Review article: nature and properties of gastro-oesophageal and extra-oesophageal refluxate

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SUMMARY

Gastric juice contains many damaging agents against which the stomach has effective defences including a mucus bilayer which generates an unstirred layer which supports surface neutralisation of acid and forms a diffusion barrier to pepsin. However, once gastric contents leave the stomach and enter the oesophagus and the upper airways the protective mechanisms are much reduced. The major aggressors in gastric refluxate are acid, pepsin and bacteria. In addition, gastric refluxate will contain, but not always, duodenal factors such as bile acids and pancreatic enzymes. Acid in the majority of reflux events will remain at a damaging level, i.e. below pH 4.0 for a significant amount of time in the oesophagus. Bile acids have been demonstrated to be damaging in many experimental models; however, great care needs to be taken when interpreting these results because the concentrations and the form of the bile acids used do not always reflect the *in vivo* situation. Pepsin is an acidic protease but has the potential to damage extra-gastric tissues at pHs up to 6.0 and will not be irreversibly inhibited until pH 7.5 or above. Trypsin, if it passes through the stomach at pH 4.0 or above or rapidly though low pH of 2 or less, will retain activity and can go on to cause damage. With the increase in the use of proton pump inhibitors to treat gastro-oesophageal reflux the elevation of gastric pH has allowed bacterial overgrowth of the stomach. Consequently, a reflux event can lead to the establishing of bacterial colonies outside of the stomach, notably into the airways and lungs.

INTRODUCTION

Gastric juice moving in a retrograde fashion out through the cardiac (lower oesophageal) sphincter and up the oesophagus is a normal physiological event. Based on measurements using 24 h ambulatory oesophageal impedance-pH monitoring of a healthy population of 72 adults, the median number of reflux events was 44 with a 75th percentile of 58 events. Interestingly, only just over half of these events were acidic (pH < 4.0) the rest being weakly acidic.[1] In our own impedance studies, we have taken 58 as the normal level of reflux events to compare normals to lung transplant patients.[2] It is clear that reflux occurs in the normal population; however, the number of events may be affected by having an impedance catheter in place.

The main aggressive agents in gastric juice are acid, pepsin, bile salts, bacteria (particularly in patients on proton pump inhibitor (PPI) therapy) and pancreatic proteolytic enzymes. With acid, the pH is likely to be below 4 for a significant time in the oesophagus, where the protective mechanisms against acid are the strongest outside of the stomach. These include peristalsis, carbonic anhydrase production of bicarbonate, heat shock protein expression, a surface layer of dead cells which

can be shed off to protect the viable cells from acid exposure and salivary mucus and bicarbonate.[3]

In terms of acid in the oesophagus, proton pump inhibitors will reduce the level and time of acid exposure in gastro-oesophageal reflux disease (GERD) patients; however, acid exposure will still tend to be above normal.[4] It is important to point out here that although PPI therapy will increase the pH and reduce the volume of gastric juice with the pH of refluxate being mainly between 5 and 7 that is weakly acidic.[5] It will not directly affect pepsin secretion and critically it will not prevent reflux.[6] Therefore on PPIs gastric juice will still contain the other damaging components.

