Ethyl pyruvate failed to reduce pro-inflammatory cytokines release following in vitro stimulation in dairy calves' whole blood.

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To my family,
who grows with this dissertation and who I love.

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ABSTRACT

Recent developments in sepsis treatment showed that control of the late mediator of inflammation, High Mobility Box group-1 (HMGB1), improves survival in animal models of endotoxemia. Ethyl pyruvate is a small-molecule inhibitor that has been shown to reduce the systemic release of HMGB1 during experimental treatment of systemic inflammation in different species. The objectives of this body of work were 1) to explore ethyl pyruvate's ability to modulate early pro-inflammatory cytokines production and immune effector function in treated calves, 2) to evaluate the safety of an administration of ethyl pyruvate infusion in neonatal calves, and 3) to explore ethyl pyruvate's ability to modulate pro-inflammatory cytokines production *in vitro* in neonatal calves' whole blood samples. The first study includes 24 calves randomly assigned to 1 of 3 treatment groups of 8 calves per group: (1) Placebo control – one liter of lactate ringer solution (2) ethyl pyruvate at 50 mg/kg infusion within one liter of lactated ringer solution; (3) ethyl pyruvate at 100 mg/kg infusion within one liter of lactated ringer solution. Treatments were given over 30-45 minutes. Blood samples were collected in EDTA and lithium heparin tubes before infusion time and at 1-, 3-, 6-, 12-, 24- and 48-hours post-infusion. Each sample was split, and one aliquot was incubated at 37°C for an hour with 1 ng/ml of *E. coli* lipopolysaccharides (*Escherichia coli* O55:B5) solution. Pro-inflammatory cytokines, TNF-α, IL-1β, IL-6, and HMBG1, were measured in plasma. Granulocyte phagocytosis activity

was measured at all time points. Ethyl pyruvate treatment did not significantly affect cytokine concentrations at 50 and 100 mg/kg.

Furthermore, evaluation of *in vitro* ethyl pyruvate at different concentrations in whole blood showed that higher concentration could increase cytokine production by immune cells and reveal a dose-dependent effect on the production of pro-inflammatory cytokines. The results of this dissertation do not support the hypothesis that ethyl pyruvate could alleviate the release of pro-inflammatory cytokines following an *in vitro* endotoxemia challenge in dairy calves. For this reason and due to possible safety concerns, it should not be used clinically in neonatal dairy calves at this point.

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LIST OF ABBREVIATIONS

AGER: Advanced glycosylation end product-specific receptor

CLP: Cecal ligation puncture

CpG: Cytosine–guanine dinucleotide

COX-2: Cyclooxygenase-2 (COX-2)

DAMPs: Damage-associated molecular pattern

ELISA: Enzyme-Linked Immunosorbent Assay

EP: Ethyl pyruvate

Fb: Fibrinogen

Glo-1: Glyoxalase -1

HMGB1: High mobility group box-1

Hp: Haptoglobin

ICAM-1: intercellular adhesion molecule-1

IFN-γ: Interferon-γ

IgA: Immunoglobulin A

IgM: Immunoglobulin M

IgG1: Immunoglobulin G1

IgG2: Immunoglobulin G2

IL-1β: Interleukin 1 beta

IL-6: Interleukin-6

IL-8: Interleukin-8

IL-10: Interleukin-10

IL-17 : Interleukin-17

iNOS: Inducible nitric oxide synthase

i.p.: Intra-peritoneal

LPS: Lipopolysaccharide

LRS: Lactated Ringer's solution

MAMPs: Microbe-associated molecular patterns

MAP: Mean arterial pressure

mM: Millimolar

NF-κB: Nuclear factor kappa B

PCT: Procalcitonin

PRRs: Pattern recognition receptors

REPS: Ringer's ethyl pyruvate solution

ROS: Reactive oxygen species

SAA: Serum amyloid A

SIRS: Systemic inflammatory response syndrome

TCA cycle: Tricarboxylic acid cycle

TLRs: Toll-like receptors

TLR2: Toll-like receptor 2

TLR4: Toll-like receptor 4

TNF-α: Tumor necrosis factor-alpha

VCAM-1: vascular cell adhesion molecule-1

INTRODUCTION

Despite significant progress over the last century in management, housing, and disease prevention, about 5% of all dairy heifers born in the United States will die before weaning (Urie et al., 2018). Several factors have been evaluated to understand the risk of morbidity and mortality in the newborn calf to reduce this high incidence of mortality. About 50% of all mortality during the first weeks of life is due to scour (Urie et al., 2018). Severe dehydration and sepsis are the main factors that lead to mortality with scour (Smith and Berchtold, 2014). Sepsis is still a leading cause of death in animal species and humans. It is mainly characterized by dysregulation of the immune system due to invasion of the bloodstream by bacteria (Rhodes et al., 2017). Development on preventing, treating, and reducing the consequences of sepsis are studied all around the globe to help clinicians. In the following review, the calf's immune system will be summarized to understand the pitfall of immunity leading to the development of sepsis during the neonatal period. Sepsis and precisely how to diagnose, treat and prevent will follow with discussion on a new molecule showing promising results in other species in treating septicemia and septic shock: ethyl pyruvate.

Immune system in dairy calves

The immune system is complex, with a capital role in animal survival. The immune system of neonatal calves is usually fully developed at birth (Cortese, 2009) but is naive to a wide variety of pathogens present in the environment (Quigley, 2002). Newborn calves are considered immunologically naïve at birth. Chase et al. 2008 reported several key factors predisposing neonatal calves to infection (Chase et al., 2008). Those are the following:

- Decreased native defense mechanisms.
    - Decreased complement activity.
    - Decreased neutrophil and macrophage activity.
    - Decreased interferon production.
    - Decreased natural killer cell function.
    - Decreased dendritic cells.

- Decreased acquired immune mechanisms.
    - Decreased lymphocyte responsiveness.
    - Neonates have TH2 response: antibody, no memory.
    - Decreased major histocompatibility complex II: Decreased antigen presented to T cells.
    - Born with no memory T or B cells.

- Antibody production decreased CD40 and decreased CD40L B-cell differentiation.
- Agammaglobulinemia: The antibody must be obtained from the mother through colostrum.

Neonatal calves are known to lack a native defence mechanism in terms of the immune system. Complement activity also decreased and reached mature function around six months of age. At birth, it will be about 50% of that in adult cows, and the circulating complement level will be reduced to 20% of adult cows' level by the first day of life. This level will slowly increase to 50% by one month of age (Firth et al., 2005). Interferon activity is similar to adult cows, but leukocyte production is reduced (Firth et al., 2005). The capacity of peripheral blood mononuclear cells from young calves to produce inducible nitrite oxide, a component of bactericidal mechanisms of phagocytic leukocytes, and interferon-γ (IFN-γ), a pivotal cytokine in cell-mediated immunity, differ substantially from the capacities of adult cattle cell (Nonnecke et al., 2003). Kampen et al. 2006 demonstrated that T-cell subpopulations are present in the peripheral blood of calves at levels comparable with adult values, while the B-cell population increases significantly with age. The decrease in the relative percentage of $\gamma\delta$ T-cells was attributable to an increase in the absolute numbers of CD4+ and CD21+ cells rather than a change in total $\gamma\delta$ T-cell numbers. Furthermore, they showed that neutrophilic granulocytes are functional and can effectively respond

in young calves from the first week of life. Phagocytic ability is reduced in neutrophils and macrophages but increases after the ingestion of colostrum (LaMotte and Eberhart, 1976, Menge et al., 1998). Moreover, the leukocyte population differs from the adult population, with an increased number of neutrophils at birth, slowly decreasing over the first months of life (Novo et al., 2015, Kim et al., 2021).

Similarly, the number of dendritic cells and circulating natural killer cells are different than the adult value, but on the contrary, neutrophil count is low at birth (Morein et al., 2002). The first is due to the absence of monocyte migration to the tissues to develop dendritic cells. The second only accounts for 3% of total lymphocyte count at one week and increases to 10% by 6 to 8 weeks of age (Kampen et al., 2006).

In calves, susceptibility to pathogens is due to the absence of primed cells, deficient number of B cells at birth, and decreased function of specific types of cells such as phagocytic cells up to 4 months of life (Kolar et al., 2020). The number of circulating B cells is significantly reduced in neonates, representing only 4% of the total lymphocytes at one week of age compared with approximately 20% to 30% in adults. The fraction of B cells in circulation increases gradually to 20% of total lymphocytes by 6 to 8 weeks of age (Kampen et al., 2006). The low number of B cells coupled with calves' endogenous

corticosteroids results in a prolonged lack of endogenous antibody response (Chase et al., 2008).

As seen, neonatal calves lack preexisting immunological memory and competent adaptive immunity. To improve innate immunity to infectious agents, neonatal calves depend on colostrum transfer due to the absence of transfer of IgG from the dam to the fetus in utero due to the type of placenta (Tizard, 2013). The ingestion of colostrum is essential for providing neonates with immunologic protection or passive immunity during at least the first 2 to 4 weeks of life (see Fig. 1). For these reasons, newborn calves can be at greater risk of infection from birth until 4-6 months of age.

Colostrum management and implication on neonatal immunity

A recent study on morbidity and mortality in pre-weaned dairy heifers in US dairy operations found that higher prevalence of respiratory, enteric or other disorders and mortality rates are observed in calves with a serum IgG concentration lower than 15 g/l, a serum total protein < 5.4 g/l, or a serum Brix score < 8,6% (Urie et al., 2018). Factors affecting colostrum absorption are the timing of feeding, colostrum quality, colostrum quantity and any factor affecting the transport of the colostrum to the site of absorption, such as reduced gastro-intestinal motility (Conneely et al., 2014, Hogan et al., 2015, Fischer et al., 2018).

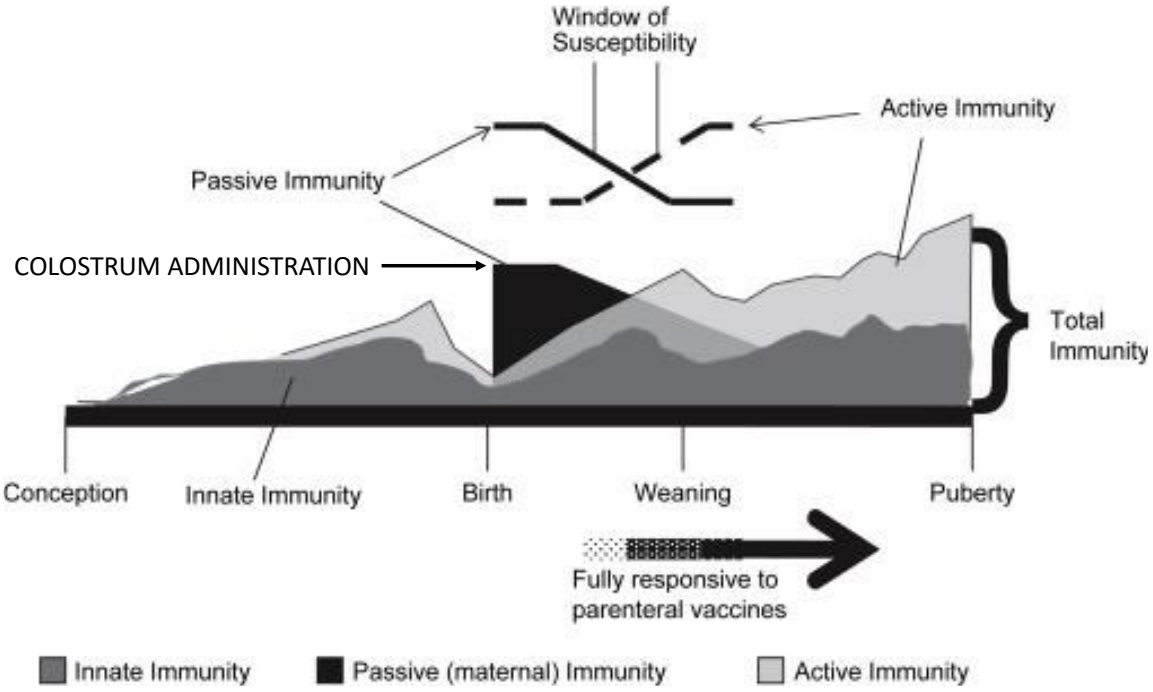


Figure 1. Evolution of passive, innate and active immunity in the calf from conception to puberty. This figure is modified from Chase et al. 2008

The actual recommendation for the optimal transfer of immunoglobulin to occur is to feed calves a first colostrum meal at 7.5% to 10% of their body weight within 2 hours after birth (House et al., 2020). After birth, the initial feed should provide 150 g of IgG calf within 2 hours. This amount of colostrum is required to ensure that 90% of calves have optimal passive transfer (Chigerwe et al., 2008).

Colostrum is an essential player in the development of immunity in calves. Bovine colostrum contains immune factors, like leukocytes and cytokines absorbed by the neonate's intestinal mucosa. In the period of immune maturation, maternal colostrum promotes temporary protection to newborns due to the passive transfer of immune factors such as immunoglobulins as calves are born agammaglobulinemic, providing an immediate antibody source (Chase et al., 2008). It has been shown that colostrum-deprived calves have only trace amounts of immunoglobulin during the first three days of life, leaving them no good protection against pathogens (Clover and Zarkower, 1980). Endogenous production of IgM in those calves only begins four days after birth and reaches a significant level after a week of life (Clover and Zarkower, 1980). Other immunoglobulins such as IgA, IgG_1 and IgG_2 only reach considerable levels at 16 to 32 days of life (Husband and Lascelles, 1975).

Pro-inflammatory cytokines IL-1β, IL-6, TNF-α and IFN-γ are also present in maternal colostrum, believing in the possibility of its absorption by the intestinal

mucosa of calves like the immunoglobulins form, reaching highest concentrations in newborns bloodstream in the first 72 h of life (Gomes et al., 2014). The role of these substances in calves' immune response is still unclear. Still, they are expected to help recruit neonatal lymphocytes into the gut to aid normal immune development (Chase et al., 2008). A study by Gomes et al. 2014 showed that healthy calves presented with concentrations not detectable of TNF-αIL-6 and IL-1β at birth, but concentration increased following colostrum administration (Gomes et al., 2014). Yamanaka et al. 2003 found similar results showing that IL-1β, IL-6, TNF-α, IFN-γ and IL-1 receptor antagonists were undetectable at birth in almost all calves but became measurable 12 hours post-colostrum feeding, reaching a peak value at 24 hours and progressively decreasing up to 4 weeks post-partum (Yamanaka et al., 2003). Those cytokines are considered to contribute to the maturation of neonatal immune functions. For example, oral administration of recombinant IL-1β to neonatal calves resulted in the appearance of the cytokine in circulation and the activation of circulating lymphocytes and neutrophils (Hagiwara et al., 2001). The high levels of two anti-inflammatory cytokines, IL-4 and transforming growth factor beta-1, were shown to suppress the local secretion of pro-inflammatory cytokines in the intestine and allow gut microbial colonization to enable the development of an excellent microbial barrier to challenge pathogen bacteria (Chase et al., 2008). Finally, Colostrum-feed calves showed increased IL-17 secretion, suggesting that maternal cells from the colostrum modulated T-cell Th17 production. This

mechanism could be responsible for a quick and efficient activation of neutrophils for bacterial clearance, making colostrum-deprived calves more likely to have clinical signs of disease (Novo et al., 2017).

Finally, colostrum contains between 1×10^6 and 2.5×10^6 somatic cells/mL (Costa et al., 2017). About 32% of these cells are viable. Gomes et al. 2011 measured the population of leukocytes in first milking colostrum. They obtained 13% of neutrophils, 16% of lymphocytes, 70% of large cells (monocytes/macrophage and epithelial cells), and 0.3% of eosinophils (Gomes et al., 2011). Libler-Tenorio et al. 2002 measure the leukocytes percentages similar to peripheral blood percentage, but with a more significant fraction of macrophages (40%–50%) and a smaller fraction of lymphocytes (22%–25%) and neutrophils (25%–37%) (Liebler-Tenorio et al., 2002). Most lymphocytes are T lymphocytes, with less than 5% being B lymphocytes. While Meganck et al. 2014 evaluated the distribution of colostral leukocytes to 25% of T lymphocytes, 3% of B lymphocytes, and 33% of macrophages (Meganck et al., 2014). Many of the cells from colostrum pass through the intestinal epithelium of calves and migrate to Peyer's patches and mesenteric lymph nodes (Liebler-Tenorio et al., 2002).

