Effects of flunixin meglumine or etodolac treatment on mucosal recovery of equine jejenum after ischemia

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Objective—To examine the effects of flunixin meglumine and etodolac treatment on recovery of ischemic-injured equine jejunal mucosa after 18 hours of reperfusion.

Animals—24 horses.

Procedure—Jejunum was exposed to 2 hours of ischemia during anesthesia. Horses received saline (0.9% NaCl) solution (12 mL, IV, q 12 h), flunixin meglumine (1.1 mg/kg, IV, q 12 h), or etodolac (23 mg/kg, IV, q 12 h). Tissue specimens were obtained from ischemic-injured and nonischemic jejunum immediately after ischemia and 18 hours after recovery from ischemia. Transepithelial electric resistance (TER) and transepithelial flux of tritium-labeled mannitol measured mucosal permeability. Denuded villous surface area and mean epithelial neutrophil count per mm² were calculated. Western blot analysis for cyclooxygenase (COX)-1 and -2 was performed. Pharmacokinetics of flunixin and etodolac and eicosanoid concentrations were determined.

Results—Ischemic-injured tissue from horses treated with flunixin and etodolac had significantly lower TER and increased permeability to mannitol, compared with that from horses treated with saline solution. Epithelial denudation after ischemia and 18 hours after recovery was not significantly different among treatments. Both COX-1 and -2 were expressed in ischemic-injured and nonischemic tissues. Ischemia caused significant upregulation of both COX isoforms. Eicosanoid concentrations were significantly lower in tissues from flunixin and etodolac-treated horses, compared with that from horses treated with saline solution.

Conclusions and Clinical Relevance—Flunixin and etodolac treatment retarded recovery of intestinal barrier function in jejunal mucosa after 18 hours of reperfusion, whereas tissues from horses treated with saline solution recovered baseline values of TER and permeability to mannitol. (Am J Vet Res 2004;65:761–769)

Intestinal disease is the leading known cause of death in horses. The estimated annual cost of intestinal disease in horses in the United States is $115,300,000. Death is largely because of strangulating intestinal disease resulting in loss of intestinal barrier function, hypovolemia, and endotoxic shock. Although ischemic intestine is resected when possible at surgery, remaining intestine may be injured as a result of distention or the surgeon's inability to detect or adequately resect all damaged intestine. Gerard et al found evidence of serosal injury and neutrophil infiltration in the proximal margins of resected ischemic intestine, indicating that not all damaged intestine had been removed.

Nonsteroidal anti-inflammatory drugs (NSAIDs), particularly flunixin meglumine, are frequently administered to horses with colic to provide analgesia and ameliorate signs of endotoxemia. Unfortunately, NSAIDs can have adverse effects. They have been found to cause gastrointestinal ulcers in horses, rats, and humans and colonic ulceration in horses and also to retard healing of injured intestinal tissue. Nonsteroidal anti-inflammatory drugs inhibit the prostaglandin (PG)-producing enzyme cyclooxygenase (COX). This enzyme produces PGH₂ in a 2-step reaction from arachidonic acid. Prostaglandins are local inflammatory hormones that are involved in leukocyte chemotaxis, nociception, vasodilatation, and other aspects of the inflammatory cascade. However, PGs also have a physiologic function in the maintenance of gastrointestinal mucosa. There are 3 principal COX isoforms. Cyclooxygenase-1 is constitutively expressed in most tissues, including the gastrointestinal tract, and is believed to be responsible for basal PG production under physiologic conditions. Alternatively, COX-2 is not detectable in most tissues under normal conditions but is upregulated in response to inflammation. However, there is some constitutive expression of COX-2 in the gastrointestinal tract and kidneys in humans. Cyclooxygenase-3 is a constitutive enzyme and a variant of the COX-1 gene; it is principally found in the cerebral cortex and heart. The function of COX-3 remains to be fully determined. Nonselective COX inhibitors such as flunixin meglumine block the action of all known COX isoforms and therefore markedly decrease PG concentrations.

