Bismuth subsalicylate reduces peptic injury of the oesophagus in rabbits

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Abstract

Bismuth subsalicylate was tested in an *in vivo* perfused rabbit model of oesophagitis for its ability to prevent the mucosal injury caused by pepsin. Treatment efficacy was assessed under both a treatment-before-injury protocol and a treatment-after-injury protocol. Oesophageal mucosal barrier function was evaluated by measuring flux rates of H⁺, K⁺, and glucose. The degree of oesophagitis was determined by gross and microscopic examination of the mucosa by several independent observers. Results showed that under both treatment protocols, bismuth subsalicylate significantly reduced the pepsin induced disruption of the mucosal barrier, as well as the morphologic changes. Bismuth subsalicylate when given after exposure to pepsin was also found to protect against the morphologic injury in a dose dependent manner. Experiments *in vitro* suggested that bismuth subsalicylate inhibits the proteolytic action of pepsin by interacting with pepsin, rather than with the pepsin substrate. We conclude that bismuth subsalicylate can protect the oesophageal mucosa against peptic injury, probably through inactivation of pepsin.

The oesophageal mucosal injury found in patients with reflux oesophagitis occurs as a result of abnormal exposure of the mucosa to the corrosive gastric contents. We have used an *in vivo* perfused rabbit model of oesophagitis to elucidate the mechanisms of oesophageal mucosal injury¹⁻⁵ and to test agents which might be useful in the prevention or treatment of such injury.⁶

The present study was designed to test the efficacy of bismuth subsalicylate in preventing experimental oesophagitis. Bismuth subsalicylate has been used for many years as an over-the-counter drug for the relief of heartburn, which is often associated with oesophagitis. Bismuth compounds have been shown to reduce experimental gastric mucosal injury in the rat,^{7,8} and they have been used successfully to treat peptic ulcer disease in man.^{9,10} Ulcer healing with bismuth compounds has been shown to be similar to cimetidine,¹¹⁻¹³ even though it lacks a significant acid neutralising capacity. Because the mucosa of the oesophagus and stomach are both exposed to the same caustic gastric contents, we investigated whether bismuth is efficacious against mucosal injury of the oesophagus.

Some of the potentially injurious components of the oesophageal refluxate fluid include hydrochloric acid (HCl), proteolytic enzymes such as pepsin, and the bile acids.³ In experimental

models of oesophagitis HCl inflicts little injury to the intact oesophageal epithelium, except at very high concentrations and after prolonged periods of exposure.^{14,16} The bile acids typically increase the oesophageal mucosal permeability, but cause only a relatively modest degree of accompanying morphologic change.^{4,6} In contrast, pepsin not only causes a significant increase in the permeability of the oesophageal mucosa, but also causes a frankly haemorrhagic, severe morphologic injury.^{3,6} The present study was therefore carried out with pepsin as the injurious agent, as it appears to be the most damaging of the components of an acidic gastroesophageal refluxate fluid.

We performed *in vivo* experiments to determine whether bismuth subsalicylate had protective effects against the pepsin induced oesophageal injury. Bismuth subsalicylate was tested in a treatment-before-injury and a treatment-after-injury protocol. After bismuth was found to be protective *in vivo*, the antipepsin activity of bismuth subsalicylate was tested *in vitro* to attempt to clarify its mechanism of action.

Methods

IN VIVO EXPERIMENTS

Oesophageal perfusion

An *in vivo* perfused rabbit model of oesophagitis, adapted from Chung¹² was used as described.^{1,3,6} In brief, New Zealand white rabbits weighing 3-5 kg were lightly anaesthetised with an intramuscular injection of ketamine/xylazine. The oesophagus was cannulated in the neck at the pharyngo-oesophageal junction and in the abdomen at the gastroesophageal junction with plastic tubing (id 0.317 mm). The oesophagus was then perfused with 45 ml of various test solutions at 10 ml/min using a peristaltic pump (Harvard Apparatus Co, Millis, MA) and a recirculating system. The perfusate solution was stirred mechanically (Haake, Berlin, Germany), and a thermoregulator (Haake) kept the temperature of the perfusate at 37°C. A pH stat/autoburette system (Radiometer, Copenhagen, Denmark) containing 0.4 N HCl constantly maintained the pH of the perfusate at 2.

