Effects of hydrochloric, valeric, and other volatile fatty acids on pathogenesis of ulcers in the nonglandular portion of the stomach of horses

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Objective—To identify in vitro effects of hydrochloric acid, valeric acid, and other volatile fatty acids (VFAs) on the pathogenesis of ulcers in the nonglandular portion of the equine stomach.

Sample Population—Gastric tissues from 13 adult horses.

Procedure—Nonglandular gastric mucosa was studied by use of Ussing chambers. Short-circuit current (Isc) and potential difference were measured and electrical resistance and conductance calculated after tissues were bathed in normal Ringer's solution (NRS) or NRS and hydrochloric, valeric, acetic, propionic, and butyric acids. Treated tissues were examined histologically.

Results—Incubation in 60mM valeric acid at pH ≤ 7.0 abruptly and irreversibly abolished Isc, which was followed by a slower decrease in resistance and an increase in conductance. Incubation in 60mM acetic, propionic, and butyric acids and, to a lesser extent, hydrochloric acid at pH ≤ 7.0 significantly decreased Isc, which was followed by an increase in resistance and a decrease in conductance.

Conclusions and Clinical Relevance—Incubation in valeric acid at pH ≤ 7.0 caused a dramatic decrease in mucosal barrier function in the nonglandular portion of the stomach. Changes in barrier function attributable to exposure to valeric acid were associated with histopathologic evidence of cellular swelling in all layers of the nonglandular mucosa. Because of its high lipid solubility, valeric acid penetrates the nonglandular gastric mucosa, resulting in inhibition of sodium transport and cellular swelling. Valeric acid and other VFAs in gastric contents may contribute to the pathogenesis of ulcers in the nonglandular portion of the stomach of horses. (*Am J Vet Res* 2003;64:413–417)

Gastric ulcers are a major health problem in horses.[1,2] Hydrochloric acid and **volatile fatty acids (VFAs)** such as acetic, propionic, and butyric acids, which are by-products of carbohydrate fermentation by gastric bacteria, can cause acid injury in the nonglandular squamous mucosa of horses.[3] We are not aware of any data on the effects of valeric acid, a longer-chain VFA, on the formation of gastric ulcers in horses.

In another study[4] conducted by our laboratory group, horses fed a diet of alfalfa hay and grain or a diet of bromegrass hay developed gastric ulcers. 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 valeric acid than horses without ulcers.

The purpose of the study reported here was to determine the in vitro effect of valeric acid, other VFAs (ie, acetic, butyric, and propionic acids), and pH on sodium transport, tissue resistance, and tissue conductance across the nonglandular stratified squamous mucosa of the equine stomach. Our objective was to elucidate the role of these factors in the pathogenesis of gastric ulcers in horses.

Materials and Methods

Sample population—Gastric tissues obtained from 13 adult horses (5 geldings, 2 stallions, and 7 mares) that were euthanized for reasons of debilitation or donation were used in the study. Information regarding sex, breed, and age of each horse was obtained.

Tissue acquisition—Horses were euthanized by lethal injection of an overdose of barbiturate.[a] Within 1 hour after horses were euthanized, 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 (NRS)**,[3] which was made in our laboratory. The mucosa was examined for gastric ulcers, which were graded by use of a scoring system for gastric ulcers in horses.[5] Nonglandular mucosa was obtained by dissecting the submucosa from the underlying muscular tissue. Specimens

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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 nonglandular gastric mucosal tissue were manipulated in Ussing chambers as described in another study[3] conducted by our laboratory group. Briefly, control tissues were bathed on the mucosal and submucosal surfaces in 10 mL of NRS. Solutions containing valeric, acetic, butyric, or propionic acids were created by substituting the sodium salt of each VFA for the equivalent amount of sodium chloride in the NRS such that the final concentration was 60mM of each respective VFA in NRS. The solution containing each specific VFA (10 mL) was added to the mucosal side of the tissue, and 10 mL of NRS was added to the submucosal side of the tissue in the Ussing chamber.

