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Acid and weakly acidic solutions impair mucosal integrity of distal exposed and proximal non-exposed human oesophagus

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ABSTRACT

Background Oesophageal mucosa dilated intercellular spaces (DIS) may be important for symptom perception in non-erosive reflux disease (NERD). Patients with NERD might have DIS even in the proximal oesophagus. We aimed to assess the effect of oesophageal perforations with acid and weakly acidic solutions on ‘exposed’ and ‘non-exposed’ oesophageal mucosa and its relationship to symptoms in healthy subjects.

Methods 14 healthy volunteers underwent upper gastrointestinal endoscopy with biopsies at 3 and 13 cm proximal to the oesophagogastric junction (OGJ). In following sessions, subjects received 30 min perfusions with neutral, weakly acidic, acidic and acidic-bile acid solutions at 5 cm above the EGJ (separated 4 weeks). Biopsies were taken 20 min after perfusions. Electron microscopy was used to measure DIS. Subjects scored heartburn during perfusions using a visual analogue scale.

Results (1) Oesophageal perfusion with acid solutions, with or without bile acids, provoked DIS in the ‘exposed’ oesophageal mucosa; (2) oesophageal perfusion with weakly acidic solutions provoked identical changes to those observed after perfusion with acid solutions; (3) distal oesophageal perforations not only provoked changes in the ‘exposed’ but also in the more proximal ‘non-exposed’ mucosa; and (4) in spite of the presence of perfusion-induced DIS, most healthy subjects did not perceive heartburn during the experiments.

Conclusions The human oesophageal mucosa is very sensitive to continuous exposure with acidic and weakly acidic solutions. In spite of the presence of intraluminal acid and DIS, healthy subjects did not experience heartburn, suggesting that NERD patients should have other critical factors underlying their symptoms.

INTRODUCTION

The pathophysiology of symptoms in gastro-oesophageal reflux disease (GORD) is not completely elucidated. In patients with oesophagitis, refluxed gastric content can reach sensory nerves endings through erosions in the oesophageal mucosa, leading to perception of heartburn. In patients with non-erosive reflux disease (NERD) the mechanism is not that obvious. In these patients, heartburn is thought to occur when acid and/or other components of the refluxed gastric content reach sensory nerve endings, through mucosal dilated intercellular spaces (DIS).1 The dilation of intercellular spaces is a marker of oesophageal mucosa cellular damage in these patients.2 Intra-epithelial nerve endings of spinal afferents are likely to be involved in the mediation of acid induced oesophageal symptoms.3

In vitro exposure of the rabbit oesophageal mucosa to acid provokes DIS4 and reduction of oesophageal acid exposure with proton pumps inhibitors (PPIs) in humans can reverse DIS5–7 suggesting that mucosal exposure to acid is critical in the development of DIS. However, NERD patients with normal oesophageal acid exposure5 and patients with well-controlled acid secretion but persistent symptoms in spite of PPI treatment may also have DIS9 suggesting that increased mucosal exposure to acid is not the only factor underlying this histological phenomenon. We recently showed that acute stress in rats10 and exposure of rabbit oesophageal mucosa to bile acids,11 can both provoke DIS. Interestingly, these two factors can be present in patients with NERD or refractory GORD.12,13 The proximal extent of reflux is an important determinant for reflux perception, particularly in patients with NERD.14 This might be due to hypersensitivity to proximal oesophageal distension but also other mucosal factors might be involved. A recent study demonstrated that patients with NERD may have DIS not only in the distal but also in the proximal oesophageal epithelium15 which might contribute to enhance perception of proximal reflux. It is unknown whether proximal DIS occurs due to direct oesophageal mucosal contact with gastric contents or involves an indirect mechanism triggered by a stimulus at a more distal oesophageal site, ie, intrinsic afferent fibres, mast cells degranulation.16,17

It has been proposed that intraluminal acid, in the presence of DIS, would provoke heartburn by activation of intra-epithelial nerve endings of spinal afferents. However, it is unknown whether these two conditions, in an otherwise healthy subject, would be enough to induce heartburn.

