Helicobacter pylori is killed by nitrite under acidic conditions

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Abstract

Background—Due to the expression of urease, *Helicobacter pylori* is able to establish itself in the human stomach under acidic conditions. A novel host defence mechanism was recently proposed, suggesting that the formation of salivary nitrite in symbiosis with facultative anaerobic bacteria in the oropharynx, is aimed at enhancing the antimicrobial activity of gastric juice.

Aims—To investigate whether the addition of nitrite in physiological concentrations influences the resistance of *H pylori* to acid.

Methods—*H pylori* cultured from fresh gastric biopsy specimens was exposed for 30 minutes to normal saline and to HCl/KCl buffer (0.2M) at pH 2 with urea (5 mM) added. The influence of potassium nitrite (50–1000 µmol/l) on bacterial survival was determined.

Results—Addition of nitrite (1 mM) to acidic solutions (pH 2) resulted in complete kill of *H pylori* within 30 minutes exposure time whereas acid alone allowed the organism to survive (p<0.001). The antimicrobial effect of nitrite at pH 2 against *H pylori* was dose dependent and complete kill of organisms occurred at concentrations ≥500 µmol/l.

Conclusion—Acidified nitrite has antibacterial activity against *H pylori*. This should prompt further research into the effect of salivary nitrite on the survival of *H pylori* in the human stomach.

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Helicobacter pylori is the commonest bacterial pathogen worldwide and more than half of the world's population of 40 years and over are colonised.[1] It causes chronic active gastritis and is associated with duodenal and gastric ulcer, and gastric malignancy.[2 3]

The majority of bacterial pathogens ingested never give rise to colonisation of the gastrointestinal tract because of the gastric acid barrier.[4] *H pylori,* however, synthesises a urease enzyme which creates an alkaline environment to protect the organism from the bactericidal effect of acid.[5 6]

Recent work from our laboratories suggested a novel host defence mechanism in the mammalian upper gastrointestinal tract. We showed the generation of salivary nitrite in the mouth through a symbiotic relationship with facultative anaerobic bacteria on the tongue surface after ingestion of dietary nitrate.[7–10] Addition of nitrite to acidic solutions in vitro achieves killing of human gut pathogens, whereas acid alone allows growth to continue.[11 12] We suggested that swallowing saliva rich in nitrite after a meal high in nitrate may enhance host defence against ingested pathogens.

In the present paper we report the antimicrobial effect of acidified nitrite on *H pylori* in vitro. Survival of organisms was studied after exposure to test solutions with acid alone, acid plus urea, and acid plus urea and nitrite. The dose dependency of the antibacterial action of nitrite at pH 2 has been determined.

Methods

PREPARATION OF THE INOCULATE

H pylori, isolated from human gastric biopsy specimens, was cultured on horse blood agar plates incubated at 37°C in an atmosphere of 10% CO_2, 5% oxygen, and 85% nitrogen ("campygas"). After three days' incubation, the bacteria were harvested and suspended in normal saline at pH 7 to give a final concentration of approximately 10^9 cells/ml (turbidity = McFarland's no 6).

EFFECT OF EXPOSURE OF *H PYLORI* TO ACID, UREA, AND NITRITE

Inoculate (1 ml) was added to 4.5 ml of 0.2 M HCl/KCl buffer at pH 2 with or without urea (5 mM) in the solution. Immediately thereafter, normal saline (1 ml) or potassium nitrite (1 ml) to reach a final concentration of 1 mmol/l was added in universal containers. As a control, the experiment was repeated with 4.5 ml normal saline at pH 7 instead of 0.2 M HCl/KCl buffer.

The samples were incubated at 37°C. After 30 minutes, aliquots of each sample were diluted with normal saline in serial 10-fold dilutions for determination of the number of colony forming units (cfu). The diluted suspensions (10 µl) were inoculated onto horse blood agar plates and incubated in an anaerobic incubator with 5% CO_2 for up to five days. Colony counts per plate were calculated as: number of colonies × [1/dilution] × [1/0.01] per ml. The lower limit of detection was 10^2 organisms/ml.

DOSE RELATIONSHIP OF THE ANTIMICROBIAL EFFECT OF NITRITE ON *H PYLORI* AT pH 2

Inoculate (1 ml) was added to 4.5 ml of 0.2 M HCl/KCl buffer at pH 2 with 5 mM urea in the solution. Immediately thereafter, potassium nitrite solution (1 ml) was added to reach final concentrations of 0, 50, 100, 200, 500, and

Table 1 Survival of H pylori (log cfu/ml (SD)) after exposure to acid (pH 2), urea (5 mmol/l), and potassium nitrite (1 mmol/l)

	Nitrite absent	Nitrite present
Control (pH 7)	6.14 (0.98) (n=5)	6.32 (1.62) (n=4)
0.2 M HCl/KCl (pH 2) plus urea (5 mmol/l)	5.06 (0.91)*** (n=5)	0† (n=5)
0.2 M HCl/KCl (pH 2) without urea	0† (n=5)	0† (n=5)

***p<0.001 versus 0.2 M HCl/KCl (pH 2) plus urea with 1 mmol/l nitrite.
†No detectable survival.

Figure 1 Antibacterial activity of nitrite against H pylori at pH 2 after 30 minutes' exposure. Experiments were carried out in triplicate (series 1, 2, and 3). No survival was detected at concentrations of nitrite ≥500 µmol/l.

