Nonsteroidal antiinflammatory drugs reduce anion secretion and increase apoptosis in equine colonic mucosa ex vivo

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OBJECTIVE
Right dorsal colitis causes chronic colic associated with long-term treatment with nonsteroidal antiinflammatory drugs (NSAIDs). This study was designed to determine if NSAIDs could inhibit anion transporters that protect against intestinal mucosal injury in other species.

ANIMALS
20 healthy horses.

METHODS
The effects of indomethacin (INDO) and firocoxib (FIR), on short-circuit current (Isc) in mucosa from the right dorsal colon (RDC) and right ventral colon (RVC) were measured in Ussing chambers by standard electrophysiological techniques. Immunohistochemical methods were used to detect apoptosis (caspase-3) with these NSAIDs and phenylbutazone (PBZ) and to locate the NKCC1 transporter.

RESULTS
The Isc in RDC and RVC incubated with INDO or FIR was increased almost 3-fold ($P < .0001$) by prostaglandin $E_2$ ($PGE_2$) through a system inhibited by loop diuretics ($P < .0001$). Although these findings and anion replacement studies were consistent with anion secretion, the RDC also displayed an Isc response suggestive of a unique transporter apparently absent in RVC or NSAID-free solutions. In RDC, FIR, INDO, and PBZ induced apoptosis in the lower half of crypts. However, significant differences in apoptotic index were recorded in the RDC between NSAID-treated and control tissues (no NSAID).

CLINICAL RELEVANCE
The effects of NSAIDs on Isc were consistent with reduced anion secretion, which could represent the pharmacological equivalent of the transport failure responsible for Cystic Fibrosis (CF) in other species. Failure of anion secretion could interfere with buffering acid from intraluminal fermentation, which could suggest a treatment target for right dorsal colitis.

Keywords: horses, right dorsal colitis, NSAIDs, anion secretion, apoptosis

Although acute and severe colic is recognized as a common cause of death in horses,
1 chronic or recurrent colic is also a life-threatening disease that can be difficult to diagnose and treat.
2 Right dorsal colitis is a well-known but poorly understood form of chronic colic, further complicated by the possibility of an iatrogenic cause.
3 Most affected horses have a current history of protracted treatment (weeks to months) for a chronic musculoskeletal disease with a nonsteroidal antiinflammatory drug (NSAID), especially phenylbutazone (PBZ).
4 This putative pharmacological risk and the consistent predilection site in the right dorsal colon (RDC) could provide clues to the management of right dorsal colitis. Diagnosis is usually presumptive, based on the history of NSAID use and colic, supported by marked
hypoalbuminemia, hypocalcemia,\textsuperscript{3,4} and ultrasonographic findings.\textsuperscript{5} Gross lesions in the RDC include mucosal ulceration, edema and hemorrhage, colonic stenosis, impaction, and even colonic rupture.\textsuperscript{5}

In a previous ex vivo study, PBZ and indomethacin were shown to inhibit anion transport, presumably both Cl\textsuperscript{−} and HCO\textsubscript{3}−, and caused apoptosis in the RDC.\textsuperscript{6} A more recent study by the authors has demonstrated that the equine RDC, but not the RVC, secretes HCO\textsubscript{3}−, probably through the cystic fibrosis transmembrane conductance regulator (CFTR), and this secretion is inhibited by PBZ.\textsuperscript{7} Possibly, HCO\textsubscript{3}− secretion could be protective in the RDC, and its inhibition by PBZ could leave the RDC vulnerable to injury from volatile fatty acids (VFAs) produced during normal hindgut fermentation. This would be analogous to the role of HCO\textsubscript{3}− secretion in protecting gastric glandular mucosa from acid injury.\textsuperscript{8} Also, defects in anion transport in other species (eg, humans and mice) associated with cystic fibrosis (CF) cause intestinal obstruction and mucosal lesions\textsuperscript{9,10} similar to those observed for right dorsal colitis.\textsuperscript{5} The intestinal lesion of CF can be attributed to failure secretion of Cl\textsuperscript{−} and HCO\textsubscript{3}−, a process essential for emptying crypts of mucus, hydrating crypt and lumen contents, maintaining the viscoelastic properties of mucus, and buffering luminal acids.\textsuperscript{9,10}

The current study was designed to demonstrate differences in anion transport between the RDC and RVC through standard electrophysiological methods in Ussing chambers. The current study was also designed to determine which cyclooxygenase (COX) isoform was involved in transporter responses and to determine if NSAIDS induced histological changes in the tissue. Our overarching hypothesis stated that NSAIDs affect RDC function in ways that could be relevant to the pathogenesis, prevention, and treatment of right dorsal colitis in horses.

