Novel repair action of vitamin C upon in vivo oxidative DNA damage

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Received 19 September 1998; received in revised form 20 October 1998

Abstract There appears to be a paucity of data examining the effect of dietary antioxidants on levels of oxidative DNA damage in vivo, limiting evidence-based assessment of antioxidant efficacy, mechanisms and recommendation for optimal intake. We have examined levels of 8-oxo-2′-deoxyguanosine (8-oxodG) in mononuclear cell DNA, serum and urine from subjects undergoing supplementation with 500 mg/day vitamin C. Significant decreases in DNA levels of 8-oxodG were seen, correlating strongly with increases in plasma vitamin C concentration. Furthermore we established a timecourse for sequential, significant increases in serum and urinary 8-oxodG levels. These results illustrate, for the first time in humans, the kinetics of 8-oxodG removal and processing in vivo, suggesting a role for vitamin C in the regulation of DNA repair enzymes and thereby demonstrating a non-scavenging antioxidant effect.

Key words: Antioxidant; Vitamin C; Antibody; 8-Oxo-2′-deoxyguanosine; DNA repair; DNA damage

1. Introduction

The effect of specific dietary antioxidants, such as vitamin C, vitamin E and carotenoids, on oxidative DNA damage has been difficult to ascertain by epidemiological studies following supplementation with fruit and vegetables [1]. It may be expected that such antioxidants would reduce the levels of oxidative DNA damage. However, there appears to be limited in vivo support for this hypothesis as a number of studies have shown no such effect following dietary supplementation with vitamins C, E or carotenoids [2–5]. Most of these studies measured urinary levels of 8-oxo-2′-deoxyguanosine (8-oxodG) as a marker and possible repair product of oxidative DNA damage, facilitating the non-invasive monitoring of in vivo oxidative stress [6–9]. 8-oxodG is understood to be a mutagenic lesion in vivo, giving rise to G→T transversions [10], and its successful repair is therefore essential. In mammalian cells, it would appear that 8-oxodG may be removed/excluded from DNA by a number of processes including: a glycosylase/apurinic lyase, hOgg1 [11,12]; an 8-oxoG endonuclease [13] and thirdly, the human MutT homologue (hMTH1) [14,15]. The latter two processes give rise to damaged deoxynucleotides which may subsequently be enzymatically hydrolysed to stable, water-soluble deoxynucleosides and finally excreted in the urine.

We have recently noted an apparent antioxidant effect in vivo for vitamin C upon DNA in peripheral blood mononuclear cells (PBMC), as measured by GC-MS [16]. A reduction in the levels of 8-oxoG was noted following supplementation which may be expected to be reflected in the urinary levels of 8-oxodG. Therefore, in this study, we have examined the effect of vitamin C supplementation upon the levels of 8-oxodG excreted in the urine of subjects collected from our previous trial [16]. We also report further HPLC-EC measurements of both 8-oxoG and 8-oxodG in the DNA of mononuclear cells collected previously and their correlation with plasma vitamin C concentration.

2. Materials and methods

2.1. Supplementation protocol

Ethical approval for the study was given by the Leicestershire Area Health Authority Ethics Committee and 30 healthy volunteer subjects, consisting of 16 females and 14 males, were subsequently recruited. The age of the subjects ranged from 17 to 49 and all gave written, informed consent. Smokers, people taking vitamin supplements and/or salicylates were excluded from the study. Fasting blood and first morning urine samples were collected from all subjects to establish baseline values. A 6-week placebo course (calcium carbonate: 500 mg/day, orally) preceded a 6-week course of vitamin C (ascorbic acid: 500 mg/day, orally). Fasting blood and first morning urine samples were taken at 3-weekly intervals up to 12 weeks and then at week 19. A further urine sample was taken at week 25. At each collection, plasma ascorbate levels were determined, PBMC-derived DNA extracted and urinary creatinine levels measured.

2.2. Measurement of plasma vitamin C by HPLC

All chemicals were of the highest purity and unless stated otherwise, were obtained from Sigma Chemical Co. (Poole, UK). The method for analysis of plasma vitamin C was based upon that of Lunec and Blake [17].