The pH of each solution used to bathe the mucosal surface was adjusted by titration with 1.0 N hydrochloric acid while measuring it with a pH electrode[b] to achieve a final pH of 1.5, 4.0, or 7.0. These pH values were chosen on the basis that 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 bathed in NRS (control solution) and VFA solution, chambers were set up with pH of 1.5, 4.0, or 7.0 on the mucosal side for a total of 3 chambersfor each VFA and 3 chambers for NRS in each experiment. The pH of each solution was measured hourly to verify stability.

Duration of each experiment was 255 minutes. Tissues were allowed to equilibrate in the NRS solution 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 was adjusted to attain a specific pH (1.5, 4.0, or 7.0); during this time, 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.

Tissue **potential difference (PD)** and **short-circuit current (Isc)** were recorded at 15-minute intervals throughout the study. Tissue PD and Isc were used to calculate tissue resistance, corrected Isc (ie, corrected for junction potentials and surface area of tissue), percentage of control Isc, and tissue conductance. All of the calculations were based on Ohm's law, as reported elsewhere.[3]

After completion of the incubation period in the Ussing chambers, the mucosa was placed in neutral-buffered 10% formalin. It was subsequently processed for routine histologic examination, as described elsewhere.[3]

Statistical analysis—Data were analyzed by use of a statistical computer program.[c] The model used for each tissue type and variable was a completely randomized design split-plot ANOVA, with treatment (solution conditions) in the main plot and time and time-by-treatment interaction in the subplot. 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 13 horses ranged from 2 to 25 years old (mean, 12.1 years), and 6 (46%) had gastric ulcers in the nonglandular mucosa. Horses had various conditions or reasons for being euthanatized, which included neurologic disease (n = 4), weight loss (4), lameness and laminitis (3), allergic small airway disease (1), and old age (1). None of the horses were treated with medications during their stay in our facility prior to being euthanatized, but treatments administered prior to arrival at our facility were not recorded. Medical conditions and prior treatments may have altered the

