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Reflux and pH: 'alkaline' components are not neutralized by gastric pH variations

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SUMMARY. **The ability of the 'alkaline' components of reflux to cause harm in vivo is still open to debate, although these components have been shown in vitro to be capable of damaging the mucosa. The precipitation of bile acids and lysolecithin that occurs at low pH values is the main reason for questioning in vivo mucosal damage. This study was undertaken to determine the composition of gastric aspirates at different original pH values and the degree of solubility of the alkaline components when pH modifications are artificially induced. The samples for chemical analysis were collected from indwelling nasogastric tubes after surgical procedures that did not involve the upper gastrointestinal tract. Bile acid and lysolecithin concentrations were assessed by means of dedicated methods. Thirty-five samples were available for bile acid evaluation and 27 for lysolecithin evaluation. Bile acid and lysolecithin assessments were repeated after pH adjustment at 2, 3.5, 5.5 and 7. For easier assessment of the results, three ranges of the original pH were selected (pH $<$ 2, 2 $\leq$ pH $<$ 5, pH $\geq$ 5). For each pH range, results were pooled together and compared with those in the other pH ranges. Bile acid concentrations were 113 $\pm$ 48, 339 $\pm$ 90 and 900 $\pm$ 303 (mean $\pm$ s.e.m. μmol/L), respectively, in the three groups selected on account of the different original pH values. Differences were significant ($p < 0.001$). Both taurine- and glycine-conjugated bile acids were represented even at pH $<$ 2. No major differences were observed in bile acid concentration with the artificially induced pH variations. Lysolecithin concentrations were 5.99 $\pm$ 3.27, 30.80 $\pm$ 8.43 and 108.37 $\pm$ 22.17 (mean $\pm$ SEM μg/ml), respectively, in the three groups selected on account of the different original pH ranges. Differences were significant ($p < 0.001$). No significant differences in lysolecithin concentration were detected with the artificially induced pH variations. In conclusion, both bile acids and lysolecithin are naturally represented in the gastric environment even at very low pH values, although their concentrations decrease on lowering of the naturally occurring pH. Given the concentration variability of bile acids and lysolecithin, further studies are needed to assess the minimal concentration capable of mucosal damage in vivo.**

INTRODUCTION

The pathogenetic role of acid reflux (HCl and pepsin) in esophageal mucosal damage has been clarified by a number of both clinical and experimental studies.[1–3]

In contrast, the harmful effects of the so-called 'alkaline' components of reflux and even their capability of refluxing into the esophagus are still questioned[4,5] and the problem of duodenogastroesophageal reflux (DGR) remains unsettled. Many studies have examined DGR from an experimental point of view. They have unequivocally shown that, among all the components which potentially reflux into the esophagus, bile acids, lysolecithin and trypsin are capable of causing esophageal mucosal damage in a variety of animals.[6–8] However, their harmful effects seem to be heavily conditioned by the environmental pH. In vitro experimental studies have shown that trypsin is inactivated at pH $<$ 6.5 and lysolecithin at pH $<$ 4. Unconjugated, glycoconjugated and tauroconjugated bile acids are precipitated and, therefore, inactivated at pH $<$ 5, 4 and 2 respectively.

More recently, an in vitro study has compared the pH dependency of the solubility of the commercially available bile acids, which have been used in all the above-mentioned animal models, with that of the bile acids in natural human bile.[9] This study seems to

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show that, in contrast to commercial bile acids, natural bile acids are all precipitated and therefore inactivated at pH < 4.[9]

Thus, as a preliminary step to improve the understanding of the noxious effects of 'alkaline' reflux, the purpose of the present study was to assess the composition of gastric aspirates at different original pH and to evaluate in vitro the solubility (as an indirect index of the harmful potential) of different naturally occurring 'alkaline' components of reflux as a function of pH.

MATERIALS AND METHODS

All reagents were of the highest purity available. HCl, KOH, hemoglobin (bovine), pepsin (porcine stomach mucosa), lysophosphatidylcholine (bovine liver), trichloroacetic acid, methanol, chloroform, NaCl, EDTA and conjugated bile acid standards were purchased from Sigma-Aldrich (Milan, Italy), silica gel H and molybdenum blue reagent from Alltech (Milan, Italy), and high-performance liquid chromatography (HPLC) reagents from Merck (Frankfurter, Germany).

