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A SCANNING ELECTRON MICROSCOPIC MORPHOLOGICAL AND SEMI-QUANTITATIVE EVALUATION OF RAT STOMACH TREATED WITH COLLOIDAL BISMUTH SUBCITRATE AND ALCOHOL

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Abstract

Scanning electron microscopy was utilised to study the effect of absolute alcohol on the normal morphology of the rat stomach, together with the gastroprotective actions of colloidal bismuth subcitrate. Studies on normal gastric morphology revealed that the major portion of the stomach was covered by a protective coating of mucus. However, there was considerable variation in the integrity of the mucosal surface of the control animals, with the loss of surface epithelial cells in some regions which may account for the variation in response to necrotising agents. The long-term administration of the gastrocytoprotective agent colloidal bismuth subcitrate resulted in a marked improvement in normal gastric integrity, compared with control tissue samples. The administration of absolute alcohol was associated with an excessive production of mucus and caused extensive damage to the gastric mucosa of control animals, resulting in destruction of the surface epithelial cells and exposure of the reticular framework. However, there was evidence that repair of this damage was underway by four hours after ethanol treatment, with a significant degree of recovery from damage occurring by 24 hours after treatment. In contrast, treatment with colloidal bismuth subcitrate prior to the administration of alcohol resulted in a significant reduction in the degree of damage induced by alcohol administration, suggesting that colloidal bismuth subcitrate has the ability to protect the stomach from the erosive action of alcohol.

Key Words: Colloidal bismuth subcitrate, alcohol, stomach, gastric damage, gastric mucosa, mucus, cytoprotection, gastric integrity, repair mechanisms, morphology.

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Introduction

The functional and morphological integrity of the mammalian stomach is maintained by complex cytoprotective mechanisms which prevent gastric injury by acid and pepsin. The major components of the gastric cytoprotective system consist of an intact mucus layer, bicarbonate secretion by the epithelial cells, the epithelial cell barrier, an adequate blood supply and the production of local hormones (Robert, 1979; Tasman-Jones, 1986; Tasman-Jones *et al*, 1987). Gastric damage occurs when there is an imbalance between these protective mechanisms and the destructive actions of acid and pepsin. The cytoprotective properties of colloidal bismuth subcitrate (CBS) are known to be effective in augmenting the healing of such damage, as well as preventing the recurrence of gastric damage (Hinsull & Bellamy, 1990).

The protective and therapeutic actions of CBS are the result of a number of its properties. CBS precipitates in an acid environment to form bismuth citrate and bismuth oxychloride, which preferentially bind to areas of damage and form a protective coating, thus preventing further erosion by the gastric luminal contents (Tasman-Jones *et al*, 1987; Koo *et al*, 1982). Other properties of CBS include the ability to stimulate mucus secretion (Bardhan, 1981), bind to pepsin (Roberts & Taylor, 1982), inhibit pyloric *Campylobacter* (Marshall *et al*, 1985) and stimulate the generation of gastric prostaglandins (Hall & van den Hoven, 1987). It is well established that short-term pretreatment of rats with CBS prevents gastric damage by ulcerogenic agents (Hall & van den Hoven, 1986: Konturek *et al*, 1987b). However, this protective action of a single CBS application is only effective for a maximum of six hours, therefore the aim of the present experiments was to carry-out a scanning electron microscope study of the effects of long-term exposure to CBS on the resistance to gastric damage, and to investigate the possible establishment of a more enduring resistance to damage.

Materials and Methods

Animal Treatment

The experimental procedures were carried-out on sixty, nine week old, WAB/Sh male rats of 200-250 g body weight, which were routinely maintained on a standard pelleted diet and tap water *ad libitum*. The animals were weight-matched into four experimental groups, one week before the commencement of the experiment. The four groups received one of the following treatments:- Group 1 consisting of 20 animals: ethanol alone; Group 2 consisting of 10 animals: CBS alone; Group 3 consisting of 20 animals: ethanol+CBS; Group 4 consisting of 10 animals: distilled water.

