The effect of vitamin C or vitamin E supplementation on basal and H2O2-induced DNA damage in human lymphocytes

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There is a wealth of epidemiological information on antioxidants and their possible prevention of disease progression but very little of the research on antioxidants has involved intervention studies. In this study, the potential protective effect of vitamin C or E supplementation \textit{in vivo} against endogenous and H2O2-induced DNA damage levels in lymphocytes was assessed. The supplementation involved fourteen healthy male and female non-smokers mean age 25\times53 (SD 1\times82) years, who were asked to supplement an otherwise unchanged diet with 1000 mg vitamin C daily for 42 d or 800 mg vitamin E daily for 42 d. DNA damage in H2O2-treated peripheral blood lymphocytes (PBL) and untreated PBL before and after supplementation, and during a 6-week washout period was assessed using an ELISA. At each sampling time-point, the red cell concentrate activities of superoxide dismutase, catalase and glutathione peroxidase were also determined. Supplementation with vitamin C or vitamin E decreased significantly H2O2-induced DNA damage in PBL, but had no effect on endogenous levels of DNA damage. The activities of the antioxidant enzymes superoxide dismutase and glutathione peroxidase were suppressed during the supplementation period. These supplementation regimens may be used to limit the possible adverse effects of reactive oxygen species (including those produced during the course of an immune response) on lymphocytes \textit{in vivo}, and so help to maintain their functional capacity.

The damaging effects of reactive oxygen species (ROS) on cellular biomolecules (proteins, lipids and nucleic acids) are well documented and the consequences of such damage have been implicated in the aetiology of a number of human disorders. Through a variety of mechanisms (Ma \textit{et al}. 1995), ROS produce a number of lesions in DNA and nucleoprotein such as base lesions, sugar lesions, single-strand breaks, abasic sites and DNA-protein cross-links (Dizdaroglu, 1993). The presence of ROS is not always detrimental to the organism: ROS have been identified as important intracellular transcription-inducers for a number of early response genes, e.g. NF-\kappa B (Meyer \textit{et al}. 1993) and they have been known to mediate signalling within T-cells (Schreck \textit{et al}. 1992).

A number of defence systems exist to combat the potentially damaging effects of ROS: enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSH-P), catalase (CAT), and non-enzymes such as caeruloplasmin, uric acid, \alpha-tocopherol, ascorbic acid and stress proteins. DNA repair processes can repair ROS-induced DNA damage and complexes such as proteasome and phospholipase A2 can remove oxidatively modified proteins and lipids respectively. Despite these extensive defence systems, biomolecule damage may still occur and persist within the cell. An accumulation of unrepaired damage, either alone or in combination with other age-related changes, may underlie the onset of a number of human disorders and even the ageing process (Strain \textit{et al}. 1991).

Lymphocytes play a central role in the immune response. They are susceptible to the DNA-damaging effects of a wide variety of agents including ROS generated by activated macrophages, neutrophils and T-lymphocytes at inflammatory foci (Metzger \textit{et al}. 1980; Gregory \textit{et al}. 1993). Research has shown that, of the spectrum of ROS generated by leucocytes at the site of immune and/or inflammatory responses, the major extracellular species causing DNA damage is H2O2 (Schraufstätter \textit{et al}. 1988). In the intracellular environ, it is clear that it is the hydroxyl radical

\textbf{Vitamin C: Vitamin E: Antioxidants: DNA damage}

\textbf{Abbreviations:} CAT, catalase; GSH-P, glutathione peroxidase; PBL, peripheral blood lymphocytes; PHA, phytohaemagglutinin; ROS, reactive oxygen species; SOD, superoxide dismutase.