The present study was designed to assess the effect of distal oesophageal perforations with acid and weakly acidic solutions in healthy subjects. The aims of the study were (1) to test the hypothesis that exposure of the oesophageal mucosa, not only to acid but also to weakly acidic solutions, can provoke DIS in the distal oesophagus of healthy human subjects; (2) to assess the spread of perfusion-induced mucosal changes from the distal ‘exposed’ to the proximal ‘non exposed’ oesophagus; and (3) to assess the relationship between symptoms perception and presence or magnitude of DIS.
METHODS

Subjects

Fourteen healthy volunteers (seven males) without upper gastrointestinal (GI) symptoms, mean age of 23±1 years (range, 19–32 years), were enrolled in the study. All subjects gave written informed consent before inclusion.

Experimental protocol

Participants underwent intraoesophageal perfusion sessions in a randomised order with at least 4 weeks washout period in between. Upper GI endoscopy with oesophageal biopsies obtained at 5 and 15 cm above the lower oesophageal sphincter (LOS) was performed approximately 20 min after the end of each perfusion. In the first session, manometry was performed to locate the proximal margin of the LOS. A single lumen perfusion catheter and a dual-pH sensor catheter were positioned trans-nasally into the oesophagus so that the perfusion point was located 5 cm proximal to the upper margin of the LOS and the two pH sensors were located 5 and 15 cm above the LOS respectively.

Perfusions were performed during 30 min at a flow rate of 2 ml/min with the subject in a semi-recumbent position. The following solutions were used: (1) neutral solution: NaCl 0.9% at pH 7.2; (2) weakly acidic solution: NaCl 0.9% at pH 5.5; (3) acidic solution: NaCl 0.9% at pH 2.0 plus 0.5 mg/ml of pepsin and (4) acidic solution+bile acid: NaCl 0.9% at pH 2.0 plus 0.5 mg/ml of pepsin plus glycocholic acid 2 mmol/l.

At the end of perfusions subjects were asked to drink 50 ml of water to stabilise the oesophageal pH above 4. Endoscopy was performed approximately 20 min after the perfusion. Two biopsies (Radial Jaw3 with needle; outside diameter 2.2 mm; Boston Scientific, Heredia, Costa Rica) were obtained from the second quadrant at the level of each pH sensor. All endoscopies were performed by a single endoscopist (RB).

Subjects underwent a separate endoscopy session to obtain biopsies at the same levels without preceding perfusion. Biopsies after exposure to weakly acidic and acidic solutions were compared to those obtained without previous perfusion and after perfusion with neutral solutions.

Analysis of the pH recording

Perfusion-induced distal oesophageal acid exposure (pH<4) was calculated from the distal pH measurement. pH monitoring at 15 cm above the LOS was used to identify proximal contamination during distal acid perfusion. Contamination was considered when proximal acid exposure was more than 10% of the perfusion time. Subjects with proximal contamination were excluded from the analysis.

Symptom score

Subjects scored heartburn or pain every 5 min during the perfusion period, using a visual analogue scale (range 0–10) (0=no sensation to 10=maximum pain intensity).

Morphological studies

Tissues were examined using both light and transmission electron microscopy. For light microscopy, tissues were fixed in 4% (w/v) paraformaldehyde. Biopsies were embedded in OCT compound (Sakura Finite USA, Torrance, California, USA) as previously described.\(^{18}\) Semi-thin sections (1 µm) were stained according to Richardson et al.\(^{19}\) Two biopsies from each protocol (one proximal and one distal) were analysed by a single histopathologist (MV) who was blinded to the solution perfused and the oesophageal site. All biopsies were assessed for total epithelial thickness, length of the papillae and basal cell layer thickness.

For transmission electron microscopy, tissues were fixed in 2.5% (w/v) glutaraldehyde in phosphate buffer. Thereafter tissues were post-fixed in 1% buffered osmium tetroxide at 4°C, and dehydrated through a graded alcohol series, then embedded in an epoxy resin. Ultrathin sections were post-stained with uranyl acetate—lead citrate. Specimens were examined and photographed using a Zeiss transmission electron microscope (Zeiss, Oberkochen, Germany). From each biopsy, intercellular spaces diameter was measured in each image. Morphometric analysis of intercellular spaces diameter was performed using a computer software program (Image-Pro MC 5.1.0.20; Media Cybernetics, Bethesda, Maryland, USA). Intercellular spaces diameter was assessed by one of the investigators (RF) blinded to the solution perfused and the oesophageal site.