By use of the concept that COX-1 is responsible for normal physiologic function, whereas COX-2 is responsible for the production of prostaglandins during inflammation, so-called gastrointestinal-safe NSAIDs have been developed that selectively inhibit COX-2. However, all available drugs inhibit both COX isoforms to a certain extent, which may be measured by a variety of COX selectivity assays. Therefore, there is a range of NSAIDs available with different selectivity for the COX isoforms. For example, etodolac and nabumetone preferentially inhibit COX-2 at low doses, but this effect is diminished at higher doses. Selectivity is also greatly dependent on the type of NSAID chosen.
of assay performed and the target species. There are no reported studies of etodolac selectivity in horses. In humans, the concentration of drug required for 50% inhibition (IC_{50}) of COX-1 and COX-2 for etodolac is 15μM and 1.4μM, respectively. This is a COX-1-to-COX-2 ratio of 10.7. Therefore, the window of selectivity (dose range in which inhibition is at least partially selective for COX-2) for etodolac is narrow; a dose of etodolac that is 10-fold that of the IC_{50} for COX-2 will result in approximately 50% inhibition of COX-1.2 Results of other studies indicate a variation in etodolac selectivity ratios from 2 to 100, depending on the assay that was performed. In horses, flunixin appears to have a slight preferential inhibition of COX-1-to-COX-2 ratio of 3.27 Results of a previous study indicate that in vitro treatment of ischemic-injured equine jejunal with flunixin meglumine retarded recovery of intestinal barrier function, whereas etodolac permitted mucosal recovery. Etodolac was chosen because of its availability on the veterinary market, although it is not yet licensed for use in horses. Horses that undergo surgery for ischemic small intestinal disease generally have severely ischemic tissue removed, and it is the remaining reperfused tissue that must undergo recovery. The purpose of the study reported here was to examine the effects of flunixin meglumine and etodolac treatment on the recovery of ischemic-injured equine jejunal mucosa after 18 hours of reperfusion.

Materials and Methods

Animals—Four groups of 6 healthy horses, 2 to 15 years of age and weighing 350 to 620 kg, were included in the study. Horses underwent a 2-week quarantine period during which time they were vaccinated and anthelmintics were administered prophylactically. Horses were housed at the university research facility for 2 to 4 months before they were used in the study. The North Carolina State University Institutional Animal Care and Use Committee approved the experimental protocol.

Surgical procedures—Cefiofur sodium (2.2 mg/kg, IV) was administered prophylactically 1 hour before surgery. Each horse was premedicated with xylazine (1.1 mg/kg, IV), followed by placement of a catheter in the left jugular vein. Anesthesia was induced with diazepam (0.1 mg/kg, IV) and ketamine (2.2 mg/kg, IV). Horses were orotracheally intubated, and a surgical plane of anesthesia was maintained with halothane vaporized in oxygen. Venous blood samples were obtained immediately before ischemia and 6 hours after the end of ischemia for measurement of eicosanoid concentrations in plasma. A midline celiotomy was performed by use of aseptic technique, and the terminal jejunum was located. Two 30-cm sections of jejunum were chosen approximately 1 m apart; the first segment was 61 cm oral to the antimesenteric band of the ileum. The local jejunal blood supply was occluded in 1 of the segments with Kelly hemostats placed over a Penrose drain to avoid damage to the blood vessels. The segment was cross-clamped with Doyen forceps to ensure that no blood flow was received from adjacent intestines. Blood supply was occluded for 2 hours. At the end of the period of ischemia, the clamps were released. At this time, 1 group of horses received saline (0.9% NaCl) solution (12 mL, IV, q 12 h) via the left jugular catheter, 1 group received flunixin meglumine (1.1 mg/kg, IV, q 12 h), and 1 group received etodolac (23 mg/kg, IV, q 12 h). The dosage of etodolac was determined on the basis of a therapeutic range extrapolated from published pharmacokinetic studies in dogs and humans and results of a preliminary study performed on 6 unrelated horses. Venous blood was sampled for pharmacokinetic analysis immediately after flunixin meglumine and etodolac were first administered and 2 and 4 hours after administration. One full-thickness wedge (1 cm) biopsy specimen was obtained from the ischemic-injured intestinal segment and 1 from nonischemic (control) jejunum located approximately 1 m oral to the ischemic segment, which had previously been unhandled. The defects were sutured with 2-0 polyglygin 910 in a Lembert pattern. The abdomen was closed in 3 layers with a simple continuous suture of 3-0 polyglatin 910 in the linea alba, a simple continuous suture of 2-0 polyglygin 910 in the subcutaneous tissues, and staples in the skin. The fourth group of 6 horses underwent surgically induced ischemia but received no treatment, and horses were not recovered from anesthesia; these horses were used to provide tissues for western blot technique and additional unrelated studies. Horses recovered from anesthesia in a padded recovery room. Each horse was moved to a stall and monitored for signs of pain, and a pain score was determined 2 hours after recovery by use of a previously established behavioral scoring system (Appendix). After pain was scored, horses in all treatment groups received butorphanol for the first 8 hours after surgery (0.05 mg/kg, IM, q 4 h) to alleviate pain. The experimental groups continued to receive flunixin meglumine or etodolac every 12 hours until they were euthanatized. Pain was scored 2 and 18 hours after the cessation of ischemia (10 hours after the last dose of butorphanol was administered), and horses were euthanatized with an overdose of sodium pentobarbital (100 mg/kg, IV). After euthanasia, ischemic-injured and nonischemic jejunal tissues were harvested for in vitro experiments.

