Effects of calcium and pH on the mucosal damage produced by deoxycholic acid in the rat colon

J J RAFTER, V W S ENG, R FURRER, A MEDLINE, AND W R BRUCE

From the Ludwig Institute for Cancer Research, Toronto, Ontario, and Department of Pathology, Toronto Western Hospital, Toronto, Ontario, Canada

SUMMARY A single pass perfusion system was used in anaesthetised, restrained rats to examine the effect of changing the composition of the perfusion fluid on the damage caused to the colonic epithelium by deoxycholic acid. Damage to the colonic surface was monitored with light microscopy, transmission and scanning electron microscopy and with measurements of deoxyribonucleic acid and carbohydrate in the perfusate. New scoring techniques for monitoring alterations in surface epithelium of light microscopy sections were used. The damaging effect of 5 mM deoxycholic acid to the colonic epithelium is inhibited by lowering the pH of the perfusion fluid from 7.9 to 5.5, or by increasing the calcium concentration from 0 to 4 mM. This inhibition is shown to be because of a decreased amount of bile acid in solution. Thus it is not the total concentration of deoxycholic acid in the colon that is responsible for the colonic damage, but the concentration in solution. Although extrapolation to the human situation must be made with caution, the concentration of bile acid in solution in the faecal water may be more relevant to colonic mucosal damage than total bile acid concentration.

The effect of bile acids and fatty acids on morphology and function of colonic tissue has been extensively studied using colon perfusion techniques.1-3 In these studies, the damaging effect of the acids on the colonic tissue has been examined under defined conditions of ion concentrations and pH. The recent observation of Wargovich et al4 that the inflammation and superficial lysis of the colonic epithelium caused by intrarectal instillations of fatty acids was largely reduced by concomitant oral administration of calcium salts raises the possibility that the damaging effect of fatty acids and bile acids may be more dependent on the local chemical environment in the colon than was previously thought.

Thus the aim of the present study was to examine in a more systematic way the effect of altering the chemistry of the perfusion fluid on the degree of damage to the rat colonic epithelium caused by bile acid. More specifically we report on how altering either the pH or the calcium concentration of the perfusion fluid alters the damage to the colonic surface caused by deoxycholic acid. In addition we describe some histological techniques, which we have developed, which can monitor subtle alterations in the morphology of the colonic mucosa which are caused by physiologically low concentrations of bile acids.

Methods

EXPERIMENTAL DESIGN

Colons were perfused in vivo in rats grouped according to the perfusion fluid used. Perfusion fluids used included: control electrolyte solutions (made up to simulate ileal effluent) at pH 7.9 with and without 4 mM calcium chloride, and at pH 5.5 without calcium; solutions containing the sodium salt of deoxycholic acid (Sigma Chemical Company, St. Louis, MO) (1, 2.5, 5 and 7.5 mM) at pH 7.9; solutions containing deoxycholic acid (5 mM) at pH 5.5 and 6.4; solutions containing deoxycholic acid (5 mM) and calcium chloride (2 mM or 4 mM) at pH 7.9. Morphological changes brought about by the perfusate solutions were assessed by light microscopy, transmission and scanning electron microscopy of the colonic mucosa. In addition the
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The perfused fluid was assayed for deoxyribonucleic acid (DNA) as a biochemical marker of epithelial and other cell loss and total carbohydrate (CHO) as a measure of total glycoproteins originating from mucus, microvilli and epithelial cells.

OPERATIVE PROCEDURE
Female Fisher 344 rats (120–150 g) which had been fasted overnight were anaesthetised with pentothal and their abdomens opened through a midline incision. An incision was made at the caecocolonic junction and the colon was flushed clear of faecal residue with Krebs-Ringer solution. This junction was then cannulated with Manosil silicone rubber tubing (id ¼ in; Manostat Tubing, New York). More tubing was inserted into the anus, giving a perfusion segment of approximately 10 cm length. The abdomen was then closed and the animals were kept under light anaesthesia and were warmed with radiant heat from an electric lamp throughout the perfusion procedure. At the end of the perfusion the animals were killed with an overdose of pentothal. The colons were removed and the length of the perfusion segment measured. Sections were prepared for the various microscopic techniques as described below.

