

Nutritional modulation of DNA repair in a human intervention study

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DNA oxidation is a potential cause of cancer in humans. It is well-known that fruits and vegetables protect against cancer, and this may be in part because they contain antioxidants, which decrease the level of oxidation of DNA. However, there are other possible mechanisms, such as an enhancement of cellular repair of this damage. A randomized cross-over study was carried out on healthy human subjects, who were given kiwifruit as a supplement to their normal diet, for 3-week periods at different 'doses', with 2-week washout periods between doses. Endogenous oxidation of bases in lymphocyte DNA, and the resistance of the DNA to oxidation *ex vivo*, were assessed using single cell gel electrophoresis (the 'comet assay'). The capacity to repair DNA base oxidation was measured with an *in vitro* test, and levels of expression of repair-related genes *OGG1* and *APE1* were assessed by semi-quantitative RT-PCR. Concentrations of dietary antioxidants were measured in plasma. The antioxidant status of plasma and of lymphocytes was increased by consumption of kiwifruit. Levels of endogenous oxidation of pyrimidines and purines in DNA were markedly decreased, and DNA repair measured on a substrate containing 8-oxo-7,8-dihydroguanine was substantially increased (without change in levels of *OGG1* or *APE1* mRNA). The magnitude of these effects was generally not related to the number of kiwifruits consumed per day. Kiwifruit provides a dual protection against oxidative DNA damage, enhancing antioxidant levels and stimulating DNA repair. It is probable that together these effects would decrease the risk of mutagenic changes leading to cancer.

Introduction

Diet certainly plays an important role in preventing cancer, but the mechanism is still not clear. Convincing epidemiological evidence links consumption of fruits and/or vegetables with decreased risk of cancer of the lung, mouth and pharynx, oesophagus, stomach, colon and rectum (1). These foods are rich in antioxidants such as vitamins C and E, carotenoids and flavonoids, which are capable of decreasing oxidative damage to DNA and thus might prevent mutation and cancer. Large-scale human intervention trials with antioxidants have, how-

ever, given equivocal results. In Linxian, China, a significant decrease in cancer incidence (especially stomach cancer) was seen after 5 years of supplementation with β -carotene, vitamin E and selenium (2); but several years of β -carotene supplementation, in two trials with smokers/asbestos workers in Finland and the USA, led to increased incidence of lung cancer (3,4). In another trial, with mainly non-smokers, β -carotene had no effect (5). Ambiguous results have also come from human trials which examine the effects of antioxidant supplements on oxidative DNA damage in white blood cells; a mixture of vitamins C and E and β -carotene led to a substantial decrease in DNA base oxidation (6), but in other trials antioxidants were ineffective (7–9).

Antioxidants in isolation may behave differently from mixtures of antioxidants and other phytochemicals as found naturally in fruits and vegetables. We therefore decided to investigate the effects of kiwifruit, a particularly rich natural source of vitamin C and other potentially active phytochemicals, on oxidative DNA damage in humans. Different 'doses' of this fruit were administered over 3-week periods to healthy volunteers. We measured concentrations of dietary antioxidants in plasma, and the resistance of lymphocyte DNA to oxidative attack *ex vivo*, as indicators of antioxidant status. Levels of endogenous oxidation of bases in lymphocyte DNA were also determined.

The second line of defence against oxidative DNA damage is DNA repair, and we therefore measured the capacity of lymphocyte extracts to incise DNA at sites of 8-oxo-7,8-dihydroguanine (8-oxoGua). The repair enzyme 8-oxoGua DNA glycosylase 1 (OGG1) removes the oxidized purine from DNA as the first step in base excision repair. OGG1 also has an AP lyase activity [converting apurinic/aprimidinic (AP) sites to breaks] but this is slow, and limits the overall rate of repair (10). APE1 is an AP endonuclease that by-passes the AP lyase activity of OGG1, enhancing OGG1 turnover (11,12); it thus has an important role in the regulation of base excision repair of oxidative DNA damage. Possible changes in expression of *OGG1* and *APE1* were examined by semi-quantitative RT-PCR.

