Topical protection of human esophageal mucosal integrity

P. Woodland,1 F. Batista-Lima,1,2 C. Lee,1 S. L. Preston,1 P. Dettmar,3 and D. Sifrim1

1Barts and the London School of Medicine and Dentistry, Queen Mary University of London, London, UK; 2Universidade Federal do Ceará, Fortaleza, Brazil; and 3Technostics, Hull, UK

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Am J Physiol Gastrointest Liver Physiol 308: G975–G980, 2015. First published April 23, 2015; doi:10.1152/ajpgi.00424.2014.—Patients with nonerosive reflux disease exhibit impaired esophageal mucosal integrity, which may underlie enhanced reflux perception. In vitro topical application of an alginate solution can protect mucosal biopsies against acid-induced changes in transepithelial electrical resistance (TER). We aimed to confirm this finding in a second model using 3D cell cultures and to assess prolonged protection in a biopsy model. We assessed the protective effect of a topically applied alginate solution 1 h after application. 3D cell cultures were grown by using an air-liquid interface and were studied in Ussing chambers. The apical surface was “protected” with 200 μl of either alginate or viscous control or was unprotected. The tissue was exposed to pH 3 + bile acid solution for 30 min and TER change was calculated. Distal esophageal mucosal biopsies were taken from 12 patients and studied in Ussing chambers. The biopsies were coated with either alginate or viscous control solution. The biopsies were then bathed in pH 7.4 solution for 1 h. The luminal chamber solution was replaced with pH 2 solution for 30 min. Percentage changes in TER were recorded. In five biopsies fluorescein-labeled alginate solution was used to allow immunohistological localization of the alginate after 1 h. In the cell culture model, alginate solution protected tissue against acid-induced change in TER. In biopsies, 60 min after protection with alginate solution, the acidic exposure caused a −8.3 ± 2.2% change in TER compared with −25.1 ± 4.5% change after protection with the viscous control (P < 0.05). Labeled alginate could be seen coating the luminal surface in all vitro. Alginate solutions can adhere to the esophageal mucosa for up to 1 h and exert a topical protectant effect. Durable topical protectants can be further explored as first-line/add-on therapies for gastroesophageal reflux disease.

GASTROESOPHAGEAL REFLUX DISEASE (GERD) is a prevalent condition affecting 10–20% of Western populations (7, 9). This is associated with a significant healthcare and financial burden. Although proton pump inhibitor (PPI) therapy has been a successful treatment for the majority of patients with GERD (especially those with erosive reflux disease), there remains a significant minority of ~30% who do not respond adequately to PPI (29). Reasons for this may include symptomatic weakly acidic reflux, bile reflux, acid breakthrough, or hypersensitivity of afferent nociceptive pathways (3). Furthermore, over recent years there have been some concerns raised about the long-term safety of PPI therapy (16). As such, there is a clinical need to develop alternative therapies for GERD.

Anatomically the esophagus is ideally placed to be amenable to topical therapies, although, with the exception of therapies for eosinophilic esophagitis (20), it has been a rarely employed strategy in esophageal disease. This is perhaps due to problems of rapid esophageal transit and uncertainties about adherence of the ingested drug to the mucosa.

The mucosa of the esophagus involves a stratified squamous epithelium that acts as a tight defensive barrier against the noxious components of the gastroesophageal refluxate. Impairment of the integrity of this mucosal barrier is thought to be of importance in the pathogenesis of gastroesophageal reflux symptoms. Patients with nonerosive reflux disease [NERD, comprising 70% of GERD (10)] can be seen to have dilated intercellular spaces between esophageal epithelial cells (28). It is hypothesized that this is a morphological representation of a defective barrier that will permit permeation of noxious substances (such as acid) into the deeper epithelium where they can stimulate nociceptive afferents. The mucosal integrity can also be demonstrated functionally, for example in terms of transepithelial electrical resistance (TER, a measure of transcellular permeability of the mucosa) (11, 27). We have recently shown that, in an ex vivo Ussing chamber model, exposure of human biopsies from NERD patients to acidic solutions containing bile acid and pepsin is able to cause a significant reduction in TER (30). This suggests that the mucosal integrity is especially vulnerable in these patients.

