PRELIMINARY SAFETY AND BIOLOGICAL EFFICACY STUDIES OF ETHYL PYRUVATE IN NORMAL ADULT HORSES

By

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ABSTRACT

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The liberation of endotoxin from gram negative bacteria plays a critical role in stimulating mononuclear cells. This leads to the cleavage of the inhibitory protein IκB from the cytosolic protein NFκB resulting in the translocation of NFκB into the nucleus of the cell culminating in up regulation of both transcription and translation of protein. The protein from the nucleus is transformed by the endoplasmic reticulum to form both pro-inflammatory and anti-inflammatory cytokines and eicosanoids (leukotrienes and prostaglandins). Mortality from sepsis in people approaches 40%. Endotoxemia causes significant morbidity in horses, including laminitis, ileus and coagulopathy. Despite current medications and fluid therapy, horses and people succumb to sepsis and endotoxemia. The search for new therapies to treat sepsis led to investigations using pyruvate derivatives. Ethyl pyruvate mitigated the cardiovascular effects of sepsis in multiple preclinical models including rodents, sheep, and swine. Ethyl pyruvate may be a novel treatment for endotoxin induced sepsis in the horse. The goal of this thesis is to provide a review of the current literature relevant to the use of ethyl pyruvate in preclinical models of sepsis and gastrointestinal ischemia and to present the data supporting ethyl pyruvate as both a safe and biologically active drug in normal adult horses.
This work is dedicated to:

Page Yaxley:
Thank you for always believing in me, for your continuous love and support as I pursue my dream, and to love life to the fullest.

My Parents:
Thank you for always believing in me, for your constant support and the inspiration to pursue my dream.

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LIST OF ABBREVIATIONS

BPM: Beats/Breaths per Minute

CBC: Complete Blood Count

CD 14: Cluster of Differentiation protein 14

CO₂: Carbon Dioxide

ECG: Electrocardiogram

FD₄: Fluorescein Dye 4

FD 70: Fluorescein Dye 70

HMGB1: High Mobility Group Box 1

H₂O: Water

H₂O₂: Peroxide

IL-1β: Interleukin 1 Beta

IL6: Interleukin 6

IL10: Interleukin 10

iNOS: Inducible Nitric Oxide Synthase

LPS: Toxic component of lipopolysaccharide

LPB: Lipoprotein Binding Protein
LRS: Lactated Ringer’s Solution

NFκB: Nuclear Factor Kappa B

NH₄⁺: Ammonium Ion

MAPK: Mitogen-activated protein kinase

O₂⁻: superoxide

OH⁻: Hydroxyl radical

ONOO⁻: Peroxynitrite

PBMC: Canine Peripheral blood mononuclear cell

p38: p38 mitogen-activated protein kinase

RelA: Rel homology domain

REPS: Ringer’s Ethyl Pyruvate Solution

TCA Cycle: Tricarboxylic Acid Cycle

TLR4: Toll Like Receptor 4

TNFα: Tumor necrosis factor alpha

VBG: Venous Blood Gas
CHAPTER 1.

A Review of the Literature

The Problem – Sepsis:

Sepsis is defined as infection with systemic inflammatory manifestations including fever, leukocytosis or leucopenia, tachycardia and tachypnea. Sepsis can be secondary to gastrointestinal ischemia, pancreatitis, pneumonia, bacteremia or any localized infection. Severe sepsis occurs when organs fail as sequel to sepsis. Septic shock is an event categorized as severe sepsis plus evidence of systemic hypotension unresponsive to fluid resuscitation. Septic shock occurs with circulatory failure due to vasodilation, capillary leak, and reduced myocardial contraction. The end result of the cardiovascular failure is decreased oxygen delivery to the tissues, increased oxygen extraction from the circulating blood and ultimately an increase in anaerobic metabolism and lactate production due to tissue dysoxia. Ultimately, organs fail and these patient dies. The incidence of fatality from sepsis is approximately 25% and this estimate rises with age and concurrent organ failure in severe sepsis. All forms of sepsis are significant healthcare dilemmas and cost millions of dollars in treatment each year world-wide. Providing appropriate medical and surgical therapy and implementation of early goal directed therapy has improved survival to discharge and decreased the occurrence of comorbidities. Improved survival in veterinary patients is due to use of modalities incorporated from human medicine, including early goal directed therapy and aggressive fluid resuscitation.
Sepsis causes a series of pathogenic events leading to the activation of mononuclear cells, cardiovascular instability, endothelial cell disruption, activation of platelets, complement and coagulation cascade activation. Sepsis leads to damaged endothelium and the release of both pro and anti-inflammatory cytokines. Fluid shifts from the vascular space into the interstitial space, resulting in edema, further compromising oxygen delivery. Vasodilation leads to inappropriate pooling of blood and plasma volume within the splanic circulation. Such fluid shifts require aggressive fluid resuscitation with crystalloid as well as colloid fluids. Physiologic parameters used to assess attainment of fluid resuscitation goals include central venous pressure or right atrial pressure, cardiac output, ventricular ejection fraction, oxygen extraction ratio, and systemic lactate production. Cardiac output is measured by thermal dilution, lithium dilution, by use of pulse contour analysis, or echocardiographic assessment. Clinicopathologic values that are commonly assessed include venous or arterial lactate, venous oxygen extraction, arterial and central venous oxygen saturation, and venous partial pressure of carbon dioxide. As global systemic changes due to sepsis and inflammatory mediator release progress, tissue hypoxia persists, myocardial depression is exacerbated, cardiac output precipitously declines, and further loss of vasoregulatory control occurs. Without emergency treatment, the patient may development hypodynamic distributive shock. Catecholamine therapies are used to improve blood pressure and venous return to the heart once appropriate vascular volume is replaced. These drugs include alpha and beta adrenergic agonist/antagonist drugs and vasopressors. These drugs increase cardiac output by increasing vasomotor tone thereby, increasing oxygen delivery to the tissue beds of vital organs.
Early instillation of other multimodal therapy for sepsis treatment in both human patients and veterinary patients include broad spectrum antimicrobial therapy, glycemic control, parenteral nutrition, nonsteroidal anti-inflammatory therapy (nonselective and selective COX drugs), oxygen therapy or ventilator therapy support, surgical intervention, reactive oxygen species scavengers, physiologic corticosteroid therapy, and immunological therapy. Sepsis results in severe uncontrolled inflammation and treatment is aimed at mitigating this inflammation response. Hyperglycemia induces release of pro-inflammatory cytokines and exacerbates the systemic inflammatory response that occurs in sepsis. Tight glycemic control and minimizing the number of peaks and troughs of blood glucose is associated with improved survival in septic patients. Early administration of antimicrobials (within 4 hours of emergency room admission) alone has significantly reduced the length of hospital stay and decreased the risk of short term mortality. There is a 7.6% increase in mortality for every hour after the initial four hours without institution of antimicrobial agents.

The pathological sequel of sepsis are largely due to up regulation of the inflammatory pathway mediated by pro-inflammatory cytokines including TNFα, IL-1β, IL-6 and High Mobility Group Box 1 protein (HMGB-1) that are released from macrophages, neutrophils, and other cells of the innate immune system. The clinical signs of sepsis occur when cell fragments, including lipopolysaccharide (LPS), flagella, and membrane components forming exotoxins, initiates the signaling cascade in monocytic cells to release pro-inflammatory mediators. Endotoxin does this by binding to lipid binding proteins (LBP) found in blood. The LPS/LBP complex interacts with pattern recognition receptors, CD14 and TLR4, on the
surface of cells triggering 2 key pro-inflammatory signaling pathways, nuclear factor kappa B (NFκB) and p38 mitogen-activated protein kinase (MAPK). Specifically, LPS/LBP binding to TLR4 causes phosphorylation and subsequent degradation of the intracellular inhibitory protein IκB, liberating NFκB. Nuclear factor κB enters the nucleus, promoting transcription and translation of acute phase inflammatory cytokines including TNFα, IL-1β, IL-6 and the late phase mediator, HMBG-1 protein. The acute phase cytokines stimulate macrophage and endothelial cells to release pro-inflammatory substances, including those involved in the generation of leukotrienes and lipoxins.

