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ORIGINAL CONTRIBUTION

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Vitamin C supplementation decreases oxidative DNA damage in mononuclear blood cells of smokers

Summary *Background* Antioxidants, in particular vitamin C, have been suggested to decrease oxidative DNA damage. Such effects have been shown in mononuclear blood cells in the first few hours after ingestion, whereas studies of longer-term effects in well-nourished humans have been mainly negative. *Aim* To investigate the antioxidant effect of vitamin C in terms of oxidative DNA damage measured by the comet assay and DNA repair measured by expression of *OGG1* mRNA in blood cells of male smokers given 2×250 mg vitamin C daily as plain or slow release tablets combined with plain release vitamin E 2×91 mg, or placebo for 4 wk. *Results* This study showed a difference in DNA protective effects between a slow release and a plain release vitamin C formulation. Ingestion of slow release vitamin C formulation was associated with fewer endonuclease III and formamidopyrimidine DNA glycosylase sensitive sites measured by the comet assay in mononuclear blood cells obtained 4 h and 8 h after a single tablet and 4 wk after two tablets a day. Ingestion of the vitamin formulation with plain release only indicated a damage-reducing effect 4 h after intake of a single tablet, and the effect was more apparent on endonuclease III than formamidopyrimidine DNA glycosylase sites. Overall the slow release tablets of vitamin C formulation had a more pronounced and a sustained protective effect on base damage compared with the plain release tablets. Plasma vitamin E was unaltered in the first 12 h after ingestion of a single tablet, suggesting that the antioxidant effect was mediated by vitamin C. Differences in plasma vitamin C levels at steady state could not explain the difference between the two vitamin C formulations, whereas wider amplitudes of plasma vitamin C were seen after ingestion of plain release formulation compared to slow release formulation. Assessment of *OGG1* mRNA levels by RT-PCR did not indicate increased expression of this DNA repair gene after 4 wk of vitamin supplementation. *Conclusion* This study suggests that long-term vitamin C supplementation at high dose, i. e. 500 mg together with vitamin E in moderate dose, 182 mg, decreases the steady-state level of oxidative DNA damage in mononuclear blood cells of smokers.

Key words oxidative stress – vitamin C – humans – smokers – OGG1 – oxidative DNA damage

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Introduction

Generation of oxidative DNA damage has been implicated in various diseases, including cancer [1]. On a theoretical basis, antioxidants, i. e. from the diet, should be able to inhibit the oxidation of biomolecules by reactive oxygen species (ROS) because of direct quenching or by modulation of the antioxidant defense system activity. ROS induce a wide range of oxidative damage by reaction with DNA, including strand breaks (SB) and oxidative damage at the base of the nucleotide [2]. Among the

latter, 7-hydro-8-oxo-2'-deoxyguanosine (8-oxodG) has been one of the most widely studied oxidative DNA lesions, probably because it is easy to measure and because it is a pre-mutagenic lesion [1]. As an alternative to the measurement of endogenous oxidative DNA damage levels, the antioxidant effect frequently is quantified from the resistance of leukocytes challenged *ex vivo* with agents producing oxidative DNA damage.

Vitamin C is regarded as the most important water-soluble antioxidant in plasma. The most common sources of vitamin C are fruits, vegetables and vitamin tablets. The recommended daily intake of vitamin C is easily achieved by ingestion of fruits and vegetables in healthy non-smoking humans. However, smokers typically have lower plasma vitamin C levels than non-smokers [3]. This is probably due to both a higher turnover and less intake of vitamin C in smokers [4]. Vitamin E is regarded as a very important lipid-soluble antioxidant that acts as a chain-breaking antioxidant in lipid membranes, thus antagonizing lipid peroxidation. Oxidized vitamin E is presumably regenerated by reaction with vitamin C. The overall antioxidant potential of vitamin C is consequently explained by both a direct scavenging effect in aqueous media and an indirect effect in lipids mediated by vitamin E recycling.

