Effect of *Helicobacter pylori* infection on colloidal bismuth subcitrate concentration in gastric mucus

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Abstract

Necropsy gastric mucus infected with *Helicobacter pylori* has a reduced capacity to concentrate colloidal bismuth subcitrate when compared with non-infected mucus. Mucus mounted in a modified in vitro diffusion chamber was bathed with colloidal bismuth subcitrate solutions at different concentrations and pH levels. Bismuth was measured by atomic absorption spectrophotometry to assess intramucosal colloidal bismuth subcitrate concentrations. Bismuth concentrations in non-infected mucus were higher than in *Helicobacter pylori* infected mucus at all experimental colloidal bismuth subcitrate concentrations and pH levels. Regardless of the infection status, the intramucosal concentration of colloidal bismuth subcitrate was dependent upon the concentration of the bathing solution and independent of the pH and the mucus thickness. Colloidial bismuth subcitrate solubility in saline solution varied with pH and was least soluble in the pH range 1.1 to 3.25 and more soluble above and below this pH range. This study suggests that *Helicobacter pylori* infection is associated with physicochemical changes in the gastric mucus with a reduction in its capacity to concentrate colloidal bismuth subcitrate. Such a reduction may compromise the attainment of optimum colloidal bismuth subcitrate concentrations necessary for its bactericidal activity.

Gastric mucus plays an important role in mucosal protection. Disruption in the integrity of the blanket of mucus has been found in individuals with peptic ulcer. *Helicobacter pylori* present in gastric mucus of the stomach or duodenal gastric metastasis is associated with gastritis and peptic ulceration. *Helicobacter pylori* resides in gastric mucus but not within epithelial cells. It may play a role in mucin breakdown. Eradication of *Helicobacter pylori* by colloidal bismuth subcitrate is followed by histological improvement of gastritis and healing of peptic ulcer. If infection recurs, ulcer relapse is frequent, particularly after one year. A combination of colloidal bismuth subcitrate with one or two antibiotics – for example, amoxicillin and/or metronidazole, is more successful than monotherapy in eradicating *Helicobacter pylori*. Ulcer relapse after colloidal bismuth subcitrate and other antibiotics may be the result of failure of the drug(s) to reach sufficient intramucosal concentrations to eradicate *Helico-

Methods

**SPECIMENS**

Mucus from 20 human stomachs was collected at necropsy within 24 hours of death. All persons (18–75 years) who had died of acute trauma or sudden unexpected medical collapse. Each stomach was opened along the greater curvature and food contents were removed by washing with normal saline. Mucus was harvested by gently scraping the gastric mucosa with a glass slide and stored at −20°C for later experiments.

Immediately before harvesting, a gastric tissue specimen was taken for *Helicobacter pylori* urease detection by the camplobacter like organism test (CLO test, Delta West Limited, Western Australia). A mucus sample was considered infected with *Helicobacter pylori* when the tissue specimen showed positive urease activity within one hour with the CLO test and non-infected when the tissue specimen showed no positive urease activity within 24 hours.

**COLLOIDAL BISMUTH SUBCITRATE CONCENTRATION IN GASTRIC MUCUS**

The modified in vitro diffusion chamber previously used for ion exchange studies on mucus was used to expose gastric mucus to colloidal bismuth subcitrate. Mucus (115 + 1 mg or 230 ± 1 mg, depending on mucus layer thickness) was held between two fine gauze mesh discs (Nybolt PA–22/17; Swiss Silk Bolting Cloth Mfg Co Ltd, Zurich, Switzerland) separated by a plastic ring to form a layer 400 µm or 800 µm thick. The mucus containing cassette was held in the chamber by two rigid mesh discs (Tetex Mono PES 130/TWL/C; Swiss Silk Bolting Cloth Mfg Co Ltd, Zurich, Switzerland). One side of the mucus was bathed...
with saline solution adjusted to pH 6.0 and the other side with filtered solutions (Filter Paper 1, Whatman International Ltd, Maidstone, England) of colloidal bismuth subcitrate (DeNoi®, Gist-Brocades, Delft, The Netherlands) at different concentrations (as bismuth) and pH's as follows:

