

Effect of firocoxib or flunixin meglumine on recovery of ischemic-injured equine jejunum

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Objective—To determine whether treatment of horses with firocoxib affects recovery of ischemic-injured jejunum, while providing effective analgesia.

Animals—18 horses.

Procedures—Horses (n = 6 horses/group) received saline (0.9% NaCl) solution (1 mL/50 kg, IV), flunixin meglumine (1.1 mg/kg, IV, q 12 h), or firocoxib (0.09 mg/kg, IV, q 24 h) before 2 hours of jejunal ischemia. Horses were monitored via pain scores and received butorphanol for analgesia. After 18 hours, ischemic-injured and control mucosa were placed in Ussing chambers for measurement of transepithelial resistance and permeability to lipopolysaccharide. Histomorphometry was used to determine denuded villus surface area. Western blots for cyclooxygenase (COX)-1 and COX-2 were performed. Plasma thromboxane B₂ and prostaglandin E₂ metabolite (PGEM) concentrations were determined.

Results—Pain scores did not significantly increase after surgery in horses receiving flunixin meglumine or firocoxib. Transepithelial resistance of ischemic-injured jejunum from horses treated with flunixin meglumine was significantly lower than in saline- or firocoxib-treated horses. Lipopolysaccharide permeability across ischemic-injured mucosa was significantly increased in horses treated with flunixin meglumine. Treatment did not affect epithelial restitution. Cyclooxygenase-1 was constitutively expressed and COX-2 was upregulated after 2 hours of ischemia. Thromboxane B₂ concentration decreased with flunixin meglumine treatment but increased with firocoxib or saline treatment. Flunixin meglumine and firocoxib prevented an increase in PGEM concentration after surgery.

Conclusions and Clinical Relevance—Flunixin meglumine retarded mucosal recovery in ischemic-injured jejunum, whereas firocoxib did not. Flunixin meglumine and firocoxib were effective visceral analgesics. Firocoxib may be advantageous in horses recovering from ischemic intestinal injury. (*Am J Vet Res* 2009;70:992–1000)

Colic is second only to old age as the leading cause of death in horses, with an overall fatality rate of 11%.¹ Death is largely a result of strangulating obstruction of the intestines, which results in disruption of intestinal barrier function, endotoxemia, hypovolemia, and shock.² Although it is usually possible to resect ischemic small intestine during surgery, the remaining proximal portion of the jejunum is commonly damaged by distention,³ and there is ongoing microscopic injury,

ABBREVIATIONS

COX	Cyclooxygenase
FITC	Fluorescein isothiocyanate
I _{sc}	Short-circuit current
LPS	Lipopolysaccharide
LSD	Least significant difference
PG	Prostaglandin
TER	Transepithelial resistance
TX	Thromboxane

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as indicated by neutrophilic infiltration during the initial 18 hours after the end of ischemia.^{4,5} Many of the signs of endotoxemia, and much of the pain associated with strangulating obstruction, are attributable to PGs elaborated by the COX isoenzymes.

The NSAIDs, particularly flunixin meglumine, are commonly used to treat horses with colic to provide analgesia and to ameliorate signs of endotoxemia.^{6,7} The NSAIDs inhibit COX isoenzymes, which are rate-limiting factors in the production of PGH₂ from arachidonic acid. Local PG synthases then modify PGH₂ to form a wide range of other prostanoids, such as PGI₂, TXA₂, and PGE₂.⁸ However, administration of NSAIDs is not without adverse effects, such as gastric ulcers and renal crest necrosis.^{9–11} Additionally, they retard the recovery

of intestinal barrier function in ischemic-injured equine jejunal mucosa^{5,12} because PGs are critical for recovery of barrier function after ischemic injury.¹³

Three COX isoforms have been identified.¹⁴ Cyclooxygenase-1 is constitutively expressed in most tissues, including the equine jejunum,⁵ and is believed to be responsible for PG production during normal physiologic processes. In contrast, COX-2 is expressed at low concentrations in most normal tissues, including the equine jejunum,⁵ but it is upregulated in response to injury. The role of COX-3, a constitutively expressed isoform, in the gastrointestinal tract has not been determined.

Different NSAIDs have differing selectivity for the COX isoforms. For example, flunixin meglumine is a nonselective inhibitor of the COX enzymes and therefore decreases PG concentrations in tissue. A COX-2 selective NSAID may be advantageous for the treatment of horses with colic because it would inhibit the COX-2 isoform, which contributes to pain and inflammation, while allowing PG-mediated intestinal repair through COX-1-associated PGs.^{5,15} Such benefits were detected with the use of the COX-2 selective inhibitor meloxicam, which can provide adequate analgesia while permitting recovery of ischemic-injured equine jejunum.¹⁵ However, an injectable solution of meloxicam suitable for use in horses with colic is not currently available in the United States.

Firocoxib is an NSAID that has excellent COX-2 selectivity in the blood of dogs, cats, and horses.^{16,17,a} In horses, firocoxib is 265 times as selective for COX-2 as it is for COX-1, and it provides substantial analgesia in horses with experimentally induced lameness.^a Additionally, firocoxib is as effective as phenylbutazone for providing analgesia in horses with lameness attributable to naturally developing osteoarthritis, with no adverse effects detected.¹⁸ Because of the selectivity for COX-2 and the musculoskeletal analgesic properties of firocoxib in horses, we believed that investigation of the effects of an injectable form of firocoxib on recovery of ischemic-injured intestine was warranted. Thus, for the study reported here, our hypothesis was that treatment with firocoxib would allow optimal recovery of mucosal barrier function in ischemic-injured equine jejunum, compared with results after treatment with flunixin meglumine, while providing effective visceral analgesia.

