

PAPERS

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

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Abstract

Background—*Helicobacter pylori* is an independent risk factor for gastric cancer, and this association may be due to the bacterium causing reactive oxygen species mediated damage to DNA in the gastric epithelium. High dietary ascorbic acid intake may protect against gastric cancer by scavenging reactive oxygen species.

Aims—To assess reactive oxygen species activity and damage in gastric mucosa in relation to gastric pathology and mucosal ascorbic acid level, and to determine the effect of *H pylori* eradication on these parameters.

Patients—Gastric biopsy specimens were obtained for analysis from 161 patients undergoing endoscopy for dyspepsia.

Methods—Reactive oxygen species activity and damage was assessed by luminol enhanced chemiluminescence and malondialdehyde equivalent estimation respectively. Ascorbic acid concentrations were measured using HPLC.

Results—Chemiluminescence and malondialdehyde levels in gastric mucosa were higher in patients with *H pylori* gastritis than in those with normal histology. Successful eradication of the bacterium led to decreases in both parameters four weeks after treatment was completed. Gastric mucosal ascorbic acid and total vitamin C concentrations were not related to mucosal histology, but correlated weakly with reactive oxygen species activity (chemiluminescence and malondialdehyde levels).

Conclusions—Data suggest that reactive oxygen species play a pathological role in *H pylori* gastritis, but mucosal ascorbic acid is not depleted in this condition.

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Keywords: *Helicobacter pylori*; gastric cancer; reactive oxygen species; ascorbic acid

There is increasing interest in the pathological role of reactive oxygen species (ROS) in diseases where inflammation is a major feature

such as rheumatoid arthritis¹ and inflammatory bowel disease.² Longstanding ulcerative colitis is a premalignant condition and ROS may play a part in the carcinogenic process. In vitro work has shown that ROS can cause a variety of DNA lesions³⁻⁵ and produce mutations in bacterial⁶⁻⁸ and mammalian cells.⁹⁻¹² ROS can also induce malignant transformation in cultured fibroblast cell lines.¹³

Helicobacter pylori infection is now recognised as an independent risk factor for gastric cancer¹⁴⁻¹⁶ and has been designated a class I carcinogen by an IARC-WHO¹⁷ committee. ROS production in association with this bacterium has been shown to occur in vitro¹⁸ and in vivo.^{19,20} ROS may play a part in *H pylori* associated gastric carcinogenesis, and it is of interest that epidemiological studies suggest a protective role for dietary ascorbic acid,^{21,22} a chemical known to be a good scavenger of ROS.^{23,24}

The presence of ROS in *H pylori* infection has so far been determined using luminol or lucigenin enhanced chemiluminescence. Although this method shows ROS to be present, it does not necessarily follow that they are responsible for tissue damage or play a pathological role.

In this study we have used the measurement of gastric mucosal malondialdehyde equivalent concentrations to assess oxidative damage to lipids, comparing the results of this technique with the luminol enhanced chemiluminescence findings in order to determine whether ROS could play a pathological role in *H pylori* infection. We have also measured mucosal ascorbic acid concentrations in order to study whether ROS generation affects levels of this vitamin, as it has previously been observed that concentrations of ascorbic acid in gastric juice are lower in the presence of chronic gastritis.^{25,26}

Patients and methods

Patients were recruited from those undergoing upper gastrointestinal endoscopy for dyspepsia in our unit. Local ethics committee permission was granted and informed consent obtained in all cases.

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Table 1 Antral chemiluminescence and malondialdehyde and vitamin C concentrations according to histological diagnosis

Histological findings	Chemiluminescence (cpm/mg)	Malondialdehyde (nmol/g)	Ascorbic acid ($\mu\text{mol/kg}$)	Total vitamin C ($\mu\text{mol/kg}$)
Normal (n=53)	1210 (467–2891)	78.9 (62.2–97.4)	441 (303–674)	553 (400–727)
Reactive gastritis (n=31)	1576 (274–2836)	88.6 (61.4–104.8)	436 (359–577)	495 (458–663)
<i>H pylori</i> gastritis (n=77)	23885 (4908–47864)*	112.3 (81.4–147.8)*	582 (347–757)	672 (394–840)

Values are expressed as median (interquartile range).