While vitamin C is a strong antioxidant *in vitro*, intervention studies in humans have produced mixed outcomes. Administration of a single high dose of vitamin C decreased the level of SB and lowered sensitivity to *ex vivo* challenge with H_2O_2 and ionizing radiation in leukocytes [5, 6]. The effect of vitamin C appeared to peak early, within 2–8 h after consumption [6]. In contrast, most of the long-term vitamin C intervention studies have reported no preventive effect on the level of oxidative DNA damage in leukocytes. Lee et al. did not find any effect on the 8-oxodG levels in leukocytes of smokers supplemented with 500 mg/d of vitamin C [7]. Also, a well-controlled trial with subjects initially eating identical meals containing 250 mg vitamin C/d followed by a 92-day period on 5 mg vitamin C/d, reported no effect on 8-oxodG by HPLC assay in mononuclear blood cells (MNBC), although the absolute levels of 8-oxodG were not reported [8]. Other intervention studies among non-smokers ingesting 60–260 mg/d did not indicate that 8-oxo-guanine assayed by GC/MS in leukocytes decreased because of vitamin C ingestion [9, 10]. However, it should be noted that the level of base damage in many studies, most of which have used GC/MS as detection system, are up to orders of magnitude higher than the steady-state level that is now considered to be the background level [11, 12]. Accordingly, results of such studies should be interpreted with caution. In three studies, the steady-state level of SB in MNBC was unaffected by vitamin C supplementation [13–15]. Since SB are unaltered in most antioxidant intervention studies, the unaltered SB levels in long-term vitamin C studies probably reflect the inability of this particular endpoint to reveal antioxidant effects [16]. In *ex vivo* challenge experiments, two studies found no effect of either X-ray or hydrogen peroxide [13, 15], whereas one study shows decreased sensitivity to hydrogen peroxide after 42 days of 1000 mg/d vitamin C consumption [14]. Collectively, the data indicate an immediate protective effect in leukocytes of vitamin C a few hours after ingestion, whereas most long-term intervention studies show null effect on various biomarkers of oxidative DNA damage. However, few of the studies included poorly nourished subjects with oxidative stress and all the studies have used plain release vitamin C tablets, which will yield large amplitudes of the plasma concentration time curve of vitamin C. However, it is conceivable that high peak concentrations may somehow affect the defense mechanisms negatively.

In this placebo-controlled parallel group study, we tested both the short-term and the long-term effect of vitamin C ingestion from two different release forms of vitamin C combined with a moderate dose of vitamin E. Vitamin E was included because most vitamin tablets ingested by humans habitually contain both vitamin C and E. Also vitamin E was included in the tablets because we wanted to cover both the direct hydrophilic antioxidant activity and the lipophilic antioxidant activity mediated by vitamin E recycling. Determined from *ex vivo* sensitivity to hydrogen peroxide, vitamin E supplementation had no antioxidant property at a dose of 400 mg/d [17], whereas it was associated with lower hydrogen peroxide sensitivity at a dose of 800 mg/d [14]. The only study investigating the dual effect of both vitamin C (350 mg/d) and vitamin E (250 mg/d) for 4 wk did not show altered level of SB, X-ray sensitivity or 8-oxodG in mononuclear blood cells [15]. We included only smokers in the study because smokers are prone to increased oxidative stress and require a higher daily vitamin C intake due to higher turnover. Also earlier studies have shown that smokers benefited more from dietary vitamin C supplementation than non-smokers [4, 6]. We compared the effects of plain release and slow release vitamin C tablets in two different treatment arms in order to test the possibility that a protective effect might be counteracted by negative effects of high peak concentrations.

Subjects and methods

Study design and subjects

The study was designed as a blinded, placebo-controlled parallel intervention study, with subjects randomized into three groups, consuming tablets with plain release or slow release of vitamin C in combination with vitamin E, or no vitamin C or E content (placebo formula-

tion). The study was approved by the local ethics committee of the municipality of Copenhagen.

