

## Antioxidant vitamin supplements do not reduce reactive oxygen species activity in *Helicobacter pylori* gastritis in the short term

Simon M. Everett\*, Ian M. Drake<sup>2</sup>, Kay L. M. White<sup>1</sup>, Nic P. Mapstone<sup>1</sup>, Douglas M. Chalmers<sup>1</sup>, Chris J. Schorah<sup>1</sup> and Anthony T. R. Axon<sup>1</sup>

<sup>1</sup>The Centre for Digestive Diseases, The General Infirmary at Leeds, Great George Street, Leeds LS1 3EX, UK

<sup>2</sup>Chorley and South Ribble District General Hospital, Preston Road, Chorley, Lancashire, PR7 1PP, UK

(Received 20 February 2001 – Revised 17 July 2001 – Accepted 28 August 2001)

Reactive oxygen species have been implicated in *Helicobacter pylori*-mediated gastric carcinogenesis, whereas diets high in antioxidant vitamins C and E are protective. We have examined the effect of vitamin C and E supplements in combination with *H. pylori* eradication on reactive oxygen species activity in *H. pylori* gastritis. *H. pylori*-positive patients were randomized into four groups: triple therapy alone (Bismuth chelate, tetracycline, and metronidazole for 2 weeks), vitamins alone (200 mg vitamin C and 50 mg vitamin E, both twice per day for 4 weeks), both treatments or neither. Plasma and mucosal ascorbic acid, malondialdehyde and reactive oxygen species were determined before and after treatment. Compared with normal controls (*n* 61), *H. pylori*-positive patients (*n* 117) had higher mucosal reactive oxygen species and malondialdehyde levels and lower plasma ascorbic acid. Plasma ascorbic acid doubled in both groups of patients receiving vitamins and mucosal levels also increased. Malondialdehyde and reactive oxygen species fell in patients in whom *H. pylori* was eradicated but vitamin supplements were not effective either alone or in combination with *H. pylori* eradication. Supplements of vitamins C and E do not significantly reduce mucosal reactive oxygen species damage in *H. pylori* gastritis.

**Ascorbic acid: Vitamin C: Vitamin E: Reactive oxygen species: Malondialdehyde: Gastric carcinoma: *Helicobacter pylori***

Gastric cancer is the second commonest cause of death from malignant disease worldwide (Parkin *et al.* 1993). Strategies to prevent this condition are therefore of great importance. It is well established that diets containing a high intake of the antioxidant vitamin C are associated with reduced risk of developing gastric cancer (Block, 1991). Vitamin E, which also has antioxidant properties, may play an additional protective role (Knekt *et al.* 1991). In recent years it has also been recognized that chronic infection of the gastric mucosa by *Helicobacter pylori* plays a pivotal role in gastric carcinogenesis (International Agency for Research on Cancer, 1994).

Infection of the gastric mucosa with *Helicobacter pylori* usually causes both an acute and chronic inflammatory cell infiltrate, leading to an increase in reactive oxygen species (ROS) (Davies *et al.* 1994). These are highly reactive compounds capable of combining with DNA in a number of potentially genotoxic ways (Jackson *et al.* 1989; Guyton & Kensler, 1993). ROS can react with the

lipid bilayer releasing peroxidation products, such as malondialdehyde. These compounds, which are also able to react with DNA, have been shown to accumulate in *H. pylori* gastritis (Marnett, 1994; Farinati *et al.* 1996; Drake *et al.* 1998). These processes could lead to alterations in the structure of DNA facilitating mutations and carcinogenesis.

In the normal stomach, ascorbic acid and total vitamin C are highly concentrated from plasma into the mucosa and (to a lesser extent) gastric juice (Sobala *et al.* 1989; Banerjee *et al.* 1994). Vitamin C exists as ascorbic acid or dehydroascorbic acid. Ascorbic acid is the reduced form of the vitamin and can act as a potent antioxidant, able to scavenge ROS in gastric mucosa (Frei, 1991; Drake *et al.* 1996). This has been proposed as one means by which it exerts an anticarcinogenic effect (Correa, 1995). Ascorbic acid may also prevent formation of *N*-nitroso compounds in gastric juice by scavenging nitrite (Mirvish *et al.* 1972). Ascorbic acid is found principally in the aqueous phase

**Abbreviation:** ROS, reactive oxygen species.

\* **Corresponding author:** Dr Simon M. Everett, fax +44 113 242 9722, email severett@doctors.net.uk

whereas vitamin E ( $\alpha$ -tocopherol) exists mainly in the lipid phase. Vitamin E regenerates ascorbic acid from its oxidised form dehydroascorbic acid and acts synergistically with ascorbic acid to prevent lipid peroxidation (Cadenas *et al.* 1996).

One way in which it may be possible to prevent carcinogenesis would be to reduce ROS damage to cellular constituents, especially DNA. It has been demonstrated that eradication of *H. pylori* leads to a reduction in ROS activity in the gastric mucosa (Drake *et al.* 1998). An alternative or additional approach might be to supplement antioxidant vitamins in the diet. The aim of the present study was, therefore, to determine whether dietary supplements of vitamins C and E are able to reduce levels of ROS and lipid peroxidation in the gastric mucosa, either alone or in combination with *H. pylori* eradication. We have randomized *H. pylori* positive patients in a 2  $\times$  2 study design to receive eradication therapy or placebo for 2 weeks followed by vitamins C and E or placebo for 4 weeks (making four separate randomized groups) and assessed ROS and lipid peroxidation before and at the end of treatment.

## Methods

### Study design

This was a randomized, double blind, controlled trial. Patients with dyspepsia aged between 18 and 80 years attending for routine out-patient endoscopy were invited to participate. Patients were excluded if any of the following were present: pregnancy or lactation, significant comorbidity, antibiotic use in the previous 6 weeks, previous attempts at *H. pylori* eradication, previous gastric surgery or a previous history of alcohol or drug abuse.