Ussing chamber studies—Jejunum was incised along the antimesenteric surface. The mucosa was stripped from the serosal muscular layer in oxygenated (95% O2 and 5% CO2) equine Ringer's solution, as prepared in another study.5 Tissue was mounted in 3.14-cm2 aperture Ussing chambers, as described in another study.8 Tissues were bathed on the serosal and mucosal sides with 10 mL of equine Ringer's solution. The solution on the serosal side contained 10 mmol of glucose/L and was osmotically balanced on the mucosal side with 10 mmol of mannitol/L. Bathing solutions were oxygenated (95% O2 and 5% CO2) and circulated in water-jacketed reservoirs at 37°C. After a 15-minute equilibration period, the spontaneous potential difference (PD) was measured by use of Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuit ed through silver-silver chloride electrodes with a voltage clamp that corrected for fluid resistance. Resistance (Ω·cm2) was calculated from the spontaneous PD and short-circuit current. If the spontaneous PD was between –1.0 and 1.0 mV, an artificial current of 100 μA was passed across the tissue for 5 seconds and PD was recorded to increase the accuracy of the measurement. Short-circuit current and PD were recorded every 15 minutes for 2 hours. Data were entered into spreadsheet sheets that calculated the transepithelial electric resistance (TER) from short-circuit current and PD by use of Ohm's law.

Mannitol flux—Mucosal-to-serosal mannitol fluxes were performed by adding tritium-labeled mannitol (H-mannitol; 10 μCi/mL) to the mucosal bathing solutions once tissues were mounted in the Ussing chambers. Time zero samples (500 μL) were taken from the reservoirs after a 15-minute equilibration period. Samples were collected at 30, 60, and 90 minutes into scintillation vials and assessed for β emission (counts/min).
Histologic examination—Mucosal samples of ischemic-injured and nonischemic (control) jejunum were obtained immediately after the 2-hour period of ischemia (biopsy specimens) and at euthanasia (18 hours after recovery from ischemia). Five-mm cross-sections taken at 300-µm intervals were stained with H&E. For each tissue, 2 investigators independently evaluated 3 sections. Three well-orientated villi and crypts were identified in each section. The length of the crypt and villus and the width at the midpoint of the villus were obtained by use of a micrometer in the eyepiece of a light microscope. In addition, the height of the epithelial covered portion of each villus was measured. Data from the 2 evaluators were pooled before any data manipulations were performed. The surface area of the villus was calculated by use of a modified formula for surface area of a cylinder. Villus surface area = \( \frac{\pi d^2}{4} \times \frac{h}{2} \), where \( \pi = 3.14 \), \( d = \) villus diameter at midpoint, and \( h = \) villus height.\(^{16}\) The denuded villus surface area was calculated from the total surface area of the villus and surface area of the villus covered by epithelium. Epithelial neutrophils were counted in a 10-µm\(^2\) grid on 3 different villous tips, and the mean count per mm\(^2\) was calculated.

Eicosanoid concentrations and pharmacokinetic measurements—Plasma obtained immediately before ischemia (0 hours) and 6 hours after the end of ischemia was frozen at –70°C and subsequently evaluated for the COX products PG\(_2\)E\(_2\), PGE\(_2\), 6-keto-PGF\(_{1\alpha}\), (a stable metabolite of PGL\(_2\)), and thromboxane B\(_2\) (TXB\(_2\); a stable metabolite of TxA\(_2\)). Assays were performed by use of ELISA kits.\(^{7}\) Only 2 horses in the group treated with saline solution had plasma samples evaluated because of problems with sample storage.

Pharmacokinetics of flunixin meglumine and etodolac were evaluated by reverse-phase high-performance liquid chromatography with UV detection on plasma samples obtained immediately after the drugs were first administered (0 hours) and at 2 and 4 hours after first administration to confirm therapeutic concentrations.

Gel electrophoresis and western blot technique—Mucosal samples of ischemic-injured and nonischemic jejunum from the group of 6 horses that were not recovered were obtained immediately after the 2-hour period of ischemia. Mucosal samples from ischemic-injured and nonischemic jejunum were obtained at euthanasia (18 hours after recovery from ischemia) from horses in the remaining 3 treatment groups. Because the experimental design examined the effect of treatment over the entire time period among groups. The repeated measurements for eicosanoid concentrations were calculated as a percentage of each horse's baseline value because of a wide variation in baseline concentrations. Mean ± SE was calculated for each treatment group. One-way ANOVA tested for the effects of treatment between the flunixin and etodolac groups; the group treated with saline solution was omitted because results were available from only 2 horses. Flunixin and etodolac pharmacokinetics were reported as mean ± SE for each treatment group and time period. Western blot densitometry measurements (COX-1 and -2 protein concentrations) were analyzed in each treatment group with a 1-way repeated-measures ANOVA for the effect of ischemia. Pain scores in each treatment group were calculated as median and range. For each statistical test, the power of the analysis was determined. A value of \( P < 0.05 \) was considered significant.

