Nonsteroidal anti-inflammatory drugs are a group of commonly used therapeutic agents with analgesic, antipyretic, and anti-inflammatory properties. The principal pharmacological effect of NSAIDs arises from the inhibition of the COXs, which convert polyunsaturated fatty acids to PGs during the inflammatory process. The COXs are membrane-bound proteins located on the surface inside the endoplasmic reticulum and on the inner and outer membranes of the nuclear membrane. Two PG endoperoxide synthase enzymes have been described: COX-1 and COX-2. However, a splice variant of COX-1 that retains intron 1—named COX-3—has also been characterized. Cyclooxygenase-1 is constitutively expressed in most tissues and is involved in the production of PGs that mediate physiologic housekeeping functions. By contrast, COX-2 is constitutively expressed in some tissues (brain, kidneys, and intestines) but is primarily an inducible enzyme that responds to cytokines, mitogens, and endotoxin in several cell types. Although COX-2 is induced during inflammation and cell proliferation, it has important physiologic functions in the brain, kidneys, and cardiovascular system.

Due to its efficacy, availability, and affordability, PBZ is the most commonly used NSAID for the treatment of osteoarthritis and musculoskeletal disorders in horses.
horses. At the recommended dose and dosing interval, PBZ is believed to be well tolerated in horses. However, recommended dosages have been reported to be associated with adverse effects, including anorexia, lethargy, oral and gastrointestinal tract ulceration, right dorsal colitis, acute necrotizing enterocolitis, renal papillary necrosis, and death. The adverse effects of PBZ may increase when administered to dehydrated animals or used concurrently with other NSAIDs. Recently, our group treated 2 horses with hematuria secondary to ulcerative cystitis that developed after long-term use of PBZ at a recommended dosage.

The objectives of the study reported here were to determine gene expressions of COX-1 and COX-2 in oral, glandular gastric, and urinary bladder mucosae in healthy horses; to determine whether oral administration of PBZ at the maximum recommended dosage daily for 7 days had an effect on gene expressions of those isoenzymes; and to evaluate the macroscopic effects of PBZ in the oral, gastric, and bladder mucosae and on clinicopathologic variables.

Materials and Methods

Horses—Twelve healthy horses (6 males and 6 females) of various breeds that ranged in age from 3 to 21 years (mean ± SD age, 13.8 ± 6.2 years) and resided at the Center for Equine Health at the University of California-Davis were included for the study. Each horse was considered to be healthy on the basis of results of physical examination, CBC, and serum biochemical analysis. These horses had not received any medication during the preceding 2 weeks. The study was approved by the Animal Care and Use Committee of the University of California.

Horses were maintained in a dry paddock with ad libitum access to water for the duration of the study. Horses were fed alfalfa hay in amounts equivalent to 1% of body weight 2 times/d. Horses were randomly assigned to receive PBZ or placebo (6 horses/group) for 7 days (designated as days 1 to 7). During the 7-day period, horses in the PBZ group received 4.4 mg/kg of PBZ mixed with corn syrup orally every 12 hours and horses in the placebo group received an equivalent amount of corn syrup alone orally every 12 hours. A physical examination of each horse was performed daily from days 0 (the day prior to commencement of the treatment period) to 8 (the day following termination of the treatment period).

Clinicopathologic evaluation—Venous blood and urine samples were collected for CBC, serum biochemical analysis, and urinalysis on days 0 and 8. For urine sample collection via catheterization, each horse was sedated with detomidine hydrochloride (0.01 mg/kg, IV); if necessary, physical restraint with a nose twitch was applied. Urine samples were evaluated visually for color and clarity and processed by use of a commercial urine analyzer for assessment of pH and concentrations of protein, glucose, ketones, bilirubin, and hemoprotein. Urine samples underwent biochemical analysis. These horses had not received any medication during the preceding 2 weeks. The study was approved by the Center for Equine Health at the University of California-Davis.


d 

Ultrasonographic evaluation—A portable ultrasound machine with a 3.5-MHz convex probe was used to measure the stomach and dorsal colon wall thicknesses in each horse on days 0 and 8. The ultrasonographic appearance of both kidneys was evaluated, and their length, width, and cortical thickness were measured.