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Over time, related decline in immune function (immunosenescence) (Pawelec et al. 1998) and genetic damage that accumulates within lymphocytes (Trainor et al. 1984; Fenech & Morley, 1985; Cole et al. 1991; Singh et al. 1991; Tates et al. 1991; King et al. 1994; Barnett & King, 1995) is thought to contribute to the age-related decline in T-cell function (Barnett & Barnett, 1998). The aim of this present study was to assess the extent of a possible protective effect of antioxidant supplementation against DNA damage and therefore accumulation of genetic damage. Thus, interventions, such as this, aimed at reducing the accumulation of genetic damage within T cells in vivo are likely to have beneficial health effects. Very little of the research on antioxidants has involved intervention studies, however Fenech (1998) used a double-blind placebo-controlled study to investigate the effect of vitamin E and folic acid on micronuclei frequency in lymphocytes. He found that an intake above the recommended daily intake of vitamin E (30 × recommended daily intake) and folic acid (10 × recommended daily intake) failed to reduce micronuclei frequency. Anderson et al. (1997) also found no effect of antioxidant supplementation on DNA. They gave supplements to subjects for 14 d with 60 mg (the recommended daily allowance), but saw no effect on H2O2-induced DNA damage in PBL using the comet assay.

In this present study, we assessed the effect of vitamin C or E supplementation in healthy, non-smoking, males and females on basal levels and H2O2-induced DNA damage in lymphocytes and endogenous antioxidant enzyme activities.

Materials and methods

Antioxidant supplementation study

Following ethical approval, fourteen healthy male and female subjects were recruited, mean age 25.53 (SD 1.82) years. All were non-smokers. Subjects were randomly assigned to two groups: seven were asked to supplement their otherwise unchanged diet with 2 × 500 mg vitamin C daily and vitamin E placebo and seven were asked to supplement their otherwise unchanged diet with 2 × 400 IU vitamin E daily (where 1 IU is equivalent to 1 mg dl-α-tocopheryl acetate) and a vitamin C placebo. The distribution of males and females was the same for each group. Supplementation was for 42 d with either regimen. Subjects then took neither supplement nor placebo for 6 weeks to allow the effect of withdrawal to be assessed (washout period). Blood samples were taken prior to supplementation, post-supplementation, midway through the washout (3 weeks) and at the end of the washout period (6 weeks). Subjects were asked to refrain from taking supplements immediately prior to blood letting to facilitate estimation of steady-state plasma levels. The antioxidant status of each subject was estimated at the time of each blood collection by measurement of GSH-P, CAT, SOD activities and vitamin C and E levels. The supplementation regimen described here was part of a larger double-blind crossover study.

Isolation and culture of peripheral blood lymphocytes

Whole blood (30 ml) was collected from each study subject by venepuncture into heparinised Vacutainers® (Ulster Anaesthetics, Belfast, Northern Ireland, UK). Mononuclear leucocytes were separated from the whole blood by density gradient centrifugation using lymphocyte separation medium (Histopaque 1077, Sigma, Poole, Dorset). Monocytes were removed from the population by incubation in a tissue culture flask for 4 h at 37°C in CO2-air (5:95, v/v) in a humidified atmosphere incubator in a tissue-culture flask at a concentration of 10^6 cells/ml. Following incubation, non-adhering cells, i.e. peripheral blood lymphocytes (PBL), were enumerated using a Neubauer counting chamber and then resuspended in fresh RPMI (Dutch modification) containing 10% fetal calf serum, 4% streptomycin (Life Technologies, Paisley, Scotland, UK) (growth medium) at a concentration of 5 × 10^5 cells/ml. PBL were stimulated to divide by the addition of phytohaemagglutinin (PHA, 0.125 mg/10 ml growth medium, Abbot Laboratories Ltd, Maidenhead, Berks., UK), for 46 h before subsequent experimentation. PHA-stimulated PBL were used in all experiments since further work (results not shown) studied the effect of antioxidant supplementation on lymphocyte DNA repair capacities following H2O2 treatment. In PBL, we performed an experiment to check that PHA stimulation and incubation of lymphocytes would not in itself affect DNA damage levels. The alkaline comet assay as described by Singh et al. (1991) was used to study the effect of incubation on both basal levels and H2O2-induced DNA damage. DNA damage was measured in freshly isolated PBL following a 24 h stimulation with PHA at 37°C in a CO2-air (5:95, v/v) humidified atmosphere and 24 h after removing PHA (a total of 46 h). DNA damage was measured as percentage tail DNA in PBL preparations. There was no significant difference between endogenous or H2O2 (200 μM)-induced DNA damage level in freshly isolated unstimulated PBL when compared to PHA-stimulated PBL at either 24 h or 46 h (results not shown).

