Evaluation of orally and rectally administered misoprostol in a low-dose endotoxin challenge in horses

by

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Abstract

Endotoxemia occurs in many equine diseases, resulting in systemic inflammatory response syndrome (SIRS). Misoprostol demonstrates *in vitro* reductions in pro-inflammatory cytokine production when stimulated by endotoxin. *In vivo* response is unknown. The objective of this study was to characterize the pharmacokinetics and pharmacodynamics of a single dose of misoprostol (5 µg/kg) administered orally (M-PO) or per rectum (M-PR) and to evaluate its effects on clinical inflammatory parameters when challenged with endotoxin intravenously (30 ng/kg IV). Horses and their treatment were randomized in a balanced 3x3 Latin square design for M-PR, M-PO, or control (CON) with minimum washout intervals of 28 days. Misoprostol plasma concentration, cytokine gene expression and production along with physical examination parameters, leukocyte counts, and blinded pain scores were obtained. Maximum plasma concentration (c_{max}) and area under the concentration-versus-time curve (AUC) were higher in M-PO treatment than M-PR treatment. Time to maximum concentration (t_{max}), disappearance half-life ($t_{½}$), and mean residence time (MRT) were longer in M-PO compared to M-PR. Wide variations in cytokine gene expression and production were appreciated between horses. Subjectively, most prominent downregulation of cytokine gene expression occurred sooner in M-PR compared to M-PO. No statistically significant differences were appreciated between M-PR, M-PO, and CON for physical exam parameters, pain score, and cytokine protein production. Values of c_{max} obtained in this study were more than 8-fold higher than those previously reported in healthy horses. Future studies should investigate how prolonged systemic misoprostol exposure may affect these parameters, and a multi-dose administration study would benefit in optimizing dosage amount and intervals of misoprostol. Lastly, comparing pharmacokinetic differences between endotoxin-challenged and unchallenged conditions in the horses used in this study is warranted.

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Table of Contents

List of Abbreviations

AUC – Area under the curve

$AUC_{0\rightarrow\infty}$ – area under the curve extrapolated to infinity

cAMP – cyclic adenosine monophosphate

CARS – compensatory anti-inflammatory response syndrome

CD – cluster of differentiation protein - cell surface proteins on phagocytes

Cl/F – apparent drug clearance

c_{max} – peak plasma drug concentration

CCR – C-C motif chemokine

COX – cyclooxygenase

CXC – C-X-C motif chemokine

CXCL - CXC ligand

CXCR – CXC receptor

DAMPs – Damage-associated molecular patterns

DIC – disseminated intravascular coagulopathy

EMS – equine metabolic syndrome

FDA – Federal Drug Administration

GPCR – G-protein coupled receptor

IFN – interferon

IL – interleukin factor

IP – interferon gamma-induced protein/interferon-inducible protein

IRF – interferon regulatory factor

LPS – lipopolysaccharide, endotoxin

LPSBP – LPS binding protein

MD2 – myeloid differentiation factor 2

MFA – misoprostol free acid

MMP – matrix metalloproteinase

mRNA – messenger ribonucleic acid

MODS – multi-organ dysfunction syndrome

MyD88 – myeloid differentiation factor 88

MRT – mean residence time of drug

NFκB – nuclear factor kappa beta

NSAID – non-steroidal anti-inflammatory drug

PAMP – pathogen associated molecular receptors

PC – prostacyclin

PG – prostaglandin

PO – per os, oral administration

PR – per rectum, administration of drug rectally

RANTES – Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted — synonymous with chemokine ligand 5 (CCL-5)

SIRS – systemic inflammatory response syndrome

SOCS-3 – suppressor of cytokine signaling-3 protein

TLR – toll-like receptor

TNF – tumor necrosis factor

TRIF – TIR domain-containing adaptor protein-inducing interferon beta

TRAM – TRIF-related adaptor adaptor molecule (also known as TICAM-2)

TIRAM – toll/interleukin—1 receptor adaptor molecule, also known as MyD88 adaptor-like protein (MAL)

$t_{½}$ – disappearance half life

t_{max} – time to peak plasma concentration

Vd/F – apparent volume of distribution

List of Tables

List of Figures

Chapter 1: Literature Review

Section 1: Definitions and importance of terminologies: Systemic inflammatory response syndrome, bacteremia, endotoxemia, sepsis

Systemic inflammatory response syndrome (SIRS) is defined as an uncontrolled, global inflammatory response that occurs in the body following an infectious or non-infectious insult.[1-3] This condition can occur from endogenous and exogenous stimuli. Exogenous stimuli are often organisms including bacteria, fungi, and viruses.[4,5] The criteria of SIRS are met by the presence of at least two components from a list of observations, including altered leukocyte count (leukopenia or leukocytosis), altered leukocyte distribution (greater than 10% band neutrophils), morphologic neutrophil changes ("toxic" changes), pyrexia/fever or hypothermia, tachycardia, or tachypnea.[5] Recent discussions have proposed that leukocytosis or elevated rectal temperature must be identified in a horse suspected of SIRS.[2] A human sepsis study identified a linear increase in the odds ratio for mortality as the number of observed SIRS criteria increased, highlighting the severity in progression and morbidity of the phenomenon.[6]

In critically ill equine patients, SIRS is highly prevalent. It is estimated that 25-41% of horses admitted for colic[7,8] and more than 30% of sick foals evaluated in the hospital are in a state of SIRS upon admission.[9,10] In adult horses, SIRS has been documented secondary to various conditions localizing to multiple body systems including gastrointestinal, respiratory, reproductive, and musculoskeletal. Specific to gastrointestinal, respiratory, and reproductive conditions, infectious or commensal/resident bacteria that may incite SIRS include beta-hemolytic streptococci, non-enteric Gram-negative bacteria, enteric Gram-negative organisms, and anaerobes.[11-14] Conditions in foals that have been associated with an increased risk for developing SIRS include perinatal asphyxia syndrome and failure of passive transfer as well as various other

conditions leading to proliferation and systemic circulation of Gram-negative or Gram-positive bacteria.[15]

The SIRS terminology is married to discrete terms including bacteremia, endotoxemia, and sepsis. It is important to emphasize that the syndrome is elicited by a wide range of pathologic insults and not limited to systemic bacterial circulation (bacteremia) and subsequent SIRS response (sepsis). As will be discussed further in the next section, endotoxin is a well-recognized cause of SIRS in horses.[1,3] Endotoxin (lipopolysaccharide, LPS) is a component of the Gram-negative bacterial cell wall, and its circulation in the bloodstream is termed endotoxemia.[1,5,16,17] Although endotoxemia is a well-cited stimulator of SIRS, other molecules categorized as pathogen associated molecular patterns (PAMPs) can elicit this condition as well, and this is made possible by their interactions with various toll-like receptors (TLRs). The recognition of PAMPs is mediated by their binding to TLRs present on immune cells and endothelial cells as they circulate systemically. Examples of these TLR and molecular pattern recognition interactions include TLR-2 recognition of Gram positive and mycobacterial products, TLR-5 recognition of the protein flagellin (which is a component of flagella allowing for bacterial motility and invasion), and TLR-9 interaction with bacterial and viral DNA components.[18] Similarly, damage associated molecular patterns (DAMPs) can also trigger SIRS following the release of specific molecules (alarmins) as a result of cell death or physiologic stress. Examples of DAMPs include histones, heat shock proteins, and glycoproteins.[2] A partial list of characterized TLRs and their activating substrates are provided in **Table 1**.

Toll-like Receptor	Primary cellular location	Stimulating PAMP	Stimulating DAMP
TLR-2	Extracellular	1. Lipoprotein peptidoglycan 2. N-acetyl glucosamine lipoteichoic acid 3. Zymosan 4. Lipoarabinomannan	1. HMGB1 2. HSP 60 3. HSP 79
TLR-3	Intracellular	Viral dsRNA	HSPs
TLR-4	Extracellular	LPS (endotoxin)	1. HMGB1 2. S-100 proteins 3. Fibrinogen 4. HSP 60 5. HSP 70
TLR-5	Extracellular	Flagellin	HSP
TLR-7	Intracellular	Viral ssRNA	
TLR-8	Intracellular	Viral ssRNA	
TLR-9	Intracellular	CpG-DNA	
RAGE	Extracellular	LPS	HMGB-1 S-100 proteins SAA AGE
NLR	Intracellular	Peptidoglycan polymers	

Table 1: Partial list of TLRs and their associated PAMPs and DAMPs, adapted from McConachie & Hart, 2016.[4]

Section 2: Pathogenesis of endotoxin induced SIRS

2a: Sources of endotoxin

Endotoxin/LPS constitutes 75% of the outer cell wall membrane in Gram-negative bacteria, which is approximately 3 to 4 million LPS molecules per cell.[19] It serves as an outer membrane barrier that is released when Gram-negative bacteria proliferate or die.[1,16] The LPS structure consists of a variable O region specific to the strain of bacteria, and conserved core polysaccharide and lipid-A regions.[17,20,21] Understanding these structures is important for targeted therapy, as discussed later. The lipid-A component is recognized as exerting the most toxic effects of Gram-negative bacteria. While all species have a profound response to the presence of endotoxin, the horse is one of the most sensitive to it.[3,16,17,20] Compared to other hindgut fermenters, such as rabbits that have documented the lethal dose of endotoxin is 3 to 10 mg/kg bodyweight, experimental studies determined the range in lethal dose in ponies was 0.2 to 0.4 mg/kg, a fifty-fold difference.[22,23]

The sources of Gram-negative bacteria can be either exogenous or endogenous. Exogenous sources of Gram-negative bacteria include opportunistic or infectious pathogens. Disease conditions that are attributed to exogenous Gram-negative bacteria include colitis, pleuropneumonia, metritis, and many others.[3,16] Commonly identified Gram-negative organisms include *Salmonella spp., Escherichia coli, Enterobacter spp., Pasteurella spp., Actinobacillus spp.,* and *Klebsiella spp.* The most significant endogenous source of Gram-negative bacteria is in the hindgut of the horse; many bacteria that reside here are Gram-negative rods, followed by Gram-positive rods and cocci, and Gram-negative cocci.[24] Common examples of Gram-negative commensal organisms include *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Pasteurellaceae*, and *Lactobacillaceae*.[11,12] Although endogenous colonic bacteria provide the hindgut fermenting horse

with a necessary means of nutrient assimilation, they can also represent a large source of lethal endotoxin, generating concentrations ranging from 2-80 μg/mL in the colon of healthy horses.[16,17]

2b. Protective mechanisms against endotoxin excess

The body utilizes several protective mechanisms to minimize LPS release, and disruption of these mechanisms can ultimately lead to their increased systemic circulation. These protective mechanisms include the natural microbiome of the gastrointestinal tract, mucosal epithelial cell function, hepatic detoxification, and anti-endotoxin antibodies.[16,17] A specific balance of bacterial populations (the gut microbiome) plays a key role in the health of the horse. The microbiome is essential for the appropriate assimilation of nutrients and is also believed to play a role in systemic immunity and behavior of the animal (ranging from cravings, reactivity to stress, and even dysphoric behavior).[25] Disruption of the microbiome may lead to overgrowth of pathogenic bacteria (pathogenic dysbiosis). This can occur from a variety of causes including selective pressures associated with antimicrobial use, physiologic stress, and dietary changes.[11,26] Specific to endotoxemia, the complex microbiome is an inherent control mechanism to prevent proliferation of pathogenic bacteria.[16,17,21]

Mucosal epithelial cells lining the gastrointestinal tract provide a barrier (through tight junctions) to prevent translocation of bacteria and their products into the systemic circulation. These cells also secrete a mucus layer which creates a medium that minimizes growth and proliferation of bacteria. Any endotoxin that successfully crosses the gastrointestinal barrier is shuttled through the portal circulation and detoxified in the liver through hepatic macrophages (Kupffer cells).[16,17] Should any residual endotoxin escape and enter the circulation, circulating anti-endotoxin antibodies bind to it, thus preventing excessive recognition and an exuberant immunologic response.[21]

Disruption of these safety mechanisms contributes to the pathophysiology of endotoxemia. Pathogen invasion and overgrowth, reduced splanchnic circulation, damage or compromise to the mucosal barrier, or impairment and avoidance of portal detoxification all represent ways in which these critical safety mechanisms can become compromised.[21] Impairment of physiologic barriers can lead to bypassing of portal detoxification, allowing LPS to divert into the lymphatics or peritoneal cavity and then subsequently into the systemic circulation.[17] Portal detoxification can be impaired or overwhelmed due to hepatic insults or increased LPS release.

2c. Recognition of LPS and its immunologic consequences

Systemic circulation of LPS leads to widespread recognition by phagocytes and can trigger global signaling, massive activation of inflammation, and subsequently SIRS. When in an unbound state, the LPS molecule is protected from recognition by phagocytes due to its amphoteric nature and subsequent ability to form micelle structures. When LPS molecules form these micelle structures, recognition of the hydrophobic lipid-A region by circulating phagocytes becomes limited. Instead, when LPS is bound by circulating LPS binding protein (LPSBP), micelle formation is prevented and recognition and response by phagocytes is rapid.[3,16,17,20] The importance of this protein was illustrated in mice whose LPSBP were inactivated by monoclonal antibody neutralization. These mice demonstrated delayed cytokine production and death when challenged with virulent Gram-negative bacteria.[27]

The association of LPS with LPSBP is important for cellular recognition and binding of LPS and for triggering key intracellular signaling processes as depicted in **Figure 1**. When bound to LPSBP, the binding of LPS to cluster of differentiation 14 (CD14), a cell surface protein present on phagocytes, is greatly facilitated. Once bound to CD14, interaction with myeloid differentiation factor 2 protein (MD2, also known as lymphocyte antigen 96) and the transmembrane receptor

TLR-4 occurs. This interaction activates the intracellular myeloid differentiation factor 88 (MyD88) pathway, and the nuclear factor kappa beta (NFκB) pathway, which is the primary contributor to the inflammatory cascades associated with SIRS.[3,5,16,17,20].

Activated TLR-4 also stimulates recruitment of the adaptor molecules TIR-domain-containing adaptor protein inducing interferon beta (TRIF) and TRIF-related adaptor molecule (TRAM), which play contrasting roles in the inflammatory process. In addition to their role in further activating NFκB and mitogen-activated protein kinases (MAPK) that are both associated with inflammatory gene transcription, they also activate anti-inflammatory actions through stimulation of interferon regulatory factor-3 (IRF-3). This activation is responsible for the release of the anti-inflammatory cytokine interleukin (IL)-10, by way of interferon (IFN)-α/β, chemokine ligand 5 (CCL-5 or RANTES), and interferon gamma-induced protein/interferon-inducible protein (IP)-10, which are downstream effects of TRIF.[3] Interestingly, depending on the type of TLR-PAMP/DAMP interaction, phagocyte activation of the same TLR can lead to variations in degree of inflammation. *In vitro*, TLR-2 and -4 activation in monocytes led to higher production of tumor necrosis factor (TNF)-α, IL-1β, and IL-10 compared to activation of TLR-3. In contrast, TLR-3 activation produced higher expressions of IFN-β, IP-10, and RANTES, which are all components of TRIF.[28] Another *in vitro* study in equine neutrophils and monocytes showed that flagellin stimulation of TLR-5 activated neutrophils, but not monocytes.

MyD88 serves two functions; the first is degrading inhibitor of nuclear factor kappa B (IκB), thereby activating NFκB, and the second is phosphorylation of MAPK. While activation of both NFκB and MAPK lead to inflammatory cytokine production,[29] NFκB has drawn particular interest because its activation results in more significant widespread effects including cytokine release, and activation of neutrophils and the cyclooxygenase (COX) pathways. Once activated,

NFκB moves into the nucleus of the phagocyte and binds to specific promotor regions of genes associated with inflammation, coagulation, and vasoactive action through various cytokines, interleukins, chemokines, and other molecules.

Although numerous cytokines contribute to the pathophysiology of SIRS, TNFα, IL-1, and IL-6, have gained particular attention for their role in triggering many of the physiologic changes associated with SIRS. These cytokines have pyrogenic effects and cause leukocyte activation and production of acute phase proteins, among other actions.[1,3,5,17] The release of these cytokines is not simultaneous, and the timing, magnitude, and duration of their release influences the degree and duration of inflammation associated with SIRS. The release of TNFα is most rapid and transient (released for approximately 1 hour after stimulation) and is followed by IL-1β (up to 20 hours after stimulation), with release of IL-6 being the most delayed and sustained (ranging from 4-20 hours after stimulation).[3] A summary of the various cytokines is shown in **Table 2.** Consequences of exuberant circulation of pro-inflammatory cytokines and uncontrolled systemic inflammation include profound immunosuppression, vascular injury, coagulopathies, and perfusion derangements.

The exaggerated inflammatory state of SIRS can lead to a severe immunosuppressed state referred to a compensatory anti-inflammatory response syndrome (CARS), which is thought to develop as a consequence of counter-regulatory mechanisms initiated to limit the exuberant pro-inflammatory state of SIRS. In patients experiencing CARS, factors that contribute to immunosuppression and increased risk of secondary infection include increased production of anti-inflammatory mediators, impaired leukocyte function (chemotaxis, phagocytosis, and killing), activation of intracellular caspases and associated lymphocyte apoptosis, and loss of dendritic cell population and function.[30]

2d. Physiologic and clinical consequences of endotoxemia and SIRS

With excess cytokine stimulation, neutrophil activity becomes prolonged and uncoordinated, ultimately resulting in global damage in the form of vascular injury and subsequent dysfunction of various organs. Manifestations of neutrophil dysfunctions include inefficient chemotaxis, increased production, release of destructive compounds (enzymes, defensins, and reactive radicals), and inhibition of neutrophil apoptosis, which further prolongs their dysfunctional actions. Inefficient chemotaxis occurs due to downregulation of chemotactic receptors and leads to a loss of specificity of neutrophil targeting. At the same time, integrins and selectins continue to promote adhesion to the endothelium and trigger widespread recruitment, sequestration, and activation of microbicidal products in post capillary venules. Increased production and release of myeloperoxidase and hydrogen peroxide by neutrophil granules further contributes to the damage to the vascular endothelium through the production of hypochlorous acid, superoxide anion, peroxynitrite radicals, other enzymes (elastase, serine protease, matrix metalloproteinases), and defensins.[31] Concurrent production of additional inflammatory mediators such as bradykinin, platelet-activating factor, C3a, C5a, and leukotriene B_4 further exacerbate vascular damage resulting in increased vascular permeability and leakage of these damaging compounds into surrounding organs.[32] Delay of neutrophil apoptosis further prolongs these actions.[33]

Coagulopathies associated with SIRS include both hyper- and hypocoagulation, which when unchecked may ultimately lead to disseminated intravascular coagulation (DIC). During the hypercoagulable phase of DIC widespread formation of micro- and macrothrombi occurs in response to inflammatory-mediated damage to the endothelium and accompanying increases in expression of proteins, such as tissue factor and plasminogen activator inhibitor, involved in

regulation of coagulation and fibrinolysis. During this phase multiple organ dysfunction syndrome (MODS) may develop in response to widespread ischemia. Persistence of this hypercoagulative state results in consumption of clotting factors, fibrinogen, and platelets, and then leads to the hypocoagulable phase of DIC characterized by widespread hemorrhage.

Perfusion alterations in SIRS arise through not only the NFκB pathway, but also through other global mechanisms. Inflammatory mediators include both vasoconstrictors, such as thromboxane A, endothelin, and activated complement proteins, as well as vasodilators, such as prostaglandins, bradykinins, and nitric oxide, among others. Global release of these mediators triggers dysfunctional distribution of blood flow throughout the body. In response, release of serotonin, epinephrine, norepinephrine, and other ino- and vasopressing agents from the renin-angiotensin-aldosterone system, can further exacerbate alterations in systemic circulation.[5] Damage associated with perfusion abnormalities is compounded with concurrent inflammatory-mediated myocardial injury, increased metabolic demands, and mitochondrial dysfunction.[34] The net effect of these processes, particularly when combined with coagulopathy and vascular damage, is distributive shock, global hypoxia, and ischemia leading to MODS.[17,32] A unique attribute in horses as a component of MODS is MODS-L (for laminitis).

Laminitis is a devastating complication of SIRS, and its prevention represents a cornerstone of supportive therapy. It is theorized that separation of the interdigitation between the third phalanx and the hoof capsule occurs in response to hypoxic events and upregulation of matrix metalloproteinases (MMPs).[35] Current research supports distal limb cryotherapy as an efficacious prevention method when the hoof temperature is below 10˚C continuously over 48-72 hours. This practice is thought to reduce the metabolic activity of the surrounding tissue along with reducing

inflammatory cytokine production, and activity of inflammatory cytokines and enzymes (e.g., MMPs).[36]

In summary, the activation of TLR-4 induced NFκB causes widespread damage uncontrolled cytokine production leading to profound perfusion derangements, severe systemic pathology, and immunosuppression. When left unchecked, complications from SIRS in horses can lead to further global damage including cardiovascular derangements, coagulopathies, multi-organ failure, laminitis, and death. As critically ill horses are often suffering from the clinical effects of SIRS, its prevention and treatment must be a primary therapeutic goal to reduce morbidity and mortality.

2e. General strategies for combating endotoxin-induced SIRS

Currently, strategic approaches for combating SIRS associated with endotoxemia are categorized into the following categories: preventing systemic endotoxin circulation, endotoxin neutralization (binding to endotoxin prior to TLR-4 binding), mitigation of inflammatory mediators, prevention of cellular activation (where a similar structure competes with TLR-4 to prevent endotoxin binding), and general supportive care.[16,17,20,37] The most attention has been placed on neutralizing endotoxin prior to immune cell activation, as well as mitigation of inflammatory mediators following activation. These will be further discussed in the next section.

