Effect of hydrochloric acid, pepsin, or taurocholate on bioelectric properties of gastric squamous mucosa in horses

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Objective—To determine the effect of pH with or without pepsin or taurocholic acid on the bioelectric properties of gastric squamous mucosa in horses.

Sample Population—Gastric tissues obtained from 16 adult horses that did not have evidence of gastric disease.

Procedure—Bioelectric properties of squamous mucosa were determined, using modified Ussing chambers. Tissues then were exposed to mucosal bath solution of pH 7.4 under neutral (pH 7.4) or acidic (pH 1.7) conditions.

Results—Exposure of mucosal sheets to an acidic pH resulted in an immediate and sustained decrease in transmembrane potential difference and calculated tissue resistance. Pepsin or taurocholic acid did not significantly affect bioelectric variables when added to a mucosal bath solution of pH 7.4. A synergistic effect between pepsin or taurocholic acid and mucosal acidification was not detected.

Conclusions and Clinical Relevance—Mucosal acidification with or without pepsin or taurocholic acid resulted in reduced tissue resistance. These data support the contention that squamous erosions or ulcers in horses are mediated, in part, by prolonged exposure of gastric squamous mucosa to luminal acid. (Am J Vet Res 2002;63:744–749).

Ulcers in the stratified squamous portion of the gastric mucosa are a serious and common problem in intensively raised or managed animals, including swine and horses. The incidence of gastric ulcers is reportedly as high as 50% in foals, 84% in yearling horses, and 81% in adult horses.1 Gastric squamous ulcers in racehorses have been associated with substantial costs for medical treatment and loss of days in training for Thoroughbreds.2 Horses used for pleasure riding or show events can also be affected, albeit they are at a slightly lower risk.1

Pathophysiologic mechanisms of squamous gastric ulcers in horses are not fully known, but prolonged exposure to acidic gastric contents is likely to be a central component.1 The importance of acidity in the development and propagation of squamous ulcers is supported by the clinical and endoscopic improvement observed with the use of acid inhibitors, including H2-receptor antagonists and proton-pump blocking agents.5

In vivo and in vitro techniques have been used to investigate the effect of acidic pH on squamous mucosa harvested from the stomach of pigs and the esophagus of rabbits, dogs, and cats.6-10 Electrochemical changes, which correlated with the amount of histologically characterized damage, have been reported in pars esophageal mucosa of pigs and esophageal mucosa of rabbits after bath solutions were acidified to pH 1.5 and 1.6, respectively. Exposure of pars esophageal mucosa of pigs to bath solutions at a slightly higher pH of 2.5 resulted in substantial electrochemical changes but did not affect histologic changes.12 There are a number of factors implicated as mediators in this pathologic response, including pH of the bath solution, duration of exposure, or addition of injurious agents. In addition to acid, conjugated and unconjugated bile salts, pepsin, trypsin, and lysolecithin have been incriminated as synergistic factors in development of squamous ulcers.13,14 Several authors have suggested that pepsin or bile salts are important contributing factors to gastroesophageal reflux disease in humans.13-15 Pepsin potentiates the ulcerogenic action of hydrochloric acid in the esophagus of rabbits13,16 and is a component of a highly reproducible model of reflux esophagitis in rabbits.17 Bile salts, specifically glycine and taurine conjugates, also may contribute to erosions or ulcers16 and are increased in gastric fluid collected from human patients with active ulcer disease.18 Berschneider et al19 observed that bile salts in conjunction with an acidic pH (ie, pH 2.5) were substantially more damaging to equine gastric squamous mucosa than acidic pH or bile salts alone.