In the present supplementation study, PHA-stimulated PBL (2 × 10^7 cells/ml) were treated with H2O2 (Table 1 details the treatment regimens) at 37°C. This temperature was used in order that repair studies be carried out over an extended timecourse (results not shown). DNA damage was

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<th>Table 1. Treatment regimens for phytohaemagglutinin-stimulated peripheral blood lymphocytes (PBL) with hydrogen peroxide</th>
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<tr>
<td><strong>Treatment</strong></td>
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<tr>
<td>PBL incubated in normal growth medium for 16 h then treated with 200 μM-H2O2</td>
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<tr>
<td>PBL incubated with 10 μM-H2O2 for 16 h</td>
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quantified in PBL pre-supplementation, post-supplementation and during a 6-week washout using ELISA.

Quantification of hydrogen peroxide-induced DNA damage

An ELISA, based on the application of a monoclonal antibody directed specifically against single-stranded DNA, was used to assess endogenous and H$_2$O$_2$-induced DNA damage levels in PBL. We used the ELISA technique which allowed actual quantification of DNA damage in a large number of cell samples simultaneously without need for extensive image analysis. This ELISA technique was chosen because it allows quantification of all types of DNA damage using a single antibody with good reproducibility. The ELISA procedure is described elsewhere by van Loon et al. (1992). Briefly, local single-strandedness (caused by single- and double-strand breaks and a number of other alkali-labile DNA lesions) was converted to more open regions of single-stranded DNA by partial alkaline unwinding. An aliquot of the same sample was unwound completely to give a 100% unwound DNA control. The amount of antibody that subsequently bound was then determined by colorimetric analysis and is directly proportional to the percentage of single-stranded DNA (% ssDNA) present in the sample. For quality control, samples from previous runs were included in each batch run to ensure reproducible results.

Antioxidant measurements

Whole blood (10 ml) from each subject was centrifuged at 717 g for 10 min immediately after collection. Plasma (1 ml) was stored at −20°C for measurement of vitamin E. Plasma (250 μl) was diluted 1:3 with 10% metaphosphoric acid and immediately frozen at −70°C for subsequent measurement of vitamin C and uric acid. After the blood was separated using Histopaque the red cell concentrate was washed twice with PBS and stored at −717°C. Intracellular vitamin C and uric acid were thawed to room temperature, centrifuged at 13 000 g for 5 min and the whole-cell extract diluted 1:10 with freshly prepared 10% metaphosphoric acid. Intracellular vitamin C levels were expressed as μg vitamin C/μg protein measured in the whole-cell extract using the Bradford (1976) assay.

Intracellular vitamin C was measured using the method of Vuilleumier & Keck (1989). This was carried out using the fluorescence option on the Cobas Bio Autoanalyser (Hoffmann-La-Rocher). Vitamin C was oxidised by ascorbate oxidase until all vitamin C was in the dehydroascorbic acid form. The dehydroascorbic acid was then reacted with a coupling reagent 1,2-phenylenediamine to give a fluorescent product, which was detected at 530-2 nm.

Vitamin E

Plasma vitamin E levels were assessed using the method of Thurnham et al. (1988). Stored plasma samples were thawed and the vitamin E was extracted in the following way: 200 μl tocopherol acetate (1 ml tocopherol acetate stock (Sigma)/100 ml ethanol) was pipetted into glass test-tubes. An aliquot (100 μl) of sodium dodecyl sulfate (10 mM) and 100 μl sample or control were added to each tube. The tubes were covered and mixed for 1 min. Heptane (1 ml) containing 0.5 g butylated hydroxytoluene/l was then added to each and the tubes mixed vigorously for 3 min. The samples were then centrifuged at 179 g for 10 min at 10°C. A portion of the upper layer (700 μl) was carefully removed, placed in glass tubes (100×17 mm) and dried under N$_2$. The samples were then reconstituted in 100 μl mobile phase (500 ml acetonitrile, 500 ml methanol, 128 ml dichloromethane, 0.01 g butylated hydroxytoluene) and vortexed. Samples were then transferred to vial inserts, ensuring no air bubbles, and placed in amber vials to be analysed by HPLC. The samples were run on a Spherisorb 5 ODS2 10 cm column (Technicol Ltd) on a Waters Wisp System (Waters; Millipore, Harrow, Middlesex, UK) with an alternating detector and a Waters Integration Package.