All patients attended for endoscopy at the same time (14.00 hours) after an overnight fast. Prior to endoscopy, a 10 ml sample of venous blood was withdrawn into a lithium heparin tube. Antral biopsies were taken for *H. pylori* culture and urease test, along with two biopsies from antrum and corpus for histological assessment. If at least two out of these three tests were positive then patients were deemed *H. pylori* positive, and *H. pylori* negative if all three were negative. Control patients were also attending for investigation of dyspepsia but had all tests negative for *H. pylori* and histologically normal gastric mucosa. Further biopsies were taken from all patients as follows: two from the antrum for luminol-enhanced chemiluminescence, one from the antrum for malondialdehyde assessment, and one from both antrum and corpus for ascorbic acid and vitamin C determination.

*H. pylori*-positive patients were randomized on a 1:1:1:1 basis to receive one of the following treatments: (1) colloidal bismuth subcitrate (120 mg) plus tetracycline (500 mg), both four times per day, plus metronidazole (400 mg) three times per day (triple therapy), followed by ascorbic acid (200 mg) plus vitamin E,  $\alpha$ -tocopherol (50 mg), both twice per day; (2) triple therapy followed by vitamin placebo; (3) triple therapy placebo followed by vitamins C and E; or (4) triple therapy placebo followed by vitamin placebo. In all cases, antibiotic (triple) therapy or its placebo was for 2 weeks followed by vitamin

supplementation or its placebo for 4 weeks. Thus, patients received triple therapy and antioxidant vitamins (triple–vitamins), triple therapy alone (triple–placebo), vitamins alone (placebo–vitamins), or neither (placebo–placebo). Consecutive patients were allocated treatment randomly according to a list kept in pharmacy. Placebo tablets (lactose) were identical in appearance to the study drugs and all drugs were dispensed by the pharmacy to maintain investigator blinding. At the end of the 6-week treatment period, patients returned for repeat endoscopy and sampling. Patients were instructed not to take tablets on the day of endoscopy. The study was approved by the Local Research Ethics Committee and all patients gave written informed consent.

### Histology

Biopsies were fixed in 10% buffered formalin. Sequential 3  $\mu$ m thick sections were cut and stained with haematoxylin and eosin and modified Giemsa's stain. Gastritis was scored from 0–3 for acute inflammation, chronic inflammation, atrophy, intestinal metaplasia and *H. pylori* infection density according to the updated Sydney classification by a single blinded histopathologist (Dixon *et al.* 1996).

### *Helicobacter pylori* culture

A single biopsy was immediately transferred to brain–heart infusion broth and incubated microaerophilically at 37°C on a VPAT plate (vancomycin, polymyxine B, amphotericin, trimethoprim) for 8 d. Plates were inspected for *H. pylori* at 4, 6 and 8 d.

### Luminol-enhanced chemiluminescence

Chemiluminescence probes such as luminol and lucigenin react with ROS to form 3-aminophthalate and *N*-methyl-acridone. The excited electrons in these compounds relax with the emission of energy as light which can be detected by a scintillation counter. Luminol has been found to be a more sensitive chemiluminescent probe than lucigenin in colonic and gastric biopsies (Simmonds *et al.* 1992; Davies *et al.* 1994). The reaction of H<sub>2</sub>O<sub>2</sub> and hypochlorite with luminol is catalysed by neutrophil-derived myeloperoxidase; inhibition of this enzyme by sodium azide leads to maximal reduction in chemiluminescence suggesting that these are the most important metabolites detected by this technique. Consistent with this, chemiluminescence results correlate with acute inflammatory scores in mucosal biopsies. Chemiluminescence is also inhibited by catalase and dimethyl superoxide suggesting detection of H<sub>2</sub>O<sub>2</sub> and hydroxyl radicals respectively. Thus, luminol was selected for the present study as it is a suitable probe to detect neutrophil-derived ROS in gastric biopsies (Drake *et al.* 1998).

Two antral biopsies from each patient were transferred immediately into pre-oxygenated PBS (pH 7.4) with added glucose (5 mM), M and C, and assayed within 3 h. Prior to weighing, each biopsy was added to a pre-counted scintillation vial containing 1 ml 75  $\mu$ M luminol solution and counted for 5 min in a liquid scintillation counter set in

the 'out of coincidence' mode. Three measurements of each sample were made and the mean calculated. The within-subject CV of this assay, based on ninety-nine pairs of biopsies, was 71%. Although high, this is consistent with previous reports of the variability of this technique, and is small in comparison with a 20-fold increase in chemiluminescence between inflamed and normal gastric mucosa shown by our results (Simmonds *et al.* 1992; Davies *et al.* 1994).

#### *Malondialdehyde*

For each patient, one antral biopsy was snap-frozen in liquid N<sub>2</sub> at the time of endoscopy, stored at -70°C, and assayed within 2 weeks by a modification of the procedure of Yagi (1976). Following thawing, blotting and weighing, each biopsy was immersed in 4 ml water. Thiobarbituric acid solution (1 ml), made by dissolving 0.167 g thiobarbituric acid solution in 50 ml water – glacial acetic acid (1:1, v/v), was then added. A set of malondialdehyde standards was also freshly prepared and added to 1 ml thiobarbituric acid solution prepared in the same way. All mixtures were heated at 100°C for 60 min. After cooling on ice, 5 ml butan-1-ol was added to extract the malondialdehyde equivalents. The tubes were centrifuged at 1100 g for 10 min to separate the aqueous and butan-1-ol phases. Fluorescence of the butan-1-ol phase at 555 nm was determined using an excitation wavelength of 515 nm. Values from the tissue specimens were compared with the standard solutions. The within-subject CV of this technique is 16.8% (based on paired biopsies from 208 patients).