Each experiment consisted of a 30 minute 'exposure period' in which the perfusate solution contained pepsin at pH 2. This was followed by a 40 minute 'flux period' in which the perfusate solution contained no pepsin, but was used for measuring transmucosal flux rates of H⁺, K⁺, and glucose. In the treatment-before-injury protocol, for 10 minutes before the initiation of the 30 minute exposure period, the oesophagus

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was perfused with either saline 145 mmol/l (control group) or saline plus bismuth subsalicylate (The Procter and Gamble Co, Cincinnati, OH) 30 mg/ml (study group), both at pH 2. At the start of the exposure period, porcine pepsin (Sigma Chemical Co, St Louis, Missouri) was added to the perfusate of both the control and study groups to obtain a final perfusate concentration of 1 mg/ml. The pH of the perfusate was maintained at 2.0 with HCl. At the end of this exposure period, the perfusate was discarded and the entire system, including the oesophagus, was irrigated with 100 ml isotonic saline in preparation for the subsequent flux period.

The exposure period in the treatment-after-injury protocol was similar to that in the treatment-before-injury protocol, except that the bismuth subsalicylate was added after an initial oesophageal exposure to pepsin. The 30 minute exposure period of both the control and study groups began with perfusion of the oesophagus with a solution containing 1 mg/ml of porcine pepsin at pH 2. Ten minutes after the initiation of the exposure period, an amount of bismuth subsalicylate was added to the perfusate solution of the study groups to obtain a concentration of either 15 mg/ml, 30 mg/ml, or 60 mg/ml of bismuth subsalicylate. Concomitantly, a quantity of pepsin and HCl was added to the perfusate to maintain the pepsin concentration at 1 mg/ml and the pH at 2. At the end of the exposure period, the perfusate was discarded and preparation was made for the flux period, as in the treatment-before-injury protocol.

The methods used during the flux period were identical for both the treatment-before-injury and the treatment-after-injury protocols. The flux solution contained 1.5 g/l polyethylene glycol (PEG, Fisher Scientific Co, Fair Lawn, NJ) 100 mCi/l ^3H -PEG (New England Nuclear, Boston, Mass), and 10 mM HCl at pH 2. The osmolality was brought to 280 mosmol/l with mannitol. A 4 ml aliquot of the flux solution was taken from the reservoir at the beginning and end of the 40 minutes flux period for later analyses of K^+ and glucose. A pH of 2 was constantly maintained in the perfusate solution with the pH stat/autoburette apparatus. After completion of the flux period, the animal was killed with an intracardiac bolus of pentobarbital and the oesophagus was excised.

Calculation of fluxes

Oesophageal mucosal permeability to ions and small molecules was determined by calculating H^+ , K^+ , and glucose flux rates. Hydrogen ion flux out of the lumen was given by the amount of HCl delivered by the pH stat/autoburette apparatus to maintain the pH of the solution at 2. Concentrations of K^+ were assayed by ion selective electrode potentials (Beckman System E4A, Beckman Instruments, Fullerton, CA), and concentrations of glucose were assayed by the hexokinase reaction (Centrifichem System 500, Union Carbide Corp, Rye, NY). Fluxes were calculated using the following formula:

$$\text{Net flux} = (C_F - C_I) \times V_I$$

where V_I is the initial volume of the perfusate at the beginning of the flux period, C_I is the initial concentration of K^+ or glucose in the perfusate solution, and C_F is the final concentration. ^3H -PEG was used as an impermeable volume marker during the initial experiments. It was not used in the later experiments because it was found to be unnecessary, as have others who have used this animal model have reported.¹⁴ Polyethylene glycol was deemed unnecessary when it was found in the initial experiments that the volume and PEG recovery rates were between 96–99%, and the volume flux was relatively low (less than 0.5 ml per experiment).