* $p < 0.0001$ v normal histology (Mann-Whitney U test).

At endoscopy, nine antral biopsy specimens and two corpus biopsy specimens were taken. Two antral specimens were used for luminol enhanced chemiluminescence and two assayed for malondialdehyde equivalent concentrations. A further two antral and two corpus biopsy specimens were examined histologically using haematoxylin and eosin, and a modified Giemsa stain to detect *H pylori*. In addition, one antral biopsy specimen was used for urease testing (CLO test) and a further specimen sent for *H pylori* culture. Finally, one antral biopsy specimen was used for mucosal total vitamin C (ascorbic acid + dehydroascorbic acid) and ascorbic acid concentration measurement using high performance liquid chromatography (HPLC).

CHEMILUMINESCENCE

Biopsy specimens were transported in phosphate buffered saline and assayed within three hours. After weighing, each specimen was added to a precounted scintillation vial containing 1 ml of 75 μmol luminol solution and counted for five minutes in a liquid scintillation counter set in the "out of coincidence" mode. The mean of the two biopsy specimens was then calculated in each case.

MALONDIALDEHYDE EQUIVALENT ESTIMATION

Biopsy specimens were stored at -70°C and assayed by a modification of the procedure of Yagi.²⁷ Briefly each sample was blot dried, weighed and added to 4 ml of water. One ml of thiobarbituric acid solution, made by dissolving 167 mg of thiobarbituric acid in a mixture of 25 ml of water and 25 ml of glacial acetic acid, was then added to each sample. A set of malondialdehyde standards was also freshly prepared and added to 1 ml of thiobarbituric acid solution in the same way. After mixing, all samples and standards were heated at 100°C for one hour. Samples and standards were cooled on ice and the malondialdehyde (MDA) equivalents extracted by adding 5 ml of butanol to each. Each tube was centrifuged at 1200 *g* for 10 minutes to separate the aqueous and organic phases and the fluorescence of the butanol phase was then read at an excitation of 515 nm and emission of 555 nm and compared with the fluorescence of the MDA standards. Duplicate samples were assayed for each subject and the average used. Measurements could be made with adequate precision down to 40 nmol/g of tissue with a blot dried weight of 1 mg of tissue, although weights were always above this and averaged 2 mg. The inter-assay coefficient of variation (precision) of the method was 10.1%.

TOTAL VITAMIN C AND ASCORBIC ACID MEASUREMENT

Biopsy specimens were stored in liquid nitrogen and assayed within two weeks. After thawing, the samples were blotted dry, then weighed. They were homogenised in 0.5–1.0 ml of 2% metaphosphoric acid and divided into two parts. Dithiothreitol was added to one part to a final concentration of 6 mg/ml for total vitamin C estimation. Both parts were centrifuged at 2000 *g*, and the supernatant analysed by HPLC using reverse phase ion pair chromatography on a C18 column.^{26, 28} Ascorbic acid was selectively measured using an electrochemical detector set at low voltage. Total vitamin C was estimated as ascorbic acid by incubating the sample containing dithiothreitol at 45°C for 120 minutes prior to analysis by HPLC in order to reduce any dehydroascorbic acid to ascorbic acid.

STATISTICS

Non-parametric tests were used. Unpaired data were compared using the Mann-Whitney test, and paired data with the Wilcoxon matched pairs, signed ranks test. Correlations were calculated using the Spearman rank test, and analysis of variance made with the Kruskal-Wallis test.

Results

There were 161 patients in the study with a mean age of 47 years (range 20–79) of whom 105 were male and 56 were female. Of these, 77 had *H pylori* associated chronic gastritis, 53 normal histology, and 31 reactive gastritis. The mean age (and age range) were as follows: *H pylori* associated gastritis 51 years (22–75), normal 40 years (20–69), and reactive gastritis 45 years (25–79). Of the *H pylori* gastritis patients, 31 (40%) smoked, compared with 15 (28%) in the normal group, and seven (23%) in the reactive gastritis group.