Series 1: Colloidal bismuth subcitrate 93 mg/l, pH 6.0, mucus layer 800 μm thick.
Series 2: Colloidal bismuth subcitrate 232 mg/l, pH 6.0, mucus layer 400 μm thick.
Series 3: Colloidal bismuth subcitrate 64 mg/l, pH 6.0, mucus layer 400 μm thick.
Series 4: Colloidal bismuth subcitrate 64 mg/l, pH 1.5, mucus layer 400 μm thick.
Series 5: Colloidal bismuth subcitrate 127 mg/l, pH 1.5, mucus layer 400 μm thick.

The solutions were continuously circulated for 90 minutes at a rate of 4.5 ml/min, room temperature 21°C.

After each experiment the excess of colloidal bismuth subcitrate solution was washed off with saline. The mucus was taken out of the chamber and dried at 110°C for four hours.

The bismuth content of the samples was measured by atomic absorption (Graphite Furnace Atomic Absorption Spectrophotometer SpectraAA-20; Melbourne, Australia) to assess the intramucosal colloidal bismuth subcitrate concentrations. Results were expressed as μg bismuth/g mucus on the assumption that all mucus samples contain 95% water. The possibility that Helicobacter pylori infection may modify the water content has not been considered in this study.

COLLOIDAL BISMUTH SUBCITRATE SOLUBILITY

Normal saline solutions were adjusted to different pH's (E512 Metrohm Herzau pH meter, Switzerland). Commercial tablets of colloidal bismuth subcitrate stripped of their outer coating were ground to a homogeneous powder. Weighed samples of the powder were added to saline to give mixtures with the same weight/volume ratio. After colloidal bismuth subcitrate was dissolved in the saline solution, samples were obtained from the supernatant and analysed for bismuth content by atomic absorption spectrophotometry. Colloidal bismuth subcitrate solubility was estimated by calculating the difference between expected bismuth level and bismuth in solution after sample preparation.

In separate experiments we observed the disintegration of commercial colloidal bismuth subcitrate tablets. Saline solutions were adjusted to different pH's. For each pH, one 60 ml test tube containing 45 ml saline and one whole commercial tablet of colloidal bismuth subcitrate was 'tumbled' in the Chiltern rotary mixer (Chiltern Scientific, Germany) at a rate of 32 'tumbles' per minute. Disintegration was assessed visually and half and total disintegration times were recorded.

To determine if the outer coating of the commercial tablet affects disintegration, tablets were stripped of their coating and exposed to saline solutions under the same conditions as above.

**STATISTICAL ANALYSIS**

Results for colloidal bismuth subcitrate concentration experiments were expressed as mean (SD). A general linear approach to the analysis of variance was used to determine the statistical significance of differences in bismuth concentration between non-infected and Helicobacter pylori infected mucus. Significant mean and interaction effects were further investigated using Tukey's procedure to analyse differences within pH's and mucus thickness. Type III sums of squares are presented because the design was unbalanced. p Values of less than 0.05 were considered significant.

**Results**

**COLLOIDAL BISMUTH SUBCITRATE CONCENTRATION IN GASTRIC MUCUS**

The Table shows mean (SD) and range bismuth concentrations in non-infected and Helicobacter pylori infected gastric mucus.

Bismuth concentrations were higher in non-infected than in Helicobacter pylori infected mucus at all experimental colloidal bismuth subcitrate concentrations and pH levels ($F_{(1,62)}=52.86$, $p=0.0001$).

Regardless of the infection status, bismuth concentrations varied in proportion to the concentration of the bathing solution and were no different at mucus thickness 400 μm or 800 μm and at pH 1.5 or 6.0 ($F_{(2,62)}=1.06$, $p=0.0389$).

In separate experiments, using unfiltered colloidal bismuth subcitrate solutions and no fine gauze mesh we found that a whitish precipitate was deposited on the mucus surface after the experiments, especially at low pH's; bismuth concentrations were up to five fold greater than bismuth levels obtained after using filtered colloidal bismuth subcitrate solutions.