Maternal cells were also found in the bloodstream of newborns, detecting peak 24 h after colostrum intake (Chase et al., 2008). Then, after 36 h, maternal cells disappeared from the bloodstream due to their migration to other tissues

and secondary lymphoid organs to help protect the newborn (Reber et al., 2006). There is evidence that maternal cells, in combination with maternal antibodies and cytokines, have a direct antimicrobial effect or can stimulate the endogenous innate immune response to infection caused by enteropathogenic *Escherichia coli*. Further, maternal cells enhance the development of innate and adaptive immune function in neonatal calves as colostrum-. Colostrum-fed calves showed an innate immune response more quickly and efficiently after natural exposure to pathogens than colostrum-deprived calves (Costa et al., 2017).

Sepsis in dairy calves

The American Thoracic Society (ATS) describes sepsis as a syndrome that occurs when severe infection results in critical illness. In the United States, it affects around 750,000 Americans annually. Sepsis occurs when a bacterial, viral, or fungal infection causes a significant response from the body's immune system. The initial reaction to sepsis shows a high heart rate, fever, or fast breathing. Then, it becomes the balance between an effective immune response and an immune response that cascades and results in adverse effects, such as shock (Fecteau et al., 2009). Systemic inflammatory response syndrome (SIRS) is the host inflammatory cascade initiated when the host defense system fails to recognize or clear the infection (Goldstein et al., 2005). The identification of SIRS in human medicine requires four criteria:

1. Core temperature abnormality (increase or decrease);

2. Heart rate abnormality (tachycardia or bradycardia);
3. Increase respiratory rate (tachypnea);
4. Leukocyte count abnormality (leucopenia or leukocytosis).

Severe sepsis develops when the infection causes damage to the main organs. Septic shock is the most severe form in which the condition causes low blood pressure and hypoperfusion, resulting in damage to multiple organs. Septic shock is a combination of variable degrees of hypovolemic (fluid losses through capillary leakage), cardiogenic (from myocardial depressant effects of sepsis), and distributive shock (decreased systemic vascular resistance) (Goldstein et al., 2005). Multiple organ dysfunction syndrome refers to a clinical situation where several organs fail (Goldstein et al., 2005). About three in every ten human patients with severe sepsis in the United States, and half of those with septic shock, would die in the hospital. In bovine medicine, the definition of septicemia has often been associated with the following criteria (Table 1).

Sepsis incidence, etiology, and risk factors in calf

Sepsis in calves is often sporadic but could become epidemic, reaching up to 30% in prevalence in specific herds (Aldridge et al., 1993, Fecteau et al., 2009) depending on the initial cause and presence of risk factors. A study from Ghent in Belgium showed a prevalence of 34.4%, with a mortality rate of 61.3% in critically ill calves presented at a teaching hospital (Pas et al., 2023). Most of the calves were Belgian Blue beef cattle. Similarly, in a group of 1400 critically ill

neonatal calves with diarrhea presented to another teaching hospital, about 20% of the calves presented showed clinical signs of septicemia (Trefz et al., 2017).

Escherichia coli is the most frequent etiologic agent found in newborn calves due to the high prevalence of colisepticemia secondary to neonatal calf diarrhea (Boccardo et al., 2017). In the study from Aldridge et al., *Escherichia coli* was the most frequently isolated organism, but gram-positive infections were found in 10%, and polymicrobial infections in 28% of the calves (Aldridge et al., 1993). Similar findings were found in four other studies showing that *Escherichia coli* and gram-negative bacteria were the most common bacteria found in blood from septicemic calves (Hariharan et al., 1992, Fecteau et al., 1997b, Lofstedt et al., 1999, Biolatti et al., 2012). A recent study by Garcia et al. showed that *Salmonella* species and *Escherichia coli* were the most common bacteria in diarrheic calves. Still, the prevalence in their study was low (9,26% or 10/108 calves) (Garcia et al., 2022) and was similar to the group of non-diarrheic calves (14,8% or 4/27 calves).

Many factors predispose calves to sepsis, such as failure of passive transfer, management deficits, adverse environmental conditions, cold stress, protein-energy malnutrition, micronutrient deficiencies, or bacterial colonization of a local site, such as the umbilicus, gastro-intestinal tract, or respiratory system (Bonelli et al., 2018). Primary risk factors in calves with sepsis have been

Table 1. Definition of septicemia in neonatal calves by Fecteau et al. 1997.

Criteria	Positive	Negative
1. Blood culture growth (rapidity)	<48 hours	>48 hours
2. Bacteria cultured	Pathogen	Contaminant
3. Complete blood count (2 of 3)	Yes	No
3.1 Total White cell count	Elevated/reduced	Normal
3.2 Fibrinogen concentration	>5 g/L (>500 mg/dL)	<5 g/L (<500 mg/dL)
3.3 Bands neutrophils (%)	>2%	<2%
2 3 criteria need to be met to consider the animal septicemic. From Fecteau G, Paré J, Van Metre DC, et al. Use of a clinical sepsis score for predicting bacteremia in neonatal dairy calves on a calf rearing farm. Can Vet J 1997;38:101–4		

identified as failure of passive transfer of immunity due to poor absorption, quality, or quantity of colostrum received on time (Biolatti et al., 2012). In a study looking at risk factors for case fatality in 25 diarrheic calves, Boccardo et al. found that mortality was mainly associated with low serum total protein concentration and weak suckle reflex (Boccardo et al., 2017). Low serum total protein can be a sign of failed transfer of passive immunity in those calves (Fecteau et al., 1997a, Fecteau et al., 1997b, Fecteau et al., 2009). Septicemia in these calves was attributed to intestinal mucosal damage caused by bacterial, viral, or parasitic gastro-intestinal infections, which allowed opportunistic gut pathogens to enter the systemic circulation. Newborn calves lack normal adult intestinal flora, leading them to be quickly colonized by virulent pathogens from the environment at birth before establishing a normal competitive flora. For this reason, a clean calving pen, a low number of bacteria in colostrum and milk replacer, a clean bottle and nipple, and other techniques to decrease environmental contamination help reduce the prevalence of septicemia following birth.

Septicemia may also come from other primary sites of infection, such as the umbilicus, joints, and lungs (Fecteau et al., 2009). Calves might also be born septicemic after in utero infection or during parturition due to prolonged parturition or during vaginal passage in the presence of vaginal infection (Pardon and Deprez, 2018). Finally, even if the immune system is competent at birth, it is

still immature compared to the adult, increasing the chance of infection or reduced clearing by the organism, as discussed in a previous section.

Immune response during sepsis

Immune response during sepsis can be characterized by a cytokine-mediated hyperinflammatory phase followed by an immune-suppressive phase (Boomer et al., 2014). Initial activation of the immune system is mediated by pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). Those usually originate from bacteria or under organisms such as fungi but could also be released by the host upon specific injury. Examples of PAMPs include lipopolysaccharides, flagellin, peptidoglycan, lipoteichoic acid, double-stranded viral DNA and unmethylated cytosine–guanine dinucleotide (CpG) motifs (Sheats, 2019). Examples of DAMPs include hyaluronan, heparan sulfate, heat shock proteins, high mobility group protein B1 (HMGB1) and adenosine triphosphate (ATP) (Sheats, 2019). The activation of pattern recognition receptors (PRRs) such as TLR4 leads to the production of numerous pro-inflammatory molecules such as TNF-α, IL-1β, IL-2, IL-6, IL-8, and IFN-γ and anti-inflammatory cytokines (IL-10) that induce a panoply of cellular responses and counter-responses. This phenomenon is called the cytokine storm. Enhanced phagocytic activity, vascular endothelial injury leading to capillary leak, synthesis of acute phase proteins by the liver, chemotaxis of leukocytes to the sites of infection and activation of the coagulation system are

some of the responses induced by the production of pro-inflammatory molecules (Boomer et al., 2014).

Different processes are associated with deregulations of the innate and adaptive immune systems during the second stage of sepsis, called immune paralysis. We see a depletion of T cells associated with systemic lymphopenia, leading to decreased adaptive immune cells and response to the invader (Boomer et al., 2014). This T-cell depletion induces cell apoptosis of immune cells, especially lymphocytes. Due to the increase in IL-10, we see a suppression in the Th1 response, leading to decreased stimulation of monocyte production of pro-inflammatory cytokines (Frazier and Hall, 2008). Cellular exhaustion is also observed in T cells secondary to high antigen load, leading to decreased T-cell functions (Yi et al., 2010). Continued stimulation of T cells leads to gradual exhaustion. Expression of PD-1 on the surface of T cells is one of the mechanisms leading to exhaustion. Exhausted T cells lose their effector function, such as IL-2 production, leading to decreased production of IFN-γ and TNF. This reduction in IFN-γ and TNF directly affects the immune system to adequately mount an immune response (Nakamori et al., 2020).

Clinical signs and septic score

Early diagnosis of septicemia remains a challenge in veterinary medicine and human neonatology. Calves with septicemia will present in two ways. Some

will present with clinical signs compatible with septicemia, those who would be discussed later, and others without any specific signs but with higher risk based on their case presentation. Classic presentation usually affects newborn calves between 2 and 6 days of age (Fecteau et al., 2009). The progression is typically rapid and most often fatal if not treated. Once in tissues or blood, endotoxin from the invading bacteria, bacterial cell wall lipopolysaccharides (LPS), leads to anorexia, abnormal thermoregulation, leukopenia, altered heart rate, reduced cardiac output and hypotension. Ultimately, collapse and death occur (Smith, 1986). Initial clinical signs are vague and nonspecific and could be due to other disease processes. Signs that can be seen are the following:

1. Abnormal rectal temperature (hyperthermia or hypothermia);
2. Weak or absent of sucking reflex;
3. Lethargy;
4. Tachycardia;
5. Tachypnea;
6. Hyperemia of the mucous membranes;
7. Scleral injection;
8. Petechia or hyphemia due to capillary fragility;
9. Slow capillary refill time diminished peripheral pulse, cold extremities, decreased urine output due to hypotension, hypoperfusion and poor cardiac output;
10. Diarrhea.

Clinical signs are not fully specific to sepsis, so clinicians developed different scoring scales to improve diagnosis. Fecteau et al. (Fecteau et al., 1997a) developed a septic score (see fig. 2). The score quantifies different parameters such as consistency of feces, level of dehydration, general behaviour, ability to stand, evidence of umbilical infection and scleral injection. This scoring method accurately predicted calves without septicemia (93%) but had a low positive predicting value for calves with septicemia (Fecteau et al., 1997a). Lofstedt et al. eventually developed two predicting models for septicemia (Lofstedt et al., 1999). The laboratory model is the first one with all possible types of predictors. The model included demographic information, physical examination findings, and clinicopathologic values for hematology, venous blood gases, serum chemistry, and immunoglobulins. Immunoglobulin concentration in serum was determined quantitatively or qualitatively using a variety of procedures, including the quantitative zinc sulfate turbidity test, sodium sulfite precipitation test, glutaraldehyde coagulation test, and radial immunodiffusion test. Failure of passive transfer of immunoglobulins was defined as an IgG concentration of <800 mg/dL by any of the tests mentioned above, a globulin concentration of <2 g/ dL (<20 g/L), or total serum protein of <5 g/dL (<50 g/L). The second, with only demographic data and physical examination results, is called a clinical model. In the end, only serum creatinine concentration, toxic changes in neutrophils >2+, failure of passive transfer, focal infection, and a poor suckle reflex were kept in

the laboratory model. The clinical model identified age of <5 days, focal infection, recumbency, and a poor suckle reflex as predictors of septicemia. At a probability cutoff of 0.3, the models had sensitivities and specificities in the 70–75% range, which was deemed acceptable by the authors. However, a positive prediction was only associated with a 52–68% probability (positive predictive value) that the calf was truly septic. The negative prediction was associated with an 83–89% probability (negative predictive value) that the calf was not septic (Lofstedt et al., 1999). Those models showed a more precise approach to identifying septic animals but are not as easy to use as previous scores and other more recently developed ones.

In a study from Trefz et al. (Trefz et al., 2016) sepsis and SIRS score were defined as follow:

SIRS: Presence on admission to the hospital with two of the following are fulfilled:

1. Presence of an abnormal leucocyte count: leucopenia or leukocytosis (reference interval, 5–12 × 109/L);
2. Hyperthermia or hypothermia (reference interval, 38.5–39.5 °C);
3. Tachycardia (> 120 beats/min);
4. Tachypnoea (> 36 breaths/min)

CRITERIA EVALUATED	RESULT OF OBSERVATION	POINTS
Focal site of infection	NO	0
	YES	1.5
Age in days	< 7 days	0
	≥ 7 days	1.2
Clinical Score (C.S)		
Hydration: 0 1 2 3		
Sclera: 0 1 2 3		
Attitude: 0 1 2 3		
Umbilicus: 0 1 2 3		
Fecal: 0 1 2 3		
Total C.S.=_____	Total C.S.	
	≤ 5	0
	> 5 and ≤ 8	2.1
	> 8	2.5
	Sepsis score (cumulative points)	

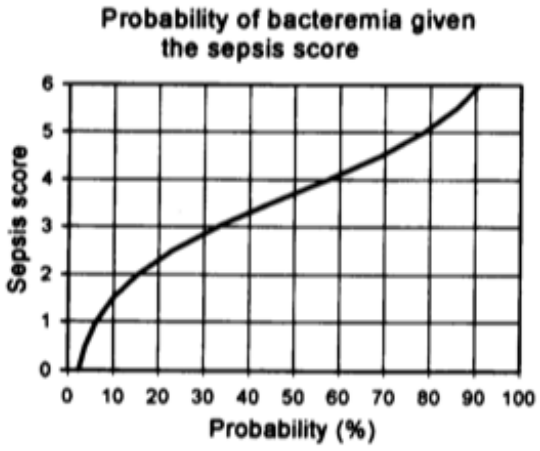


Figure 2. Scoring sheet for sepsis score and prediction of bacteremia by Fecteau et al. 1997.

Sepsis: Clinical or necropsy evidence of septicemia

1. Marked hyperemia of mucous membranes
2. Congestion/injection of episcleral vessels
3. Mucosal or sub-scleral bleeding
4. Hypopyon

Those definitions were based on previously published definitions for SIRS (Bone et al., 1992) and established reference intervals for calves (Rosenberger and Dirksen, 1990). Both scores are easy to use but have not been validated yet. Bonelli et al. (Bonelli et al., 2018) used a similar scale from Trefz in calves less than three weeks old for SIRS ((Trefz et al., 2016) and used the definition of Lofstedt et al. (Lofstedt et al., 1999) for diagnosis of septicemia. The addition of positive culture makes it more reliable as, so far, the one way to confirm sepsis is by blood culture. The score was as follows for ante and post-mortem:

Ante-mortem:

1. Positive blood culture;
2. Culture of the same bacterial agent from at least two body fluids;
3. Culture of a bacterial agent from a single joint in a calf with joint effusion involving multiple joints.

Post-mortem:

1. Morphologic changes, such as multiple disseminated abscesses of similar size, purulent vasculitis and intravascular identification of bacteria, or fibrin in multiple body cavities;
2. Bacterial isolation from heart blood;
3. Recovery of the same bacterial isolate from at least two tissues (excluding intestine).

More recently, Garcia et al. (Garcia et al., 2022)used a modified version of Fecteau et al. group (Fecteau et al., 1997a) and Pempek et al. group (Pempek et al., 2019) to only rely on easily observable signs such as depression, attitude and dehydration in their decision making (see table 2). This score still needs to be proven to help identify sepsis, but calf caretakers already use it for decision-making on using farm antimicrobials (Lowe et al., 2019, Maier et al., 2019, Garcia et al., 2022).

Blood parameters and biomarkers of sepsis

The diagnosis of sepsis in calves is often challenging. A combination of blood culture and clinical signs with a complete blood count is required for an accurate diagnosis. The pathophysiologic changes associated with inflammatory activation sepsis are dehydration and alterations in heart rate, respiratory rate, body temperature, mucous membrane status and capillary refill time, leukopenia, hypotension, and generalized weakness (Bonelli et al., 2018, House et al., 2020).

