Effects of hydrochloric, acetic, butyric, and propionic acids on pathogenesis of ulcers in the nonglandular portion of the stomach of horses

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Objective—To identify the pathogenesis of gastric ulcers by comparing injury to the nonglandular gastric mucosa of horses caused by hydrochloric acid (HCl) or volatile fatty acids (VFAs).

Sample Population—Gastric tissues from 30 horses.

Procedure—Nonglandular gastric mucosa was studied by use of Ussing chambers. Short-circuit current (Isc) and potential difference were measured and electrical resistance calculated for tissues after addition of HCl and VFAs to normal Ringer's solution (NRS). Tissues were examined histologically.

Results—Mucosa exposed to HCl in NRS (pH, 1.5) had a significant decrease in Isc, compared with Isc for mucosa exposed to NRS at pH 4.0 or 7.0. Also, exposure to 60mM acetic, propionic, and butyric acids (pH, 4.0 or 1.5) caused an immediate significant decrease in Isc. Recovery of sodium transport was detected only in samples exposed to acetic acid at pH 4.0. Recovery of sodium transport was not seen in other mucosal samples exposed to VFAs at pH ≤ 4.0.

Conclusions and Clinical Relevance—Acetic, butyric, and propionic acids and, to a lesser extent, HCl caused decreases in mucosal barrier function of the nonglandular portion of the equine stomach. Because of their lipid solubility at pH ≤ 4.0, undissociated VFAs penetrate cells in the nonglandular gastric mucosa, which causes acidification of cellular contents, inhibition of sodium transport, and cellular swelling. Results indicate that HCl alone or in combination with VFAs at gastric pH ≤ 4.0 may be important in the pathogenesis of gastric ulcers in the nonglandular portion of the stomach of horses. (*Am J Vet Res* 2003;64:404–412)

Gastric ulcers are common and represent a major health problem in horses.[1,2] The proximal third of the equine stomach is lined with nonglandular stratified squamous epithelium, and gastric ulcers are usually found in this region. The cause of these ulcers is not known but may be related to a lack of adequate barrier defenses, such as mucus and bicarbonate, which make this region susceptible to acid injury.

Hydrochloric acid, produced by the glandular mucosa and **volatile fatty acids (VFAs)**, which are by-products of carbohydrate fermentation by gastric bacteria, can cause acid injury and ulcers in the nonglandular (gastroesophageal) mucosa of pigs.[3] Using the Ussing chamber system, hydrochloric and acetic acids at a low pH (< 2.5) were found to induce irreversible acid injury to the nonglandular mucosa in vitro.[3] Also, exposer of nonglandular mucosa to 60mM acetic acid at pH ≤ 4.5 abruptly and irreversibly abolishes **short-circuit current (Isc)**, an indicator of tissue sodium transport and barrier function. This loss of sodium transport is followed by a loss of tissue electrical resistance and histopathologic changes of cellular swelling and vesicle formation in middle layers, sloughing of the outer barrier, erosion into the deeper mucosa, and ulcers. The authors of that study[3] postulated that undissociated acetic acid, because of its high lipid solubility at low pH, penetrates the outer mucosa and acidified underlying mucosal cells, resulting in loss of cellular sodium transport, which is followed by cellular swelling, necrosis, and ulcers.

Several VFAs (eg, acetic, butyric, and propionic acids) have been measured in stomach contents of horses fed hay or hay and grain diets.[4,5] Acetic acid was found in highest concentration, followed by butyric and propionic acids. Furthermore, a study[4] conducted by our laboratory group revealed that pH of stomach contents and concentrations of VFAs are important factors contributing to severity of gastric ulcers in horses. In that study, horses fed diets consisting of alfalfa hay and grain or bromegrass hay developed gastric ulcers. Use of a stepwise model constructed to determine factors important in the severity of gastric ulcers revealed that horses with ulcers had a lower gastric pH and higher concentrations of butyric and propionic acids than horses without ulcers.[4]

The purpose of the study reported here was to

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determine effects of pH and several VFAs (ie, acetic, butyric, and propionic acids) on sodium transport and tissue resistance across the nonglandular stratified squamous mucosa of the equine stomach. Our objective was to elucidate their role in the pathogenesis of gastric ulcers in horses.

Materials and Methods

Sample population—Gastric tissues obtained from 30 adult horses (12 geldings, 5 stallions, and 13 mares) that were euthanatized because of debilitation or donation were used in the study. Information regarding sex, breed, and age of each horse was obtained.

Tissue acquisition—Horses were euthanatized by lethal injection of an overdose of barbiturate.[a] Within 1 hour after horses were euthanatized, the stomach of each horse was removed and dissected along the greater curvature to expose the nonglandular mucosa. Two 50-mL conical vials were filled with gastric contents and immediately placed in a freezer at –70°C for subsequent analysis of VFA concentrations. The entire stomach, including nonglandular mucosa, was cleaned of ingesta by use of deionized water and **normal Ringer's solution**[3] (**NRS**; 142mM sodium, 124mM chloride, 25mM bicarbonate, 10mM glucose, 5mM potassium, 1.65mM HPO_4^-, 1.25mM calcium, 1.1mM magnesium, and 0.3mM H_2PO_4 [pH, 7.4]) that was made in our laboratory. The mucosa was examined for gastric ulcers; ulcers were assigned a grade on the basis of a scoring system for gastric ulcers in horses.[6] Nonglandular mucosa was obtained by dissecting the submucosa from the underlying muscular tissue. Specimens were cut into disks (approx 3.5 cm^2). A sample of nonglandular mucosa from each horse was immediately placed in neutral-buffered 10% formalin solution and subsequently used for histologic examination.

Study design—Samples of gastric mucosa were manipulated in Ussing chambers, as described in another study.[3] Nonglandular mucosa was pinned, mucosal side down, to a paraffin tray and bathed in oxygenated NRS maintained at 20 to 22°C (ie, room temperature). Each Ussing chamber had an aperture area of 7.07 cm^2. Tissues were bathed in NRS on the mucosal and submucosal surfaces. The NRS was oxygenated by circulating the solutions in water-jacket reservoirs with a gas lift and by use of 95% $O_2$and 5% CO_2.

Duration of each experiment was 255 minutes. Tissues were allowed to equilibrate in the NRS tissue baths for 15 minutes. Mucosal surface of tissues then were perfused for 180 minutes with 10 mL of NRS (control solution) or 10 mL of NRS to which a VFA had been added and that were adjusted to attain a specific pH (1.5, 4.0, or 7.0); the submucosal surface of tissues was bathed with 10 mL of NRS at pH 7.0. Luminal reservoirs were then drained and refilled with NRS (pH, 7.0), and values were recorded for an additional 60 minutes. This sequence was used for all of the experiments in which tissues were exposed to VFAs and then allowed to recover.