Smoking males were recruited by newspaper advertisement (20–65 yr and > 5 cigarettes/day). Exclusion criteria included diastolic blood pressure > 105 mmHg, bodyweight (BMI $< 20^{kg}/_{m^2}$ or BMI $> 30^{kg}/_{m^2}$), and life threatening diseases. The subjects arrived after an overnight fast in the morning for the first visit. Venous blood samples were drawn for analysis at baseline (0 h), and at several time points within the first 12 h after ingestion of one vitamin tablet (corresponding to 250 mg vitamin C + 91 mg vitamin E (100 mg α-tocopheryl acetate) or placebo (Ferrosan, Denmark)). Meals were provided during the first visit, and consisted mainly of bread, meat, and desserts (devoid of antioxidants, including vitamin C and E). The breakfast meal was ingested after the first blood sample, lunch at 1 p. m., and dinner at 6 p. m. The subjects were allowed to smoke during the study. At the end of the first visit, the subjects were instructed to ingest two tablets daily (morning and evening) for the next 4 wk. Following 4 wk supplementation fasting venous blood samples were drawn for analysis (collected approximately at the same time as the first blood sample).

Detection of oxidative DNA damage in mononuclear blood cells and urine

Venous blood samples were collected in Vacutainer CPT tubes and isolated MNBC were suspended in freezing solution (50 % FBS, 40 % RPMI, 10 % DMSO of total volume) and stored at –80 °C until analysis. For logistic reasons blood samples for oxidative DNA damage were collected after 0 h, 6 h and 4 wk for the subjects in the placebo group, and 0 h, 4 h, 8 h, and 4 wk for the subjects in the vitamin supplemented groups. The steady-state level of oxidative DNA damage in MNBC was detected by the comet assay as described previously with minor modifications [18]. Cryopreserved MNBC were thawed and washed in cold PBS, and mixed with 0.75 % low melting agarose and applied onto Gelbond slides (BioWhittaker Molecular Applications, Rockland, Me, USA). The level of DNA damage was scored in a fluorescence microscope according to five classes of damage in 100 nuclei from each sample (range of score 0–400). The levels of endonuclease III (ENDOIII) and formamidopyrimidine DNA glycosylase (FPG) sensitive sites were determined as the difference in SB between slides incubated with enzyme and buffer (concentrations of enzymes were 1 μg/ml). ENDOIII and FPG enzymes were kind gifts from Dr. Andrew Collins (University of Oslo, Norway).

Quantification of OGG1 mRNA expression in leukocytes

Total RNA from 1.5 ml full blood was isolated by Qiagen RNeasy total RNA isolation kit as recommended by the manufacturer (Qiagen, USA). Synthesis of cDNA was carried out by use of TaqMan Gold RT-PCR kit by using the recommendations of Applied Biosystems. The expression of *OGG1* mRNA, normalized to 18S RNA, was determined in leukocytes by real-time RT-PCR (Roche LightCycler). The sequence of probes and primers for *OGG1* were the same as used previously [19]: forward primer: 714F: AAA TTC CAA GGT GTG CGA CTG-3', reverse primer: 796R: GCG ATG TTG TTG TTG GAG GA-3', probe: 5'-FAM-CAA GAC CCC ATC GAA TGC CTT TTC TCT TT-TAMRA-3'. Primers and probes were obtained from Applied Biosystems, UK.

Measurement of plasma vitamin concentrations

Vitamin C was measured by HPLC with coulometric detection as previously described [20]. Drawn blood samples were immediately cold-centrifuged for exactly 2 min, stabilized with an equal amount of 10 % *meta*-phosphoric acid containing 2 mM EDTA, re-centrifuged and the supernatant was stored at –80 °C until analysis within one month. Vitamin E was analyzed by HPLC with amperometric detection following hexane extraction as described by Sattler et al. [21].

Statistics

The data set of comet assay endpoints was analyzed as the difference between values obtained at baseline and during the intervention (termed delta values), and expressed as the mean and 95 % CI for 4 h, 8 h, and 4 wk. The delta values after 6 h in the placebo group were used in the statistical analysis of the effect after 4 h and 8 h supplementation. Delta values with 95 % CI that do not include zero are considered statistically significant. The *OGG1* mRNA expression was tested by repeated measures ANOVA using time and type of tablet as independent factors. The vitamin C data were analyzed by Kruskal-Wallis non-parametric tests for differences between groups, and Wilcoxon rank test for differences between baseline and 4 wk supplementation. The statistical analysis was performed with Statistica 5.5 for Windows, Statsoft, Inc. (1997), Tulsa, OK, USA.