Table 2. Scoring criteria and descriptions for complete health assessments in diarrheic and nondiarrheic calf groups proposed by Garcia et al. 2022[1,2]

	Score			
Clinical sign	**Normal (0)**	**Mild (1)**	**Moderate (2)**	**Severe (3)**
Fecal consistency	Normal firm, sits on bedding	Semi-formed pasty, sits on bedding	Loose pasty to liquid, sits on and sifts through bedding	Watery, liquid, sifts through bedding
Dehydration	Eyes are bright, and skin feels pliable	Slight loss of skin elasticity; skin tent	Skin tent regresses between 3 and 10 s; eyes slightly regressed into orbit	Skin tent regresses in over 10 s or does not regress; eyes markedly recessed into orbit
Depression	No signs of depression	Calf suckles but not vigorously	Calf able to stand; suckling is weak or disorganized	Calf unable to stand or suckle
Nasal discharge	Normal, small amount of serous discharge	Small amount of unilateral cloudy discharge	Bilateral, cloudy, or excessive mucus discharge	Copious bilateral mucopurulent discharge
Ocular discharge	Lack of discharge	Small amount of ocular discharge	Moderate amount of bilateral discharge	Heavy ocular discharge
Ear carriage	Normal ear disposition	Ear flick or head shake	Slight unilateral droop	Head tilt or bilateral droop
Cough	None	Induced single cough	Induced repeated coughs or occasional spontaneous cough	Repeated spontaneous coughs
Joint inflammation	No inflammation present	Slight swelling; not warm or painful	Swelling with pain or heat; slight lameness	Swelling with severe pain, heat and lameness
Navel inflammation	Normal (pencil size); no heat, swelling, or discharge	Bigger than normal (width of the pointer finger); no heat, swelling, or discharge	Bigger than normal (width of 2 fingers combined); slight pain or moisture	Bigger than normal (width of 3 fingers combined); heat, pain, or malodorous discharge

1. The scoring system was adapted from previously validated studies (Fecteau et al. 1997; Pempek, 2019).
2. Original version of that table was published in Garcia et al. 2020 J. Dairy Sci. 105:807–817

A definitive diagnosis of sepsis is based on a blood culture. However, the sensitivity of blood culture can be low, and a negative result must be interpreted cautiously (Fecteau et al., 2009). In veterinary medicine, clinical signs combined with a complete blood count (CBC) and scores are considered helpful in diagnosing sepsis. However, sepsis-specific biomarkers have become a recent focus of research in both humans and veterinary species because they could potentially increase diagnostic accuracy and efficiency (Ercan et al., 2014, Fielding and Magdesian, 2015, Eichberger et al., 2022, Gude et al., 2022).

As blood culture is not always possible in the field, can be cost-limiting for some owners and is time-sensitive, it is not always practical on a farm while still recommended in a veterinary hospital. In humans, leukogram and C-reactive protein are commonly used for better accuracy in diagnosing sepsis, but still recommend assay to a lab. Detection of urine catalase activity by on-farm tests such as the Uriscreen can improve farm diagnosis. The test measures catalase activity in bacteria at a threshold of 50,000 CFU/ml. It is a helpful test for urinary tract infections. Still, septicemia as bacteriuria appeared because of bacteremia in two-thirds of human cases and as the initial cause in the other one-third (Lee et al., 1978). In adult humans with *Staphylococcus aureus* bacteremia, bacteriuria is detected within 48 hours after a positive blood culture (Lee et al., 1978). The test in LPS-inoculated colostrum-free calves shows good concordance with Uriscreen tests with bacteremia and bacteriuria, with 0.86 and

0.88, respectively (Raboisson et al., 2010). Kappa value of agreement between Uriscreen and bacteremia and bacteriuria were 0.73 and 0.76, respectively. The sensitivity of Uriscreen for bacteremia and bacteriuria was 80.0 and 86.6%, respectively, and specificity was 92.8 and 88.8%, respectively, for this study (Raboisson et al., 2010). The authors suggest that Uriscreen could be used to detect bacteremia in neonatal calves, but a large study looking at actual clinical cases is missing.

In calves, PGE2, malondialdehyde (MDA), IL-8, TNF-α, IFN-y, neopterin and procalcitonin have been evaluated in a single study (Ercan et al., 2016). The last four were significantly increased in *Escherichia coli* septicemic calves. This study used 15 septic neonatal calves with septicemic colibacillosis presented at the clinic and 15 healthy ag-matched control calves. Ercan et al. (2016) also suggest procalcitonin as the best marker as it increased four times, had a long half-time life, and remained stable at room temperature. Bonelli et al. 2018 looked at plasma procalcitonin (PCT) concentration to diagnose calves with septic systemic inflammatory syndrome (SIRS). Using a commercial ELISA assay for cattle, they found that plasma PCT concentrations in septic SIRS were four times above the value in healthy calves, similar to Ercan et al. 2016. They establish an optimal cutoff value of 67,39 pg/ml (81.0% sensitivity, 95.0% specificity) to predict SIRS (Bonelli et al., 2018). They also found a statistically significant difference in the survivor group with SIRS compared to the non-

survivor, showing that PCT could also be used as a prognostic indicator (Bonelli et al., 2018). A more recent study from Turkey looked at procalcitonin and neopterin in neonatal calves with sepsis. They found results similar to those of the procalcitonin group, Ercan et al., 2016; Bonelli et al., 2018. Neopterin was also increased about four times above the control concentration in septic calves. The exciting findings of this paper were that they followed PCT and neopterin concentration during the treatment of the calves with an antibiotic over five days, and it was possible to see a decrease of both molecules over time with healing of the animals (Akyuz and Gokce, 2021). Physical examination findings showed a positive correlation between neopterin and procalcitonin with pulse per minute and respiratory rate. Another interesting result was that in this study, calves suffering from neonatal sepsis due to a viral agent had similar values of both PCT and neopterin to calves suffering from bacterial septicemia (Akyuz and Gokce, 2021).

Kirbas et al. 2019 looked at various inflammatory markers as possible diagnostic and prognostic indicators in neonatal calves with septicemia. In their study, they found that TNF-α, IL-6, PCT, haptoglobin (Hp), and fibrinogen (Fb) concentrations were higher on day 0 in the septic group than in the control group ($P < 0.05$). After treatment (on day 15), the serum IL-6, PCT, Hp, and Fb concentrations were significantly decreased in the septic calves compared to the control calves ($P < 0.05$). The serum iron (Fe) concentrations were lower on day

0 in the septic group than in the control group ($P < 0.05$) and were higher on day 15 than on day 0 in the septic calves ($P < 0.05$). Developing a cow-side test with this molecule will be necessary to be practically valuable for on-farm decision-making.

Hypoglycemia was evaluated in a large cohort of calves presented to a teaching hospital (Trefz et al., 2016). Glycemia has the advantage of being easily measurable in the field. A total of 10,060 hospitalized calves' medical records were looked at. The calves were all less than 22 days old. Severe hypoglycemia was defined as plasma glucose concentration < 2 mmol/L (House et al., 2020). Overall, about 10% of all calves presented with severe hypoglycemia. In this study, calves with sepsis were more likely to be hypoglycemic (61%) vs normoglycemic (22%). Calves with SIRS presented a higher proportion of all sick calves in both hypoglycemic (81%) and normoglycemic (66%) groups. In this study, hypoglycemia was associated with severe disorders at admission and a lower chance of survival. The authors cautioned not to use severe hypoglycemia on arrival as the predictive value of septicemia but more a suspicion of the calf's clinical evidence of septicemia, hypothermia, acute abdominal emergencies, and history or clinical evidence of malnutrition (Trefz et al., 2016).

Therapeutical options

Sepsis therapy has three priorities:

1. Immediate stabilization of the patient (airway, breathing, circulation)
2. Removal of bacteria from the bloodstream as fast as possible and treatment of the original foci of infection
3. Support the animal during the critical phase.

The key for successful therapy for sepsis is to be early and with the appropriate treatment, meaning to choose a susceptible antibiotic. A delay in initiating appropriate treatment was associated with an increased mortality risk of roughly 6% per hour (Dellinger et al., 2013). For this reason, proper cultures should be obtained as soon as possible. Broad-spectrum antibiotics should be used until the culture results return (Evans et al., 2021). The choice and the dosage of antibiotics depend on several factors, such as the initial site of infection (for the molecule to reach the site), the spectrum wanted to be covered and, most likely, the pathogen's susceptibility to antibiotics (Evans et al., 2021). The intravenous route should always be preferred when possible (Evans et al., 2021).

Control of the inflammatory response is essential, especially in the early stage of sepsis, to control the cytokine storm. Most clinicians prefer the use of nonsteroidal anti-inflammatory drugs (NSAIDs)for animals that are in septic shock. Flunixin meglumine (0.25–0.33 mg/kg three times per day) has been widely used in bovine and equine intensive care units (Fecteau et al., 2009).

Possible side effects of aggressive NSAID treatment include abomasal ulcers and renal toxicity, particularly in dehydrated animals (Fecteau et al., 2009). The decision to use NSAIDs in any patient is based on the presence of hematologic evidence of left shift and neutrophilic toxic changes. It should be limited to 2 to 3 days of treatment because of potential toxicity. Nonsteroidal anti-inflammatory drugs should not be continued longer than they are essential for survival. Regarding anti-inflammatory drugs, corticosteroids are controversial in human and veterinary medicine (Pardon and Deprez, 2018, Evans et al., 2021). The advice of the 'surviving sepsis campaign' is against their use in sepsis or septic shock responding to fluid therapy. If fluid therapy and vasopressors fail to restore blood pressure, corticosteroid administration is suggested (Rhodes et al., 2017). The same recommendation has been found for septic calves (Fecteau et al., 2009). Surprisingly and totally in contrast to animal models where NSAIDs display beneficial effects for mortality, human studies have failed to demonstrate the clinical utility of NSAIDs in sepsis treatment (Pardon and Deprez 2018). Work on experimental endotoxemia in calves has shown no effects of corticosteroids, whereas ketoprofen completely alleviated all symptoms (Plessers et al., 2012, Plessers et al., 2016). Flunixin meglumine alone at 1.1 mg/kg or a combination of dexamethasone (2.0 mg/kg) and flunixin meglumine improve lactic acid levels, arterial bicarbonate, mean arterial pressure, pulmonary arterial pressure, and cardiac output (Margolis et al., 1987). The effect of flunixin alone was only a short

time (1 hour) compared to the combination of both products (6 hours) (Margolis et al., 1987).

Additionally, to antibiotic treatment and NSAIDs, supportive treatments for septicemic patients are critical for success. Supportive therapies for septicemic patients include providing warmth and good bedding, correction of secondary problems, intravenous fluids, vasopressor, plasma transfusion, oral or parenteral nutrition, and oxygen administration. In severe sepsis or septic shock, fluid therapy with isotonic saline is recommended at 30 mL/kg body weight in 5-20 minutes to sustain blood pressure (Dellinger et al., 2017). Glucose administration is controversial but common in pediatric medicine and likely needed in the hypoglycemic calf. Dextrose (2.5%–5%) combined with normal saline (0.9%) should be administered at a rate of at least 50 mL/kg/24 hours (House et al., 2020). Plasma transfusion (1–2 L of plasma from an adult donor negative to Bovine Leukosis Virus [BLV] or 20-40 ml/kg/day) may be used (Balcomb and Foster, 2014). Hypertonic sodium bicarbonate (8.4%) can correct metabolic acidosis if the base deficit exceeds 10 mmol/L (Fecteau et al., 2009).

Nutrition is essential to support the organism metabolism and septicemic neonates either as partial or total anorexia. As a result, they do not ingest an adequate amount of milk. If the animal refuses to nurse, tube feeding should ensure that the calf drinks at least 10% to 15% of its body weight in milk per 24

hours (Fecteau et al., 2009). The feeding schedule may involve several daily feedings (three to five). It should start with small amounts (250 to 500 ml), which should be gradually increased in quantity to prevent abdominal distention. If the gastro-intestinal system does not tolerate the tube-feeding regimen, parenteral nutrition should be considered. There is usually no need for total parenteral nutrition because most animals continue to nurse or tolerate tube feeding up to 5% of their body weight. Parenteral nutrition should be started at 30% maintenance for 24 hours to avoid possible consequences and increase as needed (House et al., 2020). Partial parenteral nutrition is less expensive, more straightforward to manage, and provides enteral nutrients to maintain gastro-intestinal system functions.

Calves suffering from hypoxia without hypercapnia may benefit from intranasal oxygen insufflation (5–10 L/h) with the help of a nasal cannula. If hypoventilation is present, mechanical ventilation becomes the treatment of choice (Evans et al., 2021). Ventilation of calves may not be feasible, depending on the economics of the situation and is not an option in the field.

Appropriate nursing care should be provided and emphasized to the client or the calf caretaker. Optimal temperature (not cold or warm) and ventilation are essential. A heat lamp or a rescue blanket can be used to support cold animals. Septicemic newborns tend to lie down most of the time, and appropriate bedding

(heavy thickness of straw) is essential to prevent skin ulceration around joint areas (Fecteau et al., 2009). Straw is superior bedding to shavings because it provides better insulation and tends to stay dry on the surface while watery feces or urine settle to the bottom of the pen. Fecal material must be washed from the perineum regularly to prevent accumulation, ulceration and myiasis. The eyes of laterally recumbent animals should be checked repeatedly for corneal ulcers (Fecteau et al., 2009). Eye lubricant can be used twice a day to decrease corneal dryness.

Novel strategies for the treatment of sepsis

As described in the previous section, conventional management of sepsis has been adequate for early sepsis resuscitation and significantly improved clinical outcomes. Recognized as the hots-mediated systemic inflammatory responses to infection, studies have focused on reducing the immune cell activation and controlling the dysregulated host responses, mainly in severe sepsis, by inhibiting specific components involved with inflammatory responses such as cytokines. Potential novel therapeutic strategies for sepsis that have been proposed so far could be classified into six categories (Zhang and Ning, 2021):

1. Targeting DAMPs
2. Targeting PAMPs
3. Targeting inflammatory mediators

4. Immune checkpoint modulation
5. Endothelial barrier stabilization
6. Restoration of vascular anticoagulation properties

Some examples of the different therapeutic strategies included extracorporeal blood-purification (Borthwick et al., 2017), TLR-4 agonist (Rice et al., 2010), nanoparticles (Spence et al., 2015) and some traditional Chinese herbs such as Kukoamine B (Liu et al., 2011)or Xuebijing (Wang et al., 2015). One particular protein acting as DAMP has been proposed in the last few years to reduce cytokine storms in COVID-19 patients (Tanwar et al., 2021). This protein, called High Mobility Group Box 1, was evaluated in the last two decades as a possible target to reduce sepsis-related mortality.

High mobility group box 1

High mobility group Box 1 protein (HMGB1) is a nuclear protein that acts as a DNA chaperone and participates in several activities in the nucleus, including transcription, replication, DNA repair and nucleosome assembly (Venereau et al., 2016). Typically, HMGB1 is mainly located in the nucleus and
Binds to chromatin but can move to the cytoplasm under various stress conditions and eventually into the extracellular space (Chen et al., 2022). HMGB1 is highly conserved through evolution and possesses 99% identity among all mammals (Bae, 2012). HMGB1 shows a crucial role in development

and reproduction as HMGB1-depleted mice would die shortly after birth or in utero, leading to lethal hypoglycemia (Calogero et al., 1999). HMGB1 has been identified as a pro-inflammatory protein secreted by innate immune cells in response to pathogenic products and released by injured or dying cells (Andersson and Tracey, 2011).

Structure and function of HMGB1

Human high-mobility group box 1 is a 215-amino-acid protein of approximately 30 kDa. Structurally, the HMGB1 protein consists of three domains: two DNA-binding domains, namely the A-box and B-box, and a negatively charged acidic tail, namely the C terminus. HMGB1 contains three redox-sensitive cysteine moieties (C23, C45 and C106) (Tang et al., 2023). Bovine HMGB1 is like humans in the difference, showing only one amino-acid change in the acidic tail (Gougeon et al., 2012). Murine HMGB1 has two differences in the acidic tail and one amino-acid change in the B box, and canine HMGB1 shows 100% homology with their human counterpart (Gougeon et al., 2012). The functions of HMGB1 depend on its subcellular location. Under normal physiological conditions, HMGB1 is found mainly in the nucleus owing to its two nuclear localization signals (NLS1 and NLS2). Nuclear HMB1 is a DNA chaperone essential in maintaining chromosome structure and functions (DNA replication, transcription, repair, and chromatin remodelling). Furthermore, the A-box (residues 38–61) of HMGB1 interacts with tumour protein p53 (TP53) to enhance its transcriptional activity.

Extracellular HMGB1 functions as a damage-associated molecular pattern (DAMPs) or danger signal under conditions of stress and mediates immune responses through interacting with various receptors, including Toll-like receptor 4 (TLR4) and advanced glycosylation end product-specific receptor (AGER or RAGE). HMGB1 has been identified as a mediator of endotoxin lethality leading to the excretion of inflammatory stimuli by macrophages and monocytes, including interferon-γ, which can exacerbate the cytokine storm in sepsis by activating NF-κB and IRF signalling pathways (Rendon-Mitchell et al., 2003). In addition, extracellular HMGB1 is a mediator of sterile inflammation by its release by necrotic cells and cells undergoing stress and cell death (Tang et al., 2023). Extracellular HMGB1 regulates immunity by forming complexes with other factors. For example, the HMGB1-LPS complex enhances TLR4-dependent cytokine production and promotes LPS uptakes by RAGE and subsequent inflammasome activation in macrophages (Deng et al., 2018). Extracellular HMGB1 also has activities that promote cell growth and tissue regeneration and, in some cases, limit bacterial proliferation.