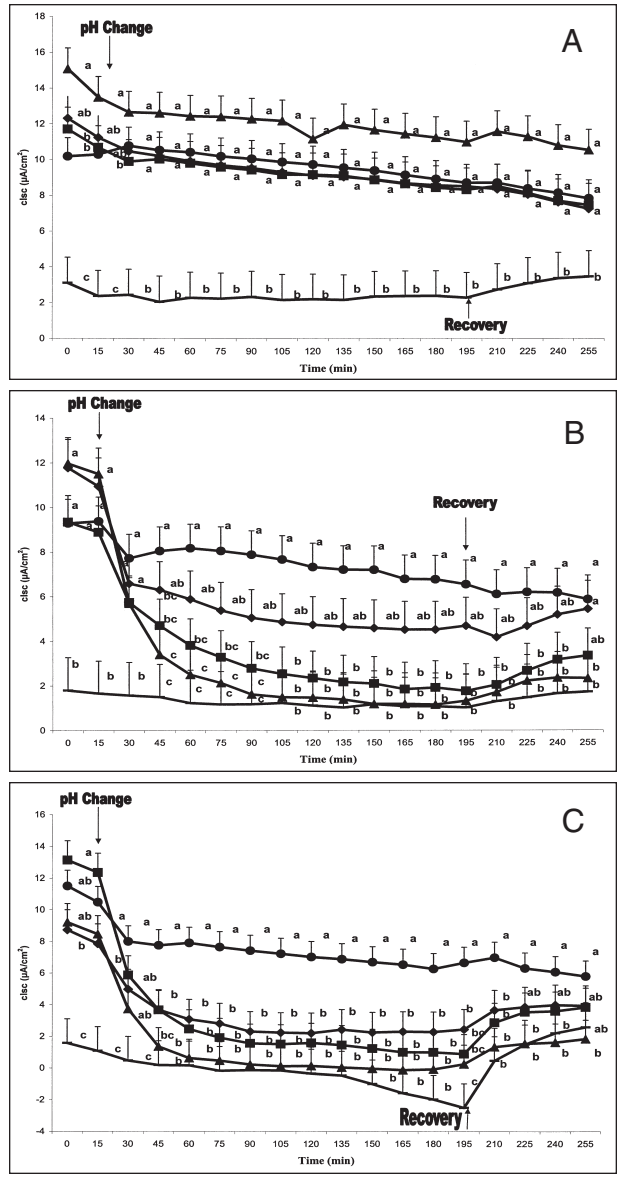

Figure 1—Mean ± SEM values for corrected short-circuit current (clsc) in samples of nonglandular squamous mucosa collected from the stomach of each of 13 horses. Tissues were placed in Ussing chambers and exposed on their mucosal surface to normal Ringer's solution (NRS; circle), 60mM acetate Ringer's solution (ARS; diamond), 60mM propionate Ringer's solution (PRS; square), 60mM butyrate Ringer's solution (BRS; triangle), or 60mM valerate Ringer's solution (VRS; line without symbols) buffered at pH 7.0 for 15 minutes. Then, pH was adjusted by addition of 1.0 N hydrochloric acid to 7.0 (A), 4.0 (B), or 1.5 (C), and tissues were incubated 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 ($P < 0.05$).

responses of the horses' tissues, because we did not control for this factor. However, NRS control tissues at each pH value were used, along with each VFA treatment, to account for variations among and within horses.

Ulcer scores were determined for 5 of 6 horses. Mean ulcer score was 1.8 (scale, 0 to 3). Thirteen tissue samples were obtained from grossly normal nonglandular mucosa at or adjacent to the margo plicatus of each horse, which is the region of the equine stomach where ulcers are commonly found and that is susceptible to acid injury. Of the 169 samples, (13 horses × 13 samples/horse), 13 (1 from each horse) were placed in neutral-buffered 10% formalin, examined by use of light microscopy, and found to be free of pathologic changes. Of the remaining 156 samples, 28 were not analyzed because of a malfunction of the Ussing chambers; thus, 128 tissue samples were evaluated. The variables measured did not differ significantly for

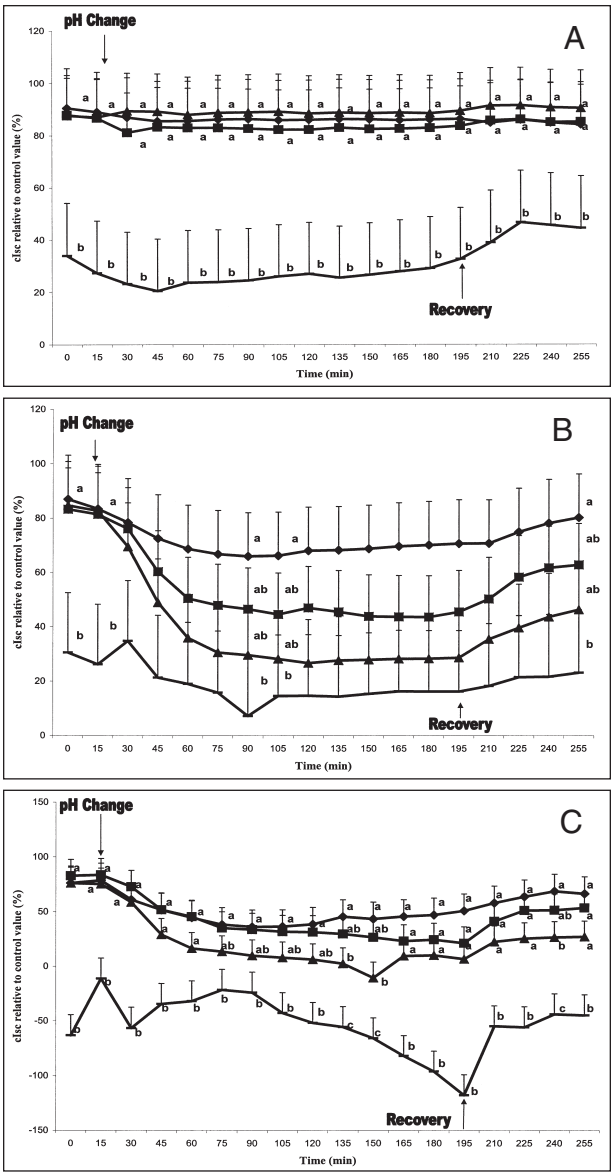

Figure 2—Mean ± SEM values for clsc expressed as a percentage of control values in samples of nonglandular squamous mucosa collected from the stomach of each of 13 horses. Tissues were placed in Ussing chambers and exposed on their mucosal side to NRS (circle), 60mM ARS (diamond), 60mM PRS (square), 60mM BRS (triangle), or 60mM VRS (line without symbols) buffered at pH 7.0 for 15 minutes. Then, pH was adjusted by addition of 1.0 N hydrochloric acid to 7.0 (A), 4.0 (B), or 1.5 (C), and tissues were incubated 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 key.