Systemic endotoxin circulation can be prevented by treating the nidus of endotoxin proliferation. Since endotoxin is released following proliferation or death of Gram-negative bacteria, this may involve targeted antimicrobial therapy based on culture and sensitivity and/or removing the source of infection wherever possible (e.g., infected umbilical remnants in septic foals).[3,16,21,37]

Supportive care is important in the treatment of endotoxemia-related SIRS. Such care primarily targets perfusion derangements and coagulopathies, as well as the prevention of laminitis. Perfusion derangements, which can occur as a result of increased vascular permeability, loss of vascular tone, and protein loss, can further worsen the horse's condition. Both crystalloid and colloid products provide benefits in SIRS mitigation. Isotonic buffered crystalloid solutions such as lactated ringer's solution (LRS), Plasmalyte®, and Normosol-R®, are used to address dehydration and electrolyte derangements while meeting maintenance fluid requirements.[3] Biologic and synthetic colloids, such as commercial plasma and hydroxyethyl starch aid in restoring oncotic pressure. Commercial plasma has additional benefits of providing natural anti-inflammatory agents and essential proteins including albumin, fibronectin, and antithrombin and when combined with heparin can be useful in treatment of coagulopathies.[3,16,37] Administration of plasma replaces coagulation factors lost through consumption and heparin may mitigate a hypercoagulable state.[1]

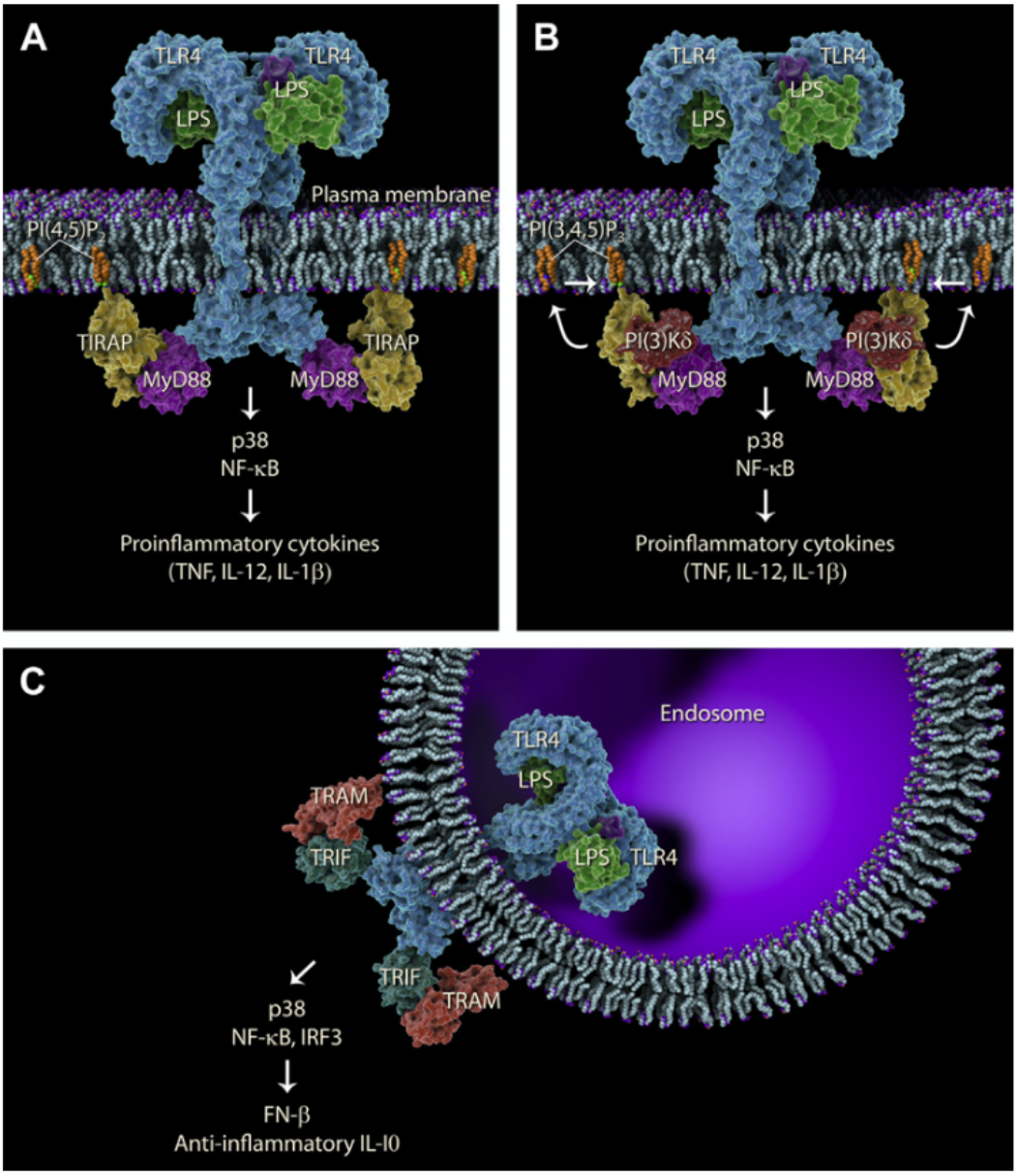


Figure 1: TLR-4 activation of MyD88, NFkB, TRIF, and TRAM. Reprinted from Veterinary Clinics of North America: Equine Practice, Vol 30, James N. Moore and Michel L. Vandenplas, Is it the Systemic Inflammatory Response Syndrome or Endotoxemia in Horses with Colic?, pp 337-351, Copyright 2014, with permission from Elsevier.

Cytokine	Main source	Main function	Influence on other mediators
TNFα	Innate and adaptive immune cells - Macrophage - Lymphocytes Fibroblasts	Induce release of other pro-inflammatory cytokines, coagulation, fever, cachexia, apoptosis	Promotes downstream upregulation of pro-inflammatory cytokines
IL-1β		Promotes coagulation, fever, hematopoiesis, leukocyte diapedesis, muscle catabolism (myalgia)	Promotes downstream upregulation of pro-inflammatory cytokines
IL-6		B & T lymphocytes proliferation mediates acute phase reaction, fever	Inhibits release of TNFα and IL-1; Promotes release of anti-inflammatory cytokine TGFβ
IL-8	Macrophages Endothelial cells	Chemokine	Neutrophil influx
IL-12	Monocyte/macrophages Neutrophils Dendritic cells	Promotes cell mediated immune response TH_1 lymphocytes	Induces release of IFN-γ
IFN-γ	NK cells Th_1 $CD8^+$ cytotoxic T cells	Antiviral activity Potential role for reversal of immunoparalysis in sepsis	Increased levels in sepsis
IL-10 (anti-inflammatory)	Immune cells of innate and adaptive immune responses	Immunosuppression impaired antigen presentation and phagocytosis	Suppresses release of pro-inflammatory cytokines and promotes sTNFR and IL-1Ra
IL-4	TH_2 lymphocytes, eosinophils, basophils	Promotes humoral immune response through differentiation of TH_0 to TH_2 lymphocytes	Induces release of IL-4 and IL-13
TGFβ	Macrophages, smooth muscle cells	Tissue repair, fibrosis, sepsis induced immunosuppression	Suppresses release of pro-inflammatory cytokines and promotes sTNFR and IL-1Ra

Table 2: Brief summary of cytokines produced following NFkB activation and their actions[4]

Section 3: Use of in vivo endotoxemia models and SIRS therapy investigations

In horses, physiologic changes and therapeutic responses to SIRS have been modeled through the administration of low doses of endotoxin (ranging from 20-250 ng/kg intravenously, or 500 ng/kg intraperitoneally). By administering 1 millionth of the anticipated lethal dose of endotoxin (200-400 μg/kg),[23] reproducible changes in clinical parameters can be transiently elicited without lasting complications. Although this study design does not represent clinical disease, where continuous endotoxin circulation occurs in varying ranges and patterns, it has provided insight into clinical effects and potential therapeutic interventions.

Low-dose endotoxin studies in horses have elucidated a variety of SIRS responses. Through these predictable effects, the model provides a means for interventional studies to assess the efficacy of therapeutic interventions while minimizing the risk of adverse complications such as laminitis. Horses exposed to endotoxin in experimental models *in vivo* have displayed similar but milder clinical signs as clinical cases including increased rectal temperature, tachycardia, tachypnea, elevated packed cell volume, hyperlactatemia, and abdominal pain.[8,38] Low-dose administration of endotoxin (20-30 ng/kg) revealed decreased plasma iron concentration and upregulation of hepatic hepcidin and IL-6 mRNA transcription compared to baseline parameters.[39] Horses with low-dose endotoxemia with concurrent hyperglycemia had minor but clinically insignificant changes in coagulation parameters compared to endotoxin alone.[40] Clinical insights have also been elucidated for horses with endocrinopathies, particularly insulin resistance. A low dose endotoxin study (20 ng/kg IV) demonstrated differences in glucose and insulin dynamics between healthy horses and those diagnosed with equine metabolic syndrome (EMS).[41] In this study, greater derangements in insulin and glucose dynamics were identified in horses with EMS when compared to their healthy counterparts following endotoxin challenge.[41]

Endotoxin neutralization occurs by preventing binding of the LPS molecule to the LPS-binding protein, thereby preventing the subsequent cascades produced by TLR-4 activation. The most commonly available neutralization therapies include hyperimmunized plasma and polymyxin B.[20] These have been well-characterized through *in vivo* low-dose endotoxin experimental models.[16,20,42,43]

Antibodies in hyperimmune plasma provide either narrow (O-antigen, individual bacterial strain-specific) or broad spectrum (core and lipid A, conserved structures) protection against endotoxin interaction with TLR-4. [16,20] *In vitro* investigations of anti-LPS hyperimmunized plasma demonstrate bactericidal efficacy against various Gram negative organisms including *Salmonella,* and *Shigella.*[44] While this theory is sound, there is mixed evidence to support its use clinically. In one study, adult horses administered hyperimmunized plasma against *E. coli* Rc mutant (J5 antibody) had significantly improved mortality rates.[45] In contrast, in another study involving 3- to 5-month-old foals, clinical and hematologic parameters were not improved when foals were challenged with LPS following administration of commercial hyperimmunized plasma against *Salmonella typhimurium* Re mutant (Endoserum®).[42] Finally, while adult horses administered hyperimmunized plasma against *E. coli* J5 prior to endotoxin administration did not demonstrate differences in peak TNFα concentrations or clinical sign manifestations, they did demonstrate lower concentrations of bound TNFα compared to saline-treated horses, which suggested reduced activity of the cytokines.[43]

Polymyxin B is a cyclic cationic peptide antibiotic which exerts its effects by forming a stable complex with the lipid-A portion of endotoxin.[42] Polymyxin B acts upon the LPS molecule itself thereby preventing recognition and subsequent binding by the TLR-4 receptor to initiate the inflammatory cascade as occurs with SIRS.[5,16,17,20,42,46-48] This prevents further amplification of all

downstream events. Dose-dependent improvements in TNFα activity in adult horses have been demonstrated.[46,48,49] While the greatest efficacy was seen when given prior to endotoxin administration, reductions in pyrexia, tachycardia, and TNFα activity were observed regardless of whether polymyxin B administration occurred 30 minutes before or after infusion of endotoxin in the horse.[46] The benefits of polymyxin B, however, must be balanced with cost considerations, as well as the potential for adverse effects including nephrotoxicity, neurotoxicity, and neuromuscular blocking.[16,20,37,50] Administration to healthy horses within the recommended dose range (1,000 to 6,000 IU/kg IV q8h) did not result in alterations in urinary GGT/creatinine ratios.[51] In contrast, higher dosages (20,000 IU/kg) or repeated administration of polymyxin (25,000 IU/kg) resulted in alterations in urinary GGT/creatinine ratios suggesting potential for renal damage.[52]

Endotoxin-mediated intracellular signaling can be mitigated to reduce production of inflammatory mediators and their subsequent systemic effects. Studies have investigated the use of corticosteroids, antibodies against TNFα, non-steroidal anti-inflammatory drugs (NSAIDs), and cyclic adenosine monophosphate (cAMP) mediators (described in Section 4). Corticosteroids are potent anti-inflammatory molecules whose use has been widely described in veterinary and medical literature.[30,53-61] Activated glucocorticoid receptors have a widespread effect in reducing inflammation that includes suppression of the NFκB and MAPK pathways.[20] Despite their potent anti-inflammatory activity, studies in horses and humans offer conflicting evidence and the use of glucocorticoids for SIRS therapy remains controversial. Adult and pediatric horses experiencing SIRS can develop hypothalamic-pituitary-adrenal axis derangements as indicated by inappropriate response to endogenous corticosteroids.[57,58,62] Corticosteroid administration may prove to be beneficial, however, in situations where exuberant inflammation has resulted in cortisol depletion.[58] Studies in humans also offer conflicting evidence supporting corticosteroid

administration to patients with SIRS, including lack of improvement in outcomes, poor response to fluid resuscitation and vasopressors, increased incidence of super infections or other complications, improved hemodynamic parameters, and better Th-1 related immune responsiveness.[61,63,64] Anti-TNFα monoclonal antibodies acquired from mice showed promise in improving clinical and hematologic parameters in miniature horses challenged with endotoxin.[65,66] However, given that the time and duration for TNFα upregulation in SIRS is brief, this therapy may have limited clinical value.[16]

Historically, NSAIDs have remained a mainstay of therapy against endotoxemia for both their analgesic and anti-inflammatory properties, with flunixin meglumine being the drug of choice.[67] Although use of NSAIDs aids in mitigating some of the effects of inflammation induced by NFκB induction, they do not target the cytokines directly responsible for SIRS.[67] NSAIDs exert their effects on the cyclooxygenase (COX) pathway, which contributes to the inflammatory response through triggering the production of prostaglandins, thromboxanes, and leukotrienes. Therefore, it only serves as a partial treatment modality. In one low-dose endotoxin study, flunixin meglumine improved clinical signs attributed to endotoxin-mediated SIRS, but did not result in reduction in TNFα concentration.[68] Furthermore, horses administered flunixin meglumine had higher IL-6 concentrations when compared to horses receiving no treatment or pentoxifylline alone.[67,68] Use of NSAIDs is not innocuous, with potential adverse effects including nephrotoxicity, GI ulceration, decreased intestinal turnover, and healing.[67,69] These effects are more prominent with non-selective COX inhibitors (such as flunixin meglumine) which not only inhibit COX-2 functions that primarily participate in inflammatory actions, but also the COX-1 functions present in healthy cells involved in normal physiologic actions.[67,69,70]

Other anti-inflammatory therapies that have been evaluated in horses include the use of ketamine and lidocaine. Horses administered subanesthetic doses of ketamine (1.5 mg/kg/h following a stepwise loading dose) 1 hour prior to LPS infusion (30 ng/kg) did not demonstrate significant differences in clinical effects including plasma TNFα concentration, thromboxane, and biochemical changes, when compared to control horses (LPS infusion without ketamine).[71] In another a continuous rate infusion (CRI) of lidocaine (1.3 mg/kg loading dose, 0.05 mg/kg/min) started prior to intraperitoneal administration of LPS (500 ng/kg) resulted in improved clinical parameters and lower serum and peritoneal TNFα activity when compared to horses receiving saline CRI.[72]

While numerous studies in horses have investigated therapeutic drug efficacy for improving clinical and physiologic parameters associated with endotoxin challenge, investigations concerning the impact that endotoxin has on drug pharmacokinetics or pharmacodynamics are lacking. Studies in other species have identified differences in drug distribution and activity that may have important applications for horses. A recent study investigated whether residues of flunixin meglumine differed between cattle receiving repeated administration of 2.2 mg/kg IV (as labeled) or IM (extra-label) flunixin in the presence or absence of endotoxin challenge (200 ng/kg LPS IV). Alterations in pharmacokinetic parameters and prolonged drug residues in milk, urine, and tissues were identified in the LPS challenged group of cattle, suggesting that the current drug withdrawal period for cattle treated for endotoxemia may be insufficient despite adherence to label instructions.[73] In goats, endotoxin challenge resulted in altered marbofloxacin pharmacokinetics compared to unchallenged animals including decreased V_d and Cl, and prolonged MRT.[74] Similarly, rabbits receiving a single dose of enrofloxacin (5 mg/kg IV) following challenge with *E. coli* endotoxin (100,000 ng/kg IV) had lower V_d, reduced Cl, and higher AUC values compared

to rabbits administered enrofloxacin without endotoxin challenge.[75] Drug-related clinical effects have been observed in humans; for example, patients with rheumatoid arthritis receiving verapamil, a calcium channel blocking antiarrhythmic drug, experienced less reduction in cardiac conductivity on ECG (measured by the interval between the P and R wave) compared to their healthy counterparts.[76]

These pharmacokinetic differences are attributed to global effects of inflammation on cell receptor expression, protein binding, and drug metabolism and excretion.[77] The concentration of albumin, a negatively-charged acute phase protein and primary plasma protein involved in drug binding, can decrease with systemic inflammation, which in turn can alter drug distribution and activity. [77] In addition to protein binding, impairment of metabolic machinery such as the enzyme cytochrome P450 3A4 and p-glycoprotein transport protein (responsible for drug metabolism, distribution, and excretion) may be inhibited in response to inflammation.[77,78] This can result in alterations in drug efficacy and increased risk of adverse events.

Section 4: The role of cyclic AMP in immunomodulation and potential therapeutic methods of cAMP modulation

The molecule cAMP is a universal regulator of cell function that has been extensively reviewed.[79] Its actions as a second messenger are mediated through G-protein coupled receptor (GPCR) activation of the stimulatory G protein α-subunit. When a ligand binds to the GPCR (e.g., an extracellular first messenger such as a neurotransmitter, hormone, chemokine, lipid mediator, or drug) guanosine diphosphate (GDP) is exchanged for guanosine triphosphate (GTP) on the Gα stimulatory protein, leading to dissociation of the beta-gamma subunit complex. The free Gα stimulatory subunit stimulates the enzyme adenylyl cyclase resulting in ATP catalysis to cAMP. A response is elicited by cAMP's role as a second messenger. Other ligands, such as leukotrienes (B_4, C_4, D_4) and chemokines (C-C motif (CC)R1-10 and CXCR1-6) may cause inhibitory actions of the GPCR (Gαi subunits) through inhibition of adenylyl cyclase and subsequently decrease cAMP production.

In the context of the immune system, promoting cAMP production or prolonged cAMP action elicits anti-inflammatory effects, while decreased production or increased degradation of cAMP promotes inflammation.[79] The actions of cAMP specifically affect phagocytes (e.g. neutrophils, monocytes, and macrophages) by modulating three specific actions: (1) inflammatory mediator production (e.g. cytokines, chemokines, and lipids), (2) phagocytosis, and (3) intracellular killing of ingested pathogens.[79,80] Inflammatory mediators, as discussed in the previous section, play key roles in modulating immune function. Increase in intracellular cAMP in phagocytes leads to decreased expression of pro-inflammatory cytokines including TNFα and IL-12, chemokines including macrophage inflammatory protein 1α and 1β, and leukotriene B_4, a pro-inflammatory lipid mediator.[79] Modulation of cAMP also affects phagocyte function including

migration and killing, as increases in cAMP suppresses complement receptors, Fcγ receptors (FcγR), and other scavenger receptors. cAMP also enhances IL-10, an anti-inflammatory cytokine, as well as the expression of suppressor of cytokine signaling-3 protein (SOCS-3) that is another control mechanism to suppress inflammation currently under investigation.[20,79]

Promotion of cAMP activity has been demonstrated with ligands including epinephrine, norepinephrine, serotonin, histamine, and COX-derived prostaglandin (PGE_2, and prostacyclin (PGI_2)). Modulation of host cell cAMP signaling has been exploited by pathogens including *Bordetella pertussis, Vibrio cholera*, and human immunodeficiency virus (HIV). In horses, it was demonstrated that cAMP modulation affected respiratory burst and adhesion of equine neutrophils.[80] Manipulating cAMP concentrations can also be achieved pharmacologically as has been demonstrated with pentoxifylline, clenbuterol, and most recently misoprostol in horses.

Pentoxifylline, a xanthine derivative similar in structure to theobromine, is a rheologic drug agent promoting flexibility of leukocytes and erythrocytes, in addition to causing vasodilation, reduced blood viscosity, plasma fibrinogen, and platelet aggregation, and increased tissue plasminogen activity. In horses, pentoxifylline is used to treat laminitis, endometritis-placentitis, and other disease conditions.[16] *In vitro* investigations of pentoxifylline demonstrated increases in intracellular cAMP concentrations by promoting phosphorylation and inhibiting phosphodiesterase activity, thereby reducing the degradation of cAMP and cGMP.[68,81,82] However, *in vivo* studies in horses have demonstrated mixed efficacy and administration as a continuous rate infusion or frequent intravenous bolus injection would be required to adequately reduce TNFα activity clinically.[81] Clenbuterol, a β_2 receptor agonist used primarily as a bronchodilator in the treatment of equine asthma, promotes increased cAMP concentrations through stimulation of adenylate cyclase and subsequent increased conversion of ATP to cAMP.[83-85] Unfortunately, as an

adrenergic agonist, clenbuterol's effects via smooth muscle relaxation may preclude its routine use in sick horses, where decrease in total peripheral resistance from β_2 action may lead to exacerbation of SIRS-induced hypotension.[20,83]

Misoprostol's uses in both human and veterinary medicine are similar, and are typically not used for treating inflammation. Misoprostol, a synthetic methyl ester analogue of PGE_1, acts upon E_2, E_3, and E_4 prostanoid receptor subtypes to exert its effects. It is approved by the Federal Drug Administration (FDA) for treatment and prevention of NSAID-related gastric and duodenal injury in humans. It has additional obstetric uses in cervical relaxation, and in conjunction with mifepristone early pregnancy termination.[86,87] Similarly in horses, misoprostol has primarily been used for aiding in gastrointestinal healing in horses with equine gastric glandular disease and has also demonstrated beneficial effects on mucosal healing and recovery following NSAID toxicity-related ulcerative colitis.[88-90] Documented actions on the prostaglandin E_2 and E_4 receptors stimulate the COX-1 pathway, mediating mucosal protection and repair.[91-93] Recent research has highlighted misoprostol's promise as an alternative therapy for treating inflammation. Antioxidative and cytoprotective effects have been cited in humans.[86,94] Studies in several species have demonstrated monocyte E_2 receptor-mediated reductions of inflammatory cytokines with misoprostol treatment.[95-97] The next sections will discuss misoprostol drug behavior investigated in humans and horses, and their potential for anti-inflammatory treatment as a cAMP modulator.

Section 5: Human pharmacokinetic studies of misoprostol

Misoprostol's pharmacokinetics have been well described for a variety of administration routes in humans, including oral, buccal, sublingual, transrectal, and transvaginal administration.[87,98-100] Once absorbed, misoprostol is rapidly metabolized and de-esterified by the liver into its biologically active metabolite, misoprostol acid.[87,91,100] Misoprostol acid is 81-89% protein bound, and depending on the route of administration demonstrates varying pharmacokinetic profiles and degrees of bioavailability. Following oral administration, misoprostol is rapidly absorbed from the stomach with peak plasma concentrations (c_{max}) occurring within 12-30 minutes and rapidly declining thereafter, with a reported half-life ($t_{½}$) of 20-40 minutes. There is some evidence in humans suggesting that food affects pharmacokinetic properties of orally administered misoprostol. In one study, the rate of absorption was reduced and in another, both rate of absorption and bioavailability were decreased.[91,101]

Clinical investigations in humans indicate that route of administration impacts drug pharmacokinetics. In one study, sublingual administration yielded the highest bioavailability when compared to oral and transvaginal routes of administration.[100] In other studies, comparison of transrectal to oral routes of administration showed a faster time to c_{max} (t_{max}) and shorter disappearance half-life ($t_{½}$) with oral administration when a 400-µg dose was administered to women ranging from 40-71 kg bodyweight (approximately 5.5-10 µg/kg dose).[98] Interestingly, transrectal and transvaginal administration yielded longer $t_{½}$ and t_{max} compared to other routes.[87,98,99] Transrectal and transvaginal administration of misoprostol did not lead to significant variations in bioavailability compared to oral administration.[87,99] Adverse effects were observed in a dose-dependent manner and were associated with sublingual or oral administration, which included nausea, vomiting, diarrhea, abdominal pain, shivering, and fever.[102,103] In contrast,

transvaginal or transrectal administration demonstrated gradual increases in plasma concentration and/or lower overall c_{max}, and had reduced or no adverse effects observed.[102]

Section 6: Equine pharmacokinetic studies of misoprostol

The pharmacokinetic profile of misoprostol (5 μg/kg) in horses has recently been described for fasted horses administered misoprostol orally (per os, PO) in corn oil and for horses administered misoprostol in water by per rectum (PR) and PO under fasted and unfasted conditions.[104,105] Comparisons between these two studies are presented in **Table 3**. Potential differences between the two equine studies may be attributed to differences in vehicle delivery of misoprostol, horse populations, or in methodology for measurement of misoprostol free acid (MFA) in plasma.

In the single-dose pharmacokinetic study, misoprostol administration by the PR route in water demonstrated higher c_{max} values when compared to both fasted and non-fasted horses administered misoprostol PO in water. Fasted horses receiving misoprostol PO in water had similar c_{max} values to those obtained in humans receiving misoprostol sublingually, which was the highest value amongst human studies reviewed.[100,104] The reported c_{max} values in fasted horses receiving misoprostol PO in water was much higher than those reported PO with corn oil.[104,105] The c_{max} values reported for nonfasted horses receiving misoprostol PO in water or horses receiving misoprostol PO in corn oil were similar and comparable to values obtained in humans after PO administration.[100,104,105]

Values of t_{max} reported in horses when misoprostol was administered PO in water were also similar to those administered PO in corn oil, and also comparable to those reported in the human literature for oral and sublingual administration.[100,104,105] In contrast, misoprostol PR in water had t_{max} that was shortest amongst any study to date.