All of the animals were deprived of food for 24 hours before the initiation of the experiment, at which time the ethanol alone and ethanol+CBS groups were given 4µl of 100% ethanol/g body weight, by oral intubation. The control and CBS alone groups received equivalent volumes of distilled water. Drinking water and food were withheld from all groups for 1 hour after treatment.

The CBS alone and ethanol+CBS groups were given 240mg colloidal bismuth subcitrate in 2ml. of distilled water/kg body weight, by oral intubation, 2 hours after the ethanol or placebo administration. This dose of CBS was repeated every 12 hours for the following 7 days, with distilled water used as the equivalent placebo treatment for the control and ethanol alone groups (Table 1).

On day seven of the experiment, a second instillation of absolute ethanol was administered, as previously described, to the fasted, ethanol alone group. Food and water were withheld for a further hour and 2ml. of distilled water/kg body weight was administered at 12-hourly intervals thereafter as a placebo for CBS treatment. The animals were killed at 2h, 4h, 24h and 7 days after the second dose of ethanol.

The ethanol + CBS group were treated in an identical manner to the ethanol alone animals, but CBS administration was continued at 12 hourly intervals. Animals from this group were killed at 2h, 4h, 24h and 7 days after the second ethanol instillation.

The CBS alone group were given 4µl of distilled water/g body weight on day seven of the experiment. The animals were maintained on the 12 hourly CBS regime for a further 7 days and killed after a total of 14 days of CBS treatment. Five animals were killed at each time interval in each experimental group (Table 1).

In a second experiment the experimental procedure was repeated to provide replicate tissue samples in order to check the accuracy of the semi-quantitative analysis.

Electron Microscopy

The animals were killed by cervical dislocation. The abdominal cavity was opened immediately and ligatures applied to the esophageal and duodenal junctions of the stomach. 0.5 ml of fixative, (consisting of 9 parts buffered neutral formalin : 1 part acetone) at 4°C , was injected into the gastric lumen. The entire stomach was removed and placed into the same fixative for an additional 30 minutes. The stomach was opened along the greater curvature and placed in fresh fixative at room temperature for an additional 17 hours. 8 mm. x 4 mm strips of tissue were removed from the fundic region, 3 mm below antral region and midway between the greater and lesser curvature on the dorsal surface. The tissue specimens were stored in 0.1 M Sorensen's phosphate buffer, pH 7.4, prior to preparation for the scanning electron microscope.

The tissue was dehydrated through a series of graded alcohols and substituted with liquid CO_2 in a Polaron Critical Point Drier. The liquid CO_2 was taken through its critical point at 1200 psi and 33°C and, after very slow venting of the gas, the specimens were removed. All specimens were mounted on aluminium stubs, with the surface epithelium facing upwards. After sputtering with gold in a Nanotech Sputterer, the specimens were viewed using a Cambridge 600 Stereoscan.

Morphology and Semi Quantitative Analysis

The gastric surface structure was examined in detail using a scanning electron microscope. In addition, morphometric studies were carried-out on the four experimental groups:- control- 2 hours after distilled water, 2 hours after ethanol alone, 2 hours after ethanol + CBS and CBS alone- 2 hours after distilled water. In the semi-quantitative analyses each microscope field was categorised into one of five main stages of tissue integrity :- 1. Mucus - the majority of the gastric surface is coated with mucus (Fig. 1); 2. Cell domes - intact epithelial cells organised around the pits to form regular domes of cells- indicative of healthy, undamaged tissue (Fig. 2); 3. Random cells - mainly intact epithelial cells but arranged in a haphazard fashion, with no obvious organisation around the pits and some evidence of cell debris- indicative of superficial damage to the gastric surface (Fig. 3); 4. Cells on reticulum - Single epithelial covering over the reticular framework - indicative of the initial stages of gastric repair involving the migration of epithelial cells to cover the exposed reticular framework (Fig. 4); 5. Bare reticulum - destruction of the epithelium to reveal the underlying reticular framework - indicative of severe erosion of the gastric surface (Fig. 5).