Materials and Methods

Horses—Eighteen horses (3 to 20 years old and weighing between 378 and 605 kg) were used in the study. Horses had no history of colic; horses were quarantined for 2 weeks and were vaccinated and received anthelmintic treatment prior to use in the study. Before surgery, a complete physical examination was performed and baseline pain scores determined by use of an established behavioral pain scoring system.¹⁹ All procedures were approved by the North Carolina State University Animal Care and Use Committee.

Surgical procedures—Horses were randomly assigned to 1 of 3 treatment groups ($n = 6$ horses/group). There were no significant differences in age or body weight

of horses among the groups. Group 1 horses received saline (0.9% NaCl) solution (1 mL/kg, IV, q 24 h). Group 2 horses received flunixin meglumine (1.1 mg/kg, IV, q 12 h). Group 3 horses received firocoxib^b (0.09 mg/kg, IV, q 24 h). Immediately before surgery, each horse was sedated by administration of xylazine (0.5 to 1 mg/kg, IV), and a catheter was placed aseptically in the left jugular vein. Prophylactic antimicrobials (ceftiofur; 2.2 mg/kg, IV, once) and the test drug for the allocated treatment group were administered immediately before anesthetic induction. Anesthesia was induced by administration of diazepam (0.1 mg/kg, IV) and ketamine hydrochloride (3 mg/kg, IV); anesthesia was maintained by administration of isoflurane vaporized in oxygen via an orotracheal tube. To provide additional analgesia, all horses received butorphanol (0.05 mg/kg, IV) immediately after anesthetic induction and at 6-hour intervals thereafter (0.05 mg/kg, IM). After routine aseptic preparation, a ventral midline approach to the abdomen was used. A 30-cm loop of jejunum was isolated and cross-clamped by use of Doyen forceps to prevent collateral blood flow. To simulate a strangulating obstruction, the local mesenteric blood supply to the jejunum was temporarily occluded for 2 hours by use of Kelly clamps placed over a Penrose drain to minimize trauma to the vessels. At the end of the 2-hour period of ischemia, all clamps were removed, and full-thickness biopsy specimens of ischemic-injured and adjacent uninjured control jejunum were obtained via enterotomy at the antimesenteric border of the jejunum. Biopsy sites were closed with an inverting suture pattern, and the abdomen was then closed in a routine manner.

Horses were allowed to recover from anesthesia in a padded recovery stall and then returned to their individual stalls, where they had access to water and were offered small amounts of hay. All horses were monitored (vital signs, gastrointestinal borborygmi, appetite, defecation, and urination) at 4, 8, and 16 hours after the end of ischemia. Additionally, at these times, pain was assessed by use of a behavioral pain scoring system.¹⁹ Group 2 horses received an additional dose of flunixin meglumine 12 hours after the end of ischemia.

Eighteen hours after the end of ischemia, each horse was euthanatized by administration of an overdose of sodium pentobarbital (100 mg/kg, IV). Immediately after horses were euthanatized, ischemic-injured and control nonischemic jejunal tissues were harvested for ex vivo experiments. Tissues were incised along the antimesenteric border, rinsed in equine Ringer's solution (114mM NaCl, 5mM KCl, 1.25mM CaCl₂, 1.1mM MgCl₂, 25mM NaHCO₃, 0.3mM NaH₂PO₄, and 1.65mM Na₂HPO₄) to remove intestinal contents, and placed in preoxygenated equine Ringer's solution for immediate transport to our laboratory.

Plasma firocoxib concentrations—Venous blood samples were collected 5 minutes and 8 and 16 hours after IV administration of firocoxib. Plasma was separated and stored at -80°C for subsequent analysis of firocoxib concentrations by use of reverse-phase high-pressure liquid chromatography with UV detection.²⁰

Plasma eicosanoid concentrations—Venous blood samples were collected into EDTA-containing tubes before surgery and 8 hours after the end of ischemia. Plas-

ma was separated and stored at -80°C for subsequent analysis. The PGE_2 concentrations were estimated by converting all its unstable metabolites to the stable metabolite 13,14-dihydro-15-keto PGA_2 for quantification by use of a competitive enzyme immunoassay.^c The TXA_2 concentrations were determined by measuring the stable metabolite TXB_2 by use of a competitive enzyme immunoassay.^d

Electrical evaluation of tissues in an Ussing chamber—The mucosa was stripped from the seromuscular layers of ischemic-injured and nonischemic control jejunal tissues in oxygenated (95% oxygen and 5% carbon dioxide) equine Ringer's solution. Ischemic and control mucosal tissues were mounted in 3.14-cm^2 aperture Ussing chambers. Tissues were bathed on the mucosal and serosal sides with 10 mL of oxygenated equine Ringer's solution. In addition, the solution used for the serosal side contained 10 mmol of glucose/L, and the solution used for the mucosal side was osmotically balanced by the addition of 10 mmol of mannitol/L. The bathing solutions were circulated and maintained at 37°C by use of water-jacketed reservoirs. After a 15-minute equilibration period, the potential difference was measured by use of Ringer-agar bridges connected to calomel electrodes, and the I_{sc} was measured by use of an automated voltage clamp. Electrical measurements were recorded every 15 minutes for 3 hours. The TER was calculated from the I_{sc} and potential difference by use of Ohm's law.

Permeability evaluations—After 30 minutes of equilibration in Ussing chambers, $83\ \mu\text{g}$ of FITC-labeled LPS (*Escherichia coli* O55:B55) was added to the mucosal bathing solutions of control and ischemic tissues. Ussing chambers used for these evaluations were protected from light. Samples ($200\ \mu\text{L}$) were obtained (in triplicate) from both the mucosal and serosal sides immediately before (time 0) and 60 and 120 minutes after addition of the FITC-LPS. A standard concentration curve was constructed by use of various concentrations of FITC-LPS (0 to $0.005\ \mu\text{g/mL}$) to determine the concentration of FITC-LPS that crossed to the serosal side of the mucosa (as determined by use of a fluorometer) after 2 hours of in vitro incubation for control and ischemic-injured loops obtained from each of the 3 groups of horses.