Cellular autophagy is a lysosome-dependent cellular process by which cells break down and recycle their cellular components. Autophagy is a crucial defense mechanism that promotes cell survival and controls infection. Cytosolic HMGB1 drives autophagy by binding to the autophagy core protein Beclin-1 (Zhu

et al., 2015). However, excessive autophagy can sometimes lead to cell death, which triggers the release of DAMPs (including HMGB1) and results in inflammation (Tang et al., 2023). HMGB1-dependent autophagy has been implicated in several inflammatory diseases, including inflammatory bowel disease and *Listeria monocytogenes* endotoxemia. In this case, cytosolic HMGB1 plays a role in defense against infection as loss of HMGB1 increases susceptibility to illness and inflammatory bowel disease (Yanai et al., 2013, Zhu et al., 2015). Cytosolic HMGB1 also enhances nucleic acid-induced immune responses owing to its DNA-binding activity.

Membrane HMGB1 may mediate neurite growth and platelet activation. In addition, translocation of HMGB1 to the cell membrane of platelets and subsequent release is involved in neutrophil extracellular trap (NET) formation during infection or tissue damage following response to oxidative stress signals (Tang et al., 2023). Platelet-derived HMGB1 is also a driver of thrombosis through interaction with TLR4 in the platelet plasma membrane (Vogel et al., 2015, Vogel et al., 2016).

Release of HMGB1 in the extracellular space

The release of HMGB1 occurs during two different mechanisms: active and passive. Passive release initiated by damage to cellular integrity is nearly instantaneous. At the same time, active secretion occurs more slowly, starting 8-12 hours after ligation with Toll-like receptors (TLRs) (see Fig. 3) (Andersson and

Tracey, 2011). Active secretions of HMGB1 occur more slowly and are secondary to cellular signal transduction through plasma membrane receptor interaction with extracellular products. The active secretion occurs when immune cells, especially monocytic cells, are exposed to microbe-associated molecular patterns (MAMPs), pathogen-associated molecular patterns (PAMPs), and other inflammatory mediators such as TNF-α, IL-1, and IFN-γ. Other cells that can actively secrete HMGB1 are neurons, astrocytes, erythroleukemia, neuroblastoma, and several tumour cells. Following activation of macrophages with LPS, HMGB1 mRNA level rises over several hours and will remain elevated for 24-48 hours post-stimulation.

For this reason, HMGB1 is considered a delayed pro-inflammatory cytokine. The passive release of HMGB1 comes from the necrotic cells and monocytes activated by exposure to apoptotic cells. HMGB1 passively releases from necrotic cells is a potent stimulator of TNF production.

High mobility group box 1 in sepsis

Bacterial endotoxin has been shown to stimulate macrophages to release HMGB1 through CD14 and TNF-α dependent mechanisms (Chen et al., 2004). HMGB1 has potent inflammatory effects and acts as a DAMP when released from the cells (Wang et al., 2014). Once released from the cell, HMGB1 trigger massive inflammatory cytokine production from immune cells secondary to stimulation of various signalling pathways by recognition by cell surface receptors

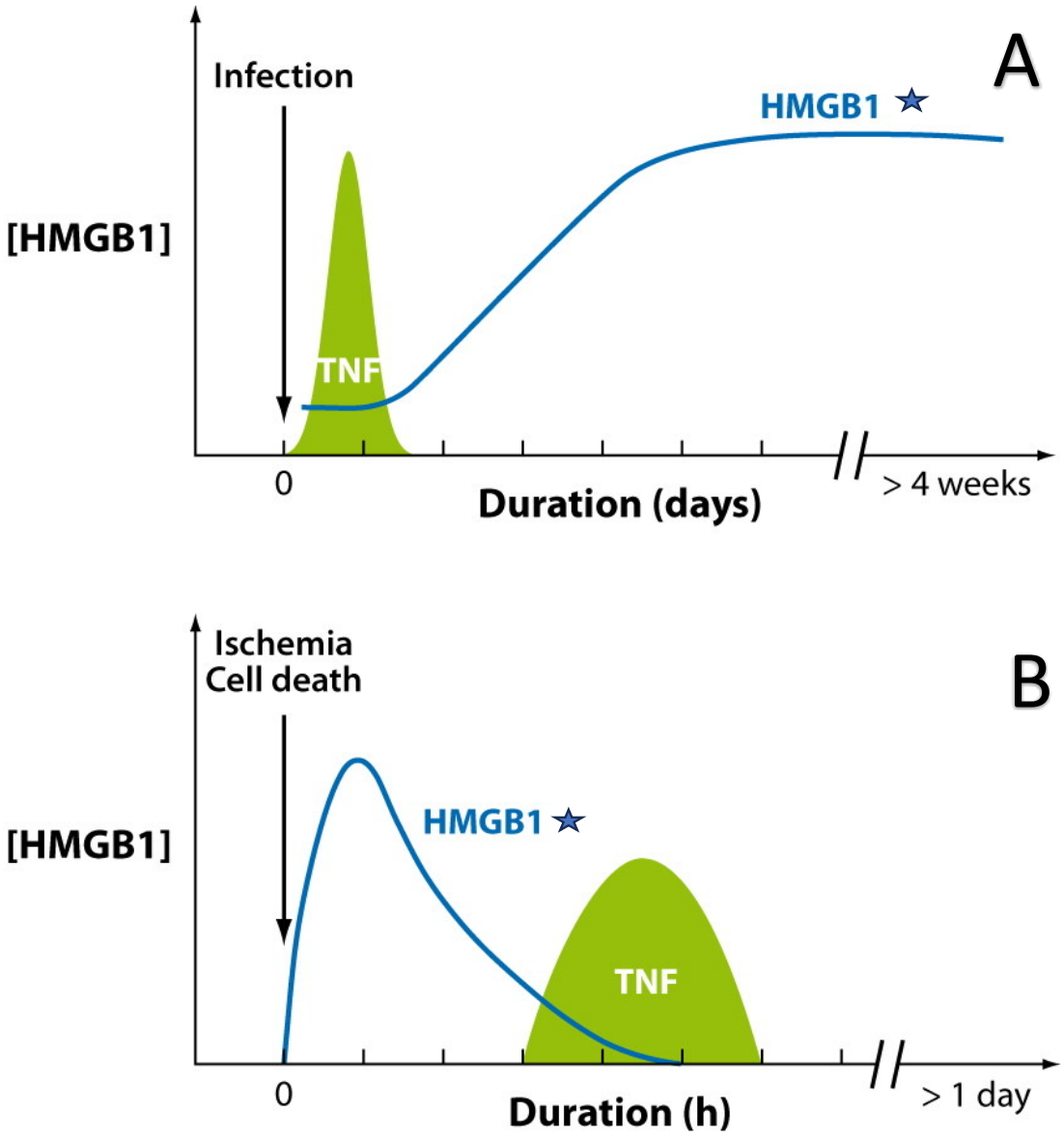


Figure 3. Release of high mobility group box-1 A) following response to infection, B) following cell death. It is modified from Andersson and Tracey 2011.

such as TLR4 and RAGE (Wang et al., 2014). The level of HMGB1 usually begins to appear 8 hours post-induction of sepsis and will peak around 16 to 32 hours in mice (Yang et al., 2015). Exposure to HMGB1 leads to the release of tumour necrosis factor (TNF), interleukin (IL)-1, IL-6, IL-8, and macrophage inflammatory protein (MIP)-1 (Yang et al., 2015). In association with RAGE, HMGB1 mediated the release of chemotaxis agents and cytokine by macrophages and endothelial cells. Anti-RAGE antibodies have been shown to partially inhibit HMGB1-induced chemokine and cytokine release in endothelial cells (Fiuza et al., 2003). TLR2 or TLR4 activation by HMGB1 is needed to activate macrophage cytokine release fully (Bae, 2012). In vitro studies have shown that HMGB1 upregulates TNF mRNA and protein expression in human primary blood mononuclear culture with an initial peak at 4 hours post-injection of HMGB1 and 10 hours post-infusion (Andersson et al., 2000). HMGB1 increases the excretion of pro-inflammatory cytokines such as TNF, IL-1, IL-6 and IL-8 but also acts on microvascular endothelial cells, leading to the production of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and increasing the inflammation at this level (Fiuza et al., 2003). At the neutrophil level, HMGB1 enhances the expression of pro-inflammatory cytokine in a NF-κB-dependent fashion, which plays a role in neutrophil activation during infection or inflammation (Bae, 2012).

Response to Gram-negative endotoxin leads to an early peak of TNF, IL-1 and IL-6, while HMGB1 releases are seen 18-24 hours after the onset of endotoxemia (Andersson and Tracey, 2003). Animals exposed to higher levels of recombinant HMGB1 develop signs of disease such as weight loss, piloerection, decreased mobility, increased somnolence, and fever (Andersson and Tracey, 2003). High doses would lead to lethality. In cattle suffering from mastitis, HMGB1 concentration has been detected in milk, showing and correlated to the severity of mastitis (Furukawa et al., 2011). In septic patients, such as those during experimental cecal perforation, HMGB1 increases in the serum between 24 and 48 hours (Yang et al., 2004). Administration of anti-HMGB1 antibodies to animals with sepsis has improved survival and helps decrease clinical signs, leading to the hypothesis that treatment reducing HMGB1 could improve patients.

Way to reduce High mobility group box 1 effect in sepsis

Blocking the release and activity of HMGB1 has been used with great success in a wide range of preclinical inflammatory disease models (for example, endotoxemia and polymicrobial sepsis), confirming that HMGB1 is an attractive therapeutic target for acute infectious and sterile inflammatory conditions. Experimental strategies that selectively target HMGB1 and TLR4 effectively reverse and prevent activation of innate immunity and significantly attenuate damage in diverse animal models. More specifically, on sepsis,

HMGB1 has been the target of choice for different disorders. Here are a few ways that have been investigated to reduce the impact of HMGB1, mainly in gram-negative sepsis.

Administration of a low dose of heparin to a level that doesn't affect coagulation has been assessed. Heparin has been found to have potent anti-inflammatory effects and present a high affinity for HMGB1. It changes the conformation of HMGB1 after binding to it, reducing the affinity of HMGB1 for its receptor (Li et al., 2015). Heparin at a dose of 0.1,1 or 10U/L was shown to reduce mortality in mice exposed to LPS by blocking the binding of HMGB1 to the receptor at the surface of macrophages and suppressing the phosphorylation of p38 and dERK1/2 but not JNK (Li et al., 2015). However, heparin alone did not affect LPS-induced production of TNF-α (Li et al., 2015). Interestingly, low doses of heparin failed to protect mice against Gram-positive sepsis, probably due to LPS and gram-positive superantigens generating pro-inflammatory cytokines by different cell lines (Andersson and Yang, 2022).

Autoantibodies against HMGB1 improved outcomes in septic shock patients (Barnay-Verdier et al., 2011). Several preclinical studies have shown some success with Gram-negative sepsis patients (Zhu et al., 2021) but failed to decrease the mortality rate in a cecal ligation puncture (CLP) sepsis model when given 36 hours post-onset of CLP.

Control of the release of acetylcholine by vagus nerve stimulation has shown promising results in chronic inflammatory processes associated with the release of HMBG1, such as rheumatoid arthritis (Koopman et al., 2016) and inflammatory bowel disease (Bonaz et al., 2016) but also in patient with experimentally induced sepsis or endotoxemia (Pavlov et al., 2018). The main problem to date is the need for surgery to implant the device for stimulation in unstable patients.

Finally, preserving mitochondrial integrity using molecules such as ethyl pyruvate seems to confer significant protection against experimental Gam-negative sepsis and endotoxemia-induce lethality in rodents (Ulloa et al., 2002) and other animal models (Yang et al., 2016). This molecule will be further discussed in the next section.

Ethyl pyruvate

Pyruvate, the anionic form of 2-oxo-propionic acid, is the final product of glycolysis and the starting substrate for the tricarboxylic acid cycle (TCA cycle), also known as Krebs cycle (Fink, 2007). Pyruvate is a known endogenous scavenger of reactive oxygen species (ROS), reducing hydrogen peroxide (H_2O_2) nonenzymatically in a reaction that yields carbon dioxide and water. In addition to H_2O_2, pyruvate can also scavenge hydroxyl radical (OH-), another highly ROS (Fink, 2004a, 2007, 2008). Pharmacological administration of pyruvate can

improve organ function in animal models of oxidant-mediated cellular injury (Fink, 2008, Kao and Fink, 2010) however, pyruvate is unstable in aqueous solution, which limits its therapeutic potential.

Aqueous pyruvate solutions undergo spontaneous aldol-like condensation reaction to form 2-hydroxy-2-methyl-4-ketoglutarate, para pyruvate, which is a potentially toxic inhibitor of mitochondrial function (Kao and Fink, 2010). Parapyruvate is a potent inhibitor of a critical step in the mitochondrial TCA cycle, preventing the formation of succinyl Coenzyme A by blocking the enzyme 2-ketoglutaric acid dehydrogenase. 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 (Kao and Fink, 2010). Initial studies by Mongan et al. 1999 using a swine hemorrhagic shock model evaluated a 30% pyruvate incorporated into a saline solution (Mongan et al., 1999). Large volumes of saline solution were given to achieve the therapeutic effects of pyruvate, resulting in serum hypernatremia. As a sequel to this study, a new fluid type was developed to administer pyruvate as a non-hyperosmolar solution. This solution was ethyl pyruvate in lactated Ringer's solution or Ringer's ethyl pyruvate (REPS). Sims et al. 2001 used that solution compared to sodium pyruvate dissolved in a Ringer-like solution and found that REPS was significantly more protective than pyruvate anion (Sims et al., 2001). Similar findings indicating that ethyl pyruvate is more effective than

pyruvate were reported by Varma et al. 1998, who compared the two compounds in an in-vitro study of redox-mediated cellular injury (Varma et al., 1998). Since that publication, more than 500 peer-reviewed papers have looked at ethyl pyruvate.

Ethyl pyruvate (EP) is the ethyl ester of pyruvate. This form is more stable than pyruvate in a buffered solution, although still labile in aqueous solution. When it is dissolved in water, EP gradually undergoes spontaneous hydrolysis to form pyruvic acid and ethanol and also gradually undergoes other reactions, including hydration to create the gem diol, ethyl-2,2-dihydroxy-propanoate as well as the ethyl ester analogue of para pyruvate (Kao and Fink, 2010). For this reason, EP solutions have been prepared just before administration to experimental animals or human subjects in many subsequent preclinical and clinical studies (Kao and Fink, 2010).

Anti-inflammatory effects of ethyl pyruvate

Several studies support EP as an effective anti-inflammatory agent (Fink, 2004b, a, 2007, 2008, Yang et al., 2016). We will discuss the broad anti-inflammatory properties of ethyl pyruvate. A specific discussion on its effects in sepsis and endotoxemia animal models will be discussed later.

The initial work that showed a possible anti-inflammatory effect of EP came from the studies carried out by Yang et al. 2002 and Venkataraman et al. 2002. (Venkataraman et al., 2002, Yang et al., 2002). They showed that a solution of EP down-regulated activation of a pro-inflammatory transcription factor, NF-kB, and reduced expression of several pro-inflammatory proteins such as TNF-α, IL-6, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) in liver and intestinal mucosa (Yang et al., 2002). On their side, Venkataraman et al. 2002 decreased circulating levels of IL-6 and nitrite/nitrate and increased IL-10 in an *Escherichia coli* LPS rat shock model (Venkataraman et al., 2002).

Further evidence for the anti-inflammatory properties of EP was provided by the results of Ulloa et al. 2002. They showed that Ep could reduce TNF-α secretion in an induced model using RAW 264.7 murine macrophage-like cells. Ep was also able to inhibit recreation of the pro-inflammatory DNA-binding protein, high mobility group box 1 (HMGB1), from those cells, but also decreasing circulating levels of HMGB1 in septic mice (Ulloa et al., 2002). In a model murine model of colitis, Davé et al. show that EP significantly decreased the content of HMGB1 in fecal material from IL-10 -/- mice and improved the severity of colitis by reducing histology score and colonic wall thickening (Dave et al., 2009). Since that discovery, many other studies have shown that EP effectively inhibits the pro-inflammatory HMGB1 in multiple models of disease,

such as ischemia-reperfusion injury (Lu et al., 2021), inflammatory bowel disease (Guo et al., 2015), hemorrhagic shock (Dong et al., 2010), myocarditis (Yu et al., 2016), pancreatitis (Yang et al., 2008, Matone et al., 2013) and several others organ injuries (Vyawahare et al., 2012, Yang et al., 2016).