If the esophageal mucosa can be topically protected against noxious components of the gastroesophageal refluxate, this may result in a lessened susceptibility to symptomatic perception of reflux events.

Sodium alginate solutions (usually in combination with antacid) are frequently used in treatment of gastroesophageal reflux disease. An alginate gastric raft may be able to reduce the number of acid reflux episodes (4, 8, 19), with this property being considered as due to the viscous barrier surface tension reducing reflux through the gastroesophageal junction. However, recent evidence suggests that the ability of the alginate raft to reduce the number of reflux episodes may be limited (6). A second important physical property of alginate rafts appears to be their ability to abolish or displace the postprandial “acid pocket” in patients with symptomatic GERD (15). It is possible that, in addition to the antacid and gastric mechanical properties of alginate-antacids, there may also be an esophageal mucosal protective effect. Alginates have been found to demonstrate bioadhesive potential, a property determined primarily by polymer chain length and the presence of ionizable groups rather than, e.g., the viscosity of the gel used (22). Specific delivery and prolonged superficial retention of a drug covering the esophageal mucosa might be desirable for treatment of GERD (and indeed other esophageal disorders such as eosinophilic esophagitis and hypersensitive esophagus). Such protective properties of alginate solutions might be effective in the treatment of GERD, where acid, pepsin, and bile acid are all believed to be important in symptom pathogenesis. We have
recently demonstrated that topical application of a sodium alginate solution to human biopsies immediately prior to acid exposure in Ussing chambers can greatly diminish the acid-induced reduction in TER (i.e., can protect the mucosa) (30). In the present study, we first wished to confirm this finding in a different model using a multilayer human esophageal cell culture system. For potential clinical translation, the duration of this protective effect is of utmost importance. If the duration of protection could last for up to 60 min, the mucosal protective effect could be of potential therapeutic benefit at times of increased reflux (e.g., in the 1 h postmeal).

In this study we aimed 1) to investigate the protective effect of a topical sodium alginate solution in a 3D human esophageal cell culture model and 2) to investigate the duration of the protective effect of a topical alginate solution in human esophageal biopsies.

MATERIALS AND METHODS

Cell culture model. Human esophageal epithelial cells were generously facilitated by Professor H. Miwa (Hyogo College of Medicine, Nishinomiya, Japan). These cells (SciCell Research Laboratories, Carlsbad, CA) were previously used to develop an air-liquid interface (ALI) culture system to study physiological properties of human esophageal mucosa (5, 17, 21). Transwell-Clear wells (Costar, Cambridge, MA) were coated with collagen, human fibronectin, and bovine serum albumin. The cells were cultured in epithelial cell medium-2 (EpiCM-2, SciCell Research Laboratories) and subcultured to Transwell-Clear wells until reaching confluence. An ALI culture was then initiated by removing the apical medium and decreasing the basal chamber medium volume to 600 ml. The ALI medium, consisting of a mixture of EpiCM-2 and DMEM (1:1), was supplemented every day, and the medium that leaked through to the apical surface was coated with 200 µl of a protectant solution. After registering baseline values, the chambers were separated. This was done such that the luminal aspect of the biopsy was exposed. Then 200 µl of a protectant solution was applied to the exposed luminal aspect of the biopsy and left on the biopsy surface for 5 min.

For each patient, two biopsies were studied: i.e., each biopsy was exposed to either the alginate solution or the viscous control. After 5 min the protectant solution was washed off with 5 ml pipetted Krebs-Henseleit pH 7.4 solution, and the chambers were rejoined and filled with Krebs-Henseleit buffer at pH 7.4. The biopsies were then allowed to reequilibrate and wash in neutral solution for a further 60 min. For each biopsy the solution in the luminal chamber was then replaced with an acidic solution (Krebs-Henseleit at pH 2 + 1 mg/ml porcine pepsin + 1 mM taurodeoxycholic acid). This acidic exposure continued for 30 min, during which time TER was continuously measured. Percentage change in TER from baseline at 30 min was calculated.

