HELICOBACTER PYLORI

Effect of physiological concentrations of vitamin C on gastric cancer cells and *Helicobacter pylori*

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Background: Gastric juice vitamin C may be protective against gastric carcinogenesis but concentrations are significantly reduced by *Helicobacter pylori* infection. We investigated the in vitro effects of vitamin C at concentrations comparable with those found in gastric juice on gastric cancer cells and *H pylori*.

Methods: Gastric cancer cell lines and various *H pylori* strains were treated with L-ascorbic acid for up to 72 hours. Cell viability, and protein and DNA synthesis were determined. Flow cytometry was used for assessment of *H pylori* adherence, cell cycle distribution, and apoptosis. *H pylori* growth and its haemagglutination activity were determined using viability count and microtitration assay.

Results: Vitamin C induced a significant dose dependent growth inhibition of gastric AGS and MKN45 cells but this effect was significantly reduced at levels similar to those in gastric juice of *H pylori* infected patients (<50 µM). Although vitamin C had no obvious effect on *H pylori* growth, haemagglutination activity, or adherence ability to gastric AGS cells compared with untreated controls, it significantly enhanced *H pylori* associated apoptosis and induced cell cycle arrest in these cells.

Conclusion: Vitamin C may inhibit gastric cancer cell growth and alter *H pylori* induced cell cycle events at concentrations comparable with those in gastric juice, but has no effect on *H pylori* growth or pathogenicity. However, the inhibitory effect on gastric cancer cells was lost at vitamin C concentrations found in patients with *H pylori* infection.

C hronic infection of *Helicobacter pylori* has been linked to the development of gastric adenocarcinoma.[1] Although a variety of factors may contribute to the carcinogenic process, gastric juice vitamin C has recently attracted many researchers.[2] This is not only because of the inter-relationship between vitamin C and *H pylori* in the stomach but also because growing evidence suggests that dietary vitamin C reduces gastric cancer risk.[3] It has been shown that vitamin C reduces the formation of *N*-nitroso compounds (NOCs) in gastric juice and scavenges reactive oxygen metabolites (ROMs) in gastric mucosa.[4] Reduced levels of vitamin C in gastric juice have been reported in *H pylori* infected patients, which return to normal after eradication of the organism.[3 5] It is thought that decreased concentrations of gastric juice vitamin C diminish its protective effect and therefore increase the risk of gastric cancer. However, evidence from epidemiological and case control studies is mainly based on vitamin C concentrations in serum or plasma[2] but there are no direct data relating low gastric juice vitamin C levels to the increased gastric cancer risk. Furthermore, most previous studies suggest that *H pylori* infection does not influence vitamin C concentrations in serum or plasma.[4 6]

Vitamin C is an acidic molecule with strong reducing activity and is an essential component of most living tissues. It has two major forms: ascorbic acid and dehydroascobic acid (DHA) which all have vitamin C activity and are interconvertible by redox chemistry.[7] It has been shown that both ascorbic acid and DHA can affect cell growth by altering cell proliferation and/or inducing cell death in various cell systems.[8 9] Ascorbic acid induced growth inhibition in various human melanoma cells[10] and apoptotic cell death in human promyelocytic leukaemic HL-60 cells[11] and in fibroblasts.[12 13] The effects were significantly enhanced in the presence of a small amounts of Cu^{2+} ions. In addition, vitamin C can inhibit various forms of T cell apoptosis.[14] Supplementation of vitamin C caused a significant reduction in colonic epithelial cell proliferation in mice.[15] Although vitamin C is present at signifi-

cantly higher levels in gastric juice and gastric mucosal cells than in plasma or serum,[2 5 16] its effect on gastric epithelial cells remains unclear.

The effect of vitamin C on *H pylori* growth and its pathogenicity has been reported by several groups. High dose vitamin C markedly inhibited *H pylori* growth, colonisation, or even led to eradication of the organism; however, concentrations required in these studies were extremely high and often 10 times or greater than physiological levels in the stomach.[17 18] As an antioxidant, vitamin C has both antioxidant and pro-oxidant activities and large doses may promote kidney stones.[19] Vitamin C is especially dangerous in the presence of high body iron stores, as iron promotes the pro-oxidant activity of vitamin C.[19] A recent study showed that vitamin C intake greater than 500 mg/day markedly increased its pro-oxidant activity and may actually induce host cell DNA damage, thereby increasing cancer risk.[20]

Hence understanding the role of physiological levels of vitamin C in the stomach is important in elucidating the protective properties of vitamin C against gastric carcinogenesis. In this study, we examined the in vitro effects of vitamin C at concentrations comparable with those found in gastric juice, on gastric cancer cell lines and *H pylori* pathogenicity to gastric epithelial cells.