When evaluating AUC, values are highest in horses receiving misoprostol PO in water and most similar between humans after transvaginal administration in one study.[99,104] In another study,

misoprostol PR in water had similar AUC as those calculated in a different study of humans receiving misoprostol PO, transvaginally, buccally, and PR.[99,100,104]

Food appears to greatly impact misoprostol's pharmacokinetic properties. Fasted horses receiving misoprostol orally mixed in water demonstrated greater c_{max}, AUC, and $t_{½}$ compared to nonfasted horses receiving the drug in water or if the drug was administered with corn oil. This suggested that food (hay or corn oil) reduces oral bioavailability of misoprostol in horses. Similar changes in bioavailability and other pharmacokinetic variables have cited in other drugs including antimicrobials, anthelmintics, and NSAIDs.[106-110] This may be due to alterations in pH between fasting/feeding states (ion trapping phenomenon), delayed gastric emptying in the presence of food, or drug binding to food.[106-110]

Adverse effects of misoprostol have been reported in both humans and horses. These effects have been attributed to alterations in smooth muscle contractility and motility leading to bowel distention manifesting as abdominal pain.[111] In horses, adverse effects of misoprostol are infrequently reported even after drug administration over a several week period. Reported adverse effects include abdominal discomfort, transient depression, soft manure, and transient pruritis.[105,112] These findings are similar to those reported in human literature where the highest incidence of adverse effects is noted after sublingual misoprostol administration, most likely due to the high peak concentrations achieved by this route.[113]

	Martin et al. EVJ 2018 Misoprostol PK & Ex-Vivo Investigation	Lopp et al. AJVR 2019 Misoprostol PK		
Routes/ Vehicle	PO-Fasted Water + Corn Syrup	PO-Fasted Water	PO-Fed Water	PR Water
T_{max} (hr)	0.39 ± 0.04	0.25 (0.17-0.75)	0.30 (0.08-1.5)	0.08
C_{max} (pg/mL)	290 ± 70	655 ± 259	352 ± 109	967 ± 492
$AUC_{0\rightarrow\infty}$ (h pg/mL)	400 ± 120	2,217 ± 955	1358 ± 891	385 ± 153
$T_{½}$ (hr.)	0.67 ± 0.20	4.13 ± 3.4	2.53 ± 1.73	0.53 ± 0.27

Table 3: Comparison between findings of two recent pharmacokinetic investigations of a single dose of misoprostol under different delivery strategies [104,105]

Section 7: Therapeutic potential of misoprostol as an anti-inflammatory drug

Recent studies suggest misoprostol may offer promise as an anti-inflammatory modality in horses.[92,93,105] Anecdotally, McCoy and Lascola have reported improvements in clinical signs of SIRS secondary to gastrointestinal disease in a clinical setting for a small group (n=5) of horses that were administered misoprostol PR three times daily over several days period. As gastrointestinal disease may preclude PO administration, PR misoprostol provides an attractive alternate route; this will be further discussed in the next section. *In vitro* studies demonstrated reduced cytokine production and neutrophil adhesion, chemotaxis, and ROS generation by harvested equine peripheral leukocytes stimulated with LPS and treated with misoprostol. Furthermore, both protein production and messenger ribonucleic acid (mRNA) transcription were reduced for IL-1β, IL-6, and TNFα, key cytokines involved in the inflammatory response to endotoxin.[92,93]

However, when this study was translated into an *ex vivo* model, LPS-stimulated leukocytes obtained from horses receiving misoprostol PO in corn oil (5 µg/kg PO) did not demonstrate TNFα mRNA inhibition.[105] These findings contrasted with a human *ex vivo* study, which reported a 29% reduction in TNFα cytokine production after treatment from baseline values.[114] The disparity between these two studies could be due to species differences as well as study designs. The horses in the *ex vivo* study were administered a single 5 µg/kg oral dose prior to harvesting of leukocytes for *ex vivo* LPS stimulation, whereas the human clinical study participants underwent 14-day courses of administration at three separate doses (100 µg, 200 µg, and 300 µg orally four times a day) with TNFα cytokine production compared between baseline values prior to the study with those at the conclusion of the three treatments several weeks later.[105,114] Therefore, the findings

reported in humans may reflect the more frequent and prolonged drug administration and represent a cumulative response, as opposed to the response to a single dose reported for horses.

Ultimately, *in vitro* and *ex vivo* experimental models, while valuable, may not demonstrate the appropriate cellular interactions that misoprostol exerts *in vivo*. Given the complexities of the inflammatory response *in vivo*, and potential differences in magnitude of TNFα stimulation when compared to *in vitro* and *ex vivo* models, *in vivo* investigation is warranted.

Section 8: Existing investigations of drug pharmacokinetics with administration by the per rectum (PR) route

Administration of medication by the PR route is utilized in both human and veterinary medicine for both local and systemic therapy.[115] In humans, reported purposes for local therapy primarily includes administration of laxatives and anti-inflammatories, while purposes for systemic therapy include administration of analgesics, anti-inflammatories, and anti-epileptic medications, among others.[115] Per rectum administration provides an alternate means of drug delivery in situations precluding oral administration, when a parenteral formulation of a drug is not available, or when parental delivery may not be technically feasible.[115] Administration PR may provide some advantages over PO. First, the environment of the rectum is typically constant and static, with minimal water and electrolyte secretion. This near neutral pH environment, in contrast to the acidic environment of the stomach, may reduce biodegradation and potential ion trapping encountered for some drugs.[115] Second, the anatomy of the rectum provides a path for drugs to bypass portal circulation and avoid first-pass metabolism by entering the middle and caudal rectal vein, which drains into the vena cava.[116] Lipophilic drugs may also drain to the surrounding mesenteric lymph nodes and enter into systemic circulation and also avoid first-pass metabolism.[115] This may allow for increased bioavailability of drugs compared to drugs administered orally.

Limitations and challenges with this route exist despite these potential benefits. First, the proximal (orad) portion of the rectum, in contrast to the distal (aborad) portion, drains into the portal system, and therefore drugs could undergo first pass metabolism depending on where deposited within the rectum; this concern is cited in humans and dogs due to the variations in patient size and overall length of these vessels.[115,117,118] It is possible that similar challenges could

be encountered in pediatric equine patients or smaller adult horse breeds. Second, in contrast to the small intestine, the rectal mucosa is not designed for absorption, as evidenced by its lack of villi or microvilli and the presence of single columnar cells with goblet cells for secretion of mucus. This lack of surface area may inhibit drug absorption.[115] Additionally, the presence of mucus and feces may limit drug absorption.[115] Finally, disease conditions may hinder the ability of drugs to be absorbed, such as systemic inflammation altering perfusion of the distal gastrointestinal tract, colitis, which can lead to increased motility (limiting the time for drug absorption to occur), or mucosal edema (increasing the distance/thickness that drugs must overcome to diffuse into circulation).[115]

In small animal veterinary medicine, rectal administration of medications is considered a viable option when oral administration poses safety concerns to the clinician, if a patient is in critical condition (e.g., administering diazepam, levetiracetam, or ketoprofen to seizure patients) or has a cardiac dysrhythmia, or when IV formulations are either not available or when parenteral administration is not feasible (e.g., pimobendan, sildenafil).[117,118] Potential differences in drug pharmacokinetics must be taken into consideration when choosing the per rectum route. A recent study of pimobendan, a phosphodiesterase 3 inhibiting inotrope and vasodilator, showed that rectal administration in dogs led to lower c_{max} and AUC, but reduced t_{max} and $t_{½}$ compared to oral administration.[117] Sildenafil, a phosphodiesterase 3 inhibiting pulmonary vasodilator used in dogs required a higher dose when given per rectum to reach similar c_{max} and $t_{½}$ and higher AUC as when given orally.[118]

In horses, rectal administration of drugs can be a valuable option for conditions that may preclude oral administration (e.g., proximal enteritis and gastrointestinal ileus).[116,119-121] As in other species, altered bioavailability associated with the per rectum route may necessitate drug

dose adjustments. Metronidazole, a nitroimidazole antimicrobial, is frequently used to target anaerobic organisms present in disease conditions such as clostridial colitis or pneumonia. Due to cost limitations associated with the parenteral formulation, this medication is typically administered orally to adult horses but may also be administered via the per rectum route at a higher dose. Rectal administration of metronidazole demonstrated similar t_{max} and $t_{½}$, but reduced c_{max} and bioavailability when compared to oral administration.[121] Importantly, bioavailability does not appear to be affected by presence of manure, eliminating the need for rectal evacuation prior to drug administration.[120] Rectal administration of altrenogest, a steroidal progestin, demonstrated decreased bioavailability and shorter $t_{½}$ when compared to oral administration at the same dose but when administered at a higher dose and frequency demonstrates comparable bioavailability as oral administration.[116] Acetylsalicylic acid is a beneficial anticoagulant with poor oral bioavailability.[122] Interestingly, in one study that did not report drug pharmacokinetics, per rectum administration of acetylsalicylic acid yielded superior inhibition of platelet thromboxane production compared to horses receiving the drug orally.[119]

Section 9: Justification for the study

In summary, SIRS is a prevalent condition observed in various equine conditions. This condition is triggered through the various arms of the NFκB pathway ultimately resulting in the clinical presentation of SIRS. To mitigate its effects, a multi-modal treatment approach is warranted. Existing therapeutic strategies are limited, and the adverse effects from utilizing these therapies (e.g., anaphylaxis due to plasma therapy, nephrotoxicity due to polymyxin or NSAID use) may further limit their use. Providing additional therapeutic modalities can aid in reducing adverse effects from individual therapies, in addition to mitigating multiple arms of the NFκB pathway. Misoprostol provides a potentially feasible and promising approach to the treatment of SIRS through direct mitigation of inflammatory cytokines and neutrophil function. While a single low-dose endotoxin model does not reflect how endotoxemia occurs in natural disease, this model elicits a predictable clinical response suitable for testing potential therapeutic efficacy.

Chapter 2: Materials and Methods

Section 1: Study Design

The following project represents a prospective, three-treatment randomized crossover study in a 3 x 3 Latin square configuration. Treatment order was assigned by randomly drawing treatment sequences (simple randomization) for each horse. Approximately 1-2 hours prior to the endotoxin and misoprostol administration, horses were instrumented with intravenous catheters in both jugular veins. The **left jugular catheter** (MILA, 14-gauge 5.25-inch length) was used for serial blood collection for measurement of plasma misoprostol concentrations and serum cytokine protein expression and for determination of peripheral blood leukocyte counts and cytokine gene expression. For each time blood was collected, 5 to 10 mL of waste blood was collected prior to sample collection to clear the catheter and extension line. Once all blood was collected for a given time point, the catheter was flushed with at least 5 mL of heparinized saline. The **right jugular catheter** (TERUMO, 14 gauge 2-inch length) was used for intravenous administration of endotoxin. A baseline sample was obtained for plasma misoprostol concentration, total and differential leukocyte counts, and serum cytokine concentrations. Horses subsequently received a single 5 μg/kg dose of misoprostol (100 μg tablets; American Health Packaging) orally (M-PO), or rectally (M-PR), or water/vehicle (CON) immediately prior to intravenous infusion of endotoxin (*Escherichia coli* O55:B5 lipopolysaccharide, 30 ng/kg, List Biological Labs) via the **right jugular catheter**. CON horses received water orally and rectally. Horses in the M-PO group received water per rectum, and horses in the M-PR group received water orally. A 28-day interval between each treatment was observed to avoid endotoxin tolerance and associated diminishment in cytokine response.[41,104] This time was also sufficient as a washout period for misoprostol's documented elimination half-life of 170 minutes for fasted horses receiving the drug orally.[104]

Figure 2 depicts sample and data collection time points for the study. A physical examination and behavioral observations were performed within 1 hour prior to misoprostol and endotoxin administration. Sample collection time points for determination of misoprostol pharmacokinetics differed between the M-PO and M-PR treatment groups and were based on previously identified differences in area-under-curves (AUC) between these routes of administration.[104] Following baseline samples obtained during instrumentation, sample collection time points for M-PO treatment horses were at 5, 10, 15, 20, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210, 240, and 360 minutes after misoprostol administration. Similarly, M-PR treatments were obtained at 3, 5, 10, 15, 20, 30, 45, 60, 75, 90, 105, 120, 150, and 180 minutes after misoprostol administration. Serum and whole blood samples were obtained for cytokine protein and gene expression measurements respectively prior to (baseline/0 hours), followed by 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 6.0, and 24 hours after completion of endotoxin infusion in both the M-PO and M-PR treatment groups. This would coincide with 60, 90, 120, 150, 180, 210, 290, and 1470 minutes after drug administration**.** Sampling time points for cytokine protein expression and for determination of peripheral blood leukocyte counts and cytokine gene expression were selected based on previously reported peaks in gene expressions of TNFα, IL-1, and IL-6 and TNFα bioactivity.[46,83,123]

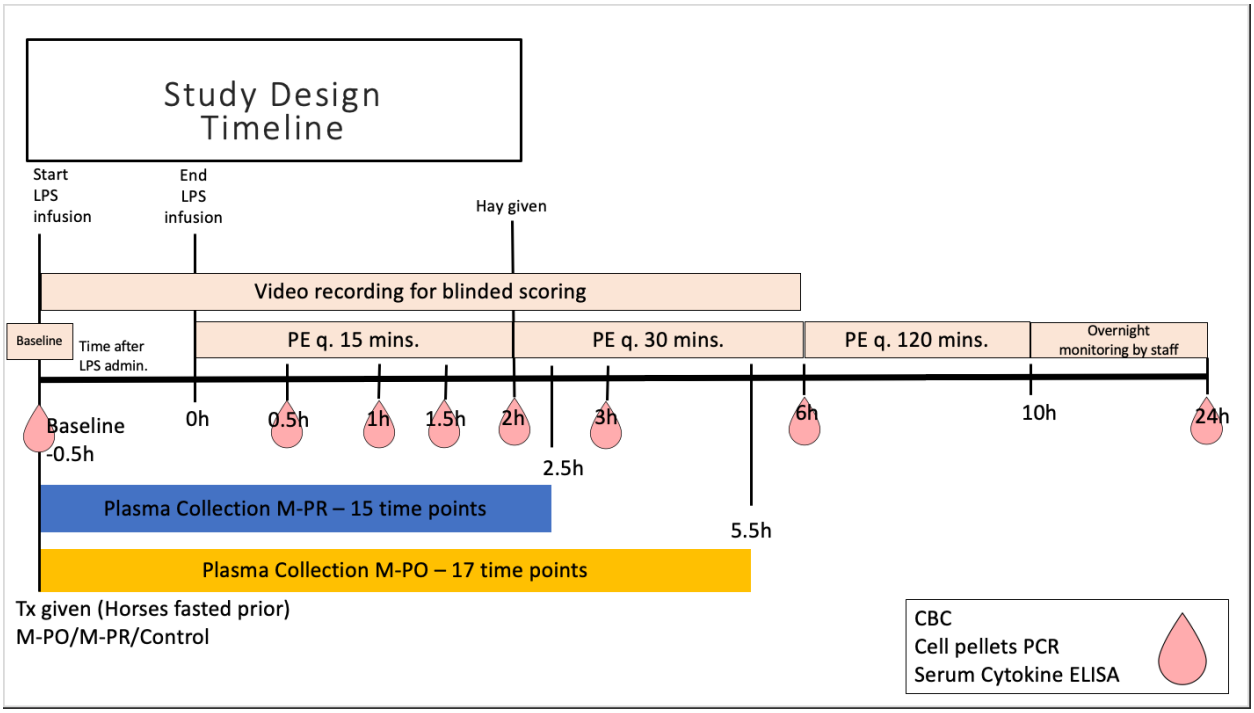


Figure 2: Study Design Timeline for Each Horse per Treatment – The timeline depicts various events involved in this study, including timing for treatments given and start of LPS infusion, sample collection points, PE/observations taken and video recording periods.

Section 2: Study Population

Six healthy adult geldings were recruited from the Auburn University teaching herd, including 5 American Quarter Horses and 1 Thoroughbred. The age range of the horses were 9-18 years. Their weights ranged from 454 to 655 kg. Horses were determined to be healthy based on history, physical examination, complete blood count, and serum biochemistry analysis prior to enrollment into the study. Horses were housed in individual stalls within the JT Vaughan Large Animal Teaching Hospital for a minimum of 18 hours prior to and for the duration of each experimental period. Horses were fasted for 12 hours prior to each experimental period. Hay consumption resumed 2 hours after endotoxin administration. Water was available ad libitum throughout the duration of the study. The diet consisted of coastal/Bermuda hay (1 flake every 6 hours) and commercial senior pelleted feed as a mash (1.5 pounds/0.7 kilograms in 2-4L of water once daily). During the washout periods, horses were housed on pasture in accordance with the standard protocol for the University resident herd.

Throughout the experimental period, monitoring included temperature, heart rate, respiratory rate, and gross observations such as colic signs, muscle fasciculations, and manure output and character. These observations were recorded every 15 minutes for the first 2 hours post-endotoxin infusion, then every 30 minutes until 6 hours post-infusion, followed by every 2 hours until 10 hours post-infusion. Changes in manure character was defined by increase in water content ranging from soft-formed to liquid. Video recordings (two minutes in duration) were also obtained to be reviewed by a blinded observer later for identification of behavioral changes and assigning of a pain score as modified from a similar study.[46] Recordings were taken at baseline, then at 0, 15, 30, 45, 60, 75, 90, 120, 150, 180, and 360 minutes after completion of endotoxin infusion. This coincides with 30, 45, 60, 75, 90, 105, 120, 150, 180, 210, and 420 minutes after treatment (misoprostol PR or PO, or control) administration. Horses were monitored overnight to ensure no

systemic changes were noted at 24 hours post-endotoxin administration before turning them back out onto pasture.

Section 3: Drug Preparation and Administration

3a. Endotoxin administration

Endotoxin solution was prepared by administering working *Escherichia coli* O55:B5 lipopolysaccharide (LPS, 10 µg/mL concentration) solution at a dose of 30 ng/kg (or 0.03 µg/kg) in 500 mL of isotonic (0.9%) saline. Immediately after administration of misoprostol or water (M-PO/M-PR/CON), the LPS mixture was given intravenously as a continuous rate infusion over 30 minutes through the dedicated right jugular vein catheter. The safety of this procedure is well-described.[40-42,45,81,82,124,125]

3b. Misoprostol administration

Manure was evacuated from all horses immediately prior to per rectum administration of water or misoprostol. For horses receiving M-PO treatments, misoprostol hydrochloride (100 µg tablets) was dissolved in 30 mL of water at a dose of 5 µg/kg and administered via 60 mL catheter-tipped oral syringe, followed by 30 mL of water through the same syringe to ensure all residual drug was administered to the horse. These horses also received 60 mL of water per rectum as described for M-PR drug administration below. For the M-PR treatment, horses were administered misoprostol at a dose of 5 µg/kg dissolved in 30 mL of water in a different 60 mL catheter-tipped oral syringe. The drug was then delivered per rectum via 16-inch (40.6 cm), 8-French red-rubber catheter inserted approximately 30 centimeters in all horses. After PR drug administration, an additional 30 mL of water was administered through the catheter to ensure delivery of the entire dose. The M-PR horses also received 60 mL of water orally via oral syringe. Lastly, CON horses

received a total of 60 mL of water orally as well as 60 mL of water rectally immediately prior to endotoxin administration as described for the M-PO and M-PR horses. The timing of treatment administration prior to endotoxin infusion was based on the expected time to peak TNFα concentration after endotoxin infusion and previously reported time to maximal misoprostol plasma concentrations.[83,104]

Section 4: Sample Collection, Processing, and Analysis

4a. Sample collection and processing

Blood collected for measurement of plasma misoprostol concentrations (10 mL volume) was immediately transferred to sodium-heparin tubes (BD Vacutainer), placed on ice until centrifugation within 15 minutes of collection. Plasma was separated by centrifugation at 400 rpm for 10 minutes at 4˚C, separated into 1 mL aliquots and immediately stored at -80˚C until analysis.

Blood collected for peripheral blood leukocyte cytokine qRT-PCR analysis (20 mL volume) was immediately transferred to sodium-heparin tubes (BD Vacutainer) for leukocyte isolation using the following a customized protocol: the buffy coat was initially collected from the blood sample by centrifugation at 400 x g for 10 minutes at 4˚C. The buffy coats from each tube were then combined and washed in 30 mL of erythrocyte lysis buffer (EL Buffer, QIAGEN). After incubation for 40 minutes with intermittent vortexing, the buffy coat-EL buffer mixture was centrifuged at 400 x g for 10 minutes at 4˚C, and the supernatant was removed. The cell pellets underwent three additional cycles of washing with 4 mL of EL buffer and centrifugation at 400 x g for 10 minutes at 4˚C to remove as many erythrocytes from the leukocyte pellet as possible. Once the erythrocytes were sufficiently removed, leukocyte cell pellets were suspended in 1.4 mL

of EL buffer before being flash-frozen in liquid nitrogen and stored in -80˚C until qRT-PCR analysis.

Blood collected for serum for cytokine protein measurements (10 mL) was immediately transferred to non-additive blood tubes (BD Vacutainer) and allowed to clot for 30 minutes before centrifugation at 800 x g for 15 minutes at room temperature. Serum was divided into 500 µL aliquots and stored at -80˚C until batch analysis.

Finally, blood collected for leukocyte and differential counts (6 mL) was transferred to potassium-EDTA tubes (BD Vacutainer) and submitted to the Auburn University Clinical Pathology service for hematologic testing.

4b. Serum cytokine protein measurements

Cytokine production (TNFα, IL-1β, and IL-6) was measured by a validated equine multiplex bead immunoassay (Equine Cytokine Magnetic Bead Panel, MILLIPLEX® MAP) read on a Luminex MAGPIX® System (Millipore Sigma) according to manufacturer directions. Briefly, a 96-well plate was prepared at room temperature with an initial wash of 200 µL of the provided wash buffer. An equal volume (25 µL each) of assay buffer, matrix buffer, and beads were added to the wells containing 25 µL of Standard, Control or Sample. The plate layout is shown in the Appendix portion of this thesis. The plates were wrapped in aluminum foil to protect from light and incubated under agitation (800 rpm) in 2-8˚C storage for 16-18 hours. The following day, the wells were washed, and the plate placed on a magnetic plate (provided by the manufacturer) where it rested for 60 seconds and decanted. Following the initial wash, detection antibodies (25 µL each) were added to each well, incubated at 20-25˚C for 1 hour, and protected from light. Streptavidin-phycoerythrin (25 µL) was added to each well and incubated at room temperature while protected from light for 30 minutes. Following another iteration of washing, wash buffer (150 µL) was added

to resuspend the proprietary beads and read using Xponent Luminex® 200™ software according to the manufacturer directions. Any samples that were above the limit of detection were repeated at 5X dilution.

4c. Peripheral blood leukocyte cytokine qRT-PCR

Prior to cDNA synthesis, RNA isolation and quantification were performed (QiAMP RNA Blood Mini Kit; QIAGEN) with modifications to the manufacturer protocol. Frozen samples were thawed, lysed, and mixed with 4mL of RLT/BME solution added to the cell pellet. These were divided between 3 QIAshredder spin columns and centrifuged for 2 minutes at maximum speed to homogenize the sample. This was repeated twice. The lysate was mixed with 70% ethanol and added to a new QIAamp spin column, and centrifuged for 15 seconds at 8,000 x g. The spin column was transferred into a separate collection tube, and 350 μL of RW1 buffer added and centrifuged for 15s at 8,000x g. Once purified, the RNA was eluted into RNase-free water. Concentration and purity of RNAs were obtained (ThermoFisher™ NanoDrop™). Synthesis of cDNA was performed using the Maxima First Strand cDNA Synthesis kit for RT-qPCR and SYBR Green/ROX qPCR Master Mix (Fisher Scientific). Equine-specific gene primer pairs and probes were designed by the McCoy laboratory group (resources from Integrated DNA Technologies, IDT). Primers are displayed in **Table 4**. Triplicate sample processing was performed on all PCR samples, along with no reverse transcriptase and no template control for each gene for each sample. Parameters set for the PCR are displayed in **Figure 3.** Normalization of the expression of genes of interest were performed to the housekeeping genes GAPDH and EF1α. Fold changes in mRNA expression were determined using the ΔΔCt method to determine relative gene expression changes of each of the cytokines measured (TNFα, IL-1β, and IL-6).