Results

Transepithelial electrical resistance—There was a significant time and treatment interaction on TER (Fig 1). No significant difference was detected for the effect of treatment over the entire time period among groups. Because the experimental design examined the effects of in vivo flunixin and etodolac treatment on
intestinal recovery from ischemia, the TER data from the first 45 minutes were analyzed to minimize the potential effects of in vitro recovery of mucosa exposed to ideal oxygenation and glucose concentration and in the absence of the COX inhibitors given in vivo. During this period, ischemic-injured tissues from horses treated with flunixin meglumine (P = 0.006) or etodolac (P = 0.04) had a significantly lower TER than ischemic-injured tissues from horses treated with saline solution; however, there was no significant difference between the flunixin meglumine and etodolac treatment groups. In nonischemic tissues, there was no significant difference in TER among treatments.

Mannitol flux—There was a significant time and treatment interaction for mannitol flux (Fig 2). Analysis of the 30-minute flux revealed that ischemic-injured tissues from horses treated with flunixin meglumine (P = 0.024) and etodolac (P = 0.014) had significantly increased permeability to mannitol, compared with tissues from horses treated with saline solution. The 60-minute flux had a significant (P = 0.038) increase in permeability in the etodolac-treated group only. The flunixin-treated group had wide variation in permeability among horses at 60 minutes. By the 90-minute flux, there was no significant difference in mucosal permeability among treatment groups. In nonischemic tissues, there was no significant difference in permeability among treatment groups at any time point.

Histologic examination—Ischemia caused epithelial denudation in all treatment groups (Fig 3). Mean denudation of ischemic tissues in all treatment groups immediately after 2 hours of ischemia was 49%.

Evidence of restitution was observed 18 hours after recovery, with a decrease in denudation from 10.2% to 18.8% in all treatment groups (Table 1). There was no significant difference among treatment groups in the amount of denudation immediately after ischemia, and there was no significant effect of treatment on the amount of denudation 18 hours after recovery. However, results of analysis within each treatment group of the amount of denudation remaining 18 hours after recovery indicated that residual denudation was significantly different from that in nonischemic tissues from horses treated with flunixin meglumine but not from those treated with saline solution or etodolac.

Histologic examination of biopsy specimens from nonischemic jejunum from all treatment groups revealed no evidence of denudation. Eighteen hours after recovery, nonischemic jejunum had a small amount of epithelial denudation (mean, 4.8%), but this was not significantly different among treatments. This value was also not significantly different from the 0% denudation observed in biopsy specimens from nonischemic jejunum within any of the treatment groups. The cause of epithelial denudation in all nonischemic tissues was unknown.

There was a significant (P = 0.001) increase in the number of infiltrating neutrophils in ischemic-injured tissues 18 hours after recovery, compared with that obtained 2 hours after ischemia (Table 2). There was no significant effect of treatment on neutrophil numbers in ischemic-injured tissues obtained 2 hours after ischemia or 18 hours after recovery. There was no significant effect of treatment on neutrophil numbers in nonischemic tissues obtained 2 hours after ischemia. However, 18 hours after recovery, neutrophil numbers were significantly (P = 0.02) higher in nonischemic tissues from horses treated with etodolac than in those treated with flunixin meglumine and saline solution.
Table 2—Mean ± SE number of neutrophils in epithelium of jejunal mucosa from horses treated with saline solution (12 mL, IV q 12 h), flunixin meglumine (1.1 mg/kg, IV, q 12 h), or etodolac (225 mg/kg, IV, q 12 h) and exposed (ischemia) or not exposed (control) to 2 hours of ischemia. Tissues were obtained immediately after ischemia (0 h) and 18 hours after recovery from ischemia.