**COLLOIDAL BISMUTH SUBCITRATE SOLUBILITY**

Figure 1 shows percentages of colloidal bismuth subcitrate solubility estimated from bismuth concentrations in supernatant samples. Solubility in solution was >81% at pH 0.5 to 1.05; <50% at pH 1.1 to 3.25 and >88% at pH 3.75 to 6.25. Minimum solubility (<15%) occurred in the pH range 1.1 to 2.1. Two points of rapid change from high precipitation to high solubility can be described, one between pH 3.25 and 3.75 and the other between pH 1.05 and 1.1.

Half disintegration times for commercial colloidal bismuth subcitrate tablets are shown in Figure 2. At pH 0.8, 1.0 and 1.1, coated colloidal
bismuth subcitrate tablets did not disintegrate even after two hours of constant 'tumbling'; disintegration times were shorter for tablets from which the coating had been stripped. There was a significant change in disintegration times between pH 1-1 and 1-2.

Discussion

Necropsy gastric mucus has the capacity to take up colloidal bismuth subcitrate depending on the concentration of colloidal bismuth subcitrate in the bathing solution. This capacity is not affected by changes in bathing solution pH if the concentration is maintained constant, but is reduced when the mucus is infected with Helicobacter pylori.

The Campylobacter like organisms test was the sole test used to diagnose Helicobacter pylori infection. This test has been found to be reliable and rapid, with at three hours similar sensitivity to Gram staining and with no false positives. A postmortem study found that the urease test is equally reliable to detect Helicobacter pylori presence when compared with other tests.

There is a paucity of literature on the behaviour of colloidal bismuth subcitrate in solution. Wieriks et al. report that colloidal bismuth subcitrate is highly soluble in water and precipitates at pH <5. Lee found 40 to 50% colloidal bismuth subcitrate retained in solution in the pH range 1-0 to 3.0. Our results are similar, with <50% colloidal bismuth subcitrate maintained in solution in the pH range 1-1 to 3-25. Minimum solubility (<15%) occurred in the range 1-1 to 2-1. The differences may be the result of variations in technique. We prepared different solutions for each pH level using commercial tablets while Lee titrated a sole sample of a 'buffered solution of colloidal bismuth subcitrate' to the different pH's.

Colloidal bismuth subcitrate dissolves and/or precipitates when exposed to an aqueous solution. In keeping with the known effects of pH on the sol gel transitions of colloids, we speculate that at pH 6-0 the bismuth subcitrate colloidal dissolves and remains in solution as negatively charged molecular complexes. When the pH is reduced to 1-5, most of the colloid would precipitate, but some bismuthoxy cations and bismuth ions as free Bi'' will be formed and remain in solution, this will be the case of the bathing solution in our experiments at pH 1-5 as the precipitated colloidal bismuth subcitrate was removed by filtration.

The ability of polyanions to selectively accumulate hydrogen ions into a zone of water immediately adjacent to the fixed charges offers a possible explanation for the in vitro concentration of colloidal bismuth subcitrate in mucus at pH 6-0. The bismuth subcitrate colloid in contact with mucus may concentrate intramuscular hydrogen ions decreasing the pH of its micro-environment to a level where it is precipitated to become a source for Bi'' and bismuthoxy formation and accumulation in mucus. In Helicobacter pylori infection, ammonia decreases the availability of hydrogen ions in mucus, thus, reducing the accumulation of bismuth cations.

The in vitro concentration of colloidal bismuth subcitrate in mucus at pH 1-5 may depend on the interaction of bismuthoxy cations and Bi'' with the negatively charged residues of sialic acid and ester sulphates present in the glycoprotein matrix. Gastric mucus contaminated with microorganisms other than Helicobacter pylori shows a diminished number of sialic acid residues. Helicobacter pylori induces changes in the physicochemical integrity of mucus and may similarly decrease these residues accounting for the reduced capacity of Helicobacter pylori infected mucus to concentrate colloidal bismuth subcitrate when compared with non-infected mucus at pH 1-5.