It should be noted that there can be difficulties in the interpretation of the results of this assay. First, aldehydes other than malondialdehyde produced in lipid peroxidation can be detected. Second, much of the malondialdehyde measured is generated by decomposition of lipid hydroperoxides during the acid-heating stage of the assay. In addition, other compounds such as bilirubin, sugars and amino acids can also be reactive towards thiobarbituric acid. Finally, malondialdehyde detected in this assay may also be generated as a byproduct of cyclooxygenase activity in platelets (Emma *et al.* 2000). However, the first two of these processes reflect other pathways in lipid peroxidation and suggest that, whilst measuring thiobarbituric acid reactive substances in this assay is not specific for malondialdehyde levels *in vivo*, it is reflective of lipid peroxidation. This is supported by previous studies that have shown significant correlations between malondialdehyde concentrations measured by this assay and both chemiluminescence and its own DNA adduct, malondialdehyde deoxyguanosine (Drake *et al.* 1998; Everett *et al.* 2001). Thus, although non-specific, results from this assay complement the chemiluminescence results and together these assays provide estimations of neutrophil-derived ROS and consequent lipid peroxidation.

#### *Ascorbic acid and total vitamin C measurements*

Venous blood samples were centrifuged. One 0.5 ml aliquot of plasma was added to 1.0 ml metaphosphoric acid (20 ml/l). A second 0.5 ml aliquot was added to 9 mg

dithiothreitol prior to addition of 1.0 ml of metaphosphoric acid (20 ml/l) for reduction of dehydroascorbic acid to ascorbic acid and subsequent analysis of total vitamin C. All samples were snap-frozen in liquid N<sub>2</sub> and stored at -70°C. Prior to analysis, samples were thawed and centrifuged at 1000 g. The supernatant fraction was analysed by HPLC using reversed-phase ion pair chromatography on a C<sub>18</sub> column (Sobala *et al.* 1991). Ascorbic acid was selectively measured using an electrochemical detector set at 350 mV. Total vitamin C (the sum of ascorbic acid and dehydroascorbic acid) was determined from the solutions initially prepared with dithiothreitol after incubation at 45°C for 120 min.

Biopsy samples were frozen in liquid N<sub>2</sub> immediately after endoscopy, stored at -70°C and assayed within 2 weeks. After thawing, biopsies were homogenized in 0.5–1.0 ml metaphosphoric acid and divided into two parts. For total vitamin C determination, dithiothreitol was added to one part to a final concentration of 6 mg/ml and incubated at 45°C for 120 min prior to analysis. HPLC was then performed for both parts as described for plasma. The within-subject CV for both ascorbic acid and total vitamin C was 5%.

#### *Statistical analysis*

Data for most variables are skewed, and in some instances normality could not be achieved by log transformation. For consistency, therefore, data are presented as median values with interquartile ranges and non-parametric statistics are used (with the exception of linear regression). For patients receiving triple therapy, results presented are for those in whom *H. pylori* was successfully eradicated, and for patients receiving placebo triple therapy, results presented are for those who remained *H. pylori* positive at follow-up. The Wilcoxon signed-ranks test was used to compare paired pre- and post-treatment data for the randomized groups. Mann-Whitney U tests were used for comparison of unpaired data and Kruskal-Wallis test was used for comparison of more than one group. Spearman's  $\rho$  was calculated for correlations. To calculate the effect of multiple variables on plasma ascorbic acid, multiple linear regression was used with log conversion of the plasma ascorbic acid to maintain normality and constant variance; data are presented as regression slope (B) with 95% CI. Two-tailed *P* values were calculated in all cases; a value <0.05 is taken to imply statistical significance. Calculations were performed using SPSS for Windows 8.0 (SPSS UK Ltd, Woking, Surrey).

## **Results**

### *Patients*

*H. pylori*-positive patients (*n* 117) were randomized to treatment. Of these, ninety-eight patients returned for the second visit. Control patients with normal gastric histology and negative *H. pylori* tests (*n* 63) were also studied. Sex, age, smoking, alcohol intake, use of non-steroidal anti-inflammatory drugs and acid suppressive therapy were recorded prior to the first endoscopy. Patients with normal

**Table 1.** Baseline characteristics for patients with normal gastric histology, *Helicobacter pylori* gastritis, and the four randomized *Helicobacter pylori*-positive groups (Mean values and ranges)

	Controls		All		Triple*+vitamins†		Placebo+vitamins†		Triple*+placebo		Placebo+placebo	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
	<i>n</i>	63		117		29		29		30		29
Age (years)	38	18–66	49***	17–75	52	17–75	48	19–68	49	22–65	49	26–70
Sex (% males)	60.3		57.3		62.1		55.2		53.3		58.6	
Smokers (%)*‡	27.8		42.6		35.7		44.4		46.2		42.9	
NSAID (%)*§	9.4		6.4		7.1		3.7		7.4		7.1	
Acid suppression (%)	26.4		27.5		25.0		25.9		33.3		28.6	

NSAID, non-steroidal anti-inflammatory drugs.

\*Triple: *H. pylori* eradication therapy comprising colloidal bismuth subcitrate, tetracycline and metronidazole for 2 weeks.

†Vitamins: ascorbic acid and  $\alpha$ -tocopherol for 4 weeks.

‡Daily smoker of cigarettes or cigars.

§Intake of NSAID, within the previous month.

|| Either H<sub>2</sub> receptor antagonist or proton pump inhibitors taken within the previous month.

\*\*\*  $P < 0.001$ , compared with normal gastric mucosa.

gastric histology were younger than *H. pylori*-positive patients ( $P < 0.001$ ), but there were no other significant differences between *H. pylori*-negative and *H. pylori*-positive patients or within the four randomized groups (Table 1).

### Baseline results

There were no significant differences in baseline measurements of malondialdehyde, chemiluminescence and plasma, antral and corpus ascorbic acid and total vitamin C in the four randomized groups (Table 2).

Antral chemiluminescence and malondialdehyde were both higher in *H. pylori*-positive patients than uninfected patients ( $P < 0.001$  for both), reflecting increased ROS activity. Malondialdehyde levels correlated significantly with chemiluminescence ( $\rho 0.2$ ,  $P = 0.009$ ).