Chemiluminescence and MDA equivalent levels were higher ($p < 0.0001$) in the mucosa of patients with *H pylori* associated chronic gastritis than in those with normal histology (table 1). There was no significant difference in the concentration of either of these indexes between patients with reactive gastritis and those with normal histology ($p = 0.70$, $p = 0.59$ respectively).

Patients with *H pylori* gastritis were graded according to the Sydney classification. Chemiluminescence varied significantly between grades 1 and 3 of inflammation (Kruskal-Wallis, $p < 0.05$) with levels being significantly higher in samples with inflammatory scores of 2 compared with those with scores of 1

Table 2 Effect of successful *H pylori* eradication treatment (n=22) on chemiluminescence and malondialdehyde and vitamin C concentrations in antral biopsy samples

	Before eradication	After eradication
Chemiluminescence (cpm/mg)	16103 (8810–478644)	1441 (646–3979)***
Malondialdehyde (nmol/g)	122.3 (84.7–135.8)	98.9 (70.9–144.6)**
Ascorbic acid (μmol/kg)	544 (341–761)	563 (347–693)
Total vitamin C (μmol/kg)	665 (406–854)	472 (325–715)

Values are expressed as median (interquartile range).

p<0.01 and *p<0.001 v before eradication (Wilcoxon).

Table 3 Effect of unsuccessful *H pylori* eradication treatment (n=17) on chemiluminescence and malondialdehyde and vitamin C concentrations in antral biopsy samples

	Before eradication	After eradication
Chemiluminescence (cpm/mg)	30301 (12112–54921)	10822 (2348–29006)
Malondialdehyde (nmol/g)	108.0 (85.1–143.1)	101.4 (81.2–109.4)
Ascorbic acid (μmol/kg)	555 (286–691)	518 (272–902)
Total vitamin C (μmol/kg)	618 (382–897)	608 (306–910)

Values are expressed as median (interquartile range).

(p<0.05). There was also significant variation in chemiluminescence between activity scores (Kruskal-Wallis, p<0.05) with levels being higher in samples with scores of 2 or 3 compared with those with scores of 0 (p<0.05).

MDA equivalent concentrations did not vary significantly between different inflammation scores (Kruskal-Wallis, p=0.20) but there was a trend for values to be greater in samples with higher activity scores (Kruskal-Wallis, p=0.07) which reached significance in samples scored 2 or 3 compared with those with a score of 0 (p<0.05).

There were no significant differences in ascorbic acid concentrations in antral mucosa between any of the histological groups, and the same was true of antral mucosal total vitamin C levels (table 1).

MDA levels did not correlate with antral mucosal concentrations of ascorbic acid (r=0.13, p=0.08), but there was, however, a weak correlation with antral total vitamin C concentration (r=0.15, p<0.05). In the case of chemiluminescence, there were weak correlations with antral ascorbic acid (r=0.19, p<0.05) and antral vitamin C (r=0.18, p=0.05).

Of the 77 patients with *H pylori* infection, 39 agreed to have a further endoscopy four weeks after completing eradication treatment. Treatment was with a two week course of tripotassium dicitratobismuthate 120 mg four times daily, tetracycline 500 mg four times daily, and metronidazole 400 mg three times daily. *H pylori* was considered to be successfully eradicated if histology, culture, and urease testing were all negative for *H pylori* at the follow up endoscopy. Using these criteria the *H pylori* in 22 patients were successfully eradicated, and 17 patients still had *H pylori* detected four weeks after treatment.

Antral levels of MDA and chemiluminescence both fell significantly in the successfully treated patients (p<0.01 and p<0.001 respectively; table 2), but there was no significant fall in MDA or chemiluminescence in those patients in whom eradication treatment had failed (p=0.37 and p=0.27 respectively; table 3). Antral mucosal concentrations of ascorbic

acid and total vitamin C did not change following eradication treatment, whether successful or not.