Results

Determined from the baseline characteristics of the subjects, the three groups did not differ with respect to vit-

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amin tablet intake, smoking status, or age (Table 1). The compliance (assessed as the number of ingested tablets divided with the total number of tablets) was 90 ± 12 %, 94 ± 11 %, 61 ± 9 % for the plain, slow, and placebo groups respectively (mean ± SD). Plasma levels of vitamins C and E before and after 4 wk supplementation are outlined in Table 2. Plasma levels of vitamins C and E decreased significantly in the placebo group after 4 wk supplementation, whereas both plasma vitamins levels increased in the two other groups. There was no difference in plasma vitamin C or E between the two supplemented groups. The time course of plasma vitamin C concentration for the plain and slow release tablets is depicted in Fig. 1. As expected the peak concentration was much higher and earlier after the plain release tablets than after the slow release formulation. A preliminary analysis of plasma samples of vitamin E from 10 subjects (6 from the slow release group and 4 from the plain release group) did not indicate elevated plasma vitamin E concentration in the first 12 h after ingestion of one tablet (data not shown).

The results of measurements of oxidative DNA damage in MNBC are outlined in Fig. 2. For the statistical analysis we calculated the difference and 95 % CI between values obtained at baseline and during the intervention (termed delta values). The differences between the baseline and values obtained after 4 h, 8 h, and 4 wk of supplementation (delta values) are shown in Table 3. Ingestion of slow release vitamin C formulation was associated with decreased levels of both ENDOIII and FPG sensitive sites relative to the baseline levels at all time points, i. e. 4 h, 8 h, and 4 wk after start of supplementation. Supplementation with plain release formulation decreased the levels of ENDOIII sensitive sites at 4 h and 8 h after the first dose, whereas the levels of FPG sensitive sites were not significantly different from baseline. The effect of plain release tablets on ENDOIII sensitive sites had disappeared after 4 wk of supplementation. The level of SB was lower only 8 h after supplementation with both the slow and the plain vitamin C formulation. Considering the small effect, this may be a chance finding. No significant changes of DNA damage were observed in the placebo group except that the level of SB was significantly increased at 6 h after the first tablet. It is, however, evident from Fig. 2 that the placebo group for unknown reasons had lower levels of DNA damage at baseline than the two intervention groups.

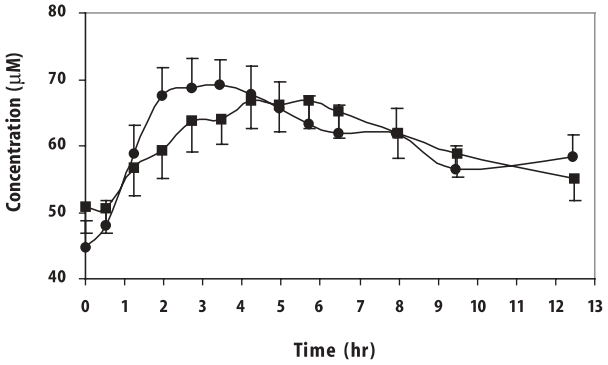

Fig 1 Plasma concentration of vitamin C in subjects supplemented with slow and plain release tablets. Points and error bars represent the mean and SEM for plain (circles) and slow (squares) release vitamin C tablets

Table 1 Characteristics of subjects at baseline (mean ± SD)

	Slow release	Plain release	Placebo	Total
Number	19	19	10	48
Age (years)	39.7 ± 12.8	38.6 ± 12.7	36.4 ± 10.6	38.6 ± 12.1
Number of subjects ingesting vitamin tablets at baseline	11	13	4	28
Alcohol (No of drinks per week)	12.2 ± 10.8	11.0 ± 13.5	16.7 ± 7.8	12.7 ± 11.5
Number cigarettes per day	16.8 ± 9.7	17.8 ± 7.3	18.3 ± 6.9	17.5 ± 8.1
Years of smoking	16.7 ± 8.5	19.4 ± 13.6	16.7 ± 11.2	17.8 ± 11.2
Smoking (packyears)	14.6 ± 13.3	17.9 ± 14.1	17.5 ± 16.3	16.5 ± 14.1

[1] There were no differences in any of the baseline characteristics between the groups ($P > 0.05$, ANOVA)