In horses, the stomach is lined dorsally with stratified squamous epithelium and ventrally with glandular acid-secreting mucosa. Prevalence of ulcers in the nonglandular squamous epithelium of the stomach in horses suggests that the protective mechanisms for this tissue are totally lacking or easily overwhelmed. Injury of this nature, and to this tissue type, is similar to that of gastroesophageal reflux disease in humans in which exposure to acid is considered to be the most important factor.18 Equine gastric squamous mucosa histologically resembles mammalian esophageal mucosa. It is stratified epithelium composed of a basal germinal layer (ie, stratum basale), stratum spinosum, stratum granulosum, and stratum corneum. The outermost layer usually is keratinized in the esophagus and variably keratinized in the stomach, depending on age and diet of the horse.20 Squamous epithelium of the fundic region of the stomach directly abuts the secre-
tory simple columnar epithelium of the cardiac gland region at the margo plicatus. Ulcers, hyperkeratosis, and erosions are frequently located at this junctional region, particularly on the lesser curvature of the stomach, because this anatomic location is likely -exposed to prolonged periods of acidity2,22 as well as duodenal contents that reflux through the pyloric sphincter (enterogastric reflux).22,23

The specific objectives of the study reported here were to determine the bioelectric properties of short-circuit current (Isc), potential difference (PD), and resistance (R) in isolated equine gastric squamous mucosa and to characterize specific responses to modulators of ion transport (ie, barium chloride, acetazolamide, amiloride, bumetanide, and ouabain). We also examined the effects of luminal acid on these bioelectric properties and evaluated the effect on those properties attributable to pepsin or a conjugated bile salt (taurocholate) in neutral or acidic conditions.

Materials and Methods

Collection of tissues—Tissue specimens were obtained from 16 adult horses (≥ 1 year old) that were euthanatized for reasons unrelated to disease of the gastrointestinal tract. None of the horses had received any medication during the 24-hour period preceding euthanasia. Immediately after IV injection of an overdose of barbiturate, the stomach was extended along the greater curvature. Gastric mucosa was removed from each horse and incised through the cardia and injection of an overdose of barbiturate, the stomach was extended along the greater curvature. Gastric mucosa was removed from each horse and incised through the cardia and

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Tissues were pinned with the mucosal side facing down in a paraffin dissecting tray and continuously bathed with Ringer's solution. Squamous mucosa was removed from muscle layers and submucosa by sharp dissection, rinsed in Ringer's solution, and mounted in modified Ussing chambers with an aperture of 1.327 cm². Tissues were bathed with 10 ml of Ringer's solution on the mucosal and serosal surfaces, and a gas mixture (95% O₂ and 5% CO₂) was continuously bubbled through the solution, using a circulating bubble lift. The water-jacketed reservoir was maintained at 37°C. Tissues were allowed to stabilize for 30 minutes in normal bicarbonate-buffered Ringer's solution (140 mM Na⁺, 6 mM K⁺, 3 mM Ca²⁺, 0.7 mM Mg²⁺, 140 mM Cl⁻, 1.3 mM HPO₄²⁻, 0.3 mM H₂PO₄⁻, 20 mM HCO₃⁻, and 10 mM glucose [pH 7.4]) before experimental manipulations.

Bath solutions for mucosal and serosal tissues were connected to calomel cell and Ag-AgCl electrodes through Ringer-agar bridges, and PD and Isc were measured, using an automatic voltage clamp.24 Tissue R was calculated from values of open-circuit PD and the ISC, using Ohm's Law.24 Tissues were short-circuited for the duration of the experiment, except for periods of 3 to 5 seconds every 30 minutes to enable us to record the open-circuit PD. The pH of bath solutions was measured at the conclusion of each experiment to confirm stability.

Liquid junction potentials were determined, by use of methods described elsewhere.24,25 These potentials result when dissimilar electrolyte concentrations are used in the bath solutions. Briefly, solutions of normal bicarbonate-buffered Ringer's solution (pH 7.4) were placed in separate glass containers and joined by a Ringer-agar bridge. Each container also was connected to a saturated KCl solution by a separate Ringer-agar bridge. The 2 containers of KCl solution were connected to a voltage clamp for measurement of PD by calomel cell electrodes. This system was balanced and zeroed to generate a junctional PD of 0.0. Use of Ringer's solution of differing pH (ie, 7.4 and 1.7; n = 4) resulted in a mean junctional potential difference of 5.325. This value was used to adjust all measured and calculated variables.