Visualization of alginate. To assess adherence of the alginate at 60 min postapplication, a further five biopsy samples were protected by a fluorescent-labeled alginate (identical to alginate used in alginate solution above). To prepare this the amine group of fluoresceinamine was covalently attached to the carboxylic group of the alginate in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC, a dehydrating agent that allows the amine bond to form). A full description of the methods can be found elsewhere (2). The experiment was conducted as described above, with the exception that after 60-min washing the biopsy was removed from the chamber and immediately fixed in 4% paraformaldehyde solution. Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI) and examined under an epifluorescent microscope. Images were taken of five sections per biopsy, five high-power fields per section (i.e., 25 images per biopsy). The thickness (in µm) of the fluorescent alginate layer in each image was calculated as the mean of five evenly spaced measurements per image, and the overall alginate layer thickness was calculated as the mean value of the 25 images.

We performed the same process in a further five biopsies using fluorescent labeled alginate but fixed the tissue for layer thickness measurement after only 15 min postapplication. This was to assess the relative change in layer thickness compared with the longer washing time.
In addition we fixed two biopsies that had been coated in unlabeled alginate and two biopsies that had not been protected at all and examined sections under epifluorescent microscopy to evaluate for any autofluorescence from the alginate solution or biopsy surface. To evaluate whether any binding was due to alginate or the vehicle it was carried in, a further three biopsies were coated with a fluorescent viscous control (fluorescein 1 mg/ml in viscous control solution) and fixed after 15-min washing (as above) for examination under epifluorescent microscopy.

**Statistical analysis.** All data are expressed as means ± SE unless otherwise stated. Single comparisons were made with unpaired Student’s t-test (parametric data) or Mann-Whitney U-test (nonparametric data) where appropriate. Multiple comparisons were made with analysis of variance followed by Bonferroni’s multiple-comparison test. Significance was declared at $P < 0.05$.

**RESULTS**

**Cell culture model.** Unprotected tissue and tissue “protected” with the control and placebo solutions had a fall in TER to 41.9 ± 8.1% ($n = 3$) and 38.4 ± 6.2% ($n = 4$) of baseline respectively after 30-min exposure to the test solution. Tissue protected with the alginate solution demonstrated very little change in TER after 30 min exposure (98.8 ± 5.1% of baseline, $n = 5$; $P < 0.05$ compared with no protection and placebo protection by ANOVA, Fig. 2).

**Biopsy Ussing chamber studies.** The mean change in TER upon exposure to an acidic solution containing bile acid and pepsin after treatment with the viscous control was $-25.1 ± 4.5\%$. After treatment with the alginate solution and 60-min washing the mean change in TER on exposure to the acidic solution containing bile acid and pepsin was $-8.3 ± 2.2\%$ ($P$ for comparison of means 0.006).

On paired analysis of results from biopsies from the same patient there was a significant reduction in the effect of acidic solution on change in TER in those biopsies treated with alginate compared with those treated with viscous control ($P = 0.004$, Fig. 3).

**Visualization and thickness of superficial alginate layer.** Fluorescein-labeled alginate could be seen to be present on the surface of each mucosal biopsy after 60 min of washing in the Ussing chamber. The mean thickness of the alginate layer on the surface of the biopsies was $4.3 ± 0.3\ \mu m$. In contrast, after only 15-min washing the mean thickness of the alginate layer was $5.7 ± 0.3\ \mu m$ (Table 1, Fig. 4).

No autofluorescence was seen from either the biopsy surface or from the unlabeled alginate solution.

After 15-min washing, no residual coating of the fluorescent control was seen on any sections.

**DISCUSSION**

This study provides evidence for esophageal mucosal adherence of a topical sodium alginate solution that is able to protect against acid/bile acid-induced impairment of mucosal integrity 1 h after its application.