Materials and methods

Subjects and study design

Fourteen volunteers (eight female, six male) were recruited by means of posters displayed in the Rowett Research Institute. They were healthy non-smokers, aged 26–54 years, and were not taking antioxidant supplements or medication. Otherwise, there were no exclusion criteria. Volunteers were requested to maintain their normal diet during the trial. Each volunteer consumed 1, 2 or 3 kiwifruits/day for successive 3-week periods separated by 2-week washout periods. Subjects were allocated randomly to three groups, each of which was given a different order of kiwifruit doses (Figure 1). Group A took 1/day in the first period, 2/day in the second and 3/day in the third; group B took 2/day, 3/day and 1/day; group C took 3/day, 1/day and 2/day. Early morning fasted blood samples were collected by venepuncture at the start of the study, at the end of each washout period, and at the end of each 3-week supplementation phase (several hours after consumption of the last kiwifruit). Plasma was separated for analysis of antioxidants, flash-frozen as aliquots in liquid nitrogen and stored at -80°C (acidified with 10%

Abbreviations: AP, apurinic/aprimidinic; APE, AP endonuclease; FPG, formamidopyrimidine DNA glycosylase; OGG1, 8-oxoGua DNA glycosylase 1; PBS, phosphate-buffered saline; 8-oxoGua, 8-oxo-7,8-dihydroguanine.

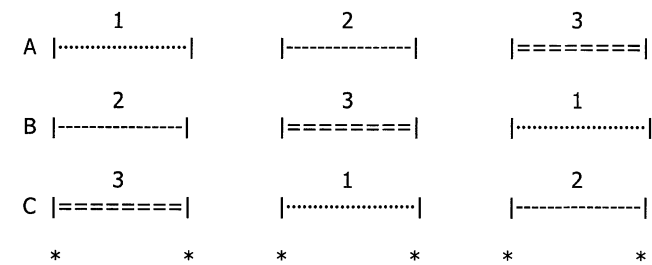


Fig. 1. Outline of intervention study. Volunteers were assigned randomly to three groups, A, B and C. They consumed 1, 2 or 3 kiwifruits/day for 3 weeks, in different orders as shown, separated by 2-week washout periods. Blood samples were taken at times indicated by asterisks.

monophosphoric acid in the case of samples for vitamin C analysis). Lymphocytes were isolated by the standard procedure of centrifugation over Lymphoprep (Nycomed, Oslo, Norway) for measurement of DNA damage and DNA repair with the comet assay. Conditions for freezing and storing the lymphocytes differed for the two assays and are described below. Persons involved in analysing the samples were not aware of their group assignment or phase in the trial (except in the case of gene expression experiments, which were carried out on samples selected on the basis of results in a previous test). The study was approved by the Grampian Research Ethics Committee.

Plasma antioxidants

Plasma vitamin C concentrations were determined by reverse phase HPLC using an ion-pairing technique with UV detection (13). Retinol, α - and β -carotene, β -cryptoxanthin, lycopene, lutein/zeaxanthin, and α - and β -tocopherols were measured by reverse phase HPLC with simultaneous UV and fluorimetric detection (14).

DNA damage estimation with the comet assay

DNA breaks were measured using the comet assay (single cell gel electrophoresis) (15). Immediately after isolation, lymphocytes were suspended in a 9:1 mixture of fetal calf serum and dimethylsulphoxide at 3×10^6 /ml. Aliquots (100 μ l) were slowly frozen to -80°C and stored in liquid nitrogen. They were thawed, centrifuged and suspended in phosphate-buffered saline (PBS). Strand breaks were introduced in certain aliquots of lymphocyte by incubating them with 100 μM H_2O_2 in PBS for 5 min on ice. The cells were washed with PBS, centrifuged, suspended (at $\sim 2 \times 10^5$ cells/ml) in 85 μ l of 1% low melting point agarose (Life Technologies, Paisley, UK) at 37°C , and placed on a glass microscope slide (precoated with agarose to aid attachment of the gels). Two gels were prepared for each sample. The gels were allowed to set at 4°C , and cells were lysed for 1 h in 2.5 M NaCl, 0.1 M Na_2EDTA , 10 mM Tris-HCl, pH 10, 1% Triton X-100 at 4°C . Lysis removes membranes, cytoplasm and most nuclear proteins, leaving DNA as nucleoids.