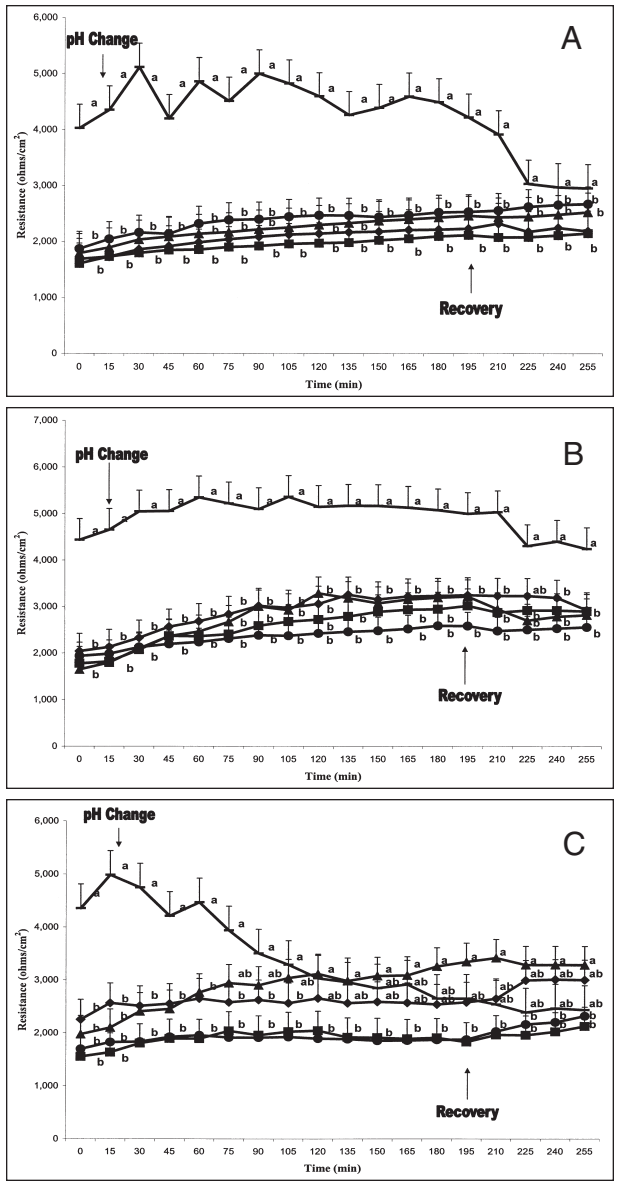

Figure 3—Mean ± SEM values for resistance in samples of nonglandular squamous mucosa collected from the stomach of each of 13 horses. Tissues were placed in Ussing chambers and exposed on their mucosal side to NRS (circle), 60mM ARS (diamond), 60mM PRS (square), 60mM BRS (triangle), or 60mM VRS (line without symbols) buffered at pH 7.0 for 15 minutes. Then, pH was adjusted by addition of 1.0 N hydrochloric acid to 7.0 (A), 4.0 (B), or 1.5 (C), and tissues were incubated 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]For each time point, mean values with different superscript letters differ significantly ($P < 0.05$).

tissues from horses with ulcers and tissues from horses without ulcers.

Mucosa exposed to valeric acid had an immediate and irreversible decrease in Isc during the first 15 minutes after onset of exposure (ie, before changing pH of the solutions). The Isc remained significantly lower throughout the exposure and recovery periods, compared with Isc values for the NRS control samples at the same pH (**Fig 1**). At pH 7.0, when the Isc was expressed as a percentage of the value for tissues in NRS at the same pH, Isc decreased 80% during exposure to valeric acid, whereas Isc decreased only 10% during exposure to acetic, propionic, and butyric acids (**Fig 2**). At pH 4.0, Isc decreased 90% during exposure to valeric acid, whereas Isc decreased 35, 55, and 75% in tissues during exposure to acetic, propionic, and butyric acids, respectively. At pH 1.5, Isc was completely abolished in tissues exposed to valeric and butyric acids and decreased 55 and 80% during exposure to acetic and propionic acids, respectively. Comparing the relative effects of VFAs on tissue Isc, acetic acid had the least effect, followed by propionic acid, butyric acid, and valeric acid. Analysis of these data suggests that VFAs with a longer carbon chain induce more damage to tissues.