Plasma ascorbic acid was significantly lower in *H. pylori*-positive patients than in *H. pylori*-negative patients ( $P = 0.02$ ) and the percentage plasma vitamin C in the reduced ascorbic acid form was also significantly lower in *H. pylori*-positive than *H. pylori*-negative patients (89.9 v. 95.3%,  $P = 0.006$ ). There were no differences in antral and corpus ascorbic acid levels between *H. pylori*-negative and -positive patients. However, ascorbic acid and total vitamin C were both significantly higher in the antrum than the corpus. This was true both for *H. pylori*-positive (584.8 v. 356.0  $\mu\text{mol/kg}$ ,  $P < 0.001$  for ascorbic acid and 681.9 v. 444.6  $\mu\text{mol/kg}$ ,  $P < 0.001$  for total vitamin C) and *H. pylori*-negative patients (451.4 v. 340.1  $\mu\text{mol/kg}$ ,  $P < 0.001$  for ascorbic acid and 572.9 v. 392.9  $\mu\text{mol/kg}$ ,  $P < 0.001$  for vitamin C). There was a significant positive correlation between chemiluminescence and antral ascorbic acid ( $\rho 0.19$ ,  $P = 0.02$ ) and total vitamin C ( $\rho 0.17$ ,  $P = 0.03$ ). No such correlation existed between malondialdehyde and either ascorbic acid or total vitamin C.

Chemiluminescence and malondialdehyde were not affected by patient sex, smoking history (smokers v. non-smokers), intake of non-steroidal anti-inflammatory drugs or use of acid-suppressive therapy. Chemiluminescence correlated significantly with age ( $\rho 0.27$ ,  $P < 0.001$ ). This correlation was not evident, however, when analysis was limited to *H. pylori*-positive patients ( $\rho 0.02$ ,  $P = 0.8$ ), suggesting that this is a result of confounding by the older age of *H. pylori*-positive patients.

Plasma ascorbic acid (16.5 v. 47.1  $\mu\text{mol/l}$ ,  $P = 0.02$ ), total plasma vitamin C (18.2 v. 50.5  $\mu\text{mol/l}$ ,  $P < 0.001$ ), antral ascorbic acid (403.7 v. 633.1  $\mu\text{mol/kg}$ ,  $P = 0.007$ ) and total antral vitamin C (538.3 v. 687.0  $\mu\text{mol/kg}$ ,  $P = 0.01$ ) were all lower in smokers than non-smokers, and plasma ascorbic acid (30.7 v. 44.9  $\mu\text{mol/l}$ ,  $P = 0.03$ ) and total plasma vitamin C (35.8 v. 47.7  $\mu\text{mol/l}$ ,  $P = 0.05$ ) were both lower in males than females. Furthermore, plasma ascorbic acid and total plasma vitamin C correlated negatively with age ( $\rho -0.21$ ,  $P = 0.008$  and  $\rho -0.21$ ,  $P = 0.007$  respectively). There were no other significant correlations between age and measures of ascorbic acid or total vitamin C. In order to determine which of age, sex, smoking and *H. pylori* infection were most important in determining plasma ascorbic acid concentrations a multiple linear regression model was constructed, with log-converted plasma ascorbic acid as the dependent factor. Age (B = -0.07, 95% CI -0.01,

**Table 2.** Pretreatment chemiluminescence, malondialdehyde, plasma, antral and corpus ascorbic acid in patients with normal gastric histology, *Helicobacter pylori* gastritis, and the four randomized *Helicobacter pylori*-positive groups\* (Median values and interquartile ranges)

	Controls			<i>Helicobacter pylori</i> -positive								
	Median	Interquartile range	All	Triple†+vitamins§		Placebo+vitamins§		Triple†+placebo		Placebo+placebo		
			Median	Interquartile range	Median	Interquartile range	Median	Interquartile range	Median	Interquartile range	Median	Interquartile range
<i>n</i>	63		117	29	29	29	30	29	30	29	29	30
Chemiluminescence (cpm/mg)	860	299–2634	15 654***	17 316	5347–71 366	19 278	3382–46 948	13 528	3276–33 194	14 591	5710–36 869	14 591
Malondialdehyde (µmol/kg)	79.0	61.5–106.4	114.4***	106.8	80.6–140.5	111.0	88.5–140.6	126.4	93.6–146.6	119.2	85.5–140.1	119.2
Plasma ascorbic acid (µmol/l)	44.3	13.3–60.8	35.2†	32.9	11.4–48.8	35.2	10.8–61.9	35.2	14.5–57.3	35.2	14.2–55.1	35.2
Antral ascorbic acid (µmol/kg)	451.4	290.1–673.4	584.8	573.5	332.2–814.8	651.8	344.1–849.4	504.2	287.3–968.1	589.9	401.4–1039.1	589.9
Corpus ascorbic acid (µmol/kg)	340.1	239.0–432.7	356.0	410.0	183.4–620.0	310.0	196.5–462.8	331.6	131.7–490.0	517.8	265.7–739.3	517.8

\* For details of subjects and procedures, see Table 1 and p. 4.

†  $P < 0.02$ , \*\*\*  $P < 0.001$ , compared with normal gastric mucosa.

‡ Triple: *H. pylori* eradication therapy comprising colloidal bismuth subcitrate, tetracycline and metronidazole for 2 weeks.

§ Vitamins: ascorbic acid and  $\alpha$ -tocopherol for 4 weeks.

–0.003,  $P=0.002$ ), female sex ( $B = 0.17$ , 95 % CI 0.06, 0.29,  $P=0.004$ ) and smoking ( $B = -0.10$ , 95 % CI –0.15, –0.05,  $P < 0.001$ ) all remained significantly predictive of plasma ascorbic acid whereas *H. pylori* infection became non-significant ( $B = 0.10$ , 95 % CI –0.03, –0.24,  $P=0.1$ ).

Sydney classification of gastritis was available for seventy-six *H. pylori*-positive patients. Mean score for acute inflammation was 1.25 (SD 0.54), for chronic inflammation was 1.80 (SD 0.69), for atrophy was 1.08 (SD 0.63), for intestinal metaplasia was 0.41 (SD 0.75) and for *H. pylori* infection density was 1.80 (SD 0.83). There were no correlations between any of these variables and either ascorbic acid or total vitamin C in plasma or mucosa. Chemiluminescence correlated positively with acute antral inflammation ( $\rho 0.40$ ,  $P < 0.001$ ), chronic antral inflammation ( $\rho 0.28$ ,  $P=0.01$ ) and antral atrophy ( $\rho 0.27$ ,  $P=0.02$ ). Surprisingly, malondialdehyde correlated negatively with acute antral inflammation ( $\rho -0.32$ ,  $P=0.006$ ) but none of the other variables of gastritis.