A semi-quantitative assessment was carried-out in the following manner: A standard magnification of x500 was used throughout the exercise and each specimen was methodically scanned at that magnification, starting at the top left of the specimen. After examination of each field, the specimen was moved one field across until the edge of the specimen was reached. At this point the specimen was moved one field down and the process repeated in the opposite direction. Each specimen usually consisted of approximately 100 microscope fields but as many standard size fields as possible were assessed and classified for each specimen . For each specimen, the number of fields in each category of integrity were expressed as a percentage of the total number of fields counted for that sample. The

Table 1 : Outline Of Experimental Regime

	CONTROL	CBS ALONE	ETHANOL ALONE	ETHANOL + CBS
Day 1	Dist.water	Dist.water	Ethanol	Ethanol
	↓	↓	↓	↓
	Dist water 12 hourly for 7 days	CBS 12 hourly for 7 days	Dist.water 12 hourly for 7 days	CBS 12 hourly for 7 days
	↓	↓	↓	↓
Day 7	Dist. water	Dist. water	Ethanol	Ethanol
	↓	↓	↓	↓
	Dist.water	CBS	Dist.water	CBS
	↓	↓	↓	↓

Animals killed from each group at 2 hours, 4 hours, 24 hours and 7 days after second ethanol/placebo exposure.

results were expressed as the mean of five samples in each experimental group. This procedure was replicated by two independent observers examining separate coded tissue specimens. A two sample *t*-test was used to test the significance of the difference between the mean values of the categories.

Results

Controls.

In the tissue taken from animals receiving neither CBS nor alcohol, over 77% of the areas examined were covered with mucus (Fig. 6). Of the remaining areas, approximately 7% fell into the cell dome and random cell categories and there were also areas classified as cells on the reticulum. Areas of bare reticulum were not apparent.

CBS alone

Animals in this group were treated with CBS at 12-hour intervals for 14 days, with the final dose administered 1 hour prior to death. This regime had no apparent ill effects on the growth or general welfare of the animals. The major difference between the control and CBS alone group lay in the 30% reduction in the number of areas covered with mucus found in the CBS group compared with the control samples (Fig. 6). About 47% of the areas examined in the CBS group were covered in mucus, with a slight increase, over control values, in the number of areas found to have cell domes. Only very rare instances of bare reticulum, indicating tissue damage, were observed. A minority of areas containing cells on the reticulum indicated recovery from this damage. The remainder of the areas examined consisted of random cells.

Ethanol alone

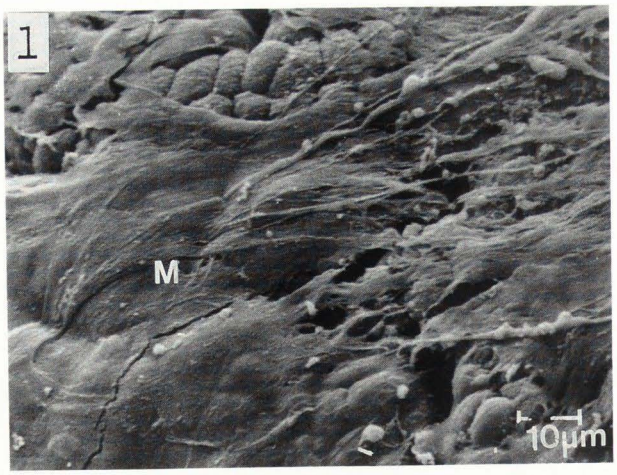
The administration of ethanol, in the absence of CBS, resulted in severe tissue damage. In animals given two doses of ethanol, at 7 days and 2 hours prior to death, the degree of tissue damage was reflected in the increase in the number of areas consisting of bare reticulum to 3.85% (Fig. 6). By far the largest number of areas were found to be covered with mucus. In this instance there were extensive sheets of mucus overhanging the edges of the tissue samples (Fig. 7). However, the 6.87% of areas containing cells on the reticulum indicated that tissue restitution was already underway. Cell domes were only observed in 2% of the areas examined, although 18.82% of the stomach surface consisted of random cells, sometimes partially covered with mucus.