The cytosolic mediator HMBG-1 protein is implicated as a late mediator of systemic inflammation. HMGB-1 is an essential cytosolic nuclear DNA binding protein that acts as a transcriptional cofactor. This cytosolic protein is markedly up regulated during a pro-inflammatory event such as sepsis or septic shock. It is a potent pro-inflammatory mediator that causes nuclear transcription, translation, and production of other potent inflammatory cytokines, prostanoids, eicosanoids, and interleukins fostering the pro-inflammatory state. HMGB-1 protein is released from macrophages or monocytes at 12-18 hours following cellular stimulation. Injection of recombinant HMGB-1 into in mice induces clinical manifestations of sepsis including multiple organ dysregulation, multiple organ failure and death. Conversely, antagonism of the HMGB-1 cytosolic protein in a rodent model of sepsis reduced direct organ damage and improved survival even when administered after a septic insult. Anti HMGB-1 protein antibodies provide significant protection against delayed endotoxin induced lethality even when administered after the acute phase cytokine responses have peaked and
Blood concentrations of HMGB-1 protein are elevated in human patients with severe sepsis. HMGB-1 concentrations are higher in non-survivors compared to survivors.  

People and animals die from sepsis despite advancements in resuscitative therapies and anti-inflammatory therapies. This fact drives the need for improved sepsis treatment. Investigations of the effectiveness of ethyl pyruvate, an alpha keto carboxylic acid derivative of pyruvic acid, as a novel anti-inflammatory therapy, continue. Derivatives of pyruvic acid including pyruvate, sodium pyruvate, methyl pyruvate, and ethyl pyruvate have all been examined in both in vitro and in vivo studies using numerous animal species in a multitude of disease models including acute endotoxemia, polymicrobial bacterial sepsis, postsurgical ileus, burn injury, acute pancreatitis, mesenteric ischemia reperfusion injury, cardiopulmonary bypass transplantation, extrahepatic biliary tract disease and obstruction, and hemorrhagic shock. Clinical use of pyruvic acid derivatives may be the next critical therapy for the successful treatment of both human and animal sepsis. These substances show promise for improved survival.

Ethyl Pyruvate:

Pyruvic acid is a simple three carbon alpha keto monocarboxylic acid that under normal physiological conditions largely exists in the extracellular fluid volume and within the cytosol of the cell. While in the cell pyruvic acid exists in its conjugated anionic form pyruvate.
Pyruvate is the final product of the aerobic glycolytic pathway and is the precursor substrate for the start of the tricarboxylic acid cycle (TCA cycle). The glycolytic pathway during anaerobic conditions still forms pyruvate where it is quickly reduced to lactate through the lactate dehydrogenase pathway within the liver.

Pyruvate has many other roles within the cell. It is both an endogenous antioxidant and free radical scavenger. Reactive oxygen species are implicated in the pathogenesis of both structural and functional tissue alterations in all forms of sepsis. They are mediators of pathological conditions such as thermal injury, hemorrhagic shock, and mesenteric ischemia/reperfusion injury. Super oxide radical anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH$^-$) and peroxynitrite (ONOO$^-$) are all examples of reactive oxygen species that cause tissue injury. Super oxide radical anions are converted to other more severe reactive oxygen species through the super oxide dismutase enzymatic pathway. Holleman et al. 1904 first recognized that endogenous pyruvic acid could reduce H$_2$O$_2$ nonenzymatically to form CO$_2$ and H$_2$O. Pyruvic acid functions as a free radical scavenger by hydrolyzing hydroxyl radicals (OH$^-$) to free water. Furthermore, the extremely liable free radical peroxynitrite (ONOO$^-$) can be reduced to ammonium (NH$_4^+$) and free water by pyruvic acid.

Pyruvate’s ability to function in these two unique capacities both as cellular energy and as a cellular protective entity through the reduction of reactive oxygen species has lead to much research in the use of pyruvate. Despite the promising therapeutic effects of pyruvate reactive oxygen species scavenging ability pyruvate has poor physiologic stability in aqueous solution. Aqueous pyruvate solutions undergo spontaneous aldol-like condensation reaction to form 2-
hydroxy-2-methyl-4-ketoglutarate, parapyruvate, which is a potentially toxic inhibitor of mitochondrial function.\textsuperscript{27,47} Parapyruvate is a potent inhibitor of a critical step in the mitochondrial TCA cycle preventing the formation of succinyl Coenzyme A.\textsuperscript{27} Parapyruvate can undergo spontaneous cyclization and dehydration to form an enolic lactone or nonenzymatic reduction within the mitochondria to form 2,4-dihydroxy-2-methylglutarate, a mitochondrial poison.\textsuperscript{47} Initial studies by Mongan \textit{et al.} 1999 using a swine hemorrhagic shock model evaluated a 30\% pyruvate incorporated into saline solution.\textsuperscript{26} In order to achieve therapeutic effects of pyruvate, large volumes of saline solution were given resulting in serum hypernatremia. As a sequel to this study a new fluid type was developed for the administration of pyruvate as a non-hyperosmolar solution. This solution was ethyl pyruvate in lactated Ringer’s solution or Ringer’s ethyl pyruvate (REPS).

Ethyl pyruvate mitigates inflammation by blocking production of pro-inflammatory cytokines as well as substances produced later in the inflammatory cascade. Nuclear factor kappa B is a central mediator in mammalian inflammatory immune responses. There are currently five known NF\kappa B proteins: RelA, p50, RelB, p52, and RelC.\textsuperscript{13,34} These proteins form a variety of homodimers and heterodimers in the cytosol of the cell. These compounds are readily held in an inactive state by the inhibitory protein I\kappa B.\textsuperscript{13,34} Once the inhibitory protein I\kappa B is cleaved off of the homodimer or heterdimer intracellular cytoplasmic mechanisms can be turned on to allow nuclear protein synthesis to occur. Mizutani \textit{et al.} 2010 showed that with preincubation of 10 mM ethyl pyruvate with nuclear extract from A549 macrophage like cells decreased binding of both the p50 and RelA protein dimers in a time dependent fashion.\textsuperscript{25} It can
be inferred from this research that ethyl pyruvate blocks the movement of cytosolic NFκB from translocation into the nucleus of the cell. Specifically ethyl pyruvate blocks both the p50 and RelA NFκB proteins disrupting the NFκB pathway and diminishing its nuclear effects. The end result is suppression of proinflammatory cytokine production.

**Ringer’s Ethyl Pyruvate In Vitro:**

Ethyl pyruvate reduces expression of pro-inflammatory cytokine genes in mononuclear cells of several species. Canine peripheral mononuclear cells (PBMC) exposed to 100ng/ml lipopolyschararide and treated simultaneously with 5mM or 10mM ethyl pyruvate had less TNFα, and IL-6 gene expression and increased IL-10 expression compared to cells not treated with ethyl pyruvate. When the canine PBMC were exposed only to LPS there was a significant increase in mRNA synthesis (150 fold increase for TNFα, a 1500 fold increase for IL-6, and a 8 fold increase for IL-10) when assayed post stimulation with LPS 100mg/ml. This study showed that ethyl pyruvate at both 5mM and 10mM concentration can significantly modulate both the pro-inflammatory and anti-inflammatory LPS response by down regulation of the pro-inflammatory cytokines TNFα, IL-6 and upregulation of anti-inflammatory cytokine IL-10.