Table 2 Plasma levels of vitamin C and E before and after 4 wk supplementation (mean ± SD)

	Plain release (n = 19)	Slow release (n = 19)	Placebo (n = 10)	P-value for difference between groups
Vitamin C (before)	45 ± 19	51 ± 18	53 ± 16	0.78
Vitamin C (after)	77 ± 17*	82 ± 3*	39 ± 18*	< 0.001
Vitamin E (before)	31.1 ± 8.5	30 ± 10.3	31 ± 7.2	0.85
Vitamin E (after)	38 ± 8.5*	36 ± 9.3*	24 ± 6.0*	0.76

*Statistically significant from samples obtained before supplementation ($P < 0.05$, Wilcoxon rank test)

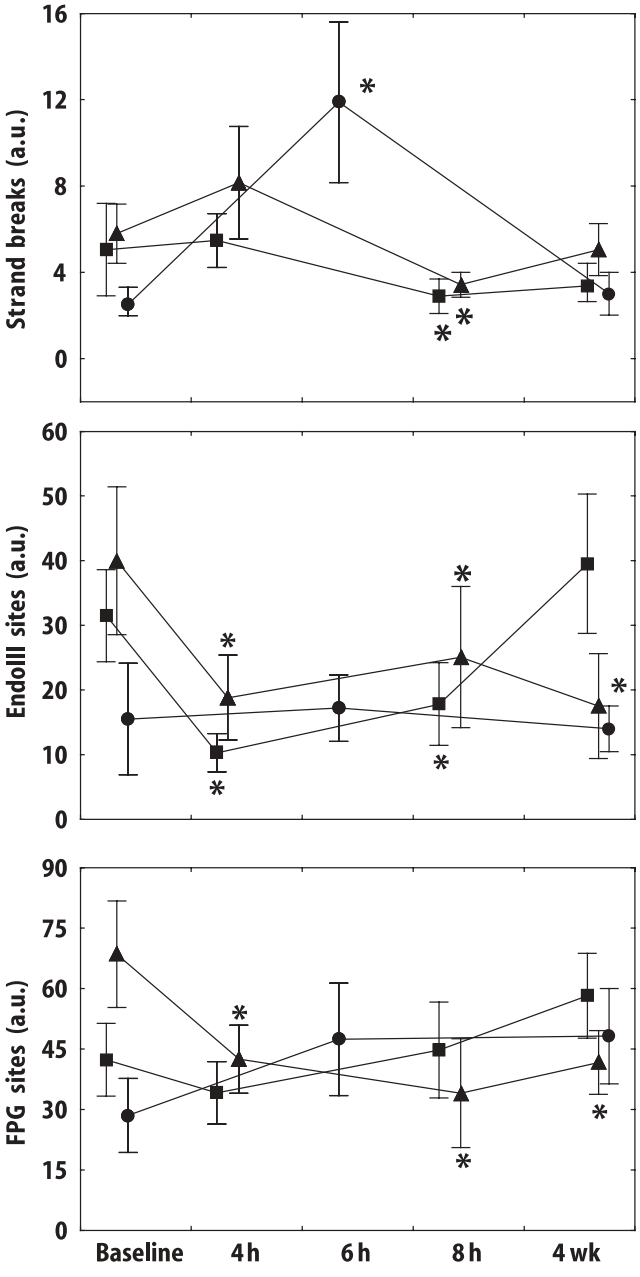

Fig 2 Level of strand breaks, endonuclease III, and FPG sensitive sites in mononuclear blood cells detected by the comet assay. Points represent the mean and SEM for plain release (squares) and slow release (triangles) vitamin C tablets, and placebo (circles).
* Denotes statistical significant from baseline values (detailed statistics of the data are provided in Table 2)

There was no association between ingestion of the two vitamin C formulations and expression of *OGG1*, determined as mRNA levels by real-time RT-PCR (Table 4). A strong period effect was observed for all three groups ($P < 0.0001$, ANOVA).