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Treatment</th>
<th>0 h</th>
<th>18 h</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>Saline solution</td>
<td>33 ± 17.7</td>
<td>33.2 ± 14.9</td>
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<tr>
<td></td>
<td>Flunixin</td>
<td>28.3 ± 11.3</td>
<td>40.3 ± 8</td>
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<tr>
<td></td>
<td>Etodolac</td>
<td>72 ± 28.5</td>
<td>88.7 ± 11*</td>
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<tr>
<td>Ischemic</td>
<td>Saline solution</td>
<td>77.5 ± 14.7</td>
<td>330.5 ± 61.5 1f</td>
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<tr>
<td></td>
<td>Flunixin</td>
<td>47 ± 11.7</td>
<td>355.5 ± 59.1</td>
</tr>
<tr>
<td></td>
<td>Etodolac</td>
<td>97 ± 19.5</td>
<td>385.8 ± 39.11</td>
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</table>

*Significantly (P < 0.05) different from control values for saline solution and flunixin at 18 hours. 1Significantly (P < 0.05) different from 0-hour values.

Eicosanoid concentrations—Baseline concentrations of each eicosanoid in plasma varied widely between horses (eg, the concentrations of TXB2 varied from 9 to 423 pg/mL). Therefore, results are reported as percentage (mean ± SE) of baseline values for each horse. Plasma concentrations of PGE2 increased 6 hours after ischemia to 275 ± 3.72% of baseline values in horses treated with saline solution. Plasma concentrations of PGE2 remained at 89 ± 5.9% of baseline values in horses treated with saline solution. Plasma concentrations of TXB2 increased 6 hours after ischemia to 3,000 ± 7.6% of baseline values in horses treated with saline solution. In horses treated with flunixin meglumine, plasma concentrations of TXB2 increased 6 hours after ischemia to 63 ± 6% of baseline values. Results for the plasma concentrations of TXB2 after 6 hours of ischemia in horses treated with etodolac were similar, with a decrease to 63 ± 3.2% of baseline values. Plasma concentration of 6-keto-PGF1α increased 6 hours after ischemia to 3,203 ± 19% of baseline values in horses treated with saline solution. In horses treated with flunixin meglumine, plasma concentrations of 6-keto-PGF1α decreased 6 hours after ischemia to 63 ± 6.4% of baseline values. Plasma concentrations of 6-keto-PGF1α in horses treated with etodolac decreased to 47.9 ± 13.2% of baseline values 6 hours after ischemia. However, there was a wide variation in values. There was no significant difference in prostanooid concentrations (mean ± SE) in plasma between the flunixin and etodolac-treated groups.

Pharmacokinetics—Mean ± SE concentrations of flunixin meglumine in plasma at 0, 2, and 4 hours after administration were 11.9 ± 1.7 µg/mL, 5.63 ± 0.37 µg/mL, and 2.67 ± 0.22 µg/mL, respectively. Mean ± SE concentrations of etodolac in plasma at 0, 2, and 4 hours after administration were 89 ± 11.4 µg/mL, 20.2 ± 2.16 µg/mL, and 4.7 ± 0.43 µg/mL, respectively.

Western blot analysis for COX-1 and -2—Both COX-1 and COX-2 were expressed in nonischemic jejunal tissues obtained 2 hours after ischemia from the group of untreated horses (Fig 4). In ischemic-injured tissues, results of densitometric analysis indicated that the amount of both COX isoforms was significantly (COX-1, P = 0.035; COX-2, P = 0.046) greater than that from nonischemic tissues.

Both COX-1 and COX-2 were also expressed in nonischemic jejunal tissues obtained 18 hours after recovery. There was no significant difference in the concentrations of COX-1 and COX-2 among treatments. Concentrations of COX-1 and COX-2 in ischemic jejunal tissues were not significantly higher than those in nonischemic tissue except for COX-2 expression in jejunal tissues from horses treated with etodolac (P < 0.001).

Pain scores—Pain scores for horses treated with flunixin meglumine and etodolac appeared similar and were different from those in horses treated with saline solution at 2 hours and 18 hours after recovery from anesthesia. Median pain scores at 2 hours were 17.5 (range, 16 to 20), 12 (range, 11 to 14), and 12 (range, 10 to 14) for horses treated with saline solution, flunixin meglumine, and etodolac, respectively. By 18 hours, pain scores had decreased in all groups to 12 (range, 11 to 16), 9 (range, 9 to 11), and 9 (range, 9 to 10) for horses treated with saline solution, flunixin meglumine, and etodolac, respectively.

Discussion

Nonsteroidal anti-inflammatory drugs are frequently used in horses with ischemic intestinal disease because they are effective analgesics and ameliorate signs of endotoxemia. However, it is important to determine whether clinical use of these drugs exacerbates intestinal injury or retards recovery. Results of a previous study indicate that in vitro treatment of ischemic-injured equine jejenum with the COX-1 and -2 inhibitor flunixin meglumine retards recovery of barrier function. The in vitro exposure of ischemic-injured jejenum to NSAIDs may not apply to the clinical setting in which an ischemic episode with subse-
quent reperfusion of the intestine occurs and NSAIDs are often administered systemically for days to ameliorate pain and endotoxemia. Nonsteroidal anti-inflammatory drugs may also add to ischemic injury during reperfusion by decreasing local blood flow caused by decreased PG production.