To measure strand breaks, the slides were immersed in 0.3 M NaOH, 1 mM Na_2EDTA for 40 min at 4°C before electrophoresis at 0.8 V/cm for 30 min at an ambient temperature of 4°C . After neutralization, gels were stained with 4',6-diamidine-2'-phenylindole dihydrochloride, and viewed by fluorescence microscopy. Nucleoid DNA extends under electrophoresis to form 'comet tails', and the relative intensity of DNA in the tail reflects DNA break frequency. Tail intensity was assessed with a visual scoring method; 100 comets selected at random were graded according to degree of damage into five classes (0–4) to give an overall score for each gel of between 0 and 400 arbitrary units. The visual score correlates closely with the mean % of DNA in the tail and with the DNA break frequency (15). H_2O_2 -induced strand breaks were estimated by subtracting comet scores of untreated cells from scores of cells treated with H_2O_2 .

For analysis of endogenous base oxidation, after the lysis stage agarose-embedded nucleoids from non- H_2O_2 -treated cells were incubated with endonuclease III (specific for oxidized pyrimidines) or with formamidopyrimidine DNA glycosylase (FPG; recognizes altered purines including 8-oxoGua) in 40 mM HEPES, 0.1 M KCl, 0.5 mM Na_2EDTA , 0.2 mg/ml bovine serum albumin, pH 8.0, or with this buffer alone, for 30 min at 37°C . Alkaline treatment and electrophoresis then followed. Net enzyme-sensitive sites, calculated by subtracting the comet score after incubation with buffer alone from the score with enzyme, indicate the extent of base oxidation (15). The enzymes were prepared as crude extracts from bacteria containing over-producing plasmid vectors, originally obtained from Dr R.Cunningham (State University of New York, USA; endonuclease III) and from Dr S.Boiteux (Institute Gustave-Roussy, Villejuif, France; FPG).

In vitro assay for DNA repair

The modification of the comet assay to measure the base excision repair capacity of a cell extract has been described (16). It depends on the incubation

Table I. Mean plasma vitamin C concentrations

Number of kiwifruits/day	Before (μM) $\pm$ SEM	After (μM) $\pm$ SEM	Increase (%)	P
1	65 $\pm$ 4	72 $\pm$ 5	11	<0.1
2	61 $\pm$ 4	73 $\pm$ 4	20	<0.01
3	61 $\pm$ 4	77 $\pm$ 3	26	<0.001

P values indicate the statistical significance of the difference (before/after kiwifruit).

of extract with substrate DNA comprising gel-embedded nucleoids from cells treated previously with a specific DNA-damaging agent.

Immediately after isolation, lymphocytes (5×10^6 in 50 μ l aliquots), in 45 mM HEPES, 0.4 M KCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, pH 7.8, were snap-frozen to -80°C . On thawing, lysis was completed by adding 12 μ l of 1% Triton X-100, and the lysate was centrifuged. The supernatant was mixed with 4 volumes of 45 mM HEPES, 0.25 mM EDTA, 2% glycerol, 0.3 mg/ml bovine serum albumin, pH 7.8 (lymphocyte extract). Substrate nucleoids were prepared from HeLa cells (a human transformed endothelial cell line), treated on ice with the photosensitizer Ro 19-8022 (Hoffmann La Roche, Basel, Switzerland) at 0.2 μM plus visible light (4 min irradiation at 330 nm from a 1000 W tungsten halogen lamp) to induce 8-oxoGua. The cells were embedded in agarose and lysed as for the standard comet assay, and then incubated (in duplicate) with 40 μ l of lymphocyte extract for 0 or 10 min at 37°C . Alkaline treatment and electrophoresis followed as in the standard comet assay. Incision rate was estimated as the increase in comet score from 0 to 10 min of incubation.