Natural components of reflux were obtained from indwelling nasogastric tubes after surgical procedures that did not involve the upper gastrointestinal tract. Aspirates were collected in the morning from fasted patients. A quantity of 20–30 ml was collected each time and placed in non-transparent-walled containers to avoid light interference. Aspirates were immediately processed. The original pH was measured using a pH-meter (Orion, model 420A). Then, samples were adjusted to pH 1, 2, 3.5, 5.5 or 7 by adding to the original aspirate HCl or KOH. The amount of added HCl or KOH (5 M) ranged between 5 and 50 µl. After pH adjustment, all samples were centrifuged at 1000 r.p.m. for 5 min in order to separate the precipitate which was present in all the samples, and proper amounts of each sample at different pH (the original pH included) were processed for the assessment of each individual component.

For a clearer statistical assessment of the results, three different ranges of the original pH were selected (pH < 2, 2 ≤ pH < 5, pH ≥ 5) and the results obtained in the evaluation of each 'alkaline' component for each individual sample were pooled together with those in the other samples with original pH in the same range and compared with those in the other pH ranges.

Bile acids

Samples for bile acid assessment were diluted 1:2 with water and processed for HPLC. A liquid chromatograph (model 235c, Perkin Elmer, Florence, Italy) was used. An Altex Ultrapore Beckman column, 4.6 mm × 25 cm, 5 µm particle size (Beckman Instruments, Fullerton, CA, USA) was used. The solvent system was methanol/0.01 M KH_2PO_4 75:25. To each liter of this mixture, 4.2 ml of 5 N NaOH were added. The pH was then adjusted to pH 5.35 by addition of H_3PO_4. The solvent was filtered through a 0.45-µm nylon filter before use. The solvent flow rate of 0.7 ml/min produced operating pressures of 13 800–15 180 kPa.

Table 1. Bile acid concentration in the samples pooled together on account of different ranges of their original pH

	Total concentration (µmol/l) Mean ± s.e.m.
pH < 2 (n = 14)	113 ± 48*
2 ≤ pH < 5 (n = 11)	336 ± 90*
pH ≥ 5 (n = 10)	900 ± 303*

*p < 0.001 (ANOVA test).

Lysolecithin

Lysophosphatidylcholine (LPC) was assayed according to the method of Tsai et al.[10] Before lipid extraction, 2 mM EDTA and 5 mM $CaCl_2$ were added to the samples of gastric aspirates to inhibit phospholipase A activity. Lipids were extracted from samples (500 µl) with methanol–chloroform (1:2). After evaporation under a stream of nitrogen, samples were redissolved in a small quantity (50–100 µl) of methanol–chloroform (1:2) and spotted in duplicate on a thin-layer chromatography (TLC) plate (silica gel H, thickness 0.5 mm) along with a known amount of a standard LPC dissolved in chloroform. The chromatogram was developed in chloroform–methanol–water (65:24:4, v/v) and, after the solvent front had ascended 20 cm (about 90 min), the plate was removed, air dried and sprayed with molybdenum blue reagent. Blue spots, indicating phospholipids, were immediately visible. The spots were quantified by densitometry (software QUANTISCAN FOR WINDOWS). LPC concentrations were estimated by comparing the areas of the samples with those of the standards. All results were expressed in µg/ml.

RESULTS

Bile acids

Different amounts of bile acids were detected in all of the 21 samples with original pH ≥ 2 and in eight out of the 14 (57.2%) samples with original pH < 2. The total concentration in the original samples was closely dependent on their pH (Table 1), and differences between the three groups selected on account of the original pH (pH < 2, 2 ≤ pH < 5, pH ≥ 5) were significant. Both taurine- and glycine-conjugated bile acids were represented in all the three groups, with a predominance of the glycine conjugated even in the

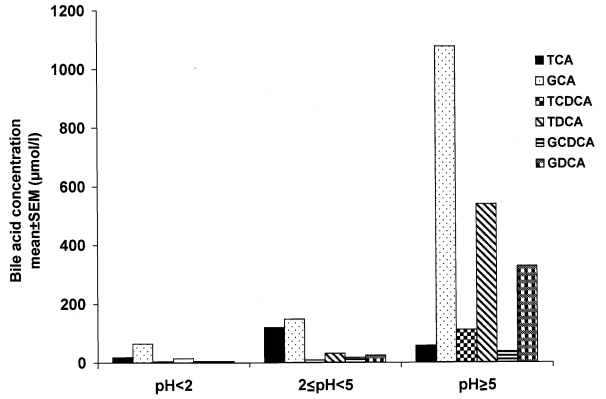

Fig. 1—Concentration of the different bile acids in the samples pooled together at different original pH values. TCA, taurocolic acid; GCA, glycocolic acid; TCDCA, taurochenodesossicholate acid; TDCA, taurodesossicholate acid; GCDCA, glycochenodesossicholate acid; GDCA, glycodesossicholate acid.