#### Six-week follow-up

The results of the follow-up at 6 weeks are summarized in Table 3. Of the initial 117 *H. pylori*-positive patients, ninety-eight (84 %) returned and could be evaluated. Of the fifty-nine patients who received eradication therapy, forty-nine returned and thirty-six were *H. pylori*-negative after 6 weeks (eradication rate 73.5 %). Four patients were indeterminate for *H. pylori* at 6 weeks and were excluded from analysis (all were *H. pylori*-positive on histology but negative on urease test and culture). *H. pylori* was eradicated in one patient in the placebo–vitamins group. There was no evidence that this patient had taken any antibiotics but high-dose vitamin C has been shown to result in eradication of *H. pylori* (Jarosz *et al.* 1998). This patient was also excluded from analysis. For all of the ensuing data, no significant changes were seen in the placebo–placebo group.

#### Plasma ascorbic acid and vitamin C

Plasma ascorbic acid and total vitamin C approximately doubled in both groups of patients that received vitamin supplements (Table 4). There were no changes in plasma ascorbic acid or total vitamin C in either group not receiving vitamin supplements and *H. pylori* eradication had no effect on plasma vitamin levels. There was, however, a significant increase in the percentage plasma vitamin C as ascorbic acid in the triple–vitamins group (86.6 to 93.3 %,  $P=0.03$ ), whereas no such change was seen in the placebo–vitamins group.

#### Tissue vitamin C and ascorbic acid

There was a significant increase in total antral vitamin C and smaller (non-significant) increases in antral ascorbic acid, corpus ascorbic acid and total corpus vitamin C in the triple–vitamins group (Table 4). In the placebo–vitamins group there were significant increases in corpus ascorbic acid and total corpus vitamin C along with smaller (non-significant) increases in antral ascorbic acid and total antral vitamin C. When all patients receiving vitamins are

**Table 3.** Follow-up of randomized patients\*

	Triple†+vitamins‡	Placebo+vitamins‡	Triple†+placebo	Placebo+placebo	Total
Randomized ( <i>n</i> )	29	29	30	29	117
Returned at 6 weeks ( <i>n</i> )	24	26	25	23	98
<i>H. pylori</i> negative at 6 weeks ( <i>n</i> )	19	1	17	0	37
Indeterminate for <i>H. pylori</i> at 6 weeks ( <i>n</i> )§	1	1	2	0	4

\* For details of subjects and procedures, see Table 1 and p. 4.

† Triple: *H. pylori* eradication therapy comprising colloidal bismuth subcitrate, tetracycline and metronidazole for 2 weeks.

‡ Vitamins: ascorbic acid and  $\alpha$ -tocopherol for 4 weeks.

§ All were *H. pylori* positive on histology but negative on urease test and culture and were excluded from analysis.

combined, regardless of eradication therapy, there were significant increases after treatment in antral ascorbic acid (586.5 to 695.6  $\mu$ mol/kg,  $P=0.03$ ), total antral vitamin C (654.7 to 824.4  $\mu$ mol/kg,  $P=0.01$ ), corpus ascorbic acid (315.7 to 529.2  $\mu$ mol/kg,  $P=0.001$ ), and total corpus vitamin C (405.4 to 617.2  $\mu$ mol/kg,  $P=0.003$ ).

#### Chemiluminescence and malondialdehyde

Chemiluminescence fell in 18/19 patients in the triple–vitamins group and in 15/17 in the triple–placebo group (Table 4). The difference between pre- and post-treatment chemiluminescence was significant for both groups ( $P<0.001$ ). In the placebo–vitamins group, chemiluminescence fell in ten but increased in thirteen patients. Although there was a small reduction in median chemiluminescence after treatment (from 23 993 to 9699 cpm/mg) this reduction was much smaller in magnitude than that seen for the *H. pylori* eradication groups and was not significant ( $P=0.6$ ). In the placebo–placebo group chemiluminescence fell in eleven and increased in ten patients ( $P=1.0$ ).

Malondialdehyde fell in 13/18 patients in the triple–placebo group. The difference was significant ( $P=0.03$ ). In the triple–vitamins group malondialdehyde levels fell in 13/16 but the difference between pre- and post-treatment values was not significant ( $P=0.1$ ). In the placebo–vitamins group, malondialdehyde fell in fifteen but increased in eight ( $P=0.4$ ) and in the placebo–placebo group it fell in ten and increased in twelve patients ( $P=0.7$ ). Thus, vitamin supplements alone did not significantly reduce either chemiluminescence or malondialdehyde, and had no additional effect to *H. pylori* eradication.

#### Discussion

There has been considerable interest in the potential for antioxidant vitamins to reduce gastric cancer risk in *H. pylori* infection by attenuating oxidative stress and oxidative DNA damage. However, the precise role of ascorbic acid and other antioxidants *in vivo* remains uncertain. Thus, vitamin C supplements have been shown to reduce gastric mucosal adduct levels in one study (Dyke *et al.* 1994) whereas in another, patients given supplements of  $\alpha$ -tocopherol and  $\beta$ -carotene were more likely to develop bronchial carcinoma than unsupplemented patients (The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group, 1994; Omenn *et al.* 1996). Furthermore, vitamin C and E supplements have been shown to reduce oxidative stress in peripheral lymphocytes and abrogate Fe-induced

oxidative stress in the rat intestine (Brennan *et al.* 2000; Srigiridhar & Nair, 2000). There is a need, therefore, to clarify the antioxidant effects of vitamin supplements in the gastric mucosa before advocating this approach.