To calculate vitamin E concentration, a response factor was determined by calculating the mean peak area following ten injections of a known tocopherol acetate standard:

\[
\text{response factor} = \frac{\text{concentration} \times \text{mean area}}{\text{dilution factor}}
\]

The response factor was then used to calculate the concentration of unknowns.

Intracellular vitamin E was not measured due to difficulties in extracting this lipid-soluble vitamin from the very limited numbers of PBL remaining following analysis of the other endpoints in the study.

Glutathione peroxidase

GSH-P activity (U/g haemoglobin) was measured using a Ransel GSH-P kit (Randox, Crumlin, Northern Ireland, UK), which is based on the method of Paglia & Valentine (1967). GSH-P catalyses the oxidation of GSH by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidised glutathione (GSSG) is immediately converted to the reduced form with concomitant oxidation of NADPH to NADP$^+$.  

...
Superoxide dismutase

SOD activity (U/g haemoglobin) was measured using a RANSOD (Randox) enzymatic colorimetric kit based on the method of Jones & Suttle (1981). The kit employs xanthine and xanthine oxidase to generate superoxide (O$_2^-$) which reacts with 2-(4-iodophenol)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. SOD activity was calculated from the degree of inhibition of this reaction.

Catalase

CAT activity was measured using the method of Aebi et al. (1974). This method measures the disintegration of H$_2$O$_2$ over time. Red cell concentrate samples were lysed in cold distilled water (1:1, v/v) and a further 1:1500 dilution performed using 50 mM-phosphate buffer (6.81 g KH$_2$PO$_4$/l; 8.9 g Na$_2$HPO$_4$2H$_2$O/100 ml; mixed 1:1.55, pH 7.0). A portion of the diluted lysate (2 ml) was transferred to a quartz cuvette and reacted with either 1.0 ml phosphate buffer (reference) or 1.0 ml freshly prepared 10 mM-H$_2$O$_2$ (30:100, v/v stock, Sigma) as the test. The decomposition of the substrate (H$_2$O$_2$) was recorded spectrophotometrically at 240 nm for 30 s. CAT activity was expressed as K/g haemoglobin and was calculated using the following equation:

$$\text{K/g haemoglobin} = 2.3/30 \log E1/E2 \times a/b \times 500,$$

where K is the rate constant of the first order reaction as defined by Aebi (1974), 2.3 is constant, 30 is 30 s, E1 is the initial absorbance, a is the dilution factor (1000), b is the amount of protein or haemoglobin (g/l), 500 is the dilution factor.

Haemoglobin

Haemoglobin was measured according to the method of Dacie & Lewis (1991) and expressed as g/dl. Measurements were converted to g/l for antioxidant enzyme calculations.

Statistics

One-way ANOVA with least significant differences was used to study the effect of supplementation, withdrawal and H$_2$O$_2$ treatments on PBL. ANOVA is a set of statistical techniques which can be used to test the null hypothesis that data are from the same population, in which the mean of a test variable is equal in several independent groups of cases defined by a single grouping variable. The post hoc sub-dialog, least significance difference was used to indicate which of the groups, if any, had significantly different values from the mean. Least significant difference uses t tests to perform all pairwise comparisons between group means. All analyses of results were carried out using SPSS for Windows (SPSS UK Ltd, Woking, Surrey, UK) statistical package.

Results

DNA damage

Endogenous levels of DNA damage remained unchanged following either vitamin C or E supplementation. There was no significant difference between levels of DNA damage in untreated PBL and levels found in PBL treated with 10 $\mu$M-H$_2$O$_2$; H$_2$O$_2$-induced (200 $\mu$M) DNA damage in PBL was significantly decreased ($P < 0.05$) following plasma vitamin C supplementation (Fig. 1) and following vitamin E supplementation (Fig. 2), when compared to H$_2$O$_2$-induced DNA damage prior to supplementation. The protective effect of vitamin C supplementation on H$_2$O$_2$-induced DNA damage persisted even through the 6-week washout. Vitamin C supplementation did not affect 10 $\mu$M-H$_2$O$_2$-induced DNA damage levels in PBL, except at the 6-week washout stage, where DNA damage was significantly less than that at pre-supplementation.