Endoscopic evaluations and biopsy specimen collection—Gastric and urinary bladder endoscopies were performed in each horse on days 0 and 8. Water and food were withheld for 4 and 12 hours, respectively, before the gastric endoscopic procedures. Each horse was sedated with detomidine hydrochloride (0.01 mg/kg, IV); for each horse, 2 samples of tissue (considered 1 biopsy specimen) were collected from the glandular mucosa of the stomach on 1 side of the stomach on day 0; on day 8, 2 samples of tissue were collected from the opposite side of the stomach.

For each horse, the side of the mouth and bladder from which urinary bladder or oral mucosa biopsy specimens were obtained on day 0 was randomly selected, and the opposite side was used for biopsy specimen collection on day 8. Oral mucosa was evaluated and characterized as apparently normal mucosa (grade 0), mild ulceration (grade 2), or severe ulceration (grade 3). A bladder biopsy specimen (2 samples of tissue) was then collected in a similar manner as the stomach biopsy specimen. On days 0 and 8, 1 oral mucosal biopsy specimen (a single sample of tissue from close to the lip commissure) was collected after the endoscopic procedures by use of disposable skin punch biopsy instruments (3 mm in diameter). For each horse, the side of the mouth and bladder from which urinary bladder or oral mucosa biopsy specimens were obtained on day 0 was randomly selected, and the opposite side was used for biopsy specimen collection on day 8.

Because of the small size of the transendoscopic biopsy forceps, 2 tissue samples were collected before and
2 tissue samples specimens were collected after PBZ administration from the stomach and bladder mucosae. The 2 biopsy specimens of each type of mucosa at each time point were considered as 1 biopsy specimen. Each biopsy specimen (stomach, bladder, and oral mucosa) obtained from each horse was placed in a plastic tube containing 1 mL of an RNA stabilization reagent. All biopsy specimens were frozen at −80°C until further processing.

**COX-1 and COX-2 mRNA analysis**—Frozen biopsy specimens were individually disrupted and homogenized in lysis buffer. The RNA was isolated by use of commercial kits in an automated system and quantified by use of a spectrophotometer. The RNA was then transformed to cDNA by use of standard 2-step synthesis of cDNA with a reverse transcriptase assay. A gDNA wipeout buffer for removal of gDNA was used in accordance with the manufacturer’s specifications. To select the most stable reference genes for the experimental conditions, primers from 8 HKGs were used. The selected reference genes belonged to different functional classes, which reduced the chance that the genes might be coregulated. Equine primers for the HKGs used in the study (glyceraldehyde 3-phosphate dehydrogenase, S-9 ribosomal region, actin, β2 microglobulin, p53, hypoxanthine phosphoribosyltransferase 1, tubulin alpha-1, ubiquitin-C, succinate dehydrogenase complex subunit A, and β-actin) have been previously published. To determine the stability of reference genes, PCR assays were performed by use of the 8 HKGs in oral, glandular, and urinary bladder mucosae specimens from 3 horses. The most stable HKGs were selected by use of commercial software as previously described. The software used calculates the gene expression stability measure (M) for a reference gene as the mean pairwise variation for that gene with all other tested reference genes. Stepwise exclusion of the gene with the highest M value allows ranking of the tested genes according to their expression stability. From the 2 most stable HKGs, the one with the lowest cycle number at the detection threshold (crossing point) was selected as the reference gene in the study.