Characterization of tissues—Techniques used to examine basal secretion and absorption of tissues included the addition of mucosal amiloride (10 µl of 10⁻⁵M; final concentration, 10⁻⁵M), serosal bumetanide (20 µl of 10⁻⁵M; final concentration, 2 × 10⁻⁵M), serosal or mucosal ouabain (10 µl of 5 × 10⁻⁵M; final concentration, 5 × 10⁻⁵M), and replacement of bicarbonate with N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) in conjunction with serosal acetazolamide (50 µl of 10⁻⁵M; final concentration, 5 × 10⁻⁵M). The effect of serosal barium chloride (500 µl of 10⁻⁵M; final concentration, 5 × 10⁻⁵M) also was evaluated at pH 7.4 and pH 1.7.

Treatments—After a 30-minute equilibration period in normal Ringer's solution, bath solutions were adjusted to the following experimental conditions: not adjusted (Ringer's solution at pH 7.4); acidified Ringer's solution, adjusted to pH 1.7 by the addition of HCl; Ringer's solution at pH 7.4 with 1 mg of pepsin/ml; Ringer's solution at pH 1.7 with 1 mg of pepsin/ml; Ringer's solution at pH 7.4 with 2.5 mM sodium taurocholate; and Ringer's solution at pH 1.7 with 2.5 mM sodium taurocholate. The PD and Isc were recorded every 30 minutes during a period of 150 minutes, after which all mucosal bathing solutions were replaced with Ringer's solution (pH 7.4), and the tissues were monitored for an additional 60 minutes.

Statistical analysis—Data were analyzed by use of an ANOVA for repeated measures with time as the repeated measure.26 Significance was designated at values of P < 0.05. Results were expressed as mean ± SEM.

Results

Results for untreated tissues—Mean ± SEM PD was 19.75 ± 1.5 mV and varied little with time in tissues maintained at pH 7.4. Mean Isc tended to decrease slowly throughout the experiment from an initial value of –11.68 ± 1.3 µA/cm² to a value of –7.45 ± 0.78 µA/cm² at 150 minutes. Consequently, calculated value for tissue R increased over the same period from an initial value of 1.798 ± 161 to 2.847 ± 312 Ω/cm².

Effects of ion transport modulators—Bioelectric variables were not significantly affected by the mucosal addition of amiloride, a transmembrane Na⁺-channel inhibitor, the serosal addition of bumetanide, a Na⁺-K⁺-2Cl⁻-transporter inhibitor, the mucosal addition of ouabain, a Na⁺-K⁺-ATPase inhibitor, or replacement of bicarbonate with HEPES and the addition of mucosal acetazolamide, a carbonic anhydrase inhibitor. Addition of ouabain to the serosal bath solution reduced mean PD from 15.77 ± 2.4 to 10.57 ± 1.63 mV and reduced mean Isc from –7.86 ± 1.3 to –4.95 ± 0.76 µA/cm²; however, it did not have a significant effect on calculated R. Addition of barium chloride to the serosal bath solution but not the mucosal bath solution resulted in a sudden and sustained large decrease in PD (17.1 ± 3.8 to 9.28 ± 2.1 mV; Fig 1) and Isc (–13.6 ± 3.0 to –5.09 ± 1.1 µA/cm²; Fig 2). There was not a significant change
Effect of acidification—Acidification of bath solutions resulted in a decrease in PD (21.5 ± 2.2 to 0.66 ± 1.5 mV; Fig 3) and ISC (–11.8 ± 2.1 to +4.4 ± 5.0 µA/cm²; Fig 4) during the period of acid exposure. A gradual but continuous decrease in PD was detected throughout the acid-exposure portion of the experiment. Following removal of acid, mean PD increased to 11.6 ± 2.0 mV, a value significantly less than the value obtained for the tissues before exposure to acid and the value for control tissues (pH 7.4) at 180 minutes (18.7 ± 1.2 mV; Table 1). After the acidic bath solution was replaced with Ringer’s solution (pH 7.4), the ISC (–7.6 ± 0.9 µA/cm²) recovered to approximately the same magnitude as for control tissues (–7.3 ± 0.9 µA/cm²). Tissue R declined steeply during the initial 30 minutes of acid exposure (from a starting value of 2,014 ± 173 to 1,675 ± 179 Ω/cm²), then decreased slowly over the remaining period to achieve a final value of 1,026 ± 179 Ω/cm². After the acidic bath solution was replaced with Ringer’s solution (pH 7.4), the R of