**Methods**

This study was conducted in 2 protocols, with 10 healthy adult horses in each. In protocol 1, tissues were harvested with horses under general anesthesia to enhance tissue viability, whereas tissues were harvested immediately after euthanasia in protocol 2.

**Protocol 1**

Ten adult light-breed horses were used that were free of systemic diseases, with no history of gastrointestinal tract diseases, and not receiving medications, particularly long-term NSAID administration or any NSAID within 3 weeks before the study. The median ages of horses were 16 years (range = 10–24 years old). Median body weights were 471 kg (range = 349–578 kg). Horses were donated to the University of Florida and kept at pasture, fed hay (2% of BW daily), and allowed ad libitum water. Feed only was withheld for 18 hours before the study. The Institutional Animal Care and Use Committee of the University of Florida approved the study protocol.

**Tissue collection**

A 14-gauge, 13.3-cm Teflon catheter was placed aseptically in the right jugular vein, immediately before induction of general anesthesia.\textsuperscript{7} Horses were sedated with 0.5 mg/kg xylazine and 0.02 mg/kg butorphanol IV and anesthesia was induced with 2.2 mg/kg IV and 0.1 mg/kg IV of ketamine and diazepam, respectively. Orotracheal intubation was performed and anesthesia was maintained by isoflurane (1% to 3%) in oxygen. After horses were positioned in left lateral recumbency, the right flank was clipped of hair and prepared for aseptic surgery. The large colon was exteriorized through a right paralumbar fossa incision and approximately 10 cm X 30 cm full-thickness segments of RDC, RVC were removed each in 3 parts. One part was assigned for Ussing chamber studies, another was further divided and placed in flasks each with a different NSAID (PBZ, FIR, or INDO), and a third was preserved in 10% neutral buffered formalin for immunohistochemical studies. Sections (2 X 5 cm) of the duodenum, jejunum, ileum and small colon were also collected for immunohistochemical studies. Tissues for Ussing chamber studies were brought to the laboratory in a similar solution as used for dissection and incubation, with a fresh solution used at each step. After all tissues were collected, each horse was euthanized with an overdose of pentobarbital sodium (88 mg/kg IV) with phentoyin.

**Electrophysiological studies**

Sheets of mucosa-submucosa of RDC and RVC were separated from the muscle layers by dissection with scissors and placed in 4°C Krebs-Ringer bicarbonate (KRB) solution containing 1 µM INDO.\textsuperscript{6} The mucosal sheets were mounted in Ussing chambers and bathed with 10 mL of the KRB and INDO solution on each tissue surface.\textsuperscript{6} For anion substitution studies, Cl\textsuperscript{−} free and HCO\textsubscript{3}− free solutions with INDO were used in place of KRB and INDO buffer (Supplementary Table S1). For all but the HCO\textsubscript{3}− free solution, the bathing solution was maintained at pH 7.4 and 38°C by circulating 95% O\textsubscript{2} and 5% CO\textsubscript{2} in flasks each with a different NSAID (PBZ, FIR, or INDO), and a third was preserved in 10% neutral buffered formalin for immunohistochemical studies. Sections (2 X 5 cm) of the duodenum, jejunum, ileum and small colon were also collected for immunohistochemical studies. Tissues for Ussing chamber studies were brought to the laboratory in a similar solution as used for dissection and incubation, with a fresh solution used at each step. After all tissues were collected, each horse was euthanized with an overdose of pentobarbital sodium (88 mg/kg IV) with phentoyin.
(G) in millisiemens per centimeter$^2$ (mS/cm$^2$) was calculated as the inverse of the transepithelial resistance (TER) and was used as a measure of barrier integrity in the colonic mucosa. The effect of the fluid resistance on the Isc was automatically corrected (World Precision Instruments). Because all tissues reached a steady state recording at 15 to 30 minutes after a chemical was added, 30 minutes was chosen as the interval between each addition. After 30 to 60 minutes of baseline recording, 1 μM PGE$_2$, (Sigma Chemical Co) was added to the chambers, and the response of the tissues was recorded every 5 minutes after that addition for 30 minutes. Then, 0.1 mM bumetanide (Sigma Chemical Co), was applied to all chambers. To investigate the effects of Na$^+$ absorption on the results, 0.1 mM amiloride was added in duplicate to INDO-incubated RDC and RVC after PGE$_2$ addition for a total of 3 horses. All additions were applied to both sides of the tissues to maximize tissue distribution to transporters throughout the mucosal layers and all treatments were in duplicate.

