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Effects of a single dose of orally and rectally administered misoprostol in an in vivo endotoxemia model in healthy adult horses

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OBJECTIVE
To describe misoprostol pharmacokinetics and anti-inflammatory efficacy when administered orally or per rectum in endotoxin-challenged horses.

ANIMALS
6 healthy geldings.

PROCEDURES
A randomized 3-treatment crossover design was performed with a minimum washout period of 28 days between treatment arms. Prior to endotoxin challenge (lipopolysaccharide, 30 ng/kg IV over 30 minutes), horses received misoprostol (5 µg/kg once) per os (M-PO) or per rectum (M-PR) or water as control (CON). Clinical parameters were evaluated and blood samples obtained to measure plasma misoprostol free acid concentration, leukocyte counts, and tumor necrosis factor-α (TNFα) and interleukin 6 (IL-6) leukocyte gene expression and serum concentrations.

RESULTS
In the M-PO treatment arm, maximum plasma concentration and area under the concentration-versus-time curve (mean ± SD) were higher (5,209 ± 3,487 pg/mL and 17,998,254 ± 13,194,420 h·pg/mL, respectively) and median (interquartile range) time to maximum concentration (25 min [18 to 34 min]) was longer than in the M-PR treatment arm (854 ± 855 pg/mL; 644,960 ± 558,866 h·pg/mL; 3 min [3 to 3.5 min]). Significant differences in clinical parameters, leukocyte counts, and TNFα or IL-6 gene expression or serum protein concentration were not detected. Downregulation of relative gene expression was appreciated for individual horses in the M-PO and M-PR treatment arms at select time points.

CLINICAL RELEVANCE
Considerable variability in measured parameters was detected among horses within and between treatment arms. Misoprostol absorption and systemic exposure after PO administration differed from previous reports in horses not administered LPS. Investigation of multidose administration of misoprostol is warranted to better evaluate efficacy as an anti-inflammatory therapeutic.

Bacterial endotoxin (lipopolysaccharide [LPS]) is considered a common trigger of systemic inflammatory response syndrome (SIRS) in horses.[1–4] An estimated 25 to 41% of horses admitted for colic,[5,6] and more than 30% of sick foals meet the diagnostic criteria of SIRS at hospital admission.[4,7–9] Unfortunately, treatments to mitigate SIRS are limited in horses. Flunixin meglumine remains a cornerstone therapeutic for the treatment of SIRS due to its ability to inhibit cyclooxygenase-mediated prostanoid synthesis associated with inflammation.[10] While flunixin meglumine improves some manifestations of SIRS, it demonstrates limited ability to reduce key inflammatory cytokines[11] and adverse effects can include gastrointestinal ulceration and nephrotoxicity.[10,12] There is a significant clinical need for novel therapeutics for the treatment of SIRS in horses. Misoprostol, a synthetic prostaglandin E_1 (PGE_1) analogue and prostanoid receptor agonist, acts through cyclic AMP (cAMP)-mediated pathways and is known to mediate gastrointestinal mucosal protection and repair.[13,14] Misoprostol has been evaluated for its

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anti-inflammatory properties in both in vitro and ex vivo studies.[15–21]

Cyclic AMP is an intracellular second messenger that helps regulate inflammation,[15–17,20–22] and the anti-inflammatory properties of the cAMP mediators clenbuterol and pentoxifylline have previously been described in horses.[23–25] Misoprostol's anti-inflammatory actions occur via E2 prostanoid receptor-mediated increases in cAMP, activation of protein kinase A, and subsequent attenuation of nuclear factor-κB (NFκB) transcriptional activity.[16,21] Single-dose studies[26] have described the pharmacokinetics of misoprostol administered per os (PO) and per rectum (PR) in fasted and fed healthy horses, and plasma concentrations reported in these studies are comparable to those described in humans. Misoprostol treatment of LPS-stimulated equine peripheral blood leukocytes in vitro resulted in inhibition of inflammatory cytokine expression and attenuation of neutrophil proinflammatory activity.[19,20] In contrast to findings in humans,[16] these in vitro results did not translate in an ex vivo experimental model in horses.[19] The effective plasma concentration of misoprostol needed for in vivo anti-inflammatory activity is unknown and, to date, investigations of misoprostol's anti-inflammatory efficacy in vivo in the horse have not been performed. Given the complexities of inflammation, in vivo evaluation is warranted.

The objectives of this study were to describe the pharmacokinetics and pharmacodynamics of a single 5-µg/kg dose of misoprostol administered PO or PR and to evaluate its efficacy in ameliorating clinical indicators and markers of inflammation in healthy adult horses experimentally challenged with LPS. We hypothesized that compared to control horses (LPS administered without misoprostol), horses administered LPS and misoprostol (PO or PR) would demonstrate reductions in expression of inflammatory cytokines and improvement in specific clinical indicators of inflammation.

Materials and Methods

Animals

Six university-owned healthy adult (age, 9 to 18 years) geldings of various breeds (body weight, 454 to 655 kg) were studied. Horses were deemed healthy based on physical examination findings, serum biochemistry analysis, and CBC performed prior to study onset. Horses were housed individually in stalls for at least 18 hours prior to and for the duration of each experimental period with ad libitum access to water. Horses were housed on pastures between experimental periods. Horses were fasted for 12 hours prior to each experimental period and until 2 hours after completion of LPS infusion. Horses were then fed 1 flake coastal bermudagrass hay and 1.5 pounds senior pelleted feed every 6 hours.

During experimental periods, horses were monitored continuously for the first 6 hours and then hourly. Physical examinations were performed prior to starting and throughout each experimental period until 24 hours after LPS administration. The study was approved by the Auburn University Institutional Animal Care and Use Committee (protocol No. 2020-3736).