Semi-quantitative PCR of OGG1 and APE1

RNA was extracted from lymphocytes using an Absolutely RNA kit (Stratagene, Amsterdam, The Netherlands). Total RNA was analysed with an Agilent Bioanalyser 2100 (Agilent Technologies, Stockport, UK) to confirm quality and quantity prior to Q-PCR. An aliquot (0.5 μg) was used for first strand cDNA synthesis at 42°C using Superscript II reverse transcriptase (Life Technologies, Paisley, UK) according to the manufacturer's instructions. PCR was performed using 18S specific primers (5'-CGGCTACCACATCCAAG-GAA-3'; 5'-GCTGGAATTACCGCGCT-3') as an internal reference (94°C for 1 min, 55°C for 1 min, 72°C for 1 min). Specific primer pairs for OGG1 (5'-AACAACAACATCGCCCGCATCACT-3'; 5'-GCTAGCCCGCCCTGTT-CTTCC-3') (96°C for 45 s, 60°C for 30 s, 74°C for 1 min) and APE1 (5'-GAGTAAGACGCGCCGAAAGAAAAA-3'; 5'-CCGAAGGAGCTGACCA-GTATTGAT) (94°C for 1 min, 55°C for 1 min, 72°C for 1 min) were designed to amplify across intronic regions. Hot start PCR was performed using 10 pmol 18S primers and 50 pmol of OGG1 or APE1 primers, 2 U Taq (Promega, Southampton, UK) in the presence of 200 μM dNTPs and 1.5 mM MgCl_2 . PCR products were verified by DNA sequencing and quantified at exponential phase of cycling using a DNA 500 chip (Agilent Technologies).

Statistics

Data were analysed by ANOVA with terms for subject and treatment (each of the six time points) and contrasts were defined in order to provide tests of the overall effect of eating kiwifruit and of each dose level.

Results

Individual dietary antioxidants were measured in plasma. Concentrations of vitamin C, before and after each phase of supplementation, are shown in Table I. The level increased significantly after 2 or 3 kiwifruits/day. Carotenoids and tocopherols, measured by HPLC, did not show consistent changes, although β -carotene was significantly higher after 2 kiwifruits/day (data not shown).

The antioxidant status of lymphocytes was assessed using a functional assay. We tested lymphocytes for their resistance to oxidative DNA damage by treating them for 5 min with 100 μM H_2O_2 on ice. DNA strand breaks were measured with the comet assay (15)—a sensitive technique in which cells are embedded in agarose, lysed with detergent and high salt to produce 'nucleoids', and then electrophoresed under alkaline conditions. Migration of DNA from the nucleoid core to form