When acetic, butyric, or propionic acids were added to the bath solution, the sodium salt of each VFA was substituted for an equivalent amount of sodium chloride in the NRS so that the final concentration was 60mM of each VFA in NRS resulting in **acetate Ringer's solution (ARS)**, **butyrate Ringer's solution (BRS)**, and **propionic Ringer's solution (PRS)**. The pH (7.0, 4.0, or 1.5) of the solution used to bathe the mucosal surface was adjusted by titration with 1.0 N hydrochloric acid while measuring with a pH electrode.[b] These values for pH were chosen because they are commonly found in the stomach of horses, and VFAs are fully dissociated (ionized) and not lipophilic at pH 7.0, partially dissociated and partially lipophilic at pH 4.0, and undissociated (nonionic) and highly lipophilic at pH 1.5. For each tissue sample in NRS (control solution) and VFA solution, chambers were set up with pH 1.5, 4.0, or 7.0 on the mucosal side for a total of 3 chambers for VFA and 3 chambers for NRS in each experiment.

Because dissimilar solutions were used in the mucosal and submucosa baths, liquid junction potentials (caused by net diffusion of sodium ions from solution to bridge to electrode, resulting in PD changes) were evident for electrical measurements. To determine their magnitude and orientation, external circuits were created by use of 2 experimental solutions as described elsewhere.[7]

Spontaneous **potential difference (PD)** was measured by use of Ringer's agar bridges, the composition of which was identical to that of the submucosal solution used to bathe the tissues. The PD was nullified by use of an automatic voltage clamp through Ag-AgCl electrodes. Tissue resistance was calculated from the open-circuit PD and from the current necessary to nullify the PD (ie, Isc). Calomel electrodes filled with and bathed in saturated KCl were used to record the values for PD and Isc. Tissue PD and Isc were recorded every 15 minutes throughout the 255-minute duration of each experiment. The pH of each bath solution was measured hourly to verify stability.

Tissue PD and Isc were then used to calculate **corrected Isc (cIsc**; corrected for junction potentials and tissue area), percentage of control Isc, and resistance. All calculations were based on Ohm's law (ie, voltage = current × resistance). Conductance was calculated as follows:

$$\text{conductance} = (\text{Isc}/7.07\ \text{cm}^2\ [\text{ie, area of the chamber}])/\text{PD}$$

Resistance was calculated as follows:

$$\text{resistance} = (1/\text{conductance}) \times 1{,}000$$

Corrected Isc was calculated as follows:

$$\text{cIsc} = \text{Isc}/7.07\ \text{cm}^2$$

Percentage of control Isc was calculated as follows:

$$\text{percentage of control Isc for each VFA} = (\text{cIsc for ARS, BRS, or PRS}/\text{cIsc for NRS}) \times 100$$

Immediately after the stomach was incised, a sample of nonglandular mucosa was collected from the margo plicatus and placed in neutral-buffered 10% formalin. This tissue was used to determine whether the nonglandular mucosa was histologically normal prior to VFA or control treatment. After bathing in the Ussing chambers, mucosa was cut into 2 pieces; 1 equal was placed in neutral-buffered 10% formalin, and the other piece was placed in NRS and frozen at –70°C for subsequent analysis of VFA concentrations. Fixed tissues were prepared for routine histologic examination and stained with H&E. Slides were examined by use of light microscopy to determine cellular swelling and necrosis.

Analysis of VFA concentrations in tissues and gastric contents—To determine uptake of VFAs into the nonglandular mucosa, the samples of frozen tissue were thawed and analyzed by use of gas chromatography in accordance with the following procedure. Tissue was removed from the NRS, weighed, minced, and placed in 5 mL of deionized water. Samples were then homogenized for 5 minutes by use of a homogenizer.[c] Samples were transferred into 15-mL polypropylene centrifuge tubes and centrifuged at 2,000 × g for 20 minutes. An aliquot (2 mL) of supernatant was placed

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into a 5-mL polypropylene test tube. Another aliquot (1 mL) of supernatant was placed in a 1.5-mL microcentrifuge tube and cooled on ice. A volume (0.2 mL) of an internal standard solution (25% phosphoric acid) was added to each aliquot, and tubes were vortexed at 13,000 rpm[d] for 20 minutes. The supernatant was removed, placed into a gas chromatography vial (approx 1 mL), and clamped with an aluminum vial closure. Vials were frozen at –70°C for subsequent analysis. To determine whether mucosal samples were absorbing the VFAs, tissue concentrations of each VFA (ie, acetic, butyric, and propionic acids) were measured in the homogenized tissue by use of the gas chromatography method described in 1 study[8] and modified in another study.[9]

To determine VFA concentrations in the gastric contents, frozen ingesta collected immediately after horses were euthanatized were thawed, and approximately 4 g of material was centrifuged[e] (1,000 × g for 15 minutes at 4°C). Supernatant (1.5 mL) was collected and added to 300 µL of 25% metaphosphoric acid (consisting of ortho-phosphoric acid[f] diluted in deionized water [5:1]) and incubated at 20 to 22°C for 30 minutes.

To determine VFA concentrations in the tissues or gastric contents, supernatant from each sample was centrifuged to remove precipitate, and 1 µL was injected into a gas chromatograph[g] with a capillary column[h] (10 m × 0.53 mm × 1 µm) packed with cross-linked polyethylene glycol-tris phosphoric acid. The gas chromatograph was equipped with a flame ionization detector, and injector temperature was 200°C, oven temperature was 80°C, and detector temperature was 250°C. The carrier gas was helium at a flow rate of 30 mL/min. Linear velocity was 26 cm/s. Head pressure was 6,894.8 Pascals. Standard solutions of acetic acid, butyric acid, and propionic acid[i] with an internal standard of 2-ethylbutyric acid[j] were used. Retention times were approximately 8.38 minutes for acetic acid, 12.68 minutes for butyric acid, 10.88 minutes for propionic acid, and 16.19 minutes for 2-ethylbutyric acid.

Statistical analysis—Data were analyzed by use of a statistical computer program.[k] The model used for each tissue type and variable was a randomized block design split-plot ANOVA with treatment (solution conditions) in the main plot and time and time-by-treatment interaction in the subplot, with each horse as a block. Time was a repeated-measures factor. Least-squares means and pooled SEM were calculated, and mean separation by use of a least-significant difference test was performed. Values of $P < 0.05$ were considered significant.

Results

The 30 horses ranged from 1 to 30 years of age (mean, 9.0 years), and 16 of 30 (53%) had gastric ulcers in the nonglandular mucosa. Horses had various debilities or reasons for being euthanatized, which included behavior problems (n = 1), melanoma (2), chronic diarrhea (2), pemphigus foliaceous (1), neurologic disease (9), lameness and laminitis (10), old age (1), recurrent uveitis (3), and pituitary adenoma (1). One horse with lameness had been treated by administration of phenylbutazone (2 g, PO, q 24 h, for 3 days) prior to being euthanatized, but other treatments administered prior to arrival at our facility were not recorded. Medical conditions and prior treatments may have altered the responses seen in the tissues of these horses, because we did not control for this factor. However, the study design (use of control samples in NRS at each pH [1.5, 4.0, and 7.0] along with each VFA treatment) was used to account for variations among and within horses.