Solutions and drugs

Porcine pepsin (porcine pepsin A, 392 units/mg solid) and glycocholic acid were purchased from Sigma Aldrich (Sigma/RBI, Bornem, Belgium) and saline solutions were from Baxter (Lessines, Belgium).

Statistics

All data are expressed as mean±SEM. A repeated-measures ANOVA was used to compare data between the different perfusion types followed by Dunnett’s post-tests to determine differences between baseline data (non perfusion) and after perfusion of different solutions. Single comparisons were performed by paired or unpaired Student t test when appropriate. Statistical analyses were performed with Graph Pad Prism 4.0. A p value of <0.05 was used to indicate significance.

RESULTS

Overall, six subjects were excluded from the study. Four subjects had proximal oesophageal contamination during distal acid perfusion and two subjects did not complete the perfusion series.

Eight subjects were included in the final analysis (five males/22.8±1.3 years). During oesophageal perfusion with the acidic solution or the acid+bile acid solution, the distal oesophageal pH was below 4 for 27.8±0.5 min and 28.2±0.4 min, respectively. There was no proximal contamination (proximal oesophageal pH was below 4 for 0.7±0.4 and 0.2±0.1 min, respectively). During oesophageal perfusion with neutral and weakly acidic solutions (pH 7.2 and pH 5.5) both the distal and the proximal oesophageal pH remained above 4 during the complete perfusion time.

Morphological studies

Light microscopy

Thirty minutes distal oesophageal perfusion with acid, weakly acidic and neutral solutions did not induce erosions. Furthermore, after perfusions, the evaluated parameters in biopsies from distal and proximal oesophageal tissues were not significantly different from those observed in biopsies obtained without previous perfusions.

Transmission electron microscopy

Intercellular space diameters in biopsies obtained without previous perfusion were 0.59±0.04 µm and 0.52±0.04 µm in the proximal and distal oesophagus respectively, (N=8, p=0.08).
Oesophagus

These diameters are comparable with those observed in healthy subjects in previous studies. The effect of distal oesophageal perfusions on intercellular space diameters in biopsies from the distal ‘exposed’ and proximal ‘non-exposed’ oesophagus is displayed in figures 1 and 2.

Compared to biopsies without previous perfusion, biopsies obtained from the ‘exposed’ distal oesophagus to acid and acid + bile solutions showed significantly enlarged intercellular spaces (≥1 μm) (figures 1A and 2). Interestingly, perfusion with a weakly acidic solution (pH 5.5) provoked an identical effect. Distal oesophageal perfusion not only provoked DIS in the ‘exposed’ oesophageal area. Weakly acidic and acid solutions also induced significant enlargement of the intercellular spaces in the most proximal ‘non-exposed’ oesophageal area (figure 1B). There was no difference in intercellular space diameters between biopsies obtained from distal and proximal oesophagus.

Unlike weakly acidic and acid solutions, perfusion of the distal oesophagus with a neutral solution (pH 7.2) did not provoke significant mucosal changes. Intercellular space diameters of the distal and proximal oesophageal mucosa were similar to those observed in biopsies without previous perfusion (figure 1).

Symptom score

None of the subjects perfused with neutral solution had discomfort or pain. In spite of having DIS in their post-perfusion mucosal biopsies, subjects perfused with the weakly acidic and acidic solutions had almost no symptoms. Their symptom score was similar to that obtained during perfusion of neutral solution (p=0.59). The median visual analogue score for each group remained at 0, indicating that the stimulus was innocuous for the majority of subjects. Only 1/8 subjects during weakly acidic perfusions and 2/8 in acidic perfusion scored symptoms (figure 3). There was no correlation between perception scores and diameter of intercellular spaces (N=8, r=0.16, p=0.59) (figure 4).