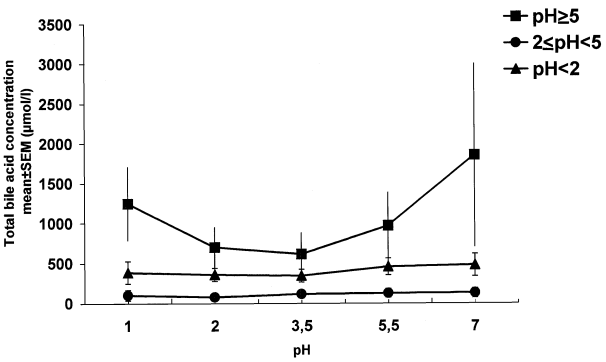

Fig. 2—Total bile acid concentration at different artificially induced pH values.

samples with original pH < 2 (Fig. 1). With the artificially induced progressive pH increase, no relevant differences in total bile acid concentration were demonstrated for samples with original pH < 2 (Fig. 2). In the samples with original pH ranging between 2 and 5, no major differences either at increased or at decreased pH were found (Fig. 2). In the samples with original pH ≥ 5, a more complex trend was observed. Stationary values (in comparison with values at original pH) were detected at intermediate pHs with consistent increases both at extremely high and low pH values, although no significant differences were reached (Fig. 2).

Lysolecithin

Lysolecithin in different amounts was detected in six of the 12 samples with pH < 2, in five of the seven samples with intermediate pH (≥2 and < 5) and in all eight samples with pH ≥ 5. The lysolecithin concentration in the original samples was closely dependent on pH, as shown by the different concentrations that were found in the different ranges of original pH (Table 2). If the threshold below which lysolecithin is

Table 2. Lysolecithin concentration in the samples pooled together on account of the different ranges of their original pH

	Total concentration (µg/ml) Mean ± s.e.m.
pH < 2 (n = 12)	5.99 ± 3.27*
2 ≤ pH < 5 (n = 7)	30.80 ± 8.43*
pH ≥ 5 (n = 8)	108.37 ± 22.17*

*p < 0.001 (ANOVA test).

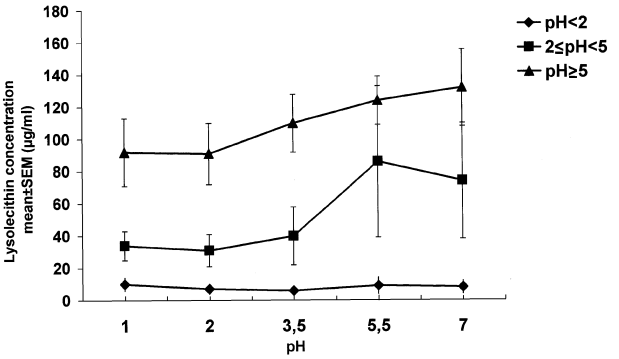

Fig. 3—Lysolecithin concentration at different artificially induced pH values.

inactivated (pH 4) is considered, eight of the 18 samples with pH ≤ 4 contained no lysolecithin, whereas different amounts of lysolecithin were shown in all the nine samples with pH > 4. Mean (± s.e.m., µg/ml) lysolecithin concentration was 17.59 (± 7.58) in the former group and 99.96 (± 20.54) in the latter (p < 0.001, ANOVA test). With the artificially induced pH changes, no significant differences in lysolecithin concentration were detected in any of the three groups distinguished upon the basis of the original pH, although a trend towards a slight increase was observed in all the three groups at the upper values of the pH scale (Fig. 3).

DISCUSSION

The main finding of this study is that variable amounts of each alkaline component of reflux are present even at pH values around 1.