2.3. Isolation of PBMC

PBMC were isolated by double density centrifugation. Briefly, whole blood, diluted with an equal volume of PBS, was added to two layers of Histopaque 1077 and 1119. Centrifugation at 700×*g*, 18°C for 30 min produced separation into five layers. Removal of the uppermost plasma/platelet layer allowed collection of the second (mononuclear cell) layer.

2.4. Extraction of PBMC DNA

This was performed essentially as described by Finnegan et al. [18], with the following modifications. RNA was digested with 20 Kunitz units RNase A (EC 3.1.27.5 from bovine pancreas) in buffer (10 mM Tris-HCl, 10 mM EDTA, 10 mM NaCl, pH 6.0) at 37°C for 1 h, prior to treatment with 3 units Pronase E (EC 3.4.24.31, protease from *Streptomyces griseus*) at 37°C, overnight. Following precipitation, washing in ethanol and removal of excess ethanol under a gentle nitrogen stream, DNA was dissolved in ultrapure water prior to quantitation by UV absorbance at 260 nm. Samples were divided into aliquots and stored at −80°C, if necessary, prior to digestion and analysis.

2.5. Formic acid digestion of DNA and HPLC-EC of 8-oxoG

To an aliquot of DNA (20 μg) in water, was added 16 nmol of 8-oxo-2,6-diaminopurine (8-oxoDAP; internal standard [19]). Samples were hydrolysed with 60% v/v formic acid in sealed, evacuated hydrolysis tubes at 140°C for 30 min. The hydrolysate was freeze-dried then reconstituted in ultrapure water and divided into two equal aliquots. Each aliquot was adjusted to pH 8.0–8.5 with 0.1 M NaOH; one

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PII: S0014-5793(98)01403-3

aliquot was treated with 0.85 mU guanase/10 μg DNA (EC 3.5.4.3, from rabbit liver) and the other with an equal volume of water at 37°C for 1 h [20]. Reversed-phase HPLC-EC was used to analyse 8-oxoG and guanine as reported by Herbert et al. [20]. Quantitation of 8-oxoG was achieved by plotting a standard curve of peak area ratio, 8-oxoG:8oxoDAP, against 8-oxoG concentration; guanine was quantitated by external calibration.

2.6. Enzymic digestion of DNA and HPLC-EC of 8-oxodG

Aliquots of DNA equivalent to 50–70 μg were freeze-dried and reconstituted in 20 mM sodium acetate, pH 4.8, containing 45 mM zinc chloride. Samples were heated in a boiling water bath for 3 min and cooled quickly on ice prior to the addition of nuclease P_1 (EC 3.1.30.1, nuclease from *Penicillium citrinum*; Calbiochem-Novabiochem, Nottingham, UK) to give 0.1 U/μg DNA and incubation of the samples at 37°C for 1 h. Samples were made alkaline by the addition of 1.5 M Tris-HCl, pH 8.0 and alkaline phosphatase (from bovine intestine; EC 3.1.3.1; Boehringer-Mannheim, Lewes, UK) added to give 0.05–0.075 U/μg DNA and incubated at 37°C for 0.5 h. Analysis of digests was performed by HPLC-EC as noted above for 8-oxoG, using the same detection parameters UV_{254nm} for 2′-deoxyguanosine and EC_{+600mV} for 8-oxodG; the mobile phase contained 10% v/v methanol. Quantitation was performed by external calibration using standards processed through exactly the same enzymic digestion procedure as the samples.

2.7. Analysis of serum 8-oxodG

Samples of whole blood were allowed to coagulate prior to centrifugation (1500×*g* for 15 min at 10°C). Serum was collected and stored at −80°C, until analysis. Following thawing the supernatants were applied to the competitive ELISA plate according to the protocol (8-OHdG check. Genox Corp., Baltimore, MD, USA) [21]. Quantitation was performed by external calibration using 8-oxodG standards.