Histologic examination—Light microscopy was used to examine biopsy specimens obtained at the end of ischemia and 18 hours after the end of ischemia from each horse. Three $5\text{-}\mu\text{m}$ -thick sections from each biopsy specimen aligned on the villus-crypt axis were stained with H&E. Total height, epithelial-covered height, and width of 3 appropriately oriented villi in each section were measured by use of a micrometer located in the eyepiece of a light microscope by 3 investigators who were unaware of the source of the tissue specimens. Total surface area denuded of epithelial cells was calculated by use of a modified equation for the surface area of a cylinder. First, total villus surface area was calculated by use of the following equation: villus surface area = $2\pi \cdot (0.5 \cdot [4/\pi]d) \cdot h$, where d is the villus width at its midpoint, and h is the villus height. The villus surface area remaining denuded of epithelium was then calcu-

lated by measuring the height of the epithelial-covered portion and subtracting that value from the total surface area. The denuded surface area was then expressed as a percentage of the total villus surface area.

SDS-PAGE and western blot analysis—Mucosal scrapings of control and ischemic-injured jejunum from each horse were obtained immediately after surgery and at the time of euthanasia; samples were snap-frozen in liquid nitrogen and stored at -80°C until analysis. One gram of each tissue sample was thawed to a temperature of 4°C ; it was then added to 2 mL of modified radioimmunoprecipitation buffer, which included the protease inhibitors aprotinin, phenylmethylsulfonyl fluoride, and sodium orthovanadate. Each sample was homogenized on ice, and the supernatant was then extracted by use of centrifugation. Protein analyses of aliquots of extracted samples were obtained, and equal concentrations of protein from each sample were mixed and boiled with sample buffer. Lysates were loaded into wells of a precast 10% SDS-polyacrylamide gel, and protein electrophoresis was performed in accordance with standard protocols.

After transfer to nitrocellulose membranes and blocking in 5% milk, membranes were cut horizontally at the approximate 50-kDa molecular-weight mark to allow separate incubation with COX primary antibody (upper part of the membrane) or β -actin primary antibody (lower part of the membrane). Incubations with the respective primary antibody were performed overnight at 4°C in a 1:200 solution of goat polyclonal COX-1 or COX-2 primary antibody^e or a 1:20,000 solution of rabbit polyclonal β -actin primary antibody.^f Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody and developed by addition of enhanced chemiluminescence reagent.^g Densitometric analysis of resulting blots was performed by use of specialized software.^h

Statistical analysis—Behavioral pain scores were examined by use of a 1-way ANOVA on ranks for the effect of treatment and time from 0 to 16 hours. When a significant interaction was detected, a pairwise multiple comparisons procedure (Tukey test) was used to identify the source of the interaction. The percentage change in TXB_2 and PGEM concentration between 0 and 8 hours was analyzed by use of a 1-way ANOVA followed by use of a multiple comparisons procedure (Holm-Sidak method). A 2-way repeated-measures ANOVA was used to analyze TER for the effect of treatment and ischemia over time. When a significant interaction was detected, a multiple comparisons procedure (Fisher LSD method) was performed. A 1-way ANOVA was performed on the ischemic-injured and control groups for the effect of treatment on LPS flux, followed by a pairwise multiple comparisons procedure (Fisher LSD method). Statistical analysis of histologic data was performed by use of a 2-way ANOVA for the effects of time and treatment, followed by use of a multiple comparisons procedure (Fisher LSD method) when a significant interaction was detected. For all tests, values of $P < 0.05$ were considered significant.

Results

Plasma firocoxib concentration—The plasma concentration of firocoxib peaked 5 minutes after IV ad-

ministration (mean \pm SEM, 0.217 ± 0.032 mg/mL). It then decreased (mean, 0.046 ± 0.005 mg/mL and 0.044 ± 0.005 mg/mL) at 8 and 16 hours after administration, respectively.

Behavioral pain scores—No horses had gross signs of pain at any time during the study. Median pain score was zero in all groups before surgery. Behavioral pain scores for horses treated with saline solution or flunixin meglumine have been published elsewhere.²¹ Experiments in these treatment groups were not repeated to avoid excessive use of horses. Horses treated with saline solution had significantly ($P = 0.002$) higher pain scores 4 and 8 hours after surgery, compared with scores for horses in that group before surgery (Figure 1). However, horses treated with flunixin meglumine or firocoxib had no significant change in postoperative pain score, compared with values before surgery for the respective groups. Additionally, at 4 hours after surgery, horses treated with saline solution had significantly higher pain scores, compared with the scores for horses treated with flunixin meglumine or firocoxib. At 8 hours after surgery, pain scores were significantly ($P = 0.033$) higher in horses treated with saline solution, compared with scores for horses treated with flunixin meglumine. Pain scores of horses treated with flunixin meglumine were not significantly different at any time point from scores of horses treated with firocoxib. Pain scores at 16 hours after surgery were not significantly different among treatment groups or from scores before surgery.

Plasma eicosanoid concentrations—Plasma concentrations of TXB₂ and PGEM varied widely among horses. Therefore, the results were expressed as the percentage change in concentration between time 0 (before surgery) and 8 hours.