Experimental design

This was a 3-treatment randomized crossover study. Treatment order was assigned by simple randomization. All horses were administered LPS in each treatment arm and were randomized to receive misoprostol PO (M-PO) or PR (M-PR) or water as a control treatment (CON) with a minimum 28-day washout period between each treatment arm to avoid endotoxin tolerance.[27] For each experimental period, horses were instrumented with 14-gauge over the needle catheters in the left jugular vein to facilitate repeated blood collection, and the right jugular vein for administration of LPS. A single 5-µg/kg dose of misoprostol (M-PO, M-PR) or similar volume of water (CON) was administered. The LPS infusion (30 ng/kg) was initiated immediately following misoprostol or water administration. Blood samples were collected for measurement of plasma misoprostol free acid (MFA) concentrations, leukogram counts, peripheral blood leukocyte cytokine gene expression, and serum cytokine concentrations. Reported clinical outcomes were derived from physical examinations, nonblinded clinical observations, and videos recorded for blinded scoring of behavioral data. For each treatment arm, baseline (time 0) blood samples and clinical outcomes were collected immediately prior to administration of misoprostol (M-PO, M-PR) or water (CON).

Misoprostol and LPS administration

For each treatment arm, manure was manually evacuated prior to administration of misoprostol (M-PO, M-PR) or water (CON). For per os administration (M-PO), misoprostol tablets (100 µg; Lupin Pharmaceuticals) were dissolved in 30 mL of water and administered via oral syringe. This was followed by administration of 30 mL of water through the same syringe for a total volume of 60 mL delivered. Water (60 mL) was also administered per rectum via 16-inch 8-French red-rubber catheter advanced approximately 30 cm into the rectum. For per rectum administration (M-PR), misoprostol tablets dissolved in 30 mL of water were infused via red-rubber catheter as described above, followed by infusion of 30 mL of water through the same syringe for a total volume of 60 mL delivered. Water (60 mL) was then administered orally via syringe. Horses in the CON treatment received water per os (60 mL) and per rectum (60 mL) as already described. Immediately following treatment administration (M-PR/M-PO/CON), LPS (10 µg/mL; List Biological Labs) was administered IV at 30 ng/kg diluted in 500 mL saline (0.9% NaCl) solution as a 30-minute continuous rate infusion.

Blood sample collection

For pharmacokinetic analysis of plasma MFA concentrations, blood was collected at 0.08, 0.17, 0.25, 0.33, 0.5, 0.75, 1, 1.25, 1.5, 1.75 2, 2.5, 3, 3.5, 4, and 6 hours after M-PO treatment and at 0.05,

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0.08, 0.17, 0.25, 0.33, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, and 3 hours after M-PR treatment. The different sampling times for M-PO and M-PR reflect reported differences in pharmacokinetic parameters between these routes of administration.[26] Samples were immediately transferred to sodium-heparin tubes, placed on ice, and centrifuged (400 X *g* for 10 minutes at 4 °C) within 15 minutes after collection. The plasma was separated into 1-mL aliquots and stored at –80 °C until analysis.

For evaluation of leukogram cell counts, cytokine gene expression, and serum cytokine concentration, blood samples were obtained at 0.5, 1, 1.5, 2, 3, 6, and 24 hours postcompletion of LPS infusion (PLI). Blood was transferred to potassium-EDTA tubes for determination of leukogram cell counts. Blood for leukocyte cytokine gene expression was transferred to sodium-heparin tubes, placed on ice, and centrifuged at 400 X *g* for 10 minutes at 4 °C. The buffy coat was then isolated, washed in erythrocyte lysis buffer (EL buffer; QIAGEN), and incubated in ice with intermittent vortexing for 40 minutes. Cell pellets underwent 3 additional cycles of washing and centrifugation and were suspended in 1.4 mL of lysis buffer, flash frozen in liquid nitrogen, and then stored at −80 °C until RNA isolation and quantitative (q)RT-PCR analysis. For serum cytokine measurement, blood was placed in a nonadditive (serum) blood tube for 30 minutes, held at room temperature to allow the blood to clot, centrifuged at 800 X *g* for 15 minutes at room temperature, and then the serum was separated and stored in 500-µL aliquots at −80 °C until batch analysis.

Clinical parameters

Physical examinations and nonblinded clinical observations were performed at baseline, upon completion of LPS infusion, every 15 minutes for the first 2 hours, every 30 minutes until 6 hours, then every 2 hours until 12 hours, and then at 24 hours PLI. Evaluated physical examination parameters included heart and respiratory rates and rectal temperature. To account for interhorse variability in baseline physical examination parameters and in total leukocyte and neutrophil cell counts, data for these parameters were evaluated as absolute values and as percent change relative to each horse's baseline value. For horses demonstrating severe or persistent (> 2 hours) effects of LPS administration including progressive abdominal discomfort, heart rate (HR) > 100 beats/min, or temperature > 39.7 °C, rescue protocols included removal from study and administration of flunixin meglumine (1.1 mg/kg IV) with additional evaluation or treatment dictated by the response to flunixin administration and specific adverse events observed.

Nonblinded clinical observations included manure output and character (formed, soft, liquid, diarrhea), colic signs, and muscle fasciculations. Videos for blinded behavioral scoring (2-minute duration) were recorded at baseline and then at 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 2.5, 3, and 6 hours PLI. Identifiers of these recorded files were scrambled by a random number generator (www.random.org). Blinded scoring was adapted from previous literature[28] to evaluate horses for behaviors consistent with discomfort including, flehmen, kicking, pawing, rolling, and other colic signs. These were scored as 1 (no signs displayed), 2 (occasional signs displayed), and 3 (continuous signs displayed) by a blinded scorer (LC). The maximum recorded score and averaged score from all video recordings during each treatment arm were reported for each horse and compared between treatment arms.

Isolation of leukocyte RNA and qRT-PCR

Preparation of samples for RNA isolation and quantification prior to cDNA synthesis was modified from the manufacturer's protocol (RNeasy Mini Kit; QIAGEN). Briefly, leukocyte pellets were homogenized using a shredder spin column (QIAshredder, QIAGEN) followed by on-column DNase digestion prior to RNA elution. Purity was determined with a spectrophotometer (NanoDrop2000; Thermo Fisher Scientific). Synthesis of cDNA was performed using a commercial kit (SuperScript First-Strand Synthesis System; Invitrogen). Primers and probes for tumor necrosis factor-α (*TNFα*), interleukin-6 (*IL-6*), and housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (*GADPH*) and elongation factor 1- (*EF1α*) were designed by 2 of the investigators (AM, SH) using a commercial tool (Integrated Data Technologies). Amplification was performed according to the following protocol: 50 °C for 1 minute, 1 time; 96 °C for 10 minutes, 1 time; and 96 °C for 15 seconds followed by 60 °C for 1 minute (with real time data collection enabled), 40 times. The relative fold changes in mRNA expression for *TNFα* and *IL-6* were determined using the ΔΔCt method.