Ulcer score was determined for 13 of 16 horses with gastric ulcers. Mean ulcer score was 1.9 (scale, 0 to 3). Thirteen tissue samples were collected from grossly normal nonglandular mucosa at or adjacent to the margo plicatus in the stomach of each horse, which is the region of the equine stomach where most ulcers develop. Of the 390 tissue samples collected from this region (30 horses × 13 tissue samples/horse), 30 (1 from each horse) were immediately placed in neutral-buffered 10% formalin for subsequent histologic evalu-

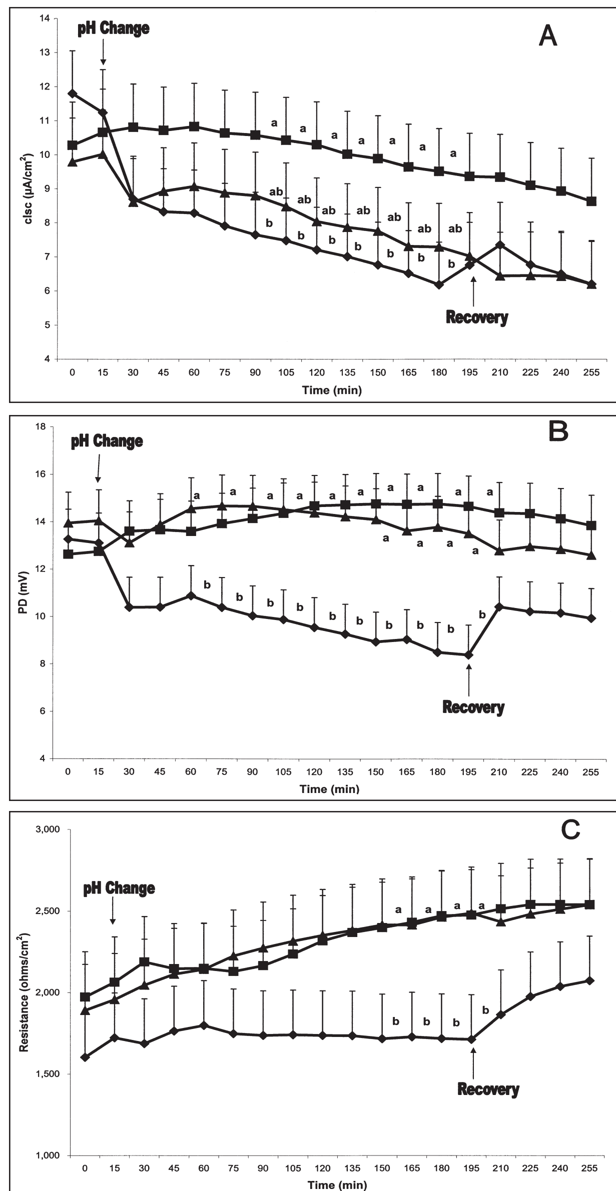

Figure 1—Mean ± SEM values for corrected short-circuit current (clsc; A), tissue potential difference (PD; B), and electrical resistance (C) in samples of nonglandular squamous mucosa collected from the stomach of each of 30 horses. Tissues were placed in Ussing chambers, allowed to equalibrate in normal Ringer's solution (NRS) buffered at pH 7.0 for 15 minutes, and then exposed on their mucosal surface to NRS at pH 1.5 (diamond), 4.0 (triangle), or 7.0 (square) and on their submucosal surface to NRS at pH 7.0. After incubation for 180 minutes, solutions were replaced with NRS at pH 7.0 (ie, recovery), and tissues were incubated for another 60 minutes. [a,b]For each time point, mean values with different superscript letters differ significantly ($P < 0.05$).

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ation. None of these tissue samples had evidence of pathologic changes. Of the remaining 360 samples, 15 were not analyzed because of a malfunction of the Ussing chambers; thus, 345 tissue samples were evaluated from the 30 horses. The variables measured did not differ significantly for tissues from horses with ulcers and tissues from horses without ulcers.

The Isc in tissues perfused with NRS at pH 1.5 decreased significantly by 75 minutes after onset of exposure, whereas the PD across the tissue decreased significantly by 30 minutes after onset of exposure, compared with values at the same time points for tissues exposed to NRS at pH 4.0 or 7.0 (**Fig 1**). On the other hand, tissue resistance was initially lower in tissues exposed to NRS at pH 1.5 and typically remained stable throughout the experimental period, whereas resistance in tissues exposed to NRS at pH 4.0 or 7.0 increased slightly and then also remained stable throughout the remainder of the experimental period. Tissue Isc was also low in the tissues exposed to NRS at pH 4.0, but this value did not differ significantly when compared with the Isc value for tissues exposed at pH 7.0 or 1.5. Tissue Isc decreased approximately 30 and 23% when exposed to NRS at pH 1.5 or 4.0, respectively, compared with Isc values for tissues exposed to NRS at pH 7.0. During the recovery phase of the experiment, Isc, PD, and resistance immediately increased in tissues exposed at pH 1.5 and did not differ significantly from values for tissues incubated at pH 4.0 or 7.0 for the 60-minute recovery period. Analysis of these data suggested that the affect of hydrochloric acid on sodium transport and barrier function in nonglandular mucosa may be reversible.

The Isc in tissues perfused with acetic, propionic, and butyric acids at pH 4.0 or 1.5 decreased significantly within 30 minutes after the pH was decreased in the mucosal solutions and remained significantly decreased throughout the 180-minute exposure period, compared with values for tissues exposed to each of the same VFAs at pH 7.0 (**Fig 2**). Furthermore, measured Isc values for tissues exposed to the VFAs were significantly lower than Isc values for tissues exposed to NRS solution (control solution) at pH 1.5 (**Fig 3**). Tissue PD decreased significantly in tissues exposed to PRS at pH 4.0, compared with PD for tissues exposed to PRS at pH 7.0, and increased significantly in tissues exposed to PRS at pH 1.5 (**Fig 4**). The Isc in tissues exposed to each