When performing this method of testing, target and reference genes are assumed to have similar efficiencies. This can be demonstrated by determining the threshold cycles for each gene at varying concentrations. If the slope of the differences in threshold cycles against the logarithm of the varying gene concentrations are near zero, they are considered to be similar in efficiencies. The formula for obtaining relative gene expressions were performed as accepted from previous literature.[126]

Gene	Forward Primer	Reverse Primer
TNFα	CTCCAGACGGTGCTTGTG	TGGAAGGCATTCGGTAACTG
IL-1β	GAGACTGACAAGATACCTGTGG	TTCCTCTTTGGGTAAGTATTGGG
IL-6	TGCAGATCAGTACCAAAGTCC	TCTTCAGCCACTCATTCTGTG
IL-10	AAAAGCTGAAGACCCTCCG	ACTCATGGCTTTGTAGACACC
GAPDH	GGCAAAGTGGATATTGTCGC	GGGTGGAATCATACTGAAACATG
EF1α	TCGTTGATATGGTTCCTGGC	GCTTTCTTGTCCACTGCTTTG

Table 4: qPCR Primer Sequences utilized. Obtained from McCoy Lab, Samantha Hammack PhD Candidate

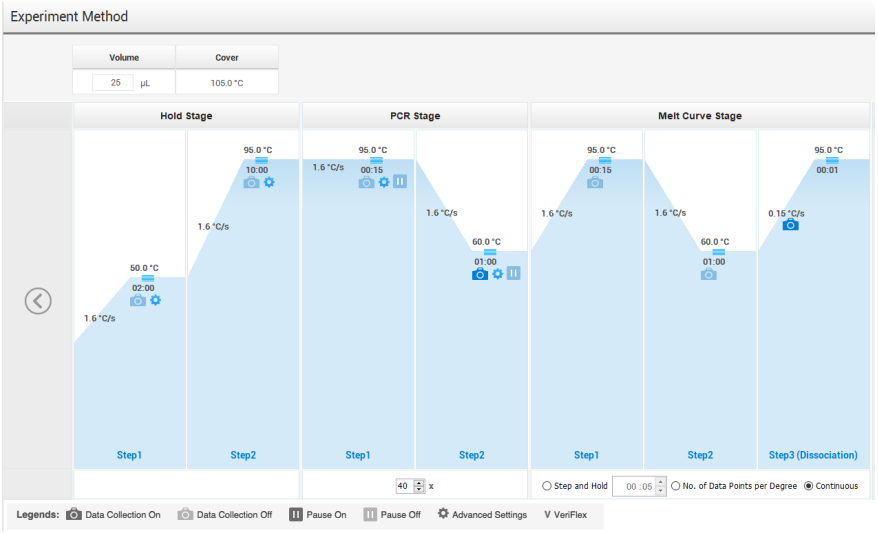


Figure 3: qPCR Parameters Setting. Obtained from McCoy Lab, Samantha Hammack DVM/PhD Candidate

4d. Misoprostol acid analysis via LC-MS/MS

This protocol has been validated for equine plasma.[104] Plasma samples were prepared for analysis by spiking 50 µL of plasma sample mixed in 1,000 µL of acetonitrile with 5 µL of misoprostol acid-d_5 standard at 100 ng/mL. This was then vortex mixed and centrifuged before the supernatant was dried and reconstituted into 100 µL of solvent prior to being subjected to LC-MS/MS analysis. Data acquisition and analysis were performed using Thermo Altis Triple Quadrupole LC-MS/MS system using Software TraceFinder 4.1. The LC separation was achieved using the Thermo Accucore Vanquish C18 column (2.1x100 mm, 1.5 µm). The mobile phase A (formic acid 0.1% in water) and mobile phase B (formic acid 0.1% in acetonitrile) was set at a flow rate of 0.3 mL/min. The following settings were established for linear gradient: 0-0.5 min. 25%B; 1-2.5 min. 100%B; 3-4.5 min. 25%B. The autosampler was set at 10˚C and the injection volume was set at 5 µL. Negative electrospray ionization (ESI) was performed for acquisition of mass spectra with ion spray voltage of -3500 volts, sheath gas of -40, auxiliary gas of 6, sweep gas of 1, ion transfer tube of 335˚C, and vaporizer of 260˚C. For quantitation, multiple reaction monitoring (MRM) was performed with the following parameters: misoprostol acid m/z 367.1 → m/z 249.0; misoprostol acid-d_5 internal standard m/z 372.1 → m/z 249.1.

Standard calibration curves were generated before performing sample analysis. This was done by using drug-free aliquots of equine plasma with commercially available MFA and MFA-d_5 that were used as the internal standards. The calibrated concentration range was 5-5,000 pg/mL. The coefficient of determination (R^2) was greater than 0.993. Lowest limit of quantitation (LLOQ) was 5 pg/mL. For misoprostol plasma concentrations of 300, 1,000, and 3,000 pg/mL, mean ± SD percentage recovery was reported 66.7 ± 9.1%, 71.0 ± 6.2%, and 72.8 ± 3.3% respectively. Within-run accuracy of misoprostol detection in plasma (expressed as mean ± SD) ranged from 96.1 ±

8.2% to 116.2 ± 3.4%, and within-run precision ranged from 4.3 to 7.8%. The between-run accuracy ranged from 98.9% ± 7.4% to 115.0 ± 5.8% (mean ± SD) and precision ranged from 3.7% to 5.4%.

4e. Pharmacokinetic and pharmacodynamic analysis

Non-compartmental pharmacokinetic modeling was analyzed by comparing plasma concentration versus time for M-PO and M-PR using Phoenix® WinNonlin v8.1 software (Certara). Calculations for area under the curve of the misoprostol acid concentration against time to infinity ($AUC_{0\rightarrow\infty}$) were calculated for each route using the log-linear trapezoidal method. Additional parameters obtained for each route of administration included: mean residence time (MRT), relative bioavailability, apparent volume of distribution (V_d/F), and apparent clearance (Cl/F). Terminal phase rate constants (λ_z) for each route were determined by linear regression, and the disappearance half-lives ($t_{½}$) were calculated as its inverse. Lastly, maximum and minimum plasma concentrations and their respective times were obtained (c_{max}, c_{min}, t_{max}, and t_{min}). To integrate the pharmacokinetic findings to pharmacodynamics, maximal plasma response (E_{max}) and 50% plasma concentration response (EC_{50}) between misoprostol concentration and serum cytokine concentrations were calculated by simple and sigmoidal modeling of the relationship effects (Phoenix®).

4f. Blinded video scoring

Videos were saved and identifiers were scrambled by a random number generator prior to scoring. Gross pain behavior – such as excessive head movements, flehmen, kicking, pawing, rolling, tail swishing, mouth playing, and stretching out – were scored as 1, 2, and 3 based on no signs displayed, occasional signs, and continuous signs, respectively. Once scores were written,

the investigators calculated each horse's mean scores over the study period for each treatment. These mean values obtained for horses for each treatment were compared for statistical difference.

Section 5: Statistical Analysis

A priori power analysis was performed (alpha = 0.05, power = 0.80) and determined six horses were sufficient to detect significant differences in TNFα cytokine protein production and other clinical indicators of endotoxemia, based on observed variability in *in vivo* endotoxin studies in horses receiving endotoxin.[40,125,127] Conservatively, 30 percent improvement in parameters following misoprostol administration was anticipated. This was extrapolated from observations in horses and other species *in vitro*.[88,92,94] Normality was assessed by Shapiro-Wilk and Anderson-Darling tests of standard normal distribution. Data are reported as mean ± SD or median (range) according to distribution. Data were removed from study if cytokine concentrations could not be obtained or were appreciably inconsistent compared to other data sets. The CV for selected values was calculated as the SD divided by the mean.

For comparisons of pharmacokinetic values between the M-PO and M-PR treatments, a paired two-sample t-test or a Wilcoxon matched-pairs signed rank test was used. Linear mixed-model analysis or 2-way repeated measure analysis of variance (ANOVA) were performed to test for significance over time and between groups for normally distributed data, with post-hoc comparisons for significant data tested with Tukey's multiple comparisons test. For non-normally distributed data a Friedman's test was used. Pearson's correlation analysis was performed to assess relationships between specific pharmacokinetic parameters (AUC, c_{max}, t_{max}) and time to onset and duration of fasciculations.

For video analysis for gross pain score, a one-way ANOVA was performed to test for significance between groups, with post-hoc comparisons for significant data tested with Tukey's multiple comparisons test. Data analysis was performed using GraphPad Prism 8.4.2 and Microsoft Excel. Significance was defined by $p < 0.05$.

Chapter 3: Results

Section 1: Unblinded gross clinical signs

Statistically significant differences for colic, manure output, and manure character were not identified between treatments for any time, but fasciculation onset time and duration were later and more abbreviated, respectively, in CON horses compared to either M-PR or M-PO. Results are summarized for each horse under each treatment in **Table 5.** Reported signs of colic included pawing, pacing, abnormal stance/stretching, and flank watching (fasciculations were included as a separate observation) and were noted in 4/6 horses in at least one of the treatment arms. One horse (Horse 2) showed moderate signs of colic shortly before the 24 hour physical examination during the M-PO treatment. This horse showed no other abnormalities on physical examination, nasogastric intubation, or trans-rectal palpation. He received a full dose of flunixin meglumine and was monitored an additional day with no other colic signs observed before returning to the herd.

Median time of onset of colic signs was 0 (range 0-0), 30 (range 0-60), and 30 (range 0-75) minutes following completion of LPS infusion for M-PR, M-PO, and CON treatments, respectively. For individual horses demonstrating signs of colic, this appeared to be more frequent in the CON group (7 instances) than M-PO (6 instances) and M-PR (3 instances). No correlation was noted between drug pharmacokinetics and the frequency of these events.

Fecal output per horse appeared to be higher for M-PR treatment (median: 5.5 piles; range: 2-7 piles) compared to the M-PO (median: 4 piles; range: 0-6 piles) and CON (median: 3 piles; range: 1-6 piles) treatments. Changes in manure character were reported during each of the three experimental conditions for 4/6 horses among all treatment groups. There was no association between change in manure character and c_{max} or AUC for misoprostol in M-PO or M-PR group.

All horses demonstrated muscle fasciculations regardless of treatment group. Median onset and duration of fasciculations post LPS-infusion for M-PR, M-PO, and CON horses were 15 (range 15-30), 37.5 (range 15-45), and 60 (range 30-120) minutes for onset and 112.5 (range 75-165), 120 (75-150) and 60 (30-120) minutes for duration, respectively. Fasciculations resolved in all horses by 180 minutes post-LPS infusion. The time of onset was significantly earlier, and the duration was significantly longer when M-PO ($P = 0.03$) and M-PR ($P = 0.004$) groups were compared to the CON group. For the M-PO and M-PR treatment groups no correlations were identified between onset of fasciculations and t_{max} ($P > 0.15$; $R^2 < 0.4$) and duration of fasciculations and AUC or c_{max} ($P > 0.2$; $R^2 < 0.4$).

	Horse Number and Treatments																	
	1			2			3			4			5			6		
	M-PR	M-PO	CON	M-PR	M-PO	CON	M-PR	M-PO	CON	M-PR	M-PO	CON	M-PR	M-PO	CON	M-PR	M-PO	CON
Fasciculation onset time (mins. after completion of LPS infusion)	15	45	90	30	15	30	15	45	60	30	15	45	15	45	0	15	30	60
Fasciculation duration (min.)	135	105	30	120	135	60	90	105	60	90	150	75	105	75	180	165	150	120
Total manure output	7	6	6	5	0	2	2	4	3	4	4	3	7	0	3	5	4	1
Altered manure consistency number	0	0	0	2	0	0	0	1	0	0	0	0	2	0	3	3	2	1
Onset of noted altered manure consistency (mins. after completion of LPS infusion)	--	--	--	15*	--	--	--	270	--	--	--	--	330	--	0	30	45	45
Total colic frequency	1	1	1	0	1	0	0	0	0	1	0	0	0	1	6	1	4	2
Onset of colic (mins. after completion of LPS infusion)	0	90	75	--	1440**	--	--	--	--	0	--	--	--	30	15	0	0	0

Table 5: Descriptive table of unblinded observations for each horse under M-PR, M-PO, and CON treatments including time of onset of fasciculations, altered manure consistency, and colic signs, duration of fasciculations, total number of manure and number of altered manure character, and total colic observations noted during the study

* Horse 2 M-PR had soft formed manure noted at baseline, but then further changed 15 minutes post LPS infusion

**Horse 2 was noted to have more significant colic signs – e.g. ***Flunixin meglumine was administered and the horse was monitored overnight. No other signs were noted 24 hours after completion of the experiment.

Section 2: Physical examination parameters

Differences in temperature, heart rate, and respiratory rate magnitudes or percentage changes between treatment groups were not identified. This included time of peak increase and duration of increase post-LPS infusion completion were considered ($p > 0.2$ for all parameters). The changes in temperature, heart rate, and respiratory rate are summarized in **Figures 4, 5,** and **6**. Baseline values were within appropriate reference intervals and differences were not identified between treatment groups. Baseline values (mean ± SD) are as follows: temperature (98.9 ± 0.5˚F); heart rate (39.8 ± 5.7 bpm), and respiratory rate (15 ± 3.5 brpm). Respiratory rate increased post LPS infusion completion ($P = 0.04$) but was outside of the reference range in only 2 horses and only during the M-PR treatment. Increases in heart rate and temperature post-LPS administration were observed for all horses and all treatment groups ($P < 0.0001$). Peak increases in heart rate were observed within 90 minutes (median 75; range 15-90) and peak increases in temperature were observed between 2-5 hours (median 3.5 hours) after completion of LPS infusion. Mean maximum heart rate (bpm) and temperature (˚F) post-LPS infusion completion for the M-PR, M-PO, and CON groups were 62 ± 14.9 and 102.3 ± 0.9; 72 ± 19.3 and 102.1 ± 0.7; 66 ± 17.5 and 101.8 ± 0.5, respectively. Significant differences in percent increase from baseline or in absolute measured values were not identified between treatment groups for respiratory rate, heart rate or temperature over the course of the experimental period or at any specific time point ($p > 0.1$ for all parameters).

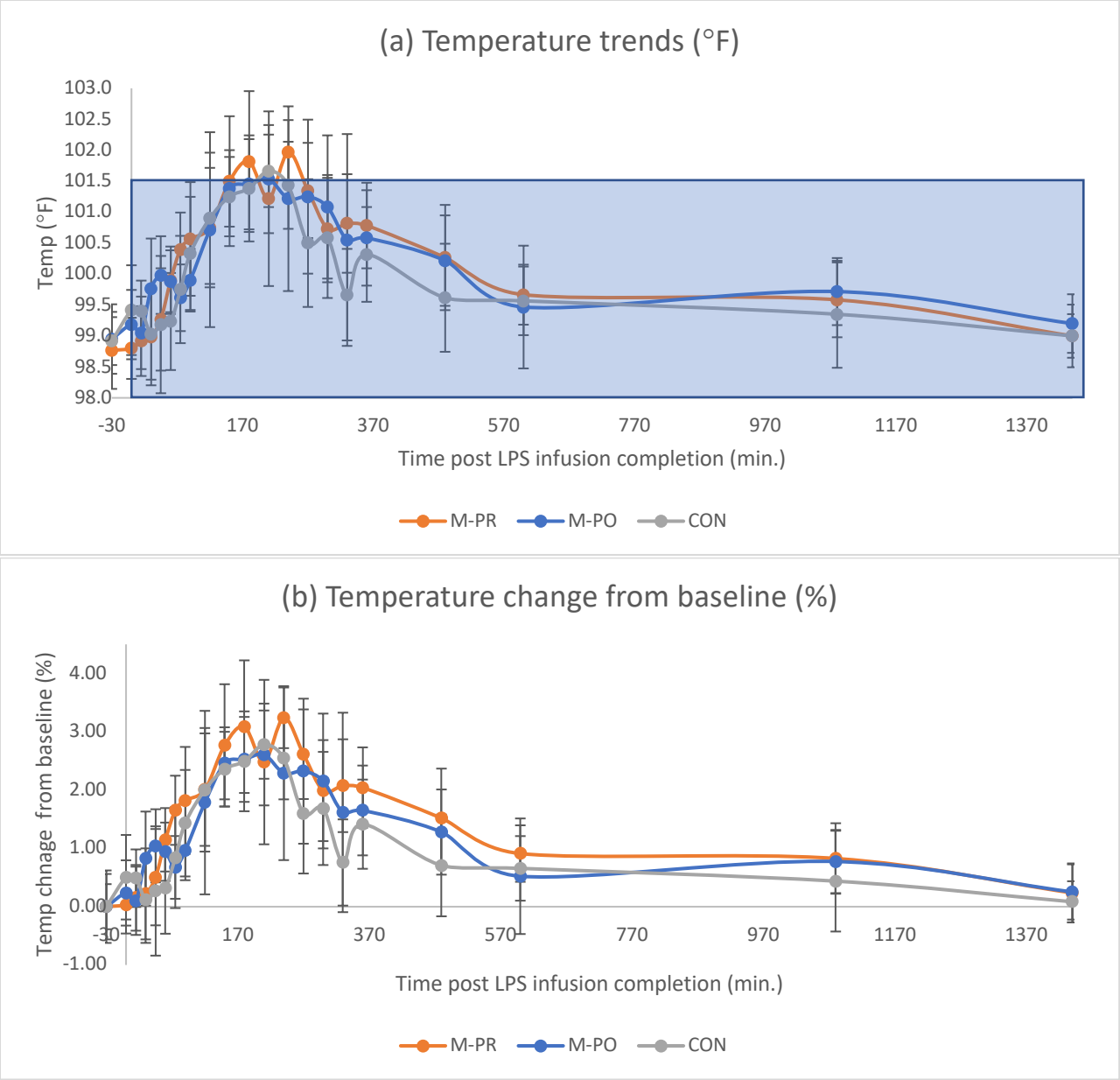


Figure 4 – a. Changes in mean rectal temperature following completion of LPS infusion for each treatment group (M-PR, M-PO, and CON). Time in minutes is expressed as following LPS infusion, therefore, -30 minutes represents baseline. The shaded region indicates the normal temperature range for healthy adult horses (98-101F). b. Temperature changes graphed according to mean percent change from baseline values for each treatment group.
Data for both graphs presented as mean ± SD. M-PR (blue line): per rectum treatment group; M-PO (orange line): oral treatment group; CON (gray line): control group.

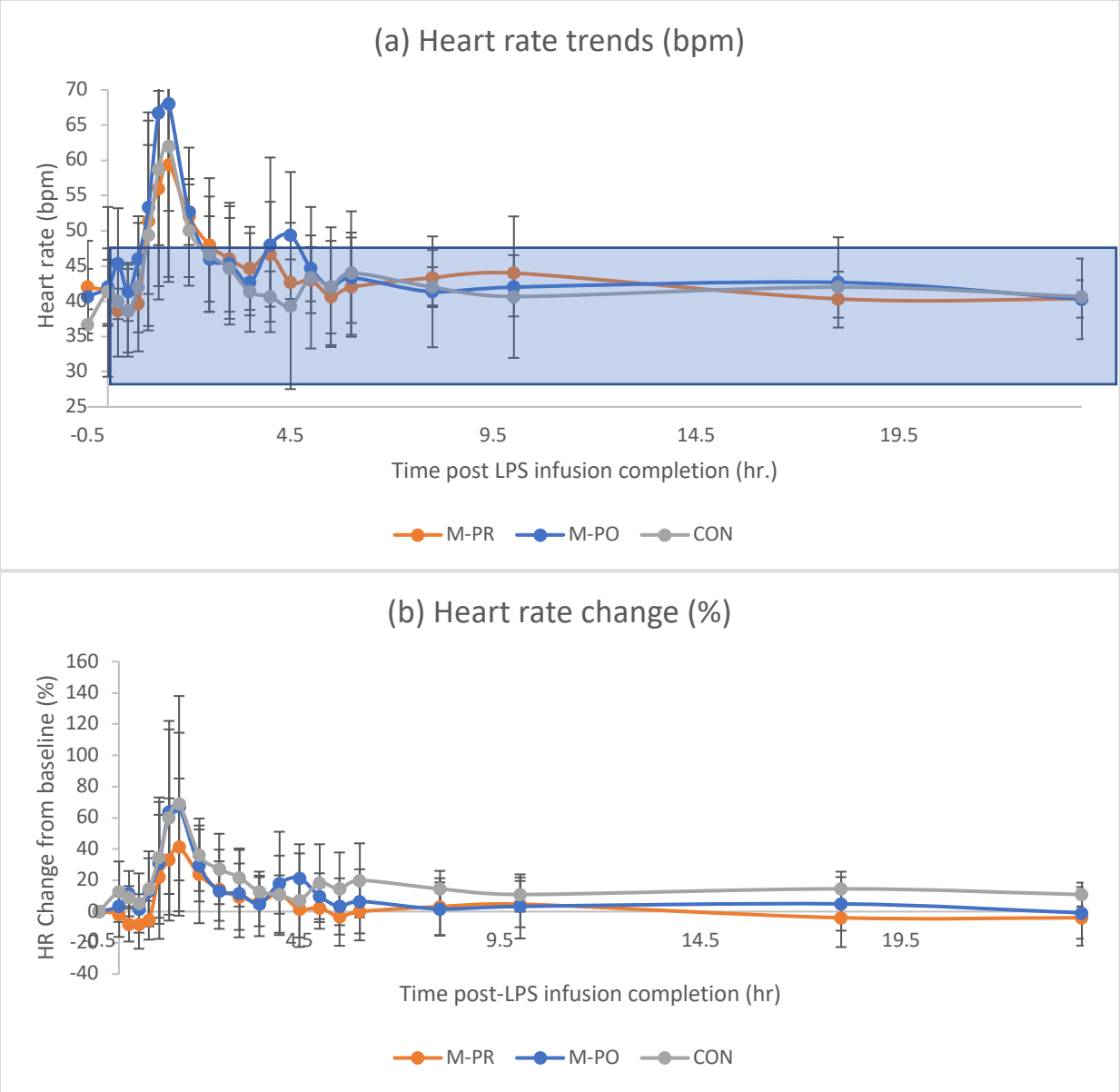


Figure 5 – a. Changes in heart rates following completion of LPS infusion for each treatment group (M-PR, M-PO, and CON). Time in minutes is expressed as following LPS infusion, therefore, -30 minutes represents baseline. The shaded region indicates the normal heart rate range for healthy adult horses (28-48 bpm). b. Heart rate changes graphed according to mean percent change from baseline values for each treatment group.
Data for both graphs presented as mean ± SD. M-PR (blue line): per rectum treatment group; M-PO (orange line): oral treatment group; CON (gray line): control group.