Serum cytokine measurement

Serum concentrations of TNFα and IL-6 proteins were measured using a multiplex bead immunoassay (Equine Cytokine Magnetic Bead Panel; Millipore Sigma) according to manufacturer directions. Briefly, an equal volume of assay buffer, matrix buffer, and beads was added to wells containing 25 µL of sample, control, or standard. Samples were incubated under agitation in 2 to 8 °C for 16 to 18 hours, and following washing were incubated at room temperature for 1 hour with detection antibody. Streptavidin-phycoerythrin was then added and incubated for an additional 30 minutes and following washing were resuspended before the plates were read under manufacturer directions (MILLIPLEX MAP with Luminex MAGPIX system; Millipore Sigma). Samples that were higher than the limit of detection were read again after a 5-fold dilution. Data were evaluated as absolute measurements of cytokine concentration at each time point PLI for all treatment arms and according to differences in concentrations for the M-PO and M-PR treatment arms as compared to the CON treatment arms.

Measurement of plasma MFA concentration

Measurement of plasma MFA concentrations was adapted from a previously described protocol[26]

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modified to enhance the lower limit of quantification (LLOQ) to 2 pg/mL. Briefly, 500 µL plasma was added to 1 mL of acetonitrile with 5 µL misoprostol acid-d_5 standard (100 ng/mL) which was then vortexed and centrifuged. The supernatant was collected, dried, and resuspended in 100 µL of solvent on a C18 column (2.1 X 100-mm, 1.5-µm particle size; Thermo Accucore Vanquish C18+ column, Thermo Fisher Scientific) prior to undergoing liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis using a triple quadrupole system (Thermo Altis Triple Quadrupole System, Thermo Fisher Scientific) and proprietary software (TraceFinder 4.1; Thermo Fisher Scientific). The data acquisition parameters were as previously described.[26]

Standard calibration curves were established prior to sample analysis using drug-free aliquots of equine plasma with commercially available MFA and MFA-d5 (Cayman Chemical) as the internal standards. The calibrated concentration range was 5 to 5,000 pg/mL. The coefficient of determination (R^2) was ≥ 0.993. The mean ± SD percentage recovery reported for misoprostol plasma concentrations of 300, 1,000, and 3,000 pg/mL was 66.7 ± 9.1%, 71.0 ± 6.2%, and 72.8 ± 3.3%, respectively. The range (mean ± SD) for within-run accuracy and precision of misoprostol detection in plasma was 96.1 ± 8.2% to 116.2 ± 3.4%, and 4.3 to 7.8%, respectively. The range (mean ± SD) for between-run accuracy and precision was 98.9% ± 7.4% to 115.0 ± 5.8% and 3.7% to 5.4%, respectively.

Pharmacokinetic analysis

Data of plasma MFA concentration-versus-time underwent noncompartmental analysis (Phoenix WinNonLin, version8.1; Cetara). For each route, values of area under the concentration-versus-time curve from time 0 to infinity ($AUC_{0\rightarrow\infty}$) and from time 0 to the last measured concentration ($AUC_{0\rightarrow last}$) were calculated using the log-linear trapezoidal method. Maximum and minimum observed concentrations and their respective times were obtained directly (C_{max}, C_{last}, t_{max}, and t_{min}). Nonlinear regression was used to obtain the slope of the terminal component of the drug-disappearance time curve. Since intravenous drug administration was not performed and the slope could not be confirmed to represent elimination, both the terminal rate constant (λ_z) and its corresponding half-life ($t_{½dis}$) could not be directly measured and were reported as disappearance. Furthermore, in the absence of IV administration, neither apparent volume of distribution (Vd) nor clearance (Cl) could be determined, and they were reported out as ratio to absolute bioavailability (F), Vd/F, and Cl/F, respectively. Other reported parameters include mean residence time (MRT) and mean concentration (C_{mean}). The relative bioavailability of PR compared to PO was determined for each horse based on F = $AUC_{M\text{-}PR}$/ $AUC_{M\text{-}PO}$. Data were subjected to dose-response analysis using nonlinear regression for all misoprostol concentrations and accompanying TNFα or IL-6 concentrations.

Statistical analysis

Statistical software packages (Graphpad Prism; SAS version 9.2) were used for all analyses. Distribution of data was evaluated for normality by the Shapiro-Wilk and Anderson-Darling tests. Normally distributed data are reported as mean ± SD, and otherwise displayed as median (interquartile range) or median (range). Significance over time was compared using a linear mixed-model analysis or 2-way repeated measure ANOVA. Nonnormally distributed data were tested for significance by Friedman's tests. Comparisons of pharmacokinetic values between M-PO and M-PR treatment arms were performed using a paired 2-sample *t* test or Wilcoxon matched-pair signed rank test. Pearson's correlation analysis was used to assess relationships between specific pharmacokinetic parameters (AUC, C_{max}, and t_{max}) and fasciculation onset and duration times. Maximum and averaged behavioral scores from video recordings were compared between M-PO, M-PR, and CON treatment arms by 1-way ANOVA. When needed, post hoc comparisons were performed using Tukey's multiple comparisons test. Significance was defined as $P < 0.05$.

Results

Clinical parameters

Muscle fasciculations were observed PLI in all horses and treatment arms. Compared to the CON treatment arm (onset: 68 ± 33 min; duration: 68 ± 30 min), time of onset was earlier and duration longer for fasciculations in the M-PO (onset: 35 ± 16 min, $P = 0.03$, 95% CI: 33 to 97; duration: 115 ± 25 min, $P = 0.03$, 95% CI: 5 to 95) and M-PR (onset: 20 ± 8 min, $P = 0.004$, 95% CI: 16 to 79; duration: 115 ± 32 min; $P = 0.04$, 95% CI: 3 to 92) treatment arms.