Ulloa *et al.* 2002 determined the effect of ethyl pyruvate on mice macrophage like-cells (RAW 264.7). The cells were stimulated with two differing doses of LPS (50ng/ml or 100ng/ml) for 20 hours. The stimulated cells were treated simultaneously with 3 1mM, 5mM, or 10mM ethyl pyruvate. HMGB-1 protein expression was significantly decreased in a dose
dependent fashion for both the 50ng/ml and the 100ng/ml LPS stimulated cells. Ethyl pyruvate (10mM) suppressed HMGB-1 protein production by greater than 75%. Ethyl pyruvate significantly decreased TNFα gene expression in both the pretreatment and post-treatment endotoxic shock model. LPS stimulated mouse mononuclear cells were incubated with a specific $^{32}$P radio labeled DNA probe containing a NFκB binding sight and analyzed with electrophoresis mobility-shift assay. The electrophoretic mobility-shift assay revealed an increased NFκB DNA binding at 15 minutes and 4 hours of incubation with endotoxin (LPS). Treatment of LPS stimulated cells with ethyl pyruvate significantly inhibited the NFκB signaling pathway for 4 hours post stimulation, indicating not a delay induction of the NFκB pathway but blockade in the activation of the pathway. The p38 mitogen-activated protein kinase pathway is an important regulatory step in the production of both pro and anti-inflammatory cytokines from LPS activated macrophages. Western blot analysis for antibodies directed at the phosphorylated form of p38 MAPK showed that ethyl pyruvate in a dose dependent fashion decreased expression of LPS phosphorylation of p38 MAPK.

The work of Ulloa et al. showed that ethyl pyruvate induced significant inhibition of both the early release of pro-inflammatory cytokines (TNFα, IL-1β, and IL-6) and the production of the late cytosolic protein mediator HMGB-1 release. When ethyl pyruvate was administered after the endotoxic stimulation ethyl pyruvate still provided protective effects, as well as, increased survivability. Ethyl pyruvate was administered as late as 24 hours after the acute cytokine release and still suppressed production of pro-inflammatory cytokines. The results of
the p38 MAPK Western Blot suppression and the electrophoresis assay for NFκB indicated that molecular effects of ethyl pyruvate on activated macrophages are through direct interference with the NFκB cell signaling pathway. Macrophage activation by endotoxin and cytokines lead to the translocation of cytosolic NFκB into the nucleus causing enhanced transcription of mRNA specifically for TNFα in the early stage of sepsis. Nuclear factor kappa B also causes phosphorylation of p38 which leads to stabilization of TNFα mRNA and increases the translation efficiency. Direct blockade of the NFκB and or p38 MAPK pathways may further explain the regulation of HMGB-1 protein production and the delayed release of this cell protein mediator in activated macrophages.

Ringer’s Ethyl Pyruvate in Septic Shock:

Ethyl pyruvate has been shown in several preclinical sepsis models to diminish morbidity and improve survival. Venkataraman et al. 2002 evaluated resuscitation with Ringer’s ethyl pyruvate or Lactated Ringer’s solution in a murine septic shock model. The rats were given intravenous Escherichia coli (E. coli) and when the mean arterial pressure decreased to less than 60 mmHg the rats were randomly assigned to be resuscitated with either the LRS or REPS. The rats resuscitated with REPS had a significantly higher mean arterial pressure, and lower blood lactate concentration, indicating both signs of improved perfusion and oxygen delivery, resulting in an increased overall survival. IL-6 pro-inflammatory gene expression decreased significantly in the REPS treatment group compared to the LRS group at 3 and 6 hours post LPS infusion, IL-10 was significantly higher in the REPS compared to LRS at 3 and 6 hours post LPS.
Infusion. Nitrite and nitrate production was significantly lower in the REPS treatment group compared to the LRS group at 3 and 6 hours post LPS infusion. There was a significant increase in TNFα expression in both treatment groups post LPS infusion. Interestingly, TNFα expression was not decreased in either the Lactated Ringer’s treatment group or the Ringer’s ethyl pyruvate treatment group at 3 and 6 hours post LPS infusion; however, TNFα has an extremely short time of release post exposure with peak release being 60 minutes post endotoxin exposure. It is not surprising that TNFα is decreased, it is expected that IL-6 would be suppressed at 3 hours and to a lesser degree at 6 hours and that IL-10 would be increased at 3 and 6 hours post LPS infusion.

Ulloa et al. 2002 showed that pretreatment of mice with ethyl pyruvate at varying doses followed by lethal infusion of LPS prevents endotoxin induced lethality through attenuation of the pro-inflammatory cytokine TNFα, IL-1β, and IL-6 pathways, prolonged survival in LPS challenged mice. Pretreatment with ethyl pyruvate significantly decreased circulating blood concentration of HMBG-1 protein twenty hours post intraperitoneal infusion of LPS. In the same study ethyl pyruvate was evaluated as a rescue therapy. Delayed administration of a fixed dose of ethyl pyruvate (40mg/kg) produced significant attenuation of endotoxemia lethality and septic peritonitis induction in a cecal ligation and puncture model. Ethyl pyruvate diminished blood concentration of HMGB-1 when administered twenty hours after LPS infusion. Ethyl pyruvate (40mg/kg) was administered intraperitoneally 24, 30, 48, and 54 hours after cecal ligation and puncture to induce septic peritonitis in mice. Ethyl pyruvate rescued mice when administered long after induction of septic peritonitis reversed clinical signs of morbidity and
improved survival with no late deaths occurring during the subsequent 3 week observation period after ethyl pyruvate administration.  

Su et al. 2007 induced sepsis using an ovine fecal peritonitis model. 0.5gr/kg feces were placed within the abdomen via typhlotomy. The fourteen sheep were then randomized to receive fluid treatment of Lactated Ringer’s with ethyl pyruvate (15mg/kg/hr) or Lactated Ringer’s solution only. Outcomes evaluated included cardiac index, stroke volume index, systemic vascular resistance, pulmonary vascular resistance, left ventricular stroke work, oxygen delivery, and oxygen extraction. Ethyl pyruvate (15mg/kg/hr) significantly delayed onset of systemic hypotension and sustained cardiac function through increased left ventricular stroke volume index. Treatment with ethyl pyruvate supported colloidal oncotic pressure, prolonged survival and delayed oliguria in a intrabdominal peritonitis sheep model. Ethyl pyruvate provided support of the cardiovascular system, decreased capillary permeability most likely through decreased endothelial damage from inflammatory cytokines, provided renal protection, and improved survival when administered after induction of septic fecal peritonitis in sheep.

Not all results support the use of pyruvic acid derivatives. Andersson et al. 2006 evaluated the hemodynamic and metabolic effects of ethyl pyruvate in a porcine endotoxic shock model. This group showed that ethyl pyruvate was ineffective in a porcine hemorrhagic shock model. In this study, they concluded that resuscitation with ethyl pyruvate had no significant advantage over resuscitation with Ringer’s Acetate. Mulier et al. 2005 and colleagues evaluated ethyl pyruvate and Lactated Ringer’s following hemorrhagic shock in pigs. They
found no significant differences in physiologic parameters between the two resuscitative groups nor was there improvement in hemodynamic endpoints or oxygen delivery and use at local tissue beds. The authors concluded that there was no significant benefit in using ethyl pyruvate as a resuscitative fluid in this hemorrhagic porcine model. Reasons for the lackluster therapeutic benefit of ethyl pyruvate in these two porcine studies potentially include inadequate dose of ethyl pyruvate or severity of the model.