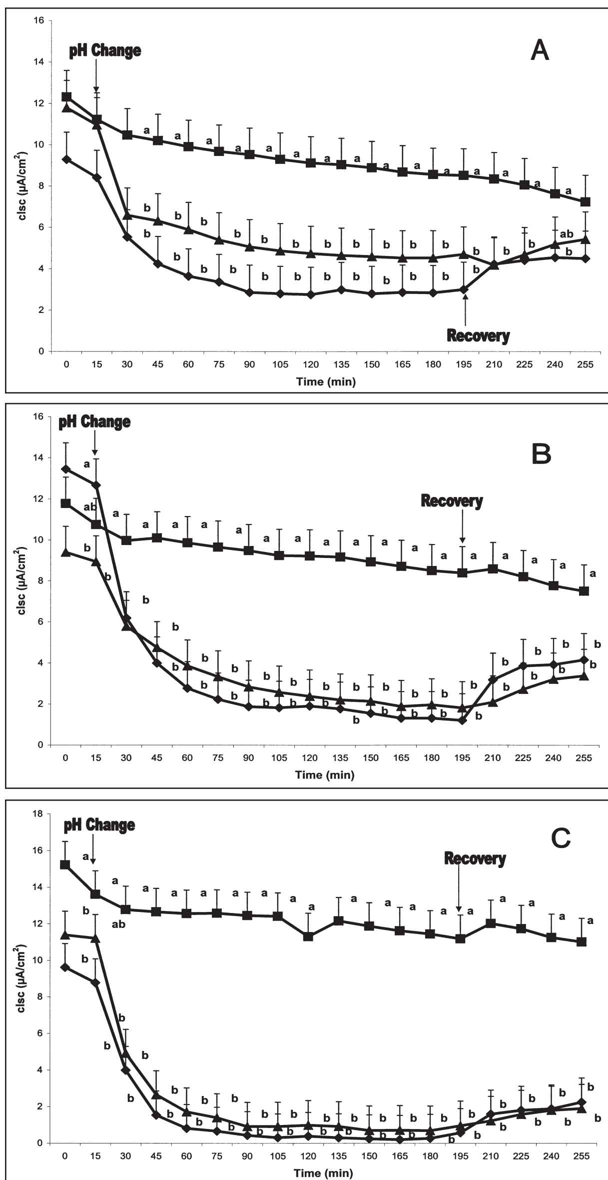

Figure 2—Mean ± SEM values for cIsc in samples of nonglandular squamous mucosa collected from the stomach of each of 30 horses. Tissues were placed in Ussing chambers and exposed on their mucosal surface to 60mM acetate Ringer's solution (ARS; A), 60mM propionate Ringer's solution (PRS; B), or 60mM butyrate Ringer's solution (BRS; C) buffered at pH 7.0. Samples were incubated for 15 minutes, then pH was adjusted by addition of 1.0 N hydrochloric acid, and tissues were incubated at pH 1.5 (diamond), 4.0 (triangle), or 7.0 (square) for another 180 minutes. The submucosal surface was exposed to NRS buffered at pH 7.0 for the duration of the experiment. Solutions were replaced with NRS buffered at pH 7.0 (ie, recovery), and tissues were incubated for another 60 minutes. *See* Figure 1 for remainder of key.

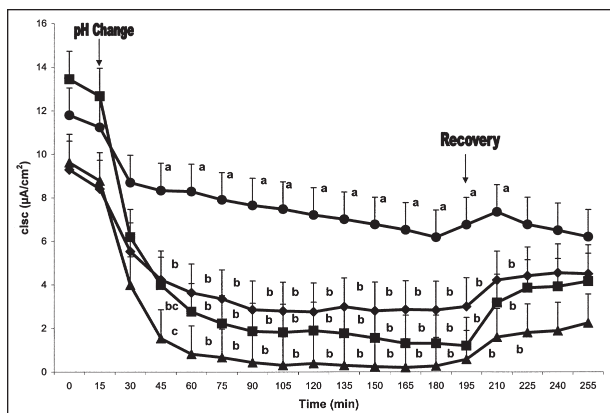

Figure 3—Mean ± SEM values for cIsc in samples of nonglandular squamous mucosa collected from the stomach of each of 30 horses. Tissues were placed in Ussing chambers and exposed on their mucosal surface to NRS (circle), 60mM ARS (diamond), 60mM PRS (square), or 60mM BRS (triangle) at pH 7.0 for 15 minutes. Then, pH was adjusted with 1.0 N hydrochloric acid, and tissues were incubated at pH 1.5 for another 180 minutes. The submucosal surface was exposed to NRS buffered at pH 7.0 for the duration of the experiment. Solutions were replaced with NRS buffered at pH 7.0 (ie, recovery), and tissues were incubated for another 60 minutes. *See* Figure 1 for remainder of key.

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of the VFAs remained significantly different throughout the recovery period at pH 1.5 and 4.0, except for tissues exposed to ARS at pH 4.0, which did not differ significantly during the last 15 minutes of the recovery period. This increase in Isc may indicate recovery in tissues exposed to ARS at pH 4.0.

The Isc was expressed as a percentage of control tissues exposed to NRS at pH 7.0. Compared with Isc values for control tissues exposed to NRS at pH 7.0, Isc decreased 30 and 60% during exposure to ARS at pH 4.0 and 1.5, respectively, decreased 55 and 80% in tissues exposed to PRS at pH 4.0 and 1.5, respectively, and decreased 80 and 100% (ie, completely abolished) in tissues exposed to BRS at pH 4.0 and 1.5, respectively (**Fig 5**). Acetic acid had the least effect, propionic acid had an intermediate effect, and butyric acid had the greatest effect on the Isc and barrier function in nonglandular gastric mucosa.

Tissue resistance significantly increased in tissues exposed to ARS or PRS at pH 4.0, compared with resistance for tissues exposed to ARS or PRS at pH 7.0 and 1.5, whereas resistance was increased significantly for tissues exposed to BRS at pH 4.0 and 1.5, compared with resistance for tissues exposed to BRS at pH 7.0 (**Fig 6**). The increase in tissue resistance was detected within 30 minutes after pH of the solution was decreased, and the increase continued throughout the remainder of the experimental period. Tissue resistance decreased slightly during the recovery period but remained increased in all tissues exposed to the VFAs at pH 1.5, except for a slight increase in resistance for the tissue exposed to PRS at pH 4.0.

Concentrations of VFAs were determined for nonglandular mucosa exposed to each of the bath solutions (**Table 1**). Nonglandular mucosa exposed to ARS had a low tissue concentration of acetic acid, and there was not a significant difference among tissue concentrations of acetic acid for any of the 3 pH values.

Tissues exposed to BRS or PRS contained significantly higher concentrations of butyric and propionic acid at pH 4.0 and 1.5, respectively, compared with those same VFA concentrations for the same tissue at pH 7.0. The highest concentrations of VFAs were found in tissues exposed to BRS at pH 4.0 and 1.5. Mean ± SEM concentration of butyric acid in nonglandular mucosal tissue exposed to BRS was significantly ($P = 0.01$) higher at pH 1.5 (0.26 ± 0.05mM) and 4.0 (0.32 ± 0.05mM), compared with the concentration in tissues exposed to BRS at pH 7.0 (0.12 ± 0.05).