MATERIALS AND METHODS
Cell culture
Gastric cancer cell lines, AGS and MKN 45, which were derived from gastric adenocarcinoma patients, were used. The cells were grown in RPMI 1640 medium containing 10% fetal

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calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml), and L-glutamine (20 mM) and were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. To assess the effects of vitamin C on gastric cancer cell growth and DNA synthesis, cells (1×10^4 cells/well in 200 µl of culture medium) were plated in the inner 60 wells of a 96 well plate. The outer wells received 200 µl of medium alone. A range of concentrations of vitamin C (L-ascorbic acid; Sigma-Aldrich Company Ltd, Dorset, UK) were added after 24 hours. Culture medium and vitamin C were renewed after 48 hours of treatment. Cell viability, and protein and DNA synthesis were examined after 72 hours of treatment. Negative control cultures were cells that received medium alone.

H pylori culture

H pylori strains NCTC 11637, SS1, and the SS1 isogenic pldA⁻ mutant (ND5— a phospholipase mutant) were used. Bacteria were grown on Columbia blood agar plates containing 5% horse blood (Oxoid, UK) for 2–3 days under microaerobic conditions (5% O_2, 15% CO_2, 80% N_2). To assess the effect of vitamin C on H pylori growth and adherence to gastric AGS cells, the bacterium (1×10^7 cfu/ml) was grown in brain-heart infusion broth containing various amounts of L-ascorbic acid at 37°C for up to 24 hours. The effect on survival after exposure to vitamin C was determined by viability count following serial 10-fold dilution on Dent's Columbia blood agar plates.

Determination of cell protein and DNA synthesis

Cell growth rates following vitamin C treatment were assessed using a colorimetric protein assay with methylene blue staining.[21] This technique is based on staining of basophilic cellular compounds (such as proteins, nucleic acid, etc) with methylene blue. Briefly, after incubation with various vitamin C concentrations, gastric AGS and MKN45 cells were washed with phosphate buffered saline (PBS, pH 7.4; the same pH was used in all subsequent experiments) and, subsequently, the cells were fixed with 10% (v/v) formalin for 10 minutes at room temperature. After removal of formalin, cells were washed and stained with methylene blue (1% w/v in borate buffer) for 10 minutes at room temperature. The excessive dye was removed by extensively washing with borate buffer. After drying, 200 µl of 0.1 M HCl was added to each well and the plate was mixed for 15 minutes at room temperature using a plate shaker to facilitate dissolution of the stain. Optical density values were read in a microplate reader at 620 nm. Results are expressed as percentage of untreated controls. Cell viability was determined by trypan blue exclusion assay. DNA synthesis in control and vitamin C treated groups was assessed using a bromodeoxyuridine (BrdU) incorporation ELISA according to the manufacturer's instructions (Boehringer Mannheim, East Sussex, UK).

Flow cytometric analysis for apoptosis and cell cycle phase distribution

The effect of H pylori on gastric cancer cell cycle phase distribution and apoptosis was assessed using flow cytometry according to a previously published method.[22] Briefly, a pool of detached and adherent cells was washed in PBS and the cells fixed in 70% ice cold (4°C) ethanol overnight. The cell pellets were resuspended in a 1 ml solution containing 200 µg/ml ribonuclease (Sigma, Dorset, UK) and 50 µg/ml propidium iodide (Sigma) for 60 minutes at 37°C. Cell cycle analysis was performed on a FACScan flow cytometer (Becton Dickinson, Oxford, UK) equipped with a FACStation and CellQuest software (Becton Dickinson Immunocytometry Systems, Oxford, UK). Debris was eliminated from the analysis using a forward angle light scatter threshold. Cell doublets and clumps were gated out of the analysis using a dot plot display of area and width. A total of 10 000 cells were analysed for each sample

and the apoptotic cells were considered to constitute the sub-G1 cell population.