Su et al. 2008 using an in vivo lipopolysaccharide challenged mouse model showed significant beneficial effects in down regulation of proinflammatory cytokine genes and suppression of the NFκB pathway. Mice were challenged with 30mg/kg LPS intraperitonially and were subsequently treated with varying doses of ethyl pyruvate (dose ranging from 0.01mg/kg-100mg/kg). There was an increase in the hazards death ratio for all doses of ethyl pyruvate (dose ranging from 0.01mg/kg-100mg/kg). There were also a significantly greater proportion of mice that died in the ethyl pyruvate treatment groups than in the two placebo treatment groups. However, 30 mg/kg LPS is a lethal endotoxin dose in mice.

**Ringer’s Ethyl Pyruvate in Gastrointestinal Ischemia:**

Sims et al. 2001 compared the use of Lactated Ringer’s solution, Ringer’s sodium pyruvate solution and Ringer’s ethyl pyruvate solution in a rodent intestinal ischemia model. Rats were pretreated with a bolus of Lactated Ringer’s solution, Ringer’s sodium pyruvate or with Ringer’s ethyl pyruvate solution. Rats received a treatment of the same solution during 60 minutes of intestinal ischemia followed by 60 minutes of reperfusion. Rats received a final fluid
therapy treatment after the surgical intervention and intestinal harvesting for tissue histology. Permeability of the intestine was also evaluated with fluorescein isothiocyanate dextran (FD$_4$). This is a low molecular weight protein (4000 kilodaltons) and easily translocates across the mucosal barrier when damage is present because of ischemia. When comparing REPS to Ringer’s sodium pyruvate solution in this murine intestinal mucosal ischemia reperfusion injury model, REPS ameliorated both the histologic mucosal structural damage and prevented increased mucosal permeability because of the ischemic tissue damage. The rats that received the control Lactated Ringer’s solution mucosal permeability and histologic structural damage were both significantly elevated 30 minutes after ischemia induction. Treatment with either Ringer’s sodium pyruvate or ethyl pyruvate ameliorated intestinal permeability during the reperfusion state. Quantitative evaluation of the histologic sections showed villous height and mucosal thickness were significantly greater during both the ischemia and reperfusion phases in rats treated with Ringer’s ethyl pyruvate solution compared with controls treated with Lactated Ringer’s solution. Mucosal injury scores were significantly lower in rats treated with Ringer’s ethyl pyruvate solution than in rats treated with Lactated Ringer’s solution. This study shows that treatment with a solution containing either sodium pyruvate or ethyl pyruvate can ameliorate histologic tissue damage, decreased mucosal injury, decreased functional damage and permeability, and lastly decreased organ dysfunction induced by ischemia reperfusion injury.

Cruz et al. 2011 evaluated the effects of ethyl pyruvate and other alpha keto carboxylic acid derivatives in a murine model of multivisceral ischemia and reperfusion injury. The results indicate that ethyl pyruvate is the most lipophilic alpha keto acid as compared to sodium
Ringer’s Ethyl Pyruvate in Post Operative Ileus:

Ethyl pyruvate is documented to be beneficial in a murine model of surgically induced ileus. Ethyl pyruvate has been shown to ameliorate cardiopulmonary effects of sepsis, help in the prevention of acute renal failure, treat both hepatic failure and cirrhosis, intestinal permeability, hemorrhagic shock, sepsis and many other ailments that lead to severe organ dysfunction and potentially death; however, this is the first article showing the potential benefit of ethyl pyruvate as a treatment of postoperative ileus of the small intestine. There was a significant decreased production of the pro-inflammatory cytokine IL-6 and inducible nitric oxide synthase (iNOS) in the smooth muscle of the small intestine when sampled 24 hours post
surgical manipulation of the bowel when comparing both a low dose (40mg/kg) and a high dose (80mg/kg) ethyl pyruvate treatment as compared to the Lactated Ringer’s group, sham surgical group and the control group. There was no significance between the two ethyl pyruvate treatment groups. Examining small intestinal transit time using the fluorescein marker FD 70, there was statistical significance in the distance travelled by the fluorescein marker FD 70 for both of the ethyl pyruvate treatment groups as compared to the Lactated Ringer’s group, however there was no statistical significant difference between the ethyl pyruvate treatment groups, the sham group or the control group. There was a significant decrease in distance traveled in the Lactated Ringer’s group when compared to the control and the sham treatment group. Finally, Harada et al. 2005 showed in an in vivo murine surgical ileus model that ethyl pyruvate in both a pretreatment and a post-treatment regime, ameliorated murine small intestine postoperative ileus after gentle small intestinal manipulation, decreased intestinal permeability, decreased the cytokine production, and supported vital cardiovascular parameters.

**Ringer’s Ethyl Pyruvate in People:**

The efficacy of ethyl pyruvate was tested in a phase two clinical trial evaluating a placebo controlled treatment of ethyl pyruvate in patients undergoing cardiopulmonary bypass. The procedure of cardiopulmonary bypass is associated with acute inflammatory response characterized by transient increases in blood concentrations of pro-inflammatory cytokines such as TNFα, IL-6, NO (nitric oxide), and complement, as well as, ischemia and reperfusion injury to the heart and other organs. During this study patients were randomized to receive 7,500mg
of ethyl pyruvate infused over 60 minutes every 6 hours for 6 total doses, or Lactated Ringer’s solution following the same dosing regimen. The study was terminated early due to a manufacturing issue with the drug container. One hundred and two subjects were enrolled in the study overall with 53 patients in the placebo group and 49 in the ethyl pyruvate treatment group. There was no significant difference between the placebo and ethyl pyruvate treated subjects in clinical parameters, systemic inflammatory markers, organ dysfunction, reported adverse effects of ethyl pyruvate, or with survival endpoints set at 14 days and 28 days. The authors note several possible reasons for the lack of clinical efficacy for ethyl pyruvate. Cardiopulmonary bypass may not cause sufficient systemic inflammation in order for effects of ethyl pyruvate to be manifested. Second, the patients undergoing cardiopulmonary bypass may not have been at a high enough risk for comorbidity and mortality to detect an effect of ethyl pyruvate treatment. Third, infusion of ethyl pyruvate may not have been over a sufficient length of time or the fixed dose may have been insufficient. Lastly, systemic inflammation may not be the most important etiology of organ injury in high risk cardiac surgery implementing cardiopulmonary bypass.

Seemingly, most of the questions that the authors raised for failure of the drug efficacy in this study have been proven in various other animal model studies implementing the pharmacotherapy ethyl pyruvate. Ethyl pyruvate has been proven in multiple animal species and models to ameliorate cytokine formation in both pre and post treatment protocols. Ethyl pyruvate has also been shown to have clinical efficacy and safety in multiple species in various disease models. Dosing regimens and dose concentration have been thoroughly researched and proven to be safe and lastly judging systemic inflammation as not being an important factor in end organ injury for cardiac bypass patients is somewhat inconceivable.
Summary:

Ethyl pyruvate may be an effective therapy for sepsis and ischemic syndromes. Ethyl pyruvate is stable in solution, highly lipid soluble and does not create a hyper osmolar state. Ethyl pyruvate is currently the most commonly investigated pyruvate derivative with proven efficacy in *in vitro* and *in vivo* preclinical disease models. Ethyl pyruvate primarily works by down regulation of the intracellular NFκB pathway. Specifically it prevents the Rel50 homodimer or heterodimer to be cleaved from the inhibitory protein IκB, blockade of the p38 MAP kinase pathway, and lastly late blockade of the HMGB-1 cytosolic protein pathway. This ultimately leads to both decreased transcription and translation of proinflammatory cytokines and eicosanoids. This decrease in production of cytokines and blockade of the HMGB-1 pathway prevents further tissue damage at the cellular level and at the global level.
REFERENCES
REFERENCES


CHAPTER 2.