2.8. Urinalysis

Collected urine samples from subjects were stored, without any additives, at −80°C in 20 ml plastic Universal tubes, until analysis. Previous studies have shown 8-oxodG to be stable in urine for at least 1 year, following freezing [3]. Following thawing and centrifugation (300×*g* for 10 min), the supernatants were applied to the competitive ELISA plate according to the protocol. In order to provide a internal reference for urine concentration, aliquots of urine supernatant were also assayed for creatinine (Department of Chemical Pathology, Leicester Royal Infirmary, Leicester, UK).

2.9. Statistical analyses

Data were analysed by the general linear model analysis of variance with subsequent comparison between means using Fisher's least significance difference test. Analyses were performed using Minitab, version 6.1 and GraphPad Prism, version 2.01.

3. Results

Supplementation of the subjects with 500 mg/day vitamin C resulted in a highly significant increase ($P < 0.001$) in plasma vitamin C compared to baseline and placebo timepoints, before returning to baseline after a 7-week washout period (Fig. 1). No significant difference was observed between placebo, baseline and washout values. Mean levels of 8-oxoG and 8-oxodG in PBMC DNA, as measured by HPLC-EC, showed a significant decrease ($P < 0.05$) from baseline and placebo, following 3 and 6 weeks of vitamin C supplementation (weeks 9 and 12, respectively). Following a 7-week washout period, levels of 8-oxoG then increased to a level not significantly different from those seen for baseline and placebo. Comparison of all 8-oxodG measurements in DNA, obtained throughout the vitamin C trial, with plasma vitamin C levels showed there to be a highly significant negative correlation (Table 1).

No significant variation in serum 8-oxodG, as determined by competitive ELISA, was seen at the baseline, placebo or week 9 (3 weeks of vitamin C supplementation) timepoints (Fig. 2). A very significant ($P < 0.001$) increase in serum 8-oxodG was measured following 6 weeks of vitamin C supplementation (week 12), which remained significantly elevated ($P < 0.001$) at the first washout timepoint (week 19). Further sampling 6 weeks later (week 25) showed that serum 8-oxodG concentration had returned to a level comparable with baseline. Analysis of serum 8-oxodG values showed a significant positive correlation with plasma vitamin C concentration (Table 1).

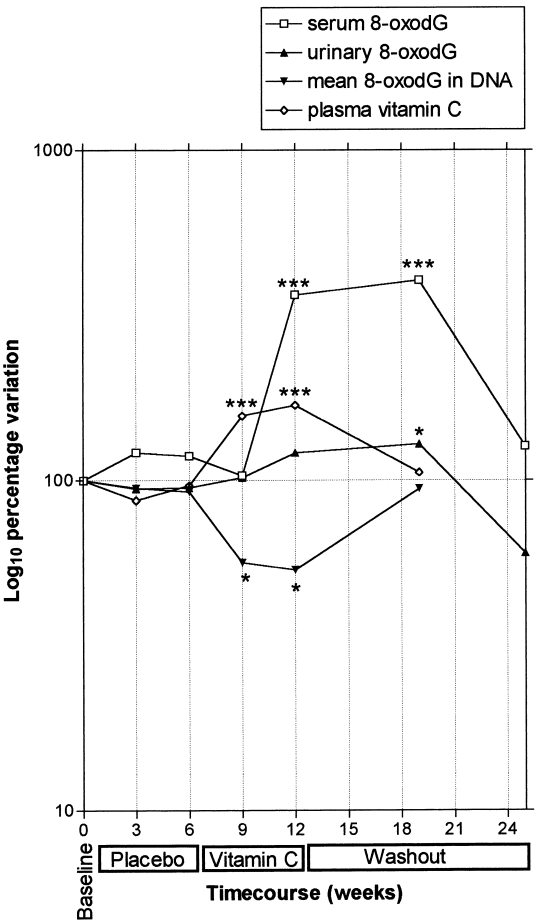

Fig. 1. Representation of in vivo 8-oxodG kinetics in cellular compartments (DNA, serum and urine) and comparison with plasma vitamin C levels. Data are expressed in terms of mean percentage variation from baseline (100%). Asterisks indicate a significant ($*P < 0.05$ and $***P < 0.001$) difference from baseline and placebo measurements.