**Apoptosis study**

To examine the effects of NSAIDs on apoptosis, approximately 2 X 2 cm of mucosa from RVC and RDC were incubated in KRB solution in beakers in a water bath at 38 °C while they were gassed with O$_2$ and CO$_2$. For each tissue (RVC, RDC), 1 beaker contained no addition, and each of the remaining 3 had 1 μM FIR, 3.24 μM PBZ, or 1 μM INDO. After 150 minutes, the tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, rehydrated, and processed to detect caspase 3 by a standard immunohistochemical technique. After routine processing for IHC with Mouse and Rabbit Specific HRP/DAB (ABC) Detection IHC Kit (Abcam) according to the manufacturer’s instructions, cleaved (CL)-Caspase-3 (Asp175; Cell Signaling Technology, Inc) was used as a primary antibody. For negative controls, only phosphate-buffered saline (PBS) was added to tissue samples. A positive reaction was evident as brown nuclei and the degree of apoptosis was determined as the apoptotic index, which is the number of apoptotic cells per 1,000 epithelial cells from surface to bottom of crypts counted manually with the 40X objective lens. Localization of staining was assessed by the principal investigator (RV) who was blinded as to the NSAID used for each slide.

**NKCC1 immunohistochemistry**

To localize NKCC1 in the colonic mucosa, a standard protocol was used for deparaffination and rehydration of paraffin-embedded tissues of 4- to 5-μm thickness mounted on silane-coated glass slides, and citrate buffer (pH 6) was used for heat-induced epitope retrieval (HIER). The tissues were dehydrated with 3% hydrogen peroxide. Immunolabeling was carried out in a humidifier chamber at room temperature using a Mouse and Rabbit Specific HRP/DAB (ABC) Detection IHC Kit according to the manufacturer’s instructions (Abcam). NKCC1 antibody (NKCC1 Rabbit anti-Human/Mouse Polyclonal; Invitrogen, Thermo Fisher Scientific) was used to detect NKCC1. Based on the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST), the epitope of anti-NKCC1 was approximately 94% homologous with both human and mouse NKCC1. For negative controls, only PBS solution was added to tissue samples. The Biotinylated Goat Anti-Polyvalent secondary antibody was used to detect the primary antibodies. The tissues were incubated with Streptavidin Peroxidase, which was polymerized directly with horseradish peroxidase and developed with 2.5% DAB as peroxidase substrate. The slides were counterstained with hematoxylin QS (Vector Laboratories). After dehydration, the slides were mounted in UltraCruz® Aqueous Mounting Medium (Santa Cruz Biotechnology, Inc) and covered with glass coverslips to be assessed under light microscopy.

**Protocol 2**

Ten adult light-breed horses that met the same inclusion criteria as in Protocol 1, with a median age of 11 years (range = 5–18 years old), and median body weights of 462.5 kg (range = 374–550 kg), were used in this phase of the study. Unlike horses in protocol 1, tissues were harvested immediately after euthanasia. Horses were sedated with 0.5 mg/kg xylazine and 0.02 mg/kg butorphanol IV and anesthesia was induced with 2.2 mg/kg IV and 0.1 mg/kg of ketamine and diazepam, respectively. Horses were then euthanized and the abdomen was opened as described above immediately after death was confirmed by cardiac auscultation, and approximately 10 cm X 30 cm full-thickness segments of RDC and RVC were removed. Mucosal sheets from RDC and RVC were collected in a 4 °C KRB solution that contained 1 μM INDO, 1 μM FIR, or no addition (control tissues). They were mounted in Ussing chambers in the same solutions as for protocol 1 and electrophysiological changes were recorded. Instead of bumetanide, furosemide (0.1 mM) was used to assess the response to a loop diuretic.