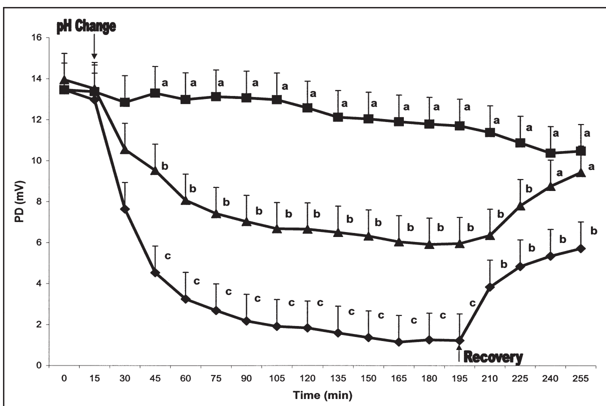
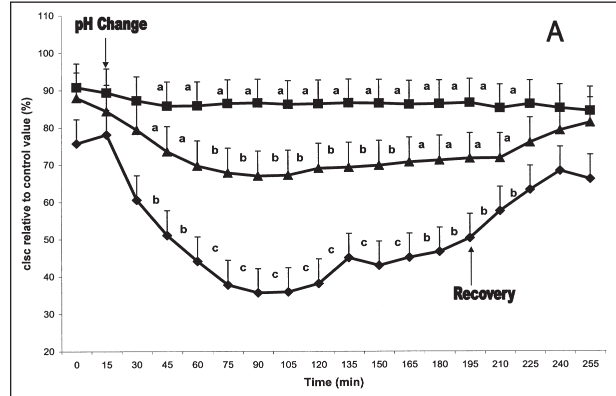
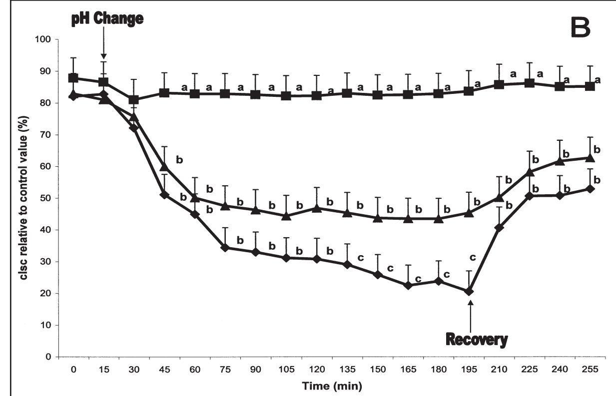


Figure 4—Mean ± SEM values for tissue PD in samples of nonglandular squamous mucosa collected from the stomach of each of 30 horses. Tissues were placed in Ussing chambers and allowed to equalibrate for 15 minutes in 60mM PRS at pH 7.0 for 15 minutes. Then, pH for the PRS that bathed the mucosal surface was adjusted to 1.5 (diamond), 4.0 (triangle), or 7.0 (square); the submucosal surface was bathed in NRS buffered at pH 7.0. Samples were incubated for 180 minutes. Solutions were replaced with NRS buffered at pH 7.0 (ie, recovery), and tissues were incubated for another 60 minutes. *See* Figure 1 for remainder of key.





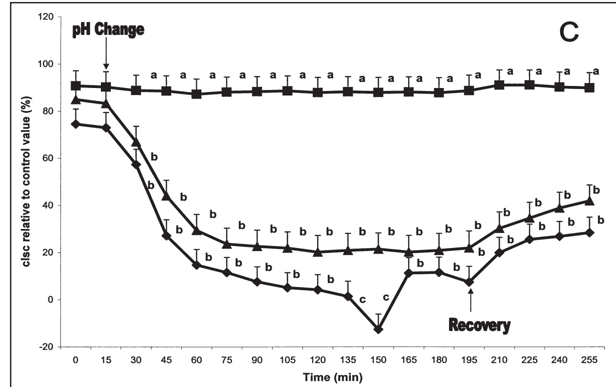


Figure 5—Mean ± SEM values for cIsc expressed as a percentage of the control values in samples of nonglandular squamous mucosa collected from the stomach of each of 30 horses. Tissues were placed in Ussing chambers and exposed on their mucosal surface to 60mM ARS (A), 60mM PRS (B), or 60mM BRS (C) at pH 7.0 for 15 minutes, then pH was adjusted with 1.0 N hydrochloric acid to pH 1.5 (diamond), 4.0 (triangle), or 7.0 (square), and tissue were incubatedexposed for another 180 minutes. The submucosal surface was exposed to NRS buffered at pH 7.0 for the duration of the experiment. Solutions were replaced with NRS buffered at pH 7.0 (ie, recovery), and tissues were incubated for another 60 minutes. [a,b,c]For each time point, mean values with different superscript letters differ significantly ($P < 0.05$).

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Mean ± SEM concentrations of VFAs in the gastric contents were determined. Horses were primarily fed grass hay during the 48 hours immediately prior to being euthanatized. We did not collect additional information about their dietary history. The VFA with the highest concentration in gastric contents of these horseswas acetic acid (16.23 ± 2.77mM), which constituted approximately 88% of the VFAs. Isobutyric (1.09 ± 0.94mM), propionic (0.67 ± 0.28mM), and butyric (0.30 ± 0.08mM) acids were found in lower concentrations in