Ethyl pyruvate mechanisms for anti-inflammatory effects

Ethyl pyruvate has been extensively studied for its anti-inflammatory effects. To date, four hypotheses have been proposed to explain its capacity to reduce inflammation:

- Decreased NF-kB-dependent signalling due to decreased intracellular glutathione concentration.
- Decreased NF-kB-dependent signalling due to covalent modification of p65 and p50.
- Decreased JAK/STAT-dependent signaling due to scavenging of ROS.
- Inhibition of the enzyme glyoxalase-1 increases intracellular levels of methylglyoxal.

Ethyl pyruvate mitigates inflammation by blocking the production of pro-inflammatory cytokines and substances produced later in the inflammatory cascade. Nuclear factor kappa B (NF-κB) is a central mediator in mammalian inflammatory immune responses. There are currently five known NF-κB proteins: p65(RelA), p50, RelB, p52, and C-Rel (Fink, 2008). These proteins form a variety

of homodimers and heterodimers in the cell's cytosol. These compounds are readily held in an inactive state by the inhibitory protein IκB (IκBa, IκBβ, IκBε and Bcl-3). Upon stimulation of the cell by a pro-inflammatory trigger such as cytokines TNF-α or IL-1β or bacterial endotoxin-like lipopolysaccharide (LPS), the IκB is phosphorylated and later cleaved. Once the inhibitory protein IκB) is cleaved off, the homodimer or heterodimer intracellular cytoplasmic mechanisms can be turned on to allow nuclear protein synthesis to occur, leading to NF-κB) activation.

The first hypothesis is that proteins from the NF-κB family share a similar characteristic motif in the DNA-binding region. This motif consists of one cysteine and three arginine residues in a RxxRxRxxC pattern. It has been shown that the sulfhydryl group of this cysteine residue is essential for DNA-binding and transcription-activating activity, mainly on protein p65 or RelA. Because of the three positively charged arginine, this region is very susceptible to oxidation (Kao and Fink, 2010). Intracellular antioxidant glutathione interferes with binding to DNA by the activated NF-κB complex. Depletion of cellular glutathione led to down-regulation of NF-κB activation in the presence of LPS or TNF-α induced inflammation (Kao and Fink, 2010). Song et al. 2004 demonstrated using stimulation of RAW 264.7 murine macrophage-like cells with LPS significantly increased intracellular levels of GSH relative to those observed in unstimulated cells. Still, this effect was prevented by co-incubation of the cells with EP (Song

et al., 2004). EP inhibited LPS-induced DNA binding by NF-kB, but, if the cells were co-incubated with the cell-permeable glutathione analogue, glutathione ethyl ester, EP-mediated inhibition of LPS-induced NF-kB activation was prevented Song, Kellum et al. 2004). These data support the notion that EP may inhibit signaling by NF-kB, partly by changing intracellular redox balance in a way that favors oxidation (mixed disulfide formation) at a key cysteine residue in one of the subunits of the pro-inflammatory transcription factor.

The second hypothesis suggests EP can suppress inflammation by acting on NF-κB proteins. Mizutani et al. 2010 showed that preincubation of 10 mM ethyl pyruvate with nuclear extract from A549 macrophage-like cells decreased the binding of both the p50 and p65 protein dimers in a time-dependent fashion (Mizutani et al., 2010). It can be inferred from this research that ethyl pyruvate blocks the movement of cytosolic NF-κB from translocation into the cell's nucleus. Specifically, ethyl pyruvate blocks the p50 and p65 NF-κB proteins, disrupting the NF-κB pathway and diminishing its nuclear effects (Mizutani et al., 2010). The result is suppression of pro-inflammatory cytokine production.

The third hypothesis proposes that EP modulates and inhibits pro-inflammatory signaling by acting as an antioxidant. This premise was supported by the fact that EP was shown to be more potent than pyruvate as an H_2O_2 and O^{2-} scavenger (Fedeli et al., 2007). Kim et al. 2008 performed several

experiments designed to test that hypothesis (Kim et al., 2008). They looked mainly at the effect of EP as a scavenger of ROS on the inhibition of JAK/STAT signaling. Janus kinase (JAK) and signal transducers and activators of transcription (STAT) proteins mediated intracellular signaling, activating man's immune and inflammatory responses. ROS generated by NADPH oxidase can initiate activation of the JAK/STAT signaling pathways. Kim et al. 2008 were able to demonstrate that EP inhibited LPS-induces expression of several JAK/STAT responsive genes (iNOS, COX-2, IL-1β, IL-6 and TNF), and inhibited LPS-induced tyrosine phosphorylation of the STAT proteins (STAT1 and STAT3) and JAK2 (Kim et al., 2008). These findings support the contention that at least some of the anti-inflammatory effects of EP are related to its ability to scavenge H_2O_2 and other ROS.

The last hypothesis is on a mechanism involving the enzyme glyoxalase-1 (Glo-1). Glo-1 is responsible for detoxifying the glycolytic byproduct methylglyoxal (MGO). MGO has been shown to down-regulated LPS-induced production of several cytokines, including TNF-αIL-1β and IL-6 in human whole blood culture (Hollenbach et al., 2008). Hollenbach et al. 2008, found that EP inhibited secretion of the same cytokines at the same level, suggesting that EP was inhibiting Glo-1, leading to intracellular accumulation of MGO (Hollenbach et al., 2008).

Ethyl pyruvate in animal models of septicemia

Ethyl pyruvate has diminished morbidity and improved survival in several preclinical sepsis models. Venkataraman et al. 2002 evaluated resuscitation with Ringer's ethyl pyruvate solution (REPS) or Lactated Ringer's solution (LRS) in a murine septic shock model (Venkataraman et al., 2002). The rats were given intravenous endotoxin (*Escherichia coli* LPS O111:B4). When the mean arterial pressure (MAP) decreased to less than 60 mmHg, the rats were randomly assigned to be resuscitated with either the LRS or REPS. Both solutions' compositions were as follows: LRS contained 109 mM NaCl, 4 mM KCl, 2.7 mM CaCl2, and 28 mM sodium L-lactate and pH ranged from 6.0 to 7.5. REPS contained 130 mM NaCl, 4 mM KCl, 2.7 mM CaCl2, and 28 mM ethyl pyruvate with a pH of around 7.0–7.2. A 3-5 ml volume of solution was administered as needed as bolus up to a total volume equal to 7% of the rat's body weight to maintain MAP above 60 mmHg. Survival time, plasma concentrations of TNF, IL-6, IL-10, nitrite/nitrate, rate of resuscitation and MAP were measured over the study period. Hypotension was seen 15 minutes following endotoxin administration. Although all rats died in this study, resuscitation with REPS significantly prolonged survival by about 2 hours (Venkataraman et al., 2002). Maintaining the MAP value above 60 mmHg requires similar fluid volume in both groups. Still, the REPS group had a significantly higher mean arterial pressure and lower blood lactate concentration, indicating improved perfusion and oxygen delivery and increased overall survival(Venkataraman et al., 2002). Plasma

concentration of TNF, IL-6, IL-10, and nitrite/nitrate increase significantly following endotoxin administration at 3- and 6-hours post-infusion (Venkataraman et al., 2002). IL-10 concentration was higher in the REPS group at 3 and 6 hours, while IL-6 and nitrite/nitrate concentrations were lower in that group compared to the LRS group. There was a significant increase in TNF expression in both treatment groups post endotoxin infusion.

Interestingly, either treatment group 3 or 6 hours post-infusion did not affect TNF expression. However, in most experimental models of acute endotoxemia, TNF is released into the circulation as a monophasic spike that becomes detectable soon after endotoxin injection and typically peaks about 60 minutes. In the present study, treatment with REPS or LRS was not initiated until about 15 minutes after the injection of LPS (Venkataraman et al., 2002). Thus, infusion of the experimental agent was delayed until after the initiation of the release of TNF. Accordingly, it is not surprising that TNF release was not affected.

Ulloa et al. 2002 showed that pretreatment of mice with ethyl pyruvate solution at different doses (40mg/kg, i.p., 4 mg/kg, i.p., or 0.4 mg/kg, i.p.) followed 30 minutes later by an injection of LPS improve survival. In this study, mice were injected with endotoxin (*Escherichia coli* LPS O111:B4) at a lethal dose (LD75) of 5 mg/kg intraperitoneal (i.p.) (Ulloa et al., 2002). The solution composition was as follows: sodium (130mM), potassium (4mM), calcium

(2.7mM), chloride (139mM), and ethyl pyruvate (28mM) with a final pH of 7.0. Solutions in mice were diluted so that each injection volume was 0.4 ml per dose. Pretreatment with a single EP at 40 mg/kg, i.p., conferred significant protection from lethal endotoxemia with a survival rate of 90% (27/40 mice). The lower doses only conferred partial protection, with 45% survival in the 4 mg/kg dosage group and no protection with the lowest dosage. Pretreatment with ethyl pyruvate significantly reduces the pro-inflammatory cytokine peak of IL-1β and IL-6 as well as TNF-α and HMGB-1, showing that ethyl pyruvate prevents endotoxin-induced lethality by attenuating the release of early (TNF-α and IL-1β) and late (HMBG-1) systemic mediators of lethality (Ulloa et al., 2002). In the same study, ethyl pyruvate was evaluated as a rescue therapy and given 4 hours after the onset of endotoxemia. Clinical signs of endotoxemia included diarrhea, piloerection, and depression. Delayed administration of a fixed dose of ethyl pyruvate (40mg/kg) produced significant attenuation of endotoxemia lethality (survival of 80%) even if the dose was given following the early peak of TNF-α noticed 1-2 hours post-induction of endotoxemia. The concentration of HMGB-1 20 measured 20 hours after the onset of endotoxemia was significantly reduced compared to the control and LPS-only group (Ulloa et al., 2002). As the rescue dose was given following an acute-phase response to endotoxin, this finding suggested that ethyl pyruvate targets late-acting mediators of endotoxin lethality, such as HMBG-1. Ethyl pyruvate's capacity to prevent lethality was also tested in a lethal sepsis model of cecal ligation and puncture. Mice were injected intraperitoneally with ethyl

pyruvate (40mg/kg) 24, 30, 48, and 54 hours after induction of septic peritonitis (Ulloa et al., 2002). 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 three weeks observation period after ethyl pyruvate administration (Ulloa et al., 2002). The protection against lethal sepsis was dose-dependent as lower dosage failed to confer any protection against death.

In a more recent study on sepsis using a murine model of cecal ligation and puncture (CPL), ethyl pyruvate ringer solution was given to mice at a 75 mg/kg dose immediately after the CPL procedure. Compared to the control group who only received ringer solution without ethyl pyruvate, ethyl pyruvate significantly attenuated the increase of IL-1β, IL-6, IL-10 and TNF-α during the first 6 hours following sepsis induction (Kang et al., 2016). The survival rate was also significantly higher in the ethyl pyruvate group at 6 hours post-induction of sepsis (80% vs 40%) (Kang et al., 2016).

As multiple organ failure is a drastic complication of sepsis, ethyl pyruvate was also evaluated to see if it can inhibit or protect against multiple organ injury to the kidney, muscle, and pancreas. Using the same dose as Ulloa et al. 2002, a group from the National Institutes of Health in Maryland looked at ethyl pyruvate's capacity to decrease acute renal failure and multiple organ damage to aged mice

as sepsis increased dramatically after 50 years of age. The group used a murine model of CLP and two doses of ethyl pyruvate (8 or 40 mg/kg) injected at 0, 6, or 12 hours after CLP surgery. The group found that a single dose of ethyl pyruvate inhibited muscle, hepatic and pancreatic injury at 24 hours. Similar findings were seen with injection at 6 and 12 hours, except that delaying the dosage of ethyl pyruvate at 12 hours had no impact on aspartate aminotransferase (AST) (Miyaji et al., 2003). Treatment with ethyl pyruvate at 8 or 40 mg/kg at any time reduced renal injury (Miyaji et al., 2003). Serum TNF-αAt 24 hours, it was decreased with both doses at 0, 6, and 12 hours after CLP surgery (Miyaji et al., 2003). Ethyl pyruvate was shown as a possible treatment for both sepsis and sepsis-induced renal and multiorgan injury. This prolonged window of opportunity is critical clinically because of the difficulty in detecting sepsis and sepsis-induced acute renal injury early in the disease process.

In addition to high mortality at the early stage, sepsis can cause sepsis-associated encephalopathy (SAE), which significantly increases the mortality of patients and largely influences the life quality of sepsis survivors. Sepsis-associated encephalopathy is a multifactorial syndrome characterized by diffuse cerebral dysfunction induced by the systemic response to the infection without clinical or laboratory evidence of direct brain infection or other types of encephalopathy (Chaudhry and Duggal, 2014). About 70% of survivors recovering from sepsis have cognitive dysfunction (Zhong et al., 2020). Ethyl

pyruvate was found to confer protection against SAE and significantly improved cognitive function during a murine CLP model (Zhong et al., 2020).

In vitro work was also performed in dogs, using canine peripheral mononuclear cells exposed to 100 ng/ml lipopolysaccharide (Escherichia coli O111: B4) (Yu et al., 2010). The cells were simultaneously treated with various concentrations of ethyl pyruvate (5 mM or 10 mM). RNA isolation was performed for cytokine gene expression of IL-6, IL-10, and TNF-α. ELISA also measured plasma cytokine concentrations in response to LPS and ethyl pyruvate. TNF-α and IL-6 were selected as representative of pro-inflammatory cytokines that are upregulated after LPS administration. IL-10 was selected as an anti-inflammatory cytokine. Lipopolysaccharide treatment produced a 150-, 1500- and 8-fold increase in TNF-α, IL-6 and IL-10 mRNA, respectively, at 3 to 6 hours (Yu et al., 2010). Cells 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 (Yu et al., 2010). In addition, the expression of IL-10 mRNA still increased 24 hours following treatment with 10 mM EP. All the cytokines in the control group (before LPS stimulation and only EP-treated) were below the detection limit. 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 downregulation of the pro-inflammatory

cytokines TNF IL-6 and upregulation of anti-inflammatory cytokine IL-10 (Yu et al., 2010).

Ethyl pyruvate is a possible approved drug for human

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 (Bennett-Guerrero et al., 2009). Cardiopulmonary bypass is an invasive procedure that diverts blood circulation away from your heart and lungs. Acute organ system support is commonly used in many heart surgeries. This technique is associated with acute inflammatory response characterized by transient increases in blood concentrations of pro-inflammatory cytokines such as TNF and IL-6 and the release of other pro-inflammatory mediators, such as nitric oxide and complement (Wan et al., 1997). During this study, patients were randomized to receive 7,500mg (roughly 150 mg/kg) of ethyl pyruvate infused over 60 minutes every 6 hours for 6 total doses or Lactated Ringer's solution following the same dosing regimen. This dose was extrapolated from a safety study in human volunteers (Fink, 2008). One hundred and two subjects were enrolled in the study. Fifty-three patients received the placebo treatment and 49 the ethyl pyruvate treatment. Unfortunately, the study failed to show any 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 (Bennett-Guerrero et al., 2009). The authors note several possible reasons for ethyl pyruvate's lack of clinical efficacy. Cardiopulmonary bypass may not cause sufficient systemic inflammation for ethyl pyruvate's effects to manifest. Second, the patients undergoing cardiopulmonary bypass may not have been at a high enough risk for comorbidity and mortality to detect the effect of ethyl pyruvate treatment. Third, the 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 crucial etiology of organ injury in high-risk cardiac surgery implementing cardiopulmonary bypass (Bennett-Guerrero et al., 2009). Despite positive results in numerous animal models, ethyl pyruvate failed to demonstrate a significant benefit to high-risk cardiac surgical human patients undergoing cardiopulmonary bypass.

Ethyl pyruvate in equine endotoxemia

Ethyl pyruvate was tested mainly in humans and rodents as animal models to humans to use in human medicine. In veterinary medicine, ethyl pyruvate was evaluated in dogs (Yu et al., 2010), swine (Hauser et al., 2005, Mulier et al., 2005, Andersson et al., 2006, Dong et al., 2010), sheep (Su et al., 2007) and bovine (Corl et al., 2010). Horses were well-studied in 2010. One of Dr. Susan Holcombe's master's student at Michigan State University, Dr. Eric Lee Schroeder, looked at the molecule during his master's degree. His initial works were on ethyl pyruvate's safety and biological efficacy in normal mature

horses. His study consisted of infusing four different doses of ethyl pyruvate (0,50, 100, and 150 mg/kg) mixed into a 1-liter bag of Lactated Ringer solution to 5 normal healthy horses in a crossover design. Another objective of his study was to stimulate equine whole blood with endotoxin from the same horse previously treated with ethyl pyruvate. He showed that ethyl pyruvate infusion was safe and that a dosage of 150 mg/kg of ethyl pyruvate was able to significantly reduce TNF-α, IL1β and IL-6 gene expression 6 hours after receiving the molecule (Schroeder et al., 2011). In vivo, the challenge was also performed in horses using 30 ng/kg of LPS (*Escherichia coli* O111:B4) and the 150 mg/kg dose of ethyl pyruvate mixed into 1 liter of lactated Ringer's solution (Jacobs et al., 2013). Ethyl pyruvate decreased gene expression of IL-6 and TNF-α, but had no impact on IL-8 and fever. Compared to flunixin meglumine, ethyl pyruvate was better at suppressing the expression of pro-inflammatory cytokines, but flunixin meglumine had a superior antipyretic effect. Both showed an excellent capacity to improve the clinical outcome of endotoxemia compared to the control group (LRS only).