PERFUSION PROCEDURE
The procedure we used was that described by Rampton et al. with minor modifications. The control electrolyte solution consisted of potassium chloride (19 mM), sodium chloride (83 mM), polyethylene glycol (PEG) 4000 (3 g/l) and TRIS (10 mM) adjusted to pH 7.9. Additional control solutions consisted of the above solution made 4 mM with respect to calcium chloride and the above solution at pH 5.5, here PIPES (15 mM) replaced the TRIS. The use of TRIS was necessitated as buffer as it did not interact with the added calcium salt. PIPES acted as a good buffer at the lower pH's tested and neither buffer had any effect on the colonic epithelium. The deoxycholic acid containing solutions were prepared by first making the initial control electrolyte solution 1, 2-5, 5 and 7-5 mM with respect to the bile acid and adjusting the pH to 7-9. The deoxycholic acid solution at 5 mM and pH 5-5 was prepared from the control solution at pH 5-5. The deoxycholic acid solution at 5 mM and pH 6-4 involved replacing the TRIS of the initial control solution with PIPES (15 mM), making it 5 mM with respect to deoxycholic acid and adjusting the pH to 6-4. The deoxycholic acid solutions at 5 mM and pH 7-9 with 2 and 4 mM calcium concentrations involved making the 5 mM deoxycholic acid in the initial control solution 2 and 4 mM with respect to calcium chloride and adjusting the pH to 7-9. All of the above solutions, without being centrifuged and maintained at 37°C, were perfused isoperistaltically at a constant rate of 0.5 ml/min. An 80 minute perfusate fraction was collected on ice from each animal. Perfusate fractions were removed from the ice bath and frozen at -20°C until the DNA and CHO assays were carried out. Each experimental group consisted of five animals.

LIGHT MICROSCOPY
At the end of the perfusion period after the colon had been excised and measured, a 1–2 cm segment of mid-colon was taken from each animal, placed on a dental wax plate, slit lengthwise, spread (stretched slightly) and fastened with needles, mucosal side up, and fixed in a solution of glutaraldehyde (2% in 0.1 mM phosphate buffer, pH 7.4) for 15–30 minutes. It was then trimmed with a razor blade into 4 approximately 5×5 mm blocks, two of which were placed in the glutaraldehyde solution, and stored at 4°C until scanning electron microscopy was performed (see below). The other two blocks were placed in a 10% buffered formalin phosphate solution overnight, embedded in paraffin, sectioned (5 μm) in the long axis of the intestine and stained with haematoxylin and eosin. The sections were then coded and examined by an independent observer who used the following scoring measures for degree of damage to the colonic epithelium. The first of these measures had as its basis the height of the surface epithelial cells and the actual distance measured was that between the top of the nucleus and the luminal surface of the cell (Fig. 1a, arrow). Three individual measurements are made, using an Image Analyzer (Micro-Plan II, GTCO Corporation) on each intercryptal bridge (as defined in Fig. 1a) and the measurements in μm averaged for 100 bridges. This quantitative measurement was designated 'distance nucleus to surface (μm)'. The second measure is based on the same criteria as the first, however it can be performed much more rapidly. In this case epithelial cells of 100 or more bridges are scored according to the distance from nucleus to luminal surface. If the majority of cells in any bridge showed any reduction with regard to this distance that bridge was scored as abnormal. This procedure gave rise to the semiquantitative measure of 'normal mucosal surface (%)'.

TRANSMISSION ELECTRON MICROSCOPY
After excision and measurement of the perfused colon, a 1–2 cm segment of mid-colon was placed directly in a fixative containing 1% glutaraldehyde+4% formaldehyde at pH 7-2 for 3 hours, postfixed in 1% osmium and 3% ferrocyanide for one hour and embedded in Epon araldite. Semithin sections were cut and stained with uranyl acetate and lead citrate and viewed in a Hitachi H-700 electron microscope.
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Methods