H pylori adherence assay

Gastric AGS cells and H pylori SS1 were used for assessment of H pylori adherence according to the method described by Dunn and colleagues.[23] Briefly, H pylori SS1 was preincubated in the presence of a range of concentrations of L-ascorbic acid for 30 or 120 minutes. Subsequently, AGS cells (5×10^6 cells) and vitamin C pretreated H pylori (5×10^8 organisms) were incubated in PBS at 37°C for one hour with agitation (150 rpm). Non-adherent bacteria were removed by centrifugation with 10 ml of 15% sucrose solution. Cells were washed once with PBS and then incubated with a 1:5 dilution of polyclonal anti-H pylori antibody (SkyTek Laboratories, Logan, USA) on ice for 30 minutes. After washing with 15 ml of PBS, cells were incubated for an additional 30 minutes on ice (4°C) in a 1:20 dilution of fluorescein isothiocyanate (FITC) conjugated goat antirabbit IgG (Sigma-Aldrich Company Ltd). The cells were subsequently washed and resuspended in 1 ml of 1% formaldehyde for flow cytometric analysis. The FACScan flow cytometer (Becton Dickinson, San Jose, USA) was used to measure bacteria adhering to AGS cells. By using a dot plot display of forward light scatter and side (90°) light scatter, the machine was gated to include single cells and to exclude cell debris and unbound bacteria. Fluorescence data were acquired in log model on a 256 channel by analysis of 10 000 individual cells. The results were expressed as percentage of fluorescent cells calculated from fluorescence frequency distribution histograms. The threshold of cells without adherent bacteria was established for each experiment, the cells being stained with subsequent two step FITC conjugated H pylori antibody staining, as described previously.

Haemagglutination assay

Red blood cells (RBCs) were obtained from defibrinated horse blood which was washed with PBS, resuspended in an equal volume of PBS, and kept at 4°C. RBCs were prepared freshly every week. For the haemagglutination assay, H pylori SS1 was pretreated with various concentrations of L-ascorbic acid in brain-heart infusion broth for up to 24 hours under microaerobic conditions. Subsequently, 50 µl of 1% RBC were mixed with 50 µl of H pylori (5×10^8 cfu/ml) in a round bottomed 96 well microtitration plate. The plates were tapped to mix the interactants, left at room temperature for 30 minutes, and the results read after further incubation overnight at 4°C according to a previously described method.[24]

Statistical analysis

Differences were determined by the Student's t test and p values <0.05 were considered significant in all analyses.

RESULTS
Effect of vitamin C on gastric AGS and MKN45 cells

Vitamin C induced significant dose dependent inhibition of cell protein and DNA synthesis in gastric AGS and MKN45 cells, as measured by methylene blue colorimetric assay and BrdU incorporation ELISA (table 1). At concentrations similar to those found in the gastric juice of healthy subjects ($\geqslant 50$ µM), vitamin C was markedly cytoxic to these cells and both protein and DNA synthesis were significantly inhibited. However, the effect was significantly reduced at levels similar to those in gastric juice of H pylori infected patients (<50 µM). Cell viability was also determined in AGS cells following 72 hours of exposure to vitamin C and a similar trend in the reduction in cell growth was observed, with 47.1 (15.9)% inhibition at a vitamin C concentration of 100 µM and 94.9 (2.8)% inhibition at 500 µM.

We further examined the relationship between cell concentration and vitamin C induced AGS cell growth inhibition. The

Table 1 Effect of vitamin C on growth and proliferation of gastric AGS and MKN45 cells

Ascorbic acid (μM)	AGS		MKN45	
	Protein	Proliferation	Protein	Proliferation
10	113 (9)	104 (3)	102 (5)	101 (4)
50	95 (10)	89 (5)*	97 (8)	90 (2)
100	59 (8)*	47 (5)*	78 (3)*	83 (2)*
200	0*	0*	38 (1)*	16 (3)*
400	0*	0*	4 (3)*	0*

Results are presented as percentage of untreated controls and each value (mean (SD)) represents an average of nine samples.
*p<0.05.