DISCUSSION

We assessed the effect of 30 min oesophageal perfusions with acid, weakly acidic and neutral solutions in healthy human subjects. The main results of our experiments were the following: (1) oesophageal perfusion with acid solutions, with or without pepsin and bile acids, provoked DIS in the ‘exposed’ distal oesophageal mucosa; (2) oesophageal perfusion with weakly acidic solutions, provoked identical changes to those observed after perfusion with acid solutions; (3) distal oesophageal perfusions not only provoked changes in the ‘exposed’ distal mucosa, but also in the more proximal ‘non-exposed’ mucosa; and (4) in spite of the presence of perfusion-induced oesophageal mucosal DIS, most healthy subjects did not perceive heartburn or pain during the experiments.

The pathophysiology of symptoms in GORD is traditionally explained by the effect of increased exposure of the damaged distal oesophageal mucosa to gastric acid. Between 60% and 70% of patients with heartburn do not have distal oesophageal erosions and are classified as having NERD. In patients with NERD, the mechanism for symptoms is not yet completely elucidated. These patients do not have macroscopic erosions, but they frequently present microscopic mucosal DIS. This feature has been proposed as the missing link in the pathogenesis of symptoms in NERD. Barlow and Orlando suggested that DIS enable the diffusion of refluxed gastric acid into the intercellular space. Through DIS, acid can reach and activate chemosensitive nociceptors that transmit signals via the spinal cord to the brain resulting in perception of heartburn.

The development of DIS has been traditionally attributed to increased exposure of the distal oesophageal mucosa to acid. Prolonged exposure of oesophageal mucosa to acid is important in the pathogenesis of oesophagitis and Barrett’s oesophagus. However, patients with NERD not always have increased mucosal exposure to acid and DIS has been found in both, NERD patients with and without increased oesophageal acid exposure.

Understanding the mechanisms for development of DIS can be useful to design treatments to reduce heartburn in patients with NERD. Experimental oesophageal acid perfusion in animal studies and human volunteers could provoke DIS. Bove et al perfused acid solutions (pH 1.1) in healthy subjects and found DIS in the distal oesophageal biopsies using light microscopy. Our study confirmed these findings using acid solutions at pH 2 and detection of DIS by transmission electron microscopy. Additionally, we studied a possible synergistic effect of bile acids on the impairment of oesophageal mucosa integrity induced by acid. It is known that a subgroup of NERD patients may have increased acid and bile exposure as compared to normal subjects. Recent in vitro experiments in our laboratory, using rabbit oesophageal mucosa, showed that addition of glycocholic acid to acid solutions increased oesophageal mucosal permeability and further reduced mucosal electrical resistance.

Against this hypothesis, a study by Calabrese et al found no differences in prevalence of DIS between patients with only acid reflux and patients with acid and bile reflux as detected with Bilitec 2000 (Synectics, Stockholm, Sweden). Our current results are in line with those findings. Perfusion of the distal oesophagus with acid solutions containing bile acids (glycocholic...
2 mmol/l) induced similar changes in intercellular spaces than those observed after perfusion with acid solution alone. These data suggest that in NERD patients with clear increased oesophageal acid exposure the presence of bile acids plays a minor role in the pathogenesis of symptoms. NERD patients with increased acid exposure are the best responders to PPI treatment.26

DIS was detected in NERD patients without increased oesophageal acid exposure and in NERD patients that persisted symptomatic in spite of a good acid control with PPI.9 We hypothesised that, in these patients, the persistence of weakly acidic reflux containing bile acids and/or other gastric juice components, may induce or maintain both DIS and symptoms. Recent in vitro experiments in our laboratory showed that exposure of the rabbit oesophageal mucosa to weakly acidic solutions with bile acids can provoke increased oesophageal permeability and DIS.27 The results of the present study, in healthy human subjects, are in line with our previous results in vitro. Oesophageal perfusion with weakly acidic solutions (pH 5.5) induced DIS of similar magnitude than that provoked by acid solutions. These data suggest that weakly acidic reflux in NERD patients with normal acid exposure or in patients ‘on’ PPIs, may contribute to symptoms by affecting oesophageal mucosa integrity.