Total carbohydrate was undertaken on the perfusates using the phenol-sulphuric acid method of Dubois et al. and DNA was measured fluorometrically. These results were expressed as μg/cm²/10 min. To determine the actual concentration of deoxycholic acid in solution, as opposed to total concentration – that is including precipitated bile acid, in the various perfusion fluids, two techniques were used. The first involved centrifugation (300xg) of the perfusion fluid and a bile acid analysis on the resulting supernatant. The second technique involved dialysis. Dialysis bags were prepared by filling 6 cm cellulose dialysis tubing (Spectrapore, cut off mol wt 12 000–14 000, Spectrum Medical, Los Angeles, California) with 10% of dextran (wt/vol) (average mol wt 60 000–90 000 daltons, Sigma Chemical Company) in deionized water as an oncotic agent, and tying off the ends. This bag was suspended in 50 ml stirred perfusion fluid for 24 h when a bile acid analysis was done on the contents of the bag. The bile acids were determined enzymatically using 3-hydroxysteroid dehydrogenase.

Statistical methods

Results are expressed as mean values ± standard deviations. To assess the correlations between the various histological and chemical measures of degree of mucosal damage used, Spearman’s correlation analyses were used.

Results

Effect of perfusing deoxycholic acid on the colonic epithelium of the rat

In order to thoroughly characterise the effect of perfusing the bile acid, deoxycholic acid, on the rat colonic epithelium we used light microscopy, transmission and scanning electron microscopy. These results are shown in Figure 1.

Light microscopy

Figure 1a shows a section of mucosa from a colon which was perfused with the control electrolyte solution alone. The crypts are readily discernible and the bridges between the crypts are composed of tall columnar cells with oval shaped nuclei with their long axes perpendicular to the luminal surface and the distance from the top of these nuclei to the...

Fig. 1(a) Photomicrograph of rat colon perfused with control electrolyte solution revealing several intercryptal bridges (B) covered by tall columnar cells with basal oval nuclei. Note, distance nucleus to luminal surface (arrow). H and E.

(b) Photomicrograph of colon perfused with 2.5 mM deoxycholate showing a more cuboidal appearance of surface cells. Nuclei appear more rounded (arrow) and distance nucleus to luminal surface markedly reduced as compared with Fig. 1a. H and E.

(c) Photomicrograph of colon perfused with 5.0 mM deoxycholate showing erosion of surface epithelium with exfoliation of cells into lumen. Basement membrane in bridge (B) is exposed to lumen. H and E.

(d) Transmission electron micrograph of rat colon perfused with control solution showing numerous microvilli (MV) in a parallel arrangement, covered in part by a glycoalyx. The nuclei are oval with their long axes perpendicular to the lumen. Anchoring filaments (arrows) are prominent.

(e) Altered surface cells after perfusion with 2.5 mM deoxycholate. The microvilli (MV) are fewer with varying sizes and shapes and devoid of glycoalyx. The nuclei (N) are more rounded. The mitochondria (arrows) are located close to the luminal surface and their matrix is less dense as compared to figure (d).

Anchoring filaments are not seen. (f) Rat colon after perfusion with 5.0 mM deoxycholate. There is extensive damage to surface epithelial cells with exfoliation of cells into the lumen (EC). Remaining cells show a virtual loss of microvilli (arrow). The distance from the basement membrane to the lumen is markedly decreased (solid bar) as is the distance between the nucleus (N) and lumen. Focal denudation is not seen in this photomicrograph. Capillary (C).

(g) Scanning electron micrograph of surface of rat colon perfused with control solution showing intercryptal bridges (B) well covered with normal cells on which microvilli are evident. (h) Surface of colon after perfusion with 2.5 mM deoxycholate. Strands of mucus are evident on the surface. (i) Colon surface after perfusion with 5.0 mM deoxycholate. Mouths of crypts are much more obvious as a result of loss of epithelial cells from the intercryptal bridges (B). Leakage of leukocytes onto surface is also evident.
luminal surface is approximately 8 μm. Figure 1b shows a section from a colon perfused with fluid containing 2-5 mM deoxycholic acid. An alteration in the surface epithelium is evident. The cells making up the bridges between the crypts have lost their tall columnar appearance and have become more cuboidal. In addition the nuclei reveal irregular shapes and the distance from the top of the nuclei to the luminal surface is reduced as compared with Fig. 1a. Figure 1c shows the damage evident in the epithelium perfused with 5 mM deoxycholic acid. There is considerable erosion of cells from the intercryptal bridges leaving the lamina propria exposed to the lumen in certain places. Considerable numbers of cells and cell fragments have been exfoliated from the surface and are present within the lumen or loosely attached to the mucosa. When the number of cells were counted from the bottom to the top of the crypts it was obvious that the crypt height in the colons exposed to 2-5 mM bile acid was significantly shortened when compared with control colons (33-7±2-9 vs 36-0±3-0, 0-02>p>0-01) as was the crypt height in the colons exposed to 5-0 mM bile acid (23-6±5-1 vs 36-0±3-0, p<0-0001). (Values are means±standard deviations and n=30.)