Table 2 pH values in different ascorbic acid solutions (n=3)

	pH value
0.5 M Ascorbic acid stock solution	2.25 (0.2)
Medium* alone (supplier's data)	7.3 (0.3)
Medium alone (measured)	7.4 (0.2)
Medium containing 500 μM ascorbic acid	7.4 (0.3)
Medium containing 1000 μM ascorbic acid	7.3 (0.2)

Values are (mean (SD)).
*RPMI 1640 (Sigma R0883).

Figure 1 Effect of ascorbic acid (vit C) on induction of *Helicobacter pylori* induced gastric AGS cell apoptosis. Gastric AGS cells were infected with *H pylori* NCTC11637 in the presence or absence of 400 µM ascorbic acid for 48 hours. Apoptosis was assessed by flow cytometry with propidium iodide staining and the results are the average of three experiments. ***p<0.001.

Figure 2 Effect of ascorbic acid (vitamin C) on *Helicobacter pylori* associated gastric AGS cell cycle phase distribution. Gastric AGS cells were infected with *H pylori* NCTC11637 in the presence or absence of 400 µM ascorbic acid for 48 hours. Cell cycle phase distribution (gap 0/gap 1 phases (G0/G1), S phase, and gap 2/mitosis (G2/M) phases) of surviving cells was analysed by flow cytometry with propidium iodide staining, and the results are the average of three experiments. **p<0.01; ***p<0.0002.

Figure 3 Effect of vitamin C on *Helicobacter pylori* growth. *H pylori* strains SS1 and ND5 were exposed to various concentrations of vitamin C (0–400 µM) for 24 hours. Viability counts of *H pylori* were performed on Dent's Columbia blood agar plates. The results are an average of two experiments.

effect of vitamin C on AGS cell viability was compared between cells treated in 24 well plates (1×10^5 cells/ml seeding concentration) and those in 96 well plates (1×10^4 cells/ml seeding concentration). After 72 hours of incubation, vitamin C induced only 32.5 (2.4)% inhibition in 24 well plates at a vitamin C concentration of 400 µM but 94.3 (3.7)% inhibition in 96 well plates at a vitamin C concentration of 200 μM (p<0.0001). This finding suggests that gastric cell concentration is an important determinant in vitamin C induced in vitro cell damage.

As an acidic molecule, addition of vitamin C may influence the culture medium pH and therefore affect cell growth.[25] The pH values of various vitamin C solutions involved in the study were determined using a pH meter (ABB Kent-Teylor Ltd, UK). There was no obvious alteration in pH values between original culture medium and those with various amounts of vitamin C (table 2).

Effect of vitamin C on *H pylori* induced cell cycle phase distribution

Following demonstration that vitamin C reduces growth and DNA synthesis in gastric cancer cell lines, we further examined its effect on *H pylori* associated gastric cell cycle events. *H pylori* NCTC11637 were incubated with AGS cells in the absence or presence of L-ascorbic acid (400 µM) for 48 hours. Treatment with vitamin C showed a further enhancement of apoptosis induced by *H pylori* (p<0.001) (fig 1). Analysis of surviving cells by flow cytometry showed an increased cell number in G0/G1 (gap 0/gap 1 phases) cell cycle phases (p<0.0002) and decreased cell number in G2/M (gap 2/mitosis) phases (p<0.01) compared with cells treated with *H pylori* alone, indicating that vitamin C promoted G0/G1 cell cycle arrest in *H pylori* infected cells (fig 2).

Effect of vitamin C on *H pylori* growth

To examine the effect of vitamin C on *H pylori* growth at concentrations comparable with those found in gastric juice, we exposed *H pylori* strains SS1 and ND5 to a range of concentrations of L-ascorbic acid (0–400 µM) for 24 hours. As shown in fig 3, the viability counts of both SS1 and ND5 were similar to
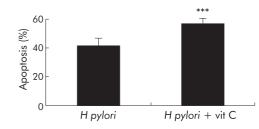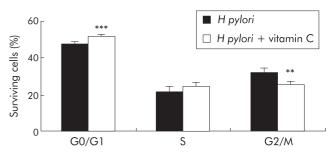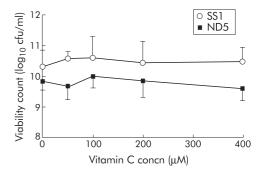
untreated controls following vitamin C treatment, suggesting that vitamin C at these concentrations had no effect on *H pylori* growth.