MAJOR AGGRESSORS IN GASTRIC JUICE WITH POTENTIAL TO DAMAGE EXTRA-OESOPHAGEAL TISSUES

Bile acids

Bile acids can be found in gastric juice and are present as a result of reflux from the duodenum through the pylorus into the stomach (duodenogastric reflux). Bile acids have been measured in fasting gastric juice using an enzymatic method based on a 3α hydroxydehydrogenase, which acts on the hydroxyl group of the bile acid steroid ring and in the process releasing reduced NAD. The NADH is then reacted with nitrotetrazolium blue to produce formazan cataylsed by diaphorase. The resulting blue colour development can be quantified at 540 nm. This assay can be made more sensitive by using thio-NAD^+ in the presence of excess NADH, which results in enzyme cycling and the rate of formation of thio-NADH can be determined directly by a change in absorbance at 405 nm, with a claimed lower detection limit of 1 μmol/L. Using the above methods fasting gastric bile acid levels ranged from 0 to 150 μmol/L, median 0 μmol/L (n = 20) and 0–410 μmol/L, median 100 μmol/L (n = 14) in controls and oesophagitis patients respectively.[7] In our studies, using the enzymatic method, in fasting gastric juice of patients undergoing routine upper GI endoscopy the values ranged from 10 to 10 000 μmol/L with a median of 55 μmol/L and only seven of the 60 samples having bile acid levels above 500 μmol/L (see Table 1). It is likely that gastric juice bile acid concentrations increase in the postprandial period. This can be implied from the increases in bile acid concentrations in oesophageal aspirates increasing from a mean of 6 to 12 μmol/L and 10 to 60 μmol/L from fasting to postprandial in controls and GERD patients respectively.[8] Therefore reflux after a meal is potentially more damaging than in the fasting state. Based on the levels of bile acids present in gastric juice and the normal serum range 0–10 μmol/L. Along with the manufacturers claim for the enzymatic assay of a detection limit of 1 μmol/L, in our hands 2 μmol/L and the findings of Klokkenburg *et al.*[9] that 'outcomes lower than 5 μmol/L may not be reliable'. Bile acid levels in the oesophagus would be measurable and present at potentially damaging levels in the oesophagus but once the refluxate has left the oesophagus and entered the pharynx/larynx and has been diluted by upper airway and salivary secretions, in many cases bile acids will be below detectable levels. In particular detection is unlikely if the refluxate is aspirated into the lungs and collected by bronchoalveolar lavage which involves a 100–200 times dilution with saline. We would recommend that more sensitive quantitation methods are used for extra-oesophageal refluxate, e.g. HPLC which can determine levels of individual bile acids with a limit of detection of 0.07–0.6 μmol/L and quantification in the range of 0.2–1.8 μmol/L.[10] However, the most sensitive detection system available is tandem mass spectrometry with a quantitation limit of 0.1 μmol/L and with pre-extraction using a C18SPE column eluted with methanol the limit can be improved to 1 nmol/L.

Bile acids have been demonstrated in many studies to be damaging to oesophageal and extra-oesophageal tissues. In a perfused rabbit oesophagus model, bile acids at concentrations up to 5 mmol/L have been shown to damage the mucosa particularly at low pH with the taurine conjugated bile acids, taurodeoxycholate and taurocholate causing increased permeability to hydrogen ions and mucosal damage, while un-conjugated ones did not.[11, 12] Bile salts have the potential to damage tissues as far away from the stomach as the lungs. Exposure of type II pneumocytes in culture to chenodeoxycholate in μmol/L concentrations caused increases in cell membrane permeability and decreased cell viability.[13] In our laboratory, we have demonstrated that lithocholate (the free acid) concentrations above 10 μmol/L cause significant cell death of primary bronchial epithelial cells in culture. Further recent studies by Farre *et al.*,[14] using rabbit oesophageal mucosa set up in Ussing chambers have confirmed that the conjugated bile acid sodium salts glycocholate and taurodeoxycholate at concentrations between 0.5 and 5 mmol/L, at pH 2.0 damaged the mucosa as seen by decreased electrical resistance. Interestingly, they further demonstrated that these conjugated bile acids and un-conjugated deoxycholate caused damage at higher pHs of 5.0 and 7.4 when used at the higher

Table 1 | Analysis of fasting total bile acids in gastric juice from routine endoscopy patients