Primers for the target genes COX-1 and COX-2 have been previously published. The primers were designed in regions spanning 2 exons to prevent gDNA amplification. Real-time PCR assays were performed with a real-time detection system involving detection of dye intercalation. Reaction samples had a final volume of 10 µL and contained 5 µL of SYBR green I master mix, 0.5µM each primer, 0.2 U of uracil glycosylase, 1 µL of cDNA, and water. Amplification conditions were 37°C for 10 minutes, 95°C for 5 minutes, and 45 cycles of 95°C for 5 seconds, 55°C for 10 seconds, and 72°C for 6 seconds. To ensure specificity of amplifications and to detect primer-dimer formation dissociation, curves of the melting temperature were created and evaluated by heating the samples from 65°C to 95°C in 0.5°C increments with a dwell time at each temperature of 10 seconds; fluorescence was continuously monitored. All samples were run in triplicate, and nontemplate negative controls were included. Relative quantification to the most stable HKG was performed with commercial software by use of the E-method as recently described. The E-method produces accurate relative quantification data by compensating for differences in target and reference-gene amplification by use of specific gene efficiencies.

By use of the aforementioned procedures, biopsy specimens of oral, glandular gastric, and bladder mucosae from 5 placebo- and 6 PBZ-treated horses collected on days 0 and 8 were tested for COX-1 and COX-2 gene expressions. A placebo-treated horse was removed from the study after a cystic calculus was detected during baseline endoscopic examination of the urinary bladder. In addition, 7 randomly selected biopsy specimens (from 7 horses) collected on day 0 were used to investigate gene expressions of COX-1 and COX-2 in the different tissues (ie, oral, gastric glandular, and bladder mucosae) in healthy horses.

**Statistical analysis**—Comparison of the expressions of COX-1 and COX-2 in the specimens of oral cavity, glandular gastric, and bladder mucosae collected from 7 horses on day 0 was performed by use of a 1-way ANOVA, followed by the Bonferroni post hoc test. Results obtained from the urinalyses, CBCs, serum biochemical analyses, and ultrasonographic measurements were analyzed by use of a 2-way repeated-measures ANOVA for the effects of time and group. Results regarding gene expressions and ulcer severity grades in the oral, gastric glandular, and urinary bladder mucosae were analyzed via nonparametric methods. Gene expression data and ulcer severity grades were analyzed between groups on days 0 and 8 by use of a Mann-Whitney U test and within groups on day 0 versus day 8 by use of a Wilcoxon signed rank test. Statistical analysis was performed with commercial software. Values of P < 0.05 were considered significant.

**Results**

**Horses**—One of the placebo-treated horses was removed from the study after a cystic calculus was detected during baseline cystoscopic examination. Data from the remaining 11 horses were analyzed (placebo group, n = 5; PBZ group, 6). On day 0, the physical examinations, CBCs, serum biochemical analyses, and urinalyses revealed no abnormalities in any of the 11 horses. Physical examination results were considered normal for all horses throughout the duration of the study. None of the horses developed ulceration of the oral mucosa during the study period.

**Clinicopathologic evaluation**—For each of the 11 horses, results of a CBC, serum biochemical analysis, and urinalysis performed on days 0 and 8 were within reference limits. Furthermore, there was no significant difference in BUN and serum creatinine, total protein, or albumin concentrations within groups (day 0 vs day 8 data) or between groups (day 0 data comparisons and also day 8 data comparisons).

**Ultrasonographic evaluation**—Wall thicknesses of the stomach and colon and renal length, width, and cortical thickness were within reference limits. There was no significant difference in any of these variables within groups (day 0 vs day 8 data) or between groups (day 0 data comparisons and also day 8 data comparisons).
Endoscopic evaluations—Glandular gastric and urinary bladder biopsy specimens were collected during endoscopic examinations from 5 placebo-treated and 6 PBZ-treated horses on days 0 and 8. Gastric and bladder biopsy specimens were easily collected from the horses during sedation and with the physical restraint used. Bleeding at all biopsy sites immediately after specimen collection was minimal. On day 0, gastroscopy revealed mild gastric ulceration (grade 2) of the squamous mucosa near the margo plicatus at the lesser curvature in 2 horses (1 from each group). After 7 days of placebo treatment, 2 additional horses had developed grade 1 gastric lesions in the squamous mucosa. After 7 days of PBZ administration, 5 of 6 horses had lesions in the squamous gastric mucosa, 2 of which had developed grade 4 gastric lesions. The 2 horses with grade 4 lesions also developed ulcers in the glandular mucosa. With regard to mean gastric ulcer severity grades, there was no significant difference between groups at day 0 or 8. Findings within the placebo group (day 0 vs day 8) did not differ significantly; however, after administration of PBZ for 7 days, mean gastric ulcer severity grade did change significantly from day 0 findings (Figure 1).