Effect of vitamin C on *H pylori* adherence ability to gastric epithelial cells

The ability to attach to the host cell is an essential step in pathogenesis for many bacteria. Previous studies have shown that vitamin C at high concentrations reduced the colonisation of *H pylori* to gastric mucosa.[17] We assessed the effect of vitamin C on *H pylori* adherence to gastric AGS cells by pre-exposing *H pylori* to a range of concentrations of L-ascorbic acid (0–400 µM) for 30 or 120 minutes. Flow cytometric analysis showed that there was no significant difference in the adherence to gastric AGS cells for either 30 or 120 minutes of vitamin C pretreated *H pylori* compared with untreated *H pylori* (table 3), suggesting that vitamin C had no effect on the ability of *H pylori* to adhere to gastric AGS cells.

Table 3 Effect of vitamin C on the ability of *Helicobacter pylori* to adhere to gastric AGS cells

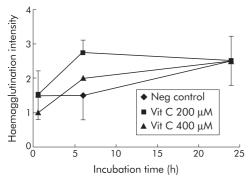| Vitamin C | Adhesion rate (% control (SD)) | |
	30 min	120 min
100 µM	102.4 (3.2)	99.8 (6.7)
200 µM	96.1 (6.7)	102.8 (4.6)
400 µM	93.1 (15.4)	103.5 (8.9)

*Results are the average of five experiments.

Figure 4 Effect of vitamin C on *Helicobacter pylori* haemagglutination activity. Horse red blood cells (1%) and ascorbic acid pretreated SS1 (5×10^{8} cfu/ml) were incubated in an equal volume (50 µl each) in a round bottomed 96 well microtitration plate. The plates were left at room temperature for 30 minutes and the results were read after further incubation overnight at 4°C.

Effect of vitamin C on *H pylori* haemagglutination activity

Bacterial attachment to host cells is mediated by adhesins or ligands which can be assessed by haemagglutination assay.[24] To further examine the possible effect of vitamin C on *H pylori* pathogenicity and to confirm our results regarding the effect of vitamin C on *H pylori* adherence, *H pylori* haemagglutinating activity following addition of L-ascorbic acid was determined. As shown in fig 4, regardless of the dose of vitamin C or duration of pretreatment, *H pylori* induced haemagglutination in all wells. This suggests that vitamin C at concentrations of 0–400 µM had no obvious effect on the haemagglutination activity of *H pylori*.

DISCUSSION

Vitamin C is thought to be an important local protective factor against gastric carcinogenesis. *H pylori* infection induces marked infiltration of inflammatory cells within the gastric mucosa; these inflammatory cells such as neutrophils and monocytes synthesise and release copious amounts of toxic ROMs[26] which may be neutralised by vitamin C and other antioxidants in the stomach. Gastric epithelial cells actively secrete ascorbic acid, the reduced form of vitamin C, into gastric lumen, where there is a significantly higher concentration than that in plasma in healthy subjects.[27 28] There is variation in gastric juice concentrations of vitamin C (ascorbic acid alone or plus DHA) in healthy subjects according to the methods and subjects involved in the studies. Although low levels have been reported,[29] most studies in the fasting stomach have detected ascorbic acid concentrations in the range 50 to 400 µM. However, gastric juice vitamin C is significantly reduced or even absent in *H pylori* infection.[5 16 27 28 30 31] This reduction in gastric juice vitamin C concentration is restored to normal after *H pylori* eradication, indicating that the defect in vitamin C transport and *H pylori* induced gastric cell damage is reversible.[3] For the present study, we set 0–400 µM ascorbic

acid as the physiological range which would include healthy subjects and those with *H pylori* infection.