Vitamin E supplementation had no effect on levels of DNA damage in untreated PBL or 10 $\mu$M-H$_2$O$_2$-induced levels of DNA damage. Immediately following vitamin E supplementation, levels of 200 $\mu$M-H$_2$O$_2$-induced DNA damage were not significantly different when compared with damage induced pre-supplementation. However, 3 weeks following the withdrawal of vitamin E supplementation, levels of 200 $\mu$M-H$_2$O$_2$-induced DNA damage were significantly lower when compared to induced damage at both pre- and post-vitamin E-supplementation sampling points.

Antioxidants

Plasma vitamin C levels increased significantly following supplementation with 1000 mg vitamin C daily for 42 d

![Fig. 1. Endogenous and H$_2$O$_2$-induced DNA damage levels in peripheral blood lymphocytes (PBL) from subjects (n 7) supplemented with vitamin C (2 x 500 mg/d) followed by a 6-week washout (WWO). I, Control, untreated PBL; I, PBL treated with 200 $\mu$M-H$_2$O$_2$ only; I, PBL treated with 10 $\mu$M-H$_2$O$_2$ only (see Table 1). Values are mean DNA damage (expressed as % single-stranded (ss)DNA) with standard errors of the means represented by vertical bars. Values following treatments are compared statistically to damage levels present pre- and post-supplementation and during washout samples: mean values were significantly different from the same treatment pre-supplementation (ANOVA): *P < 0.05; mean values were significantly different from the same treatment immediately post-supplementation (ANOVA): †P < 0.05.](https://www.cambridge.org/core/terms)
The distribution of males and females was equal for each group. Mean values were significantly different from pre-supplementation values (‘within’ subject ANOVA): *P < 0.05.

Table 2. Plasma levels of vitamin C and vitamin E in subjects supplemented with vitamin C or vitamin E for 42 d followed by a 6-week washout (WWO) period

<table>
<thead>
<tr>
<th>Timepoint</th>
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<td>(2 × 500 mg/d and placebo)</td>
<td>70 ± 36</td>
<td>105 ± 23*</td>
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<td>75 ± 88</td>
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<td>Vitamin E</td>
<td>27 ± 63</td>
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Discussion and conclusions

Following supplementation with vitamin C, levels of vitamin C in plasma and within stimulated PBL were increased significantly (P < 0.05). The levels were decreased significantly 3 weeks post-supplementation, indicating that watersoluble vitamin C had been washed out of the plasma at least. These data must be viewed in the light of results from another as yet unpublished study using an identical supplementation regimen (LA Brennan, GM Morris, GR Wasson, BM Hannigan and YA Barnett, unpublished results). We showed that while elevated plasma vitamin C levels were washed out by 3 weeks, intracellular levels remained significantly increased (P < 0.05) compared to pre-supplemented levels for up to 6 weeks following withdrawal of the vitamin C supplement (Table 4). This work showed that µmolar amounts of vitamin C were accumulated by PBL following supplementation even though plasma levels decreased significantly by 3 weeks following withdrawal of the supplementation. Thus, in the present study, decreased levels of 200 µM-H_{2}O_{2}-induced DNA damage in PBL following supplementation may be attributed to the increased scavenging of H_{2}O_{2}-derived ROS by increased plasma and cellular vitamin C. This effect was still seen 3 weeks following the withdrawal of this supplementation. The accumulation and maintenance of µmolar amounts of vitamin C within PBL would explain this persistent damage-reducing effect. Sweetman et al. (1997) showed that ascorbic acid added to culture medium resulted in increased resistance to H_{2}O_{2}-induced DNA damage in Raji lymphoblastoid cells, and they found that ascorbic acid was more effective when added 24 h before treatment, presumably to allow for intracellular accumulation.

We did not find any effect of vitamin E supplementation on plasma vitamin C levels. The vitamin E level in plasma was increased significantly following supplementation and vitamin C or E resulted in a significant decrease in SOD activity (Table 3). Following withdrawal of vitamin C, SOD returned to baseline levels within 6 weeks. GSH-P activity was significantly decreased following vitamin C supplementation, and remained depressed even after the 6-week washout. CAT activity did not appear to be affected by either supplementation regimen.