**Statistical methods**

The normality of the data was tested by visual inspection (histogram, QQ plots, and density distributions) and by the Shapiro-Wilk test. Homogeneity of variances was tested by Levene’s test for equality of variances (between the groups of the between-subjects’ factors) or Mauchly’s test of sphericity (between within-subjects’ groups). No significant outliers were evident. The independence of observations was observed when 1-way ANOVA was performed. A statistical software program (R program, Version i386 3.5.2) was used for analyses. Changes in Isc were compared for each type of colon in each incubation condition before (baseline), after the addition of PGE$_2$; and before and after the addition of loop diuretics throughout the experiment by use of repeated measures ANOVA. Tukey’s HSD and Bonferroni methods were used as the post hoc.
procedures to detect differences between means. The Kruskal-Wallis test was used for analyzing the apoptotic index; \( P < .05 \) was considered significant in all analyses. No statistical comparisons were made between data obtained in protocol 1 compared with protocol 2.

**Results**

**Protocol 1**

In the RVC in KRB + INDO buffer, exogenous PGE\(_2\) significantly increased mean Isc (\( P < .0001; \text{Table 1, Figure 1} \)). This PGE\(_2\)-induced Isc increase was significantly decreased by bumetanide in all tissues (\( P < .0001; \text{Table 1, Figure 1} \)). In the RDC, 2 responses were recorded with PGE\(_2\), RDC-1, which was similar to the RVC response, and RDC-2, in which PGE\(_2\) caused a brief downward deflection in the recording (Table 1, Figure 1). This was followed by a recovery that produced a peak significantly lower (\( P < .0001 \)) than recorded in the RVC (Table 1, Figure 1). The RDC-1 response only accounted for 20% of all responses in the RDC (n = 2) while the RDC-2 response accounted for 80% (n = 8).

A Cl\(^-\)-free solution (Supplementary Table S1) eliminated responses to all additions in the RDC and RVC (Table 1). A HCO\(_3\)-free solution (Supplementary Table S1) eliminated responses to all additions in the RDC, but did not affect Isc increases in the RVC (Table 1). Amiloride had no effects on the Isc following PGE\(_2\) in INDO-incubated RDC and RVC.

The increase in mean conductance throughout the study in all solutions was not significant, but conductance at baseline was higher for RVC than RVC (Supplementary Table S2). In a Cl\(^-\)-free solution, the conductance was significantly greater (\( P < .05 \)) for the same solution.

**Apoptosis study**

In RDC incubated in FIR, INDO, and PBZ, caspase-3 staining as evidence of apoptosis was located in the lower half of crypt cells (Figure 2) with all NSAIDs. However, significant differences in apoptotic index were recorded between all NSAID-treated and control tissues (no NSAID) in the RDC (Table 2). In the RVC, the apoptotic index of the control tissues differed significantly from INDO- and PBZ-incubated tissues (\( P < .05 \)), but not FIR-incubated tissues (Table 2).

**Immunohistochemistry**

Staining for NKCC1 was almost exclusively in goblet cells from the base of crypts (Figure 3) to the mucosal surface in the RVC, RDC, duodenum, jejunum, ileum, and small colon.

**Protocol 2**

Electrophysiological recordings and responses to modifiers were qualitatively similar in tissues collected after euthanasia in RVC and RDC in protocol 2 compared to segments collected under general anesthesia in protocol 1. Also, furosemide elicited a similar Isc response in all tissues in protocol 2.

| Table 1—Effect of buffer solutions and modifiers on Isc in protocol 1. |
|----------------|----------------|----------------|----------------|----------------|----------------|
| Solution        | Segment        | Baseline Isc (µA/cm\(^2\)) | After PGE\(_2\) Isc (µA/cm\(^2\)) | Before bumetanide Isc (µA/cm\(^2\)) | After bumetanide Isc (µA/cm\(^2\)) |
| KRB + Indo      | RDC-1 (n = 2)  | 21.91 ± 21.59          | 56.64 ± 6.88*          | 52.43 ± 12.83*          | 33.63 ± 1.88*          |
|                 | RDC-2 (n = 8)  | 22.84 ± 13.22          | 9.88 ± 10.53          | 27.21 ± 7.66           | 16.13 ± 5.47           |
| Cl\(^-\) + Indo | RVC (n = 2)    | 26.06 ± 11.17          | 59.39 ± 16.74        | 59.00 ± 17.36           | 32.69 ± 16.67*        |
| Cl\(^-\) free + Indo | RDC (n = 6)  | 38.57 ± 21.66          | 47.35 ± 23.61        | 46.24 ± 22.36           | 36.21 ± 20.48         |
| HCO\(_3\) - free + Indo | RVC (n = 6)  | 32.82 ± 10.27          | 41.37 ± 10.73        | 40.41 ± 10.65           | 34.59 ± 13.13         |
|                 | RVC (n = 6)    | 21.98 ± 4.95           | 37.54 ± 10.31        | 36.43 ± 9.11            | 27.58 ± 15.56         |
|                 | RVC (n = 6)    | 27.21 ± 5.44*          | 54.65 ± 14.77*       | 52.36 ± 16.32           | 49.34 ± 15.30*        |