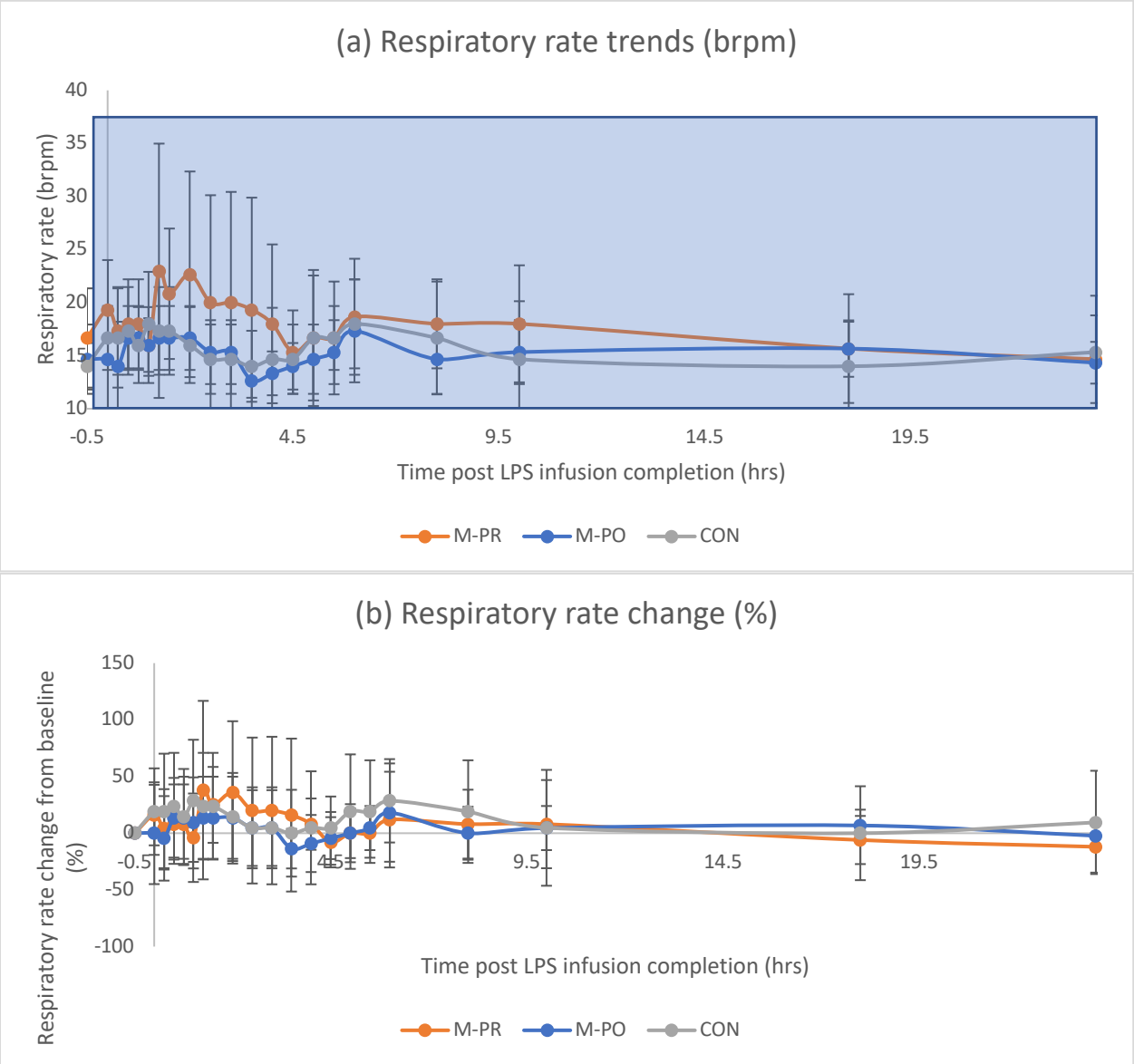
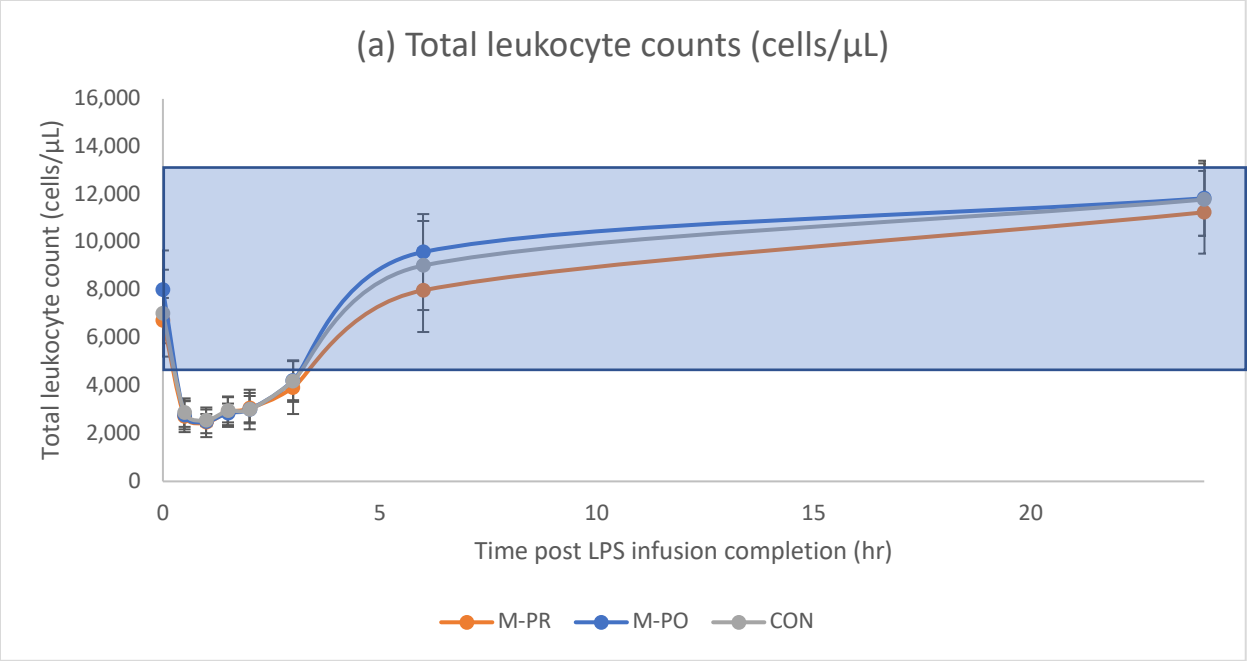


Figure 6 – a. Changes in mean respiratory rates following completion of LPS infusion for each treatment group (M-PR, M-PO, and CON). Time in minutes is expressed as following LPS infusion, therefore, -30 minutes represents baseline. The shaded region indicates the normal respiratory rate range for healthy adult horses (8-40 brpm). b. Respiratory rate changes graphed according to mean percent change from baseline values for each treatment group.
Data for both graphs presented as mean ± SD. M-PR (blue line): per rectum treatment group; M-PO (orange line): oral treatment group; CON (gray line): control group.

Section 3: Total leukocyte, neutrophil lymphocyte counts

No significant differences were identified between various treatment groups across time points or cumulatively (area under the curve for each treatment groups across time points) for changes in total leukocyte, segmented neutrophil, and lymphocyte counts. These trends are presented in **Figures 7, 8,** and **9**. Similar to physical examination parameters, at baseline, parameters for all horses were within normal reference ranges for total leukocyte count (mean ± SD: 7,263 ± 1,531 cells/μL), neutrophil count (mean ± SD: 4,540 ± 1,355 cells/μL), and lymphocyte count (mean: 2,401 ± 1,051 cells/μL). Over the course of the experimental period leukocyte, segmented neutrophil, and lymphocyte counts changed significantly ($p < 0.001$) with observed ranges of 1,530-14,290 cells/μL, 100-12,004 cells/μL, and 239-5,081 cells/μL respectively. Compared to baseline values, significant reductions in leukocyte count were noted between 30 and 120 minutes for the M-PR ($p < 0.04$) and CON ($p < 0.03$) groups and between 30 and 180 minutes for the M-PO group ($p < 0.03$). Neutrophils were significantly decreased from baseline between 30 and 120 minutes for M-PR group ($p < 0.01$), 30 and 360 minutes for the M-PO group ($p< 0.04$ all) and at 90 minutes for the CON group ($p = 0.03$). Neutrophil count was significantly increased from baseline at 24 hours in the M-PR ($p = 0.002$) and CON ($p = 0.01$) groups. Relative to baseline values, changes in lymphocyte counts were mild and only significant for the M-PO group at 90 minutes ($p = 0.03$) and the M-PR group at 120 and 360 minutes ($p < 0.03$).



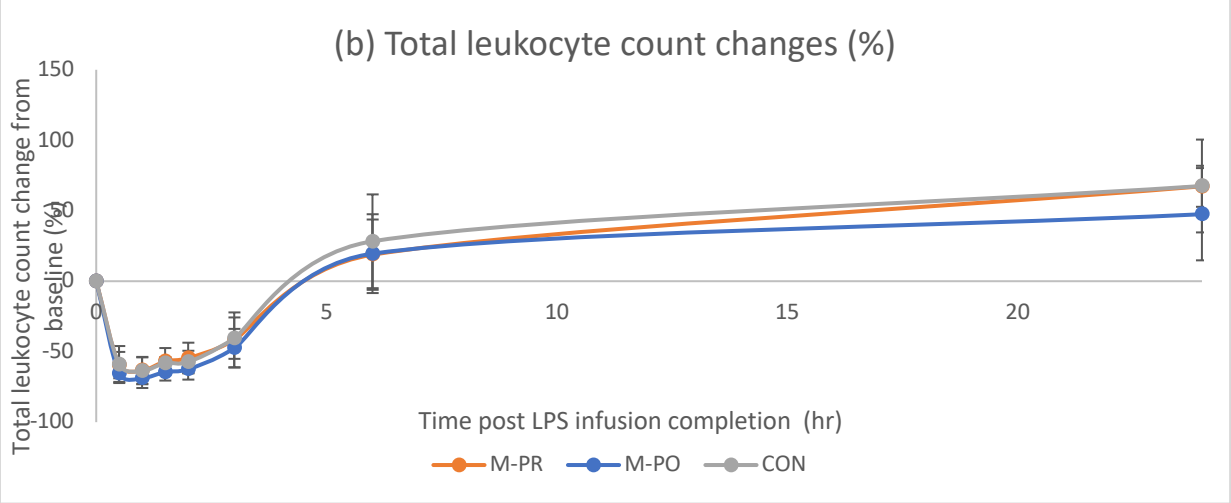
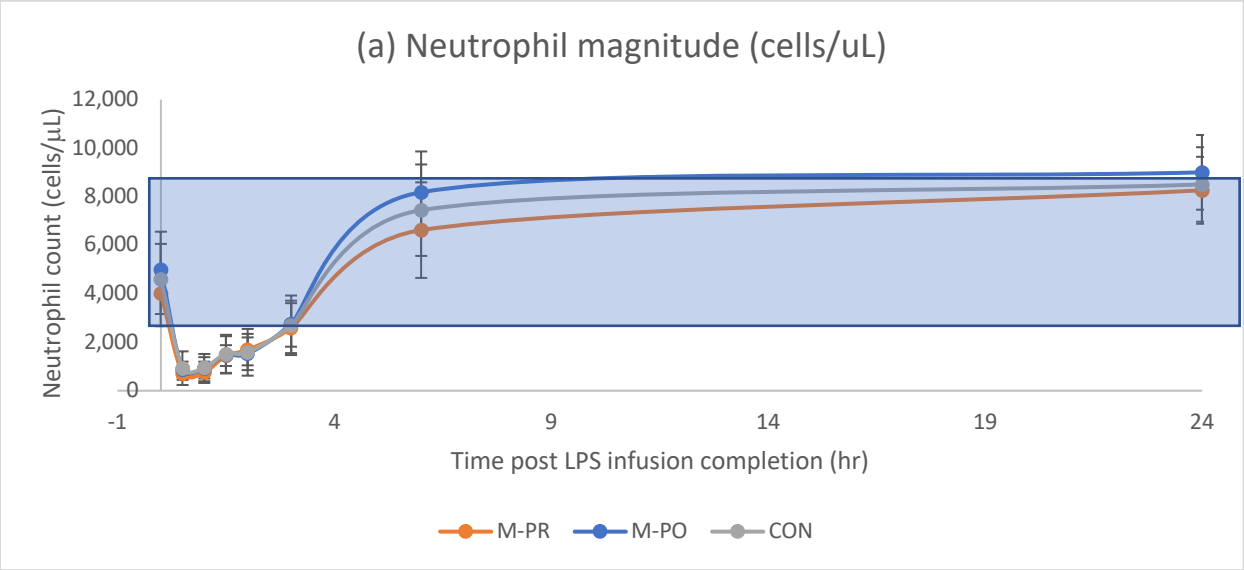


Figure 7 – a. Changes in mean total leukocyte counts following completion of LPS infusion for each treatment group (M-PR, M-PO, and CON). Time in minutes is expressed as following LPS infusion, therefore, -30 minutes represents baseline. The shaded region indicates the normal total leukocyte count range for healthy adult horses (5,400-14,300 WBC/µL). b. Total leukocyte count changes graphed according to mean percent change from baseline values for each treatment group. Data for both graphs presented as mean ± SD. M-PR (blue line): per rectum treatment group; M-PO (orange line): oral treatment group; CON (gray line): control group.



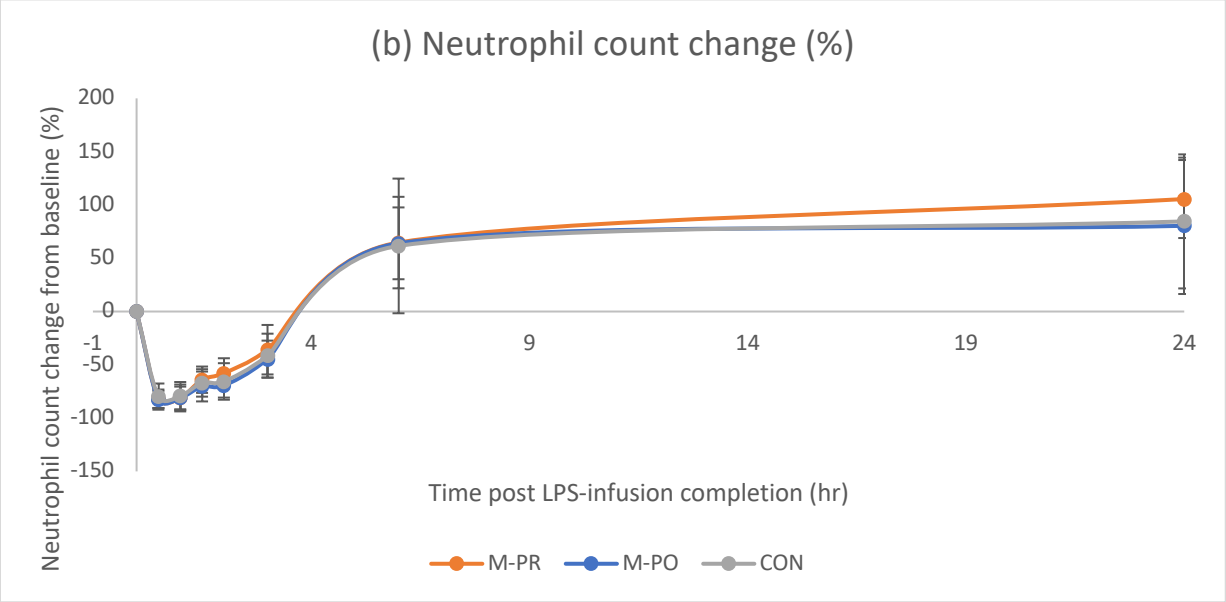


Figure 8 – a. Changes in segmented neutrophil counts following completion of LPS infusion for each treatment group (M-PR, M-PO, and CON). Time in minutes is expressed as following LPS infusion, therefore, -30 minutes represents baseline. The shaded region indicates the normal segmented neutrophil count range for healthy adult horses (2,300-8,600 segmented neutrophils/µL). b. Segmented neutrophil changes graphed according to mean percent change from baseline values for each treatment group.
Data for both graphs presented as mean ± SD. M-PR (blue line): per rectum treatment group; M-PO (orange line): oral treatment group; CON (gray line): control group.

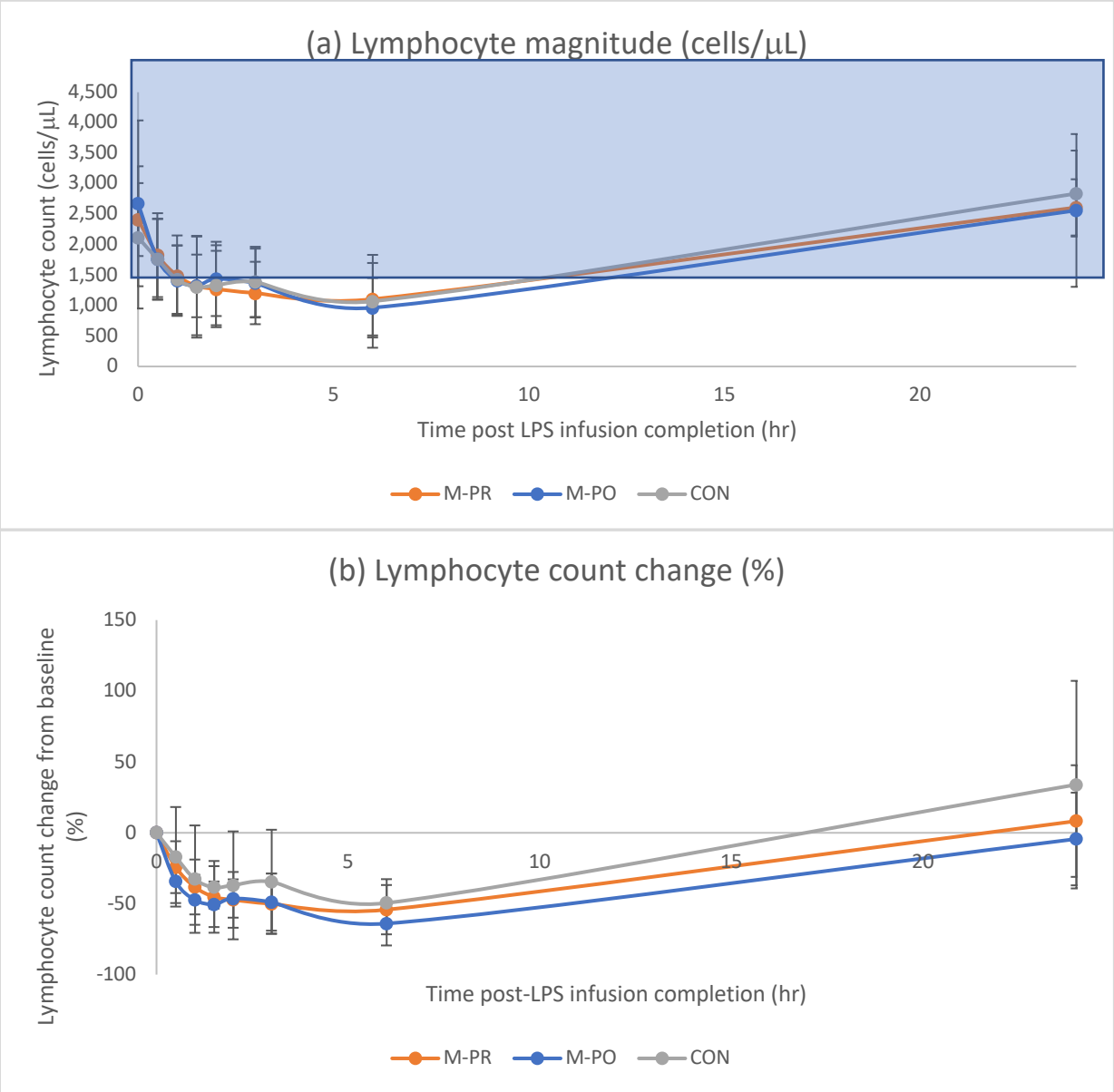


Figure 9 – a. Changes in lymphocyte count following completion of LPS infusion for each treatment group (M-PR, M-PO, and CON). Time in minutes is expressed as following LPS infusion, therefore, -30 minutes represents baseline. The shaded region indicates the normal lymphocyte count range for healthy adult horses (1,500-7.700 lymphocytes/µL). b. Lymphocyte changes graphed according to mean percent change from baseline values for each treatment group. Data for both graphs presented as mean ± SD. M-PR (blue line): per rectum treatment group; M-PO (orange line): oral treatment group; CON (gray line): control group.

Section 4: Blinded pain assessment results

Pain scores were averaged across all horse for each of the treatment groups. Over the course of the study, the mean ± SD of the pain scores for M-PR, M-PO, and CON treatments were 2.10 ± 0.33, 2.04 ± 0.24, 1.90 ± 0.36 respectively and were not statistically significant between all treatments ($p = 0.29$).

Section 5: Pharmacokinetic data

Compared to M-PR treatment, horses in M-PO had higher c_{max}, AUC, and Vd/F, longer t_{max}, $t_{½}$, and MRT and lower Cl/F in the face of large variations measured between horses. Plasma pharmacokinetic parameters for MFA are summarized in **Table 6**. Plasma concentration versus time curves for misoprostol free acid (MFA) were generated for the M-PO and M-PR experimental groups and are displayed in **Figure 10**. A greater than 6-fold increase in c_{max} was observed between M-PO and M-PR ($p < 0.001$). Similarly, $AUC_{0\rightarrow\infty}$, values were more than 20-fold greater for M-PO compared to M-PR ($p< 0.001$). The t_{max} was significantly shorter for the M-PR condition ($p = 0.005$), as were $t_{½}$ and MRT ($p < 0.02$). The M-PO group demonstrated a greater Vd/F ($p = 0.03$) and reduced Cl/F ($p = 0.02$) in comparison to the M-PR group. Relative bioavailability of misoprostol for the M-PR group compared to the M-PO group was 5.00 ± 3.34% (range 0.83% to 8.77%).

Variations in plasma misoprostol concentrations were appreciable between horses for both M-PR and M-PO treatments. Within the M-PO group, the coefficient of variation (CV) for c_{max} and $AUC_{0\rightarrow\infty}$ were 67% and 73% respectively with c_{max} values ranging from 1,664 pg/mL to 10,226 pg/mL and $AUC_{0\rightarrow\infty}$ values ranging from 5,013,960 to 41,107,860 h pg/mL. For the M-PR group the CVs for these parameters were 100% and 91% respectively with c_{max} values ranging from 268 pg/mL to 2,580 pg/mL and $AUC_{0\rightarrow\infty}$ values ranging from 161,580 to 1,746,540 h pg/mL.

For M-PO treatments, all six horses had detectable plasma misoprostol concentrations until 150 minutes post-drug administration, and 2/6 of the horses had detectable concentrations up to 240 minutes post-administration. In contrast all 6 horses in M-PR treatments had detectable plasma misoprostol concentrations until 45 minutes post-drug administration, with only 1/6 horse having detectable concentrations 120 minutes post-drug administration (**Table 7**). Mean ± SD percentage of the $AUC_{0-\infty}$ that was extrapolated was 2.2 ± 2.1% for the M-PO group and 1.9 ± 1.6% for the M-PR group.

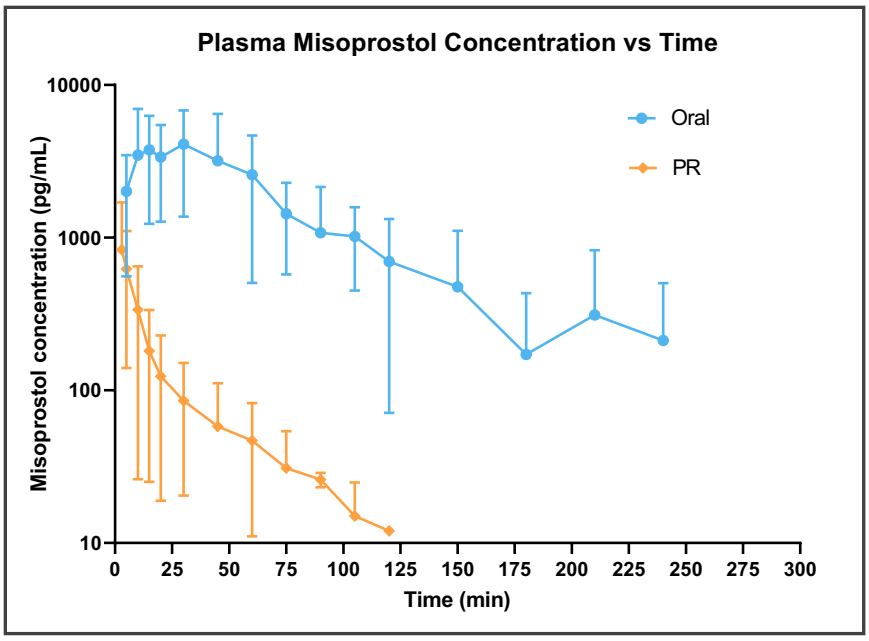


Figure 10: Concentration against time curve after a single dose of orally (M-PO) or rectally (M-PR) administered misoprostol in 6 adult horses.