Transmission electron microscopy
The effects of deoxycholic acid are shown in Figures 1e and f. In comparison with tissue not exposed to bile acids (Fig. 1d), the mucosal surface is markedly altered. The most prominent features of the luminal cells involve the marked decrease in the number and shortening of microvilli accompanied by an apparent loss in anchoring filaments and glycoalyx. The mitochondria are situated close to the luminal surface in Figure 1e and many reveal a less dense matrix as compared with the normal. The nuclei are more rounded in shape, but the intercellular membranes and junctions appear intact. The distance between the upper aspect of the nuclear membrane and the luminal surface is reduced. The colons of animals receiving 5 mM deoxycholic acid show more extensive damage including loss of microvilli (Fig. 1f) and focal denudation of the cells leaving the capillaries exposed to the lumen is often observed.

Scanning electron microscopy
Figure 1g shows the surface of a colon which has been exposed to the control electrolyte solution alone. Here it is clear that the intercryptal regions are well covered with normal cells on which the microvilli are evident. The 'barnacle-like' appearance in Figure 1i is typical of colonic surfaces which have been exposed to 5 mM deoxycholic acid. Here the mouths of the crypts are much more obvious as there has been a substantial loss of epithelial cells from the intercryptal areas. In addition there appears to be extensive leakage of leucocytes from the capillaries just below the basement membrane in the areas which have been eroded free of epithelial cells. Strands of mucous are also evident on the damaged surface. Figure 1h which shows intermediate damage has been exposed to 2-5 mM deoxycholic acid in the perfusion fluid.

In order to obtain a more quantitative measure of the degree of damage caused by the bile acid to the colonic epithelium, we developed scoring techniques based on the appearance of the light microscopy sections referred to as 'distance nucleus to surface (μm)' and 'normal mucosal surface (%)' (see Methods). As is evident from Figure 2 the measure, distance nucleus to surface (μM), decreased almost linearly with increasing concentration of bile acid. As is also shown in Figure 2, the second of these measures correlated with concentration of bile acid being perfused through the colon with the number

![Graph](https://via.placeholder.com/150)

**Fig. 2.** Dose response curves of two different measures of damage to the colonic epithelium (based on appearance of light microscopic sections) with deoxycholic acid concentrations in perfusion fluid. Top: measure based on distance (μm) from tops of nuclei to luminal surface utilising an image analyser. Bottom: measure based on same parameter but scored by an observer on the basis of the appearance of the cells on the intercryptal bridges. Each point is the mean of 5 values with the bar representing ± one standard deviation.
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of intercryptal bridges scored as normal decreasing as the concentration of bile acid is increased. It is evident that the second technique, which could be performed quite rapidly, correlated highly with the quantitative measure (with Spearman correlation coefficient equal to 0.91, p<0.0001). In the studies described below, we thus monitored the degree of damage to the colonic epithelium from the histological point of view with the measure ‘normal mucosal surface (%)’ alone.

In order to correlate the above morphological alterations with known biochemical markers for mucosal damage, we also monitored the concentrations of CHO and DNA in the perfusates. As can be seen from Figure 3, both the concentrations of CHO and DNA in the perfusates increase in a dose-dependent manner with increasing concentrations of deoxycholic acid in the perfusion fluid. Also evident from Figure 3 is the good correlation between the concentration of CHO in the perfusate and mucosal damage as measured by ‘normal mucosal surface’

![Graph](image)

**Fig. 3** Dose response curves of one of the measures of damage to the colonic epithelium (based on appearance of light microscopic sections) described in legend to Fig. 2 (top) and two chemical assays of damage to the colonic epithelium—that is, total carbohydrate (CHO) (middle) and DNA (bottom) in perfusates, with deoxycholic acid concentration in perfusion fluid. Points represented as for Fig. 2.