Determination of oesophagitis index

Immediately after removal, the oesophagus was fixed in 10% formalin. A photograph of the fixed specimen was taken for later grading of the degree of gross morphologic injury. Histologic slides were prepared from sections taken from the proximal, central, and distal portions of the esophagus and stained with haematoxylin and eosin. Photographs and slides of all specimens were scored for the severity of oesophagitis by several observers unaware of the treatments given, using a system previously described.^{3,6} In brief, the gross oesophagitis index was determined by the following criteria: 1=normal appearance; 2=erythema or other abnormal appearance, but no haemorrhage; 3=non-confluent mucosal haemorrhage; 4=confluent intramural haemorrhages (Fig 1). The microscopic oesophagitis index was scored by these

Figure 1: Grades of gross oesophagitis. Numbers correspond to the gross oesophagitis index (GOI).

Figure 2: Grades of microscopic oesophagitis. Numbers correspond to the microscopic oesophagitis index (MOI).

criteria: 1=normal oesophagus; 2=submucosal oedema or separation of epithelial layers; 3=focal areas of intramural haemorrhage or partial epithelial loss; 4=large areas of haemorrhage or complete epithelial desquamation (Fig 2).

In vitro experiments

A series of *in vitro* experiments were performed to determine whether bismuth subsalicylate could inhibit the proteolytic action of pepsin on haemoglobin, and if so, whether this inhibition was caused by the interaction of bismuth subsalicylate with pepsin or its substrate. The biochemical principle underlying this experiment was that if an enzyme inhibitor acts by binding to the substrate molecule, then the inhibition observed at a low substrate concentration can be reduced by providing more substrate to the reaction. Alternatively, if the inhibition is caused by the inhibitor binding to the enzyme, then the inhibition will not be reduced by increasing the concentration of substrate.¹⁷ In the experiment, 50 mg/ml pepsin was dissolved in one of the following solutions: (a) HCl at pH 2, or (b) HCl plus 30 mg/ml bismuth subsalicylate at pH 2. The peptic activity of these solutions was then assayed by spectrophotometrically measuring the amount of tyrosine released from 2.5 ml of 1, 2, 3, or 4 g% solutions of bovine haemoglobin substrate.¹⁸

Statistical analysis

Each experimental group contained between five and nine animals. All results are expressed as the mean $\pm$ one standard error of the mean (SE). The differences between means of groups of continuous data (H^+ , K^+ , and glucose flux rates) were assessed for statistical significance ($p < 0.05$) using the Student's unpaired *t* test. Non-parametric data (gross and microscopic

oesophagitis grades) were compared for significant differences ($p < 0.05$) with the Mann-Whitney test.

Results

IN VIVO EXPERIMENTS

Treatment-before-injury protocol

When administered before exposure of the oesophageal mucosa to pepsin, bismuth subsalicylate reduced both the permeability changes and the morphologic injury caused by pepsin. As shown in Table I, significantly lower transmucosal flux rates of H^+ , K^+ , and glucose occurred in the group treated with bismuth subsalicylate than in the untreated group ($p < 0.05$). Treatment with bismuth subsalicylate before pepsin exposure also significantly reduced the morphologic injury, as reflected by both the gross and microscopic oesophagitis indices. The gross oesophagitis index of the untreated control group was 3.2 (0.2), compared with the group treated with bismuth subsalicylate, which was graded as 2.0 (0.3) ($p < 0.01$, Fig 3). Similarly, the microscopic oesophagitis index was reduced from 3.1 (0.2) in the untreated group to 2.1 (0.2) in the group treated with bismuth subsalicylate ($p < 0.001$, Fig 3).