Variable	M-PO	M- PR
t_{max} (min)*	25 (10-45)[a]	3 (3-5)[b]
c_{max} (pg/mL)	5,209 ± 3,487 [a]	853.83 ± 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]	644,960.4 ± 558,866 [b]
AUC_{all} (h pg/mL)	17,467,344 ± 12,449,328	633,860.4 ± 552,758
$t_{½}$ (min)	40 ± 21[a]	9 ± 7 [b]
λ_z (min.$^{-1}$)	0.021 ± 0.008 [a]	0.111 ± 0.084 [b]
c_{mean} (pg/mL)	829 ± 601	60 ± 52
MRT (min)	59 ± 13	15 ± 9
Vd/F (mL/kg)	1.3 ± 0.8 [a]	0.8 ± 0.3 [b]
Cl/F (mL/h/kg)	1.6 ± 1.3[a]	5.5 ± 4.3 [b]

Table 6: Plasma pharmacokinetic variables (mean ± SD unless otherwise specified) for misoprostol oral (M-PO) or rectal (M-PR) administration to 6 adult horses. t_{max}: time to maximum plasma concentration; c_{max}: maximum plasma concentration; $AUC_{0\rightarrow\infty}$: area under the plasma concentration versus time curve extrapolated to infinity; $t_{½}$: disappearance half-life; λ_z: disappearance rate constant; MRT: mean residence time; Vd/F: apparent volume of distribution, not adjusted for bioavailability; Cl/F: apparent clearance, not adjusted for bioavailability
Different superscripts between columns signify statistically significant differences ($p<0.05$)
*listed as median (range)

Time	M-PR	M-PO
3	833 ± 868 (6)	NR
5	624 ± 484 (6)	2014 ± 1457 (6)
10	337 ± 311 (6)	3477 ± 3494 (6)
15	181 ± 156 (6)	3756 ± 2524 (6)
20	124 ± 105 (6)	3371 ± 2096 (6)
30	86 ± 65 (6)	4090 ± 2716 (6)
45	58 ± 53 (6)	3190 ± 3273 (6)
60	47 ± 36 (5)	2586 ± 2081 (6)
75	31 ±23 (4)	1433 ± 858 (6)
90	26 ± 3 (2)	1076 ± 1073 (6)
105	15 ± 10 (2)	1017 ± 566 (6)
120	12 ± 0 (1)	697 ± 626 (6)
150	ND	476 ± 633 (6)
180	ND	172 ± 262 (4)
210	ND	312 ± 516 (3)
240	ND	212 ± 292 (2)

Table 7 – Mean +/- SD plasma MFA concentrations at various points after 6 adult horses received a single dose of misoprostol (5 ug/kg) administered fasted PO and PR in a crossover study design. Numbers in parentheses signify the number of horses included in the data.
ND = not determined because values of all 6 horses were below the LLOQ (5 pg/mL)
NR = not recorded – data was not obtained for horses in M-PO group
*By 360 minutes, all horses had values below the LLOQ

Section 6: RT-PCR Gene expression

Downregulation in relative gene expression of cytokines were appreciated for both M-PO and M-PR in relation to CON, with large variations in magnitude and peak time of downregulation noted between horses. Horses receiving M-PR had the most pronounced downregulation of both TNFα and IL-6 gene expression at 0.5h (60 mins post drug-administration), whereas M-PO treated horses had the most pronounced downregulation of gene expression of these two cytokines at 6h and 0.5h respectively post-LPS infusion completion.

Changes in cytokine gene expression (TNFα, IL-6, IL-1β) for the misoprostol treatment groups (M-PR or M-PO) relative to the control group are depicted in **Figures 11** and **12** (TNFα and IL-6 comparisons amongst all horses). Individual horse gene transcription changes for TNFα, IL-6, IL-1β of M-PO in relation to CON are displayed in **Figures 13-15. Figures 16** and **17** depict individual gene transcription changes for M-PR in relation to CON for TNFα and IL-6**.** For the M-PR group IL-1β analysis was not performed due to insufficient funds. Variability in misoprostol pharmacokinetic parameters does not correlate with observed inter-horse variability in gene expression. For the M-PO treatment group, data for horse 3 represents 7 sample points, as the sample for 0.5h post-LPS administration during M-PO treatment was lost during processing. For all other horses, all time points are represented. Data that represent at least a 2-fold change in up-regulation or down regulation relative to the control group are considered significant as indicated in the figures.

Downregulation in TNFα gene expression after M-PO treatment ranged from 2.06 to 7.16-fold decreases compared to CON. Downregulation in IL-6 gene expression after M-PO treatment in these same horses ranged from 2.3 to 7.01-fold decreases compared to CON. The time point 6h

post-LPS infusion completion had the largest proportion of horses with downregulation of TNFα (5/6 horses), IL-6 (3/6 horses), and IL-1β (2/6 horses) gene expression.

Downregulation in TNFα gene expression after M-PR treatment ranged from 2.38 to 11.64-fold decreases compared to CON. Downregulation in IL-6 gene expression after M-PR treatment in these same horses ranged from 2.00 to 10.91-fold decreases compared to CON. The time point 0.5h post-LPS infusion completion had the highest proportion of downregulation in TNFα (5/6 horses) and IL-6 (4/6 horses) gene expression.

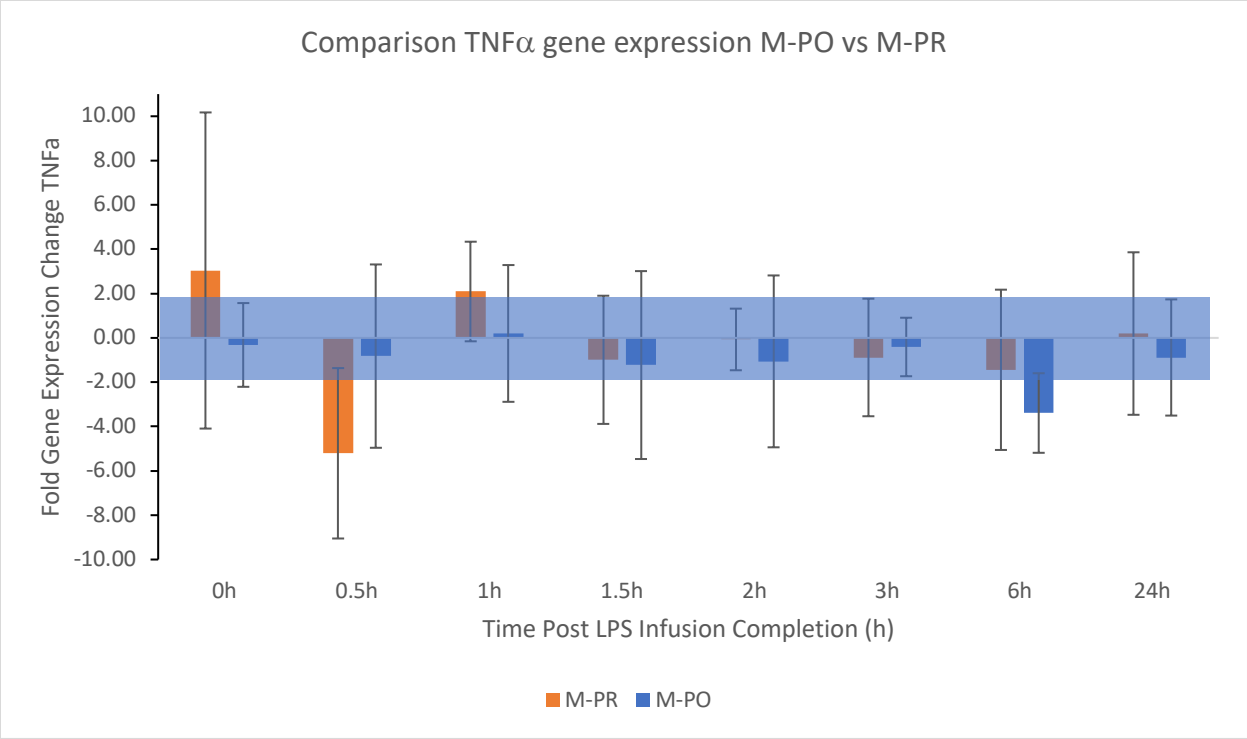


Fig 11 – Bar graph indicates changes in gene expression of TNFα across time between all horses comparing M-PR (blue bar) and M-PO (orange bar) against CON. Time 0h indicates baseline prior to LPS infusion, and each time point thereafter indicating hours post-infusion completion. A blue shaded region indicates areas of non-significant gene expression changes (two-fold upregulation or downregulation).

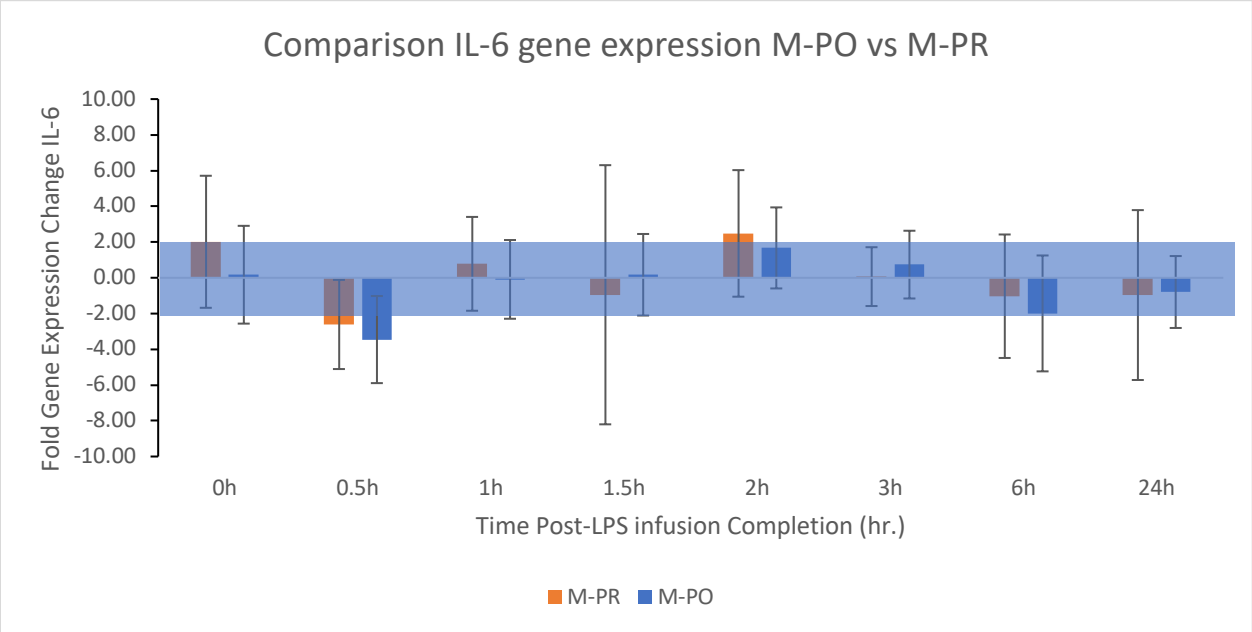


Fig 12 – Bar graph indicates mean ± SD fold changes in gene expression of IL-6 across time between all horses comparing M-PR (blue bar) and M-PO (orange bar) against CON. Time 0h indicates baseline prior to LPS infusion, and each time point thereafter indicating hours post-LPS infusion completion. A blue shaded region indicates areas of non-significant gene expression changes (two-fold upregulation or downregulation).

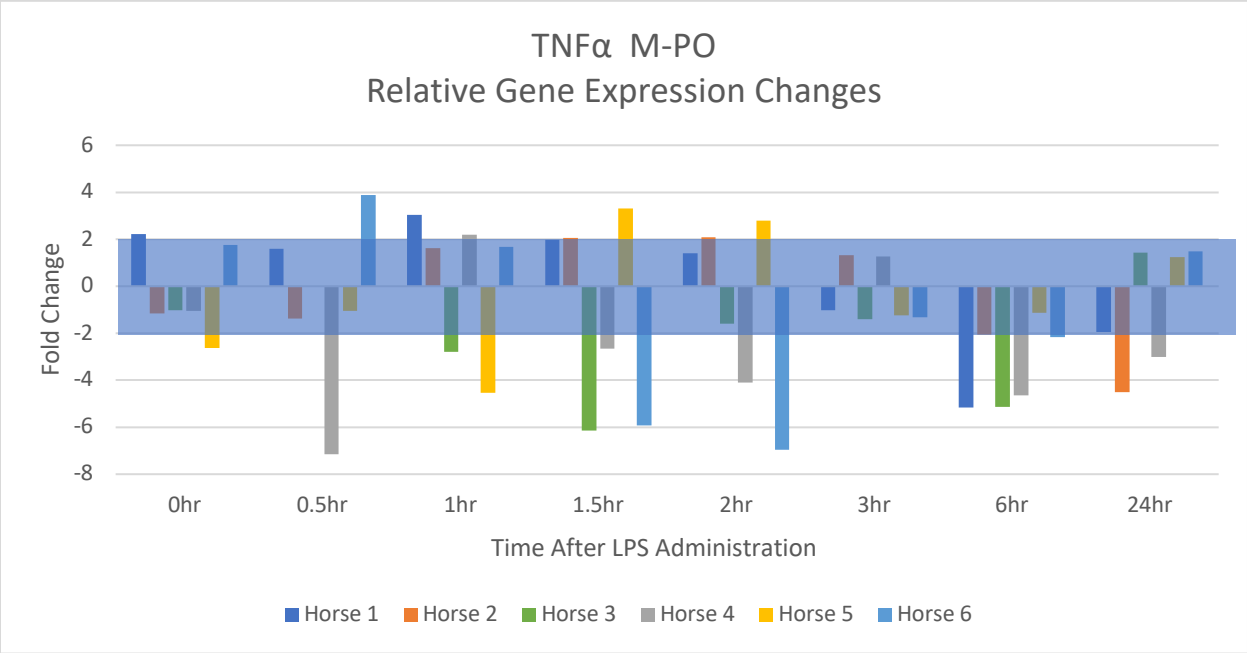


Fig 13 – Bar graph indicates changes in gene expression of TNFα across time comparing horses receiving M-PO treatments compared to CON treatments. Time 0h indicates baseline prior to LPS infusion, and each time point thereafter indicating hours post-infusion completion. Each bar represents an individual horse as indicated by the figure legend. A blue shaded region indicates areas of non-significant gene expression changes (two-fold upregulation or downregulation).

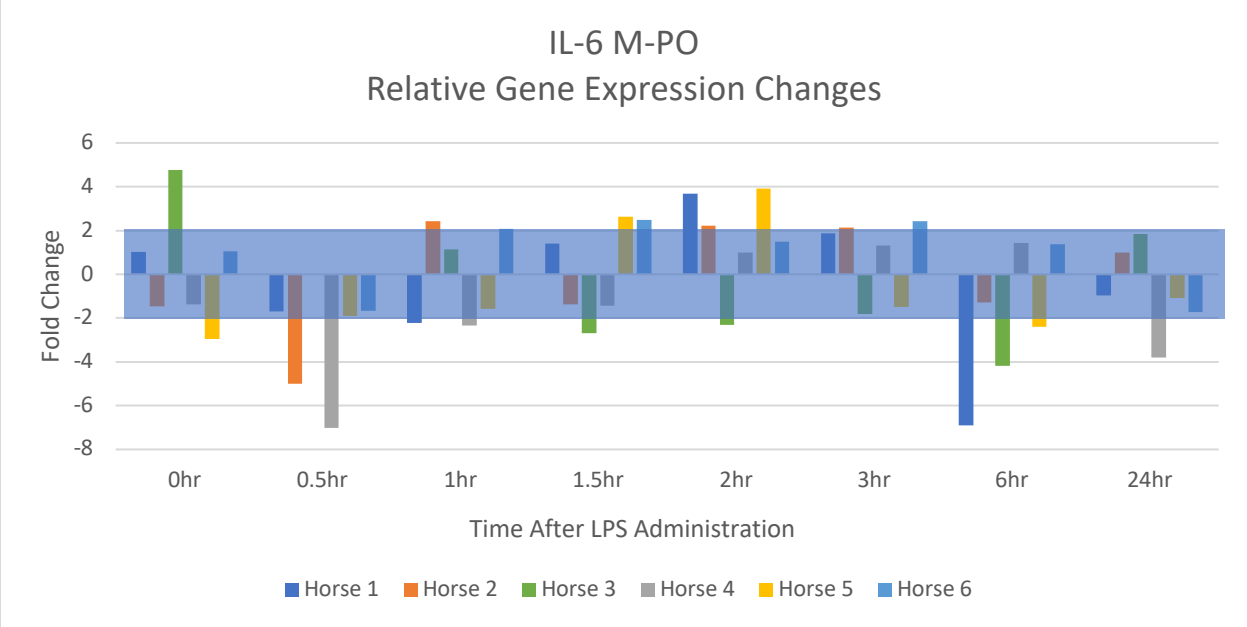


Fig 14 – Bar graph indicates changes in gene expression of IL-6 across time comparing horses receiving M-PO treatments compared to CON treatments. Time 0h indicates baseline prior to LPS infusion, and each time point thereafter indicating hours post-infusion completion. Each bar represents an individual horse as indicated by the figure legend. A blue shaded region indicates areas of non-significant gene expression changes (two-fold upregulation or downregulation).

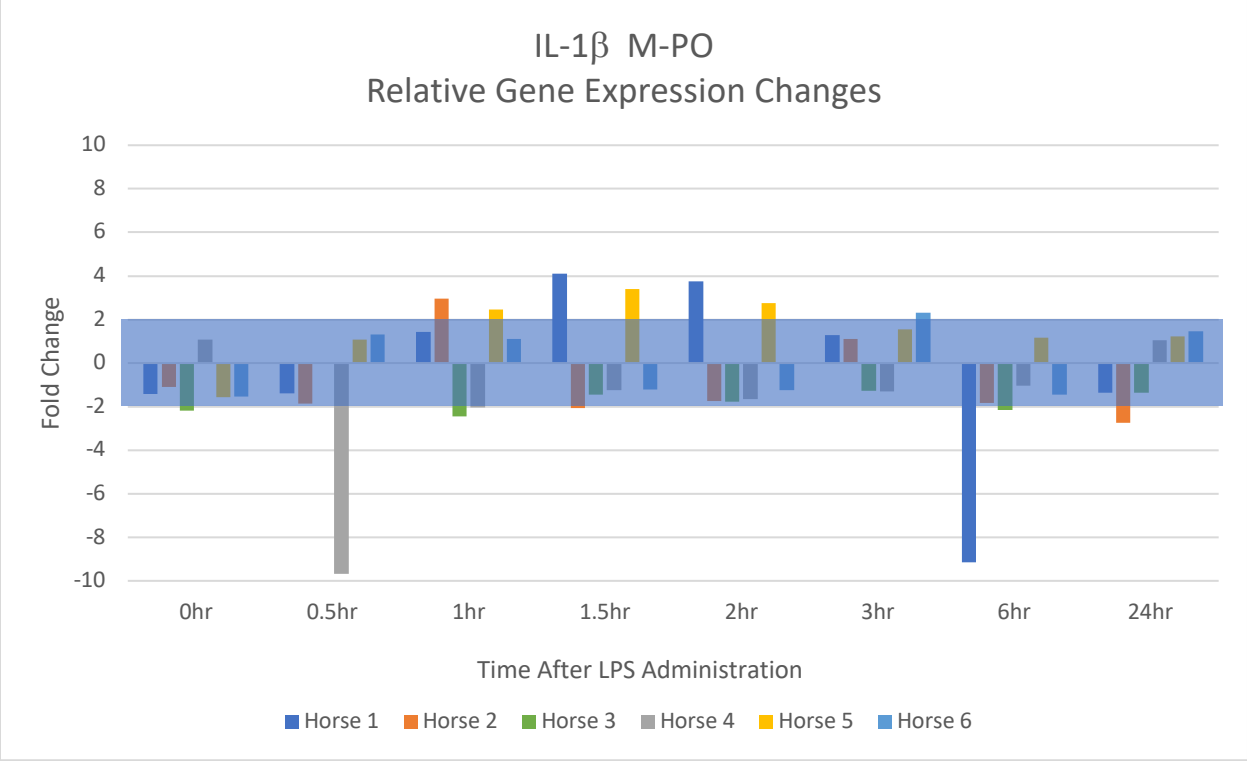


Fig 15 – Bar graph indicates changes in gene expression of IL-1β across time comparing horses receiving M-PO treatments compared to CON treatments. Time 0h indicates baseline prior to LPS infusion, and each time point thereafter indicating hours post-infusion completion. Each bar represents an individual horse as indicated by the figure legend. A blue shaded region indicates areas of non-significant gene expression changes (two-fold upregulation or downregulation).

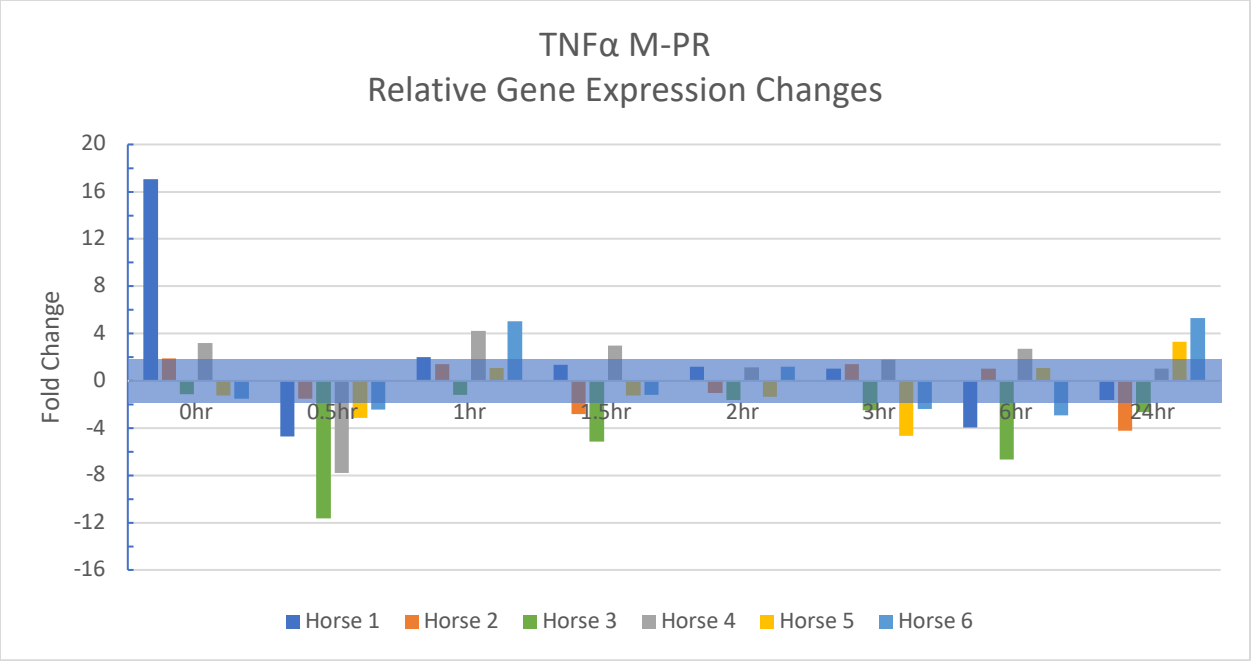


Fig 16 – Bar graph indicates changes in gene expression of TNFα across time comparing horses receiving M-PR treatments compared to CON treatments. Time 0h indicates baseline prior to LPS infusion, and each time point thereafter indicating hours post-infusion completion. Each bar represents an individual horse as indicated by the figure legend. A blue shaded region indicates areas of non-significant gene expression changes (two-fold upregulation or downregulation).