At each pH, resistance was significantly higher in tissues exposed to valeric acid, compared with values for tissues exposed to other VFAs and NRS alone (**Fig 3**). However, at pH 1.5, resistance significantly decreased in tissues exposed to valeric acid during the initial 45 minutes after exposure at pH 1.5, compared with resistance for tissues exposed at pH ≥ 4.0. Tissue resistance was significantly increased within 75 minutes after onset of exposure at pH 1.5, compared with resistance for butyric acid for tissues exposed to other VFAs and NRS at pH 1.5. At pH 1.5, resistance continued to decrease in tissues exposed to valeric acid during the recovery period. At pH 1.5, conductance was increased in mucosa exposed to valeric acid, compared with values for tissues exposed at a higher pH (≥ 4.0); however, conductance values did not differ significantly until the recovery period (**Fig 4**). At pH ≥ 4.0, conductance remained low throughout the experimental period. At the lowest pH (1.5), tissue permeability (ie, conductance) continued to increase during the recovery period. Loss of tissue resistance and increased permeability (as indicated by increased conductance) indicate loss of function of the tissue barrier.

Histologic examination of the nonglandular mucosa exposed to NRS at pH 7.0 did not reveal cellular swelling in any layers, whereas mucosa exposed to valeric acid had cellular swelling in the **stratum corneum** (**SC**), **stratum transitionale** (**ST**), **stratum**

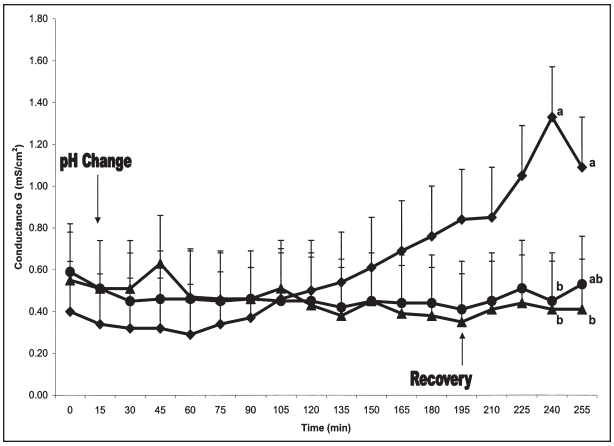

Figure 4—Mean ± SEM values for conductance in samples of nonglandular squamous mucosa collected from the stomach of each of 13 horses. Tissues were placed in Ussing chambers and exposed on their mucosal side to 60mM VRS buffered at pH 7.0 for 30 minutes. Then, pH was adjusted by addition of 1.0 N hydrochloric acid to 1.5 (diamond), 4.0 (triangle), or 7.0 (square), and tissues were incubated 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 3 for remainder of key.