Table 1—Effect of acidic conditions with or without pepsin or bile salt in bath solutions on mean ± SEM transmembrane potential difference (PD) for equine gastric mucosa

<table>
<thead>
<tr>
<th>pH</th>
<th>Before treatment</th>
<th>During treatment</th>
<th>After treatment</th>
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<tr>
<td></td>
<td>t=0 min</td>
<td>t=150 min</td>
<td>t=30 min</td>
</tr>
<tr>
<td>7.4</td>
<td>19.75 ± 1.10</td>
<td>19.84 ± 1.50</td>
<td>19.73 ± 1.20</td>
</tr>
<tr>
<td>1.7</td>
<td>21.5 ± 2.19</td>
<td>21.47 ± 1.95</td>
<td>21.51 ± 1.98</td>
</tr>
<tr>
<td>7.4 + pepsin*</td>
<td>20.45 ± 2.41</td>
<td>20.19 ± 1.13</td>
<td>20.68 ± 1.58</td>
</tr>
<tr>
<td>1.7 + pepsin*</td>
<td>18.75 ± 1.74</td>
<td>18.08 ± 2.67</td>
<td>18.66 ± 2.71</td>
</tr>
<tr>
<td>7.4 + bile salt†</td>
<td>20.46 ± 3.02</td>
<td>19.98 ± 1.51</td>
<td>20.13 ± 1.22</td>
</tr>
<tr>
<td>1.7 + bile salt†</td>
<td>19.98 ± 1.51</td>
<td>19.38 ± 1.27</td>
<td>19.13 ± 1.22</td>
</tr>
</tbody>
</table>

*Addition of 1 mg of pepsin/ml. †Addition of 2.5 mM taurocholic acid. *Within a row, values with different superscript letters differ significantly (P < 0.05).
Effect of acidification and pepsin—A bath solution of pH 1.7 with the addition of pepsin (1 mg/ml) resulted in a decrease of PD from an initial value of 18.75 ± 1.74 to 0.19 ± 1.13 mV after 150 minutes of exposure (Table 1). Mean Isc decreased from –9.98 ± 1.49 to –0.32 ± 1.46 µA/cm² with variable response between individual tissues. Calculated R decreased from 2,130 ± 264 to 1,139 ± 317 Ω·cm² at 150 minutes after initiation of acid and pepsin exposure, but it rebounded after removal of acid (1,587 ± 141 Ω·cm² after 30 minutes of recovery; Table 2).

Effect of acidification and taurocholate—Mean PD decreased from 20.0 ± 1.5 to –0.38 ± 1.3 mV at 150 minutes and failed to return to values similar to those of the control tissues after 30 minutes of recovery at pH 7.4 (Table 1). Initial mean Isc was –11.87 ± 2.64 µA/cm², which steadily decreased to +1.09 ± 2.64 µA/cm² after 150 minutes of acid exposure. Calculated R also decreased from an initial value of 2,033 ± 333 to 1,023 ± 348 Ω·cm² after 150 minutes of acid exposure. This value then increased negligibly after replacement of the bath solution with Ringer’s solution (pH 7.4; Table 2).

Effect of pepsin—We did not detect significant differences between tissues maintained at pH 7.4 with pepsin and those in bath solutions at pH 7.4 without pepsin.

Effect of taurocholate—We did not detect significant differences between tissues maintained at pH 7.4 with taurocholate and those in bath solutions at pH 7.4 without taurocholate.

Effect of acidification and barium chloride—The magnitude and rate of decrease of PD (Fig 1) and Isc (Fig 2) after acidification were greater in serosal tissues when the tissues had received prior treatment with barium chloride.

Discussion

Resting bioelectric properties of untreated equine gastric squamous epithelium reported here are comparable to those of esophageal squamous epithelium from rabbits and pars esophageal squamous mucosa from pigs. High inherent electrical resistance is a characteristic of barrier epithelia, as is low resting Isc, which is indicative of a tissue with minimal active transport properties. Measurement of PD and Isc in epithelial tissues, using the Ussing chamber system, enables investigators to identify ion transport or electrical regulatory properties of these tissues. Potential difference is a measurement of charge moving from a higher to a lower potential, usually across a membrane, and is expressed in units of volts. The membrane potential exists as a result of active or passive flow of ions (eg, sodium, potassium, calcium, chloride) across or through a membrane barrier. Short-circuit current is the measurement of the current necessary to be introduced into the system that will reduce that potential difference to zero.