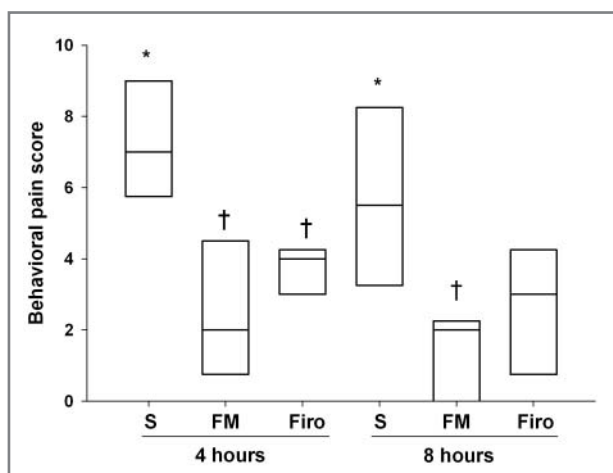


Figure 1—Box plots of behavioral pain scores 4 and 8 hours after the end of 2 hours of jejunal ischemia for horses ($n = 6$ horses/group) treated with saline (0.9% NaCl) solution (S; 1 mL/kg, IV, q 24 h), flunixin meglumine (FM; 1.1 mg/kg, IV, q 12 h), or firocoxib (Firo; 0.09 mg/kg, IV, q 24 h). The upper and lower limits of each box represent the 75th and 25th percentiles, respectively, and the horizontal line in each box represents the median. *Within a treatment group, the score is significantly ($P = 0.002$) greater than the preoperative score for that treatment group. †Within a time point, the score is significantly ($P < 0.05$) lower than the score for the horses treated with saline solution.

Plasma TXB₂ concentrations increased significantly ($P = 0.016$) by 8 hours after ischemia in horses treated with firocoxib (mean \pm SEM, $110.1 \pm 70.5\%$), compared with the concentrations in horses treated with flunixin meglumine, which decreased by 8 hours after ischemia (mean, $-61.5 \pm 12.9\%$). Plasma TXB₂ concentrations in horses treated with saline solution increased (mean, $39.8 \pm 29.7\%$) by 8 hours after ischemia, which was not significantly different from the percentage change for the other 2 treatment groups.

Plasma PGEM concentrations were significantly ($P = 0.005$) increased at 8 hours after ischemia (compared

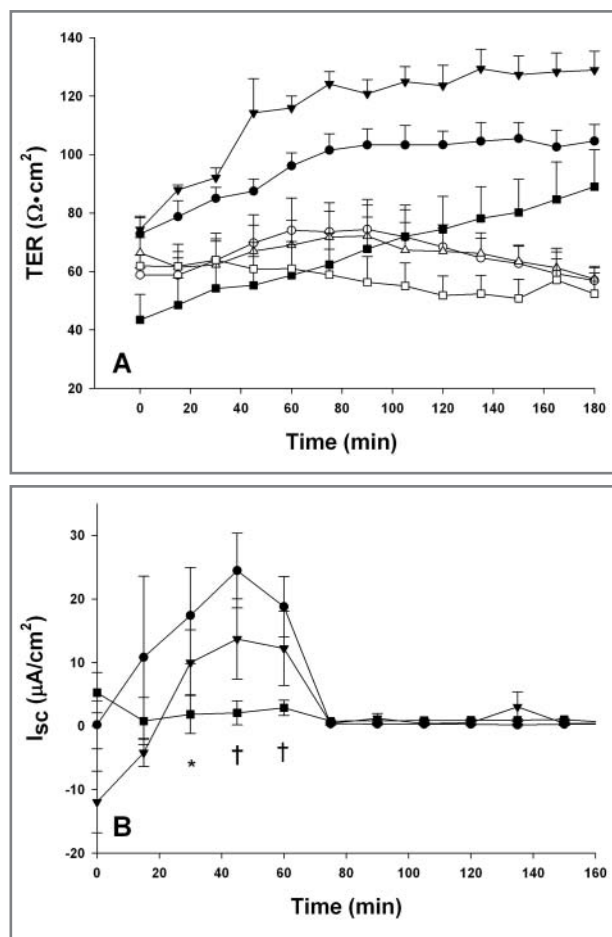


Figure 2—Mean \pm SEM TER (A) and I_{sc} (B) for jejunal mucosa obtained 18 hours after ischemic injury from horses ($n = 6$ horses/group) treated with saline solution, flunixin meglumine, or firocoxib. A—The TER of control mucosa (saline solution [white circles], flunixin meglumine [white squares], and firocoxib [white triangles]) did not vary among treatment groups. The TER of ischemic-injured mucosa from horses treated with saline solution (black circles) or firocoxib (black triangles), and the TER of ischemic-injured mucosa from horses treated with flunixin meglumine (black squares) was significantly ($P < 0.009$) lower than the TER of ischemic-injured mucosa from horses treated with saline solution or firocoxib. B—*Within a time point, the I_{sc} for ischemic-injured mucosa obtained from horses treated with flunixin meglumine (black squares) is significantly ($P = 0.006$) lower than the I_{sc} for ischemic-injured mucosa obtained from horses treated with saline solution (black circles). †Within a time point, the I_{sc} for ischemic-injured mucosa obtained from horses treated with flunixin meglumine is significantly ($P < 0.027$) lower than the I_{sc} for mucosa obtained from horses treated with saline solution or firocoxib (black triangles). See Figure 1 for remainder of key.

with concentrations before surgery) in horses treated with saline solution (mean \pm SEM, $55.8 \pm 12.5\%$), compared with the change for horses treated with flunixin meglumine or firocoxib. Plasma PGEM concentrations remained virtually unchanged after ischemia in horses treated with flunixin meglumine or firocoxib (mean change, $-2.9 \pm 14.3\%$ and $-3.5 \pm 11.1\%$, respectively).