GJ sample	pH	TBA (μM)	GJ sample	pH	TBA (μM)	GJ sample	pH	TBA (μM)
1	2.3	360	21	2.8	n.d.	41	1.8	40
2	7.8	n.d.	22	2.9	80	42	1.5	200
3		1160	23	3.5	n.d.	43	1.6	240
4	4.8	40	24	1.6	80	44	2.4	n.d.
5	2.2	n.d.	25	1.7	140	45	6.6	250
6	1.9	220	26	2.6	80	46	4.8	130
7	7.5	10010	27	1.6	160	47	2.0	20
8	3.9	n.d.	28	7.5	130	48	1.4	40
9	8.4	20	29	7.9	40	49	5.5	480
10	2.2	100	30	4.1	n.d.	50	4.1	420
11	1.8	330	31	1.6	70	51	4.7	n.d.
12	2.9	n.d.	32	1.8	n.d.	52	8.4	n.d.
13	6.7	20	33	1.6	n.d.	53	1.6	40
14	1.7	890	34	1.4	150	54	5.1	n.d.
15	4.7	8030	35	7.6	140	55	5.2	3060
16	6.9	30	36	1.5	30	56	6.0	10
17	0.8	n.d.	37	2.1	220	57	1.7	20
18	1.9	750	38	1.4	20	58	1.6	320
19	1.5	120	39	7.5	6450	59	2.2	n.d.
20	6.6	160	40	1.8	n.d.	60	1.7	n.d.

n.d., not detectable (detection limit 10 μM); TBA, total bile acids.

Mean: 55 (10–10 010) μM. Bile acid concentration was measured using the 3α-hydroxysteroid dehydrogenase assay.

mmol/L concentrations, 2 and above in the presence of 1 mg/mL pepsin. From the above studies, bile acids are damaging in the mmol/L range; however, there is conflicting data as to the levels of bile acids reaching the extra-gastric tissues.

In addition, in experimental assessments of bile acid damage, the form of bile acids *in vivo* needs to be carefully considered. Bile acids exist in several forms, as free acids, bile salts with sodium and potassium, conjugated with glycine or taurine and conjugated bile salts with sodium or potassium. Bile salts are synthesised in the liver from cholesterol. The two primary bile acids are cholic acid and chenodeoxycholic acid, having three and two hydroxyl groups present on the steroid rings respectively. In the colon, cholic acid and chenodeoxycholic acid are converted to the secondary bile acids deoxycholic acid and lithocholic acid by bacteria, with two and one hydroxyl groups present on the steroid rings respectively. Consequently lithocholate with only one hydroxyl group is the most lipid soluble and potentially the most damaging to cells. In the liver, bile acids are conjugated to glycine or taurine. Any free bile acids and conjugated bile acids are converted to sodium or potassium salts in the alkaline conditions of hepatic bile. The question therefore is what is the distribution of bile acids in bile and in different regions of the GI lumen and in what form are they present?

In human, bile only a trace of free bile acids are present and glycine conjugates make up around 75% and taurine conjugates around 25% (Table 2).[15] Bile acids detected and quantitated in aspirated duodenal fluid were conjugated, with the free bile acid, cholic acid being below the level of detection (0.07–0.6 μmol/L). Conjugated bile acids were present in the following percentages; glycodeoxycholate 48%, glycochenodeoxycholate 20%, glycocholate 15%, taurochenodeoxycholate 8% and taurocholate 6%. In gastric aspirates again conjugates predominate with no free cholic acid detectable. The conjugated bile acids were as follows: glycodeoxycholate 13%, glycochenodeoxycholate 39%, glycol-cholate 35%, taurochenodeoxycholate 4% and taurocholate 6%.[10] In human oesophageal aspirates, again no free bile acids have been

Table 2 | Bile acid properties

	Solubility in H_2O at RT (μM/L)	p*K*a	% in human bile
Free bile acids			
CA	242	5.2	Trace
DCA	100	5.02	Trace
CDCA	142	4.98	Trace
Na^+ salts			
CA	116–349 × 10^3		
DCA	48–241 × 10^3		
CDCA	48 × 10^3		
Glycine conjugated			
GCA	53	3.88	30
GDCA	17.5	3.88	15
GCDCA	17.6	3.87	30
Na^+ salts			
GCA	410 × 10^3		
GDCA	20–100 × 10^3		
GCDCA	110 × 10^3		
Taurine conjugated			
TCA	14 × 10^3	<2	10
TDCA	82 × 10^3	<2	10
TCDCA	n/a but expected in the range of other taurine conjugates	<2	5
Na^+ salts			
TCA	470 × 10^3		
TDCA	38–500 × 10^3		
TCDCA	193 × 10^3		

CA, cholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; GCA, glyco-cholic acid; GDCA, glyco-deoxycholic acid; GCDCA, glyco-chenodeoxycholic acid; TCA, tauro-cholic acid; TDCA, tauro-deoxycholic acid; TCDCA, tauro-chenodeoxycholic acid; RT, room temperature.