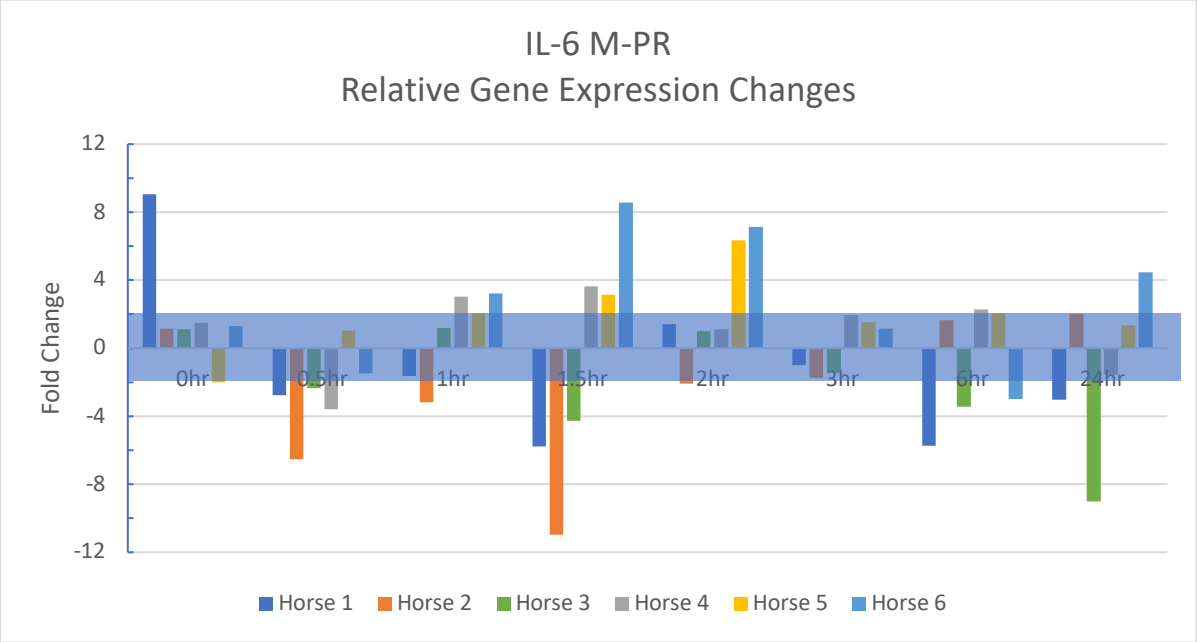


Fig 17 – Bar graph indicates changes in gene expression of IL-6 across time comparing horses receiving M-PR treatments compared to CON treatments. Time 0h indicates baseline prior to LPS infusion, and each time point thereafter indicating hours post-infusion completion. Each bar represents an individual horse as indicated by the figure legend. A blue shaded region indicates areas of non-significant gene expression changes (two-fold upregulation or downregulation).

Section 7: ELISA Multiplex cytokine production

For TNFα and IL-6, all horses had significantly different values from baseline ($p<0.001$) post-LPS infusion for all treatments, and these values returned to baseline by 24h post LPS-infusion completion. No differences were identified between treatment groups at any time point ($p=0.738$ for TNFα; $p=0.6898$ for IL-6). There were no significant differences noted between M-PO and M-PR treatments when values were normalized to CON ($p=0.3350$ for TNFα; $p=0.8058$ for IL-6). Changes in IL-1β were minimal in each horse across all time points and for all treatments and observed increases or decreases were not significant over time of the study ($p=0.3742$) or between treatment groups ($p=0.8209$). **Figures 18** and **19** show changes in TNFα cytokine production for M-PR and M-PO respectively. Likewise, **Figures 20** and **21** show changes in IL-6 cytokine production for M-PR and M-PO respectively. **Figures 22, 23,** and **24** show the changes in TNFα, IL-6, and IL-1β respectively, comparing all treatments across time post-LPS infusion, with (a) representing absolute magnitude in change measured for each of the cytokines for M-PR, M-PO, and CON, and (b) representing relative amount of increase or decrease in M-PO and M-PR values normalized against CON for TNFα, IL-6, and IL-1β respectively. Horse 4 was removed from data inclusion as no detectable cytokines were measured.

Median (range) values for peak TNFα cytokine production for M-PR, M-PO, and CON were 6,008 pg/mL (949-13,969 pg/mL), 3,951 pg/mL (1461-6,870 pg/mL), and 2,351 pg/mL (1,083-12,854 pg/mL) respectively. The highest magnitude of IL-6 cytokine production occurred at 3h post-LPS infusion completion (exception was Horse 4 for M-PO and CON, that was removed as previously mentioned). Median (range) values for peak IL-6 cytokine production for M-PR, M-PO, and CON were 122 pg/mL (87-335 pg/mL), 135 pg/mL (119-247 pg/mL), and 168 pg/mL (57-388 pg/mL) respectively.

When cytokine protein expression for M-PR and M-PO treatments were evaluated relative to values for the control group, the largest reduction of TNFα cytokine protein expression [median (range)] was 770 pg/mL (5-3,539 pg/mL) and 1,463 (187-9,269 pg/mL) for M-PR and M-PO, respectively. The time [median (range)] post LPS administration at which this peak decrease in TNFα cytokine protein expression occurred was 60 minutes (0-120 minutes) and 90 minutes (60-120 minutes) for the M-PR and M-PO treatments, respectively. For IL-6, the greatest reduction of IL-6 cytokine protein expression relative to control horses for the M-PR and M-PO treatment groups was 57 pg/mL (13-107 pg/mL) and 27 pg/mL (14-269 pg/mL), respectively, occurring at 120 minutes (90-180 minutes) for the M-PR group and 180 minutes (90-180 minutes) for the M-PO group. A relationship between c_{max}, AUC, or MRT and the observed increases or decreases in in cytokine protein expression relative to the control group was not apparent.

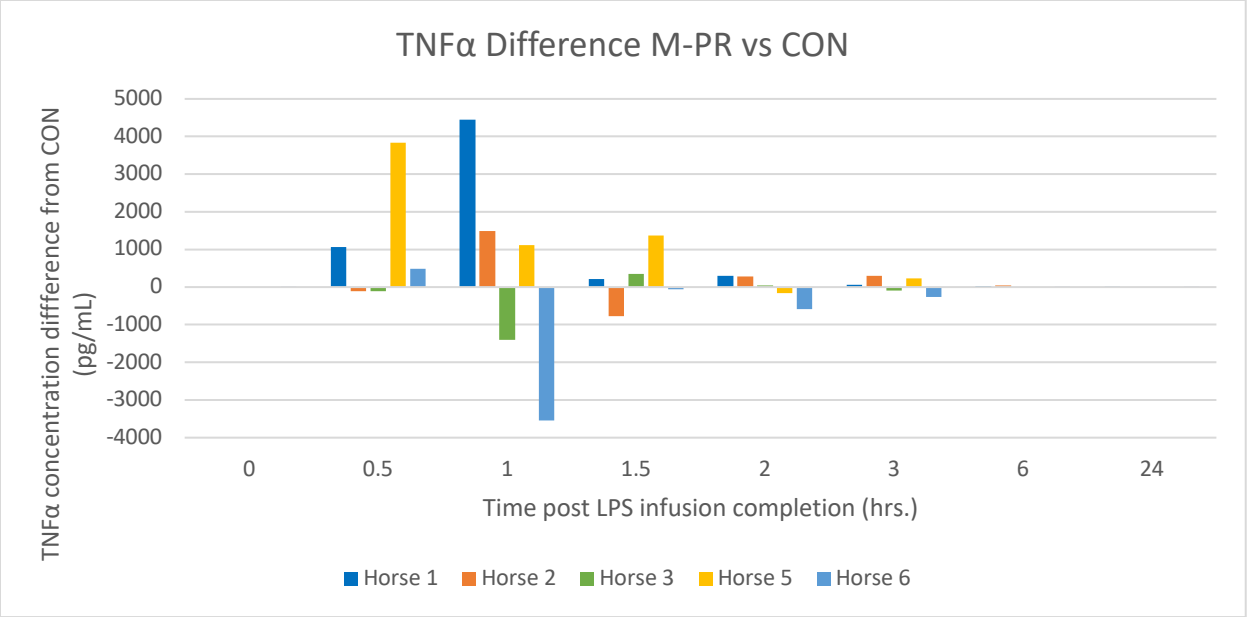


Fig 18 – Bar graph indicates changes in protein production of TNFα across time of horses receiving M-PR treatments as a difference to CON treatments. Time 0h indicates baseline prior to LPS infusion, and each time point thereafter indicating hours post-infusion completion. Each bar represents an individual horse as indicated by the figure legend.

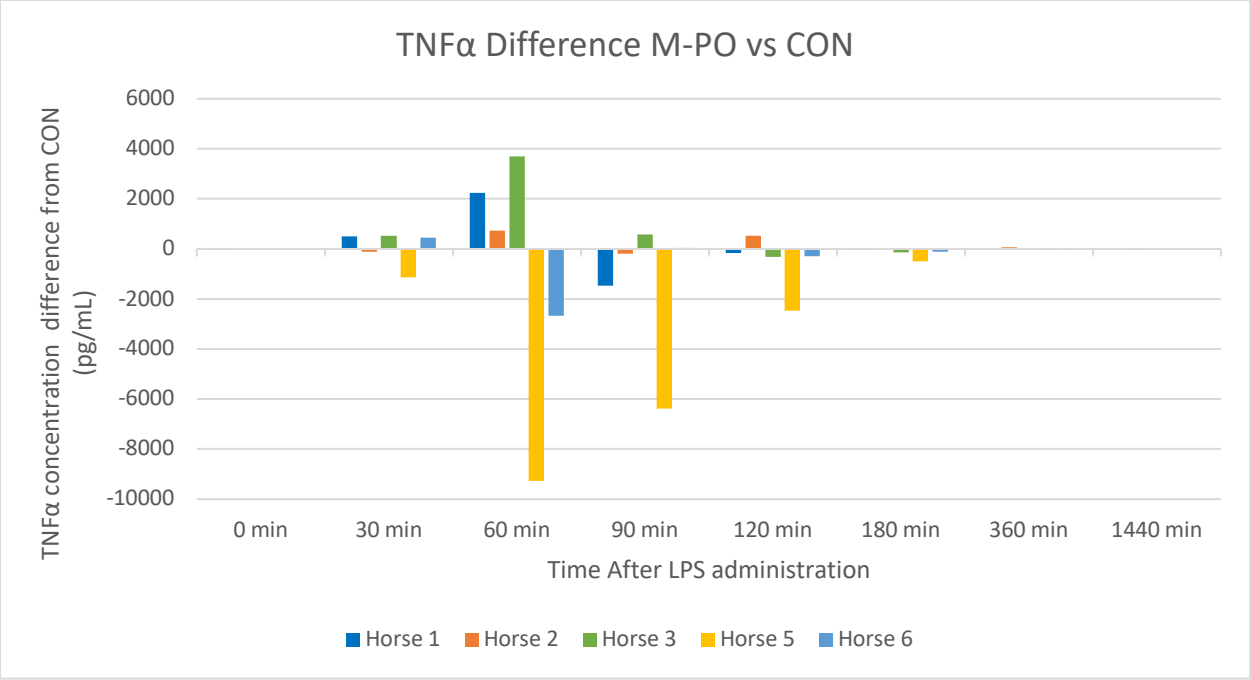


Fig 19 – Bar graph indicates changes in protein production of TNFα across time of horses receiving M-PO treatments as a difference to CON treatments. Time 0h indicates baseline prior to LPS infusion, and each time point thereafter indicating hours post-infusion completion. Each bar represents an individual horse as indicated by the figure legend.

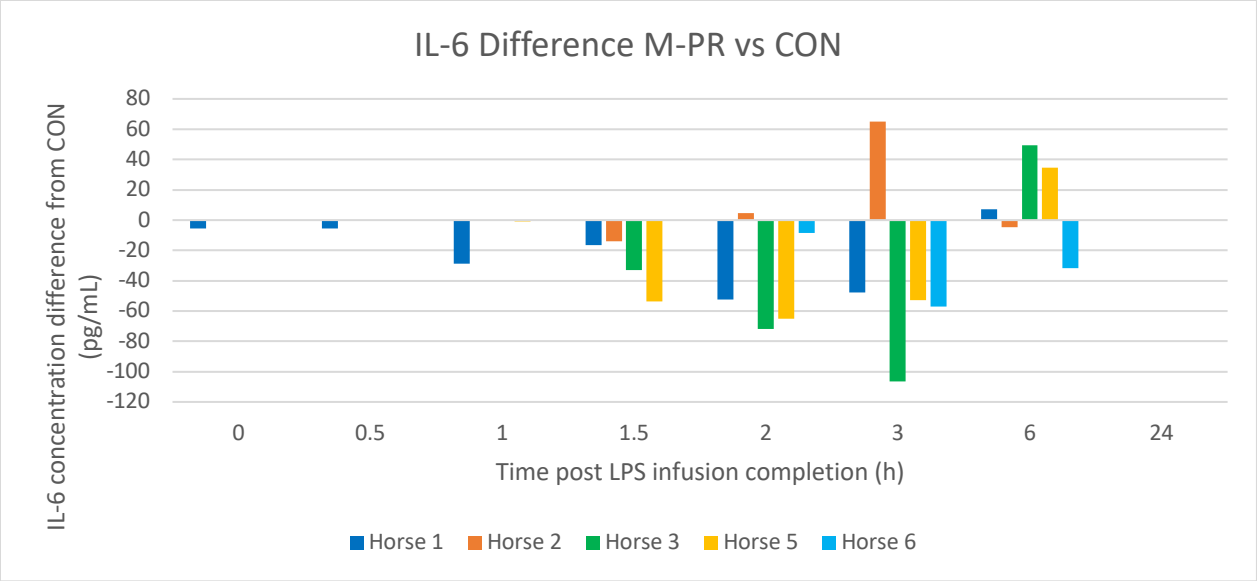


Fig 20 – Bar graph indicates changes in protein production of IL-6 across time of horses receiving M-PR treatments as a difference to CON treatments. Time 0h indicates baseline prior to LPS infusion, and each time point thereafter indicating hours post-infusion completion. Each bar represents an individual horse as indicated by the figure legend.

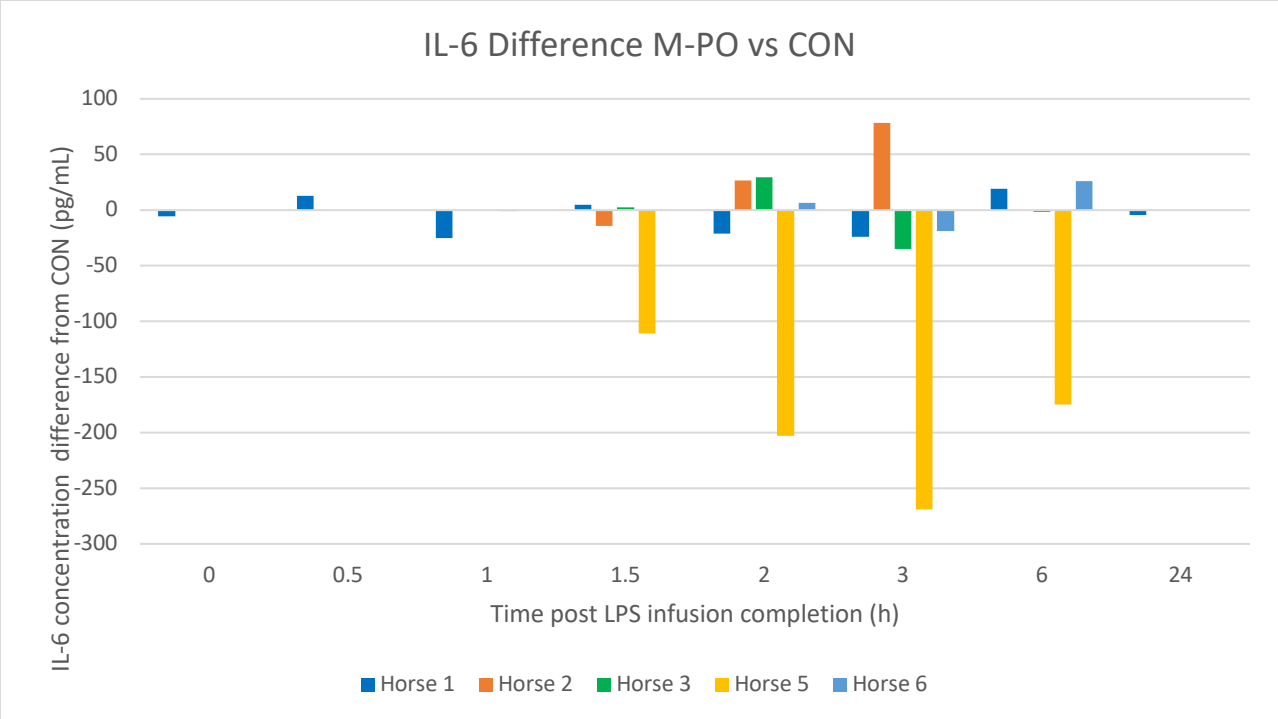


Fig 21 – Bar graph indicates changes in protein production of IL-6 across time of horses receiving M-PO treatments as a difference to CON treatments. Time 0h indicates baseline prior to LPS infusion, and each time point thereafter indicating hours post-infusion completion. Each bar represents an individual horse as indicated by the figure legend.

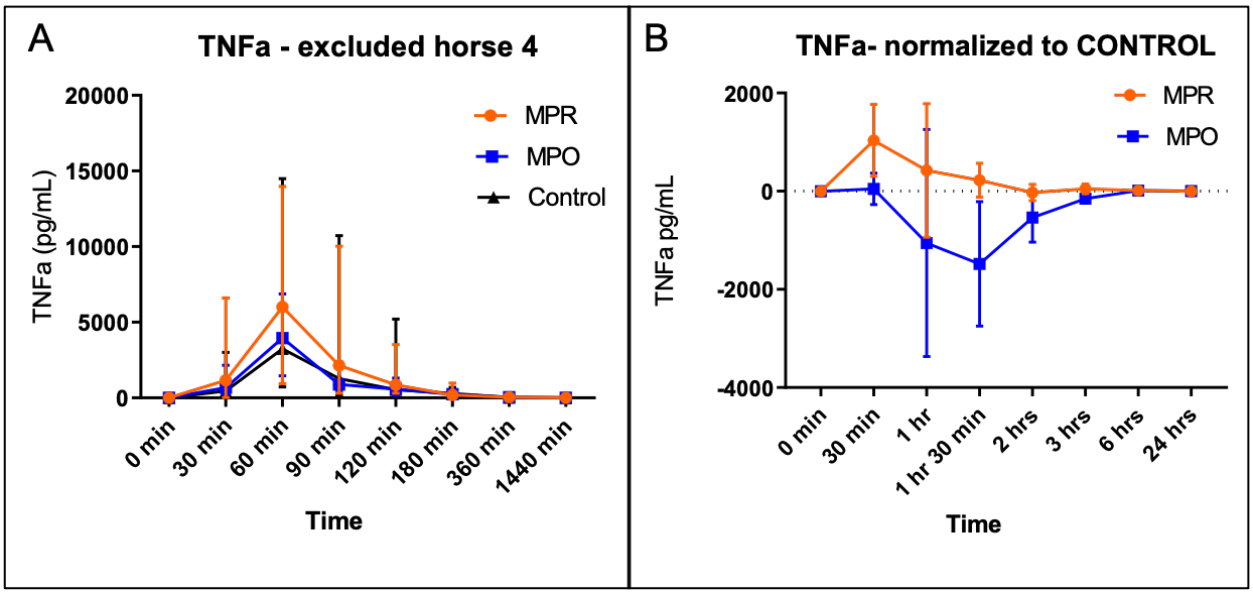


Figure 22 – (a) Changes in absolute TNF alpha cytokine production amongst all horses across time-post LPS infusion completion for M-PR, M-PO, and CON treatments. (b) Changes in TNF alpha cytokine production as a difference to CON treatments for M-PR and M-PO treatments. Horse 4 was excluded from compilation as it was found to be an outlier.

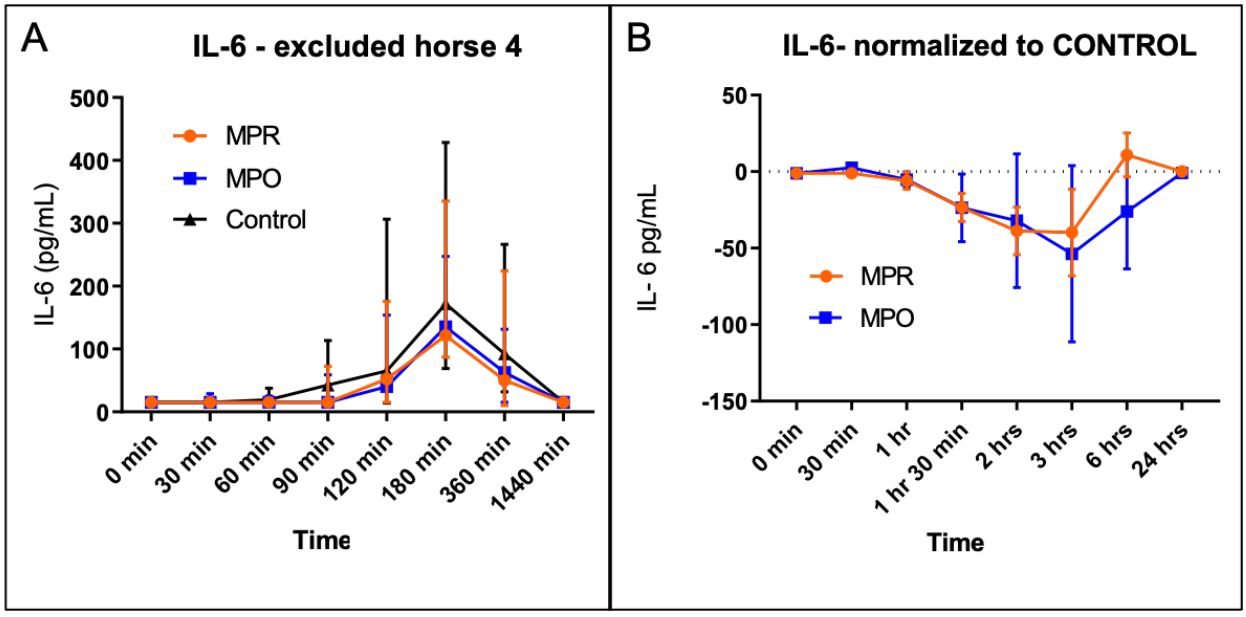


Figure 23 – (a) Changes in absolute IL-6 cytokine production amongst all horses across time-post LPS infusion completion for M-PR, M-PO, and CON treatments. (b) Changes in IL-6 cytokine production as a difference to CON treatments for M-PR and M-PO treatments. Horse 4 was excluded from compilation as it was found to be an outlier.

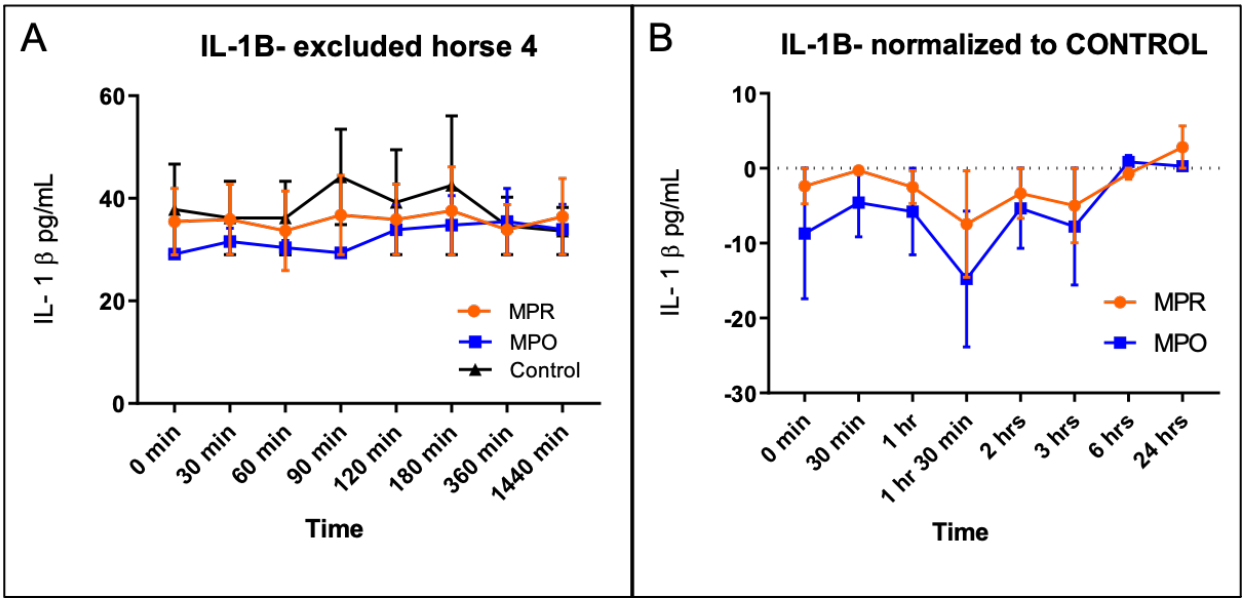


Figure 24 –(a) Changes in absolute IL-1β cytokine production amongst all horses across time-post LPS infusion completion for M-PR, M-PO, and CON treatments. (b) Changes in IL-1β cytokine production as a difference to CON treatments for M-PR and M-PO treatments. Horse 4 was excluded from compilation as it was found to be an outlier.