Electrical evaluation—The TER did not differ significantly for the control jejunum from horses treated with saline solution, flunixin meglumine, or firocoxib during the in vitro incubation period. There was also no significant difference in TER of ischemic-injured mucosa from horses treated with flunixin meglumine and control tissues from all treatment groups during the entire in vitro incubation period (Figure 2). The TER for control and ischemic-injured mucosa from horses treated with saline solution or flunixin meglumine has been reported elsewhere.²¹ The TER was significantly increased in ischemic-injured mucosa from horses treated with saline solution ($P = 0.009$) or firocoxib ($P < 0.001$), compared with the TER in ischemic-injured mucosa from horses treated with flunixin meglumine. The TER in ischemic-injured mucosa from horses treated with saline solution or firocoxib was significantly ($P = 0.008$) increased, compared with the TER for all control mucosa, during the in vitro recovery period.

Immediately after ischemic-injured mucosa was stripped from the seromuscular layer and mounted in Ussing chambers, an increase in I_{sc} , an indicator of chloride secretion, was detected in tissues from horses treated with saline solution or firocoxib (Figure 2). The I_{sc} remained virtually unchanged in mucosa from horses treated with flunixin meglumine and was significantly ($P = 0.006$) lower than that of horses treated with saline solution at 30 minutes and significantly ($P < 0.027$) lower than that of horses treated with saline solution or firocoxib at 45 and 60 minutes. After the initial 60-minute in vitro period, I_{sc} returned to values before incubation and was not significantly differ-

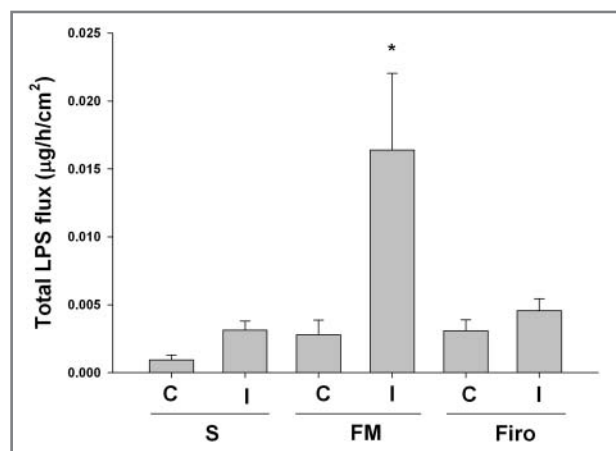


Figure 3—Amount of LPS crossing from the mucosal to serosal side of equine jejunal mucosa mounted in Ussing chambers. Mucosa was harvested from jejunum that was uninjured (control jejunum [C]) or subjected to 2 hours of ischemia by occlusion of the local blood supply (ischemic-injured jejunum [I]) from horses ($n = 6$ horses/group) treated with saline solution, flunixin meglumine, or firocoxib. *Value is significantly ($P < 0.006$) greater than the value for all other groups. See Figure 1 for remainder of key.

ent among treatment groups for the remainder of the experiment.

Permeability evaluation—The LPS flux was significantly ($P < 0.006$) increased across ischemic-injured mucosa from horses treated with flunixin meglumine, compared with the flux across control mucosa from all treatment groups. The LPS flux across ischemic-injured mucosa from horses treated with flunixin meglumine was also significantly ($P < 0.004$) increased, compared with the flux across ischemic-injured mucosa from horses treated with saline solution or firocoxib (Figure 3). In the horses treated with saline solution or firocoxib, LPS flux across ischemic-injured mucosa did not differ significantly from that for the respective control mucosa. The results of the LPS flux across control and ischemic-injured mucosa from horses treated with saline solution or flunixin meglumine have been reported elsewhere.²¹

Histologic examination—No denudation of villi was detected in sections of biopsy specimens obtained from control jejunum at the end of the 2-hour ischemic period. The percentage of villus denudation after 2 hours of ischemia did not differ significantly among the 3 treatment groups. Therefore, the data were pooled to provide a mean \pm SEM overall villus denudation of $39.8 \pm 3.8\%$ after 2 hours of ischemia. By 18 hours, restitution was almost complete, with a reduction in the percentage denudation to a mean of $8.7 \pm 8.3\%$ in horses treated with saline solution, $9.5 \pm 8.0\%$ in horses treated with flunixin meglumine, and $0 \pm 0\%$ in horses treated with firocoxib (Figure 4). The percentage of villus denudation did not differ significantly among treatment groups after 18 hours of recovery. Mild epithelial loss ($< 0.5\%$) was detected in control jejunum at 18 hours after surgery (data not shown).

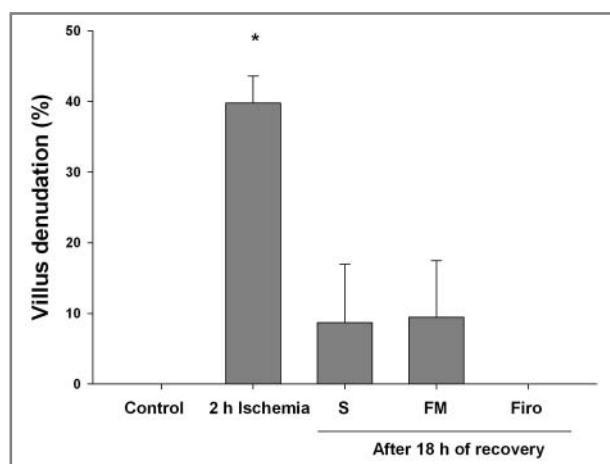


Figure 4—Mean \pm SEM percentage of villus denudation measured in biopsy samples obtained from horses ($n = 6$ horses/group) treated with saline solution, flunixin meglumine, or firocoxib. Biopsy samples were obtained from ischemic-injured jejunum after 2 hours of ischemia (2 h ischemia) and adjacent uninjured jejunum (Control). No significant difference was detected among treatment groups at this time point, so the data were pooled. Biopsy samples obtained after 18 hours of recovery from ischemia did not differ significantly ($P > 0.05$) in restitution among treatment groups at this time point. *Value is significantly ($P < 0.001$) greater than the value for all other groups. See Figure 1 for remainder of key.