Treatment-after-injury protocol

The efficacy of bismuth subsalicylate in reducing the oesophageal injury when administered after exposure to pepsin was similar to that observed when it was administered before the pepsin exposure. Flux rates of H^+ , K^+ , and glucose were significantly lower in the groups exposed to 15 mg/ml, 30 mg/ml, and 60 mg/ml of bismuth subsalicylate than those in the untreated control group ($p < 0.05$, Table II). Bismuth subsalicylate

TABLE I Transmucosal flux rates in the treatment-before-injury experiments

	Flux rate†		
	H ⁺ ($\mu\text{mol}/10\text{ min}$)	K ⁺ ($\mu\text{mol}/10\text{ min}$)	Glucose ($\mu\text{mol}/10\text{ min}$)
No treatment	53 (6)	7 (1)	5 (1)
BSS treatment	28 (3)*	3 (1)*	3 (1)*

Results are expressed as mean (SE). Bismuth subsalicylate 30 mg/ml.

*Significantly different from no treatment group, $p < 0.05$;

†Direction of flux: H⁺=out of lumen, K⁺, glucose=into lumen.

also reduced both the gross and microscopic esophageal indices in a dose dependent manner (Fig 4). The gross esophagitis index of the untreated group was 3.4 (0.2), while the treated groups were graded as 2.5 (0.2), 2.3 (0.2), and 1.8 (0.2) for bismuth subsalicylate concentrations of 15 mg/ml, 30 mg/ml, and 60 mg/ml, respectively ($p < 0.05$). Similarly, the microscopic esophagitis index was reduced from 3.4 (0.3) in the untreated group to 2.5 (0.4), 2.3 (0.2), and 1.8 (0.3) for the same concentrations of bismuth subsalicylate ($p < 0.05$).

In vitro experiments

In vitro experiments were done to determine if bismuth subsalicylate interacted predominantly with pepsin or its substrate. Peptic activity of acidic solutions containing either pepsin alone or pepsin plus bismuth subsalicylate was assayed in solutions containing haemoglobin substrate concentrations of 1, 2, 3, and 4 g%. The results are shown in Figure 5. When assayed in a 1% haemoglobin substrate solution, peptic activity

TABLE II Transmucosal flux rates in the treatment-after-injury experiments

	Flux rate†		
	H ⁺ ($\mu\text{mol}/10\text{ min}$)	K ⁺ ($\mu\text{mol}/10\text{ min}$)	Glucose ($\mu\text{mol}/10\text{ min}$)
No treatment	61 (5)	7 (1)	8 (2)
BSS 15 mg/ml	49 (5)*	4 (1)*	4 (1)*
BSS 30 mg/ml	46 (5)*	3 (1)*	5 (1)*
BSS 60 mg/ml	43 (7)*	4 (1)*	5 (1)*

Results are expressed as mean (SE). Bismuth subsalicylate.

*Significantly different from no treatment group; †Direction of flux: H⁺=out of lumen, K⁺, glucose=into lumen.

Figure 3: Gross and microscopic esophagitis indices from experiments in which bismuth subsalicylate was administered before pepsin exposure. * = Significantly different ($p < 0.01$) from the control group, which was exposed to pepsin but with no bismuth subsalicylate treatment.

Figure 4: Gross and microscopic esophagitis indices from experiments in which bismuth subsalicylate was administered 10 minutes after pepsin exposure. * = Significantly different ($p < 0.05$) from the control group, which was exposed to pepsin but with no BSS treatment.

of the solution containing pepsin plus bismuth subsalicylate was about half the peptic activity of the solution containing only pepsin. As the substrate concentration was increased, peptic activity of the solution containing pepsin plus bismuth subsalicylate did not increase. These results suggest an interaction of bismuth subsalicylate primarily with pepsin *in vitro*. It is also possible, however, that bismuth subsalicylate could exert some of its protective effect *in vivo* by binding to mucosal proteinaceous substrates for which it has a higher affinity than haemoglobin.