Discussion

We have shown increased chemiluminescence in the antral mucosa of patients with *H pylori* associated chronic gastritis compared with those with normal histology. This confirms previous work.²⁰ However, we have also shown that MDA concentrations are higher in the *H pylori* associated chronic gastritis group. Since MDA production could reflect oxidative damage to lipids, this supports the hypothesis that ROS do play a pathological role in this condition. We found no difference in chemiluminescence or MDA levels in the mucosa of patients with reactive gastritis compared with those with normal histology. This is consistent with the view that ROS are produced primarily by neutrophils which are much more prominent in chronic gastritis than in reactive gastritis.

In the Sydney system activity scores reflect the degree of neutrophil infiltration and inflammatory scores are based on lymphocyte and plasma cell levels. The trends for increased chemiluminescence and MDA equivalent concentrations with higher activity grades are again consistent with neutrophils being the chief source of ROS in gastritis. The weaker statistical linking of MDA and chemiluminescence with inflammation scores is not surprising, as lymphocytes and plasma cells are thought to be less important as sources of ROS.

The importance of *H pylori* as the initiating agent for neutrophil infiltration and subsequent ROS production is shown by our eradication results. MDA concentrations and chemiluminescence levels both fell significantly in the mucosa of patients in whom *H pylori* was successfully eradicated, but not in those in whom eradication treatment failed. Thus *H pylori* infection is likely to be a cause of increased ROS generation and damage rather than an association.

MDA concentrations must always be interpreted with caution as lipid oxidation as a result of cell death from any cause can lead to increased MDA levels. However, cell proliferation (and presumably also cell death) is increased in the postsurgical stomach where biliary reactive gastritis is usual²⁹; MDA levels were not however increased in our patients with reactive gastritis. This suggests that cell death is not an important source of MDA in this study and we believe that here, MDA levels are likely to be a reflection of oxidative changes occurring as a primary event leading to cell damage, rather than as a consequence of cell death. This is supported by the fact that MDA concentrations are correlated with chemiluminescence levels, when all 161 cases are taken together (r=0.30, p<0.001). As it is generally believed that luminol dependent chemiluminescence is a reliable measure of important ROS²⁰ produced in the respiratory burst of granulocytes and macrophages,^{30,31} the correlation between chemiluminescence and MDA levels is further evidence that MDA reflects ROS mediated lipid peroxidation.

We were unable to show any relation between the mucosal concentrations of ascorbic acid or total vitamin C and the histological findings. This confirms earlier work.^{28 32}

The weak correlations between ROS activity (as measured by MDA and chemiluminescence) and antral concentrations of ascorbic acid or total vitamin C could constitute a type 2 error. If not, then it may be that concentrations of ascorbic acid (the active antioxidant component of vitamin C) are higher in the infected gastric mucosa either as a protective response to increased oxidative stress or because of the influx of inflammatory cells which contain higher vitamin C concentrations than those reported here for normal gastric mucosa.³³ Whatever the explanation, the ascorbic acid/vitamin C data provide no support for the argument that an increase in mucosal ascorbic acid (for example by dietary manipulation) would be an effective way of reducing ROS damage as high levels are not depleted during increased ROS generation, suggesting that ascorbate is either not involved in quenching ROS, or that normal metabolism adequately sustains mucosal ascorbate reserves by rapid reduction of dehydroascorbic acid created by ROS oxidation of ascorbic acid. However, other antioxidant nutrients (for example, vitamin E, carotenoids) may be better at limiting such damage, but initial investigations suggest that like ascorbic acid, mucosal levels of these antioxidants are not affected by *H pylori* associated gastritis.³⁴

In conclusion, the findings suggest that ROS play a pathological role in *H pylori* associated gastritis. If ROS are implicated in gastric carcinogenesis, eradication of *H pylori* could reduce gastric cancer risk. If we accept that ROS could contribute to *H pylori* mediated carcinogenesis, experimental work showing oxidative DNA damage in this condition is now needed.

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