Active sodium absorption across a mucosal surface has been documented in the squamous epithelia of the esophagus in rabbits, stomach in pigs, and forestomach in ruminants. Activity of ATPase has been determined in the stratum basale of equine gastric squamous mucosa by use of histochemical techniques, providing indirect evidence of active sodium transport. Using limited methods, we observed a decrease in Isc and PD after the addition of ouabain (an inhibitor of the Na⁺-K⁺-ATPase pump) to the serosal bath solution, but we did not observe an appreciable response to addition of the sodium channel blocker amiloride to the mucosal bath solution. It is possible that the low dose of amiloride had minimal contact with the most metabolically active cells in the stratum basale when added to the bath solution. The addition of bumetamide to the serosal bath solution to block the Na⁺-2Cl⁻-K⁺ cotransporter also did not have an effect on measurable bioelectric properties, nor did addition of HEPES in place of bicarbonate in the serosal bath solution. An electroneutral paired basolateral Na⁺-H⁺ exchanger has been reported in the squamous epithelium of the esophagus of rabbits. This exchanger is important in the maintenance of intracellular pH, and up-regulation of the exchanger through repeated exposure to acid may mediate a hyperproliferative response of esophageal squamous epithelium in humans, predisposing them to a condition known as Barrett’s esophagus. Equine gastric squamous mucosa may contain the basolateral Na⁺-H⁺ exchanger, but because of its electroneutral nature, we were unable to investigate it using the techniques available to us.

The addition of barium chloride to the basolateral or serosal side of the tissue resulted in a sudden decrease in PD and Isc, indicating barium-sensitive basolateral potassium channels, similar to those described in esophageal mucosa of rabbits. Potassium channels are important for active sodium absorption across the apical surface of the epithelium, maintenance of cell volume, and control of intracellular pH.

Acidification of the luminal bathing solution to a pH of 1.7 after treatment of the serosal bath solution with barium chloride resulted in greater and more rapid decreases in PD and R than for acid exposure alone. It is likely that blockade of the basolateral potassium channel, accomplished by disturbing a homeostatic mechanism responsible for regulation of the intracellular environment, made the cells more vulnerable to the adverse effects of low extracellular pH.

Squamous mucosa of the stomach or esophagus is

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Table 2—Effect of acidic conditions with or without pepsin or bile salt in bath solutions on mean ± SEM calculated tissue resistance (R) for equine gastric mucosa

<table>
<thead>
<tr>
<th>pH</th>
<th>Before treatment</th>
<th>During treatment</th>
<th>After treatment</th>
</tr>
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<tbody>
<tr>
<td>pH 7.4</td>
<td>1,798 ± 161</td>
<td>2,847 ± 312</td>
<td>2,789 ± 364</td>
</tr>
<tr>
<td>pH 1.7</td>
<td>2,014 ± 170</td>
<td>1,026 ± 179</td>
<td>1,610 ± 212</td>
</tr>
<tr>
<td>pH 1.7 + pepsin</td>
<td>1,813 ± 131</td>
<td>2,985 ± 244</td>
<td>2,832 ± 302</td>
</tr>
<tr>
<td>pH 7.4 + bile salt</td>
<td>2,130 ± 264</td>
<td>1,135 ± 316</td>
<td>1,588 ± 141</td>
</tr>
<tr>
<td>pH 7.4 + bile salt</td>
<td>1,544 ± 202</td>
<td>2,579 ± 433</td>
<td>2,212 ± 520</td>
</tr>
<tr>
<td>pH 1.7 + bile salt</td>
<td>2,033 ± 334</td>
<td>1,023 ± 348</td>
<td>1,363 ± 219</td>
</tr>
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</table>