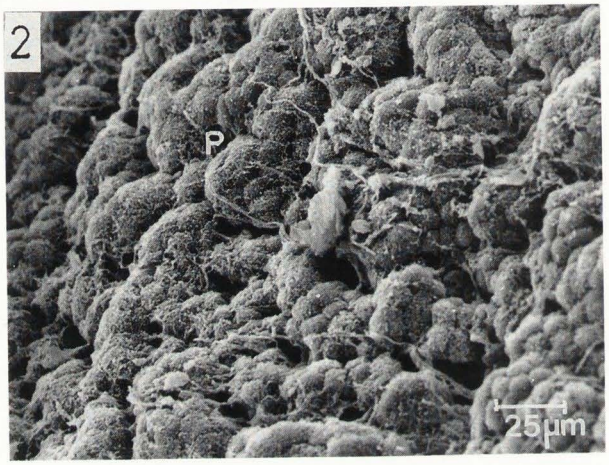
At 4 hours after the second ethanol instillation, morphological studies alone indicated some reduction in the degree of damage observed at 2 hours. Areas consisting of bare reticulum were less obvious, whilst cells on the reticulum were much more in evidence than in the 2 hour group, indicating initial recovery from damage. Once again, a large number of areas was covered in mucus, with an absence of cells domes but the presence of some regions of random cells partially covered with mucus. There were also regions where red blood cell rouleaux were observed on the surface of the tissue (Fig. 8). Overall, the picture at 4 hours after ethanol treatment is similar to that seen at 2 hours, but with indications of tissue regeneration.

At 24 hours after the final ethanol treatment instances of bare reticulum were rare, but areas containing cells on the reticulum were more evident, indicating a significant degree of recovery from the ethanol damage found in the previous two groups. The amount of mucus present, together with the presence of some areas containing cell domes, further indicated a return to normality.

Ethanol + CBS

Following their initial ethanol instillation, animals in this group were treated with CBS at 12-hour intervals. At 7 days a second dose of ethanol was administered and CBS treatment continued. The degree of damage induced by the second dose of gastric ethanol was significantly reduced by CBS treatment. Comparison of the results presented in figure 6 show that there was little difference between the morphometric analyses of tissue treated with CBS alone and that treated with CBS followed by ethanol. When





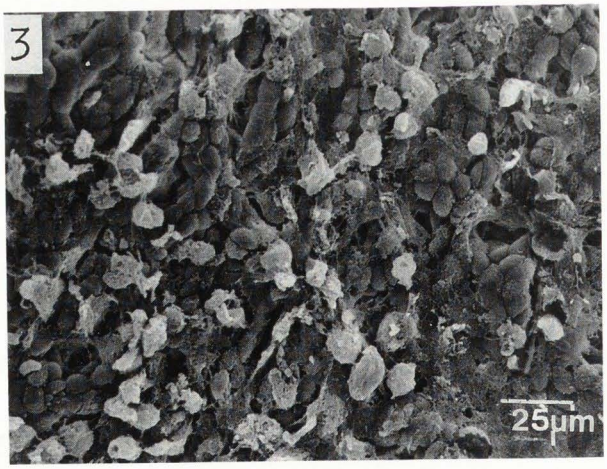
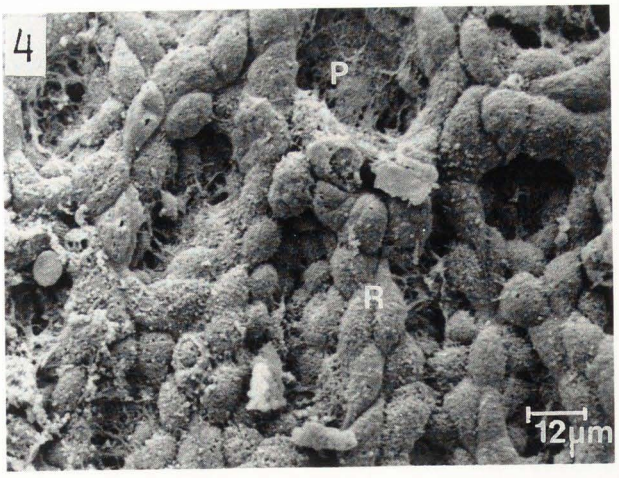