Changes in manure character characterized as single bouts of liquid or soft manure were observed in 2/6 horses in M-PO and CON and 3/6 horses in the M-PR treatment arms. During each treatment arm, 2/6 horses demonstrated transient signs of mild colic (flehmen, response, stretching out, or intermittent pawing) that resolved within 90 minutes PLI. For 1 horse, colic and changes in manure were observed in each treatment arm. Averaged behavioral scores were 6 ± 1 (M-PO), 7 ± 2 (M-PR), and 5 ± 1 (CON). Maximum scores were 11 ± 3 (M-PO), 11 ± 3 (M-PR), and 11 ± 3 (CON). Differences in averaged ($P = 0.41$) or maximum ($P = 0.95$) score were not observed between treatment arms.

There were no significant differences in HR, temperature, or RR (respiratory rate) between treatment arms at any time point for either absolute values or percent change from baseline. Increases PLI in HR ($P < 0.001$ all) and temperature ($P < 0.001$ all) were identified in each treatment arm. For HR, maximum values [median (range)] and maximum percent increase (mean ± SD) relative to baseline were observed within 1.5 hours PLI and were 62 (48-84) beats/min and 49 ± 38%, 68 (52-100) beats/min and 82 ± 55%, and 60 (52-100) beats/min and

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87 ± 69% for the M-PR, M-PO, and CON treatment arms, respectively. Maximum temperatures and maximum percentage increase over baseline were observed within 3 hours PLI and were 39.0 (38.6 to 39.7) °C and 3.6 ± 0.65%, 38.9 (38.4 to 38.8) °C, and 3.2 ± 0.64%, and 38.7 (39.4 to 39.2) °C and 3 ± 0.84% for the M-PR, M-PO, and CON treatment arms, respectively. Changes in RR were variable and not significant.

Leukogram changes

There were no significant differences in total leukocyte (P = 0.52) and neutrophil (P = 0.82) counts between treatment arms at any time point. Similarly, there were no differences between treatment arms in percent change from baseline at any time point for total leukocyte (P = 0.46) or neutrophil (p = 0.93) counts **(Supplemental Figures S1 and S2)**. Baseline total leukocyte counts (cells/µL) were 6,727 ± 947, 8,027 ± 1,633 and 7,037 ± 1,817 for the M-PR, M-PO, and CON treatment arms. Baseline neutrophil counts (cells/µL) for the M-PR, M-PO, and CON treatment arms were 4,021 ± 859, 4,991 ± 1,064, and 4,608 ± 1,949. In all treatment arms, neutrophil (P < 0.001) and total leukocyte (P < 0.004) counts significantly decreased between 0.5 and 1.5 hours PLI with observed peak percent reductions from baseline of 82 ± 10% and 66 ± 8.4%, respectively. Total leukocyte and neutrophil counts (P = 0.002 both) were significantly increased by 64 ± 28% and 102 ± 53% at 24 hours PLI when compared to baseline values.

Cytokine gene expression

Fold changes in *TNFα* and *IL-6* gene expression for individual horses in the M-PR and M-PO treatments relative to CON treatment are depicted **(Figures 1** and **2)**. For the M-PO treatment arm, the 0.5-hour PLI time point is excluded for horse 3 as that sample was lost during processing. Data that represent at least a 2-fold change in relative gene expression (up- or downregulation) were considered biologically significant. Relative gene expression was highly variable between horses and across time points for both *TNFα* and *IL-6*. At time 0 (baseline), minimal changes in relative gene expression were observed for most horses, with the notable exception of horse 1 in the M-PR treatment arm, that demonstrated a 17-fold upregulation of *TNFα* and 9-fold upregulation of *IL-6* gene expression (relative to baseline under the CON treatment) that was not maintained for the remainder of the treatment period.

Downregulation in relative gene expression of *TNFα* and *IL-6* was appreciated for individual horses in both the M-PO and M-PR treatment arms with considerable variation existing among horses in timing and magnitude of downregulation. In the M-PO treatment arm, overall fold-reduction in *TNFα* relative gene expression ranged from 2.06 to 7.16

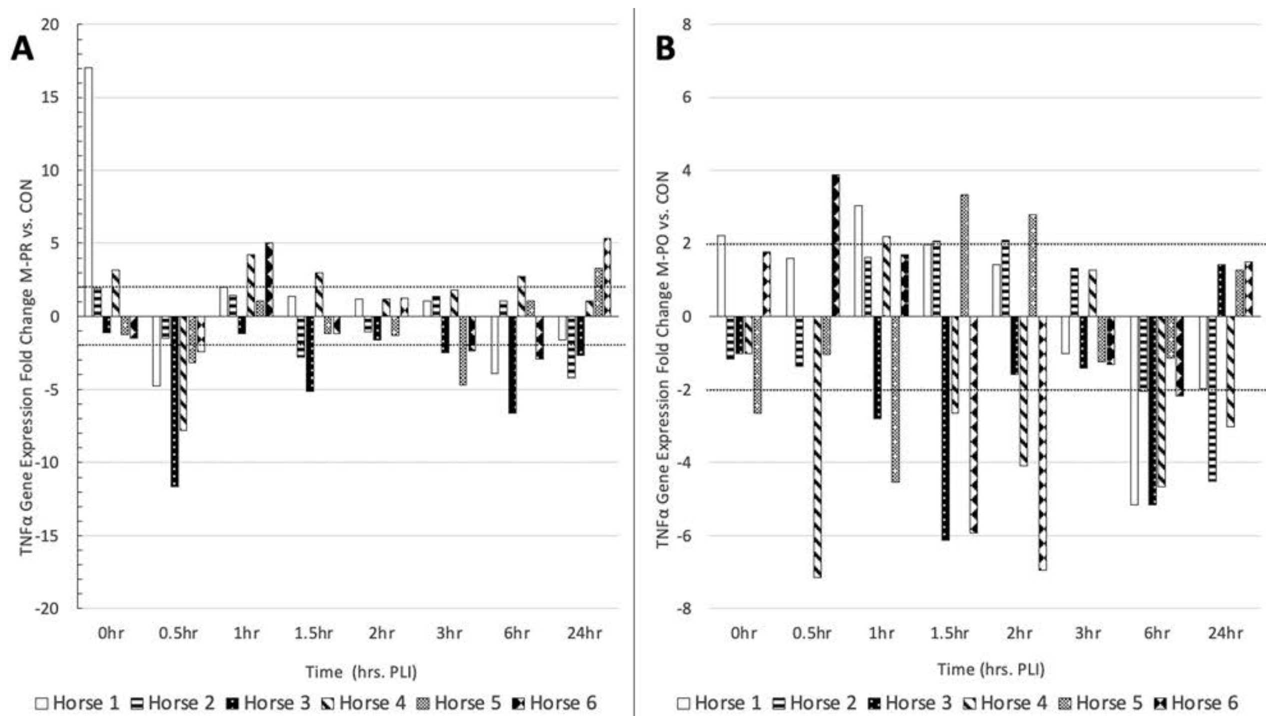

Figure 1—Bar graphs indicating individual horse fold change in tumor necrosis factor-α (*TNFα*) gene expression across time for misoprostol administered per rectum (M-PR) relative to control (CON) treatment arm (A), and misoprostol administered per os (M-PO) relative to CON treatment arm (B). The dotted line indicates areas of nonsignificant fold-changes in gene expression changes (≤ 2-fold upregulation or downregulation). LPI = Postcompletion of LPS infusion.