In another in vitro experiment, ethyl pyruvate reduced pro-inflammatory gene expression following incubation with LPS-stimulated equine monocytes (Cook et al., 2011). Ethyl pyruvate reduced IL-8 expression at a concentration of 5mM and reduced expression of Il-8, TNF-α and COX-2 at 10 mM. Surprisingly, it did not affect IL-1β and IL-6 gene expression, but only in incubation time for 1

hour. Bauquier et al. compared four compounds with potential anti-endotoxic effects, namely rolipram, azithromycin, ethyl pyruvate and metformin, using in vitro equine whole blood stimulated with bacterial lipopolysaccharide (*Escherichia coli* O55:B5) (Bauquier et al., 2015). In this study, all molecules could inhibit TNF-α and IL-1β production. Inhibition of both cytokines was concentration-dependent with an inhibition activity of IL-1β by ethyl pyruvate at 10 mM). However, almost complete inhibition at 30 mM showed an importance in dosage requirement for anti-inflammatory response with ethyl pyruvate.

Recently, during a placebo-controlled randomized multicenter study, ethyl pyruvate was administered to horses following anesthesia recovery for colic due to large colon volvulus of more than 360° (Johnson et al., 2020). The infusion was performed over 60 minutes, and 150 mg/kg was used within one liter of lactated ringer solution. The control group received 1 liter of lactated ringer solution over the same period. No adverse events were reported during the infusion of the drug. Unfortunately, the treatment failed to show any significant effect on clinical variables measured during the study period (heart rate, PCV, L-lactate concentration) and survival. Still, the authors reported an underpowered study limiting the impact of their results (Johnson et al., 2020).

Ethyl pyruvate in ruminants

The efficacy of ethyl pyruvate against endotoxemia was tested twice in ruminants. The first study was conducted in anesthetized sheep using a large

animal model of septic shock resulting from fecal peritonitis (Su et al., 2007). In this study, sheep received only 15 mg/kg per hour of ethyl pyruvate combined with Ringer lactate solution or Ringer lactate solution. In this study, the authors were interested in seeing if ethyl pyruvate Ringer solution prolongs survival time and maintains clinical values (heart rate mean arterial pressure, cardiac index, systemic vascular resistance, and lactic acid concentration). The main findings were that ethyl pyruvate was able to delay the onset of hypotension (27 vs 15 hours) and oliguria (24 vs 16 hours) with prolonged survival time (median time of 29,5 vs 17 hours), showing potential use of ethyl pyruvate in the treatment of severe sepsis and septic shock (Su et al., 2007). Arterial IL-6 concentrations were also lower in the ethyl pyruvate group.

In the bovine species, the effect of ethyl pyruvate on the production of vascular pro-inflammatory mediators was evaluated using an in vitro model of coliform mastitis (Corl et al., 2010). Cultured bovine mammary endothelial cells (BMEC) were stimulated with endotoxin (50 ng/ml of *Escherichia coli* O111:B4) for an initial 1 hour, followed by an additional 1 hour of incubation with ethyl pyruvate at 0, 5 or 10 mM for RNA isolation or 2 hours for protein isolation. Treatment of endotoxin-stimulated BMEC with ethyl pyruvate showed a significant reduction in IL-6, IL-8, and intercellular adhesion molecule 1 gene expression. Expression of cyclooxygenase 2 and 15-lipoxygenase 1 was also reduced. The ability of ethyl pyruvate to effectively inhibit gene and protein

expression of potent vascular pro-inflammatory mediators in vitro warrants further investigations to assess its in vivo efficacy. As ethyl pyruvate is safe for human consumption, it is an attractive therapeutic option in the face of endotoxin-induced inflammatory response diseases such as coliform mastitis and sepsis.

Conclusion of literature review

Ethyl pyruvate may be an effective therapy for sepsis in calves. It is stable in solution, highly lipid soluble and does not create a hyperosmolar state following infusion. Ethyl pyruvate has been proven to facilitate cytokine production and release in pre- and post-treatment protocols in multiple animal species and models. Ethyl pyruvate has also shown clinical efficacy and safety in numerous species in various disease models. Dosing regimens and dose concentration have been thoroughly researched and proven safe, and lastly, judging systemic inflammation.

CHAPTER I
EFFECT OF ETHYL PYRUVATE ON THE INFLAMMATORY RESPONSE AND NEUTROPHIL FUNCTION IN NEONATAL CALVES

A version of this chapter will be submitted to the Journal of Dairy Science.

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Abstract

Septicemia or endotoxemia is a vital disease leading to severe inflammatory response syndrome and multiple organ failure in humans and animals. Many high-consequence diseases of cattle, including neonatal diarrhea and septicemia, have an underlying pathophysiologic mechanism of endotoxemia. Controlling the overwhelming inflammatory response to lipopolysaccharides is critical to reducing organ damage and improving clinical outcomes. Ethyl pyruvate is a small-molecule inhibitor that has shown promise in

experimental treatment of systemic inflammation in laboratory and livestock species. Its proposed mechanism is via inhibition of transcriptional regulators of the inflammatory response. This study aimed to explore ethyl pyruvate's ability to modulate early pro-inflammatory cytokine production and immune effector function in treated calves. Calves were randomly assigned to 1 of 3 treatment groups of 8 calves per group: (1) Placebo control – one liter of lactate ringer solution (2) ethyl pyruvate at 50 mg/kg infusion within one liter of lactated ringer solution; (3) ethyl pyruvate at 100 mg/kg infusion within one liter of lactated ringer solution. Treatments were given over 30 minutes. Blood samples were collected in EDTA tubes before infusion, 6-, 12-, 24, and 48 hours post-infusion. Each sample was split, and one aliquot was incubated at 37 C for an hour with 1 ng/ml of *E. coli* lipopolysaccharides (*Escherichia coli* O55:B5) solution. Pro-inflammatory cytokines, TNF-α, IL-1β, IL-6, and HMGB1, were measured in plasma. The capacity of granulocytes to efficiently phagocytize fluorescently labelled *E. Coli* was measured at all time points. Except for tachypnea, when the infusion rate was too fast, no side effects were associated with the infusion of ethyl pyruvate. Treatment with LPS alone or co-incubated LPS with ethyl pyruvate significantly elevated IL-6 ($p < 0.0001$ and $p < 0.0001$) and TNF-α ($p < 0.0001$ and $p < 0.001$, respectively). Incubation with LPS alone significantly elevated HMGB1 ($p<0.02$) and L-1β ($p < 0.0001$, however, when co-incubated with ethyl pyruvate, no significant effect was observed. Pre-treatment with ethyl pyruvate demonstrated no significant impact on the production of pro-

inflammatory cytokines or phagocytosis efficiency. Results of this study suggest that ethyl pyruvate is safe with few side effects when infused in a one-liter LRS bolus to neonatal dairy calves but could show severe side effects at higher concentrations and rates. Furthermore, we observed a limited impact of ethyl pyruvate in reducing cellular inflammation, limiting our recommendation to use this compound in the dairy industry.
Keywords: ethyl pyruvate, dairy calves, endotoxemia, high mobility group box 1

Introduction

Neonatal calf diarrhea is the leading cause of death in pre-weaned dairy heifers (Urie et al., 2018). Severe intestinal barrier damage can lead to bacterial translocation, bacteremia, and lipopolysaccharides (LPS) release into the blood. Foundational therapy for calves exhibiting signs of endotoxemia includes antimicrobials, fluids and electrolyte restoration, and controlling the inflammatory response. Treatments for controlling inflammation in livestock are limited and consist primarily of NSAIDs, such as flunixin meglumine. In the US, flunixin is the only approved treatment for inflammation caused by endotoxemia. NSAIDs inhibit cyclooxygenase enzymes, reduce the production of inflammatory mediators and indirectly alleviate the clinical signs of endotoxemia. Their targets, however, are far downstream from the initial cellular responses to LPS (Constable, 2009). Therefore, their overall effect is limited. In addition, administration of NSAIDs for extended periods may lead to complications, such

as abomasal ulcers and renal injury. Novel anti-inflammatory therapies with improved safety characteristics that affect more relevant targets are needed. Ethyl pyruvate (EP) is a simple derivative of pyruvate, the metabolic intermediary of glycolysis and the citric acid cycle. Pyruvate is an essential natural scavenger of reactive oxygen species (ROS, but is pharmacologically unstable and of limited therapeutic value (Fink, 2004b, 2008). EP, however, retains the effects of its parent molecule against oxidant-mediated injury, remains stable and demonstrates additional distinct attenuation of NF-κB and HGMB1-dependent signalling pathways (Fink, 2008). In light of these characteristics, EP has been investigated for efficacy in multiple disease models and has shown promising results in the treatment of sepsis, acute respiratory distress, viral myocarditis, acute pancreatitis, alcoholic liver injury in laboratory species and post-operative endotoxemia in horses (Fink, 2004b, 2008, Jacobs et al., 2013).

EP ameliorates inflammation by directly disrupting the induction of early inflammatory signalling pathways. One of inflammation's most critical early regulators is the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) family of transcriptional regulators. When activated by cellular injury or pathogen recognition receptors, NF-kB translocates into the nucleus, binds to DNA at specific response elements and induces gene expression of inflammatory cytokines, adhesion molecules, growth factors, and inducible enzymes such as cyclooxygenase-2 and iNOS. Han et al. demonstrated that EP disrupts essential

DNA binding sites within the NF-kB subunit p65, thereby inhibiting its ability to induce gene activation (Han et al., 2005). An additional target of EP is the nuclear protein high mobility group box-1 (HMGB1). Like NF-kB, this protein is activated by cellular injury but predominantly via TLR4 activation. Its primary function is to induce and amplify the production of inflammatory mediators and, like NF-kB, has been implicated as a critical regulator of inflammation in several inflammatory disease models, including sepsis. While a mechanism for EP inhibition of HGMB1 has not been postulated, EP treatment was shown to reduce HMGB1 concentration and improve survival in LPS-challenged macrophages and mice (Ulloa et al., 2002, Fink, 2008).

Despite an extensive body of experimental work, there have been limited clinical trials seeking to determine the efficacy of EP in natural diseases. In veterinary medicine, EP downregulated global expression of TNF-α and IL-6 mRNA in LPS-challenged dogs (Yu et al., 2010). Likewise, EP administration at 150 mg/kg to healthy horses was deemed safe and capable of diminishing the inflammatory response following LPS infusion (Schroeder et al., 2011, Jacobs et al., 2013). In sheep receiving induced peritonitis, EP (15 mg/kg per hr. constant rate infusion) delayed the development of organ dysfunction and prolonged survival following surgery (Su et al., 2007). Corl et al. reported significantly reduced gene expression of IL-6, IL-8, and intercellular adhesion molecule 1, as well as expression of eicosanoid-producing enzymes, including cyclooxygenase

2 and 15-lipoxygenase 1, in bovine mammary endothelial cells after treatment with LPS as a model of coliform mastitis (Corl et al., 2010). However, to date, no studies have been performed on live cattle.

Therefore, the primary objectives of the current study were to demonstrate the safety of EP administration in neonatal calves at 2 concentrations and determine its effect on pro-inflammatory cytokines production and the function of immune cells isolated from treated calves. We hypothesized that ethyl pyruvate was safe and biologically active in healthy neonatal calves. We expected that cells isolated from treated calves would demonstrate dose-dependent disruption of inflammatory signal induction when exposed to LPS in vitro. This would quell the production of critical inflammatory biomarkers (TNF-α, IL-1β, IL-6, HMGB1). Additionally, neutrophils from treated calves will have less impaired phagocytosis when compared to non-treated cells. The demonstration of *in vitro* efficacy will be used as preliminary justification for prospective challenge studies and future clinical trials in natural disease outbreaks.

Materials and Methods

Animals

The study protocol was reviewed and approved under the ethics guidelines of the University of Tennessee Institutional Animal Care and Use

Committee (IACUC). The study was a controlled and randomized clinical trial performed in June 2022. Twenty-four healthy Holstein male or Holstein-Angus crossed female calves were acquired for this study, 10-21 days of age. Calves were transported to East Tennessee AgResearch and Education Center (ETREC) – Little River Dairy Unit in Walland, Tennessee, and randomly allocated into three groups of 8 calves, balanced by sex and age. Calves were group housed by treatment in 5 m x 10 m pens bedded with sawdust. Calves were fed 3 quarts of milk twice daily by the farm employees. Before enrollment, a complete physical examination was performed to rule out concurrent illnesses, and an acclimation time of 5 days was provided to ensure that no calves develop disease before the study period. Any calf showing signs of infection up to the day of the initiation of ethyl pyruvate infusion was removed from the study and replaced by a healthy calf of a similar age and sex.

Experimental protocol

The three groups (A–C, n=8/groups) consisted of 1 of 3 concentrations of EP (A = 0 mg/kg (control), B = 50 mg/kg, or C = 100 mg/kg). These concentrations were weight-based interpolations from those reported in lab animal and equine studies with ethyl pyruvate. A short-term intravenous jugular catheter (Terumo Surflash IV Catheters 16gx2") was aseptically placed on the evening before initiation to collect whole blood and drug infusion. Samples were

collected at baseline immediately before infusion (0 hours) and 1-, 3-, 6-, 12-, 24- and 48-hours following treatment. Blood was taken via the jugular catheter using a three-syringes technique. A flush syringe containing 5 ml of 0.9% heparinized saline flush solution was used to flush the catheter and check its patency. Using the same syringe, blood was taken by aspirating 5 ml of blood into the syringe. Keeping the syringe attached to the catheter hub, the sample was pushed to reinfuse the blood into the catheter. The aspiration and reinfusion were repeated at least three times and aspirated one last time (retaining the blood sample). The syringe was placed aside. A new syringe was attached to draw the sample for analysis. The sample was transferred to the different sampling tubes by puncture through the tube cap. Blood in the first syringe was reinfused into the catheter. If the blood has clotted in the syringe, discard the sample. A new syringe was used to flush the catheter with up to 10 ml of 0.9% heparinized sodium chloride solution or more as needed to inject enough flush solution to clear residual blood from the catheter. Blood samples were collected in three EDTA sample tubes (BD Vacutainer™, 2 ml) for ELISA assays and one lithium heparin tube (BD Vacutainer™, 2 ml) for phagocytosis activity. All treatments were blinded to the research team. An individual not involved in sample collection or data analysis prepared the solutions of each concentration and labelled treatments A – C in a random allotment. The body weight of each calf was recorded before preparation and used to calculate the appropriate individual dose. Ethyl pyruvate is a novel therapy, and therefore, administration warranted euthanasia, according to state

law, at the end of the study period for the two groups treated with EP. Calves were euthanized via a captive bolt, followed by an overdose of IV potassium chloride. The remaining placebo-treated calves were reinstituted in the university dairy herd.

Assessment of health and clinical monitoring

Total body weight was collected at arrival, on the day of the infusion and at the end of the study period. Feed intakes were assessed twice daily by the farm personnel. Each calf received a complete physical examination, including rectal temperature, respiratory rate, cardiac rate, fecal score, and attitude assessment (Poulsen and McGuirk, 2009, Lowe et al., 2019). On the day of EP administration, a physical examination was performed with sample collection at 0-, 1-, 3-, 6-, 12-, 24-, and 48 hours following infusion and twice daily throughout the remaining study period. Any abnormalities were recorded and assessed as potential adverse events.

Preparation of ethyl pyruvate

Solutions of EP (Ethyl pyruvate 98% purity (E47808), Sigma-Aldrich) were prepared up to fifteen minutes before infusion in a 1-liter bag of lactated ringer solution (Lactated Ringer's Injection, Nova-Tech, Patterson Veterinary) under

sterile conditions. A 0.2-micron disc filter was used to ensure sterility. Each concentration was finished to a total volume of 1000 ml in sterile lactated ringer solution. Each solution was prepared so that calves received consistent total volumes and the appropriate concentrations by treatment allocation. The solution was administered over 30-45 minutes using a macro drip set line through the intravenous catheter into the jugular vein of the calf. Each calf was examined during the infusion for signs of toxicity (i.e., increased respiratory rate, increased respiratory effort, collapse, or cough), and the rate was decreased if signs were observed. A maximum of 6 calves per infusion time were started, with three investigators supervising a set of two calves for the duration of the infusion. Following collection of blood samples in sample tubes, samples were put on ice for transportation to the lab. At the lab, one tube was centrifugated at 2,500 x *g* at 4C and plasma was collected and stored at -80C until subsequent analyses were performed.