Preliminary Safety and Biological Efficacy Studies of Ethyl Pyruvate in Normal Adult Horses

By

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Summary:

Reasons for Performing the Study: Endotoxemia causes substantial morbidity and mortality in horses with colic and sepsis. Ethyl pyruvate is a novel anti-inflammatory medication that improved survival in preclinical models of severe sepsis endotoxemia, and intestinal ischemia and reperfusion in rodents, swine, sheep and dogs, and may be a useful medication in horses.

Hypothesis: Ethyl pyruvate has no adverse effects in normal horses and is biologically active based on suppression of pro-inflammatory gene expression in endotoxin stimulated whole blood, in vitro.

Methods: Physical examinations, neurologic examinations, behavior scores, electrocardiograms, and clinicopathologic tests were performed on 5 normal healthy horses receiving 4 different doses of ethyl pyruvate. Doses included 0, 50, 100, and 150 mg/kg administered in a randomized crossover design with a 2 week wash out period between doses. Biological efficacy was assessed by stimulating whole blood with endotoxin from the horses that received ethyl pyruvate prior to and 1 and 6 hours after drug infusion. Gene expression for TNFα, IL-1β, and IL-6 was assessed.

Results: There were no effects of drug or dose (0, 50, 100 or 150mg/kg) on any of the physical or neurologic examination, behavior factors, electrocardiogram, or clinical pathologic results collected from any of the horses. All parameters measured remained within the normal reference range. There was a significant reduction in TNFα, IL-1β and IL-6 gene expression in endotoxin.
stimulated whole blood from horses 6 hours after receiving 150 mg/kg ethyl pyruvate. There were no detectable effects on gene expression of any of the other doses of ethyl pyruvate tested.

**Conclusion:** We were unable to detect any detrimental effects of ethyl pyruvate administration in normal horses. Ethyl pyruvate significantly decreased proinflammatory gene expression in endotoxin stimulated blood 6 hours after drug administration.

**Clinical Relevance:** Ethyl pyruvate may be a safe, effective medication in endotoxemic horses.
Introduction:

Endotoxemia is a common and devastating sequel to colic and is associated with significant morbidity and mortality in horses. Between 25 - 41% of horses admitted to university hospitals had measurable endotoxin in their plasma at the time of hospital admission. Endotoxin is part of the lipopolysaccharide molecule that is found in the cell membrane of gram-negative bacteria and is liberated during cell replication or death. Endotoxin and bacteria are present in the gastrointestinal tract of healthy horses without adverse effects due to the presence of a competent mucosal barrier. This barrier includes the mucus layer covering the enterocytes, epithelium, and tight junctions between the enterocytes that prevent absorption of endotoxin or translocation of bacteria into the systemic circulation. The intestinal mucosal barrier can be damaged by ischemia or inflammation associated with many forms of colic. Disruption of the mucosal barrier allows endotoxin to cross the epithelium and reach the systemic circulation. The resulting endotoxemia and the ensuing systemic inflammatory response can lead to hypovolemia, a form of distributive shock, end organ failure, including laminitis, and eventually death.

The clinical signs of endotoxemia occur when endotoxin initiates the signaling cascade in monocylic cells to release pro-inflammatory mediators. Endotoxin binds to lipid binding proteins found in blood. This complex interacts with pattern recognition receptors, CD14 and TLR4, on the surface of cells triggering 2 key pro-inflammatory signaling pathways, nuclear factor kappa B and p38 MAPK. Specifically, LPS/LBP binding to TLR4 receptor causes
phosphorylation and subsequent degradation of the intracellular inhibitory protein IκB, liberating NFκB. Nuclear factor kappa B enters the nucleus, promoting transcription and translation of inflammatory cytokines including TNFα, IL-1β and IL-6. These acute phase cytokines stimulate macrophage and endothelial cells to release pro-inflammatory substances, including those involved in the generation of leukotrienes and lipoxins. A medication that would moderate the inflammatory process initiated by endotoxin as early as possible in the signaling cascade may be therapeutically beneficial in horses with endotoxemia.

Ethyl pyruvate, a stable lipophilic pyruvate derivative, was developed and first evaluated in Dr. Mitchell Fink’s laboratory. Sims et. al. 2001 studied the potential benefits of ethyl pyruvate in rats exposed to visceral ischemia and reperfusion. Intestinal mucosal injury was ameliorated by ethyl pyruvate in Lactated Ringer’s solution compared to Lactated Ringer’s solution alone. The anti-inflammatory potential of ethyl pyruvate was demonstrated by Yang and colleagues using a rodent hemorrhagic shock model. Resuscitation with ethyl pyruvate blocked the activation of NFκB as well as subsequent production of pro-inflammatory mediators including TNFα and IL-6. Ulloa and colleagues showed that ethyl pyruvate inhibited the production of TNFα by RAW 264.7 murine macrophage-like cells following stimulation with LPS. Next, they showed that treatment with ethyl pyruvate improved survival in mice challenged with a lethal dose of LPS. Finally, these investigators demonstrated that ethyl pyruvate improved survival in mice following cecal ligation and puncture to induce sepsis even when the drug was administered 24 hours after the onset of clinical signs. Following these
seeminal experiments, ethyl pyruvate has been tested extensively in multiple disease models and species. It improved survival as well as organ function in pre-clinical models of severe sepsis and endotoxemia, acute respiratory distress syndrome, hemorrhagic shock and stroke when it was administered within 12 - 24 hours of disease induction in rodents, and hemorrhagic shock in swine. Ethyl pyruvate failed to improve hemodynamic indices in patients during cardiopulmonary bypass in a phase II clinical trial. Interestingly, in a porcine hemorrhagic shock model, ethyl pyruvate in Hextend did not improve hemodynamics compared to Hextend alone; however, resuscitation with ethyl pyruvate preserved intestinal morphology, maintained serum bacterial endotoxin levels similar to those of controls, and prevented up regulation of the intrinsic coagulation pathway.

Ethyl pyruvate’s mechanism of action is not completely understood. It inhibits binding of NFκB to nuclear DNA thereby decreasing expression of pro-inflammatory cytokines including TNFα, IL-1β, and IL-6. It is also an antioxidant and reactive oxygen species scavenger. Based on these effects, ethyl pyruvate may be a useful medication for horses with colic and endotoxemia, and other syndromes that provoke a systemic inflammatory response. We hypothesize that ethyl pyruvate has no adverse effects in normal horses and is biologically active based on suppression of pro-inflammatory gene expression in endotoxin stimulated whole blood, in vitro. The objectives of the current study were to determine the effects of ethyl pyruvate administration on normal horses by assessing physical and clinicopathologic factors following administration of 4 doses of ethyl pyruvate in a randomized, crossover design. Efficacy of ethyl pyruvate treatment was tested by determining the effects of
ethyl pyruvate on pro-inflammatory gene expression of whole blood from horses receiving ethyl pyruvate following \textit{in vitro} stimulation with endotoxin.