Alkaline components have been shown both clinically and experimentally[6–8] to be harmful to the esophageal mucosa. Clinical evidence is represented by the very severe esophagitis that follows total gastrectomy (suggesting complete anacidity) with esophagoduodenal anastomosis or, more recently noted, with esophagojejunal anastomosis without a complete exclusion of the esophagus from 'alkaline' reflux.[11,12] The frequent occurrence of esophagitis was the main reason for abandoning these types of reconstruction. Moreover, esophagitis has been shown in another situation with complete anacidity, i.e. in the presence of atrophic gastritis associated with pernicious anemia.[13]

Glyco- and tauroconjugated bile acids have been reported to be harmful to the esophageal mucosa at acid pH (pH below 4 and even down to pH 2 for tauro-conjugated).[6] In contrast, deconjugated bile acids are more damaging at higher pH levels (pH between 5 and 8).[6] Lysolecithin has also been shown to cause mucosal damage when in an acidic environment,[14] whereas damage caused by trypsin is relevant only in the absence of acid.[15] Whereas for bile acids the concentration threshold is established at 1 mmol/l,[16] for lysolecithin the minimal concentration above which mucosal damage occurs is unknown.

However, all these experimental studies have utilized commercial products in mono- or two-product mixtures. This, indeed, does not reproduce the naturally occurring situation. A more realistic experimental model should utilize gastric aspirates with known composition because the different alkaline and acid components of reflux seem to reciprocally interact and synergistically enhance their effects on the esophageal mucosa. This means that the minimal noxious concentrations of bile acids and lysolecithin may be much lower in vivo than in vitro. However, a preliminary condition for the harmful potential of each 'alkaline' component is its permanence in solution. In this respect, our findings, which show that bile acids and lysolecithin are still in solution down to pH 1, seem important. These data appear even more relevant in view of the previously reported evidence of a nearly complete precipitation of the naturally occurring bile acids in human bile at pH < 4.[9] Moreover, they suggest that bile acids and lysolecithin may be important for mucosal damage as they are both in solution and not inactivated at low pH. Although it has not been studied in the present paper, trypsin is completely inactivated at low pH, exerts its proteolytic activity at pH around 7, and is probably the main, if not the only, damaging agent of reflux in achlorhydric conditions, such as total gastrectomy and atrophic gastritis associated with pernicious anemia.

Therefore, because this study has shown that concentrations of bile acids and lysolecithin decrease with lowering of pH and that variable concentrations can be found also at very low pH, the principal unresolved problem is whether at pH around 2 (usual gastric environmental pH) their concentration is still such as to be capable of mucosal damage. However, although up to the present time values for minimal noxious concentrations have been obtained with commercial products in mono- or two-product mixtures, studies with gastric aspirates, as opposed to commercial products, are needed. This because the interaction occurring in vivo between the different 'alkaline' and acid components enhances the harmful potential of each of them. As yet, it is not known what is the in vivo threshold concentration for each component that is capable of damaging the mucosa when reflux consists of a mixture of HCl, pepsin, bile acids, trypsin and lysolecithin. The harmful potential of the mixture depends upon the fact that each single component interacts with the others and affects their physicochemical properties, such as solubility, lipophilicity and detergent capability. Moreover, even more complex interactions take place. In this respect, pH again plays a major role. Luminal acidity is essential to determine intracellular bile acid accumulation, which in turn seems the main determinant for bile acid cytotoxic capability.[17]

In addition to the persistence in solution at low pH values of bile acids and lysolecithin, another finding of the present study is the role of pH in determining the concentration of each component. More specifically relevant in this respect is the original pH, i.e. the pH at the time of aspiration of gastric content. Only minor concentration changes are caused following artificially induced pH variations. This finding suggest that early stabilization of physicochemical properties of the gastric content occurs, and that these are not later modified to any great extent by pH changes.

If we consider that all the 'alkaline' components may be represented in the refluxate, and that both their concentration (as shown in the present paper) and capacity to cause damage (as previously experimentally shown) depend upon the original pH, the advantages of simultaneously detecting 'alkaline' reflux and acidity by means of simultaneous Bilitec- and pH monitoring are patent.[18]

In conclusion, in addition to the well-known finding that, for about 2.5 h during the 24-h day, gastric pH is above 4, even in normal subjects, our finding that the 'alkaline' components of reflux are present in solution even at low pH values supports the possibility of interaction of the alkaline components with esophageal and gastric mucosa even in primary refluxers.

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