![Graph](image)

**Fig. 4** Effect of pH of perfusion fluid on three measures of damage to the colonic epithelium of the rat—that is normal mucosal surface (%) and carbohydrate (CHO) and DNA in the perfusates. Solid line (——) represents perfusion with 5.0 mM deoxycholic acid and broken line (––––) with control electrolyte solution alone. It is evident that lowering the pH from 7.9 to 5.5 returns the three measures of damage to the control values. Points represented as for Fig. 2.
Modification of damage to the colonic epithelium caused by deoxycholic acid by altering the chemistry of the perfusion fluid

Effect of pH

In this experiment we monitored the degree of colonic damage with the three measures, normal mucosal surface (%) and concentrations of CHO and DNA in the perfusates. In Figure 4 we see that perfusion of the colons with 5 mM deoxycholic acid at pH 7.9 results in a marked degree of damage according to the above three measures when compared to the control group which contains no bile acid in the perfusion fluid. Also evident from this figure is the fact that decreasing the pH of the perfusion fluid from 7.9 to 5.5 appears to return the three measures of colonic damage to the control or undamaged values. Decreasing the pH of the control perfusion fluid had no effect on any of the three measures of damage. This indicates that by decreasing the pH in the colon we can essentially counteract the damage caused to the epithelial surface by the 5 mM bile acid.

Effect of calcium concentration

The degree of colonic epithelial damage was monitored using the same three measures as were used in the pH experiment. The three measures indicate considerable damage to the colonic epithelium caused by 5 mM deoxycholic acid in the perfusion fluid, which is at pH 7.9 and 0 mM calcium concentration, when compared with control values (Fig. 5). Figure 5 also shows that if the calcium concentration is increased up to 4 mM in the perfusion fluid containing the 5 mM bile acid, the measures of damage return to the control or undamaged values. Increasing the calcium concentration to the same extent in the control perfusion fluid has no effect on any of the measures for colonic damage. Thus the damage to the epithelial surface caused with 5 mM bile acid can be prevented by increasing the calcium concentration in the colon.

The actual concentrations of deoxycholic acid in solution in the various perfusion fluids discussed above are presented in the Table.

Discussion

In the present study we have been able to show that

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*Perfusion fluid centrifuged at 300xg and bile acid analysis performed on supernatant.
†Bile acid analysis carried out on contents of dialysis bag containing 10% dextran which had been suspended in perfusion fluid for 24 hours.
‡Deoxycholic acid concentrations are means of duplicates.
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Perfusing the rat colon with deoxycholic acid brings about morphological alterations in the surface epithelial cells in a dose dependent manner. The morphological alterations monitored by the microscopic techniques outlined above were paralleled by the appearance of the biochemical markers in the colon perfusates – that is, DNA as a marker for exfoliated cells and CHO as a marker for glycoprotein originating from mucin, microvilli and epithelial cell cytoplasm. The initial alteration in the appearance of the surface epithelial cells, effected by low concentrations of bile acid, involved a change from a columnar to a more rounded or cuboidal shape. As even at these low concentrations of bile acid in the colon DNA was present in the perfusates and a decrease in crypt height was observed, we interpret the altered cellular shape of the epithelial cells as indicating that the exfoliated cells are replaced by less differentiated cells moving up from the top of the crypts in an effort to keep the intercryptal bridges covered. At higher concentrations of bile acids in the colon – that is, 5 mM, however, the integrity of the intercryptal areas is not maintained and the basement membrane becomes exposed to the luminal contents. We also describe new techniques to quantify the degree of surface damage to the colonic mucosa based on the appearance of the light microscopy sections. The measures derived from the various techniques correlate well with one another and with concentration of bile acid in the colon. The most quantitative of the measures – that is, distance nucleus to surface (μm), enables alterations in the epithelial cells due to bile acid concentrations in the 0–1 mM range to be detected (see Fig. 2). Thus we would predict that these scoring techniques might be useful in future studies for detecting subtle morphological changes in colon surface epithelial cells brought about by low concentrations of bile acids or other damaging agents. Our data, therefore, confirms that which is well established in the literature – that is, that perfusion of the colons of a number of species, including man, with bile acids brings about marked morphological changes in the colonic mucosa which are often associated with changes in fluid and electrolyte secretion. In contrast with our findings, however, it has been reported that perfusion of rat colon with 1 mM deoxycholic acid in vivo did not cause morphological changes in the mucosa. One explanation for this discrepancy might be that our scoring techniques are more sensitive to subtle alterations in colonic epithelial cells than those used in the above studies. Another recent study which examined the effect of deoxycholic acid on the perfused rat colon indicated that even during extreme changes in mucosal permeability induced by 8 mM deoxycholate, no epithelial lesions exposing the basement membrane to the luminal contents could be detected. This is in stark contrast with our data where we see considerable erosion of cells and exposure of the basement membrane even at 5 mM concentrations of deoxycholate. Interestingly, the perfusion solution in this study contained 1.25 mM calcium chloride.