This is the first study to have randomized *H. pylori*-infected patients to vitamin C and E supplements and eradication therapy, alone and in combination, and examine the effect of these treatments on ROS activity in gastric biopsies. There was no specific determination of compliance but plasma ascorbic acid more than doubled in the vitamin-supplemented patients to concentrations that are considered to be the plateau for ascorbic acid. This is good evidence that the tablets were taken largely as prescribed, and that the dose of ascorbic acid, at seven times the recommended daily intake, was sufficient for the purposes of the study (Levine *et al.* 1996).

Our baseline data closely reflect previous studies, some from our own department (Sobala *et al.* 1989; Banerjee *et al.* 1994; Davies *et al.* 1994; Farinati *et al.* 1996; Drake *et al.* 1998). ROS and malondialdehyde levels were elevated in *H. pylori* infection and malondialdehyde concentrations correlated significantly with chemiluminescence. Ascorbic acid and total vitamin C were highly concentrated in the mucosa, and levels were not affected by *H. pylori* infection. Plasma ascorbic acid, total vitamin C, and the percentage plasma vitamin C as ascorbic acid were all significantly diminished in *H. pylori*-positive patients compared with controls. This latter finding is in contrast with previous work and probably reflects the larger number of patients in our present study (Phull *et al.* 1998). It is unlikely, however, that the reduction in plasma ascorbic acid is a direct effect of *H. pylori* infection since levels did not alter after eradication of the organism. A lack of independent effect was confirmed by multiple linear regression. It is more likely that the difference reflects different dietary intake or other factors such as socio-economic status. This is reflected by the lower proportion of smokers in the control group, and is consistent with studies of nutritional factors and *H. pylori* infection rates in Colombian children (Goodman *et al.* 1997).

Eradication of *H. pylori* resulted in expected reductions in mucosal chemiluminescence and malondialdehyde concentrations, in line with previous studies (Farinati *et al.* 1996; Waring *et al.* 1996; Drake *et al.* 1998). Vitamin supplements, either alone or in concert with *H. pylori* eradication, led to increases in tissue and plasma ascorbic acid and total vitamin C concentrations. However, supplements had a negligible effect on tissue chemiluminescence and malondialdehyde. It is possible that a small effect of vitamin supplements on ROS

**Table 4.** Paired plasma, antral and corpus ascorbic acid and total vitamin C, chemiluminescence and malondialdehyde for patients with *Helicobacter pylori* gastritis, before and 6 weeks after treatment\*

(Median values and interquartile ranges)

Time (weeks)	Triple†+vitamins‡ (n 19)§				Placebo+vitamins‡ (n 24)				Triple†+placebo (n 17)§				Placebo+placebo (n 23)			
	0		6		0		6		0		6		0		6	
	Median	Interquartile range	Median	Interquartile range	Median	Interquartile range	Median	Interquartile range	Median	Interquartile range	Median	Interquartile range	Median	Interquartile range	Median	Interquartile range
Plasma ascorbic acid (μmol/l)	29.8	10.8–44.3	65.9§§	53.4–90.8	36.3	11.4–61.9	74.9***	58.5–81.8	23.6	7.1–58.2	24.4	9.1–63.6	35.2	14.2–65.3	31.2	16.5–53.9
Plasma total vitamin C (μmol/l)	39.7	13.6–48.3	73.2††	56.2–94.8	40.3	14.2–68.1	78.9***	69.8–85.7	33.2	8.2–63.0	34.6	10.2–68.1	39.7	18.7–62.5	37.5	20.4–60.8
Antral ascorbic acid (μmol/kg)	546.2	266.3–739.8	691.6	418.5–973.2	647.9	343.5–855.7	699.5	496.3–1071.4	543.4	328.1–974.9	424.1	271.4–715.4	634.2	341.2–855.7	692.7	360.6–946.0
Antral total Vitamin C (μmol/kg)	568.9	371.9–853.4	760.9††	530.3–1151.5	719.4	445.7–995.4	854.0	607.5–1188.4	536.0	382.7–1101.0	498.0	356.0–804.6	668.9	427.0–965.8	859.1	644.5–1060.1
Corpus ascorbic acid (μmol/kg)	405.4	168.6–534.3	473.5	316.3–670.0	304.3	189.1–472.4	541.1**	241.9–794.4	392.9	80.1–493.4	231.7	84.6–449.1	458.8	254.9–679.1	383.8	170.3–546.2
Corpus total vitamin C (μmol/kg)	480.4	290.7–725.1	562.7	440.6–813.7	403.1	270.8–652.4	663.2†††	353.2–988.5	433.2	178.9–608.1	370.2	135.1–533.7	548.5	295.8–804.0	516.1	367.4–689.9
Chemiluminescence (c.p.m./mg)	17 316	11 270–106 386	684***	374–1264	23 993	6 698–54 525	9 699	1 899–31 820	9 593	2 504–36 966	9 19***	278–1 449	14 591	5 849–35 237	13 151	4 301–41 690
Malondialdehyde (μmol/kg)	97.7	77.3–140.5	89.8	68.7–131.1	124.5	97.9–141.9	115.7	98.6–113.1	126.3	98.1–146.6	92.8§§	70.1–113.9	121.9	85.1–137.1	112.3	87.8–135.7

\* For details of subjects and procedures, see Table 1 and p. 4.

† Triple: *H. pylori* eradication therapy comprising colloidal bismuth subcitrate, tetracycline and metronidazole for 2 weeks.

‡ Vitamins: ascorbic acid and α-tocopherol for 4 weeks.

§ For patients receiving triple therapy, data are for those in whom *H. pylori* was successfully eradicated.|| For patients receiving placebo triple therapy, data are for those who remained *H. pylori* positive.Median values were significantly different from paired pre-treatment values (wilcoxon signed ranks test): †† $P=0.04$ , ††† $P=0.03$ , §§ $P=0.02$ , \*\* $P=0.01$ , ††† $P=0.004$ , \*\*\* $P<0.001$ . All other paired comparisons were non-significant.

activity has been missed due to the relatively small numbers in each group and the large CV for chemiluminescence. It is clear from the results, however, that any effect of vitamins is far less than eradication of *H. pylori*. The reduction in chemiluminescence after treatment of the infection was several orders of magnitude greater than that of vitamin supplements and it would be difficult to demonstrate an effect over and above this. Likewise, the results relating to malondialdehyde indicate that the most effective treatment is *H. pylori* eradication and vitamin supplements do not add to this effect.