Levels of urinary 8-oxodG were also quantitated by competitive ELISA and expressed relative to pmol of creatinine to eliminate variations in urine concentration (Fig. 3). The mean values of urinary 8-oxodG levels suggested an upward trend from week 12, reaching statistical significance ($P < 0.05$) at week 19, compared to the baseline and placebo values (Fig. 3). No statistically significant increase was seen during the vitamin C supplementation phase, at either week 9 or week 12. Likewise, no significant changes were observed during the baseline or placebo phases of the trial. Urine samples, provided by subjects at week 25, showed a decrease from the values seen at week 19. No significant correlation with plasma

Table 1
Correlation analysis data between plasma vitamin C levels, oxidative DNA damage, serum and plasma 8-oxodG levels

Analysis parameter	Oxidative DNA lesion/method of analysis					
	8-oxoA (GC-MS)	8-oxoG (GC-MS)	8-oxoG (HPLC-EC)	8-oxodG (HPLC-EC)	Serum 8-oxodG (ELISA)	Urinary 8-oxodG (ELISA)
Pearson r	0.42	−0.40	−0.38	−0.25	0.20	0.09
P value	<0.0001	<0.0001	<0.0001	<0.04	<0.04	$=0.34$
P value summary	***	***	***	*	*	ns
Significant correlation with plasma vitamin C?	Yes	Yes	Yes	Yes	Yes	No

DNA levels of damage were measured by either GC-MS or HPLC-EC; serum and urinary 8-oxodG were determined by ELISA. Statistical analyses were performed using Minitab, version 6.1 and GraphPad Prism, version 2.01.

vitamin C levels was seen. With the exception of week 9, which showed a significant decrease, urinary levels of creatinine remained constant throughout the trial. Both intra- and inter-assay variability of the competitive ELISA analysis of serum and urine were shown to be $<10\%$. The coefficient of variation of a routinely measured quality control standard for the ELISA assay was also $<10\%$.

4. Discussion

We have examined the effect of dietary supplementation with vitamin C on DNA levels of 8-oxoG, measured either as the base or as the deoxynucleoside, along with serum and urinary levels of 8-oxodG. Urinary 8-oxodG is considered a biomarker for the rate of oxidative damage to DNA [22,23], with elevated levels of urinary oxodG and, by implication, DNA damage having been noted in individuals with malignant disease compared with healthy controls [24–26]. Such findings further support urinary 8-oxodG output to be reflective of in vivo oxidative stress. Our study is the first report examining the effect of vitamin C supplementation upon both serum and urinary levels of 8-oxodG in normal, healthy individuals. There also appears to be no literature precedent for the measurement of serum 8-oxodG, which was facilitated, without enrichment, by the use of competitive ELISA. Comparison of our basal urinary 8-oxodG values with those derived from HPLC-based methodology reveal that higher levels are detected using the ELISA kit. Explanation for this is based on our experimental evidence that the antibody has the potential to bind 8-oxodG-containing oligonucleotides (unpublished results) and 8-oxoguanosine [27], both putative urinary competitors of the monoclonal antibody [28,29] and, we propose, pertinent markers of oxidative stress. Full characterisation of the monoclonal antibody used and its specificity for 8-oxodG has been reported by Toyokuni et al. [27].

Although there is evidence to suggest that dietary vitamin C insufficiency may give rise to increased levels of oxidative DNA damage in vivo [30], there have been few reports of clinical trials which support this hypothesis. Indeed, Duthie et al. [31], whilst showing a reduction in oxidative DNA damage upon supplementation with a mixture of antioxidants, suggested that vitamin C was not responsible for this effect. This finding would appear to be supported by Daube et al. [32] who showed no association between plasma vitamin C concentration and placental 8-oxodG levels. In contrast, we have recently shown that dietary supplementation with vitamin C reduces 8-oxoG levels in the DNA of circulating PBMC [16]. The potential for vitamin C to exert such an effect is supported by Fraga et al. [30]. However, the apparent antioxidant effect, seen previously in PBMC [16], occurred simultaneously with a pro-oxidant effect, evident from an increase in 8-oxoadenine (8-oxoA) levels. Analysis of the results for DNA levels of 8-oxodG and 8-oxoG, described here, showed highly significant negative correlations with plasma vitamin C levels. These findings further support the strong negative correlation between 8-oxoG and plasma vitamin C, along with a positive correlation for 8-oxoA [33].