The solubilities are in water ~pH 5.0 at RT. The data in this table are taken from or calculated from figures in references 15 and 17–22.

found with 60% glycocholate, 16% glycodeoxycholate and 15%, glycochenodeoxycholate the remaining ~10% being made up of taurine conjugates and glycolithocholate.[8] It appears from the above studies that free bile acids should not be used in experimental models of reflux. The last question relating to bile acids is their damaging potential. Do they have a direct detergent effect on the cell membrane or do they need to enter the cell? Based on the p*K*a values in Table 2, the glycine conjugates, which make up 70%, would be uncharged at pHs below 4, the values occurring in refluxate. Glycine conjugates would therefore be hydrophobic and lipid membrane soluble. They could then accumulate inside the cell, as once inside they would ionise, become hydrophilic at pH 7.4 and be trapped inside the cell. As taurine conjugates have p*K*a values below pH 2, only a small fraction will be protonated (uncharged) at pHs between 2 and above. So these are unlikely to accumulate inside cells as a result of a reflux event. Another point to consider is that at very low pH, bile acids will precipitate and therefore cease to be damaging. It is also possible that some charged bile acids may be actively taken up by upper GI tract mucosal cells, e.g. in the oesophagus by scavenging receptors.

A situation where un-conjugated bile acids may become important is in patients on acid suppression therapy, where bacterial overgrowth occurs in the stomach and these bacteria could deconjugate the bile acids.[16] Then bile acids with p*K*a values around 5 could damage mucosa by entering cells at pHs in the weakly acid reflux range above pH 5.

The direct detergent effect of bile acids requires a hydrophobic and a hydrophilic region and is affected by solubility and p*K*a, as detergent behaviour is altered by ionisation state. Therefore taurine conjugates would be expected to have a topical detergent effect across the whole pH range of refluxate. Taurine conjugates are so called acid resistant detergents.

Pepsins

Pepsins are by definition acidic proteases and have maximal activity against a protein substrate such as haemoglobin between pH 1.9 and 3.6.[23] This wide range is because human gastric juice contains eight different pepsins. The major pepsin is pepsin 3, making up 70.3 ± 2.6% of the total.[24] Pepsin 3 has a pH optimum between 2.4 and 2.8 against haemoglobin. It is important to note that human pepsin 3 retains between 5% and 10% of its activity against purified protein at pH 5.0 and 10% or more at pH 4.0 and retains measurable activity up to pH 6.0. In addition, pepsins ability to damage tissue also extends up to pH 6.0 as seen in the pig larynx model.[3, 25] The ability to degrade and damage mucosal tissue up to pH 6.0 is important when considering reflux from the stomach into the upper airways and lungs as reflux events in the weakly acidic range, pH 5.0 and above have been widely reported.[14] Consequently, pepsin must be considered a potential damaging agent up to pH 6.0. A further consideration is that pepsin could be taken

up by the mucosal cells of the aerodigestive tract and cause intracellular damage in regions of low pH inside the cell, e.g. lysosomes.[26] Although pepsin is inactive above pH 6.0, it is still un-denatured. In gastric juice, pepsin retains its native structure up to pH 7.5 and possibly higher. Therefore, pepsin present in a refluxate could bind to the mucosa, remains native but inactive after neutralisation of the refluxate and be reactivated by a subsequent reflux event below pH 6.0 or by the passage over the mucosa of an acidic drink, e.g. cola pH 2.5–2.8. The presence of pepsin in the refluxate makes it a potential biomarker of reflux as well as a factor in the aetiology of tissue damage. Unlike the measurement of bile acids, there is a sensitive pepsin ELISA available with a lower quantitation limit of 1 ng/mL.[6]