We investigated whether the in vitro effect of NSAID treatment was prolonged after an 18-hour in vivo recovery of jejunum from ischemia. Because flunixin meglumine is 3 times more selective for COX-1 in horses, we also examined whether a drug that reportedly preferentially inhibits COX-2 (etodolac) would permit enough PG production for adequate recovery. Results of our study indicated that both flunixin meglumine and etodolac treatment significantly retarded recovery of intestinal barrier function in equine jejunum that had undergone a period of ischemia followed by 18 hours of reperfusion, whereas horses treated with saline solution recovered baseline values of TER and permeability to mannitol.

Because the experimental design examined the effects of in vivo flunixin meglumine and etodolac treatment on intestinal recovery from ischemia, the TER data from the first 45 minutes were analyzed to minimize possible in vitro recovery of mucosa exposed to ideal oxygenation and glucose concentration within Ussing chambers and in the absence of the COX inhibitors given in vivo. Results of a previous study examining the effects of NSAIDs on the in vitro recovery of equine jejunum exposed to 1 hour of ischemia indicated that the initial TER (before NSAID treatment) of the ischemic-injured jejunum was approximately 40 Ω·cm². In our study, portions of the jejunum were exposed to 2 hours of ischemia; therefore, the level of injury should be more severe. Thus, in an 18-hour in vivo recovery period, the TER of ischemic-injured jejunum recovered from a probable value of ≤40 Ω·cm² to 65 Ω·cm² in tissues from horses treated with saline solution and only 45 to 50 Ω·cm² in tissues from horses treated with etodolac and flunixin meglumine. After the in vitro (no longer exposed to NSAIDs) period of 2 hours on the Ussing chamber, the TER in all treatment groups had recovered to between 60 to 70 Ω·cm², indicating that there was an in vitro recovery response superimposed on the 18-hour in vivo recovery response. Hence, it was concluded that the data obtained early in the in vitro period were a valid representation of the effects of in vivo NSAID treatment.

Although etodolac preferentially inhibits COX-2 in certain species, it appears that this drug does not permit sufficient PG production in horses for adequate recovery from ischemia at 18 hours. There are several reasons that could account for this, including a lack of selectivity of etodolac for COX-2 in horses (thereby potentially serving as a nonselective COX inhibitor) at the dosage administered. An alternative explanation is that prostanoids induced by COX-2 are important for mucosal recovery, as has been suggested in other studies.

The pharmacokinetic data on flunixin meglumine agree with results of previous studies and so were within the therapeutic range. Etodolac concentrations in plasma were similar to those from a separate unpublished preliminary pharmacokinetic study in horses (range, 9.73 to 19.18 µg/mL; mean, 15.97 µg/mL). However, there is no currently published therapeutic range for etodolac in horses.

Blood samples were obtained to assess COX activity by measuring prostanoid concentrations in plasma. Although there was a large reduction in the concentrations of 6-keto-PGF₁α, TXB₂, and PGE₂ in response to treatment with etodolac or flunixin meglumine, compared with that after treatment with saline solution, there was no significant difference in the concentrations of these prostanoids between NSAID treatment groups. Thromboxane B₂ in whole blood has been used as an index of COX-1 activity because of the colocalization of COX-1 with thromboxane synthase in platelets. This indicates that the action of etodolac at the dose used is not COX-2 selective. In humans, etodolac selectivity for COX-2 is only approximately 10-fold, meaning that etodolac inhibits COX-2 only 10 times more than it inhibits COX-1. In the study reported here, the concentration of etodolac conferring COX-2 selectivity in horses may have been exceeded at 23 mg/kg. This is in contrast to results of a previous in vitro study, in which etodolac administered to tissues at a concentration of 2.7 X 10⁻⁷ M did not inhibit production of TXB₂, whereas it did partially inhibit PGE₂ and 6-keto-PGF₁α production, suggesting a COX-2 selective profile.

However, it can be argued that prostanoid analyses are not specific for select COX isoforms because both COX-1 and COX-2 produce the same intermediary prostanoïd (PGH₂) and local synthases modify PGH₂ to form the specific prostanoids measured. Therefore, the prostanoids induced depend on which synthase enzyme is associated with which COX isoenzymes, a fact that may be tissue dependent.