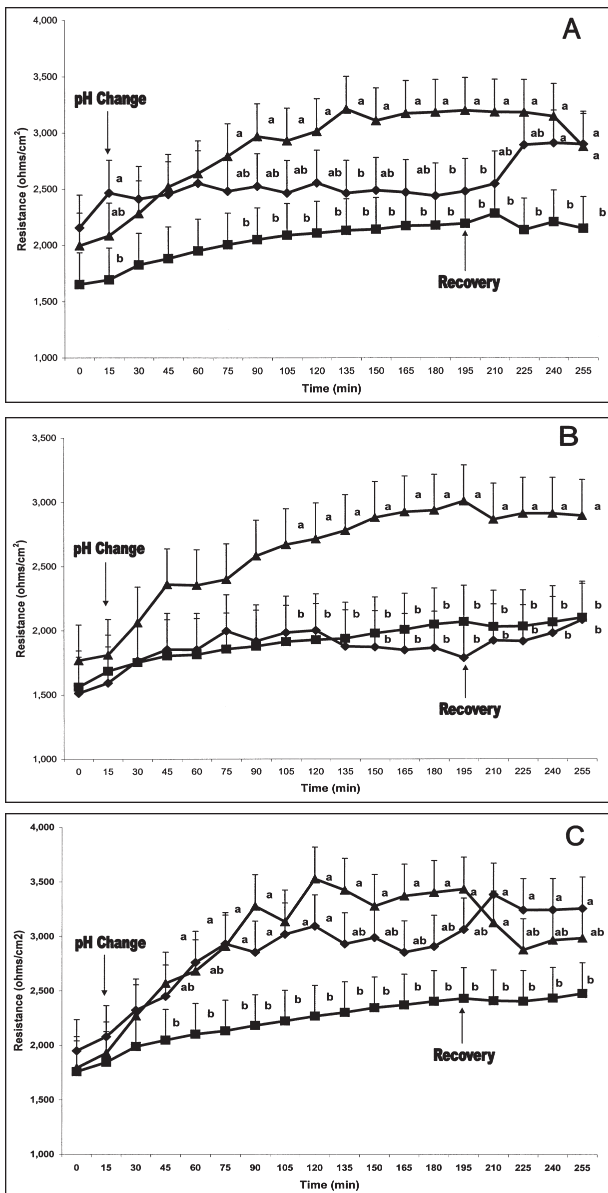

Figure 6—Mean ± SEM values for tissue resistance in samples of nonglandular squamous mucosa collected from the stomach of each of 30 horses. Tissues were placed in Ussing chambers and exposed on their mucosal side to 60mM ARS (A), 60mM PRS (B), or 60mM BRS (C) at pH 7.0 for 15 minutes, Then, pH was adjusted with 1.0 N hydrochloric acid to 1.5 (diamond), 4.0 (triangle), or 7.0 (square), and tissues were inoculated for another 180 minutes. The submucosal surface was exposed to NRS buffered at pH 7.0 for the duration of the experiment. At 195 minutes, solutions were replaced with NRS buffered at pH 7.0 (ie, recovery), and tissues were incubated for another 60 minutes. *See* Figure 1 for remainder of key.

Table 1—Mean ± SEM tissue concentrations of volatile fatty acids after exposure of samples of equine nonglandular squamous mucosal tissue to various solutions at pH 4.0 or 1.5 in an Ussing chamber

Bath solution	Acetic acid (mmol/L)	Butyric acid (mmol/L)	Propionic acid (mmol/L)
NRS (n = 82)	0.32 ± 0.03	0.05 ± 0.06[a]	0.16 ± 0.06[a]
ARS (n = 80)	0.38 ± 0.02	0.04 ± 0.06[a]	0.16 ± 0.06[a]
BRS (n = 80)	0.31 ± 0.03	0.79 ± 0.06[b]	0.27 ± 0.06[a]
PRS (n = 84)	0.33 ± 0.04	0.06 ± 0.06[a]	0.58 ± 0.06[b]

[a,b]Within a column, values with different superscript letters differ significantly ($P = 0.01$).

NRS = Normal Ringer's solution. ARS = 60mM Acetic acid in NRS. BRS = 60mM Butyric acid in NRS. PRS = 60mM Propionic acid in NRS. n = Number of tissue samples.

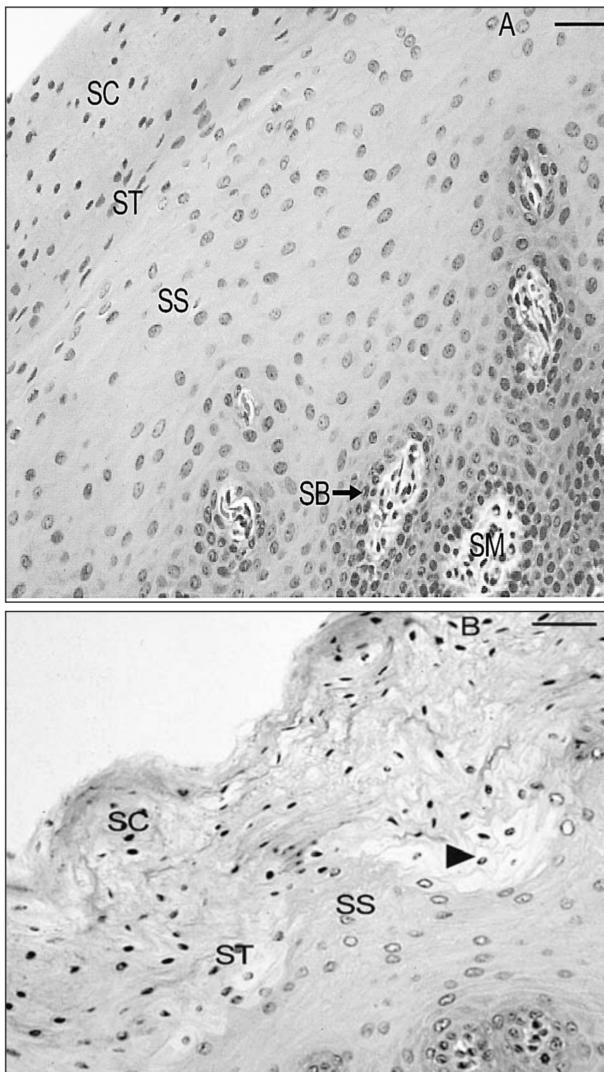

Figure 7—Photomicrograph of tissue sections of equine nonglandular squamous mucosa after incubation for 255 minutes in NRS at pH 7.0 on the mucosal and submucosal surfaces (A) and after incubation for 180 minutes in NRS at pH 1.5 on the mucosal side and pH 7.0 on the submucosal side (B). Notice that the stratum corneum (SC), stratum transitionale (ST), stratum spinosum (SS), stratum basale (SB [arrow]), and submucosa (SM) in the sample incubated at pH 7.0 have a normal appearance, but there is pallor and disruption indicative of cellular swelling in the SC, ST, and superficial SS (arrowhead) in the sample incubated at pH 1.5. H&E stain; bar = 40 µm.

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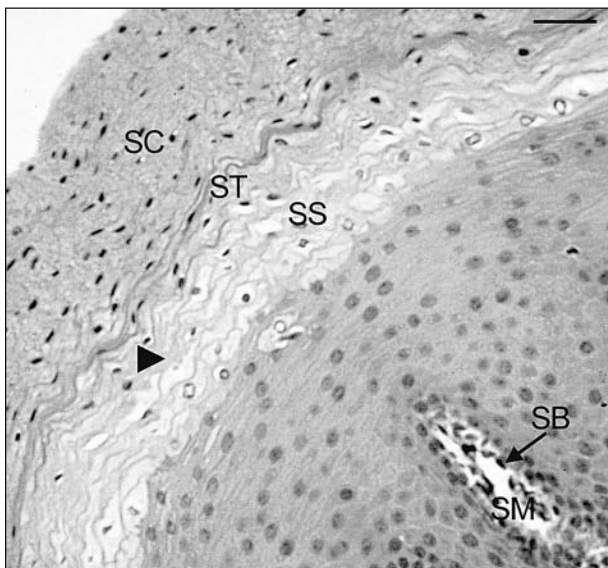

Figure 8—Photomicrograph of tissue sections of equine nonglandular squamous mucosa after incubation for 180 minutes in 60mM BRS at pH 1.5 on the mucosal surface and NRS at pH 7.0 on the submucosal surface. Notice the normal appearance of the SC and the pallor and disruption in the ST and superficial SS. The pallor is indicative of cellular swelling (arrowhead). H&E stain; bar = 40 µm.

the gastric contents. We also detected isovaleric (0.09 ± 0.17mM) and valeric (0.02 ± 0.08) acids in the gastric contents. We did not detect a correlation between VFA concentrations in gastric contents and evidence of gastric ulcers.