Pancreatic proteolytic enzymes

Pancreatic exocrine secretions contain several proteolytic enzymes including trypsin and chymotrypsin. Trypsin has been the most studied.[27, 28] It is secreted as trypsinogen and activated by an intestinal enzyme, enteropeptidase. The activation involves removal of a 6 amino acid aspartate rich peptide. This is unlike the conversion of pepsinogen to pepsin which is auto-catalytic and pH dependent. Trypsin has a pH range of activity between 6 and 10 with optima around pH 8. For trypsin to have a major role in damage to the aerodigestive tract it must pass through the stomach from the duodenum without being inactivated and it must reach the oesophagus and above at pHs within its activity range. Rat models demonstrate the damaging potential to the oesophagus of trypsin. In models where duodenal contents are introduced into the oesophagus by means of an oesophagogastroduodenostomy, where the duodenum is joined to the stomach above the pylorus and to the oesophagus. That is a situation where duodenal contents do not pass through an intact stomach. This gastroduodenal reflux model induced oesophageal erosions and ulcer formation within 8 weeks of surgery and the use of trypsin inhibitors reduced the damage.[29] Also in humans with distal gastrectomy reconstructed with an anastomosis between the remaining stomach and the duodenum, trypsin activity was elevated in oesophageal washings, pH range 5.2–7.2 average 6.3, in patients with severe ooesophagitis.[30] However, in both these situations duodenal juice has not passed through a normal intact stomach. When it has passed through an intact stomach, only seven of 365 oesophageal aspirated contained active trypsin and the level of trypsin activity was only above 20 μg/mL when the pH was above 4.6.[28] In our studies, trypsin activity in human gastric juice correlated with the pH of the juice, i.e. the higher the pH the higher the trypsin level. In further experiments, trypsin was stable when exposed to pepsin at pH 4.0 for 6 h but destroyed by incubation with pepsin at pH 2.2 for 4 h. Consequently, for trypsin to be a major source of damage to tissues above the stomach, it must pass through the stomach at a pH above 2.2, a situation present in patients on PPI therapy.

Bacteria/bacterial products

We have shown in experiments with human primary lung epithelial cells that human gastric juice at 1/100 to 1/1000 dilution with media (therefore pH will be 7.4) could cause almost 100% cell death and dialysis and filtration reduced the level of cell death. Recently Mertens *et al.*[5] using primary bronchial cells from one patient have exposed them to diluted gastric juice collected from 11 patients on or off PPI therapy having routine endoscopy. Gastric juice from patients on PPI therapy produced a significantly higher Il-8 response than patients off PPI and that the pH of gastric juice as collected significantly correlated with Il-8 production. Filtration of the gastric juice significantly reduced the Il-8 response. These results imply a role for constituents in gastric juice of patients on PPI therapy, that cause significant inflammatory responses, that are removed at least in part by filtration. As on PPI therapy the gastric pH would be sufficiently high to permit some bacterial overgrowth in the stomach, candidates for these constituents are bacteria and bacterial products such as endotoxins.

CONCLUSIONS

In refluxate:

(i) Acid is damaging to the oesophagus but once outside the oesophagus it will be rapidly neutralised.
(ii) Pepsin can damage all extra-gastric tissues at pHs up to 6.
(iii) Bile acids are potentially damaging but most models do not use the *in vivo* form of bile acids. The levels of bile acids outside of the stomach and oesophagus are difficult to quantitate accurately.
(iv) Trypsin could cause damage if it retains activity after passing through the stomach.
(v) Bacteria and bacterial products can cause tissue damage if they have survived exposure to gastric juice.

Acid can be controlled by PPI therapy all the other damaging factors remain potentially damaging on PPI therapy and may have their damaging ability enhanced by the increase in the pH of gastric juice.

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