\textbf{Materials and Methods:}

\textbf{Horses:} The study was conducted in accordance with the Animal Care and Use Committee at Michigan State University. Five mares, from 12 – 22 years old, with a mean weight of 512 ± 42 kg, were used in the study. There were 2 Quarter horses, 1 Thoroughbred and 2 Standardbreds. The results of physical, behavioral, and neurologic examinations, complete blood counts (CBC), serum biochemical profiles, and venous blood gas (VBG) analyses were normal for all horses. Horses were housed in two large 2-3 acre pastures with access to ad libitum grass and water. They were fed grass hay twice daily. The experiments were performed in a barn adjacent to the pasture. The windows and doors of the barn remained open during the experiment.

\textbf{Experimental protocol:} The morning of each protocol, the horses were led from the pasture and placed in individual box stalls with access to fresh water and mixed grass hay. Protocols were performed on 2 – 4 horses each time such that no horse was in the barn alone. First, physical, neurologic, and behavioral examinations were performed on each horse. Preliminary blood samples were then obtained for CBC, serum chemistry, and VBG analysis by jugular venopuncture. Three small areas of hair were clipped for adhesion of leads for a telemetric electrocardiogram (ECG) (1) and a 15 second tracing was made. Intravenous catheters were placed in the jugular vein of each horse. Horses received an infusion of 1 liter of Lactated Ringer’s solution (LRS) with 0, 50, 100, or 150 mg/kg ethyl pyruvate (2) infused over 60
minutes. During the infusion, physical examinations and ECG recordings were performed every 15 minutes. At the end of the infusion, a VBG was obtained. Physical and behavioral examinations and ECGs were performed hourly for the next 5 hours and at 12 and 24 hours post drug infusion. Neurologic examinations were repeated at 24 hours post drug infusion. Blood for serum chemistry and CBC was obtained 24 hours after drug administration. Blood was also drawn prior to drug infusion (0 hour), at the end of the drug infusion (1 hour), and 6 hours after the start of the infusion (6 hour) for stimulation and RNA isolation and purification.

The doses of ethyl pyruvate were administered in a blinded, randomized, cross-over design with a 2 week wash out phase between doses such that each horse received 0, 50, 100, and 150 mg/kg ethyl pyruvate during the study. Data collection occurred from June 10 – August 23, 2009 during 4 separate periods separated by 2 weeks.

**Instrumentation:** Telemetric ECG: Three adhesive ECG patches were placed over the left heart based between the 3rd and 4th intercostal space, on the left side of the neck near the caudal cervical spine, and just dorsal to the scapula. The transmitter was placed in a nylon pouch and secured around the horse’s neck with snaps and white medical tape. The receiver (3) was placed in the barn isle. Intravenous catheter: The hair over the left jugular vein was clipped and aseptically prepared in a routine fashion using betadine scrub and 70% isopropyl alcohol. The cutaneous and subcutaneous tissues were blocked with 2 mls 2% lidocaine solution and a 14 gauge 5.25 inch Abbocath (4) was steriley placed and securely sutured to the skin. The catheter and extension set (5) were flushed with heparinized saline solution to maintain patency of the catheter. The catheter was used for drug infusion and for blood sample collection.
**Drug Preparation and Infusion:** Ethyl pyruvate 9 M stock solution was sterilely filtered with a 0.2 micron filter (6) and added to 1 liter bags of Lactated Ringer’s solution. The fluid bags were coded such that the investigators administering the medication and performing the examinations were unaware of the drug concentration. The infusions were given within 30 minutes of drug preparation. Doses were randomly assigned. Ethyl pyruvate doses (0, 50, 100, and 150 mg/kg) were chosen based on effective doses reported in rodents, swine, and people.¹⁰

**Clinical Assessment:** Clinical assessment of each horse during the protocol was based on physical, neurologic, and behavioral examination. Physical examination included rectal temperature, oral mucus membrane color, texture, and capillary refill time, heart and respiratory rate and auscultation, gastrointestinal auscultation, palpation of digital pulses, assessment of urination and defecation. The horses were monitored for behavioral changes before, during and after infusion of the drug. The behavior scoring system used was previously validated in the horse.¹⁹ Briefly, the behavioral scoring system documents evidence of pain or distress in the horse. Head position, ear position, response to another horse, response to the stall door opening, response to being approached, response to lifting of the feet and response to grain were monitored. A score was given for each of these categories with 0 being completely normal to 3 representing evidence of continuous pain. Finally, neurologic examinations were performed on the horses. A neurologic score was developed based on evidence of cranial nerve deficits, proprioception deficits, gait deficits at the walk and trot and while circling and backing, cutaneous sensation, and the ability to urinate and defecate. Scores were from 0 – 5 with 0 = no neurologic dysfunction, 1 = deficits were barely perceptible and the signs were exacerbated with elevation of the head, 2 = evidence of neurologic deficits at the walk, 3 = deficits are noted at
rest, walking and the horse may fall with elevation of the head, 4 = falls at normal gaits, and 5 =
recumbent horse. Because these horses are part of a teaching herd, they were assessed daily in
the pasture for abnormal behavior for 9 months following the experiment. The jugular veins
were examined monthly for evidence of thrombophlebitis.

**Clinicopathologic assessment:** The **CBC** included total protein, hematocrit, and fibrinogen, total
number of red blood cells, platelets, white blood cells, neutrophils, lymphocytes, and monocytes.
**Serum chemistry** measured blood urea nitrogen, creatinine, sodium, potassium, chloride, total
calculator, phosphorus, serum total protein, albumin, total bilirubin, indirect bilirubin, direct
bilirubin, alkaline phosphotase, γ-glutamyl transferase, creatinine kinase, triglycerides, and
-glucose. **Venous blood gas** analysis included measured and calculated values of pH, partial
pressure of carbon dioxide, partial pressure of oxygen, bicarbonate, non protein bound (free)
calcium, non protein bound magnesium and lactate.

**In vitro LPS stimulation of whole blood:** Blood was collected from the intravenous catheter into
EDTA tubes prior to drug administration, at the end of the 60 minute drug infusion (1 hour), and
6 hours after the start of the drug infusion (6 hour). Whole blood in EDTA was divided into 5 ml
aliquots and placed in 50 ml conical tubes. Lipopolysaccharide (**E. coli** O111:B4 LPS) (7) was
reconstituted in RPMI (8) to 1 mg/ml and stored in 10 μl aliquots at -20°F. Prior to use, LPS was
diluted in RPMI and mixed by vortexing. Preliminary experiments were performed and doses of
LPS from 0.05 – 1 ng/ml were used to stimulate aliquots of equine whole blood for 60 minutes
(data not shown). The dose 0.1 ng/ml LPS stimulated pro-inflammatory gene expression for
TNF-α, IL-1β, and IL-6 by approximately 45, 100, and 95 fold compared to equine whole blood
treated with the same volume of RPMI without LPS, fresh LPS was prepared for each stimulation (0, 1, and 6 hours). The same volume of RPMI was added to control samples.