Our study showed 1) A 3D human esophageal cell culture model is able to demonstrate the protective effect of alginate solutions when the tissue is exposed to an acidic solution, as previously seen in an esophageal biopsy model. 2) A topically applied sodium alginate solution is able to prevent reductions in TER caused by in vitro exposure of human esophageal biopsies to acidic solution containing pepsin and bile acid, even after 60 min of application and washing. 3) This effect appears to be due to a persistent adherence of alginate to the luminal mucosal surface. As such, these in vitro results suggest the theoretical possibility that a strategically timed (e.g., immediately postprandial) application of topical alginate-containing solution may be able to reduce mucosal injury from reflux.

We were able to reproduce our original findings of the short-duration mucosal protective effect of alginate solutions.

**Table 1. Thickness of alginate layer on biopsy surface**

<table>
<thead>
<tr>
<th>Biopsy Number</th>
<th>Mean Thickness of Alginate Layer (μm) after 15 min Washing</th>
<th>Mean Thickness of Alginate Layer (μm) after 60 min Washing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.1</td>
<td>5.1</td>
</tr>
<tr>
<td>2</td>
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</tr>
<tr>
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<tr>
<td>5</td>
<td>4.6</td>
<td>4.3</td>
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</tbody>
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that we saw in human biopsies. That the cell culture model was able to reproduce this gives us greater confidence that this in vitro effect is valid. This 3D stratified squamous epithelium model has been shown to be similar to esophageal epithelium in terms of morphology, molecular marker expression, and barrier function (17). It is of note, however, that the cell culture is more vulnerable to acid damage than native biopsy tissue, and this is why a less aggressive test solution was used with this model. We do not see this increased vulnerability as a weakness of the model, since it will enable it to serve as a sensitive evaluator of mucosal protection. This would not have been possible if the model were more resistant to acid.

In the study care was taken to control for the viscosity of the control solution because this could be a confounding factor if high viscosity was causing a physical barrier against the test solutions. To overcome this, a placebo solution of similar viscosity to the alginate solution (but lacking alginate or antacid) was used. The viscosity measured was actually very slightly higher in the viscous control than in the alginate solution (albeit in relative terms the results are very similar). This rejects the possibility that it is viscosity alone that protects the mucosa against access by the acid solution. Furthermore, in this study care was taken to be thorough in washing off “pretreatment” solutions after applying them, both with a fast 5-ml pipette wash, then with a 15-min (in the case of the cell culture experiments) or 60-min (in the case of the human biopsy experiments) period in neutral solution within the Ussing chamber before exposure to the acidic solution. If the alginate solution was an electrical insulator then application could have resulted in an artifactual high TER. However, the alginate solution was an excellent electrical conductor, with conductance that was in fact greater than seen in the control solution. This study has shown that, in the biopsy model, the protective effect after 60 min is less than was seen after only the 15-min washing period we had previously demonstrated, and that this corresponds to a slightly thinner layer of alginate lining the mucosa. To compare, in our previous study we had assessed alginate protection against the same acid insult after a washing period of only 5 min. Here the change in TER after alginate protection was −1% (−5.9 to 3.9) (30). As such it can be seen that the protective effect diminishes with increased washing time and thinning of the protective layer.

We have previously demonstrated that the protective effect of the alginate solution is due to the sodium alginate component, not the antacid component (30). Further evidence of this was that only the labeled alginate, not the fluorescent viscous control, could be seen to have mucosal adherence after washing.

In the presence of acid, alginates precipitate, forming a gel that may confer benefit via its physical rather than chemical properties. Alginates are natural polysaccharide polymers isolated from brown seaweed. Chemically they are copolymers of α-L-guluronic and β-D-mannuronic acid residues connected by 1:4 glycosidic linkages. The gel that is formed when alginates encounter low pH or calcium is able to form a physical raft on top of the gastric juice. This floating capability is often enhanced by the inclusion of bicarbonate, which facilitates the production of CO2 in the acid stomach environment, which is proposed to turn the raft into a foam that aids buoyancy (14).