Discussion

In this study, we show that ingestion of a regular 250 mg vitamin C tablet produces a transient protective effect on the level of ENDOIII sensitive sites, whereas tablets with slow release of vitamin C produce a long-term protective effect that is evident on both FPG and ENDOIII sensitive sites. Importantly, the results reported here support the theory that the antioxidant effect of plain release vitamin C tablets is immediate. The tablet formulation also contained plain-release vitamin E in moderate amounts. Determined by the comet assay, a high dose of vitamin C (1 g) was associated with a marked decrease in H_2O_2-sensitivity 2–4 h after ingestion, whereas high vitamin E (1 g) dose only had effect at later time points, e. g. 18 h and 24 h after ingestion [6]. It is therefore likely that the immediate effect of the vitamin supplementation is entirely due to vitamin C, which is also absorbed much faster than vitamin E. The presence of plain release vitamin E in the tablets does not explain the antioxidant effect exerted by the slow release vitamin C formulation after 4 wk supplementation. Additionally, the vitamin E amounts supplied in this study were lower compared with other studies that have investigated the antioxidant effect of vitamin E either as a single antioxidant or in combination with other antioxidants. Most have investigated daily vitamin E doses in excess of 280 mg [14, 17, 22, 23]. This indicates that the reduction of endogenous oxidative DNA damage seen in this study is likely to be due to vitamin C. The differences between the two forms of vitamin C tablets and the unchanged levels in the parallel placebo group indicate that our findings are not due to a period effect.

Since we found no difference in expression of *OGG1* mRNA levels, the lower number of FPG sensitive sites in the slow release vitamin C group cannot be explained by an upregulation of this DNA repair system at the level of transcription or stabilization of mRNA (however putative post-translational regulations of this DNA repair system are not detected by mRNA RT-PCR analysis). Bearing this limitation in mind, we suggest that the protective effect of slow release vitamin C is an antioxidant effect rather than one of increased DNA repair. The repair of FPG sensitive sites *in vitro* in primary lymphocytes proceeds with a half-life of less than one hour [24]. This indicates that with a proficient antioxidant effect of vitamin C, most lesions should have been removed after 4 h. However, the subjects were allowed to smoke during the trial and considering that endogenous generation of oxidative DNA damage is an inevitable fact of aerobic life, the level of FPG sites 4 h after ingestion may reflect a steady-state level of oxidative DNA damage in vitamin C-supplemented subjects (i. e. less than 40 a. u. score). This also indicates that the repair activity is sufficiently high to confer fast alterations in the level of oxidative DNA damage if antioxidants are ingested. We have no

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Table 3 Alteration in oxidative DNA damage in mononuclear blood cells after antioxidant supplementation (mean and 95 % CI)[1, 2]

	Slow release	Plain release	Placebo
Strand breaks			
4 h	2.4 (0.04;4.7)	0.42 (–1.9;2.8)	
6 h			11.9 (9.6;14.2)
8 h	–2.4 (–3.9;–0.9)	–2.15 (–3.6;–0.7)	
4 wk	–0.7 (–2.5:1.1)	–1.7 (–3.5;0.1)	0.5 (–1.3;2.3)
ENDOIII sensitive sites			
4 h	–21.2 (–29.9;–12.4)	–21.2 (–30.0;–12.5)	
6 h			1.7 (–8.2;11.6)
8 h	–14.9 (–24.8;–5.0)	–13.7 (–23.6;–3.8)	
4 wk	–22.5 (–33.3;–11.7)	8.0 (–2.8;18.8)	–1.5 (–12.3;9.3)
FPG sensitive sites			
4 h	–26.1 (–34.5;–17.2)	–8.2 (–17.1;0.7)	
6 h			18.9 (6.6;31.2)
8 h	–34.5 (–46.8;–22.2)	2.4 (–9.9;14.7)	
4 wk	–26.8 (–33.4;–16.3)	15.9 (5.4;26.4)	19.7 (9.2;30.2)

[1] The delta values in oxidative DNA damage are calculated as the differences in score between the baseline values and values obtained after 4 h, 8 h, or 4 wk of vitamin C supplementation. The delta value for the placebo group is calculated for 6 h and 4 wk. The delta value of the placebo group after 6 h has been used in the calculation of 95 % CI for delta values after 4 h and 8 h in the supplementation groups. The delta values are calculated from the original data presented in Fig. 2

[2] The delta values are statistically significant if the 95 % CIs do not include zero

Table 4 Expression of OGG1 in leukocytes[1]