Figure 2 pH in universal containers at the end of the experiment versus number of surviving H pylori (starting pH=2 in all experiments).

1000 µmol/l. As a control, the experiment was repeated with 4.5 ml normal saline at pH 7 instead of 0.2 M HCl/KCl buffer at pH 2.

The containers were incubated at 37°C. After 30 minutes, samples of each of the containers were taken and diluted with normal saline in serial 10-fold dilutions for cfu determination. The diluted suspensions were inoculated onto horse blood agar plates and incubated in an anaerobic incubator with 5% CO_2 for up to five days. Colony counts per plate were calculated as: number of colonies × [1/dilution] × [1/0.01] per ml. The lower limit of detection was 10^2 organisms/ml.

The pH in the universal containers was measured with a glass pH electrode before and after each 30 minute incubation of *H pylori* in acidified nitrite. The experiments were carried out in triplicate.

Results

Table 1 shows the effects of exposure of *H pylori* to acid, urea, and nitrite. The control experiments showed some 5×10^6 cfu/ml after 30 minutes exposure at pH 7 in the universal containers; a similar survival rate was observed when 1 mM potassium nitrite was present. The organisms were also able to survive, although in smaller numbers, after exposure to pH 2 in the presence of urea. However, the addition of 1 mM potassium nitrite at pH 2 plus urea resulted in complete kill (p<0.001). Without urea, *H pylori* was unable to survive exposure at pH 2 even in the absence of nitrite.

Figure 1 shows that the antimicrobial effect of nitrite is dose dependent. Increased kill was seen after addition of 0.05 and 0.1 mM of nitrite in all three experiments. Although the same isolate was used, the inoculum of the first series seemed markedly more sensitive to the mechanism than those in the other two series. No survival was detected in the first series at concentrations ≥0.2 mmol/l, and in the other two series at ≥0.5 mmol/l. The difference in sensitivity of the inoculates is most likely due to differences in viability which was also apparent when the inoculates were exposed to acid alone for 30 minutes. Whereas 1×10^6 cfu/ml were recovered after inoculum 1 had been exposed to normal saline at pH 7, only 3×10^4 cfu/ml survived after exposure to HCl/KCl buffer at pH 2, a killing of 97% of organisms due to acid alone. These figures were 3×10^5 at pH 7 versus 1×10^5 at pH 2 for the second, and 1×10^6 at pH 7 versus 4×10^5 at pH 2 for the third inoculum, representing killings of only 60–70% of organisms due to the addition of acid.

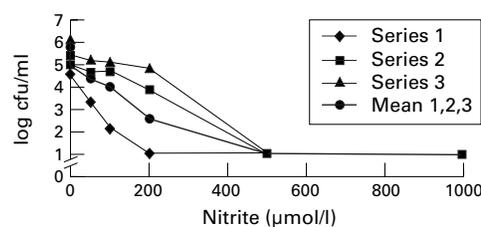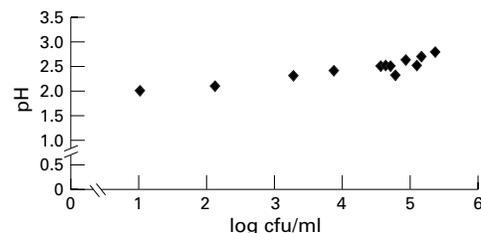The pH was 2 in all universal containers at the start of exposure. A rise in pH was observed at the end of the exposure time in those containers where survival of *H pylori* was observed. The pH at the end of the experiments in relation to the number of surviving organisms is plotted in fig 2.

Discussion

The experiment, summarised in table 1, showed good survival at pH 7. No antibacterial effect of the addition of 1 mM potassium nitrite was observed at this pH. *H pylori* were killed at pH 2 unless urea was present in the solution. The hydrolysis of urea to ammonia and bicarbonate mediated by bacterial urease, neutralises hydrogen ions penetrating the cell wall[6]:

$$\begin{array}{c} NH_2 \\ | \\ C=O + 2H_2O + H^+ \xrightarrow{urease} 2NH_4^+ + HCO_3^- \\ | \\ NH_2 \end{array}$$
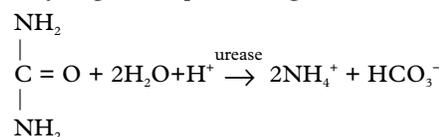
However, even in the presence of urea, the organism seemed to be unable to survive when 1 mM of nitrite was added to the medium. Figure 1 shows the (negative) dose dependent relationship between nitrite and the number of surviving micro-organisms at pH 2.