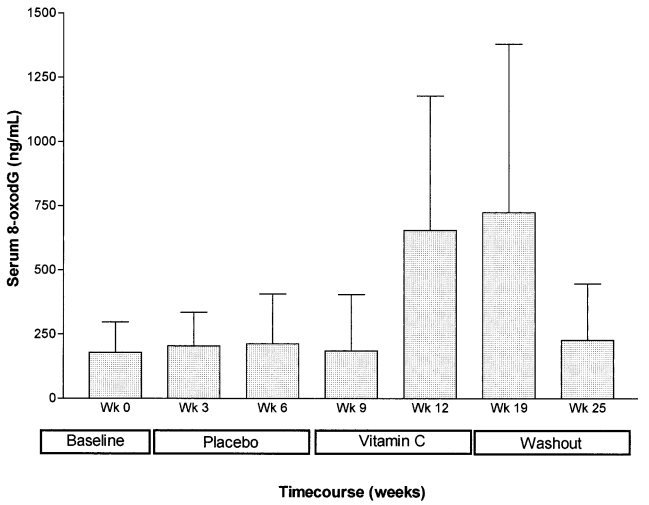

Fig. 2. Levels of 8-oxodG, determined by competitive ELISA (8-OHdG check, Genox Corp., Baltimore, MD, USA [21]), in serum from subjects undergoing vitamin C supplementation. Values represent the mean and standard deviation for each sampling timepoint.

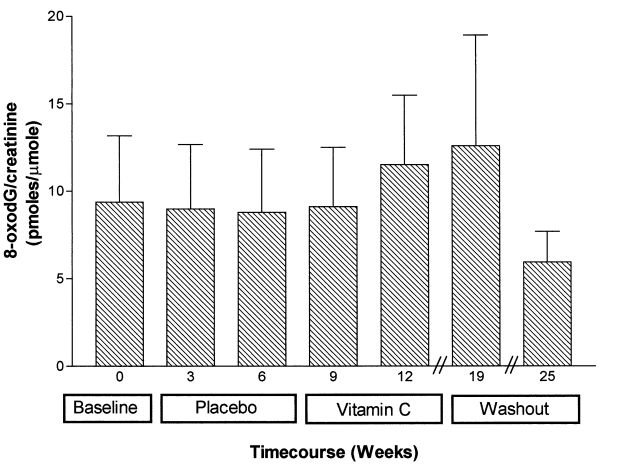

Fig. 3. Levels of 8-oxodG, determined by competitive ELISA (8-OHdG check, Genox Corp., Baltimore, MD, USA [21]), in urine from subjects undergoing vitamin C supplementation. Values represent the mean and standard deviation for each sampling timepoint.

