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D15EQ-018 EVALUATION OF MISOPROSTOL AS A NOVEL ANTI-INFLAMMATORY DRUG IN HORSES

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Non Technical Summary
Inflammation is initiated by host cell production of pro-inflammatory mediators triggered by microbial molecules such as lipopolysaccharide (LPS). These mediators amplify the inflammatory response and initiate neutrophil recruitment into tissues and their subsequent activation. However, dysregulated production of pro-inflammatory mediators resulting in massive migration of neutrophils and wholesale

activation is quite damaging to tissues (1). Neutrophils account for much of the damage during inflammation in many important diseases of horses. Our laboratory focuses on identifying key molecules that regulate the inflammatory response to discover new targets for anti-inflammatory therapy to treat serious equine diseases such as sepsis, laminitis, colitis, ischemic colic. One such molecule is cAMP. Pharmacological elevation of intracellular cAMP in leukocytes dampens cellular responses stimulated by LPS and other molecules that trigger inflammation (2,3). However, many drugs that elevate intracellular cAMP have severe side effects that limit their use as anti-inflammatory drugs. Our challenge is to develop therapeutic strategies to elevate intracellular cAMP with sufficient specificity to block the production of pro-inflammatory mediators and the deleterious effects of neutrophils during inflammation without unwanted side effects.

Animal Health Component

50%

Research Effort Categories

Basic

50%

Applied

50%

Developmental

(N/A)

Classification

Knowledge Area (KA)	Subject of Investigation (SOI)	Field of Science (FOS)	Percent
315	3810	1180	100%

Knowledge Area
315 - Animal Welfare/Well-Being and Protection;

Subject Of Investigation
3810 - Horses, ponies, and mules;

Field Of Science
1180 - Pharmacology;

Keywords
equine
misoprostol
anit-inflammatory

Goals / Objectives

The specific hypothesis to be tested is: The intracellular cAMP elevating synthetic prostaglandin E misoprostol will inhibit pro-inflammatory mediator production in leukocytes stimulated by LPS and inhibit activation of neutrophil migration and effector functions. Moreover, we hypothesize that the concentrations that inhibit leukocyte activation are safely achievable in vivo in horses administered oral misoprostol. We will test this hypothesis with the following four Objectives: 1) Determine the effect of misoprostol on LPS stimulated equine leukocyte pro-inflammatory mediator production and neutrophil functional activation in vitro; 2) Determine whether treatment with misoprostol after stimulation has been initiated affects equine leukocyte production of pro-inflammatory mediators or neutrophil activation; 3) Determine the pharmacokinetics of misoprostol in healthy adult horses; 4) Determine the effect of oral administration of misoprostol on LPS stimulated

equine leukocyte pro-inflammatory mediator production and neutrophil functional activation ex vivo.

Project Methods
One: Determine effect of misoprostol on LPS stimulated equine leukocyte pro-inflammatory mediator production and neutrophil functional activation in vitroLeukocyte rich plasma prepared from blood collected from healthy horses not on medications as described (28) and transferred to 15 ml conical tube for use in assays for mediator secretion and expression. Leukocytes primed with GMCSF (100 ng/ml) and pretreated with vehicle or misoprostol (50μM) for 30 min. Leukocytes will be stimulated with LPS (100 ng/ml) for 2, 4, 6, 8, and 18 hrs. We will determine the effects of range of concentrations of misoprostol (1nM-100μM) on mediator secretion after 4 hrs stimulation with LPS to generate a concentration response curve for each mediator. Commercially available validated ELISA kits will measure cellular supernatant (plasma) concentrations of TNFα (Thermo Scientific Co), IL-1β (Kingfisher Biotech), IL-6 (Kingfisher Biotech), and IL-8 (Genorise Scientific, Inc.) in triplicate for each horse according to manufacturer instructions.Use quantitative PCR as described (28) to assess effect of misoprostol on equine leukocyte TNFα, IL-1β, IL-6, IL-8 gene expression (mRNA content) in equine leukocytes stimulated with LPS as above. Comparative threshold cycle (Ct) analysis will be used calculating mean fold changes (2-ΔΔCt) in mRNA transcript abundance in total RNA extracted from equine leukocytes treated as above. PCR product size analyzed on 1.5% agarose gel stained with ethidium bromide and sequenced to confirm product is expected amplicon. Gene-specific primers for equine TNFα, IL-1β, IL-6, IL-8, and housekeeping gene GAPDH are published and validated as described (28). Glyceraldehyde 3-phosphate dehydrogenase was the housekeeping gene based on comparison of variability of Ct values obtained for β actin, GAPDH, and β2 microglobulin primer pairs amongst several equine leukocyte test samples. Samples without DNA will serve as controls for DNA contamination.Assess effect of misoprostol treatment on equine neutrophil migration using transmembrane migration instrument (ChemoTx, Neuroprobe) as described (29). Equine neutrophils loaded with fluorescent dye calcein and pre-incubated with vehicle or misoprostol (1nM-100μM to determine a concentration response curve for effect of misoprostol). Pretreatment with PI-3 kinase inhibitor wortmannin (100 nM) serves as positive control for ability to inhibit migration. Migration of 10,000 neutrophils across 3 μm pore filters in response to platelet activating factor (100 nM), leukotriene B4 (100 nM), and recombinant human IL-8 (100 ng/ml) will be quantitated by measuring fluorescence in lower chamber after 1 hour of chemotaxis. Fluorescence in lower chamber will be measured using fluorescence plate reader. Transmembrane migration will be expressed as percentage of cells migrating to the lower chamber calculated from standard curve relating fluorescence to cell number generated for each experiment. We have correlated fluorescence measurements with manual counts of equine neutrophils in lower chamber (not shown). Horse data will be expressed as mean +/- SD of triplicate wells.Determine effect misoprostol treatment on ROI production using dihydrorhodamine-123 (DHR-123) method as described (29). Equine neutrophils pretreated with vehicle or misoprostol (1nM-100μM). Cells settle on surfaces coated with β2 integrin substrate fibrinogen. DHR-123 (Sigma) will be added prior to stimulation. Cells stimulated with pre-determined optimal concentrations of LPS (100 ng/ml), leukotriene B4 (100 nM), platelet activating factor (100 nM), rhIL-8 (10 ng/ml), or rhTNFα (10 ng/ml). Cells are stimulated by allowing to adhere to surfaces coated with immobilized immune complexes. Treatment with β2 integrin antibody IB4 serves as positive control for ability to inhibit ROI production. ROI-induced oxidation of DHR-123