It has been proposed that vitamin C may have a major role in preventing gastric cancer by inhibiting the formation of NOCs and neutralising the toxicity of ROMs in the stomach. However, previous studies have suggested that vitamin C may also directly interfere with the synthesis of host cell DNA, RNA, and protein, and thereby inhibit cell proliferation and induce cell death in some cell systems.[32 33] The relationship between gastric epithelial cell growth and vitamin C, especially at concentrations of vitamin C comparable with those present in gastric juice, remains unknown. To examine the effect of vitamin C on gastric cancer cells, three methods were used to assess cell growth and proliferation following vitamin C treatment. We observed an obvious dose dependent inhibition of growth for both AGS and MKN45 cells. As cell viability decreased, protein and DNA synthesis were inhibited. These data indicate that vitamin C is capable of inducing gastric cancer cell growth inhibition, which may be related to the effects on cell protein and DNA synthesis. Whether the inhibition is specific for gastric cancer cells or also occurs in normal gastric epithelial cells is not known. However, current evidence suggests that vitamin C metabolism in neoplastic and non-neoplastic cells is remarkably different. The ascorbic acid secretion mechanisms may be impaired in tumour cells as loss of intracellular ascorbic acid from some gastric cancer cells, including AGS cells, is slower than that seen in normal gastric epithelial cells.[34] There is also evidence that vitamin C concentrations are higher in neoplasms compared with the adjacent normal tissue.[35 36]

Because vitamin C is an acidic molecule and might alter the pH of culture medium and inhibit cell growth, we examined this possibility and confirmed that pH was unchanged following addition of vitamin C (table 2). Furthermore, as reported in other cell systems, we found that the gastric cell density used in these experiments was important in determining the degree of cell growth inhibition.[8] We compared the cell viability of gastric AGS cells following vitamin C exposure in 96 well and 24 well plates and found that high cell concentrations exhibited less cell growth inhibition than low concentrations, suggesting that cell concentration is an important determinant in vitamin C induced gastric cancer cell growth inhibition.

The protective role of gastric juice vitamin C against gastric carcinogenesis could also be related to a direct effect on *H pylori*. Infection with the bacteria causes significant reduction of gastric juice vitamin C concentration through the development of chronic gastritis and/or *H pylori* oxidase activity.[3 37] On the other hand, vitamin C may also be capable of inhibiting *H pylori* growth. Jarosz *et al* treated *H pylori* positive dyspeptic patients with vitamin C 5 g daily for four weeks. They showed that *H pylori* infection was eradicated in eight of 27 patients (30%) and gastric juice vitamin C level was significantly higher in patients who underwent successful clearance of the bacteria than in those with persistent *H pylori* infection.[18] A recent study in Mongolian gerbils also suggests that supplementation with vitamin C 10 mg daily (equivalent to 12 g daily in humans), reduced colonisation of *H pylori*.[17] Thus a pharmacological supplement of vitamin C may be of value in the control of *H pylori* infection whereas physiological levels of vitamin C in gastric juice may be important in the prevention of *H pylori* infection and gastric carcinogenesis. We examined therefore the effect of vitamin C at physiological levels on *H pylori* growth and its pathogenicity. It has been suggested that vitamin C is extremely unstable in the presence of *H pylori* and can be degraded within 30 minutes.[17 37 38] Hence we examined the effect of vitamin C at 30 minutes and 24 hours after treatment. The effect of vitamin C on *H pylori* growth was tested at different concentrations ranging from 0 to 400 µM and we found that vitamin C, in this concentration range, showed no effect on *H pylori* growth. A recent study using a regular MIC

determination by the agar dilution method showed similar results.[17] Furthermore, there was also no effect on the ability of *H pylori* to adhere to gastric AGS cells or on haemagglutinating activity of the bacteria. Data from the present study therefore suggest that vitamin C at levels comparable with those found in gastric juice have no effect on *H pylori* growth and the ability to adhere to gastric epithelial cells.

We also observed that vitamin C enhanced induction of apoptosis and increased the percentage of cells in the G0/G1 phases of the cell cycle in *H pylori* infected gastric AGS cells. The underlying mechanisms remain unclear. However, it is unlikely to be related to antioxidant activity as vitamin C is rapidly broken down in the presence of *H pylori*. We assessed the effect after 48 hours of exposure to vitamin C and *H pylori* which makes it most likely that these observation were due to vitamin C oxidative products rather than native vitamin C. Clearly, further studies are needed to examine the possible mechanisms by which vitamin C has these effects on gastric cancer cells.

In conclusion, vitamin C may inhibit the growth of gastric cancer cells at concentrations comparable with those found in normal gastric juice but we found no effect on *H pylori* growth or the ability to adhere to gastric epithelial cells. However, the inhibitory effect on gastric cancer cells may be lost as vitamin C concentrations decrease during *H pylori* infection.

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