Cystoscopically, all 11 horses for which data were subsequently analyzed appeared to have a normal urinary bladder mucosa on day 0. On day 8, cystoscopy revealed areas of pinpoint redness that corresponded to the previous biopsy sites in 7 horses (2 from the placebo group and 5 from the PBZ group). In the remaining 4 horses (3 from the placebo group and 1 from the PBZ group), the previous biopsy sites were not evident on day 8. No other mucosal treatment–associated alterations were observed.

COX-1 and COX-2 mRNA analysis—The 2 more stable HKGs for the purposes of this study were ubiquitin-C and glyceraldehyde 3-phosphate dehydrogenase. All results of gene expression were reported in terms of relative expression to ubiquitin-C gene expression. Oral, glandular gastric, and urinary bladder mucosa biopsy specimens obtained before commencement of experimental treatments in 7 of the 11 healthy horses in Figure 1. For each horse, 1 biopsy specimen collected from the oral mucosa and 2 small tissue samples were collected from the glandular gastric and urinary bladder mucosae; the latter pairs of samples were each considered as 1 biopsy specimen. Values (which are unitless) indicate gene expression relative to expression of the HKG ubiquitin-C. *For a given isoenzyme, columns with different letters are significantly different (P < 0.05) different.
Discussion

Cyclooxygenase-1 was expressed in the oral, glandular gastric, and bladder mucosa biopsy specimens collected from healthy horses in the present study. It has been suggested that COX-1 provides PGs that are required for homeostatic functions, including cytoprotection and hemostasis. However, its expression may differ between tissues as indicated by the data obtained in the present study, in which higher COX-1 gene expression was detected in the glandular gastric and bladder mucosa before and after treatment; at each time point, the latter pairs of samples were each considered as 1 biopsy specimen. Values (which are unitless) indicate gene expression relative to expression of the HKG ubiquitin-C. For either isoenzyme in any tissue, no significant differences were evident within groups (day 0 vs day 8) or between groups (day 0 data comparisons and day 8 data comparisons).

Cyclooxygenase-1 and -2 are bifunctional enzymes that mediate 2 reactions: the double deoxygenation of arachidonic acid to PGG2 and the reduction of PGG2 to PGH2. Arachidonic acid oxygenation occurs in the COX active site, and PGG2 reduction occurs in the peroxidase active site. With the exception of acetylsalicylic acid, all other COX-2 inhibitors bind to proteins in a noncovalent manner in the COX active site. Therefore, NSAIDs do not act by reducing the expression of COX protein but instead by blocking the enzyme function. We expected an increase in COX gene expression as a response to the NSAID-associated decrease in COX protein activity in the horses of the present study. However, neither COX-1 nor COX-2 gene expression was affected by the administration of PBZ. This is in agreement with results of a study in dogs, in which no effect on protein expression for COX-1 in the pyloric and duodenal mucosa was detected after 3-day treatments with each of 3 NSAIDs (acetylsalicylic acid, carprofen, and deracoxib). However, in that study, COX-2 protein expression in the duodenal mucosa in the dogs receiving acetylsalicylic acid increased, compared with findings during treatment with carprofen or deracoxib. Differences in the NSAID used, administration protocols, species, and tissues make direct comparisons between studies difficult. It has been suggested that COX-2 has a role in mucosal protection. A study in rats revealed a rapid upregulation of COX-2 after oral administration of acetylsalicylic acid or indomethacin. To avoid misinterpretation of results in the present study, biopsy specimens were collected from opposing oral, gastric, and urinary bladder sites on days 0 and 8 and from areas where no active ulceration was observed. However, it is unknown whether upregulation of COX-2 gene expression in ulcerated or reddened areas in the stomach and urinary bladder occurs as a mechanism to provide mucosal protection and healing. A limitation of the present study was that the small number of horses used of PBZ, even when the drug is administered IV. Therefore, although expressed at lesser amounts than the amounts in the glandular gastric or bladder mucosa, PGs should be actively involved in the protection of the oral mucosa.