See Table 1 for key.
inherently resistant to luminal acid, primarily because of the thickness of the stratum corneum combined with its high electrical resistance and tight epithelial junctions. Cellular ion-transport properties also are likely to be important in controlling intracellular pH, which was documented by treatment of the serosal bath solution with barium chloride prior to acidification of the bath solution. Prolonged exposure to a bath solution with a pH of 1.7 had a damaging effect on gastric squamous epithelium, as measured by PD and calculated R. There was a sudden decrease in values for measured variables followed by a more sustained decrease during the period of acid exposure. After removal of acid, tissues had reduced R, compared with values for control mucosal tissues, indicating sustained damage. The effect of mucosal acidification on equine gastric squamous mucosa was greater than that reported by others; however, the site of tissue collection was not described in detail in that report and may have differed from the site selected for our study. Notably, the principal difference between these 2 studies was the pH of the bath solution; we used a solution with pH 1.7, whereas Berschneider et al used a solution with pH 2.5. Both are within the range of physiologic pH described for equine gastric fluid.24

Acid-induced bioelectric changes consistent with tissue damage were not induced in porcine squamous mucosa until mucosal pH was < 1.5.16 Esophageal mucosa of rabbits was resistant to mucosal acidification at pH 3.0 but damaged when pH was lowered to 1.5.17 The proposed mechanism for acid-induced injury of squamous mucosa involves an initial increase in mucosal permeability (paracellularly or directly through the cell) followed by inhibition of active sodium transport and loss of osmolar regulatory abilities. Swelling of epithelial cells leads to vesicle formation and sloughing of overlying mucosal layers with exposure of underlying interstitial tissue that is vulnerable to perpetuated acid-induced injury.7

The addition of pepsin or taurocholate to the bath solution maintained at pH 7.4 did not have an effect on any of the measured variables. The concentration of pepsin used in this study was determined on the basis of concentrations in gastric fluid obtained from cannulated horses.1 Pepsinogen, an inactive protease precursor to pepsin, also has been isolated from equine gastric mucosa. Conversion to pepsin is a pH-dependent process, with peak conversion at pH 2.0.45 Proteolytic activity of the enzyme is also greatest when luminal pH is < 3.0. Consequently, changes in mucosal bioelectric properties would not be expected when the enzyme is added to a fluid of pH 7.4. In contrast to studies that used esophageal tissues obtained from rabbits,17 dogs,18 or cats,14 we were unable to document a synergistic effect of pepsin when added to a bath solution of pH 1.7. It is possible that the pH used in our study was too severe for this tissue type to enable us to identify subtle changes induced by pepsin. Alternatively, the concentration of pepsin used may have been inadequate with respect to physiologic conditions.

Entero gastric reflux of small intestinal contents, including bile salts, has been documented in horses.15,46 Total concentration of bile salts ranging from 21 to 77 µmol/L has been reported in gastric fluid from horses from which food has been withheld.4 In another study,50 bile salt concentrations < 200 µmol/L were reported in fed horses, with mean concentrations between 225 and 440 µmol/L in horses from which food was withheld. Taurocholate, a taurine conjugate of cholate, is hepatically synthesized and secreted into bile.35 Conjugated bile salts have a lower pH than unconjugated salts. The pKₐ of conjugated taurocholate and taurodeoxycholate is 1.9, whereas the pKₐ of unconjugated cholate is 3, and the pKₐ of unconjugated deoxycholate is 3.3.45 At a pH near its pKₐ, approximately 50% of a weak acid will be ionized and soluble, and as the pH decreases below the pKₐ (such as in the gastric environment), more of the compound becomes nonionized, insoluble, and lipophilic, potentially allowing entry into epithelial cells. Therefore, when taurocholate is added to a solution with high pH, it becomes predominately ionized and not easily absorbed,44 and similar to the situation for pepsin, an effect at pH 7.4 was not anticipated nor observed. Based on its pKₐ and on limited data from horses and other species, we predicted a synergistic effect of taurocholate in a bath solution with low pH.20,25 Unfortunately, as stated previously, the low pH selected in the study reported here may have overshadowed any effect of the bile acid.

Acidic pH has a substantial in vitro corrosive effect on gastric squamous mucosa of horses. Prolonged exposure of mucosa to acidic luminal contents may be a critical factor in the development of squamous erosions and ulcers in horses.