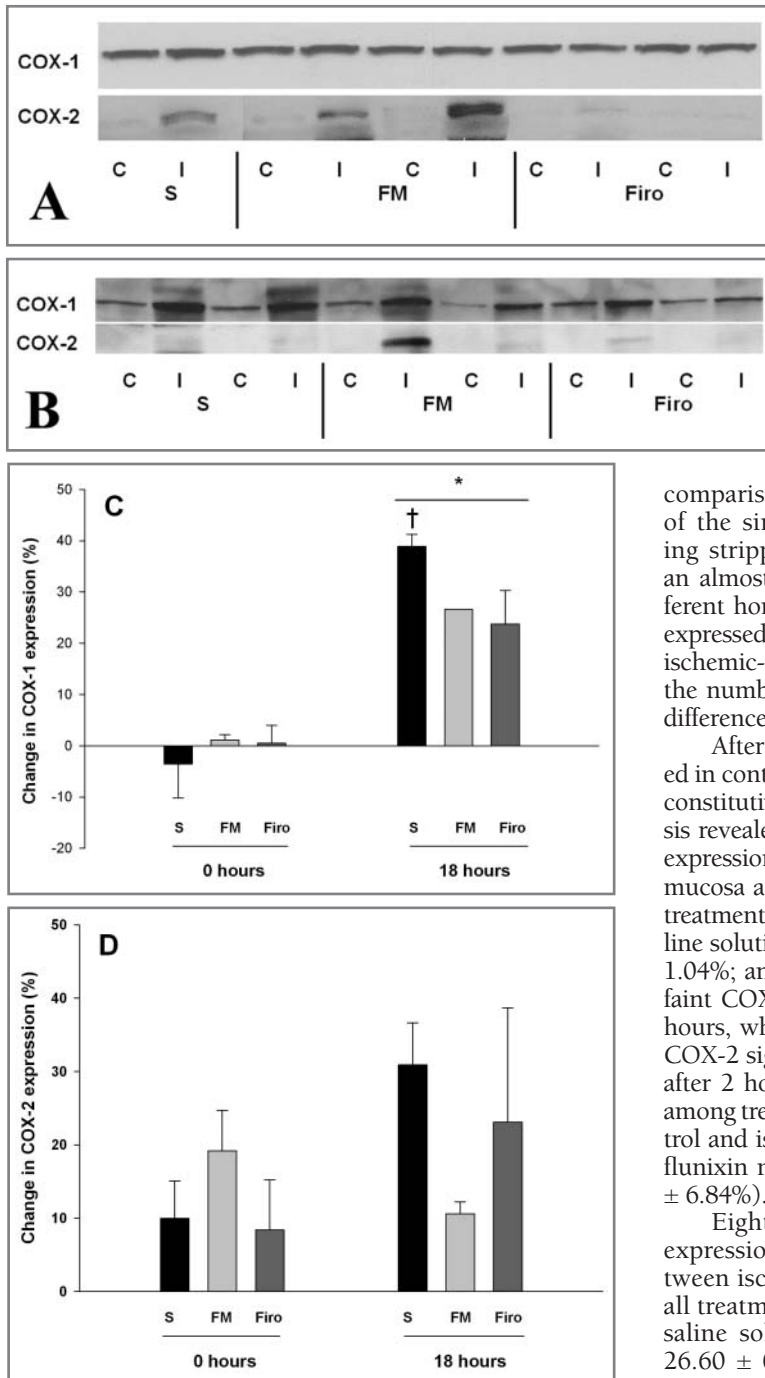


Figure 5—Photographs of western blots of COX-1 and COX-2 expression in uninjured (control) and ischemic-injured mucosa after 2 hours of ischemia (0 hours [A]) or after 2 hours of ischemia followed by 18 hours of recovery (18 hours [B]) and graphs of the percentage change in expression of COX-1 (C) and COX-2 (D) between uninjured (control) and ischemic-injured mucosa as determined on the basis of densitometric analysis of western blots. Mucosal samples were obtained from representative horses treated with saline solution, flunixin meglumine, or firocoxib. A and B—Notice that COX-1 is constitutively expressed, as indicated by its presence in uninjured (control) jejunum at time 0, but both COX-1 and COX-2 appear to be upregulated in ischemic-injured mucosa after 18 hours of recovery. C—*Values are significantly ($P \leq 0.003$) greater than those at 0 hours in all treatment groups. †Within this time point, the value differs significantly ($P = 0.025$) from the value for horses treated with firocoxib. D—Values did not differ significantly ($P > 0.05$) between 0 and 18 hours or among treatment groups. See Figures 1 and 3 for remainder of key.

Western blot analyses—Samples from 3 representative horses in each treatment group were evaluated from control (uninjured) and ischemic-injured mucosa obtained at the end of the ischemic period (0 hours of recovery) and immediately after horses were euthanized (18 hours of recovery). Densitometric analysis of β -actin bands revealed no significant difference across all lanes, which confirmed equal protein loading. Blots were probed for COX-2, then stripped and probed for COX-1. Multiple bands of various molecular weights were detected with the COX-2 antibody. The band immediately below the 75-kDa molecular-weight marker was considered to be COX-2. This was also confirmed by

comparison of the selected COX-2 band to the position of the single clear band detected on the corresponding stripped COX-1 blots, which were found to be in an almost identical position. Because samples from different horses were evaluated on different gels, data were expressed as the percentage change between control and ischemic-injured mucosa for each horse, rather than as the number of absolute densitometry units, to allow for differences in transfer and developing between the blots.