Stimulated blood was incubated for 60 minutes in a water bath at 37°C. Following stimulation, RNA was extracted using the Qiagen QIAamp RNA Blood Mini KIT (9). Briefly, samples were placed on ice and 25 ml of erythrocyte lysis buffer was added. Samples were vortexed briefly twice and incubated on ice for 15 minutes and then centrifuged for 10 minutes at 4°C at 400-x g. Supernatant was discarded leaving a leukocyte pellet. Ten ml of lysis buffer was added and mixed by vortexing. Samples were centrifuged again at the same setting. Supernatant was discarded. Six hundred microliters of Buffer RLT (pre mixed with β-mercaptoethanol) was added to each leukocyte pellet. Samples were vortexed vigorously to suspend the pellet and pipetted into 2 ml tubes. Total RNA was extracted using a commercially available kit in accordance with the manufacturer’s instructions. The RNase-Free DNase set (QIAGEN) was used in conjunction with the QIAamp RNA Blood Mini kit to eliminate genomic DNA. Sample purity and concentration was evaluated with a NanoDrop 1000 spectrophotometer (10). Only samples with 260:280-nm absorbance ratios between 2.0 and 2.2 were used to synthesize cDNA using the High Capacity cDNA Reverse Transcription Kit with Rnase inhibitor (11).

**PCR primers:** Three endogenous control genes, β actin, β2 microglobulin and ubiquitin were used and TNFα, IL-1β, IL-6 were evaluated. The design parameters for each target gene were tested extensively, resulting in 100% efficiency when measured over a 6-log dilution range in samples that are free of PCR inhibitors. (See Table 1 for the probes and primers for these genes).
**Quantitative Real-Time-PCR:** Real-time PCR was performed in a 7500 Fast Real-Time PCR system (12) using custom designed TaqMan MGB probes from Applied Biosystems (13). Polymerase chain reactions were performed in triplicate using a 20µl reaction mixture per well, containing 10µl of TaqMan Fast Universal PCR Master Mix (2X) (14), 1µl of (20X) Custom TaqMan® Gene Expression Assay Mix (15), 20ng cDNA, and the balance as nuclease-free water. Polymerase chain reaction was carried out for targeted genes with the reaction mixture described above. Changes in gene expression were calculated by relative quantification using the $2^{-\Delta\Delta C_t}$ method. Fold changes in gene expression for each sample were calculated as $2^{-\Delta C_t}$. Untreated blood from each horse (blood drawn prior to drug infusion) was used as the calibrator or control sample. The geometric average of the 3 endogenous control genes was subtracted from the target gene to determine the ΔCt.

**Statistical Analysis:** The continuous data that was collected from physical examinations (rectal temperature, heart rate, respiratory rate) and factors from the complete blood count, serum chemistry, and venous blood gas (Response Variables) were analyzed using SAS PROC MIXED (16) according to the model:

$$Y = \mu + \text{Dose} + \text{Period} + \text{Horse} + \text{Err}_1 + \text{Time} + \text{Dose} \times \text{Time} + \text{Err}_2$$

Where Y was the Response Variable, Dose, Period, and Time were fixed factors, and Horse was a random factor. Differences determined by the Mixed Procedure were analyzed by Least Squares Means. Data was analyzed for factor interaction of treatment, time, period and time
treatment interaction by Least Squared Means. Categorical data collected from physical examinations including capillary refill time, gastrointestinal sounds and mucous membrane color, the behavioral and neurologic examination scores were not analyzed because no changes were ever noted and the scores were identical for all horses at all doses. Pro-inflammatory gene expression data was analyzed using 2-way repeated measures ANOVA with horse, drug, and time as factors using the ΔCt values. ANOVA differences were analyzed by Fisher’s LSD test. Significance is set at P < 0.05.

Results:
All horses completed the protocol successfully. There was no significant effect of ethyl pyruvate at 0, 50, 100, or 150 mg/kg on any of the parameters measured including physical, neurologic, and behavioral examinations, and clinicopathologic variables. All physical examination parameters, electrocardiograms, clinicopathologic variables remained within the normal reference ranges. No cardiac arrhythmias were detected. All behavioral and neurologic scores remained 0 through the study, regardless of drug dose administered. Horses were normal 12 months after the experiments and no abnormalities of the jugular veins were detected.

There was a significant effect of time on heart rate. The mean heart rate (48.0 ± 4.4 beats per minute {BPM}) was significantly higher prior to drug infusion (time 0) compared to 2 hours (42.5 ± 4.3 BPM), 3 hours (41.3 ± 4.1 BPM), 4 hours (41.3 ± 4.0 BPM), 5 hours (41.4 ± 3.8 BPM), 6 hours (41.2 ± 4.4 BPM), 12 hours (40.8 ± 4.3 BPM), and 24 hours (39.1 ± 4.4 BPM) post drug infusion, p < 0.0001. There was also an effect of time on mean rectal temperature. Rectal temperature at 30 minutes (37.7 ± 0.22 ºC) was significantly higher than the mean rectal temperature prior to the start of the drug infusion (37.2 ± 0.3 ºC). Mean rectal temperature at 24
hours post drug administration (36.9 ± 0.2°C) was significantly lower than mean rectal
temperature at 30 minutes, p< 0.001. There was an effect of time on plasma lactate, p=0.026.
Mean plasma lactate prior to drug infusion was 0.48 ± 0.14 mmol/dl compared to 1.16 ± 0.14
following the 60 minute drug infusion. There was an effect of treatment period on mean
respiratory rate. The mean respiratory rate during the first treatment period (20.2 ± 1.2 breaths
per minute {BPM}) was significantly higher than the mean respiratory rates during the third
treatment period (16.8 ± 2.1 BPM) and during the fourth treatment period (15.7 ± 1.8 BPM).

Endotoxin stimulation significantly increased TNFα, IL-1β, and IL-6 mRNA expression
compared to the non-stimulated whole blood sample, (p<0.001) (Figures 1 - 3). Ethyl pyruvate
at 150 mg/kg blunted the pro-inflammatory cytokine response to endotoxin stimulation by
significantly reducing mRNA expression of TNFα, IL-1β, and IL-6 at 6 hours post treatment
(Figures 1 – 3), p<0.05. Mean increase in TNFα, IL-1β, and IL-6 expression, respectively, for
untreated blood stimulated with LPS was 63.9 ± 25.4 , 83.8 ± 27.7, and 251.1 ± 133 fold
compared to the non-LPS stimulated blood sample. One hour after 150 mg/kg ethyl pyruvate,
blood samples stimulated with endotoxin had TNFα, IL-1β, and IL-6 gene expression,
respectively of 53.1 ± 22.1, 62.4 ± 21.7, and 151.7 ± 63.8 fold increase compared to non-
stimulated sample. Six hours after treatment with 150 mg/kg ethyl pyruvate, LPS stimulation
only increased TNFα, IL-1β, and IL-6 expression by 18.6 ± 12.2, 18.2 ± 10.0, and 35.9 ± 20.9
fold, respectively, compared to the non-stimulated blood. There was no significant effect of
ethyl pyruvate at 0, 50, or 100 mg/kg on mRNA gene expression for TNFα, IL-1β, and IL-6
following endotoxin stimulation.
Discussion:

The results of this study suggest that ethyl pyruvate is safe when administered to normal horses up to 150 mg/kg at <2.5 mg/kg/min. There were no significant effects of ethyl pyruvate on any of the physical, neurologic, behavioral, or clinicopathological factors measured or assessed and all indices remained within the normal reference ranges for each value examined. Four doses of ethyl pyruvate were administered, from 0 – 150 mg/kg. The doses were based on effective doses used in rodents, swine and people, between 25 – 100 mg/kg. The 150 mg/kg dose was meant to be 50% higher than the anticipated most effective dose of 100 mg/kg. However, the minimal effective dose in normal horses in this study was 150 mg/kg. Additional studies should be conducted to determine a safe dose range by administering ethyl pyruvate to horses at a dose that exceeds the effective dose and induces evidence of toxicity. Subjective assessments and neurologic examinations were performed because adverse reactions to ethyl pyruvate include hind limb rigidity and weakness as well as ataxia in rodents administered high doses (900mg/kg), (M. Fink, personal communication). In the current study, the drug was infused over 60 minutes so that infusion rate did not exceed 2.5 mg/kg/min. Blurred vision in people was associated with rapid infusion (> 5 mg/kg/min) that subsided as soon as the infusion was stopped, (M. Fink, personal communication). Should ethyl pyruvate be adopted as an efficacious anti-endotoxic therapy, its use in horses anesthetized for surgical correction of gastrointestinal incarceration may be useful. Therefore, repeating this study with much higher doses, faster administration, as well as assessment of hemodynamic and cardiopulmonary effects in anesthetized horses is warranted.