Results of the western blot analyses indicated that in equine jejunal tissue, COX-1 and COX-2 are constitutively expressed, although COX-2 is typically regarded as an inducible enzyme in gastrointestinal mucosa. Vogiagis et al identified mRNA from both COX-1 and COX-2 in normal stomach tissue in rats. An alternative interpretation in the present study is that the apparent constitutive expression of COX-2 may reflect induction caused by ischemia in adjacent tissue. Tissue for western blot analysis sampled immediately after ischemia was from the untreated group of horses that were not recovered. Biopsies were not obtained in these horses, and nonischemic jejunum was harvested at least 2 m oral to the ischemic jejunum, where the intestine was deep in the abdomen during the period of ischemia, not adjacent to the ischemic section. Therefore, the authors refute this theory for the samples obtained during surgery. However, in the samples obtained 18 hours after recovery from ischemia, there probably was an effect caused by inflammation of the adjacent ischemic-injured intestine.

In the study reported here, both COX isoforms were upregulated in response to ischemia, although
COX-1 is typically regarded as constitutive in gastrointestinal mucosa. Upregulation of COX-1 has been reported in microglial cells in the brain in response to injury. Results of that study challenge the paradigm that COX-2 is the sole inflammatory isoform of the enzyme that is upregulated in response to injury. Results of densitometry 18 hours after recovery indicated that there was only a significant increase in COX-2 expression in etodolac-treated horses. Expression may have been upregulated in the other treatment groups, but we were unable to detect a difference because the power of the ANOVA test used was only 0.2 (less than the desired power of 0.8) and probably resulted from insufficient numbers of horses to discern smaller differences in other treatment groups. Another reason for no detectable increase in the COX enzymes in ischemic tissue 18 hours after recovery may be that COX concentrations are no longer increased because the tissue has partially recovered.

There was no significant difference detected in the extent of villus denudation between treatment groups immediately after ischemia and 18 hours after recovery. Resutition occurred during the recovery period, but after 18 hours of recovery from ischemia, epithelial restitution was not complete (denudation was 10.2% to 18.8%). The amount of residual denudation in mucosa after ischemia, compared with nonischemic tissue within each treatment group, was significantly increased in tissues from horses treated with flunixin meglumine but not in that from horses treated with etodolac or saline solution. This indicates that there was significant histologic damage in tissues from horses treated with flunixin meglumine, which may have contributed to the increased TER and permeability to H-mannitol. However, etodolac treatment significantly decreased TER and increased mannitol permeability despite no significant residual epithelial denudation, and the effects of flunixin meglumine and etodolac treatment on TER were not significantly different from each other. Therefore, changes resulting in increased permeability of jejunal tissue from horses treated with etodolac appear to be solely caused by changes in the paracellular pathway that is regulated by interepithelial tight junctions. Changes in the permeability of jejunal tissue from horses treated with flunixin meglumine appear to be mainly caused by the effect on tight junctions, despite significant (18.8%) epithelial denudation. Results of a study on bile-injured porcine ileum indicate that prostaglandin-mediated regulation, and not restitution, of paracellular permeability (tight junctions) was mainly responsible for the recovery of TER. The paracellular pathway contributes a large surface area that is potentially permeable to small molecules. Even when the epithelium was 42% denuded in that study, the paracellular pathway provided the bulk of the surface area for these molecules to cross. After stimulation of restitution from 49% to 100% by use of growth factors, the TER of porcine ileum did not recover to baseline values without endogenous prostaglandin-mediated closure of tight junctions. Results of a previous study in porcine ileum indicate that rescaling of tight junctions during recovery from ischemic injury appears to be largely regulated by PGs.

In the present study, tissues that underwent ischemia were able to fully recover normal indices of TER and mannitol permeability within 18 hours in horses treated with saline solution despite neutrophil infiltration and residual denudation, suggesting compensation by adjacent restituted epithelium, possibly as a result of heightened apposition of tight junctions. The lack of complete restitution in all tissues that underwent ischemia may have been caused by inflammation, considering the significant increase in mucosal neutrophil numbers in those tissues 18 hours after recovery. Results of a study with porcine ileum indicate that mucosal repair events can be inhibited by neutrophil infiltration 6 to 18 hours after injury. The neutrophils migrate between restituting epithelial cells and release reactive oxygen metabolites, which damage the epithelium.

Flunixin meglumine and etodolac may have enhanced ischemic injury in addition to inhibiting recovery. Drugs were administered immediately after 2 hours of ischemia to reflect a clinically relevant scenario, but the peak drug concentrations occurring at the time of reperfusion could have resulted in greater inhibition of PG synthesis, thus resulting in decreased blood flow. Therefore, it is conceivable that increases in mucosal permeability to mannitol in the presence of flunixin or etodolac during the recovery period could have been caused by increased mucosal damage during reperfusion, rather than inhibition of mucosal repair. However, the extent of histologic damage 18 hours after recovery in tissues from horses treated with NSAIDs was not significantly different from that in horses treated with saline solution in the absence of NSAIDs.