Our study provides an experimental basis to suggest that a further mechanism of action of alginate preparations might act as a topical mucosal protectant against acidic solutions. A number of attempts to develop topical therapies for GERD have been considered. Perhaps one of the most well known is sucralfate. Sucralfate is a salt of aluminum hydroxide and sucrose octasulfate. It is believed to have cytoprotective properties and has been shown to have modest clinical therapeutic benefit in reflux disease and in healing of gastric ulcers without causing a meaningful change in gastric pH (12, 13). A limitation of sucralfate as a therapy for GERD is its relatively poor mucosal adherence. A scintigraphic imaging study demonstrated a significant limitation of sucralfate in offering esophageal mucosal protection in the absence of erosion. This coating study revealed the failure of sucralfate to remain on the esophageal wall after application in 70% of subjects (25).

It is of interest to consider the mechanism by which alginate solutions may protect the esophageal mucosa. Along with their mechanical properties at the gastroesophageal junction and on the acid pocket, alginates have been found to demonstrate bioadhesive potential, a property determined primarily by polymer chain length and the presence of ionizable groups rather than, e.g., the viscosity of the gel used. Furthermore, they appear to become adhesive on hydration (as occurs upon entering the gastrointestinal tract). An in vitro porcine model of esophageal mucosal retention suggested adhesion is due to alginate-mucosal interaction since there was no retention on a cellular acetate model (1). A further study using a porcine in vitro model determined that the high molecular weight poly-
mbers exhibited better bioadhesion than low molecular weight polymers (with ~40 vs. 20% retention at 20 min) (23). It has also been shown that the nature of the vehicle used for the alginate preparation can influence bioadhesive properties. Suspensions containing a vehicle that required a low level of dilution to initiate swelling (such as glycerol) are more muco- \red{c}retentive in in vivo studies (18). When adhered to the esophageal epithelium, the alginate solution may enable protection against refluxate damage. In vitro diffusion studies (using a dialysis membrane) have shown that the presence of alginates significantly reduces acid and pepsin diffusion across the membrane compared with control (26). The alginate-based formulation of Gaviscon Advance (Reckitt Benckiser) has been found, in vitro, to inhibit pepsin activity and to reduce pepsin and bile acid (taurocholate, glycocholate, and deoxycholate) diffusion across an artificial membrane (24). Since we know that mucosal integrity appears to be impaired by the presence of acid, pepsin, and bile acid, it would be possible that the impact Gaviscon has on diffusion and activity of these substances plays a role in the protective effect seen in this study. The bioadhesive properties appear to have enabled protection to be present after active and passive washing phases totaling more than 60 min.

Limitations of this study must be considered. Firstly, the model is an in vitro model and as such is not a true reflection of physiological conditions. In vivo, gravity, saliva swallowing, and mucus could all act to dislodge the alginate from the esophageal tissue. To overcome this we used a relatively vigorous washing technique to ensure that the alginate was not adhering to the mucosa because of stasis. We also need to be aware that the effect on integrity does not necessarily translate to a therapeutic effect in vivo. It does, however, suggest a potential for an exciting treatment approach to GERD treatment based on topical mucosal protection. Furthermore, the methods we have reported can be the basis for screening of new products (whether alginate or nonalginate) to assess their adherence and protective effects before translating into the vivo situation.

In summary, this study demonstrates that alginate-based topical application can act, in vitro, to protect human esophageal mucosa against acid-induced damage for a prolonged period after application. This appears to be due to bioadherence on the surface of the mucosa.

DISCLOSURES

D. Sifrim receives a research grant from Reckitt Benckiser, Hull, UK. P. Dettmar works for Technostics Ltd, a company that conducts commercial healthcare product development and consultancy.

AUTHOR CONTRIBUTIONS

P.W., P.D., and D.S. conception and design of research; P.W., F.B.-L., C.L., and S.L.P. performed experiments; P.W., F.B.-L., C.L., and P.D. analyzed data; P.W. and D.S. interpreted results of experiments; P.W. and C.L. prepared and S.L.P. performed experiments; P.W., F.B.-L., C.L., and P.D. analyzed data. P.W., F.B.-L., C.L., and P.D. analyzed data. P.W., F.B.-L., C.L., and P.D. wrote the manuscript; D.S. approved final version of manuscript.

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