assessed by measuring fluorescence in wells every 15 min for 120 min described (29). Data for horses are expressed as mean +/- SD of triplicate wells.Two: Determine whether treatment with misoprostol after stimulation has been initiated affects equine leukocyte production of pro-inflammatory mediators or neutrophil activation.Experiments performed as Objective 1 except misoprostol will be added after stimulation with activator is initiated. For proinflammatory mediator production, misoprostol will be added at 0.5, 1, 2, 3, and 4 hours after LPS. For neutrophil migration and ROI production assays, misoprostol will be added at 1, 3, 5, 10, 15, or 30 min after the activator. Data will be analyzed as 1. The IC50 for any affect post-stimulation misoprostol effect will be determined and compared with pretreatment IC50 values.Three: Determine pharmacokinetics of misoprostol in healthy adult horses.Experimental Design: Horses brought into stalls the night prior to experiment and jugular catheters are aseptically placed (a small area over the jugular vein will be clipped and aseptically prepared). Skin and subcutaneous tissue are anesthetized with 2% lidocaine hydrochloride. Catheters are placed and sutured in position. If horse becomes anxious during this procedure, horse will be sedated with xylazine (0.2-0.3 mg/kg IV once). For oral administration, commercially available tablets (Cytotec®, Pfizer, 200μg) will be administered by dissolving tablets in 60 mL catheter tip syringe and mixing with corn syrup to final volume of 60 mL. Drug will be administered in interdental space to back of tongue and horse's head held elevated for 30 seconds to ensure drug delivery. The dose administered will be 5 μg/kg PO (dose commonly used in clinical cases at NCSU-CVM hospital). Blood samples (6 ml) for evaluation of plasma misoprostol concentrations will be collected from jugular vein catheter and placed into heparinized tubes immediately prior to drug administration and at 10, 20, 30, 45, 60, and 90 minutes and at 2, 4, 6, 8, 12 and 24 hours post-administration. Heparinized samples are placed on ice, and centrifuged at 2,000 x g. Plasma will be aspirated, transferred to cryotubes, and stored at -80°C until analysis. After each blood sample collection, catheter will be flushed with 10 ml heparinized saline.Misoprostol plasma concentrations are determined by ultra-high pressure liquid chromatography with tandem mass spectrometry (UPLC-MS/MS) as previously described (30). Analysis will be performed at Large Animal Analysis Laboratory at NCSU-CVM.Four: Determine effect of misoprostol on LPS stimulated equine leukocyte pro-inflammatory mediator production and neutrophil functional activation ex vivo.Oral administration of misoprostol will be performed in 6 horses as in 3. Heparinized blood will be collected at time of peak plasma concentration of misoprostol is expected (determined in 3). Blood collected 30 min and 1 hr before and 1 hr after expected peak to account for individual variations in misoprostol pharmacokinetics. Leukocyte rich plasma prepared from collected blood and mediator secretion and expression in response to LPS stimulation will be performed as in 1. Neutrophil ROI production performed as described above with modifications. DHR-123 will be added to leukocyte rich plasma and cells then stimulated with LPS (100 ng/ml), leukotriene B4 (100 nM), platelet activating factor (100 nM), rhIL-8 (10 ng/ml), rhTNFα (10 ng/ml), or aggregated immune complexes. Oxidation of DHR-123 determined in neutrophils (and other types of leukocytes) using flow cytometric assay to measure intracellular fluorescence as described (31). Assays for neutrophil migration cannot be used in model because neutrophils must be purified and process removes misoprostol. Plasma misoprostol concentration in samples will be measured to correlate effects with plasma drug concentration. Data will be analyzed as 1.