	Baseline	Baseline after 4 wk supplementation
Slow release	5.4 ± 3.2 (19)	3.5 ± 1.5 (19)
Plain release	5.5 ± 6.0 (18)	2.8 ± 1.3 (18)
Placebo	9.9 ± 12.2 (10)	2.5 ± 1.7 (10)
Total	6.4 ± 7.0 (47)	3.0 ± 1.5 (47)[2]

[1] The data are expressed as mean ± SD, and number of subjects in brackets

[2] The expression of OGG1 mRNA was reduced after 4 wk of supplementation in all the groups ($P < 0.0001$, ANOVA) . There was no statistically significant interaction between the type of tablet and time point, and there was no single factor effect of the type of tablet

obvious explanation of the period effect of the *OGG1* mRNA level. Interestingly, we also observed a period effect in the level of *OGG1* mRNA expression in a fruit and vegetable withdrawal study, although this was increased throughout the study [19]. Moreover, period effects of oxidative DNA damage have been observed in some placebo-controlled intervention trials, using parallel or crossover designs [10, 23, 25, 26]. This shows the importance of using strong experimental placebo-controlled designs with parallel interventions to identify antioxidant effects in human molecular epidemiological studies. Thus, we can rule out that there are any major changes in OGG1 activity related to the intervention in the present study. Other repair systems, like nucleotide excision repair, were not investigated in this study and may or may not be altered.

The baseline plasma vitamin C level in this study was 48.9 μM, which is among the lowest reported for biomarker-based intervention studies investigating oxidative DNA damage. The supplementation increased the plasma vitamin C concentration above 60 μM in a few hours after ingestion, and even higher plasma concentrations were detected as baseline levels after 4 wk of supplementation. The formulations also contained vitamin E and ensured that this lipid soluble antioxidant was present in a sufficient amount. Several of the previous studies showing null effects have enrolled subjects with > 60 μM plasma vitamin C at baseline [7, 8, 13]. The protective effect observed in the present study is likely due to the subjects being under increased oxidative stress (smokers). In this respect, it is worth noting that the number of ENDOIII and FPG sensitive sites at baseline were markedly higher than we normally observe among healthy nonsmokers, i. e. usually less than 10 arbitrary units [27]. The aggregated data indicate that vitamin C supplementation confers no protective effect in terms of oxidative DNA damage if the plasma concentration is already high at baseline. This view is supported by the null effect studies of urinary 8-oxodG excretion using subjects with initial plasma vitamin C concentrations above 60 μM [28, 29]. The lack of antioxidant effect in subjects with high plasma concentration of vitamin C probably results from vitamin C being largely excreted or not absorbed in these individuals [30].

Although we only observed protective effects in terms of oxidative DNA damage in the vitamin C supplemented groups, the level of oxidative DNA damage in MNBC did not appear to be related to the steady-state plasma level of vitamin C. Most other studies of oxidative DNA damage have used tablet formulations that were similar to the plain release vitamin C tablet. In agreement with these, the results of plain release vitamin C in this study support

the notion that the effect of vitamin C is short-lived. Interestingly, the slow release vitamin C formulation overtly decreased the level of ENDOIII and FPG sensitive sites without direct relationship with the plasma ascorbate concentration. We cannot rule out that the difference between the two vitamin C supplemented groups is a chance finding, and the results warrant further investigations before firm conclusions can be made. Nevertheless, it is possible that high peak concentrations of vitamin C may be prooxidant, result in greater loss of vitamin C through urinary excretion or cause downregulation of other antioxidant defense systems. A large number of genes are regulated by oxidative stress [31], although the exact importance of this has not been determined *in vivo*. Whereas vitamin C has well known prooxidant capacities in the presence of transition metals *in vitro*, convincing evidence of such effects *in vivo* is very limited [32], and may not be relevant because of the *in vivo* mechanisms to avoid free transition metals.

In conclusion, this study has shown that vitamin C supplementation decreases the level of oxidative DNA damage in samples obtained within a few hours after ingestion, whereas only vitamin C ingested as a slow release formulation provides a protective effect after long-term supplementation. The difference between slow and plain release vitamin C warrants further investigations before firm conclusions can be made.

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