After 2 hours of ischemia (time 0), COX-1 was detected in control samples from all horses, which confirmed its constitutive expression (Figure 5). Densitometric analysis revealed that there was virtually no change in COX-1 expression in ischemic-injured mucosa relative to control mucosa at 0 hours, with no significant difference among treatment groups (mean \pm SEM percentage change: saline solution, $-3.54 \pm 6.60\%$; flunixin meglumine, $1.14 \pm 1.04\%$; and firocoxib, $0.49 \pm 3.53\%$). In contrast, only a faint COX-2 signal was detected in control mucosa at 0 hours, which indicated low constitutive expression. The COX-2 signal was detectable in ischemic-injured mucosa after 2 hours of ischemia, with no significant difference among treatment groups (percentage change between control and ischemic mucosa: saline solution, $9.94 \pm 5.14\%$; flunixin meglumine, $19.19 \pm 5.50\%$; and firocoxib, $8.38 \pm 6.84\%$).

Eighteen hours after the end of ischemia, COX-1 expression was significantly ($P \leq 0.003$) increased between ischemic-injured mucosa and control mucosa in all treatment groups (mean \pm SEM percentage increase: saline solution, $38.89 \pm 2.35\%$; flunixin meglumine, $26.60 \pm 0.03\%$; and firocoxib, $23.72 \pm 6.57\%$; Figure 5). The percentage increase in COX-1 expression in ischemic-injured tissue was significantly ($P = 0.025$) greater in horses treated with saline solution than in horses treated with firocoxib. Expression of COX-2 was increased in ischemic-injured mucosa, compared with expression in control mucosa, in all treatment groups after 18 hours of recovery, although there was no significant difference among groups (mean percentage increase: saline solution, $30.90 \pm 5.74\%$; flunixin meglumine, $10.61 \pm 1.62\%$; and firocoxib, $23.10 \pm 15.56\%$).

Discussion

Flunixin meglumine is currently the drug most commonly administered after colic surgery for its an-

algic effects and because it reduces clinical signs of endotoxemia.⁷ Providing adequate analgesia after colic surgery can reduce postsurgical weight loss and duration of hospitalization²²; therefore, it is a critical part of postoperative management. However, in the study reported here, similar to results for other studies,^{5,15} we found that although flunixin meglumine provided effective visceral analgesia, it retarded recovery of ischemic-injured jejunum. Typically, ischemic-injured mucosa has a higher TER than control tissue after 18 hours of recovery.¹⁵ This overshoot phenomenon is likely attributable to the PGs produced in response to ischemic injury stimulating closure of the paracellular spaces in the crypts during recovery^{13,23,24} and is exaggerated as a result of the high density of tight junctions in crypt epithelium.^{25,26} This increase in TER can be detected in horses treated with saline solution, but it is inhibited by treatment with flunixin meglumine.

Of particular concern from a clinical perspective is the increase in LPS flux across ischemic mucosa obtained from horses treated with flunixin meglumine. Horses are particularly sensitive to endotoxin absorption, with administration of only 0.03 µg/kg causing tachypnea, tachycardia, fever, and reductions in gastrointestinal tract motility.²⁷ In the *in vitro* study reported here, there was absorption of 0.0164 µg of LPS/h/cm² of jejunal mucosa obtained from horses treated with flunixin meglumine. The time for complete recovery of barrier function in horses treated with flunixin meglumine is unknown, but on the basis of the current data, it is obviously > 18 hours. Therefore, it is possible that in a clinical situation, even a small residual area of injured mucosa could potentially result in substantial absorption of endotoxin in horses treated with flunixin meglumine. However, treatment with firocoxib did allow recovery of the mucosal barrier, as indicated by a high TER and low permeability to LPS, which were not significantly different from values measured in horses treated with saline solution.

Recovery of the epithelial barrier after ischemic injury initially requires villus contraction and restitution of the epithelium to cover the denuded basement membrane.²⁸ We found that restitution was almost complete after 18 hours of recovery and was not affected by treatment. Therefore, the reduction in TER and increase in LPS flux in ischemic-injured jejunum obtained from horses treated with flunixin meglumine were unlikely to be attributable to inhibition of this phase of healing. The relative lack of importance of restitution in recovery of TER has been reported in bile-injured porcine ileum.²⁴ Analysis of results of that study²⁴ and another study²⁹ of ischemic-injured porcine ileum suggests that it is more likely the subsequent phase in recovery of the epithelial barrier (ie, closure of the paracellular space) that is critical for recovery of barrier function. Other studies²⁹⁻³² in ischemic-injured porcine ileum have determined that this phase of repair is mediated by COX-derived PGs through stimulation of chloride secretion and inhibition of electroneutral sodium absorption. Inhibiting the production of COX-derived PGs by administration of a nonselective COX inhibitor, such as flunixin meglumine, would retard closure of the paracellular space. This effect of flunixin meglumine can

be evaluated by measuring the I_{sc} generated by chloride secretion in response to endogenous tissue PGs, which are released when the mucosa is stripped from the seromuscular layer.³³ In the study reported here, we determined that chloride secretion increased during the first 60 minutes of *in vitro* incubation of mucosa from horses treated with saline solution or firocoxib, but that this increase was inhibited in tissues from horses treated with flunixin meglumine. Analysis of these data suggested that the effect of flunixin meglumine on recovery of TER and paracellular permeability to LPS in ischemic-injured equine jejunum may be attributable to inhibition of COX-1-derived PGs and, ultimately, prevention of closure of the paracellular space. Conversely, in tissues from horses treated with firocoxib or saline solution, I_{sc} was increased after stripping of the mucosa, which suggested that COX-1-derived PGs were produced and that they stimulated closure of the paracellular space. This would imply that firocoxib allows sufficient production of COX-1-derived PGs to allow mucosal recovery in horses.