Histologic examination of samples of nonglandular mucosa exposed to NRS and the various VFAs at pH 7.0 did not reveal evidence of cellular swelling or necrosis in any of the various tissue layers **stratum corneum [SC]**, **stratum transitionale [ST]**, **stratum spinosum [SS]**, stratum basale, and submucosa; **Fig 7**. Mucosa exposed to NRS at pH 1.5 had cellular swelling and a mottled appearance in the superficial SC and ST, whereas mucosa samples exposed to ARS, BRS, and PRS at pH 1.5 or 4.0 had cellular swelling in the ST and spinosum subjacent to the SC or throughout the SS. Cellular swelling of cells in the SS was most dramatic in samples exposed to BRS at pH 4.0 and 1.5 and less apparent in samples exposed to PRS at pH 4.0 and 1.5 (**Fig 8**).

Discussion

The prevalence of gastric ulcers in adult performance horses reportedly ranges from 60 to 90% and varies with age, breed, and intensity of training.[1,2] The prevalence of gastric ulcers in the horses in the study reported here was 16 of 30 (53%), and mean gastric ulcer score was 1.9 on a scale of 0 to 3. Prevalence and severity of gastric ulcers in this population of older horses (mean age, 9.0 years) was surprising, considering the horses were not in training and not used for performance events. However, most of the horses were euthanatized because of clinical signs related to an underlying medical disease or musculoskeletal lameness. Such clinical disease could have predisposed them to a high prevalence of gastric ulcers, because the incidence of gastric ulcers is reportedly high in horses with clinical signs of disease.[10] Also, clinical disease and prior treatment of horses in the study reported here could have affected the tissue responses to the hydrochloric acid and VFAs in NRS; however, NRS control tissues at each pH were used for each horse, and results from those tissues were found to be comparable among horses.

Results of the study reported here indicated that acetic, propionic, and butyric acids at a low pH (≤ 4.0) induce more severe functional and histopathologic changes in the nonglandular mucosa of the equine stomach than are induced by hydrochloric acid alone (Fig 3). In this study, exposure of tissue to VFAs at a low pH (≤ 4.0) induced functional mucosal damage manifested by a decrease in sodium transport (ie, Isc) and increase in tissue resistance. These changes were associated with histologic evidence of cellular swelling in the nonglandular epithelium.

Explanation for changes in electrical measurements of the nonglandular mucosa in horses is based on results of studies[3,7,11-16] of tissues of other organs and species. Values for Isc and resistance reported here are similar to those for rabbit esophageal epithelium, porcine nonglandular mucosa, and porcine colon.[3,7,11] This means that similar ion transport and diffusion barrier mechanisms are in place in the nonglandular mucosa of horses. Short-circuit current is a direct indicator of active ion transport and, therefore, an indicator of epithelial function and tissue viability.[3] Experiments with frog skin,[12,13] rumen epithelium,[14] and rabbit esophagus[15] have revealed that Isc is equal to net sodium transport across tissues. In rumen epithelium and rabbit esophageal epithelium, it was found that the viable layers immediately beneath the SC function in sodium transport and are powered by intracellular sodium pumps (Na^+-K^+ ATPases).[14,15] A study[16] of the gastroesophageal mucosa of pigs confirmed that current flow across nonglandular epithelium is attributable to sodium transport and that sodium transport is primarily in the ST and SS layers of the nonglandular mucosa. Furthermore, ATPases have been histochemically located in the SS layer in equine nonglandular mucosa, which consists of nonstratified squamous epithelium.[17]

In the study reported here, acetic, propionic, and butyric acids, and to a lesser extent, hydrochloric acid, in NRS significantly ($P = 0.01$) decreased sodium transport when nonglandular mucosa was exposed at pH 1.5 and 4.0 (Fig 3), and exposed tissues did not recover to initial values except for mild recovery detected in tissues exposed to ARS at pH 4.0. Hydrochloric acid at pH 1.5 and, to a lesser extent, pH 4.0 mildly decreased sodium transport across the nonglandular tissue by approximately 25% after 75 minutes of exposure, whereas tissue resistance at pH 1.5 was initially low and remained stable throughout the experimental period. Although tissue resistance did not change in tissues exposed to NRS at pH 1.5, PD was significantly decreased within 15 minutes after onset of exposure. These data are similar to results of studies in porcine gastroesophageal mucosa[3] and experimentally induced injury in rabbit esophageal epithelium.[7] In the study reported here and studies[3,7] in

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rabbits and pigs, exposure to hydrochloric acid at pH 1.5 resulted in an immediate decrease in tissue PD (crude indicator of tissue resistance). However, tissue resistance was initially lower and remained significantly lower throughout the experimental period. The rapid change in PD and, to a lesser extent, the lower resistance in the tissues exposed to hydrochloric acid indicate a change in barrier function, which was then followed by a decrease in tissue Isc 45 to 60 minutes later. Analysis of these data suggests that hydrogen ions initially cause an increase in outer barrier permeability (decrease in tissue PD and resistance), thus allowing hydrochloric acid to diffuse into the deeper sodium-transporting cell layers (ie, ST and SS), which were then acidified, leading to cellular swelling and a subsequent decrease in Isc. Histopathologic changes of cellular swelling in the superficial and deeper layers and the mottled appearance seen in the tissues of the study reported here support this theory (Fig 7). However, although resistance remained significantly lower at higher pH than resistance for control tissues (pH, 7.0), it did not change significantly throughout the study period. This would suggest that equine nonglandular squamous mucosa may be more resistant to acid damage than porcine gastroesophageal or rabbit esophageal mucosa. An explanation for this difference in tissue resistance is not apparent and is not related to the gastric secretory patterns in these species, because horses, pigs, and rabbits constantly secrete gastric acid. Other factors such as tissue thickness, intracellular bicarbonate concentration, or tight intercellular junctions may play a role in this difference in tissue resistance.

The effect of hydrogen ions on sodium transport and barrier function appears to be reversible, because Isc and resistance were not significantly different from values in control tissues during the recovery period (Fig 1). Thus, a high pH (> 4.0) in the stomach of horses may decrease severity of damage induced by hydrochloric acid, whereas prolonged exposure (> 180 minutes) of the nonglandular mucosa to acid conditions (pH ≤ 4) may lead to acid injury and gastric ulcers. This could be the reason that horses that are not fed for prolonged periods develop gastric ulcers.[18]

In contrast to the mild decrease in Isc, PD, and resistance detected in tissues exposed to hydrochloric acid, tissues exposed to the VFAs had an immediate and irreversible decrease in Isc (ie, sodium transport) and an increase in resistance during the 180-minute exposure period. This decrease in sodium transport appears to be dependent on the chain length of the VFA as well as the concentration of VFA in the tissue. Exposure of tissues to acetic acid (2 carbons) decreased Isc by 30 to 60%, whereas exposure to propionic acid (3 carbons) decreased Isc by 55 to 80%, and exposure to butyric acid (4 carbons) decreased Isc by 80 to 112% at pH 1.5 and 4.0, respectively. Furthermore, tissue concentrations of butyric acid were the highest, followed by propionic acid and then acetic acid. Mild recovery in tissue Isc was only detected for tissues exposed to acetic acid at pH 4.0, which is consistent with data collected after porcine gastroesophageal mucosa was exposed to acidic conditions for 75 minutes.[3] Histopathologic changes confirmed that there was cellular swelling in the layers subjacent to the SC.