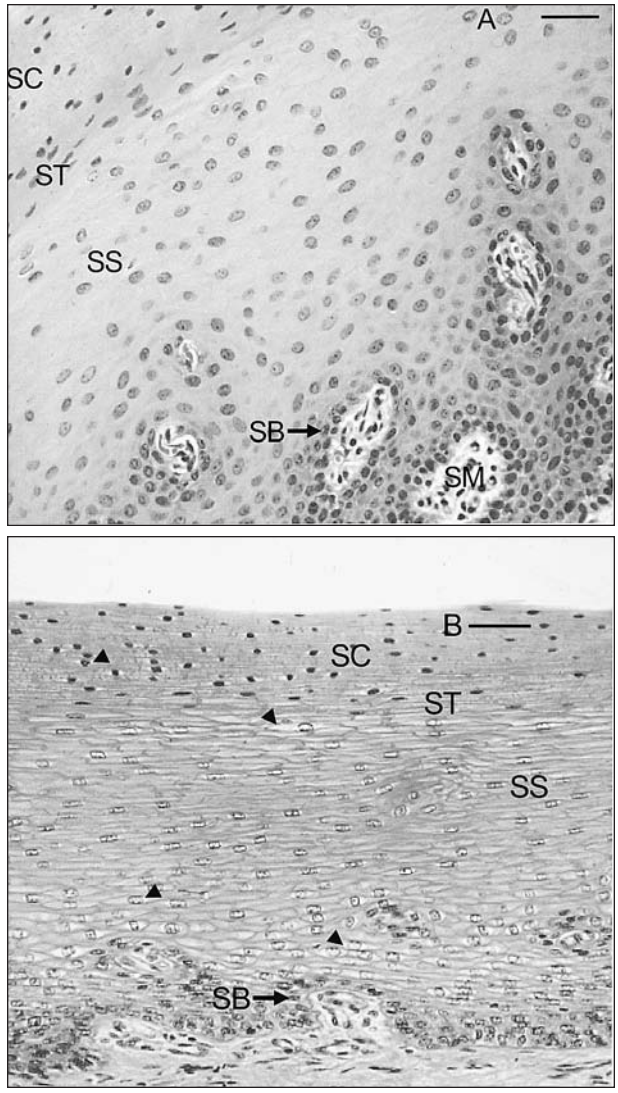

Figure 5—Photomicrograph of tissue sections of equine nonglandular squamous mucosa after incubation for 255 minutes in NRS at pH 7.0 on the mucosal and submucosal sides (A) or 60mM VRS at pH 1.5 on the mucosal surface and NRS at pH 7.0 on the submucosal surface (B). Notice the pallor and disruption of the stratum corneum (SC), stratum transitionale (ST), stratum spinosum (SS), stratum basale (SB [arrow]) and submucosa (SM); pallor denotes cellular swelling (arrowheads). H&E stain; bar = 40 μm in panel A and 80 μm in panel B.

spinosum (SS), and stratum basale at all of the pH values (**Fig 5**). Also, tissues exposed to acetic, propionic, and butyric acids had cellular swelling predominantly in the layers subjacent to the SC (ie, the ST and SS) at pH ≤ 4.0, as has been reported in another study.[3] We did not detect cellular swelling in tissues exposed to acetic, propionic, or butyric acid at pH 7.0.

Discussion

Prevalence of gastric ulcers in the horses in the study reported here was 6 of 13 (46%), and mean gastric ulcer score was 1.8 (scale, 0 to 3). The prevalence and severity of gastric ulcers in these horses were similar to those in another report[3] for similarly aged horses donated because of underlying medical diseases and musculoskeletal lameness. Illness could have predisposed the horses to a high prevalence of gastric ulcers, because the incidence of gastric ulcers reportedly is high in horses with clinical signs of disease.[6]

Analysis of results of the study reported here indicates that incubation of tissues in valeric acid at pH 1.5 or 4.0 induced more functional and histopathologic changes in the nonglandular mucosal barrier than did incubation with acetic, propionic, or butyric acids at pH 1.5 or 4.0. Valeric acid induced functional mucosal damage that was manifested as a decrease in sodium transport (Isc), a loss of tissue resistance, and an increase in tissue permeability (conductance) at pH 1.5. These changes were associated with histologic evidence of cellular swelling throughout all layers of the nonglandular squamous mucosa of the equine stomach.

In the study reported here, the decrease in Isc and initial increase in resistance in the mucosa exposed to valeric acid were similar to those in another study[3] of equine nonglandular mucosa exposed to other VFAs. However, during the initial 45 minutes after exposure at pH 1.5, resistance was significantly decreased, compared with initial values. From these data, this initially high resistance for each tissue exposed to valeric acid at each pH may be attributable to rapid cellular swelling (increased intracellular space) and loss of intercellular space in all mucosal cell layers, because cellular swelling was seen on histologic examination, even in tissues incubated at pH 7.0. This rapid cellular swelling may have been the initial response of the nonglandular mucosa to exposure to valeric acid.