The failure of antioxidant vitamin supplements to significantly effect levels of ROS is not surprising, because, even in the inflamed stomach, ascorbic acid is highly concentrated into the mucosa. Concentrations of the ascorbyl radical are increased in the mucosa of *H. pylori* gastritis, but its concentration remains considerably lower than ascorbic acid (Drake *et al.* 1996). This lends support to an antioxidant role for ascorbic acid but suggests that, even in the presence of continued inflammation, the scavenging capability of ascorbic acid is replete. Supplements are only likely to be of use when ascorbic acid stores are significantly diminished.

A second consideration is that ascorbic acid can act not only as antioxidant but also, in the presence of transition metals, as pro-oxidant (Halliwell & Gutteridge, 1998). In keeping with this, and consistent with earlier work, we found that antral mucosal ascorbic acid had a weak positive correlation with chemiluminescence (Drake *et al.* 1998; Everett *et al.* 2001). Alternative explanations for this relationship include an influx of ascorbic acid into the mucosa in response to oxidative stress or as a consequence of the rich content of ascorbic acid found in leucocytes. However, the possibility of ascorbic acid acting as pro-oxidant *in vivo* suggests that, depending on the circumstances, vitamin supplements might have either a beneficial or a detrimental effect (Herbert, 1994).

It is worthy of comment that epidemiological data mainly support a protective effect of high fruit and vegetable intake rather than antioxidants *per se*. Our results do not conflict with this observation, which may result from the presence of other compounds such as folic acid or the balance between different micronutrients in fruits and vegetables (La Vecchia *et al.* 1994). Furthermore, the results of the present study do not rule out the possibility that antioxidant vitamin supplements may prevent the development of gastritis. Indeed it has already been observed that diets high in vitamin C may prevent infection with *H. pylori* (Goodman *et al.* 1997; Jarosz *et al.* 1998). Vitamin supplements could also have a beneficial effect on the formation of *N*-nitroso compounds in gastric juice. However, although it is tempting to speculate that longer and larger doses of antioxidants may yet be shown to be beneficial, the potential for harm is also present. Not only is ascorbic acid potentially pro-oxidant but recent *in vitro* work has also shown that vitamin C may mediate formation of genotoxins from lipid hydroperoxides in the absence of transition metal ions (Lee *et al.* 2001). These issues may only be settled by large population studies or using direct measurements of DNA damage.

In conclusion, we have found that dietary supplementation of antioxidant vitamin supplements do not reduce ROS activity in the gastric mucosa. Oxidative stress, and hence oxidative DNA damage, in the gastric mucosa are only likely to be reduced if *H. pylori* is eradicated, regardless of dietary manipulation.

### Acknowledgements

This study was supported by a grant from the Ivy Hobson Trust for Cancer Research.

### References

- Banerjee S, Hawksby C, Miller S, Dahill S, Beattie A & McColl K (1994) Effect of *Helicobacter pylori* and its eradication on gastric juice ascorbic acid. *Gut* **35**, 317–322.
- Block G (1991) Vitamin C and cancer prevention: the epidemiologic evidence. *American Journal of Clinical Nutrition* **53**, 270S–282S.
- Brennan LA, Morris GM, Wasson GR, Hannigan BM & Barnett YA (2000) The effect of vitamin C or vitamin E supplementation on basal and H<sub>2</sub>O<sub>2</sub> induced DNA damage in human lymphocytes. *British Journal of Nutrition* **84**, 195–202.
- Cadenas S, Lertsiri S, Otsuka M, Barja G & Miyazawa T (1996) Phospholipid hydroperoxides and lipid peroxidation in liver and plasma ODS rats supplemented with alpha-tocopherol and ascorbic acid. *Free Radical Research* **24**, 485–493.
- Correa P (1995) The role of antioxidants in gastric carcinogenesis. *Critical Reviews in Food Science and Nutrition* **35**, 59–64.
- Davies GR, Simmonds NJ, Stevens TRJ, Sheaff MT, Banatvala N, Laurenson IF, Blake DR & Rampton DS (1994) *Helicobacter pylori* stimulates antral mucosal reactive oxygen metabolite production *in vivo*. *Gut* **35**, 179–185.
- Dixon MF, Genta RM & Yardley JH (1996) Correa P and the participants of the International Workshop on the Histopathology of Gastritis Classification and grading of gastritis. The updated Sydney system. *American Journal of Surgical Pathology* **20**, 1161–1181.
- Drake I, Davies M, Mapstone N, Dixon M, Schorah C, White K, Chalmers D & Axon A (1996) Ascorbic acid may protect against human gastric cancer by scavenging mucosal oxygen radicals. *Carcinogenesis* **17**, 559–562.
- Drake I, Mapstone N, Schorah C, White K, Chalmers D, Dixon M & Axon A (1998) Reactive oxygen species activity and lipid peroxidation in *Helicobacter pylori* associated gastritis: relation to gastric mucosal ascorbic acid concentrations and effect of *H. pylori* eradication. *Gut* **42**, 768–771.
- Dyke G, Craven J, Hall R & Garner R (1994) Effect of vitamin C supplementation on gastric mucosal DNA damage. *Carcinogenesis* **15**, 291–295.
- Emma AM & Fitzgerald GA (2000) Indices of lipid peroxidation *in vivo*: strengths and limitations. *Free Radical Biology and Medicine* **28**, 1745–1750.
- Everett SM, Singh R, Leuratti C, White KLM, Neville P, Greenwood D, Marnett LJ, Schorah CJ, Forman D, Shuker D & Axon ATR (2001) Levels of malondialdehyde-deoxyguanosine in the gastric mucosa - relationship with lipid peroxidation, ascorbic acid and *Helicobacter pylori*. *Cancer Epidemiology and Biomarkers* **10**, 369–376.
- Farinati F, Della Libera G, Cardin R, Molari A, Plebani M, Rugge M, Di Mario F & Naccarato R (1996) Gastric antioxidant, nitrites, and mucosal lipoperoxidation in chronic gastritis and *Helicobacter pylori* infection. *Journal of Clinical Gastroenterology* **22**, 275–281.