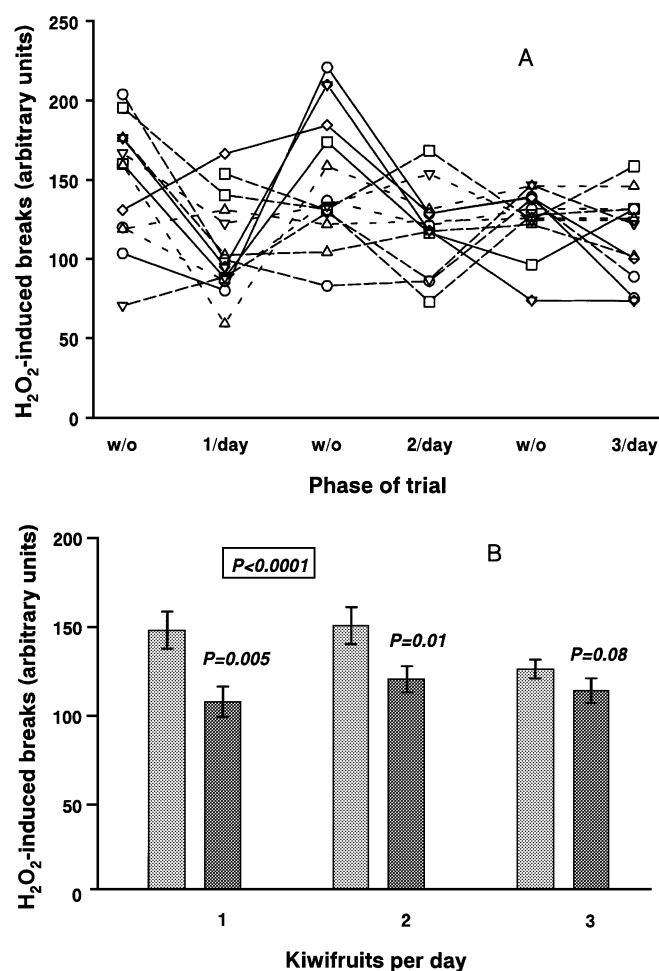


Fig. 2. Sensitivity of lymphocytes to oxidative attack by H_2O_2 *in vitro*, indicated by strand breakage measured with the comet assay. One hundred comets from each of two duplicate gels were analysed visually on a scale of 0–4, from no detectable tail to almost all DNA in tail. The overall score, between 0 and 400, is related to the DNA break frequency. (A) Individual values from 14 subjects. The data are presented in the order 1, 2 and 3/day for all subjects. The actual order varied and is indicated by the style of the line: long dashes (1, 2 and 3/day); solid (2, 3 and 1/day); short dashes (3, 1 and 2/day). (B) Mean comet assay scores ($\pm$ SEM) before (light shading) and after (dark shading) each kiwifruit supplementation phase. *P* values (0.1 or less) are given for each dose: the boxed *P* value refers to pooled data (before supplementation compared with after supplementation, regardless of dose).

a comet-like image indicates the presence of DNA breaks. Figure 2A shows the comet scores for all subjects throughout the trial; a pattern is discernible, with higher levels of damage in washout samples, and this is seen more clearly when expressed as mean scores (Figure 2B). Significantly lower levels of DNA breaks in the lymphocytes taken after consumption of kiwifruit, indicate an increased antioxidant capacity.

An enhanced antioxidant status in lymphocytes should protect DNA from endogenous oxidation, too. We therefore measured the background levels of oxidative damage, using a modification of the comet assay in which oxidized bases are converted to breaks by incubation of the nucleoids with lesion-specific repair endonucleases. As seen in Figures 3 and 4, after kiwifruit consumption there were substantial and significant decreases in both oxidized pyrimidines and altered purines, compared with the levels before supplementation.