Tissue resistance increased at pH 1.5, compared with resistance for control tissues exposed to NRS at pH 7.0 or 4.0. Electrical resistance is a barrier function of the stomach of horses. Electrical resistance ranges from 1,500 to 3,000 Ω/cm^2, which is greater than that measured in the ruminant forestomach epithelium.[14] Application of 30mM hydrochloric acid at pH 1.5 for as long as 3 hours is necessary to change resistance and result in histologic damage to pig and rabbit nonglandular mucosa in vitro.[3,19] In the study in horses reported here, 30mM hydrochloric acid at pH 1.5 did not dramatically alter tissue resistance during the 180-minute exposure period.

In the study reported here, Isc decreased immediately in VFA-exposed tissues, which was then followed by an increase in resistance. These results agree with those of another study[3] in which tissues exposed to acetate at pH ≤ 4.5 underwent rapid and irreversible abolishment of Isc before any changes in resistance were detected. Because VFAs are less ionic and more lipophilic at lower pH (ie, pH ≤ 4.0), this implies that the nonglandular mucosa is highly permeable to the lipid-soluble form of the weak electrolyte (acetate) and indicates that VFAs can penetrate the tissue beneath even without changes in the permeability of the outer barrier.

The proposed sequence of events that lead to tissue injury associated with VFAs can be characterized. Undissociated (nonionic) VFAs (at pH ≤ 4.0) penetrate the outer barrier layers and are taken up by living cells subadjacent to the SC (perhaps the VFAs are transported into the cells at the same time as sodium). Intracellular pH is higher in these cells, compared with pH of the extracellular fluid, so continued intracellular accumulation and dissociation of the weak acid acidifies the intracellular contents. This disrupts sodium transport (as measured by Isc) and regulation of cell volume. Therefore, the continuous uptake of water and sodium across the basal membrane cannot be alleviated by sodium pumping or potassium leakage through the basolateral membrane, which results in cellular swelling and necrosis. Cellular swelling in the SS leads to an increase in resistance and, eventually, a decrease in resistance and sloughing of the outer barrier. There may be an initial effect of damage induced by hydrogen ions, because PD across the tissue decreased initially, despite the fact that tissue resistance increased. This initial loss of barrier function may enable sodium and water to enter these underlying layers, hastening the process.

The increased resistance of nonglandular gastric mucosa of horses exposed to these VFAs differs from data reported in pigs. In a study in pigs,[3] tissues were exposed to acetic acid for only 75 minutes, and cellular swelling, necrosis, and separation of the superficial mucosa were apparent during histologic examination. In the study reported here, there was evidence of cellular swelling but not of necrosis or sloughing of mucosa. However, in a preliminary experiment in our laboratory, we exposed equine nonglandular gastric mucosa to 60mM butyric acid at pH 1.5 for 12 hours; there was an increase in resistance for 4 hours after onset of expo-

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sure, followed by a progressive decrease in resistance in the tissues during the entire 12 hours of exposure (data not shown). The initial increase in resistance in the tissues of horses in that preliminary experiment may have been attributable to cellular swelling (identified histologically) causing a decrease in intercellular space. An initial increase in resistance was evident in the gastroesophageal mucosa of pigs prior to a decrease in resistance.[3] Thus, analysis of data for horses revealed a much longer period of increased resistance, compared with results in porcine gastroesophageal mucosa, which had a decrease in resistance and an increase in conductance. Thus, nonglandular mucosa of horses may be more resistant to VFA damage and may be able to maintain barrier function longer than porcine gastroesophageal or rabbit esophageal mucosa.

Although acetic, butyric, or propionic acids were not found in high concentrations (60mM) in the gastric contents of the horses reported here, it is possible that the VFAs were volatilized prior to collection or had already been metabolized to form carbon dioxide, β-hydroxybutyrate, or acetoacetate. Also, the horses in this study were not on a highly fermentable grain diet, in contrast to horses in another study[4] in which higher VFA concentrations were found in gastric contents. Diets higher in fermentable carbohydrates would have led to higher concentrations of VFAs in gastric contents of our horses, as has been described elsewhere.[4]

Some absorption was evident in tissues exposed to PRS or BRS, because tissues exposed to propionic and butyric acids had higher concentrations of these VFAs at the end of the study, compared with concentrations of other VFAs. In another study,[20] investigators examined the mucosal uptake and serosal release of VFAs and used the differences between variables as an indicator of intraepithelial metabolism and VFA accumulation. In that study, it was found that when propionate (15 mmol/L) was the only VFA included in solutions used to bathe the mucosa, mean mucosal concentrations decreased to approximately 13.5mM, and serosal concentrations increased to 0.8 mmol/L, resulting in a mean mucosal loss of 18 µmol/h and a mean serosal gain of 9 µmol/h. Serosal release was equal to approximately half of mucosal uptake, and the authors of that study[20] believed that this indicated substantial intraepithelial metabolism or accumulation.

Analysis of results of the study reported here suggests that hydrochloric acid or VFAs, which are fermentation products of carbohydrates, at a low pH (≤ 4) can cause functional damage to the nonglandular mucosa of the stomach of horses. There is damage to barrier function and sodium transport mechanisms in the living layers immediately deep to the SC of the nonglandular mucosa, which undermines the superficial mucosa and leads to eventual sloughing and ulcers. The synergistic effects of hydrochloric acid and VFAs may be the reason that diets high in fermentable carbohydrates have been implicated in the cause of gastric ulcers in horses.

[a]Beuthanasia, Schering-Plough Corp, Kenilworth, NJ.
[b]Accumet A-10 portable pH meter, Fisher Scientific Co, Pittsburgh, Pa.
[c]Polytron, Brinkmann Instruments, Westbury, NY.
[d]Vortex-Genie, Scientific Industries Inc, Bohemia, NY.
[e]Model J-21A, Beckman Instruments, Palo Alto, Calif.
[f]Ortho-phosphoric acid, Fisher Scientific Co, Pittsburgh, Pa.
[g]Hewlett Packard model 5890 gas chromatograph, Hewlett Packard, Avondale, Pa.
[h]HP-FFAP capillary column, Hewlett Packard, Avondale, Pa.
[i]Acetic, butyric, and propionic acid standards, Alltech Associates Inc, Deerfield, Ill.
[j]2-ethylbutyric acid, Eastman Kodak Co, Rochester, NY.
[k]SAS version 8.02, SAS Institute Inc, Cary, NC.

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