LPS challenge preparation

Commercially acquired LPS (E. coli O55:B5 LPS (L2880), Sigma-Aldrich) was prepared in RPMI 1640 (Gibo^{TM}, ThermoFisher Scientific) to a dose of 1 mg/ml by adding 1 ml of RPMI. On the day of the study, using the 1 mg/ml solution of LPS, a 1 ng/ml solution was made using subsequent dilution in 50 ml conical vial to obtain a 1 ng/ml solution of LPS in RPMI 1640. Using one of the

blood samples in EDTA solution, 2 ml of LPS (1ng/ml) was added to each sample, followed by incubation in a water bath at 37°C for 60 minutes. Immediately following incubation, all stimulated blood samples were centrifuged at 2,500 x *g* for 10 minutes at 4°C, and the plasma sample was stored at -80°C for future ELISA assays to be performed after collecting all samples.

PrPro-inflammatory cytokines

Pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and HMGB1 were measured in plasma in duplicate from LPS-challenged and sham-challenged aliquots. Samples were thawed rapidly at room temperature and were kept on ice until analyzed. For each sample, 100μL of plasma was used per experiment. The day before analysis, ELISA plates were prepared to detect IL-1β and IL-6. Plates were prepared using 8-Well Polystyrene Strip (Pierce™ 8-Well Polystyrene Strip Plates, Corner Notch, No. 15031, Thermofisher™). All reagents and buffers were to equilibrate to room temperature (22-25°C) before use. The coating antibody for IL-1β and IL-6 was diluted at 1:100 in carbonate-bicarbonate buffer by adding 110μL coating antibody to 10.89mL of carbonate-bicarbonate buffer solution (Carbonate/Bicarbonate Buffer, No. 28382, Thermofisher™). A volume of 100μL of diluted Coating Antibody was added to each well using a multichannel micropipette. The plates were covered with plate sealer (Sealing tape for 96-Well plates, No. 15036, Thermofisher™) and incubated overnight at room

temperature. The next day, the coating antibody solution was aspirated with the multichannel pipette and 300µL of blocking buffer (ELISA Blocker Blocking Buffer, No. N502, Thermofisher™) was added to each well. Plates were once more covered with a plate sealer and incubated for 1 hour at room temperature. The blocking buffer was aspirated, and the assay was run. ELISA testing was performed according to the manufacturer's instructions (IL-6 Bovine Uncoated ELISA Kit, Invitrogen, ThermoFisher Scientific; IL-1 beta Bovine Uncoated ELISA Kit Invitrogen, ThermoFisher Scientific; TNF alpha Bovine ELISA Kit, Invitrogen, ThermoFisher Scientific; Bovine high-mobility group box 1 ELISA Kit, Mybiosource). For IL-6 and IL-1beta, ELISA assays were run using the premade plate. Standards were reconstituted with reagent diluent at 10,000 pg/ml or IL-6 and 4,000 pg/ml for Il-1β. Standards were diluted 1:2, and 100µL of standards and samples were added to each well in duplicate. The plate was covered and incubated for 1 hour at room temperature. The wash procedure was performed three times following aspiration of the well content. 300µL of wash buffer was used per well at each time. The plate was dried on an absorbent paper towel before the next step. The detection antibody was diluted in reagent diluent by adding 110µL of detection antibody to 10,89ml of reagent diluent (1:100). 100µL of detection antibody solution was added to each well. The plate was covered with a plate sealer and incubated for another 1 hour at room temperature. Another cycle of plate wash was performed as previously described. Streptavidin-HRP was diluted into reagent diluent by adding 30µL of Streptavidin-

HRP to 12 ml of reagent diluent (1:400). 100μL of diluted streptavidin-DRP was added to each plate. The plate was covered with a plate sealer and incubated for another 30 minutes at room temperature. Another cycle of plate wash was performed as previously described. 100μL of substrate solution was added to each well. The plate was covered with a plate sealer and incubated for another 20 minutes at room temperature in the dark. 100μL of stop solution was added to each well to stop the reaction. Absorbance was measured using a plate reader. For TNF- α, a similar process was used. ELISA plate provided with the kit was used. Standard were reconstituted with reagent diluent at 30 ng/ml concentration. Standards were diluted 1:2 (0, 0.123, 0.307, 0.768, 1.92, 4.8, 12 and 30 ng/ml), and 100μf standards and experimental samples were added to each well in duplicate. The plate was covered and incubated for 2.5 hours at room temperature with gentle shaking. The wash procedure was performed four times following aspiration of the well content. 300μL of wash buffer was used per well at each time. The plate was dried on an absorbent paper towel after the last wash. Biotin conjugate 80-fold with assay diluent B. 100μL of prepared biotin conjugate solution was added to each plate. The plate was covered with a plate sealer and incubated for another 1 hour at room temperature with gentle shaking. Another cycle of plate wash was performed as previously described. Streptavidin-HRP was diluted 600-fold by adding assay diluent B. 100μL of diluted streptavidin-DRP solution was added to each well. The plate was covered with a plate sealer and incubated for another 45 minutes at room temperature

with gentle shaking. The solution was discarded, and another cycle of plate wash was performed as previously described. 100μL of TMS substrate solution was added to each well. The plate was covered with a plate sealer and incubated for another 30 minutes at room temperature in the dark with gentle shaking. 50μL of stop solution was added to each well to stop the reaction. Absorbance was measured using a plate reader. For the HMGB1 assay, 100μL of standards (0, 5, 10, 25, 50 and 100 ng/ml) and experimental samples were added to the plate, all in duplicate. The same volume of PBS was added to the blank sample. All except for blank control, 50μL of the conjugate enzyme were added to each well. The plate was covered and incubated for 1 hour at 37°C. Manual washing was performed following aspiration of the plate contents and using a wash solution to fill the well completely. The process was repeated five times, and the plate was dried by hitting the plate onto absorbent paper towels until no moisture appeared. 50μL of Substrate A and B were added to each well, and the plate was covered and incubated in the dark for 15-20 minutes at 37°C. 50μL of stop solution was added to each well. The intensity of the reaction signal was read with an ELISA plate reader at 450nm and 550nm 30 minutes after stopping the reaction. It was correlated to the standard curve to determine the sample concentration. For HMGB1 and TNF- α, the signal was only read at 450nm. Standard curves for each plate were made using Prism 10 (GraphPad Software, Boston, MA) (see Appendix at the end of the dissertation). Due to extreme variation in the TNF-α standard curve, a mean standard curve was used for extrapolating data. Minimal

limit detection according to the company pamphlet for HMGB1, IL-6, IL1-β and TNF-α ELISA assay are 1.0 ng/ml, 78.1 pg/ml, 31.3 pg/ml and 0.1 ng/ml respectively. Data below the detection limit were considered as a zero value for analysis.

Neutrophil function evaluation

Granulocytes, including neutrophils, were isolated from heparin anticoagulant whole blood and neutrophil phagocytosis was performed using pHrodo™ Green E. coli BioParticles™ Phagocytosis Kit for Flow Cytometry (ThermoFisher). Briefly, whole blood samples from previously treated calves in blood collection tubes containing heparin anticoagulant were placed on ice for 10 minutes before use. PHrodo™ BioParticles® were conjugated in 2.2 mL Buffer B and placed on ice for 10 minutes before use. Two tubes of each positive control, negative control, and experimental sample were prepared using 100 μL of whole blood per tube. A volume of 20 μL of pHrodo™ BioParticles® conjugate solution was added to all positive and experimental samples. One tube from each control and experimental sample was placed on ice (4°C), and the other tube was placed in a 37°C water bath for 15 minutes. Lysis Buffer A (100 μL) was added to all tubes to lyse red blood cells, vortexed briefly, and incubated at room temperature for 5 minutes. One mL of Buffer B was added to all tubes, vortexed briefly, and incubated at room temperature for 5 minutes. Centrifugation was performed at

350 × *g* for 5 minutes at room temperature. The supernatant was discarded, and the cell pellets were resuspended in 1 mL Wash Buffer. The centrifugation was repeated once, and the supernatant was discarded once again. The remaining cell pellet was resuspended in 0.5 mL of Wash Buffer for flow cytometry analysis. Flow cytometry analysis was performed immediately using a flow cytometer (Attune NxT, Invitrogen). The negative control sample was used to set voltages and to locate the white blood cell scatter pattern. Gating on the granulocyte population was performed. Using the positive control sample, voltage and other experimental settings were adjusted. A minimum of 35,000 events were counted per sample. Percentage of phagocytosis was performed in all the experimental samples incubated on ice (4°C) and 37°C.

Data analysis

The sample size was calculated using G*Power software (version 3.1) with an alpha of 0.05 and a power of 0.80. According to previous reports by our lab and others, IL-6 has demonstrated the most significant variability in cattle. Using an internally obtained effects size of 0.25, a deviation of 0.155, an equal number of animals per treatment suggests a minimum sample size of 8 calves per treatment. The 0.25 was a 25% reduction in IL-6 concentration, as reported in the rat study.

The effects of LPS, ethyl pyruvate and time on plasma cytokine concentration were examined using mixed model analysis with individual subjects as the random block factor. Rank data transformation was applied when diagnostic analysis on residuals violated normality and equal variance assumptions using the Shapiro–Wilk test and Levene's test. Post hoc multiple comparisons were performed with Tukey's adjustment. Statistical significance was identified at $p<0.05$. Analyses were conducted in SAS 9.4 TS1M8 (SAS Institute Inc., Cary, NC). Cytometric results were expressed using the percentage of cells showing fluorescence. The rate of cells showing fluorescence was compared to sham and non-treated controls using one-way repeated measure ANOVA.

Results

Effect of infusion of ethyl pyruvate

The infusion of ethyl pyruvate in 1 L LRS showed no severe adverse effects. While administering the infusion, two calves, only from the highest treatment group (100mg/kg), showed increased respiratory rate and coughing if the infusion rate was above 5 drops/second. Adjustment of the rate improved clinical signs. Rectal temperature, heart rate, respiratory rate, milk consumption and fecal score stayed normal during the study. However, six calves developed thrombophlebitis 48 hours post-placement of the intravenous jugular catheter,

and the catheters were removed. Calves were kept in the study, and their last sample was taken by direct venipuncture using a 10 cc syringe in the opposite jugular vein. Of those six calves, three were in the control group, two in the 50 mg/kg group and one in the 100 mg/kg group. Two calves that developed high fevers and clinical signs consistent with pneumonia on study day 0 were removed from the study and replaced with additional calves.

PrPro-inflammatory cytokines

In vitro, exposure to LPS proved a sufficient inflammatory challenge as all cytokine analyses demonstrated significantly increased concentrations compared to non-challenged samples. HMGB1 showed a significant elevation in concentration ($p<0.02$) following *in vitro* exposure to LPS among all time points and treatments (figure 4). EP treatment produced no significant observed effect on HMGB1 concentrations.

In addition, *in vitro,* LPS significantly increased the concentration of IL-6 ($p <0.0001$) at all time points (figure 5). Ethyl pyruvate at both concentrations administered demonstrated an additive inflammatory effect on IL-6 concentrations when combined with *in vitro* exposure to LPS with an overall increase compared to control samples with and without LPS exposure or samples from treated calves without LPS exposure ($p <0.0001$).

In vitro, exposure to LPS among all calves showed increased concentration of IL-1β ($p < 0.0001$) (figure 6). However, calves receiving either 50 or 100 mg/kg EP demonstrated decreased IL-1β concentration starting 1-hour post-infusion up to 48 hours post-infusion compared to time 0 in the LPS exposed groups. Calves treated with the highest concentration of EP (100 mg/kg) demonstrated a reduction in IL-1β concentration even in the absence of LPS stimulation.

Concentrations of TNF-α were significantly higher ($p < 0.0001$) while comparing each group following LPS exposure (figure 7). In vitro exposure to LPS in samples from calves treated with 50 mg/kg EP showed a significant increase in TNF-α ($p < 0.00$), but only compared to unexposed samples treated with 100 mg/kg EP. Interestingly, while stimulated with LPS, the increase of TNF-α was higher at time 0 in the 50 mg/kg group while more elevated in the 1-hour group in the 100 mg/kg and similar in the 0 mg/kg group.

Phagocytosis

When looking at results for phagocytosis at 37°C, phagocytosis activity was constant over the study period but did decrease at 12 hours post-infusion for all groups (figure 8). Even though phagocytic efficiency dropped among all treatment groups at 12 hours, EP treatment at both 50 mg/kg and 100 mg/kg increased uptake of labelled bacteria at time points 1 h ($p < 0.01$), 24 h ($p <$

0.001), and 48 h ($p< 0.002$) hours post-infusion. Phagocytosis activity was also higher at 24 h compared to 6 h post-infusion ($p<0.01$) in all groups, showing an increase in label cells on flow cytometry.

A time-by-treatment interaction was only significant within the 100 mg/kg treated calves at 24 hours after recovering from the overall reduction in phagocytosis at 12 hours.

Discussion

In this study, no significant adverse effects were noted following the administration of ethyl pyruvate. However, the infusion rate seems to be critical. An initial pilot study performed by the authors using a concentration of 200 mg/kg injected as bolus infusion resulted in rapid tachypnea, respiratory distress, convulsion, muscle tremors and death of the animal. However, when instilled in a liter of LRS and infused, the exact concentration only produced an elevated respiratory rate and effort that could be modulated by increasing or decreasing the infusion rate. Once the infusion was complete, all clinical signs were resolved within minutes. Based on these findings, we selected a maximum dosage of 100 mg/kg ethyl pyruvate for use in the principal study here to reduce possible adverse effects. In the current study, tachypnea was seen only in the treatment group receiving the highest dose (100 mg/kg of ethyl pyruvate) when the infusion rate increased and only in a limited number of calves. Human subjects receiving

ethyl pyruvate at an infusion rate above 5 mg/kg bwt/min reported blurred vision, and, like our observations, the side effects resolved after the infusion was stopped (Schroeder et al., 2011). Based on these observations, Schroeder et al. used a maximum rate of 2.5 mg/kg bwt/min for their horse study (Schroeder et al., 2011). In their study, they found no adverse effect associated with the infusion of ethyl pyruvate. However, throughout the experiment, they noticed significant differences in rectal temperature, heart rate, and respiratory and lactate concentration in the ethyl pyruvate group. Still, all values remained within the normal reference range (Schroeder et al., 2011). The same protocol using a dose of 150 mg/kg of ethyl pyruvate in a 1-liter bag of LRS was used in a multicenter placebo-controlled randomized study of ethyl pyruvate in horses following surgical treatment for ≥ 360◦ large colon volvulus, and no adverse effects were noted during or after infusion (Johnson et al., 2020). Similar findings were found on anesthetized horses (Munoz et al., 2022). If ethyl pyruvate is to be further studied in calves, the infusion rate should not exceed 2.5 mg/kg bwt/min. In the current study, calves were infused over approximately 30 minutes without adverse effects. Field use may be challenging for larger animals because of longer infusion times. Therefore, application to adult cattle may require a different drug formulation, such as combining it with slow-release agents, which could be given subcutaneously.

The results presented herein calves were not completely different than those reported in horses treated with ethyl pyruvate. Cytokine response following exposure to LPS after one hour following EP infusion showed no significant difference compared to time 0 for TNF-α, IL-1β and IL-6, similar to Schroeder et al. 2011 study. The main difference with that study was that values of TNF-α, IL-1β and IL-6 decreased significantly 6-hour post-infusion compared to time 0 and 1, 6 hour in the equine patient while the calf showed a non-significant decrease for TNF-α, IL-1β. One of the major differences in the experiment protocol compared to that species is that the authors looked at gene expression using real-time quantitative polymerase chain reaction (Q-PCR) (Schroeder et al., 2011, Jacobs et al., 2013). In this study, we decided to measure the direct level of cytokines by ELISA. The first method involves the measurement of cytokines mRNA transcript abundance. This method allowed for the detection of relatively small sample amounts. The major disadvantage of using that technique is that the presence of RNA does not always accurately correlate to protein levels (Amsen et al., 2009). Other disadvantages included isolating cellular sources of cytokines and not reaching the detection threshold while only a few cells were present in the sample. We examined the protein secretions using enzyme-linked immunosorbent assay (ELISA) for those reasons. This method is straightforward and quantitative, but the disadvantage of needing sufficient samples to obtain accurate protein levels is that it sometimes leads to underestimating actual cytokine levels (Amsen et al., 2009). We may not have enough detection levels

for our IL-6 as many of the samples came back lower than the detection limit, limiting our interpretation of this cytokine. MRNA cytokine values and protein concentration should be considered if the budget allows it.