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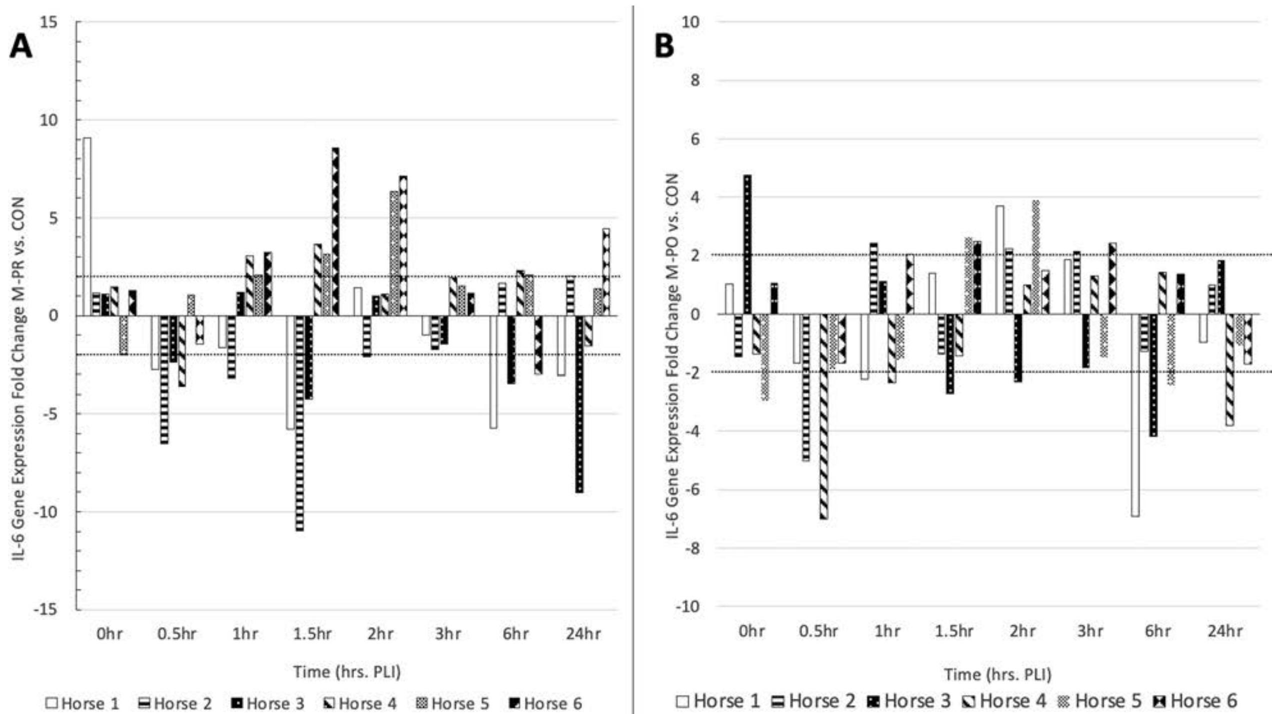

Figure 2—Bar graphs indicating individual horse fold change in interleukin 6 (IL-6) gene expression across time for M-PR relative to CON treatment arm (A) and M-PO relative to CON treatment arm (B). The dotted line indicates areas of nonsignificant fold-changes in gene expression changes (≤ 2-fold upregulation or downregulation).

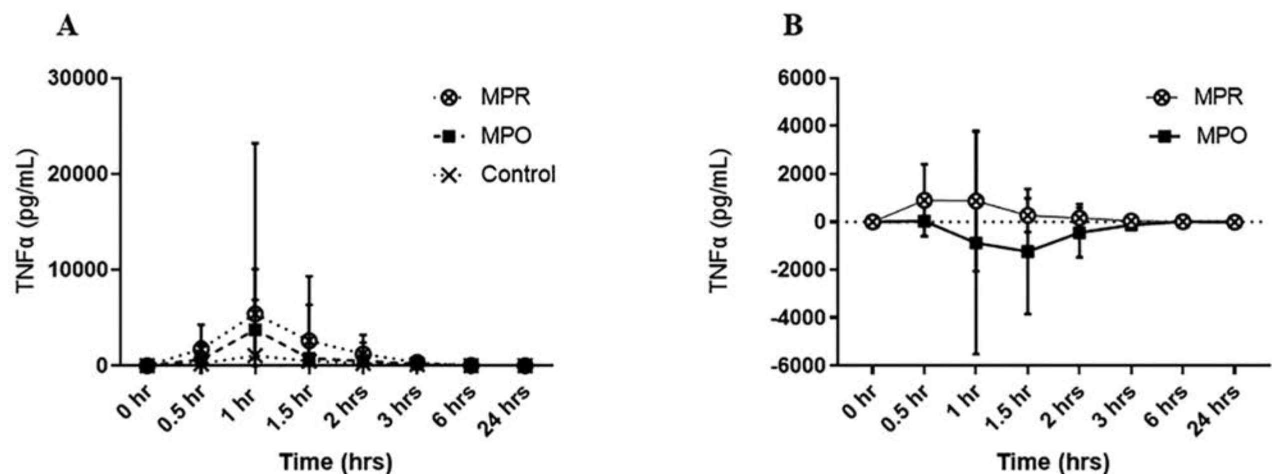

Figure 3—Line graphs indicating TNFα serum concentrations (mean ± SD) post-LPS infusion for M-PR, M-PO, and CON treatment arms (A) and displayed as differences in concentrations for M-PR treatment arm compared to CON treatment arm (CON time *x* – M-PR time *x*) and for M-PO treatment arm compared to CON treatment arm (CON time *x* – M-PO time *x*) (B). The dashed line represents TNFα concentrations for the CON treatment arm set to zero.