Figure 1. Classification of Tissue Integrity - Mucus. The major part of the gastric luminal surface covered by mucus. M - Mucus.

Figure 2. Classification of Tissue Integrity - Cell Domes. The luminal surface consists of epithelial cells constituting a well-organised tissue structure. P - Pit.

Figure 3. Classification of Tissue Integrity - Random Cells. Intact epithelial cells but forming a disorganised tissue structure.



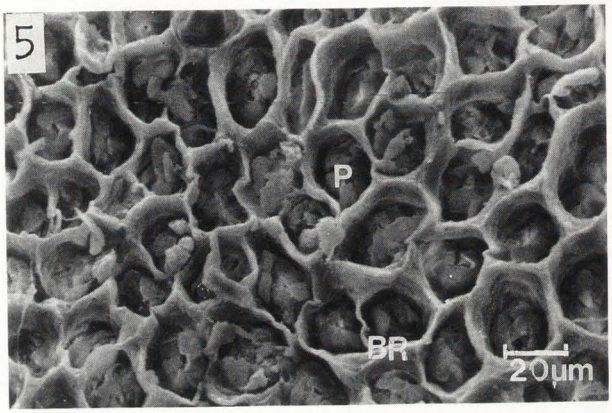


Figure 4. Classification of Tissue Integrity - Cells on Reticulum. Epithelial cells covering the reticular framework, indicating possible tissue regeneration. P - Pit. R - Cells covering reticulum.

Figure 5. Classification of Tissue Integrity - Bare Reticulum. Destruction of the epithelium to reveal the underlying reticular framework, indicating grossly damaged tissue. P - Pit. BR - Bare reticulum.