Isc (µA/cm\(^2\)) as means ± SD. Baseline is the Isc when PGE\(_2\) is added (see Figure 1). The responses after PGE\(_2\) are the peak (RDC-1) or nadir (RDC-2) response to this addition. Before bumetanide is the Isc at 30 minutes after PGE\(_2\) and when bumetanide is added (see Figure 1). After bumetanide is the Isc 30 minutes later. Values in each row with different superscript letters are significantly different (\( P < .05 \)). Values with different symbols in each column are significantly different (\( P < .05 \)) for the same solution.

**Figure 1**—Changes in mean Isc (µA/cm\(^2\)) to demonstrate RDC-1 and RDC-2 as 2 separate responses in the RDC to PGE\(_2\) added to tissues in a solution containing indo. The times of addition (arrows) immediately follow the recording indicated. The SD for each data point is omitted for clarity but is included in Table 1 with statistical analyses.
as bumetanide in protocol 1. When incubated in KRB buffer without an NSAID (control), both RVC and RDC had similar Isc at baseline, and transport modifiers did not significantly change Isc (Table 3). The NSAID-related RDC-2 response to PGE₂ evident in protocol 1 was also evident in protocol 2 and with prevalence rates of 70% for INDO and 50% for FIR (Table 3). With both INDO and FIR, the RDC-2 response to PGE₂ was significantly different (P < .05) to the RDC-1 and RVC responses (Table 3). The response to PGE₂ was also significantly different (P < .05) to baseline for RDC-1, RDC-2, and RVC with INDO and FIR. The decrease in Isc induced by furosemide was significant (P < .05) for INDO and FIR in RDC-2, but not for RDC-1 with INDO (Table 3). The mean conductance throughout the study was not significantly different (P < .05) between incubation conditions (Supplementary Table S3).

Table 2—Apoptotic index in protocol 1.

<table>
<thead>
<tr>
<th>Segment</th>
<th>Control</th>
<th>FIR</th>
<th>Indo</th>
<th>PBZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>RDC (n = 5)</td>
<td>132 ± 139.71ᵃ</td>
<td>424 ± 193.08ᵇ</td>
<td>484 ± 105.26ᵇ</td>
<td>456 ± 145.88ᵇ</td>
</tr>
<tr>
<td>RVC (n = 5)</td>
<td>80 ± 73.49ᵃ</td>
<td>156 ± 32.86ᵇ</td>
<td>448 ± 152.71ᵇ</td>
<td>448 ± 165.89ᵇ</td>
</tr>
</tbody>
</table>

The apoptotic index as means ± SD was defined as the number of apoptotic cells per 1,000 epithelial cells counted manually with the 40X objective lens from the muscularis mucosa to the luminal surface. Control was KRB solution without an NSAID. Indo = Indomethacin; FIR = Firocoxib; PBZ = Phenylbutazone. Values across each row with superscript letters are significantly different from other tissues with a different letter, at P < .05 for RDC and P < .01 for RVC.