with 5/6 horses demonstrating ≥ 2-fold reduction in *TNFα* gene expression at 6h PLI. Overall fold-reductions in *IL-6* relative gene expression ranged from 2.22 to 7.01 with 3/6 horses demonstrating ≥ 2-fold reduction at 6 hours. In the M-PR treatment arm, 5/6 horses demonstrated a ≥ 2-fold reduction (range, 2.43 to 11.64-fold reduction) in *TNFα* gene expression and 4/6 horses demonstrated a ≥ 2-fold reduction (range, 2.36-6.53-fold reduction) in *IL-6* gene expression at 0.5 hours PLI with 3/6 horses demonstrating reductions in both *IL-6* and *TNFα* at this time point.

Serum cytokine concentrations

Changes in TNFα and IL-6 protein concentrations for all treatment arms and the differences in cytokine concentration relative to the CON treatment arm for the M-PR and M-PO treatment arms are displayed **(Figures 3** and **4)**. In all treatment arms, TNFα ($P < 0.001$) and IL-6 ($P < 0.001$) concentrations increased

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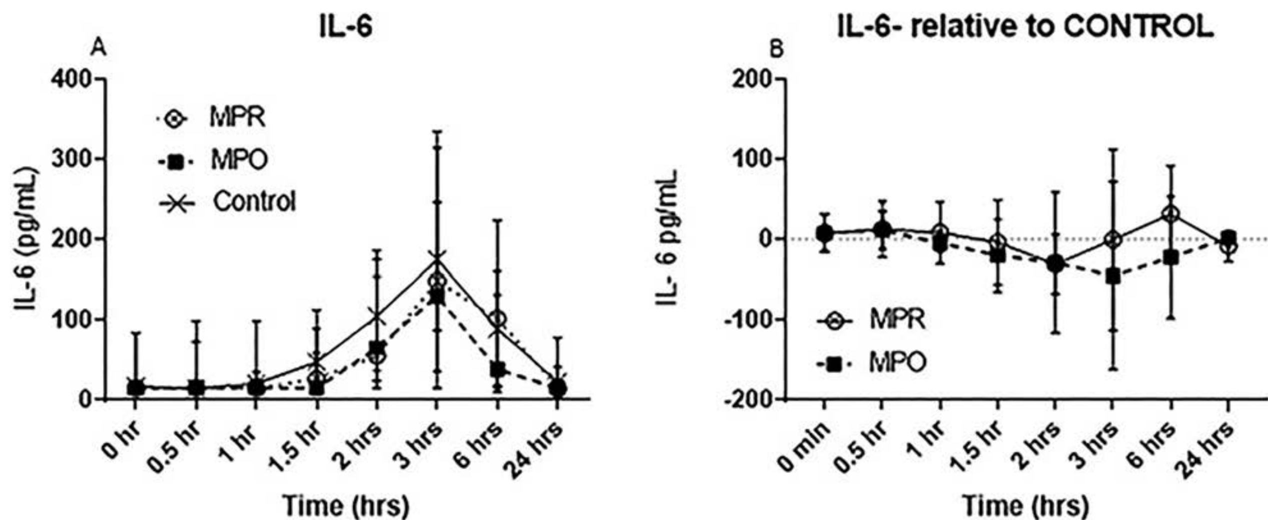

Figure 4—Line graphs indicating IL-6 serum concentrations (mean ± SD) post-LPS infusion for M-PR, M-PO, and CON treatment arms (A) and displayed as differences in concentrations for M-PR treatment arm compared to CON treatment arm (CON time *x* – M-PR time *x*) and for M-PO treatment arm compared to CON treatment arm (CON time *x* – M-PO time *x*) (B). The dashed line represents IL-6 concentrations for the CON treatment arm set to zero.

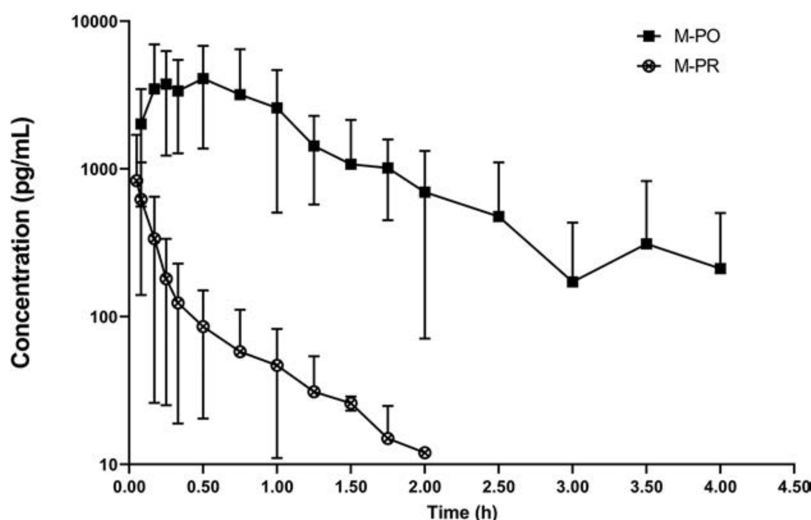

Figure 5—Mean plasma misoprostol free acid (MFA) concentration-versus-time curves for 6 healthy adult horses that received a single dose of misoprostol (5 µg/kg) administered M-PO (rectangle) and M-PR (hatched circle) immediately prior to LPS (30 ng/kg) administration in a crossover study design. Concentrations were measured before (time 0) and at 0.08, 0.17, 0.25, 0.33, 0.5, 0.75, 1, 1.25, 1.5, 1.75 2, 2.5, 3, 3.5, 4, and 6 hours after M-PO treatment, and at 0.05, 0.08, 0.17, 0.25, 0.33, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, and 3 hours after M-PR treatment however, data are shown for the first 4.5 hours only because concentrations were below the lower limit of quantification of the assay (2 pg/mL) at all subsequent measurement points when misoprostol was administered PO and after the 2-h point when it was administered PR. Error bars represent SD.

significantly from baseline, peaking at 1 hour PLI for TNFα and by 3 hours PLI for IL-6. Maximum concentrations for the M-PR, M-PO, and CON treatment arms were 5,404 ± 4,681 pg/mL, 3,657 ± 2,613 pg/mL, and 5,254 ± 4,633 pg/mL for TNFα and 177 ± 91 pg/mL, 145 ± 52 pg/mL, and 191 ± 122 pg/mL for IL-6. Differences compared to baseline cytokine concentrations were not observed for either TNFα or IL-6 at 24-hours PLI. Cytokine concentrations did not differ between treatment arms at any time point nor were differences in achieved peak concentrations identified.