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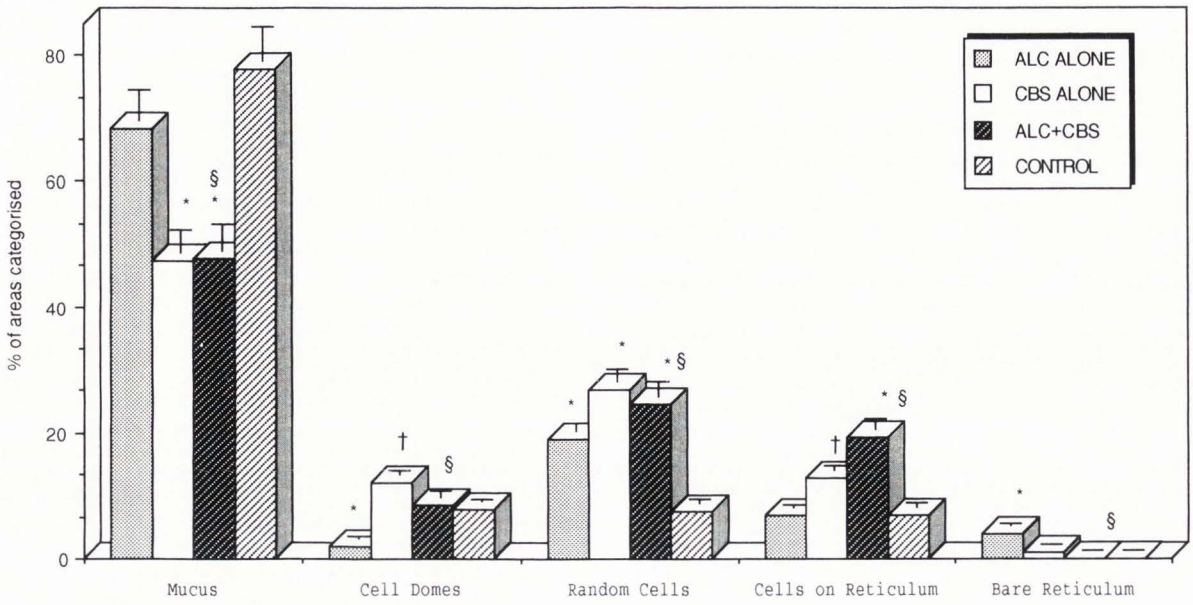
the animals were pretreated with CBS there was little indication of the increased mucus output or deep erosions seen with ethanol administration alone. The number of areas covered with mucus was almost identical to that observed in animals treated with CBS alone with no indication of the increased production of mucus found in the animals treated with ethanol alone (Fig. 6). The number of areas consisting of well organised cell domes was also comparable with control values whilst no areas of bare reticulum were found. A high proportion of the areas were found to contain cells on the reticulum, indicating repair to damaged areas. Generally, the tissue appeared to be in a much healthier state than in animals not receiving CBS.

Figure 6



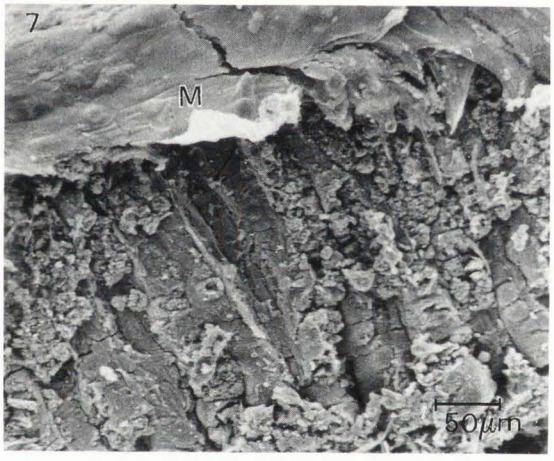
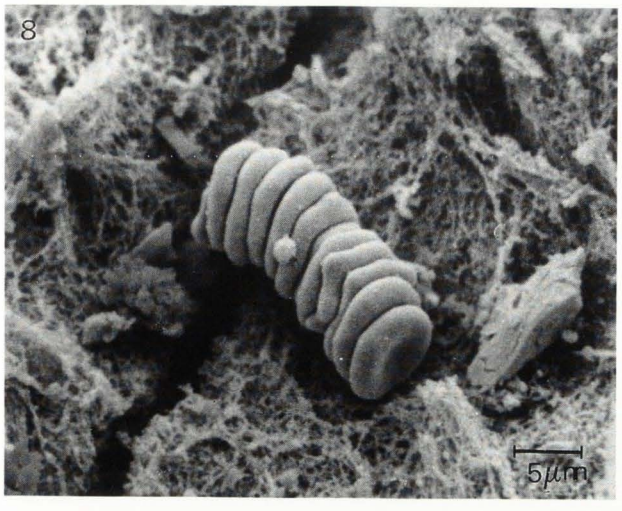


Figure 6. Distribution of areas of gastric integrity, expressed as a percentage of the total number of areas categorised for each experimental group. Results expressed as mean ± standard error. Two sample t-test statistical analysis :- * - significantly different from control values , $p < 0.001$; § - significantly different from control values, $p < 0.02$; † - significantly different from alcohol alone values, $p < 0.001$.