Within 45 minutes after exposure at pH 1.5, tissues exposed to valeric acid had a significant decrease in resistance, which was followed by an increase in conductance. The decrease in tissue resistance and increase in tissue conductance continued after removal of the valeric acid and were indicative of loss of function of the mucosal barrier. Loss of function by the tissue barrier and increased permeability in response to exposure to VFAs are similar to acid injury reported[7] in porcine gastroesophageal mucosa exposed to 60mM acetic acid at pH ≤ 4.5. However, valeric acid appears to cause more acid injury than other VFAs. Analysis of these data suggests that valeric acid at pH 1.50 or 4.0 diffuses rapidly into the nonglandular mucosa and acidifies cellular contents and uncouples sodium transport, which results in cellular swelling. At pH 1.5, function of the mucosal cell barrier is compromised, which leads to increased permeability and eventual necrosis and ulcers. The mechanism by which valeric acid causes more acid injury is unknown, but it may be attributable to its lipid solubility at pH ≥ 4.0. However, the **dissociation constant (pKa)** value for valeric acid (ie, pKa of 4.82) is similar to that of the other VFAs used in this study.

Valeric acid appears to have a more profound effect on function of the nonglandular gastric mucosa barrier than is evident when the other VFAs or hydrochloric acid alone were used. In contrast to the mild decrease in Isc and resistance seen in the tissues exposed to hydrochloric acid and the decrease in Isc and increase in resistance seen with acetic, propionic, and butyric acids, tissues exposed to valeric acid had a dramatic and irreversible decrease in sodium transport (Isc) and resistance and an increase in conductance shortly after onset of exposure at pH 1.5. This profound effect of valeric acid on barrier function may have been attributable to the length of its carbon chain and relative tissue concentration.[3] Because of its longer carbon chain (5 carbons), valeric acid may be more lipid soluble and, thus, accumulate inside mucosal epithelium more readily than shorter chain VFAs at a higher pH. There appears to be a relationship between length of carbon chain and effect on sodium transport and barrier function in tissues obtained from the nonglandular mucosa of horses.[3]

Analysis of the results of the study reported here suggests that valeric acid, a fermentation product of carbohydrates, causes functional damage to the nonglandular mucosa of the stomach of horses. Sodium transport mechanisms, barrier function, and permeability of the nonglandular mucosal epithelium are damaged. This damage is accelerated at an acidic pH (≤ 1.5) and leads to cellular swelling in all layers of the gastric nonglandular squamous mucosa. Analysis of these data suggests that valeric acid at pH ≤ 7.0 has a potent effect on function of the nonglandular mucosal barrier of horses and may be the reason that some gastric ulcers persist despite adequate acid control.

[a]Beuthanasia, Schering-Plough Corp, Kenilworth, NJ.
[b]Accumet A-10 portable pH meter, Fisher Scientific, Pittsburgh, Pa.
[c]SAS version 8.02, SAS Institute Inc, Cary, NC.

References

1. Hammand CJ, Mason DK, Watkins KL. Gastric ulceration in mature thoroughbred horses. *Equine Vet J* 1986;18:284–287.

2. Vatistas NJ, Snyder JR, Carlson G, et al. Epidemiological study of gastric ulceration in the thoroughbred racehorse: 202 horses (1992–1993), in *Proceedings*. Annu Meet Am Assoc Equine Pract 1994;4:125–126.

3. Nadeau JA, Andrews FM, Patton CS, et al. Effects of hydrochloric, acetic, butyric and propionic acids on pathogenesis of ulcers in the nonglandular portion of the stomach of horses. *Am J Vet Res* 2003;64:404–412.

4. Nadeau JA, Andrews FM, Mathew AG, et al. Evaluation of diet as a cause of gastric ulcers in horses. *Am J Vet Res* 2000;61:784–790.

5. Andrews FM, Sifferman RL, Bernard W, et al. Efficacy of omeprazole paste in the treatment and prevention of gastric ulcers in horses. *Equine Vet J* 1999;29:81–86.

6. Murray MJ, Grodinsky C, Anderson CW, et al. Gastric ulcers in horses: a comparison of endoscopic findings in horses with and without clinical signs. *Equine Vet J Suppl* 1989;7:68–72.

7. Argenzio RA, Eisemann J. Mechanisms of acid injury in porcine gastroesophageal mucosa. *Am J Vet Res* 1996;57:564–573.