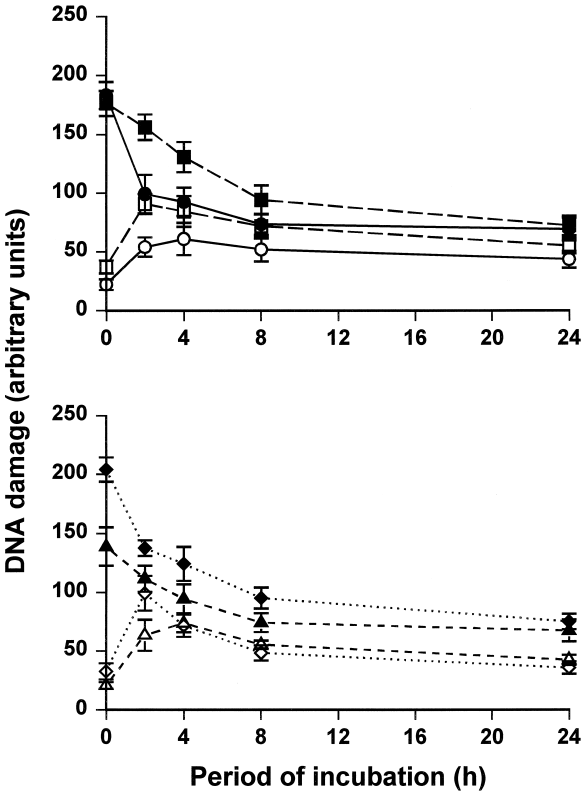

Fig. 6 Recovery of lymphocytes from DNA damage inflicted by H_2O_2: β-carotene supplementation phase. Solid symbols (upper two curves in each panel) represent lymphocytes from the β-carotene phase of the trial, incubated for up to 24 h after treatment with 100 μM H_2O_2. Open symbols (lower two curves in each panel) represent control cells, not treated with H_2O_2. The upper panel shows lymphocytes from baseline samples (squares) and after 1 week of supplementation (circles); the lower panel shows lymphocytes after wash-out for 1 week (triangles) and 3 weeks (diamonds). Bars indicate SE; n=8.

β-Carotene supplementation

On supplementation with β-carotene, a strong increase in mean plasma β-carotene concentration was seen (Fig. 5); only one subject (the same as showed a small response to lutein) apparently was unable to take up this carotenoid. Plasma β-carotene concentrations fell back to baseline by the end of the 3 week wash-out period. The increase in plasma β-carotene was associated with a significantly enhanced recovery from DNA damage (Fig. 6a) – as well as a lower level of damage in the control, untreated cells. After one week of wash-out (when plasma β-carotene levels were still elevated), the damage induced by H_2O_2 was less than in the lymphocytes from the start of the trial (Fig. 6b) and it is difficult to discern whether there is any difference in recovery from damage at these two times.

Discussion

Our earlier work [10] reported an apparent effect of β-carotene supplementation on recovery from oxidative DNA damage, in the two individuals (out of 4) who showed substantial increases in plasma levels of the carotenoid. This was suggestive, but not conclusive evidence. We have now carried out a more comprehensive study, of three different carotenoids, in larger groups of subjects, and with a longer period of supplementation as well as wash-out periods following supplementation. We can confirm the enhancing effect of β-carotene; in this group, all but one subject showed a significant increase in plasma β-carotene after one week of supplementation, and overall the apparent ability of lymphocytes collected at this time to rejoin H_2O_2-induced DNA strand breaks was significantly greater in the first few hours of incubation, compared with lymphocytes taken before supplementation or after wash-out. By 2 h (in the supplemented lymphocytes) about 50% of the induced damage had disappeared, compared with only about 10% in the baseline lymphocytes.

Lycopene supplementation also enables lymphocytes to recover more quickly from damage. In this case, it was necessary to exclude from the analysis those individuals whose plasma lycopene levels did not increase. Lycopene (from these capsules) is taken up less effectively than other carotenoids, some individuals showing no detectable increase in serum concentration after several weeks' supplementation (B. Olmedilla, personal communication).

In all three groups, the control lymphocytes, not treated with H_2O_2, show an accumulation of DNA breaks during the first few hours of incubation. The accumulation is less marked in the lymphocytes collected at the end of the pe-

riod of lycopene or β-carotene supplementation. It seems likely that this damage is oxidative in origin (though in principle strand breaks can arise from diverse causes), and that it results from sudden exposure of cells to an atmosphere in which oxygen tension is several times higher than that in the blood. In this case, the protection against damage afforded by the supplement of carotenoid is probably simply an antioxidant effect, rather than a stimulation of DNA repair. The H_2O_2-treated lymphocytes are also exposed to atmospheric oxygen, of course, and the continuing input of damage from this source, added to that from H_2O_2, probably accounts for the apparent slowness of repair in lymphocytes compared with cultured cells (see Introduction); the latter are apparently adapted to a higher oxygen tension. Furthermore, the apparent enhancement of repair of induced damage by β-carotene and lycopene is probably no more than protection against further atmospheric oxygen damage.

It is, perhaps, surprising that such a substantial enhancement of recovery from DNA damage (or protection against further oxidation) results from a fairly modest increase in plasma concentration of one carotenoid. It is possible that accumulation of the supplemented carotenoid occurs in the lymphocytes, giving a higher effective concentration than is indicated by measurements of plasma levels. The lack of influence of lutein supplementation – even though the increase in plasma concentration is almost three-fold on average – is curious, and suggests that carotenoid-specific effects are important.

The steady-state level of damage measured in freshly isolated lymphocytes represents a balance established between input of damage and its repair by cellular enzymes. The repair element in the equation is crucially important; the same steady-state level could arise from fast repair of a high input of damage, or slow repair of a low input of damage. Until a method is devised to measure DNA repair rates without this added complication of oxidation by atmospheric oxygen, we will not be able to understand fully how the balance is achieved.

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