Table 3—Effect of buffer solutions and modifiers on Isc in protocol 2.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Segment</th>
<th>Baseline Isc (µA/cm²)</th>
<th>Isc After PGE₂ (µA/cm²)</th>
<th>Isc Before furosemide (µA/cm²)</th>
<th>Isc After furosemide (µA/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRB no NSAID</td>
<td>RDC-1 (n = 6)</td>
<td>13.94 ± 17.24</td>
<td>36.28 ± 18.64</td>
<td>35.69 ± 18.21</td>
<td>26.70 ± 13.54</td>
</tr>
<tr>
<td></td>
<td>RVC (n = 6)</td>
<td>29.28 ± 19.54</td>
<td>40.41 ± 21.13</td>
<td>39.31 ± 20.61</td>
<td>25.07 ± 13.01</td>
</tr>
<tr>
<td>KRB +1 µM Indo</td>
<td>RDC-1 (n = 3)</td>
<td>17.70 ± 12.55ᵃ</td>
<td>55.16 ± 22.7⁰</td>
<td>54.57 ± 22.50ᵇ</td>
<td>33.78 ± 16.31ᵇ</td>
</tr>
<tr>
<td></td>
<td>RDC-2 (n = 7)</td>
<td>16.25 ± 9.59ᵃ</td>
<td>7.02 ± 2.52ᵃ</td>
<td>38.75 ± 21.12ᶜ</td>
<td>25.41 ± 11.34ᶜ</td>
</tr>
<tr>
<td></td>
<td>RVC (n = 10)</td>
<td>17.83 ± 9.14ᵃ</td>
<td>52.57 ± 25.64ᵃ</td>
<td>52.30 ± 25.55ᵇ</td>
<td>25.66 ± 15.71ᵃ</td>
</tr>
<tr>
<td>KRB +1 µM FIR</td>
<td>RDC-1 (n = 5)</td>
<td>20.53 ± 15.28ᵃ</td>
<td>34.55 ± 13.11ᵃ</td>
<td>34.16 ± 12.80ᵇ</td>
<td>21.50 ± 13.63ᵃ</td>
</tr>
<tr>
<td></td>
<td>RDC-2 (n = 5)</td>
<td>15.31 ± 9.91ᵃ</td>
<td>7.61 ± 6.55ᵇ</td>
<td>42.57 ± 26.31ᵃ</td>
<td>28.50 ± 8.20ᵃ</td>
</tr>
<tr>
<td></td>
<td>RVC (n = 10)</td>
<td>31.11 ± 13.49ᵃ</td>
<td>48.58 ± 14.52ᵇ</td>
<td>47.43 ± 14.27ᵇ</td>
<td>27.97 ± 10.25ᵃ</td>
</tr>
</tbody>
</table>

Isc (µA/cm²) as means ± SD and as peak or nadir values for each response (see Figure 1 and Table 1 for explanations about the timing of additions). Values in each row with different superscript letters are significantly different (P < .05). Values with different symbols in each column are significantly different (P < .05) for the same solution.
Discussion

The major findings of this study were different Isc responses to PGE₂ and NSAIDs between equine RDC and RVC ex vivo (Tables 1 and 3, Figure 1). A negative deflection in Isc was recorded and labeled as RDC-2 (Tables 1 and 3, Figure 1), which could be associated with several different electrogenic transport processes. This Isc response has not been previously described, as far as the authors are aware. Additional findings were apoptosis in crypt cells in response to FIR, INDO, and PBZ in RDC but not with FIR in RVC (Table 2, Figure 2). Also, conductance, which reflects permeability to ions and low-molecular weight substances, was significantly greater for RDC than RVC in protocol 1, which could suggest a greater degree of leakiness in RDC (Supplementary Table S2). These findings, combined with previous evidence of HCO₃⁻ secretion in equine RDC, identify some unique features of RDC physiology that could be relevant to the role of NSAIDs in the pathogenesis of right dorsal colitis. Specifically, the findings support our hypothesis that NSAIDs could inhibit the secretion of anions, such as Cl⁻ and HCO₃⁻, and failure to secrete HCO₃⁻ could leave the colon vulnerable to injury from volatile fatty acids produced during normal hindgut fermentation. This would be the pharmacological equivalent to CF in other species (eg, humans and mice), which is also associated with intestinal mucosal lesions.⁹,¹⁰