Pharmacokinetic and pharmacodynamic results

Plasma concentrations of MFA were detectable in all horses until 150 minutes (M-PO) and 45 minutes (M-PR) postdrug administration and were below LLOQ for all horses after 240 and 120 minutes for the M-PO and M-PR treatment arms, respectively. Plasma misoprostol concentration-against-time curves were generated for each treatment arm **(Figure 5)** and pharmacokinetic parameters for the M-PO and M-PR treatment arms are summarized **(Table 1)**. The mean relative bioavailability of misoprostol in the M-PR treatment arm was 5.00 ± 3.34% (range, 0.83% to 8.77%) compared with bioavailability in the M-PO treatment arm.

Values of C_{max} for M-PO were more than 6-fold higher than M-PR ($P < 0.001$). Similarly, M-PO had more than 20-fold higher $AUC_{0\to\infty}$ than M-PR ($P < 0.001$). Both M-PO and M-PR demonstrated large variations in C_{max} and $AUC_{0\to\infty}$ among horses. Coefficient of variation (CV) of C_{max} for M-PO and M-PR was 67% and 100%, respectively, with ranges of 1,664 to 10,226 pg/mL for M-PO and 268 to 2,580 pg/mL for M-PR. For $AUC_{0\to\infty}$, CV for M-PO and M-PR were 73% and 91%, respectively. Ranges in $AUC_{0\to\infty}$ were 5,013,960 to 41,107,860 h·pg/mL for M-PO and 161,580 to

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Table 1—Plasma pharmacokinetic values for misoprostol following per os (M-PO) or per rectum (M-PR) administration to 6 adult horses.

Variable	M-PO	M- PR
t_{max} (min)	25 (18-34)[a]	3 (3-3.5)[b]
	10-45	3-5
t_{last} (min)	150 (150-240)[a]	45 (56-109)[b]
	195-240	75-120
C_{max} (pg/mL)	5,209 ± 3,487[a]	854 ± 855[b]
C_{last} (pg/mL)	135 ± 155	8.83 ± 4.07
$AUC_{0\rightarrow\infty}$ (h·pg/mL)	17,998,254 ± 13,194,420[a]	644,960 ± 558,866[b]
$AUC_{0\rightarrow last}$ (h·pg/mL)	17,467,344 ± 12,449,328[a]	633,860 ± 552,758[b]
$t_{½dis}$ (min)	40 ± 21[a]	9 ± 7[b]
λ_z (min^{-1})	0.02 ± 0.0[a]	0.11 ± 0.08[b]
C_{mean} (pg/mL)	829 ± 601[a]	60 ± 52[b]
MRT (min)	59 ± 13[a]	15 ± 9[b]
Vd/F (mL/kg)	1.3 ± 0.8[a]	12 ± 9[b]
Cl/F (mL/h/kg)	1.7 ± 1.3[a]	45 ± 35[b]

Values are reported as mean ± SD for all parameters except $t_{½dis}$, which is reported as harmonic mean ± pseudoSD and t_{max} and t_{last}, which are reported as median (interquartile range) and range.

λ_z = Terminal rate constant. $AUC_{0\rightarrow\infty}$ = Area under the concentration-versus-time curve from time 0 to infinity. $AUC_{0\rightarrow last}$ = Area under the concentration-versus-time curve from time 0 to the last measured concentration. Cl/F = Apparent clearance, corrected for bioavailability. C_{mean} = Mean plasma concentration during study period. C_{max} and C_{last} = Maximum observed and last observed plasma concentration' MRT = Mean residence time. $t_{½dis}$ = Disappearance half-life. t_{max} and t_{last} = Time to maximum and time to last measured plasma concentration. Vd/F = Apparent volume of distribution, corrected for bioavailability.

[a,b]Within a row, values with different superscript letters are significantly different ($P < 0.05$)

1,746,540 h·pg/mL for M-PR. The M-PR treatment arm had greater Vd/F Cl/F ($P < 0.001$ both) compared to the M-PO treatment arm. The t_{max} and MRT for M-PO were significantly longer ($P = 0.005$ and $P < 0.02$, respectively) than M-PR.

For the M-PO and M-PR treatment arms, correlations were not identified between t_{max}, C_{max}, MRT, or $AUC_{0\rightarrow\infty}$ and time of onset or duration of fasciculations, changes in temperature, HR, RR, neutrophil counts, or observed cytokine concentrations ($P > 0.15$ all; $R^2 < 0.4$ all).

Discussion

In the present study, we investigated misoprostol's pharmacokinetic and anti-inflammatory characteristics in the context of single low-dose endotoxin challenge in vivo. To our knowledge, similar experimental studies with misoprostol have not been performed in horses. Compared to PO administration, horses receiving misoprostol PR had lower C_{max} and AUC with shorter t_{max}, $t_{½dis}$, and MRT. The values for these parameters obtained in our study, particularly after PO administration, are several-fold higher from previous pharmacokinetic studies[18,26,29–32] in both horses and humans. Contrary to our hypothesis, we did not detect improved clinical outcomes or attenuation in leukocyte reduction or inflammatory cytokine production associated with misoprostol administration in horses challenged with endotoxin. Gene expression changes and altered protein production of both *TNFα* and *IL-6* could be appreciated in individual horses well after the LLOQ of plasma misoprostol concentration was reached.