Discussion

The findings of this study show that bismuth subsalicylate can significantly reduce the severity of the esophageal mucosal injury caused by pepsin. Administration of bismuth subsalicylate to rabbits, at concentrations similar to that in commercially available products (Pepto-Bismol, 17.5 mg/ml), diminished the permeability changes and the morphologic injury induced by pepsin. This protection was observed whether bismuth subsalicylate was given before or after the esophageal mucosa was exposed to pepsin. Additionally it was noted that, when given after exposure to pepsin, bismuth subsalicylate suppressed the pepsin mediated esophageal morphologic injury in a dose dependent manner.

Figure 5: *In vitro* pepsin assay. Significantly lower pepsin activity was measured when bismuth subsalicylate was present than when it was not (* = $p < 0.05$). Bismuth subsalicylate apparently interacts directly with pepsin, rather than the substrate, because increasing the substrate concentration did not increase the peptic activity.

There are several possible mechanisms by which this protection could occur. Bismuth compounds are known to complex with proteins. Bismuth subsalicylate could therefore interfere with the peptic digestion of the oesophageal mucosa by complexing with either pepsin or with the pepsin substrate protein in the mucosa. Our *in vitro* experiment would indicate that the former occurs and, indeed, pepsin inactivation by bismuth compounds has been described by others.^{12 19 20}

Alternatively, we could not rule out the possibility of a topical protective effect of bismuth subsalicylate by binding to the oesophageal mucosa in addition to the pepsin inactivation. It has been suggested that bismuth subsalicylate and other bismuth salts encourage gastric and duodenal ulcers to heal because of their ability to bind with various glycoproteins and mucopolysaccharides at the ulcer base. Bismuth compounds are thought to form a barrier over the base of an ulcer which protects it from the noxious luminal contents.²¹⁻²⁵ When complexed with gastric mucus, bismuth has been shown to drastically retard the migration of hydrogen ions.²⁶ These local effects have not yet been described in the oesophagus, but could have contributed to the protective effect observed *in vitro* in the present study. In a clinical setting administration of bismuth subsalicylate in a liquid form (Pepto Bismol, Proctor and Gamble), which could coat the oesophagus during swallowing, would be desirable to take advantage of any topical properties.

Bismuth is reported to exhibit other interesting properties which are pertinent to its potential applicability to the treatment of esophagitis. Colloidal bismuth was recently shown to suppress the activity and output of pepsin in patients with duodenal and gastric ulcers.²⁷ This effect was still present 24 hours after the medication had been discontinued, suggesting the inhibition was sustained.²⁸ A long lasting agent would be desirable in treating reflux oesophagitis, where protection is frequently needed during periods of nocturnal reflux.²⁹ In addition, its activity against campylobacter like organisms may be beneficial, as these bacteria may contribute to the pathogenesis of oesophagitis in some cases.³⁰

Current therapies for oesophagitis have traditionally included measures aimed at reducing reflux, either by increasing lower oesophageal sphincter tone or decreasing intra-abdominal pressure. Furthermore, the mainstay of therapy rests with reduction of gastric acidity. None of the existing therapies, however, are specifically directed at diminishing peptic injury of the mucosa, even though part of the efficacy of antacids may derive from a reduction of the refluxate pepsin activity through a pH rise. We recently found that the mucosal protective agent sucralfate was highly effective in preventing experimental peptic oesophagitis in the rabbit.⁶ This benefit was attributed to a topical protection when it was found that sucralfate did not inactivate pepsin. The present study suggests that, through direct inactivation of pepsin, bismuth subsalicylate could provide an added benefit to a clinical therapeutic regimen which might include a combination of agents acting

to prevent mucosal injury by different mechanisms.

In conclusion, these studies indicate that bismuth subsalicylate can prevent the oesophageal mucosal injury caused by pepsin. Its effect derives, at least in part, from its capacity to interact with pepsin.

In conducting the research described in this report, the investigators adhered to the *Guide for laboratory facilities and care* as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council. This work was funded in part by Proctor and Gamble. The authors acknowledge the assistance of Barbara L. Bass, Raj Lakshman, and his staff, and William Andrews.

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