The actual ion movements responsible for Isc in this study are unknown, although studies of radiolabeled Cl⁻ fluxes would support a role for anion secretion in equine RVC and RDC, most likely through CFTR. Unfortunately, ³⁶Cl is no longer available for flux studies and direct measurement of HCO₃⁻ was not feasible under conditions of the current study.⁷ The inhibitory effects of loop diuretics on the PGE₂-induced increase in Isc (Tables 1 and 3, Figure 1) would support a role for Cl⁻ entry into cells through NKCC1 and subsequent secretion through CFTR in Isc responses. The available evidence would suggest that CFTR can transport both Cl⁻ and HCO₃⁻ but with different affinities between colon segments.⁶ The diminished responses to PGE₂ and bumetanide in solutions in which Cl⁻ or HCO₃⁻ were excluded are consistent with the dependence of these responses to anions (Table 2). The failure of RDC to respond significantly in an HCO₃⁻ free buffer (Table 2) supports previous findings that the RDC has Cl⁻ independent HCO₃⁻ secretion that is weak or absent in RVC.⁷ The present study confirmed the previous finding by Clarke and Argenzio¹¹ that amiloride did not affect Isc in INDO-incubated tissues, evidence that Isc responses were not produced by electrogenic Na⁺ absorption through the epithelial Na⁺ channel (ENaC).¹⁶

The unique and transient RDC-2 response (Table 2) could represent either anion absorption or cation secretion, the latter possibly an electrogenic K⁺ secretion through an apical K⁺ channel.¹⁷ This K⁺ secretion could be stimulated by cyclic AMP, also responsible for enhancing anion secretion through CFTR.¹⁸,¹⁹ The cotransporter NKCC1 is not only a critical system for Cl⁻ secretion, but it also facilitates K⁺ entry into the cell to drive its secretion in the distal colon. Secretion of K⁺ could increase the electrical gradient opposing anion delivery into the cell thereby reducing anion uptake for subsequent secretion through CFTR. This would be consistent with the significantly (P < .05) lower Isc peak induced by PGE₂ in RDC-2 compared with RDC-1 and RVC (Table 1, Figure 1). However, cellular hyperpolarization induced by mucosal K⁺ secretion can also drive agonist-induced anion secretion through CFTR.²⁰

In diet-response studies in ponies, the intraluminal concentration of K⁺ peaked in the RDC and subsequent segments at all times after meal ingestion. These changes are consistent with a meal-induced K⁺ secretion in these segments, which could be investigated in future ex vivo studies by ion replacement and K⁺ channel blockers.¹³,²⁰ The absence of RDC-2 in Cl⁻ and HCO₃⁻ replacement studies suggests that anions might also play a role in this response. Whether or not RDC-1 or RDC-2 is linked to the risk of right dorsal colitis is unknown, although the variation in prevalence of these responses between horses (Tables 1 and 3) could be consistent with an idiopathic predisposition to the disease in some horses.¹⁴,²² Possibly, the 2 transporters coexist in the same cells but with different degrees of expression, so that one predominates under certain conditions.

Another possible explanation for RDC-2 was an aberrant systemic response to tissue collection from the anesthetized horse, which was the method selected in this and previous studies to improve tissue viability and eliminate possible adverse effects of euthanasia solution on tissue function. However, no apparent difference in the prevalence of RDC-2 was detected when tissue was collected before or after euthanasia, thereby eliminating any potential effect from the conditions of tissue collection.

Indomethacin was selected as an NSAID for the current study because it has been widely used in other species and systems and produces similar results as PBZ in equine colon.⁶ the NSAID most commonly implicated in right dorsal colitis.¹³,²² Like most NSAIDs used in equine medicine,²³ it is also a nonselective COX inhibitor.¹²-¹⁴ Therefore, it reduces inflammation caused by inducible COX-2, but also inhibits COX-1, which is constitutive and responsible for protective and reparative roles.¹²-¹⁴ It is possible that COX-2 was activated by an inflammatory process induced by tissue harvesting and submucosal dissection for ex vivo studies, and baseline endogenous PGE₂ activity in response to this inflammation would explain the insignificant response to added PGE₂ in NSAID-free solutions (protocol 2). The COX-2 selective, or COX-1 sparing, NSAID, FIR was studied because it might be a safe alternative to PBZ in the management of musculoskeletal pain.²⁴ In the current study, FIR had a similar effect on Isc as INDO in both colon segments, which would suggest a role for COX-2 in anion secretion. However, the distinction between COX activities might be complicated in horses, based on evidence that FIR can induce gastric ulceration²⁵ and sufficiently alter colonic
mucosal function to cause dysbiosis.26 A limitation of the current study is the potential for the selected concentration of FIR to be sufficiently high to inhibit COX-1 activity, an effect that could be determined by measuring the activity of this enzyme at different concentrations of FIR.27