A lower steady-state level of DNA oxidation could result

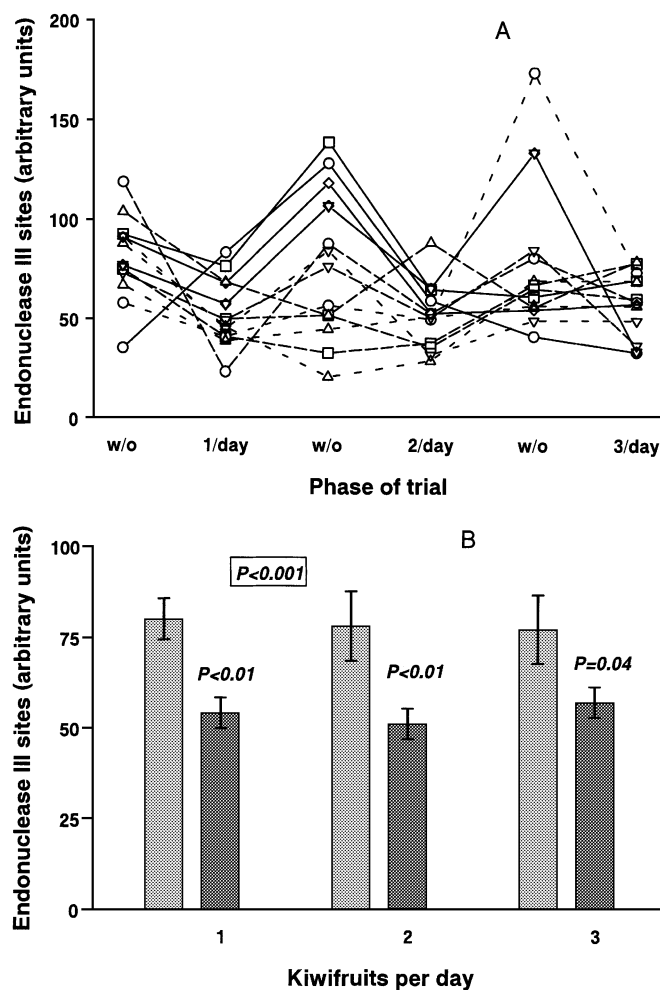


Fig. 3. Endogenous oxidation of pyrimidines in lymphocyte DNA. Repair enzyme endonuclease III was used in combination with the comet assay to make breaks at sites of oxidized pyrimidines. (A) Individual values from 14 subjects. (B) Mean comet assay scores ($\pm$ SEM). Other details are as for Figure 2.

from an increased rate of DNA repair, as well as from enhanced antioxidant status. The capacity for incision activity at oxidized purines in DNA was monitored in lymphocyte extracts using an *in vitro* method—a modified version of the comet assay (16). As with the other biomarkers, incision rates were measured before and after each kiwifruit supplementation phase. Individual rates plotted over the three phases (Figure 5A) show clear fluctuations with, in general, higher rates after kiwifruit supplementation. Figure 5B shows mean incision rates. A significant increase in repair was seen at each dose, and after three kiwifruits, the rate increased by two-thirds.

The possibility that the increase in repair incision activity resulted from enhanced expression of repair genes was investigated using semi-quantitative PCR. Three subjects were selected whose lymphocytes showed consistent and substantial increases in repair activity after kiwifruit supplementation. Total RNA isolated from the lymphocytes was reverse-transcribed and amplified using specific primers for *OGG1*, *APE1* and 18S (ribosomal RNA). Concentrations of PCR product from *OGG1* and *APE1* RNA were expressed relative to the yield from 18S. Figure 6 indicates that the levels of *OGG1* and *APE1* RNA do not change as a result of kiwifruit supplementation.