The effects of time on heart rate, rectal temperature, and plasma lactate, and the effect of study period on respiratory rate are unclear. Although significantly different, all values remained
within the normal reference range. The highest mean heart rate was measured prior to drug infusion and decreased over the 24 hour period. This likely occurred because horses acclimated to the barn over time and they were the most relaxed when they were at pasture at 12 and 24 hours post drug infusion. Rectal temperature was highest at 30 minutes after the start of the infusion compared to prior to the start of the infusion or 24 hours post infusion. These differences were likely caused by the warmer temperature inside the barn compared to the pasture, though the barn temperature was not measured. The plasma lactate values prior to drug infusion were significantly lower compared to immediately after infusion. This small increase in lactate may have been due to the lactate in the LRS. Samples were taken immediately after the end of the infusion and there likely was a small amount of lactate in circulation that had not yet been metabolized. The increase in lactate was similar for the 0 dose (from 0.056 to 1.2 mmol/dl) and the 150 mg/kg dose (0.42 to 1.4 mmol/dl). Finally, there was an effect of study period on respiratory rate. Respiratory rate during the second period was higher compared to the third and forth periods. The cause of the increased respiratory rate is unclear.

Efficacy of ethyl pyruvate in horses is supported by suppression of pro-inflammatory gene expression in whole blood from horses that received 150 mg/kg ethyl pyruvate stimulated in vitro with endotoxin. Gene expression of TNFα, IL-1β, and IL-6 was significantly suppressed at 6 hours following ethyl pyruvate infusion. Previous studies of horses receiving endotoxin infusion, in vivo, showed that TNFα and IL-1β gene expression increased significantly and was maximized at 60 minutes after endotoxin infusion while IL-6 peaked at 90 minutes post infusion. Based on this time course, whole blood samples in the current study were stimulated with LPS for 60 minutes, anticipating maximum gene expression for acute phase cytokines. Blood was sampled prior to ethyl pyruvate administration, at the end of the 60 minute infusion,
and 5 hours later (or 6 hours from the start of the infusion) in an attempt to determine the time course for ethyl pyruvate’s suppression of pro-inflammatory genes. Significant reduction in expression for any of the genes studied was not detected until 6 hours post infusion, though expression was decreased for all genes at the end of the drug infusion. Further investigations detailing duration of effective pro-inflammatory gene suppression, important for developing appropriate dosing intervals, is warranted. It is unclear why only the highest dose (150 mg/kg) of ethyl pyruvate was biologically active. Differences between the experiments in horses and work in rodents and swine include species distinctions and variations in the experimental model, specifically assessment of ethyl pyruvate’s effectiveness in vitro vs. in vivo. Other studies report efficacy of ethyl pyruvate following disease induction in vivo or its effects in vitro on mononuclear and endothelial cell cultures. In the study reported here, horses received ethyl pyruvate and then blood was drawn and stimulated with endotoxin, in vitro. This was an essential first step so that a minimally effective dose could be determined in horses. An obvious next step is to test the efficacy of ethyl pyruvate, in vivo, in horses following administration of endotoxin.

The results of this study suggest that ethyl pyruvate is biologically active in horses and may be efficacious in horses with endotoxemia and related systemic inflammatory response due to its ability to suppress the expression of TNFα, IL-1β, and IL-6. Elevated levels of pro-inflammatory cytokines have been measured in horses with acute abdominal crisis as well as research horses receiving endotoxin infusion. Elevated peritoneal fluid and blood concentrations of TNFα and IL-6 were associated with non-survival in horses with colic, suggesting that these cytokines are logical therapeutic targets. The results of this study suggest
that ethyl pyruvate is a promising, novel anti-inflammatory medication with biological efficacy in horses. This medication was safe in normal adult horses at the doses and infusion rates tested.
Figure 1. Graphical representation of TNFα gene expression with fold change on the Y axis, time and treatment on the X axis.

**Figure 1** Ethyl pyruvate diminished endotoxin induced TNFα expression. TNFα gene expression was measured in whole blood before ethyl pyruvate treatment, with (+) and without (-) 60 minutes 0.1ng/ml LPS stimulation, (0 hours). Whole blood was stimulated with 0.1 ng/ml LPS following 60 minutes of 150 mg/kg ethyl pyruvate infusion (1 hour) and 6 hours after the start of the drug infusion (6 hour). Data is presented as means ± standard error of the means and different letters indicate significant differences between the means, p < 0.05.
Figure 2. Graphical representation of IL-1β gene expression with fold change on the Y axis, time and treatment on the X axis.

IL-1β gene expression was measured in whole blood before ethyl pyruvate treatment, with (+) and without (-) 60 minutes 0.1ng/ml LPS stimulation, (0 hours). Whole blood was stimulated with 0.1 ng/ml LPS following 60 minutes of 150 mg/kg ethyl pyruvate infusion (1 hour) and 6 hours after the start of the drug infusion (6 hour). Ethyl pyruvate diminished endotoxin induced IL-1β expression. Data is presented as means ± standard error of the means and different letters indicate significant differences between the means, p < 0.05.
Figure 3. Graphical representation of IL-6 gene expression with fold change on the Y axis, time and treatment on the X axis.

IL-6 gene expression was measured in whole blood before ethyl pyruvate treatment, with (+) and without (-) 60 minutes 0.1ng/ml LPS stimulation, (0 hours). Whole blood was stimulated with 0.1 ng/ml LPS following 60 minutes of 150 mg/kg ethyl pyruvate infusion (1 hour) and 6 hours after the start of the drug infusion (6 hour). Ethyl pyruvate diminished endotoxin induced IL-6 expression. Data is presented as means ± standard error of the means and different letters indicate significant differences between the means, p < 0.05.
MANUFACTURER ADDRESSES


2. Ethyl pyruvate\(^{98}\%\) (100 gram bottle) Sigma-Aldrich, St. Louis, MO.


4. Abbocath catheters 14 gauge 5.25 inch Becton Dickson Infusion Therapy Systems, Franklin Lakes NJ.

5. Extension set Microbore Extension Set 20 inch Life Shield Latex Free Hospira Inc. Lake Forest, IL

6. MillexGV syringe filter 0.22 \(\mu\)m Millipore Cork, Ireland.

7. LPS, \textit{E. coli} O111:B4 LPS, Sigma-Aldrich, St Louis, MO

8. RPMI-1640 Sigma-Aldrich, St. Louis, MO.

10. NanoDrop 1000 spectrophotometer, NanoDrop Products, Wilmington, DE.

11. High Capacity cDNA Reverse Transcription Kit with RNA inhibitor, Applied Biosystems, Foster City, CA.

12. 7500 Fast Real-Time PCR system Applied Biosystems, Foster City, CA.

13. TaqMan MGB Applied Biosystems, Foster City, CA.

14. TaqMan Fast Universal PCR Master Mix (2X) Applied Biosystems, Foster City, CA.

15. Gene Expression Assay Mix, Applied Biosystems, Foster City, CA.

REFERENCES
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