- Frei B (1991) Ascorbic acid protects lipids in human plasma and low density lipoprotein against oxidative damage. *American Journal of Clinical Nutrition* **54**, 1113S–1118S.
- Goodman K, Correa P, Tengana Aux H, DeLany J & Collazos T (1997) Nutritional factors and *Helicobacter pylori* infection in Colombian children. *Journal of Pediatric Gastroenterology and Nutrition* **25**, 507–515.
- Guyton KZ & Kensler TW (1993) Oxidative mechanisms in carcinogenesis. *British Medical Bulletin* **49**, 523–544.
- Halliwell B & Gutteridge J (1998) *Free Radicals in Biology and Medicine*, Oxford: Oxford University Press.
- Herbert V (1994) The antioxidant supplement myth. *American Journal of Clinical Nutrition* **60**, 157–158.
- International Agency for Research on Cancer (1994) *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Shistosomes, Liver Flukes and Helicobacter pylori. Monograph 61*, Lyon: IARC Scientific Publications.
- Jackson JH, Gajewski E, Schraufstatter IU, Hyslop PA, Fuciarelli AF, Cochrane CG & Dizdaroglu M (1989) Damage to the bases in DNA induced by stimulated human neutrophils. *Journal of Clinical Investigation* **84**, 1644–1649.
- Jarosz M, Dzieniszewski J, Dabrowska-Ufniarz E, Wartanowicz M, Ziemlariski S & Reed PI (1998) Effects of high vitamin C doses on *Helicobacter pylori* infection and total vitamin C concentration in gastric juice. *European Journal of Cancer Prevention* **7**, 449–454.
- Knekt P, Aromaa A, Maatela J, Aaran R, Nikkari T, Hakama M, Hakulinen T, Peto R & Teppo L (1991) Vitamin E and cancer prevention. *American Journal of Clinical Nutrition* **53**, 283S–286S.
- La Vecchia C, Ferraroni M, D'Avanzo B, Decarli A & Franceschi S (1994) Selected micronutrient intake and the risk of gastric cancer. *Cancer, Epidemiology, Biomarkers and Prevention* **3**, 393–398.
- Lee SH, Oe T & Blair IA (2001) Vitamin C-induced decomposition of lipid hydroperoxides to endogenous genotoxins. *Science* **292**, 2083–2086.
- Levine M, Conry-Cantilena C, Wang Y, Welch R, Washko P, Dhariwal K, Park J, Laxarev A, Graumilch J, King J & Cantilena L (1996) Vitamin C pharmacokinetics in healthy volunteers: Evidence for a recommended dietary allowance. *Proceedings of the National Academy of Science, USA* **93**, 3704–3709.
- Marnett LJ (1994) DNA adducts of  $\alpha$ ,  $\beta$ -unsaturated aldehydes and dicarbonyl compounds. In *DNA Adducts: Identification and Biological Significance*, Vol 125, pp. 151–163 [K Hemminki, A Dipple, DEG Shuker, FF Kadlubar, D Segerback and H Bartsch, editors]. Lyon: IARC Scientific Publications.
- Mirvish S, Wallcave L, Eagen M & Shubik P (1972) Ascorbate–Nitrite reaction: Possible means of blocking the formation of carcinogenic N-nitroso compounds. *Science* **177**, 65–67.
- Omenn G, Goodman G, Thornquist M, Balmes J, Cullen MR, Glass A, Keogh JP, Meyskens FL Jr, Valanis B, Williams JH Jr, Barnhart S, Cherniack MG, Brodtkin CA & Hammar S (1996) Risk factors for lung cancer and for intervention effects in CARET, the Beta-Carotene and Retinal Efficacy Trial. *Journal of the National Cancer Institute* **88**, 1550–1559.
- Parkin DM, Pisani P & Ferlay J (1993) Estimates of worldwide incidence of eighteen major cancers in 1985. *International Journal of Cancer* **54**, 594–606.
- Phull P, Price A, Thornmiley M, Green C & Jacyna M (1998) Plasma free radical activity and antioxidant vitamin levels in dyspeptic patients: correlation with smoking and *Helicobacter pylori* infection. *European Journal of Gastroenterology and Hepatology* **10**, 573–577.
- Simmonds N, Allen R, Stevens T, Van Someren N, Blake D & Rampton D (1992) Chemiluminescence assay of mucosal reactive oxygen metabolites in inflammatory bowel disease. *Gastroenterology* **103**, 186–196.
- Sobala G, Schorah C, Sanderson M, Dixon M, Tompkins D, Godwin P & Axon A (1989) Ascorbic acid in the human stomach. *Gastroenterology* **97**, 357–363.
- Sobala GM, Pignatelli B, Schorah CJ, Bartsch H, Sanderson M, Dixon MF, Shire S, King RFG & Axon ATR (1991) Levels of nitrite, nitrate, N-nitroso compounds, ascorbic acid and total bile acids in gastric juice of patients with and without precancerous conditions of the stomach. *Carcinogenesis* **12**, 193–198.
- Srigiridhar K & Nair KM (2000) Supplementation with  $\alpha$ -tocopherol or a combination of  $\alpha$ -tocopherol and ascorbic acid protects the gastrointestinal tract of iron-deficient rats against iron-induced oxidative damage during iron repletion. *British Journal of Nutrition* **84**, 165–173.
- The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group (1994) The effects of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. *New England Journal of Medicine* **12**, 289–303.
- Waring A, Drake I, Schorah C, White K, Lynch D, Axon A & Dixon M (1996) Ascorbic acid and total vitamin C concentrations in plasma, gastric juice, and gastrointestinal mucosa: effects of gastritis and oral supplementation. *Gut* **38**, 171–176.
- Yagi K (1976) A simple fluorimetric assay for lipoperoxide in blood plasma. *Biochemical Medicine* **15**, 212–216.