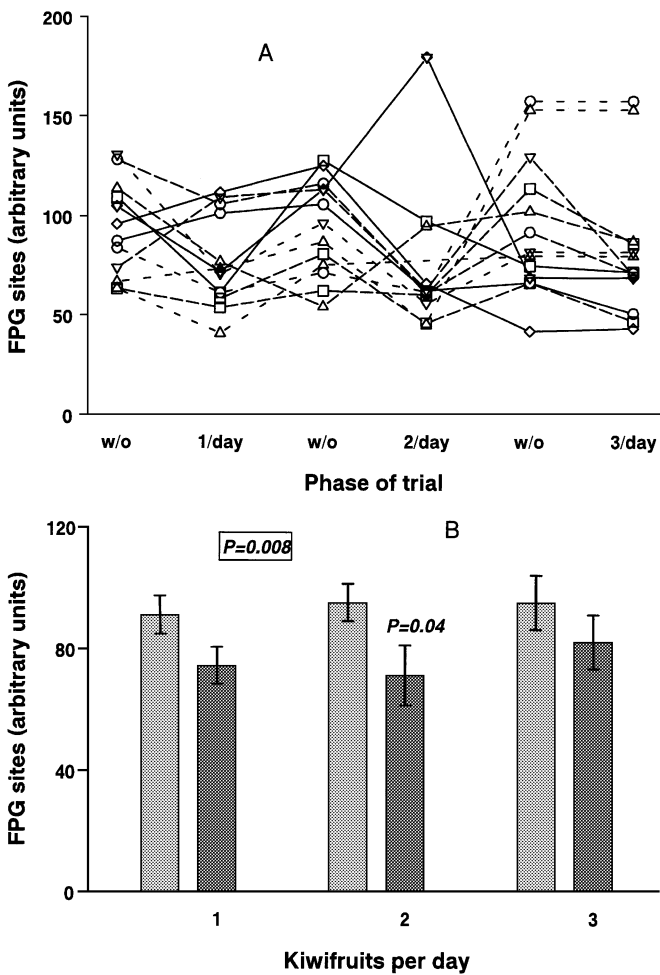


Fig. 4. Endogenous oxidation of purines in lymphocyte DNA. Repair enzyme FPG was used in combination with the comet assay to make breaks at sites of oxidized purines. (A) Individual values from 14 subjects. (B) Mean comet assay scores ($\pm$ SEM). Other details are as for Figure 2.

Discussion

Oxidative DNA damage, as measured in lymphocytes, is maintained in a dynamic steady-state by antioxidant defences, which control input of damage, together with cellular DNA repair, which removes the damage that occurs in spite of antioxidant protection. OGG1, the eukaryotic counterpart of FPG, excises 8-oxoGua as the first step in base excision repair. There are few reports on either the extent of inter-individual variation in repair capacity, or the susceptibility of repair in humans to exogenous stimulation or inhibition. Only recently have reliable methods to study repair at this level become available (16,17). In the assay used here (16), a whole-cell extract from lymphocytes is tested for its ability to incise the DNA of gel-embedded nucleoids from cells treated previously with the photosensitizer Ro 19-8022 and visible light to induce 8-oxoGua. Accumulation of breaks during a 10-min incubation is an index of the repair incision capacity of the extract. This assay was tested previously on wild-type and *Ogg1*-mouse cell lines, and found to be highly specific; no activity was detected in extract from the *Ogg1*-cells (16). The activity from human lymphocyte extract is stable; a linear accumulation of breaks was seen over 40 min (V.Harrington, unpublished).

There are no previous reports of changes in base excision repair activity associated with consumption of a particular

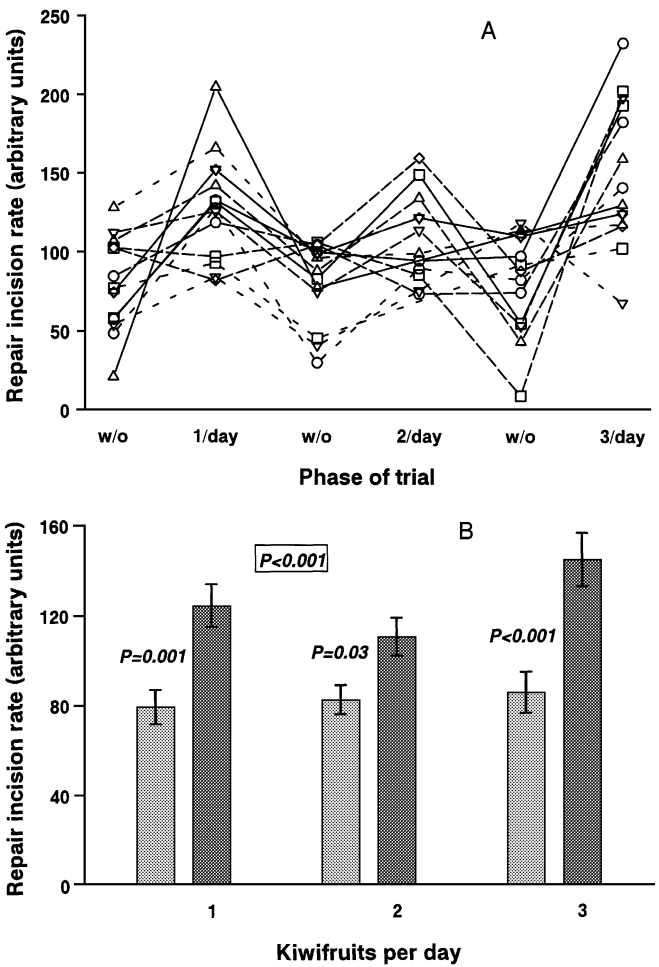


Fig. 5. DNA repair: incision activity of lymphocyte extract at 8-oxoGua residues in gel-embedded nucleoid DNA measured using the comet assay. (A) Individual incision rates from 14 subjects. (B) Mean incision rates ($\pm$ SEM). Other details are as for Figure 2.