Figure 7. Sheets of mucus overhanging the edges of tissue from animals given alcohol alone 2 hours prior to death. M - Mucus.

Figure 8. Red blood cell rouleau on the gastric luminal surface of tissue from animals given alcohol alone 4 hours prior to death (Alcohol Alone).



Discussion

It is well established that mucus plays an important role in maintaining the functional integrity of gastric mucosa (Allen & Garner, 1980). It was to be expected, therefore, that the major part of the stomach surface of control animals would be covered with mucus. However, tissue processing may have disrupted part of the mucus layer. In the areas not covered by mucus it was apparent that there was considerable variation in the integrity of the gastric epithelial surface with some indication of epithelial cell loss. This complies with earlier work which showed that damage, in the form of loss of surface epithelial cells, can occur after food intake (Grant *et al*, 1953). This diversity in gastric integrity may result in a variation across the mucosa in the response to necrotising agents such as ethanol.

In comparison with control values, CBS administration alone resulted in a threefold increase in the number of areas with random cells and proportionally smaller increases in areas with cell domes and cells on the reticulum. This again illustrated the variation in the surface structure of the stomach. There was, however, little indication that long-term treatment with CBS resulted in damage to the cellular integrity of the gastric mucosal surface despite the apparent depletion of the protective mucosal layer. These findings concur with earlier work at the light microscope level showing that the long-term administration of CBS results in an increase in the number and alcohol resistance of the gastric mucosal epithelial cells (Hinsull & Bellamy, 1990). An apparent decrease in the number of mucus covered areas after CBS treatment, but this may have been due to shrinkage of the mucosal layer during tissue preparation. Previous reports have shown that short-term CBS treatment results in increased mucus secretion (Hollanders *et al*, 1983).

The administration of ethanol alone resulted in extensive gastric damage. This concurs with the numerous reports on the action of a single dose of absolute alcohol on the stomach (Hinsull & Bellamy, 1990). Several workers have reported that the administration of necrotising agents results in the production of copious amounts of mucus in the areas of gastric injury, which acts as an initial barrier to prevent further erosion of the damaged area (Sellers *et al*, 1987). This was apparent in the present experiments from the sheets of mucus found overhanging the tissue samples. The results of light microscope studies on tissue samples from adjacent regions to those used for the present experiments, together with the evidence from previous reports, suggest that the mucus may cover underlying areas of acute damage (Hinsull & Bellamy, 1990; Sellers *et al*, 1987). In regions not masked by mucus there was an increase in the number of areas where bare reticulum was observed. This indicated the destruction of the epithelial cellular population to reveal the laminal scaffold and denoted severe erosion of the gastric lining. Although gastric damage appeared to be severe, morphological studies indicated that the gastric repair mechanism was active at four hours after exposure to ethanol, and by twenty-four hours there were signs of a significant degree of damage repair. This time scale is in agreement with that of previous work using light microscopy which showed that the initial restitution of the gastric surface involves existing epithelial cells, located in the neck region, migrating to the luminal surface to cover the damaged region. This prevents further erosion of the damaged area by gastric acid and pepsin. There follows a period of epithelial cell proliferative activity in the neck region to replenish the depleted epithelial cell population (Ito & Lacy, 1985).