By contrast, COX-2 was not expressed in the oral mucosa biopsy specimens obtained in the present study but was expressed in the glandular gastric mucosa of the stomach and the mucosa of the urinary bladder. Cyclooxygenase-2 has been traditionally described to have a role in PG formation during pathological states such as inflammation and carcinogenesis. However, results of recent studies have challenged those findings and identified constitutive COX-2 expression in the brain, kidneys, and female reproductive tract of mammals. In addition, COX-2 is constitutively expressed in the mucosa of the small intestine of horses. Moreover, the reported cardiotoxic effects associated with the prolonged use of selective COX-2 inhibitors support a homeostatic role for COX-2. In the present study, the finding that COX-2 was constitutively expressed in the glandular gastric and bladder mucosa supported the suggestion that COX-2 may have some housekeeping role in the proximal portion of the gastrointestinal and urinary tracts.
could have affected the power of the test (the probability of correctly rejecting a false null hypothesis), thereby limiting our ability to detect a significant difference between groups.

In the horses used in the present study, no difference in serum total protein or albumin concentration was evident after 7 days of PBZ treatment. This is similar to results of a previous study, in which the same dose, frequency, and route of PBZ administration were investigated and no changes were found in serum total protein or albumin concentration after a 5-day treatment period.

Renal papillary or medullary crest necrosis has been described as an adverse effect of various dosages of PBZ used in horses and foals in clinical and experimental settings. Development of renal papillary necrosis is due to impaired blood supply, particularly in the medulla of the kidneys. Inhibition of PG synthesis by NSAIDs results in decreased ability of the kidneys to autoregulate blood flow. Although the problem is exacerbated by concurrent dehydration, renal crest necrosis has been observed in the kidneys of clinically normal horses receiving recommended dosages of PBZ. In addition, results of urinalysis, fractional assessment of urinary protein or albumin concentration after a 5-day treatment period.

Equine clinicians should judiciously and carefully make recommendations for the use of PBZ. There is an extreme susceptibility to the drug in some patients. The development of acute necrotizing enterocolitis, with severe hypoproteinemia and hypoaalbuminemia, in 2 horses after 5 and 7 days of PBZ administration at the same dosage and route used in the present study has been reported. Veterinarians prescribing PBZ for horses that are exposed to stressful conditions for long periods of time should consider administration of agents with proven efficacy and fewer adverse effects as an alternative (eg, selective COX-2 inhibitors). Because the use of selective COX inhibitors still allows PG production by COX-1, selective or partial COX inhibitors may have less deleterious effects on mucosal health. In a study comparing firocoxib and PBZ in horses with osteoarthritis, both agents had comparable efficacy. In addition, no adverse effects have been observed in horses after 30 days of oral administration of firocoxib at the manufacturer's recommended dosage. The traditional role of COX-2 as being only an inducible enzyme in the mediation of pain or inflammation may not be completely true. The observation of COX-2 gene and protein expression in several normal tissues suggests a physiologic role for this enzyme. However, increased COX-2 expression is associated with jejunal inflammation in horses. In addition, the treatment of ischemia-injured jejunum of horses with a nonselective COX inhibitor delayed mucosal recovery, but treatment with selective COX-2 inhibitors did not. The exact role of the COX isoenzymes in the gastrointestinal tract remains to be determined, as does the clinical relevance of the results of the present study, which have suggested that horses express COX-2 constitutively in the glandular gastric and bladder mucosa but not in the oral mucosa. Nevertheless, it is important to note that administration of PBZ for 7 days at a recommended dosage did not affect COX gene expression in healthy horses.

References