There was some evidence of epithelial denudation in nonischemic tissues 18 hours after recovery (approx 5%). The amount of denudation in those tissues was not significantly different from that in nonischemic tissues obtained at biopsy. The denudation may have been caused by handling at surgery because it was necessary to obtain biopsies, or it may have been caused by inflammation in the adjacent, ischemic-injured jejunum. However, neutrophil numbers were not significantly different in nonischemic tissues 18 hours after recovery, compared with those in nonischemic tissues obtained at biopsy.

Pain scores for horses treated with NSAIDs were less than those for horses treated with saline solution, which was as expected. The time points when pain scores were obtained were before or at least 10 hours after butorphanol administration, indicating that the pain scores were likely an accurate reflection of the analgesic properties of the NSAIDs alone. There may have been residual concentrations of butorphanol in horses 18 hours after recovery from ischemia, but butorphanol was administered at a consistent concentration in all treatment groups. On the basis of the conditions of this study, it appears that flunixin and etodolac are equally effective analgesics. Butorphanol is a partial µ opioid agonist. Stimulation of µ receptors causes decreased intestinal permeability, particularly during inflammation. This may have affected all of our horses, but comparison of results between treatment groups is still valid.
Although results of our study confirm that non-selective inhibition of PG production by flunixin meglumine retards mucosal recovery, we cannot be certain of the precise role of COX-1 and COX-2 because of the potential lack of selectivity of etodolac. Thus, it is possible that etodolac inhibits mucosal PG production to the same extent as flunixin meglumine, resulting in inhibition of mucosal recovery. The concentrations of systemic prostanoids would tend to support this premise. Alternatively, results of a previous study\(^{39,42}\) suggest that etodolac may be preferentially inhibiting COX-2, but that prostanooids produced by the COX-2 enzyme are required for full mucosal recovery. The latter is suggested by results of studies\(^{39,42}\) in other species in which selective inhibition of COX-2 reduced gastroduodenal epithelial repair. To answer these questions conclusively, a highly selective COX-2 inhibitor in horses (verified by selectivity assays) will be required.

Although the objective of this study was to examine the effects of flunixin meglumine and etodolac treatment in vivo, the effect of these drugs on intestinal barrier function may not outweigh their beneficial analgesic, anti-inflammatory, and antiendotoxic effects in the early postoperative period.

References

33. Sellon DC, Roberts MC, Blisklager A, et al. Effects of continuous intravenous infusion of butorphanol in horses after...


### Appendix

**Behavioral pain scoring system**

<table>
<thead>
<tr>
<th>Behavior category</th>
<th>Behavioral score to be assigned for each category</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gross pain</td>
<td>None</td>
<td>NA</td>
<td>Occasional</td>
<td>Continuous</td>
<td></td>
</tr>
<tr>
<td>Head position</td>
<td>Above withers</td>
<td>NA</td>
<td>At withers</td>
<td>Below withers</td>
<td></td>
</tr>
<tr>
<td>Ear position</td>
<td>Forward, frequent movement</td>
<td>NA</td>
<td>Slightly back, little movement</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td>At door watching environment</td>
<td>Standing in middle, facing front of stall</td>
<td>Standing in middle, facing sides of stall</td>
<td>Standing in middle, facing back of stall</td>
<td></td>
</tr>
<tr>
<td>Spontaneous locomotion</td>
<td>Moves freely</td>
<td>Occasional steps</td>
<td>NA</td>
<td>No movement</td>
<td></td>
</tr>
<tr>
<td>Response to another horse</td>
<td>Ears forward, head up, moves to door</td>
<td>Ears forward, head up, no movement to door</td>
<td>Ear flick, no movement to door</td>
<td>No response</td>
<td></td>
</tr>
<tr>
<td>Response to open door</td>
<td>Moves to door</td>
<td>Looks at door</td>
<td>NA</td>
<td>No response</td>
<td></td>
</tr>
<tr>
<td>Response to approach</td>
<td>Moves to observer, ears forward</td>
<td>Looks at observer, ears forward</td>
<td>Moves away</td>
<td>Does not move, ears back</td>
<td></td>
</tr>
<tr>
<td>Lifting feet</td>
<td>Freely when asked</td>
<td>After mild encouragement</td>
<td>NA</td>
<td>Unwilling</td>
<td></td>
</tr>
</tbody>
</table>

Scores are added to give a total subjective pain score.
NA = Not applicable.