Further evidence of the COX-1-sparing effect of firocoxib was evident in the results for the plasma prostanoicid concentrations. Thromboxane A₂ is produced in platelets solely by COX-1, with no contribution from COX-2.³⁴ Measurement of its stable metabolite, TXB₂, in whole blood and plasma has been used as a determinant of COX-1 activity.^{35,36} We found that treatment with firocoxib allowed an increase in plasma TXB₂ concentration after surgery, whereas this increase was inhibited by treatment with flunixin meglumine. This suggested that firocoxib has a sparing effect on the generation of COX-1-derived TXA₂, whereas production of TXA₂ is inhibited by flunixin meglumine. The enzyme PGE₂ synthase is responsible for the conversion of COX-derived PGH₂ to PGE₂.^{37,38} There are several forms of PGE synthase, but the enzyme is preferentially coupled to COX-2, rather than to COX-1^{37,38}; therefore, measurement of the PGE₂ concentration in blood and plasma is primarily used as an indicator of COX-2 activity.³⁶ Analysis of our results indicated that treatment with flunixin meglumine or firocoxib inhibited the postsurgical increase in plasma PGE₂ concentrations detected in horses treated with saline solution, which implied that both drugs inhibit the COX-2 isoform.

Western blot analysis in our study confirmed the findings of other studies,^{5,15} namely, that COX-1 is constitutively expressed in equine jejunum as evident by its detection in control tissues at the time of ischemia and after 18 hours of recovery. The COX-1 protein was upregulated in ischemic-injured tissue after 18 hours of recovery in all treatment groups, which suggested that it is not purely a constitutive enzyme. An increase in COX-1 has also been reported in microglia after cerebral ischemia³⁹ and has been found in ischemic equine jejunum at this time point in an aforementioned study.¹⁵ This increase in COX-1 in ischemic-injured mucosa at 18 hours was partly abolished by treatment with firocoxib, which is a finding that has been reported¹⁵ for meloxicam, an NSAID with selectivity for COX-2. However, the increase in TER and reduction in LPS flux found in firocoxib-treated horses indicated that firocoxib still permitted sufficient production of PGs to

allow mucosal recovery, and this reduction in COX-1 may have been a result of alternative anti-inflammatory mechanisms of NSAIDs.⁴⁰

In contrast to COX-1, COX-2 appeared to be rapidly upregulated after ischemic injury, with increased amounts in ischemic tissue after only 2 hours of ischemia. This rapid increase in COX-2 protein concentrations by 2 hours has also been found in rats after intestinal ischemia induced by occlusion of the superior mesenteric artery.⁴¹ The COX-2–derived PGs contribute to intestinal inflammation and injury after small intestinal ischemia-reperfusion; therefore, COX-2 is a potential therapeutic target.⁴¹ Although no differences were detected among treatment groups for COX-2 expression, the power of this test was 0.091 (less than the desired power of 0.8), which was probably attributable to the small number of horses analyzed in each treatment group. Therefore, it is possible that there are differences in the effects of the treatments on COX-2 expression, but we were unable to detect it.

The goat polyclonal COX-2 antibody that we used resulted in several strong false-positive signals of proteins of various molecular weights. This has been described as a complication of COX-2 immunoblotting and underscores the importance of the use of accurate molecular-weight markers when identifying the COX-2 signal.⁴² The use of a real-time quantitative PCR assay to identify COX-2 mRNA would be an appropriate alternative to immunoblotting for COX-2, and its use in the determination of COX-2 mRNA expression in equine laminae has been reported.⁴³

Firocoxib remained at detectable concentrations in plasma 16 hours after IV administration and appeared to have a long elimination phase. This suggested that the dose used in this study is appropriate for once-daily IV administration of this drug in clinically normal horses. However, horses with colic commonly have compromised cardiovascular function, such as reduced cardiac output⁴⁴ and hypotension,⁴⁵ which may affect elimination and metabolism of drugs and necessitate a change in the dose or frequency of administration.⁴⁶ Therefore, a pharmacokinetic study of firocoxib in horses with colic would be advisable.

An established behavioral pain scoring system¹⁹ was used in our study as a more objective method to assess the visceral analgesic effects of the various treatments. Although pain scores in horses treated with saline solution were significantly increased 4 and 8 hours after the end of ischemia, compared with preoperative values, no gross evidence of pain was detected at any time. Firocoxib is effective as a somatic analgesic in horses because it can reduce pain associated with osteoarthritis.¹⁸ In the study reported here, postoperative pain scores were not significantly increased, compared with preoperative values, in horses treated with firocoxib, which suggested that this treatment provides effective visceral analgesia.

On the basis of the data obtained in this study, the IV formulation of firocoxib appeared to be selective for COX-2 in horses. Administration of firocoxib resulted in effective visceral analgesia while permitting recovery of mucosal barrier function in ischemic-injured jejunum. Additional in vivo studies are indicated to deter-

mine its suitability for use as an alternative to flunixin meglumine in postoperative colic patients with ischemic-injured intestine.

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- c. Prostaglandin E metabolite EIA kit, Cayman Chemical, Ann Arbor, Mich.
- d. Thromboxane B₂ EIA kit, Cayman Chemical, Ann Arbor, Mich.
- e. SantaCruz Biotechnology Inc, Santa Cruz, Calif.
- f. AbCam plc, Cambridge, Mass.
- g. ECL western blotting substrate, Pierce Chemical Co, Rockford, Ill.
- h. SigmaScan Pro 5, Systat Software Inc, San Jose, Calif.

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