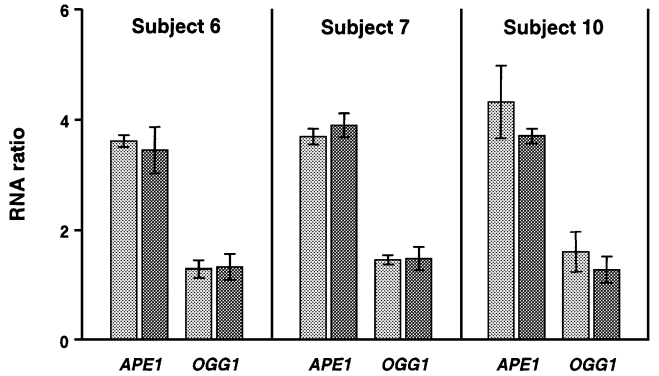


Fig. 6. Effect of kiwifruit on expression of repair-related genes. Levels of *APE1* and *OGG1* mRNA were estimated in lymphocytes from three subjects taken before (light shading) and after (dark shading) kiwifruit consumption. The amounts of cDNA generated with specific primers for 18S, *APE1* and *OGG1* in the exponential phase of PCR were quantified, and the average yield (from at least three PCR reactions) is expressed here for *APE1* and *OGG1* relative to 18S. Bars indicate SEM.

food, although supplementation with the antioxidant coenzyme Q₁₀ resulted in increased repair activity (assessed using the same modified comet assay) but no change in the level of DNA base oxidation (18). A recent investigation of cultured cells treated with crocidolite asbestos (19) showed an increase

in 8-oxoGua in DNA, and a delayed increase in OGG1 activity (up to 2.6-fold). The level of *OGG1* mRNA also increased, by as much as 7.5-fold.

We reported previously a decrease in H₂O₂-induced DNA damage after a single, very large dose of kiwifruit juice (20). The novel findings in the present work, with lower doses over a longer period, are the decrease in endogenous DNA damage, and the increase in incision activity at oxidized purines in DNA. The unprecedented effect of fruit on DNA repair appears not to be mediated by a change in gene expression of *OGG1* or of *APE1* and may instead result from an increased stability of the OGG1 protein or availability of an unknown co-factor. It is conceivable that repair enzymes are susceptible to inhibition by reactive oxygen species and that a decrease in the latter resulting from exposure to antioxidants gives rise to the higher rates of repair. Whatever the explanation, increased repair together with increased antioxidant capacity can account for the significant decreases in endogenous oxidative DNA damage.

In general, the magnitude of these effects of kiwifruit is not related to the number of fruits consumed. There is no obvious explanation for this. There were no significant correlations between individual changes in plasma vitamin C levels (arguably a marker for intake of phytochemicals from kiwifruit) and changes in repair activity or DNA oxidation. Nor were there correlations between individual repair rates and levels of damage.

It is important to note that the effects of kiwifruit on DNA damage and repair were seen after relatively small supplements of kiwifruit, easily attainable as part of a balanced diet. We deliberately did not impose restrictions on consumption of other fruits during the trial, as we are interested in the effects of including kiwifruit in the normal diet. Kiwifruit is the only fruit tested so far. It is likely that other fruits provide a similar dual protection against damage. Meanwhile, consumption of kiwifruit may be an effective way to protect against a kind of DNA damage that has been shown to cause mutations through miscoding (21) and that therefore might be responsible for initiating carcinogenesis.

Acknowledgements

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