Apoptosis was induced by INDO and PBZ in RDC and RVC, although the effect of FIR on the apoptotic index was significant only in the RDC (Table 2). Apoptosis can be induced by NSAIDs in rodent colon, which might explain the ability of these drugs to reduce colorectal cancer or aberrant crypt formation.28 Apoptosis is usually regarded as a minor injury, with loss of individual epithelial cells, causing small defects that are capable of rapid repair without an inflammatory response.15,29,30 It has been described in the ischemic equine colon as part of epithelial repair,31 but has also been implicated in the loss of integrity in equine intestinal mucosa.32 Also, NSAIDs could be detrimental to the repair of apoptotic and other mucosal defects because of the apparent need for PGE2 to direct intestinal stem cells, progenitor cells, and/or immature enterocytes in the repair process.33

Apoptosis develops predominantly in the bottom half of mucosal crypts (Figure 2), which could indicate premature loss of cells before they develop full secretory function. It could also place cellular components of apoptotic cells and fragments within the crypt lumen, where their removal could be dependent on flushing from this locus by secretion of water and mucus by crypt cells. Inhibition of this process by NSAIDs could allow mucus plugging, microbacterial invasion, inflammation, and abscessation within the crypts.34,35 The design of the study and method of detecting apoptosis did not allow identification of the cells targeted by this response, such as stem cells, and this should be addressed in future studies.

Although 2 loop diuretics were used in the current study to inhibit NKCC1, bumetanide, and furosemide, the latter was chosen for protocol 2 because of its greater solubility. The main function of NKCC1 is basolateral uptake of Na+, K+ and Cl− by colonocytes to provide the driving force for Cl− secretion through apical CFTR.11,34 and entail an osmotic flow of water and electrolytes into the crypt lumen. As in rats,35 NKCC1 transporters were strongly expressed in goblet cells, consistent with a role for NKCC1-mediated fluid secretion in normal mucus hydration, alkalization, flow, and bacterial clearance in crypts.36 Although it would be expected also in colonocytes to support their transport functions, its distribution in these cells was sparse.

A limitation of this study was the failure of immunohistochemistry to identify CFTR in cells and the failure to use Western blot analysis to identify and quantify candidate protein transporters in the colon. As in previous experiments in equine RDC,7 CFTR staining was weak and nonspecific compared with the more intense CFTR pattern in colon crypts in other species.16 Weak expression of CFTR could be explained by low sensitivity/ specificity to anti-CFTR antibodies,37 expression in different molecular weights depending on the degree of glycosylation,38–41 degradation by bacterial enzymes,42–45 and sensitivity to low temperatures.5,7,41 two sets of conditions that applied to immunohistochemical methods in the current study. Glycosylation is not necessary for CFTR function38–41 and findings in our electrophysiological study were consistent with functional CFTR. Another study limitation is the lack of information about the relevance of the ex vivo concentrations used in the current study to the in vivo condition. Concentrations selected were based on previous ex vivo studies,6 pharmacokinetic studies,12–14 and from a dose-response curve for INDO,11 whereas FIR was added at a similar concentration as INDO. Selection of concentration is complicated by the long half-life of FIR and the high degree of protein binding in vivo for all NSAIDs.12

In conclusion, the findings of this study demonstrated that NSAIDs can block anion secretion in the equine colon and that a unique but uncharacterized transporter in the RDC appears to be involved in the process. The anions most likely involved are HCO3−, which could buffer VFAs produced by microbial fermentation,45 and Cl−, which could enhance water and mucus flow. Apoptosis induced by NSAIDs could further decrease tissue integrity, crypt clearance, and mucosal barrier function.15 Results of the present study could also provide some guidance to potential treatments,4 such as the use of misoprostol46 to restore anion secretion in the manner demonstrated by PGE2. Recent studies on dietary or oral provision of HCO3− have identified a potential method to replace this critical intraluminal buffer if its secretion is impaired in the RDC.45,47,48 The findings of this study are also consistent with the synergistic effects of 2 important conditions in the pathogenesis of right dorsal colitis, specifically the use of PBZ in dehydrated horses.3,49 Because large volumes of water and ions are required for gastrointestinal tract secretion,50 colonic secretions could decrease in dehydrated horses, which could exacerbate the inhibitory response to NSAID. Consequently, anion secretion could be reduced to the point of causing mucosal injury, similar to that induced by failed anion secretion in CF.

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Disclosures

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**Supplementary Materials**

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