Another explanation for the differences in intramuscular bismuth concentrations is an alteration of composition and structure of mucus caused by continued glycoprotein and lipid digestion by Helicobacter pylori enzymes between death, harvesting of mucus samples and the experiments.

We postulate that the precipitated and soluble forms of colloidal bismuth subcitrate have different therapeutic actions. Gastric ulcer healing is related to the formation of a protective layer of precipitated colloidal bismuth subcitrate bonded to serous secretions on the ulcer crater. An acid pH of the lumen should aid precipitation of
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The layer. Helicobacter pylori associated gastritis and duodenal ulcer healing depends on the capacity of mucus (mucus of gastric metaplasia in the case of duodenal ulcer) to concentrate bismuth cations either in solution or mobilised from bismuth subcitrate colloid in contact with mucus (depending on luminal pH).

The concentrations of bismuth in infected mucus at pH 1–5 (14 to 32 μg/g; 16 to 36 μg/g calculated as Bi₂Os) observed in the present study will not be representative of the in vivo situation because they were obtained after 90 minutes of bathing with solutions at a constant concentration. In vivo, a favourable intragastric pH of about 1–5, i.e., gastric emptying of 40% or more for liquid meals after 20 minutes, 1,3 15 the diluting effects of food and the long disintegration time of commercial tablets at pH <1–1 (Fig 2) will restrict the intragastric availability of colloidal bismuth subcitrate in solution.

Intramuscular bismuth concentrations falling below the mean inhibitory concentration of colloidal bismuth subcitrate for Helicobacter pylori (4 to 32 μg/ml in vitro,14–16 19) would explain the failure of colloidal bismuth subcitrate to always eliminate the organism. Bismuth concentrations in endoscopic biopsy samples after an oral dose of colloidal bismuth subcitrate20–22 will contain mucus-surface precipitated colloidal bismuth subcitrate and will not represent intramuscular bismuth subcitrate.

The clinical efficacy of colloidal bismuth subcitrate in healing peptic ulcers and gastritis is dependent on multiple mechanisms. Eradication of Helicobacter pylori is an important factor. Resolution of the gastritis when associated with peptic ulcer will play an important role in ulcer healing, possibly through humoral mechanisms.

Small intramuscular colloidal bismuth subcitrate concentrations either in gastric and/or duodenal mucus may aid healing through stimulation of prostaglandin production,5 6 15 enhancement in the activity of epidermal growth factor,15 17 inhibition of the protease and lipase activities of Helicobacter pylori towards gastric glycoproteins,18 19 and possible detachment of microorganisms from the gastric epithelium.17

Preliminary results of a study conducted by Salmon23 showed increased early (four weeks) healing of duodenal ulcers treated with a combination of colloidal bismuth subcitrate and cimetidine when compared with colloidal bismuth subcitrate or cimetidine alone. Maseo et al.24 observed the existence of some duodenal ulcer patients with intragastric pH values steadily ranging around 1–0 throughout the 24 hours, regardless of meals and of the time of the day. As Figure 1 shows, at both pH’s (3.75 to 6.25 and 1.0), colloidal bismuth subcitrate solubility will be favoured but, as speculated, the colloidal bismuth subcitrate forms in solution will be different. Further studies will be necessary to determine if the intragastric pH is important for a better success in eradicating Helicobacter pylori.

The ecological niche for Helicobacter pylori is the gastric mucus. For any antibacterial agent to eradicate it, it must be able to penetrate the mucus layer in sufficient concentrations. We have shown that gastric mucus possesses the capacity to concentrate colloidal bismuth subcitrate and that this capacity is reduced when Helicobacter pylori are present. In vivo, the reduced capacity of mucus to concentrate colloidal bismuth subcitrate may compromise the attainment of optimal intramuscular colloidal bismuth subcitrate concentrations necessary for its bactericidal activity. The behaviour of colloidal bismuth subcitrate in acidic solutions suggests that the intragastric availability of the drug in solution may be low, further restricting satisfactory antibacterial intramuscular concentrations.

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