The administration of CBS prior to exposure to ethanol resulted in a dramatic decrease in the degree of gastric damage. A number of previous reports have suggested that CBS is an effective gastroprotectant which acts by precipitating in the acidic gastric environment to form a diffusion barrier to hydrogen ions, as well as inactivating pepsin and binding bile salts (Koo *et al*, 1982; Roberts & Taylor, 1982; Stiel & Peters, 1983; Lee, 1982). However, these reports are based on the administration of a single dose of CBS immediately before exposure to the necrotising agent and it is well established that this protective action is only effective for a maximum of 6 hours (Konturek *et al*, 1987b). In the present experimental regime, CBS was given 12 hours prior to the second ethanol dose and it is therefore unlikely that a physical coating of CBS on the gastric surface could account for the reduction in damage observed in these experiments. However, the increased gastric integrity observed with the application of CBS alone, together with the previously reported increases in epithelial cell density after long-term CBS treatment, could render the tissue more resistant to ethanol damage. The underlying mechanisms associated with the increased resistance to gastric damage observed after long-term CBS treatment have not been elaborated but the involvement of other gastric protective agents, such as prostaglandins and epidermal growth factor (EGF) cannot be discounted. (Konturek *et al*, 1987a; Konturek *et al*, 1988; Terano *et al*, 1987; Tygat *et al*, 1986).

The present results suggest that repeated administration of CBS results in an alteration in gastric architecture such that the mucosal surface has an increased resistance to adverse actions of alcohol. This action of CBS does not appear to be related to the formation of a physical barrier or to the increased secretion of mucus but may be mediated by a more fundamental mechanism involving such factors as EGF or prostaglandins.

Acknowledgements

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Discussion with Reviewers

C. Tasman Jones: The important and reproducible result from this study is the convincing evidence to show that colloidal bismuth subcitrate administered prior to the administration of alcohol significantly reduced damage to the epithelial surface of the stomach. The studies of the mucus layer are not interpretable because the authors have made no effort to prevent the very well documented alteration of the mucus layer which occurs during the normal preparative dehydration phases for light and electron microscopy.

Authors: The authors thank this reviewer for highlighting the problems in interpreting the findings on the gastric mucus layer. We are well aware of the alterations to the mucus layer which can result from tissue processing, but unfortunately, techniques such as antibody stabilisation of the mucus were not available to us when these experiments were carried-out. However, in the discussion we have called attention to the effect of tissue processing on the mucus layer and have concentrated on the effects of alcohol and colloidal bismuth subcitrate on the gastric cellular populations, without drawing any conclusions on the actions of these substances on the mucus.

Reviewer II: It has been suggested that there is a negative correlation between the amount of mucus and the lesion formation in the stomach. From the data, the amount of adherent mucus does not reflect the integrity of the stomach because the alcohol alone had more mucus than the CBS + alcohol group. What is the author's comment about this discrepancy?

Authors: We have stated in the previous paragraph that it is difficult to interpret the findings on the amount of mucus present in any of the experimental groups because of the disruption caused by tissue processing. However, it is well established that copious amounts of mucus are secreted in association with alcohol induced necrosis. In the CBS treated animals the degree of damage induced by alcohol administration was significantly lower than in the placebo treated animals, consequently the amount of mucus produced in response to gastric damage was lower than in the animals treated with alcohol alone. References to the production of mucus in response to alcohol administration are already included in the text.

A. J. Spencer: Are there any side effects from long-term treatment with CBS?

Authors: In the present experiments the long-term administration of CBS had no apparent

adverse effects on the general welfare, food and water intake or body weights of the experimental animals. However, a number of toxic effects have been attributed to bismuth compounds in humans. These include: nephropathy, encephalopathy, osteoarthropathy, gingivitis, stomatitis and colitis. These adverse effects were observed after ingestion of very high doses of inorganic bismuth salts. Reports on the adverse effects of colloidal bismuth subcitrate have mainly concerned cases of voluntary overdose in combination with other substances such as Paracetamol [Slikkerveer, A., de Wolff, F. A. (1989) Pharmacokinetics and toxicity of bismuth compounds. *Med. Toxicol. Adverse Drug Exp.* **4**, 303-323].

A. J. Spencer: Is there any difference between the amount of damage observed after the first and second treatments with ethanol alone and ethanol with CBS?
Authors: There is no significant difference in the amount of damage observed after the first and second alcohol treatments in the control animals.