Loft et al. [3] report that the intake of vitamin C is not associated with urinary 8-oxodG excretion. These results appear to contrast with our findings, which show that following 6 weeks of vitamin C supplementation (week 12), levels of urinary 8-oxodG have begun to rise and become significantly raised by week 19 (washout), 7 weeks following cessation of supplementation. This occurs whilst levels of 8-oxoG in the DNA of PBMC decrease during supplementation and return to basal levels at washout. The discrepancy between our result for urinary 8-oxodG and that described by Loft et al. [3] may be explained by dose. We supplemented our subjects with vitamin doses of 500 mg/day, compared to an average intake of 72 mg/day used by Loft et al. [3]. We have previously shown 500 mg/day to increase mean plasma vitamin C levels by 60%, compared to baseline [16], suggesting that 500 mg/day is more likely to be effective at increasing plasma vitamin C levels. An alternative explanation for the discrepancy may come from a recent study by Priemé et al. [4]. This study showed a 10.2% increase in 8-oxodG excretion following supplementation with 250 mg ascorbate (twice daily for 2 months) which did not achieve statistical significance. In agreement with this, we also note a non-significant, 21.9% increase following 500 mg/day for 6 weeks. However, in contrast to the study by Priemé et al. [4], we continued to collect and assay urine many weeks after cessation of vitamin C and subsequently noted a significant 30.0% increase in urinary 8-oxodG, 7 weeks post cessation of vitamin C. Clearly it is important to maintain sampling, even when plasma vitamin C levels have returned to baseline. Examination of serum 8-oxodG levels show a similar trend to that seen in the urine, only achieving significance 3 weeks in advance of the urine. It is likely that this time delay between appearing in serum and urine is reflective of the in vivo kinetics of 8-oxodG distribution between these two compartments, a facet of 8-oxodG processing previously unaddressed. The delay would also explain the lack of correlation between urinary 8-oxodG and plasma vitamin C, as the changes seen were completely out of phase.

First examination of the mean results for 8-oxodG in DNA suggests that vitamin C is acting as an antioxidant by scavenging potentially damaging oxygen free radicals and preventing the formation of 8-oxoG, whilst steady-state DNA repair removes existing lesions. However, there would appear to be limited experimental evidence for vitamin C acting as a free radical scavenger in vivo, with most evidence for this property coming from in vitro studies [34]. Nevertheless, on the basis that vitamin C does act as an antioxidant one would expect serum and urinary levels of 8-oxodG derived from DNA and other possible cellular sources of 8-oxodG to decrease. Instead an increase is seen, suggesting an apparent pro-oxidant effect upon deoxyguanosine moieties, similar to that previously noted for adenine in DNA [16]. There are a number of possible sources for 8-oxodG in the urine, regulated by enzymic processes [13–15,29]. In consideration of this, several explanations may account for the increase in serum and urinary 8-oxodG. Firstly, ascorbate may be acting as a pro-oxidant for guanine moieties not contained in DNA, e.g. dGTP, giving rise to 8-oxodGTP which can be processed to 8-oxodG and excreted [35]. Secondly, ascorbate may promote the 'purging' of 8-oxodG, derived from dGTP and/or DNA, from the cellular environment. In both cases, this would suggest that vitamin C is having some form of residual effect, detectable long after plasma values have returned to baseline, and that it is the processing of these lesions which explains the delay between their removal and appearance in the urine.

Another explanation may be that vitamin C is not acting as an antioxidant per se but promotes the removal of 8-oxodG from the DNA and/or nucleotide pool, via the upregulation of repair enzymes, previously postulated by Rehman et al. [36], perhaps induced by vitamin C's pro-oxidant properties. There is a precedent for the redox regulation of DNA repair enzymes [37], inclusion of which into our hypothesis and coupled with a concomitant pro-oxidant effect may present some explanation for the decrease in DNA levels of 8-oxoG/8-oxodG reported here and previously [16] whilst serum/urine levels of 8-oxodG increase. The increase in 8-oxoA [16] may be explained if repair of this lesion had not been stimulated. Indeed, recent work has shown that 8-oxoA, following its in situ formation rather than by mis-incorporation into DNA, is a poor substrate for yOgg1, the yeast homologue of the human hOgg1 enzyme [38]. The latter scenario is best supported by current literature and offers the most reasonable explanation for the observed experimental events.

In conclusion, this study shows that vitamin C supplementation has significant influence upon DNA, serum and urinary 8-oxodG levels in vivo and has promoted the hypothesis that vitamin C, perhaps through its redox properties [16], stimulates the repair of 8-oxodG in DNA/nucleotide pool.

Acknowledgements: The authors gratefully acknowledge financial assistance from the Ministry of Agriculture, Fisheries and Food, UK and Leicester Royal Infirmary NHS Trust. Technical assistance from Mr P. Whitaker, Miss B. Kaur and Mr S. Mohammad along with phlebotomy by members of Chemical Pathology (Leicester Royal Infirmary NHS Trust) is much appreciated.

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