A recent study by Caviglia et al showed that NERD patients may have DIS not only close to the gastro-oesophageal junction but also in the more proximal oesophagus, less exposed to gastric refluxates.15 It was unclear whether the presence of DIS in the proximal oesophagus was due to direct exposure of the proximal mucosa to reflux or to an indirect mechanism triggered by distal oesophageal events. The present study shows that DIS in the proximal oesophagus may involve such indirect mechanism. In our study, we excluded those subjects with ‘contamination’ of the proximal oesophagus during distal perfusions and we found DIS in the proximal biopsies even when the proximal acid exposure was minimal. Although such indirect mechanism, acting at long distance from the affected area, has never been described in the oesophagus, a similar phenomenon has been reported in other areas of the gastrointestinal tract.28

Figure 2  Electron microscopy pictures of oesophageal mucosa after exposure to different test solutions. (A) non-perfused, (B) acidic solution, (C) acidic solution plus bile acid and (D) weakly acidic solution. Note the presence of dilated intercellular spaces in B, C and D as compared to biopsies obtained without previous perfusion. Scale bar=7 μm.

Figure 3  Visual analogue scale pain scores recorded by subjects following 30 min distal oesophageal perfusion. Thick lines indicate median visual analogue scale score for each solution.

Figure 4  Correlation between intercellular space diameters and perception scores evaluated with visual analogue scale during perfusion with different test solutions.
Oesophagus

It is known that oesophageal mucosa acidification can induce mast cell degranulation, activation of capsaicin-sensitive afferent neurons and release of neurokinines. These or other mediators may be involved in the spreading of DIS towards the proximal oesophagus. The presence of DIS in the proximal oesophageal mucosa can contribute to the hypersensitivity observed in NERD patients to proximal reflux episodes. This effect is likely to be synergistic with central factors such as stress and spinal—cortical sensitisation that also affect sensitivity. Experimental acidification of the distal oesophagus in healthy subjects induces hypersensitivity of the proximal oesophagus to electrical stimulation. Such hypersensitivity is currently considered as centrally mediated (spinal—cortical), and also involves a specific activation pattern of the autonomic nervous system. The contribution of proximal oesophageal impaired mucosal in hypersensitivity to electrical stimuli is currently under investigation. It is possible that breakdown of the epithelial barrier may permit increased nociception to electrical stimulation by enhanced stimulation of primary afferents.

In spite of the presence of perfusion-induced distal and proximal oesophageal mucosa DIS; most healthy subjects did not perceive heartburn or pain during the experiments. This finding suggests that other factors, not only DIS, are necessary for the generation of symptoms in patients with NERD. For example, upregulation of transient receptor potential vanilloid 1 (TRPV-1) in nerve fibres and other proton-gated ion channels such as ASICs (acid-sensing ion channels) and P2X purinergic receptors may be relevant for symptom perception.

Limitations of our study were (1) the small number of subjects who underwent all perfusion periods; (2) our evaluation of symptoms perception was limited to the perfusion period; and (3) 30 min of perfusion is more than single episodes of reflux exposure in healthy subjects or patients with NERD. We have chosen 30 min exposure to produce measurable effects, but we acknowledge that multiple short distal acid exposures can produce and maintain oesophageal hypersensitivity. DIS was present in biopsies obtained 20 min after the end of perfusions; it is possible that symptoms might occur later during a re-exposure to acid. Further experiments are needed to better understand the relationship between DIS, acid exposure and symptoms.

In conclusion, the human oesophageal mucosa is very sensitive to continuous exposure to acidic solutions. Both acid and weakly acidic solutions can provoke DIS. These mucosal changes can occur also in the more proximal ‘non-exposed’ oesophagus. However, in spite of the presence of perfusion-induced oesophageal mucosa DIS and intraluminal acid, most healthy subjects did not experience heartburn suggesting that other factors are critical in the pathophysiology of symptoms in NERD.

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Competing interests None.

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Contributors Ricardo Farre and Fernando Fornari contributed equally to the paper.

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