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Mean values were significantly different from pre-supplementation values (‘within’ subject ANOVA): *P < 0.05.

† The distribution of males and females was equal for each group.
the levels fell significantly during the washout period following withdrawal of vitamin E. Levels of DNA damage induced by 200 μM H₂O₂ were decreased significantly at 3 weeks following the withdrawal of vitamin E. The bio-kinetics and tissue absorption of vitamin E have not been well studied in human subjects. Animal studies show that there are fast and slow turnover tissues and certain tissues such as adipose, liver and adrenal tissue accumulate vitamin E (Packer, 1992), but there is little or no information such as adipose, liver and adrenal tissue accumulate vitamin E (Packer, 1992), but there is little or no information involving lymphocytes. It is possible that lymphocytes may have taken some time to accumulate and incorporate the excess vitamin into the cell membranes. Such measurements were not possible as part of this study due to the large numbers of PBL required for accurate intracellular vitamin E determination.

Supplementation with vitamin C decreased both SOD and GSH-P activities measured in red cell concentrates, however, CAT activity was unaffected. Following withdrawal of vitamin C SOD returned to baseline while activity of GSH-P remained significantly lower than its pre-supplemented activity at least for the duration of the study. The decreased activity of these red cell concentrate antioxidant enzymes may indicate an increase in the global antioxidant capacity caused by an increase in vitamin C levels which was concomitant with decreased H₂O₂-induced DNA damage. Vitamin E supplementation significantly decreased red cell concentrate SOD activity but had no effect on GSH-P activity. As SOD dismutes O₂⁻ it generates H₂O₂ and thus, it would seem logical that vitamin E may affect SOD activity as an overall antioxidant strategy.

Duthie et al. (1996) have also shown reduced H₂O₂-induced DNA damage following vitamin C or β-carotene supplementation, but this was shown in unstimulated PBL. Pohl & Reidy (1989) found that vitamin C protected against chromosomal damage caused by bleomycin in human lymphocytes ex vivo. Vitamin C is known to be a first-line defence antioxidant mopping up hydroperoxide (HO₂⁻), the hydroxyl radical (OH⁻) and singlet oxygen (O₂Δg). Vitamin C levels in leucocytes have been found to be eighty times higher than plasma levels (Moser, 1987) and concentrations of up to 1500 μM have been recorded in the cornea and aqueous humour of the eye (Frei, 1989). In this study, vitamin C conferred significant intracellular protection against H₂O₂-induced DNA damage. It has been suggested that certain cells, subjected to high levels of endogenous free radical production, may have the capacity to actively concentrate...
vitamin C to afford cellular protection against the potential biomolecule damaging effects of free radicals (Washko et al., 1989).

Vitamin E did not show a protective effect against H$_2$O$_2$-induced DNA damage until 3 weeks following the withdrawal of vitamin E supplementation. We have suggested that this may have been due to time taken to accumulate and incorporate the vitamin into lymphocyte cell membranes. Vitamin E is a powerful chain-breaking antioxidant, primarily preventing lipid peroxidation by breaking the chain of events leading to the formation of hydroperoxides. This action should also lead to a reduction in DNA damage since the intermediate products of lipid peroxidation include lipid peroxides, which can cause strand breaks in DNA (Cheeseman, 1993).

In conclusion, supplementation with vitamin C or vitamin E afforded protection against 200 µM H$_2$O$_2$-induced DNA damage, despite a decrease in the activities of antioxidant enzymes SOD and GSH-P. There was no effect of either supplementation regimen on endogenous or 10 µM H$_2$O$_2$-induced levels of DNA damage and, even though SOD and GSH-P activities remained depressed, there was no detrimental effect (in terms of endogenous, 10 µM- or 200 µM H$_2$O$_2$-induced DNA damage) of withdrawal of the supplementation. The protective effect of both vitamins remained for at least 3 weeks following withdrawal. In the case of vitamin C supplementation, this protection may be a result of boosted intracellular levels of ascorbic acid. These results are interesting in light of the possible accumulation of DNA damage in T cells following participation in immune responses where high doses of ROS are generated. It has not yet been shown whether vitamin C and E supplementation might help protect against the age-related decline in T-cell-mediated immune responses.

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References


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