Chapter 4: Discussion

The goal of this study was to characterize the pharmacokinetics and anti-inflammatory effects of misoprostol in an *in vivo* study in healthy, adult horses challenged with low-dose intravenous endotoxin. The administered dosage of 30 ng/kg infused IV over 30 minutes described in various studies elicited the predicted response,[39,40] though large inter-horse variability within and between treatments was appreciated. Our study hypothesis that misoprostol administration PO or PR would result in mitigation of clinical signs related to SIRS (e.g., rectal temperature, heart rate, respiratory rate, leukogram changes) or reduced cytokine protein production, was not supported. Within individual horses, significant downregulation of TNFα and IL-6 gene expression compared to CON were appreciated beyond the lowest limit of quantification of misoprostol for both M-PO and M-PR treatments. The only difference noted from clinical observations in this study was that faster onset and longer duration of fasciculations occurred in both misoprostol treatment routes compared to CON. Similar to a previous study investigating misoprostol administration to adult horses, we identified differences in several pharmacokinetic parameters between misoprostol PR and PO.[104] Similar to the previous study, horses receiving misoprostol PO had prolonged t_{max}, $t_{½}$, and MRT, as well as higher AUC and Cl/F compared to misoprostol PR.[104] In contrast, c_{max} and AUC obtained were several-folds higher in magnitude, and Vd/F and c_{max} values were higher for M-PO than M-PR treatments, which differed from previously published literature in horses.[104,105]

Predictable changes in temperature, heart rate, respiratory rate, and leukocyte (total and individual) counts between pre- and post-endotoxin challenge were observed for all treatment groups in this study as expected based on previous literature.[46,82] However, there were no differences in these parameters after misoprostol treatments were compared to control treatment.

While lack of differences in systemic leukocyte trends do not support previously observed *in vitro* inhibition to neutrophil chemotaxis, it is possible that misoprostol may still inhibit neutrophil products (such as ROS and other microbicidal substances), which was not investigated in this study.[93] Administration of oral clenbuterol, a β_2-agonist which increases cAMP production, prior to endotoxin challenge did correspond to decreased peak temperature and peak TNFα compared to control horses. In contrast, pentoxifylline administered 30 minutes after LPS induction did not result in differences in any inflammatory or coagulation parameters compared to LPS induction alone, and this observation was attributed to timing as a potential confounding factor.[82] Similar to the study with clenbuterol, the timing of misoprostol administration relative to the end of LPS infusion was selected for this study after consideration of previously obtained c_{max} and t_{max} values and in relation to anticipated peak TNFα activity time.[42,46,68,82,104,123,128] The cytokine TNFα plays a central role in SIRS activation and a variety of inflammatory conditions, and has been used to evaluate anti-inflammatory efficacy based on its role in leukocyte margination, activation, and the associated clinical signs of SIRS previously discussed.[68,82] This timing was optimized for PO administration, but not for PR administration which reached t_{max} much sooner than the anticipated peak TNFα concentration.

The mechanism for the faster onset and longer duration of fasciculations noted in horses in M-PO and M-PR compared to CON is unknown. Fasciculations, presumably a manifestation of abdominal cramping, have been documented following endotoxin administration in horses.[46] Clinical endotoxemia and the experimental administration of LPS can result in variable signs of abdominal discomfort and may be a result of cytokine release (particularly TNFα) leading to decreased threshold for nociceptor activation. Colic and gastrointestinal cramping have also been reported as infrequent adverse effects of misoprostol administration in individual

horses.[89,104,105,111,112] The proposed mechanism of this is gas accumulation and subsequent intestinal distention due to prostaglandin's effects on gastrointestinal motility.[105,111] In humans, routes of administration of misoprostol associated with greater c_{max} and shorter t_{max} were more likely to elicit severe manifestation of adverse effects.[87,100,113] In this study, no such association between measured clinical and pharmacokinetic parameters were observed. Thus, while misoprostol did not appear to mitigate these recognized effects of LPS, it is unlikely that the effects were exacerbated with misoprostol administration.

Determining the effects of misoprostol on gene expression and protein production changes in this study were challenging. A wide variation in gene expression and protein production of inflammatory cytokines were present among horses in this study, and changes in gene expression did not correlate with subsequent protein production in individual horses. Cytokine measurements between individual horses also differed greatly between treatments. Variability in gene expression was also noted in previous *ex vivo* investigations of misoprostol in horses.[105] These unique manifestations in response to misoprostol may be caused by the wide variability in drug absorption for both routes, and variation in individual horse responses to experimental LPS and drug administration (pharmacogenomics).[129]

It was particularly interesting to note the pronounced and prolonged effects in gene expression and cytokine production noted in individual horses during this study particularly for M-PR treatments after only a single dose. Such trends were appreciated beyond times at which MFA concentration was below the lowest limit of quantitation for individual horses. One explanation for this observation is that drug accumulation within the leukocytes lead to lack of free measurable drug, and its accumulation within the leukocytes lead to the observed gene transcription differences. Previous studies utilizing cAMP inhibitors such as pentoxifylline and

clenbuterol did not investigate effects of gene expression and similar prolonged investigations of pharmacologic intervention of inflammatory cytokine gene expression are scant in equine medicine.

Cytokine gene expression profiles in horses have been performed comparing ethyl pyruvate's effects on TNFα and IL-6 gene expression under low-dose endotoxin challenge *in vivo*. This only demonstrated gene expression changes up to 3 hours and returned to baseline values by 6 hours following LPS infusion, and at most observed a 3-fold reduction by ethyl pyruvate treatment compared to pre-endotoxin administration values.[128] In a recent investigation of IV meloxicam administration in donkeys, TNFα mRNA expression returned to baseline by the end of the study (3h post LPS infusion), while IL-6 activity remained higher than baseline at this same time for both control and treatment group.[130] Another study demonstrated that IL-8 expression remained upregulated by 2-fold compared to baseline expression up to 48 hours following colic surgery.[131] This study compared gene expression changes between sham (CON) and misoprostol administration (M-PR/M-PO) at individual time points, where up to 7-fold gene expression reductions were observed in comparison to CON. These findings suggest that gene expression measurements should be obtained for longer periods following endotoxin infusion.

It is also possible that a more prolonged drug exposure in horses (e.g. a multi-dose regimen) is required for anti-inflammatory effects to be observed. Misoprostol's ability to cause LPS-induced cytokine modulation was observed following a 62-day course of misoprostol in humans.[114] It would be interesting to note whether differences in gene expression and cytokine production exist and if they would correlate if misoprostol were administered over longer study periods with prolonged exposure. This warrants further investigation in a multi-dose pharmacokinetics study under an endotoxin challenge model.

Under endotoxin-challenged conditions, horses receiving misoprostol PO had longer values of t_{max}, $t_{½}$, and MRT, and higher values of c_{max} and AUC compared to PR. Misoprostol PR had greater apparent volume of distribution and clearance compared to PO. Orally administered medications must transit through the esophagus and stomach before finally reaching the small intestines, the optimal site of absorption. Once absorbed, the drug is processed through the liver (first pass metabolism) before entering systemic circulation. In contrast, drugs administered rectally can bypass first pass metabolism.[115] These pattern in t_{max}, AUC, $t_{½}$, MRT, and Cl/F seen in the current study are similar in trends to the previous study on healthy horses that were not influenced by endotoxin.[104]

Some differences were noted in this study compared to previous findings in healthy horses not challenged by endotoxin. Compared to the previous study, c_{max} was greater after M-PO treatment and Vd/F was higher after M-PR treatment.[104] Additionally the overall magnitude in AUC and c_{max} are profoundly greater in this study compared to those by previous investigators.[104,105] The differences in c_{max} may be a sampling issue. The true c_{max} in M-PR may be greater than M-PO, but may not be captured as it may be earlier than the 3-minute post-administration time assigned for the first sample point collected for M-PR. It is also possible that the pronounced comparative differences between M-PO and M-PR and the overall magnitude differences anticipated from previous literature reflects the impact of inflammation on drug behavior. Under non-inflammatory conditions, drugs administered orally undergo first pass metabolism. Inflammation can alter hepatic function through downregulation of cytochrome P_{450} enzymes responsible for drug metabolism, thus allowing for less drug degradation.[77] Systemic inflammation may also increase mucosal permeability, thus facilitating more drug absorption through PO routes, or inhibit absorption from PR routes through changes in motility (diarrhea or

ileus) and secretion (ion trapping).[132] These events can lead to pronounced or diminished absorptions of drugs. Inflammation can also affect first pass metabolism, systemic perfusion, and receptor availability which may also affect response to treatment or potential to adverse response.[77,78] While this study obtained a much higher c_{max} and AUC after PO administration than those from previous literature, it is unknown whether modification of misoprostol dosage would be warranted for future investigations of misoprostol for SIRS therapy.[104,105]

The different magnitudes in c_{max}, AUC, $t_{½}$, and MRT between this study with horses challenged with endotoxin and the previous studies without endotoxin challenges are speculative. However, differences in pharmacokinetic profiles for drugs between inflammatory and non-inflammatory states have been demonstrated in other species.[74,75,78,133] Although unlikely, it is possible that these are purely idiosyncratic or geographic differences between the two populations of horses recruited in the different studies.[129] Comparison of these parameters within the same population of horses is needed to demonstrate that endotoxin-induced inflammation in this study did in fact alter pharmacokinetic parameters of misoprostol. This finding would strengthen previous published studies demonstrating drug metabolism and effects are different between physiologically stable and unstable conditions (e.g., systemic inflammation). These effects may influence treatment efficacy or risk of adverse effects.[77,78] The large inter-horse variations in this study highlights the importance of each horse serving as its own control and emphasizes the need of determining the pharmacokinetic profiles of our research horses when receiving M-PO or M-PR without endotoxin challenge.

Although no statistical correlation in gene expression, protein production, and pharmacokinetic parameters could be determined, individual horses appeared to show some patterns. Potential confounding factors include inadequate sampling times and intervals, which

may have failed to capture the appropriate relationship to show drug influence on gene expression and protein production. Additionally, as gene expression changes were observed 24 hours after LPS infusion completion and well after the lowest measurable drug concentration, it is possible that prolonged systemic misoprostol circulation may lead to more statistically appreciable trends.

This study has several limitations. First, although the low-dose endotoxin challenge model provides a predictable and measurable method for studying interventional therapy in a safe and ethical manner, it is not an accurate reflection of what occurs clinically. A single, low-dose administration of endotoxin is not synonymous to conditions such as pleuropneumonia, colitis, metritis, and neonatal sepsis. Initiation of SIRS can occur not only from endotoxin recognition by TLR-4, but also other by other PAMP and TLR interaction in a mixed infection. One could argue that endotoxin administration as a continuous rate infusion may better reflect clinical disease conditions as in a clinical setting, endotoxin (and other PAMPs) is continuously released until the source of bacteria are addressed; however, this approach may increase the likelihood of complications such as laminitis, coagulopathies, hypoperfusion, ileus, and multi-organ dysfunction. Therefore, to fully elucidate the clinical value of misoprostol as a therapy for mitigating SIRS a controlled clinical trial will be required.

Second, the single dose administration of misoprostol performed in this study does not reflect the application of this drug in a clinical setting. This may explain the lack of significant differences in physical examination parameters (both blinded and unblinded), along with cytokine gene expression and protein production. The dosage and frequency of misoprostol administration has yet to be standardized for equine medicine. The dose of 5 μg/kg is extrapolated from human studies[102,114] and has been commonly accepted to be effective for treating certain gastrointestinal conditions when given two to three times per day.[90,112] Drug plasma concentrations in horses are

similar to or exceed those reported in humans, and it is unlikely that a higher dose would be advantageous. However, ideal dose intervals and duration of administration are unknown in horses and may vary according to the specific clinical problem. In this study, a single dose of misoprostol did not produce consistent nor statistically significant differences in measured variables within the study period. This may be a result of timing of misoprostol and LPS administration. The timings were selected based on determining peak gene expression and production of cytokines in relation to previously studied peak systemic misoprostol concentration but may not have been optimal. Therefore, a future multi-dose study would be beneficial in both determining the true anti-inflammatory effects of misoprostol for horses, and optimizing misoprostol's therapeutic drug dosage and interval, both for PO and PR administration.

Third, the accuracy of administration of per rectum dosage is unknown. Optimization of misoprostol absorption PR was attempted based on rectal evacuation as well as consistent drug deposition. While the presence of feces in the rectum has been cited to reduce drug absorption in many studies, very few currently investigate or demonstrate differences in PK parameters in the presence of feces.[119,121] Given that food was previously demonstrated to affect pharmacokinetics of misoprostol, rectal evacuation was performed in this study. However, confirmation is worthwhile given no difference in pharmacokinetic profiles were observed for metronidazole between per rectum administration with or without feces.[104,120,121] Additionally, while the similarity in horse sizes of this study allowed for likely a consistent location of drug deposition, to what degree the misoprostol actually bypassed portal circulation by this route was unknown. If drug is administered far orad in the rectum, this would in fact cause more drug to reach portal circulation, thus undergoing first pass metabolism. Optimizing location of deposition for horses of varying breeds may provide useful insight on drug efficacy and absorption.

Fourth, this study is limited in horse number and the signalment of the study group enrolled, which may have contributed to the lack of statistical significance and correlation between pharmacokinetic, gene expression, and cytokine production parameters. Given the wide individual variations in these variables, statistical significance may not have been appreciable despite *a priori* power analysis.

Current strategies to mitigate SIRS are limited in scope, and multi-modal therapeutic approaches are highly desirable to minimize both the effects of SIRS and any adverse effects of the existing therapeutics. While there may be situations that may preclude its use such as reproductive status (pregnant or breeding mares) or cost, misoprostol's actions as a cAMP mediator shows promise. Misoprostol may work synergistically with existing therapeutics such as polymyxin B and flunixin meglumine in the context of endotoxin-induced SIRS: polymyxin B binding to endotoxin could minimize further SIRS initiation, while flunixin meglumine could improve perfusion by COX inhibition and misoprostol could act by cAMP to mediate neutrophil action (ROS generation, chemotaxis) and cytokine release.

In conclusion, this project successfully demonstrated measurable differences in cytokine gene transcription after misoprostol administered PO and PR (compared to the control condition) in individual healthy horses challenged with a low-dose intravenous endotoxin *in vivo* mode. We were unable to demonstrate statistically significant differences in cytokine protein production, leukogram changes, or gross observations between treated and control conditions. Pharmacokinetic parameters obtained in this project were different from previous studies performed in resting healthy horses from a different population, which has potential implications including the possible increased risk for drug-related adverse effects, differences in anticipated clearance of drug residues (both in high performing athletic horses undergoing drug screening, or

meat residues in food animal species), and drug efficacy (potentially increasing or decreasing drug efficacy). Future studies should characterize the pharmacokinetic behavior of misoprostol administered PO and PR in the current population in a non-challenged state to appropriately compare differences and develop appropriate drug doses and administration intervals for a multi-dose study in both endotoxin challenged and unchallenged scenarios. A lower and more frequent dose and interval than the published 5 μg/kg administered every 12 hours, as is currently used for treatment of equine glandular gastric disease,[90] may be appropriate for SIRS patients. Ultimately, controlled clinical trials (misoprostol by itself as well as with other therapeutics) are warranted to determine the cost-benefit and therapeutic efficacy of misoprostol in mitigating SIRS and improving clinical outcome in associated equine diseases.

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Appendix 1: Plate layout for ELISA Multiplex

Technique and volumes are as outlined in Materials and Methods section for ELISA Multiplex Assay in 2.4b of this thesis. For all plate layouts below: QC denotes quality control, AB denotes assay buffer, SM denotes serum matrix. For samples: first number denotes the horse number, the second alphabet (A, B, or C) denotes treatment where where A is M-PR, B is M-PO, and C is CON; third numbers denote time point in hours following LPS infusion. Therefore 1A1.0 would mean Horse 1, Treatment M-PR, 1 hour post LPS infusion completion.

PLATE 1 Serum volume: 25uL AB: Assay Buffer SM: Serum Matrix

	1	2	3	4	5	6	7	8	9	10	11	12
A	**AB: 25**	Std2: 25	Std5: 25	QC1: 25	1A1.0: 25	1A3: 25	1B0.5: 25	1B2: 25	1C0: 25	1C1.5: 25	1C6: 25	2A1.0: 25
	SM: 25	SM: 25	SM: 25	SM: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25
B	**AB: 25**	Std3: 25	Std5: 25	QC1: 25	1A1.0: 25	1A3: 25	1B0.5: 25	1B2: 25	1C0: 25	1C1.5: 25	1C6: 25	2A1.0: 25
	SM: 25	SM: 25	SM: 25	SM: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25
C	**AB: 25**	Std3: 25	Std6: 25	QC2: 25	1A1.0(x5): 25	1A6: 25	1B1.0: 25	1B3: 25	1C0.5: 25	1C1.5(x5): 25	1C24: 25	2A1.0(x5): 25
	SM: 25	SM: 25	SM: 25	SM: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25
D	Std1: 25	Std3: 25	Std6: 25	QC2: 25	1A1.0(x5): 25	1A6: 25	1B1.0: 25	1B3: 25	1C0.5: 25	1C1.5(x5): 25	1C24: 25	2A1.0(x5): 25
	SM: 25	SM: 25	SM: 25	SM: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25
E	Std1: 25	Std4: 25	Std6: 25	1A0: 25	1A1.5: 25	1A24: 25	1B1.0(x5): 25	1B6: 25	1C1.0:25	1C2: 25	2A0: 25	2A1.5: 25
	SM: 25	SM: 25	SM: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25
F	Std1: 25	Std4: 25		1A0: 25	1A1.5: 25	1A24: 25	1B1.0(x5): 25	1B6:2 5	1C1.0: 25	1C2: 25	2A0: 25	2A1.5: 25
	SM: 25	SM: 25		AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25
	Beads: 25	Beads: 25		Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25
G	Std2: 25	Std4: 25		1A0.5: 25	1A2: 25	1B0: 25	1B1.5: 25	1B24: 25	1C1.0(x5): 25	1C3: 25	2A0.5: 25	2A2: 25
	SM: 25	SM: 25		AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25
	Beads: 25	Beads: 25		Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25
H	Std2: 25	Std5: 25		1A0.5: 25	1A2: 25	1B0: 25	1B1.5: 25	1B24: 25	1C1.0(x5): 25	1C3: 25	2A0.5: 25	2A2: 25
	SM: 25	SM: 25		AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25
	Beads: 25	Beads: 25		Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25

PLATE 2		Serum volume: 25uL		AB: Assay Buffer		SM: Serum Matrix					
	1	2	3	4	5	6	7	8	9	10	11	12
A	QC1: 25	2A24	2B1.5	2B24	2C1.5	2C24	3A1.5	3A24	3B1.0(x5)	3B6	3C1.0	3C2
	SM: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25
B	QC1: 25	2A24	2B1.5	2B24	2C1.5	2C24	3A1.5	3A24	3B1.0(x5)	3B6	3C1.0	3C2
	SM: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25
C	QC2: 25	2B0	2B2	2C0	2C2	3A0	3A2	3B0	3B1.5	3B24	3C1.0(x5)	3C3
	SM: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25
D	QC2: 25	2B0	2B2	2C0	2C2	3A0	3A2	3B0	3B1.5	3B24	3C1.0(x5)	3C3
	SM: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25
E	2A3	2B0.5	2B3	2C0.5	2C3	3A0.5	3A3	3B0.5	3B2	3C0	3C1.5	3C6
	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25
F	2A3	2B0.5	2B3	2C0.5	2C3	3A0.5	3A3	3B0.5	3B2	3C0	3C1.5	3C6
	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25
G	2A6	2B1.0	2B6	2C1.0	2C6	3A1.0	3A6	3B1.0	3B3	3C0.5	3C1.5(x5)	3C24
	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25
H	2A6	2B1.0	2B6	2C1.0	2C6	3A1.0	3A6	3B1.0	3B3	3C0.5	3C1.5(x5)	3C24
	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25

PLATE 3		Serum volume: 25uL		AB: Assay Buffer		SM: Serum Matrix					
	1	2	3	4	5	6	7	8	9	10	11	12
A	QC1: 25	4A1.0	4A3	4B0.5	4B2	4C0	4C2	5A0	5A1.0(x5)	5A3	5B0.5	5B3
	SM: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25
B	QC1: 25	4A1.0	4A3	4B0.5	4B2	4C0	4C2	5A0	5A1.0(x5)	5A3	5B0.5	5B3
	SM: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25
C	QC2: 25	4A1.0(x5)	4A6	4B1.0	4B3	4C0.5	4C3	5A0.5	5A1.5	5A6	5B1.0	5B6
	SM: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25
D	QC2: 25	4A1.0(x5)	4A6	4B1.0	4B3	4C0.5	4C3	5A0.5	5A1.5	5A6	5B1.0	5B6
	SM: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25
E	4A0	4A1.5	4A24	4B1.0(x5)	4B6	4C1.0	4C6	5A0.5(x5)	5A1.5(x5)	5A24	5B1.5	5B24
	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25
F	4A0	4A1.5	4A24	4B1.0(x5)	4B6	4C1.0	4C6	5A0.5(x5)	5A1.5(x5)	5A24	5B1.5	5B24
	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25
G	4A0.5	4A2	4B0	4B1.5	4B24	4C1.5	4C24	5A1.0	5A2	5B0	5B2	
	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	
H	4A0.5	4A2	4B0	4B1.5	4B24	4C1.5	4C24	5A1.0	5A2	5B0	5B2	
	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	

PLATE 4		Serum volume: 25uL		AB: Assay Buffer		SM: Serum Matrix					
	1	2	3	4	5	6	7	8	9	10	11	12
A	QC1: 25	5C1.0	5C2	6A0	6A1.5	6A24	6B1.0(x5)	6B6	6C1.0	6C3		
	SM: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25		
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25		
B	QC1: 25	5C1.0	5C2	6A0	6A1.5	6A24	6B1.0(x5)	6B6	6C1.0	6C3		
	SM: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25		
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25		
C	QC2: 25	5C1.0(x5)	5C3	6A0.5	6A2	6B0	6B1.5	6B24	6C1.0(x5)	6C6		
	SM: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25		
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25		
D	QC2: 25	5C1.0(x5)	5C3	6A0.5	6A2	6B0	6B1.5	6B24	6C1.0(x5)	6C6		
	SM: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25		
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25		
E	5C0	5C1.5	5C6	6A1.0	6A3	6B0.5	6B2	6C0	6C1.5	6C24		
	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25		
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25		
F	5C0	5C1.5	5C6	6A1.0	6A3	6B0.5	6B2	6C0	6C1.5	6C24		
	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25		
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25		
G	5C0.5	5C1.5(x5)	5C24	6A1.0(x5)	6A6	6B1.0	6B3	6C0.5	6C2			
	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25			
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25			
H	5C0.5	5C1.5(x5)	5C24	6A1.0(x5)	6A6	6B1.0	6B3	6C0.5	6C2			
	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25	AB: 25			
	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25	Beads: 25			