In an additional series of experiments we were able to show that by decreasing the pH of the perfusion fluid from 7.9 to 5.5 the damage which was normally caused to the colonic epithelium by 5 mM deoxycholate was largely prevented. The most plausible explanation for this effect would be that as we dropped below the pKa of deoxycholate (6.58) the solubility of the bile acid decreased markedly. A similar observation has been made by McJunkin et al who showed that a high correlation exists between faecal pH and per cent of faecal dihydroxy bile acids in aequous solution. We were also able to prevent the damaging effect of the 5 mM bile acid by increasing the calcium concentration of the perfusion fluid to 4 mM. Presumably with increasing calcium concentration we are achieving increased formation of non-irritating, non-soluble calcium soaps. Indeed, increased calcium soap formation has been confirmed in several animal studies where quantitative measurements have been made during concomitant ingestion of fat and calcium salts. In addition, dietary supplementation with calcium has been shown to inhibit irritation to colonic mucosa caused by intrarectally administered bile acid and fatty acid. In our study, the bile acid concentrations in the various perfusion fluids at different pHs and calcium concentrations were determined. Two methods were used – that is, centrifugation and dialysis, both of which have been used for preparation of faecal water (see below). There was good correlation between the two methods. When we plotted the degree of damage caused to the colon by those perfusion fluids against the bile acid concentrations obtained for the various perfusion fluids, the points fell along the curve for the deoxycholate in perfusion fluid versus colonic damage (Fig. 6). This indicates that it is not the total concentration of bile acid perfusing through the colon that is responsible for the damage to the epithelial cells but that fraction which is free to penetrate the dialysis bag – that is, the fraction of bile acid that is in solution in the aqueous phase. While we cannot rule out the possibility that insoluble bile acid may dissolve within the gut lumen during perfusion, the results presented in Fig. 6 indicate that if this is happening it is happening only to a minor extent. While the concentration of deoxycholate – that is, 5 mM, used in the above perfusion studies might
appear high, similar total bile acid concentrations have been found in the aqueous phase of stools from patients with ileal resection.\textsuperscript{19}

Although extrapolation from the above colon perfusion studies to the human situation must be made with extreme caution, we would suggest that it is not the total faecal bile acid concentration which is the most important factor in relation to mucosal damage in the colon, but the concentration of bile acid in the faecal water. We would also suggest that any dietary modification which would bring about a decrease in faecal water bile acid concentration, such as by lowering the pH or increasing colonic calcium concentration or some other mechanism, might result in a decrease of this colonic mucosal damage. A relationship between mucosal damage, as caused by bile acids, and development of colon cancer has already been suggested.\textsuperscript{20} A number of studies\textsuperscript{21-23} have indicated that increased total faecal bile acids is associated with increased risk for colon cancer although certain other studies\textsuperscript{24-25} have not been able to make this association. This discrepancy might possibly be avoided if one determined the concentration of bile acid in the faecal water rather than the total faecal bile acid concentration. In addition, mucosal damage, brought about by exposure to raised concentrations of bile acids in solution, may well be responsible for other diseases affecting the colonic epithelium in addition to colon cancer.

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