As expected, predictable changes in temperature, heart rate, respiratory rate, WBC, and neutrophil counts were observed after LPS administration for all horses in this study.[23,27,28, 32–34] Previous in vitro studies utilizing equine leukocytes demonstrated misoprostol-mediated inhibition of neutrophil chemotaxis along with inhibition of inflammatory cytokine expression and production.[19,20] The cause of neutropenia following endotoxin administration is attributed to margination of neutrophils from the circulating pool following upregulation of adhesion molecules,[35] and therefore, in vitro findings were thought to translate in vivo by mitigation in the severity of neutropenia, which was not observed in our study. It is possible that misoprostol more specifically inhibits neutrophil reactive oxygen species production or respiratory burst activity, as has been demonstrated in previous in vitro studies,[19,20] but this was not evaluated in this study.

In this study, there was no observed relationship between changes in leukocyte gene expression and serum protein concentrations for TNFα and IL-6 after misoprostol administration. This may be attributable to several factors, foremost of which is the considerable variability noted between horses in our study. Similar to our findings, previous ex vivo work demonstrated large variations in *TNFα* mRNA production between individual horse leukocytes stimulated with LPS.[18] However, it is noteworthy that individual horses in our study displayed pronounced and prolonged alterations in gene expression and cytokine production for both the M-PO and M-PR treatment arms despite the fact that plasma MFA concentrations reached the LLOQ for most horses at 1.5 h after drug administration in the M-PR and at 3.5 hours in the M-PO treatment arms, respectively (Table 1). These apparent "late effects" may be better

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appreciated with more prolonged drug exposure in horses (eg, a multidose regimen), as LPS-induced TNFα modulation ex vivo was observed following a 14-day course of misoprostol administered every 6 hours in humans.[16] Finally, while changes in serum concentrations of TNFα and IL-6 may result from changes in leukocyte gene expression, they may also reflect cytokine production from other sources sensitive to LPS, such as endothelial cells.[35]

As recently reported for horses without LPS challenge,[26] we identified more rapid absorption of misoprostol after PR administration but greater systemic exposure after PO administration (Table 1) with an observed bioavailability of PR relative to PO administration of approximately 5%. A potential advantage of PR administration is a drug's ability bypass first-pass metabolism.[36] This can result in superior systemic drug exposure compared to PO administration.[36,37] Factors that could influence PR drug absorption include location of drug deposition within the rectum and fecal material.[36,38] In this study, PR drug administration, including rectal evacuation, was standardized across horses and passage of manure was not observed in any horse until well after t_{max} had been achieved. The physiology of PR drug absorption is less clearly defined in the horse[36] and thus somewhat less predictable, which may also contribute to poor bioavailability of PR relative to PO administration observed for misoprostol in this and the previous study[26] and reported for other drugs.[38,39]

There are some notable differences between our study and the previous study by Lopp et al[26] While C_{max} and AUC are similar between studies for PR administration, C_{max} reported by Lopp et al[26] after PO administration was 967 ± 492 pg/mL, and when converted to units comparable to our study, their reported AUC was 3,859,200 ± 1,296,000 (h·pg/mL). This is approximately 8- and 4.5-fold lower than C_{max} and AUC reported for the M-PO treatment arm in this study. Interestingly, our reported CV for C_{max} and AUC was approximately twice that reported by Lopp et al[26] suggesting greater variability in misoprostol absorption and exposure in this study. It is unknown whether these pharmacokinetic differences can be attributed to LPS administration as our study did not include a treatment arm administered misoprostol without LPS. Studies[40,41] in other species suggest that LPS can alter various pharmacokinetic parameters. Systemic inflammation can alter drug pharmacokinetics and pharmacodynamics by modifying cell receptor expression, protein binding, and drug absorption, metabolism, distribution, and excretion[42] and might affect gastrointestinal absorption by altering mucosal permeability or gastrointestinal motility and secretory action.[43] If LPS did impact misoprostol absorption in this study, the more pronounced pharmacokinetic differences in the M-PO treatment arm could be attributed to the slower absorption of misoprostol after PO administration, which would have provided a greater exposure to any physiologic effects of LPS during the drug absorption phase.

Variably reported adverse effects associated with misoprostol administration in horses include abdominal discomfort and diarrhea.[18] In humans, these are dose and drug absorption dependent. In this study an association between pharmacokinetic parameters and the frequency, duration, or onset of signs of colic, changes in manure character, and muscle fasciculations was not observed in the M-PO and M-PR treatment arms. Furthermore, adverse effects associated with LPS administration are similar to those of misoprostol and individual horses in all treatment arms, including CON, demonstrated these signs. Thus, while it is impossible to rule out the contribution of misoprostol, it is likely that observed colic, changes in manure, and fasciculations were attributable primarily to LPS administration.

There are limitations to this study. Our low- and single-dose model for administration of LPS does not reflect SIRS associated with natural disease where the duration and degree of exposure to LPS or other proinflammatory molecules may be increased and unpredictable. Administration of a higher LPS doses to horses is described but may increase the risk of more severe clinical sequelae.[44,45] The 30-ng/kg dose chosen for this study is extensively used in equine endotoxemia models,[23, 27,28,32,34] including repeated measure study designs, where its suitability for avoiding endotoxin tolerance is reported.[27]

Similarly, the duration, frequency, and timing of misoprostol administration relative to LPS administration used in this study do not reflect the clinical setting where repeated administration of misoprostol possibly after SIRS onset may occur and thus may not have optimized potential anti-inflammatory actions of misoprostol or our ability to detect them. In humans, administration of misoprostol every 3 hours is associated with increased systemic drug exposure,[30] and it is possible that repeated administration of misoprostol influences its anti-inflammatory effects.[16] Finally, the considerable variability between horses may have precluded our ability to appreciate statistically significant differences between treatment arms in this study.

In conclusion, this study identified notable differences in drug absorption and systemic exposure in horses administered misoprostol PO or PR immediately prior to LPS administration. Additionally, appreciable changes in *TNFα* and *IL-6* gene expression and serum protein concentrations were identified in individual horses at select time points after misoprostol administration in the face of an LPS challenge. Large interhorse variability precluded our ability to conclusively determine the effects of misoprostol on cytokine production. Investigation of repeated dose administration of misoprostol with or without the combination of other anti-inflammatory medications is warranted to improve understanding of the anti-inflammatory effects of misoprostol in horses.

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The authors declare that there are no conflicts of interests to disclose.

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Supplementary Materials

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