Acidification of nitrite caused generation of reactive intermediates of nitrogen that have cytotoxic properties:

$$NO_2^- + H^+ \underset{pK_a=3.42}{\Leftrightarrow} HNO_2$$
$$2HNO_2 \Leftrightarrow H_2O + N_2O_3$$
$$N_2O_3 \Leftrightarrow NO\cdot + NO_2\cdot$$
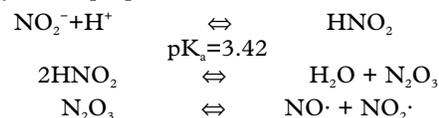
Nitric oxide inhibits respiratory chain enzymes through inactivation of iron-sulphur complexes,[13] and disrupts DNA replication by inhibiting ribonucleotide reductase.[14] Its toxicity has been shown for a rapidly expanding list of micro-organisms[15] as well as for tumour cells.[16] However, experiments with NO donor compounds have shown little antibacterial activity of NO itself,[17] and its toxic effects are more likely to be accomplished via the

formation of peroxynitrite in the presence of superoxide,[18] the oxygen dependent generation of the nitrogen dioxide radical when nitric oxide concentrations are high,[19] and/or still uncharacterised nitrogen species.[20] It seems most likely that the antibacterial activity of acidified nitrite is due to an additive contribution of reactive intermediates of nitrogen.[21]

In addition to the uncertainty about the identity of the reactive intermediate(s) of nitrogen responsible for the antimicrobial activity observed, the data in this paper leave room for the interpretation that the kill of *H pylori* by acidified nitrite is not due to antibacterial action per se but is merely a result of inhibition of the urease enzyme. The demonstration of antimicrobial activity of acidified nitrite against other gut pathogens that are urease negative,[12] and against the yeast *Candida albicans*,[11] offers support for a true antibacterial action, but clearly further research is needed into the causal relationship between acidified nitrite and death of micro-organisms.

The selection of the experimental range of nitrite concentrations in this paper is based on values of salivary nitrite as they have been reported for the past 30 years.[22] The concentration of nitrite in human saliva varies from 0.05 to 1 mmol/l, depending on dietary intake of nitrate. Gastric nitrite concentrations are significantly lower than salivary concentrations because of the formation of nitrous acid (HNO_2), which reacts to generate other oxides of nitrogen as indicated above. Nitric oxide and nitrogen dioxide escape into the gaseous phase. Lundberg *et al* showed the production of nitric oxide in gastric headspace gas after a nitrate meal in human volunteers.[23] In this journal, we recently reported a rise in gastric nitrite concentrations and nitric oxide production after a nitrate drink (2 mmol) in human volunteers.[24] Salivary nitrite concentrations increased from about 50 µmol/l to 800 µmol/l and gastric nitrite concentrations from less than 20 µmol/l to more than 100 µmol/l. We also showed a rise in gastric headspace gas nitric oxide concentration from less than 20 parts per million to a maximum of 291 parts per million. The increase in gastric nitric oxide production was sustained for more than two hours after nitrate ingestion. The depletion of nitrite due to the formation of other oxides of nitrogen is the main cause of the discrepancy between salivary and gastric nitrite concentrations, which cannot be explained by dilution of saliva with gastric contents alone. The data presented in this paper show no effect of nitrite at neutral pH on the survival of *H pylori*. This implies that reactive oxides of nitrogen rather than nitrite itself were responsible for the antibacterial action of acidified nitrite. The generation of these reactive compounds in the stomach is dependent on the continuous supply of nitrite by the swallowing of saliva.

Snepar *et al*[25] showed that treatment with cimetidine has a significant effect in raising gastric microbial titres. The number of organisms present in gastric secretions correlates directly with the gastric pH. A similar increase was observed by Verdu *et al*[26] in the concentration of nitrate reducing bacteria after treatment with omeprazole. Although the authors could not show a concurrent rise in gastric nitrite concentrations, it is possible that overgrowth of these bacteria could have a role in the reduction of *H pylori* after acid inhibitory treatment.

Foods which contain a high concentration of nitrate are green leafy vegetables. Since increasing the dietary intake of nitrate will result in increased salivary nitrite, ingestion of foods rich in nitrate may protect against colonisation of the stomach by *H pylori*. There is no epidemiological evidence that people with a high nitrate intake might have a reduced prevalence of the organism. On the contrary, transmission of the infection has been related to consumption of uncooked vegetables,[27] and the infection is acquired earlier with a high percentage of the adult population infected in developing countries where nitrate intake is expected to be relatively high.[1] However, no investigations have been conducted to investigate the relation between dietary nitrate intake and survival of *H pylori*.

Whether or not the antimicrobial mechanism of acidified nitrite against *H pylori* is active in vivo, its demonstration in vitro should prompt further study of the role of the oxides of nitrogen in this infection.

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