Another possibility of not detecting a sufficient decrease in interleukin release could be related to the timing of the endotoxemia challenge. A recent study evaluating several cytokines releases following an intravenous lipopolysaccharide challenge in beef steer showed that TNF-α concentration peak 1-hour post-challenge while IL-1β peak 2 hours post-challenge (Smock et al., 2023). For that reason, it is possible that we missed the period when IL-1β peak and could have seen a difference. This difference could also be potential for IL-6 concentration. Another reason to see no statistical difference in our samples, especially for IL-1β and IL-6, is due to a large amount of value under the detectable limit. Several possibilities could explain this high amount of variation between samples. First, before the challenge, we did not control the different samples for neutrophils and macrophage numbers by cell count. It is possible that a sample with a lower value could be related to a lower number of cytokines-producing cells. Another possible reason could be individual susceptibility to endotoxemia, as previously reported in cattle (Jacobsen et al., 2005). Despite controlling for hematologic parameters, individual susceptibility could not be influenced. In our samples, despite some outliers, no animal was identified as a poor or heavier responder in the face of the LPS challenge.

This study's initial choice of ethyl pyruvate included 0, 50, 100 and 200 mg/kg. The 200 mg/kg was based on being a little superior to the equine studies, which found that a dose of 150 mg/kg was the only significant dosage in this species (Schroeder et al., 2011). Following the pilot study, we only kept the 0, 50, and 100 mg/kg dosages to control the infusion time to about 30 minutes. The reasoning behind that decision was to keep an option for field practitioners to allow treatment on the farm. Another reason was that in the sheep study using a model of induced peritonitis, the functional dose was 15 mg/kg of ethyl pyruvate solution (Su et al., 2007). We hypothesize at that time that a calf will behave more like another ruminant than an equine. It is possible that we found no significant difference in our study due to the adequate anti-inflammatory dosage being too low. A minimum dose of 150 mg/kg with a longer infusion time could be tried to optimize the effect of ethyl pyruvate. In this case, a longer infusion time should be considered to allow a higher concentration of ethyl pyruvate without side effects.

In the only other bovine study that looked at the effect of ethyl pyruvate, ethyl pyruvate showed decreased expression of IL-1β, IL-6 and IL-8 I compared to the control group. However, the fold change compared to baseline concentrations for both dosages showed a more modest increase in concentration. IL-1β concentrations for both dosages of EP were lower at all time points compared to baseline concentration, though not significantly different.

Similarly, when compared to the control group, mean values were all lower at 1 to 12 hours in both treatment groups. This finding is interesting as we could see an effect in reducing IL-1β up to 12 hours following ethyl pyruvate infusion) with additional power. The TNF-α data are also interesting. While looking at the dosage of 50 mg/kg, we see a non-significant reduction from time 0 to time 1 hour. This reduction is a value of interest with TNF- (known to peak around 1 hour post-response to an inflammatory stimulus (Venkataraman et al., 2002). Finally, we see no significant decrease in HMGB1 during the study period using either control group. One possible pitfall in our study is that HMGB1 is a late mediator of inflammation, and therefore, the design of the experiment was not optimal for seeing a statistical difference. There is a chance that using an in vivo endotoxemia challenge, we could see a decrease in HMGB1 concentration between 12 and 24 hours compared to non-treated animals, as previously seen with a rodent model of endotoxemia (Ulloa et al., 2002).

Conclusion

The result of this study suggests further investigation of the systemic effect of ethyl pyruvate. The study results show no effect of ethyl pyruvate on releasing pro-inflammatory cytokines. Experimental protocol should be redefined to allow better evaluation of the different cytokines, especially HMBG1. The ability of ethyl pyruvate to effectively inhibit gene and protein expression of pro-inflammatory cytokines *in vitro* warrants further investigations to assess its *in vivo*

efficacy. Given the possible side effects, an infusion at a maximum rate of 2.5 mg/kg btw/min would be considered safe for additional study in the live calf. Still, it should not be recommended at this time for in-field use due to possible severe side effects if not well controlled. Even if ethyl pyruvate is safe for human consumption, the result of this study could not support the use of this molecule in the dairy industry.

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Conflict of interests

The authors have no competing interests to disclose.

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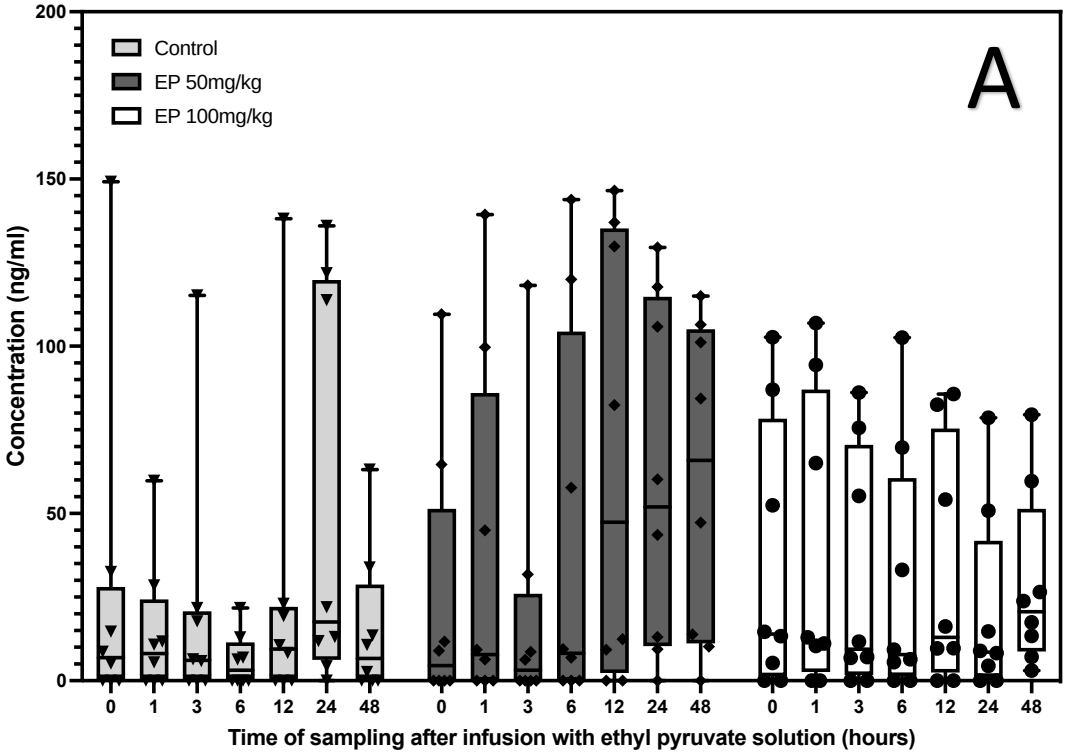
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Appendix

Figure 4. Whisker plot of the concentration of HMGB1 concentration (ng/ml) at each time point for calves infused with ethyl pyruvate at 0 mg/kg, 50 mg/kg, or 100 mg/kg. Time 0 represents pre-infusion sampling. The upper graph (A) displays the concentration of HMGB1 without in vitro LPS exposure. The lower graph shows the concentration of HMGB1 in the same blood sample following exposure to E. coli O55:B5 LPS for 60 minutes as an in vitro inflammatory challenge (B). The range of the data set for each time point is in between bars. The upper bar represents the maximum value, while the lower bar represents the minimum value.


A
Control
EP 50mg/kg
EP 100mg/kg
Concentration (ng/ml)
200
150
100
50
0
0 1 3 6 12 24 48 0 1 3 6 12 24 48 0 1 3 6 12 24 48
Time of sampling after infusion with ethyl pyruvate solution (hours)

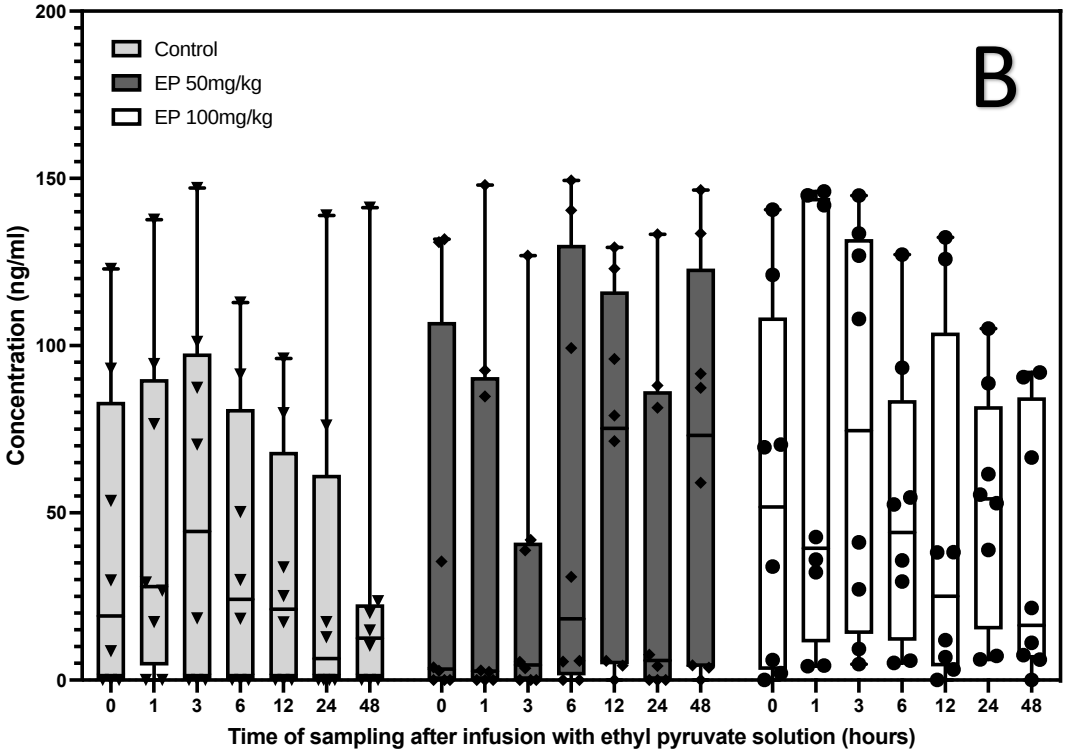
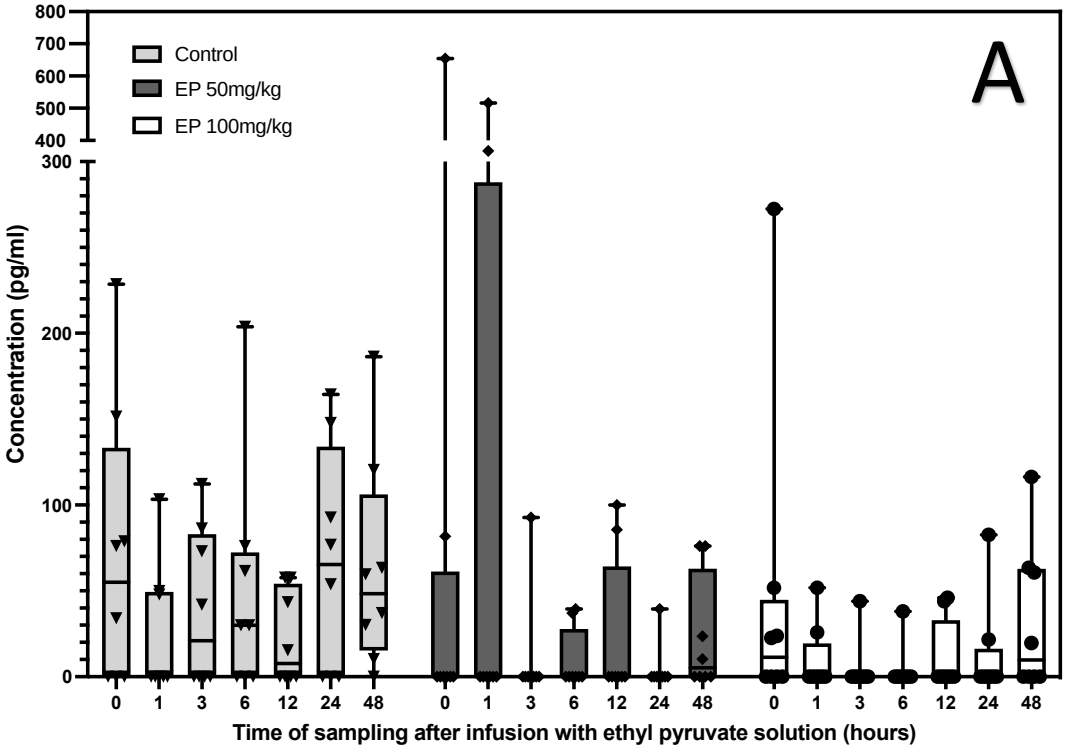

B
Control
EP 50mg/kg
EP 100mg/kg
Concentration (ng/ml)
200
150
100
50
0
0 1 3 6 12 24 48 0 1 3 6 12 24 48 0 1 3 6 12 24 48
Time of sampling after infusion with ethyl pyruvate solution (hours)

Figure 5. Whisker plots the IL-6 concentration (pg/ml) at each time point for calves infused with ethyl pyruvate at 0 mg/kg, 50 mg/kg, or 100 mg/kg. Time 0 represents pre-infusion sampling. The upper graph (A) displays the concentration of IL-6 without in vitro LPS exposure. The lower graph shows the concentration of IL-6 in the same blood sample following exposure to E. coli O55:B5 LPS for 60 minutes as an in vitro inflammatory challenge (B). The range of the data set for each time point is in between bars. The upper bar represents the maximum value, while the lower bar represents the minimum value.


A
Control
EP 50mg/kg
EP 100mg/kg
Concentration (pg/ml)
800
700
600
500
400
300
200
100
0
0 1 3 6 12 24 48 0 1 3 6 12 24 48 0 1 3 6 12 24 48
Time of sampling after infusion with ethyl pyruvate solution (hours)

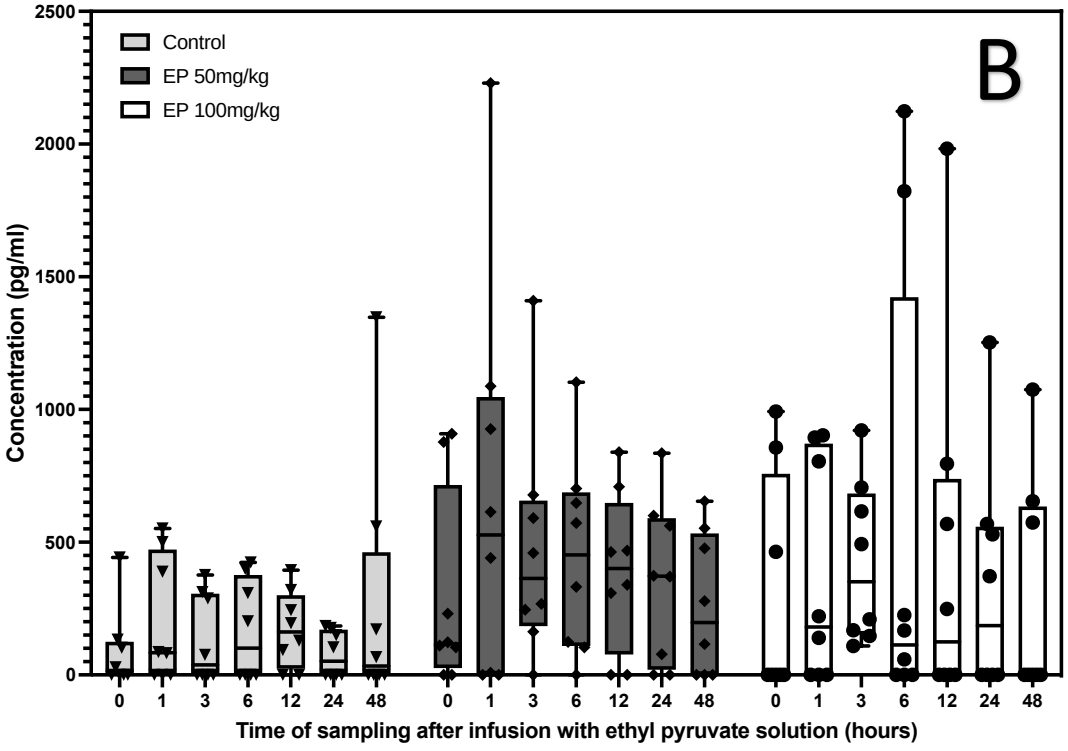
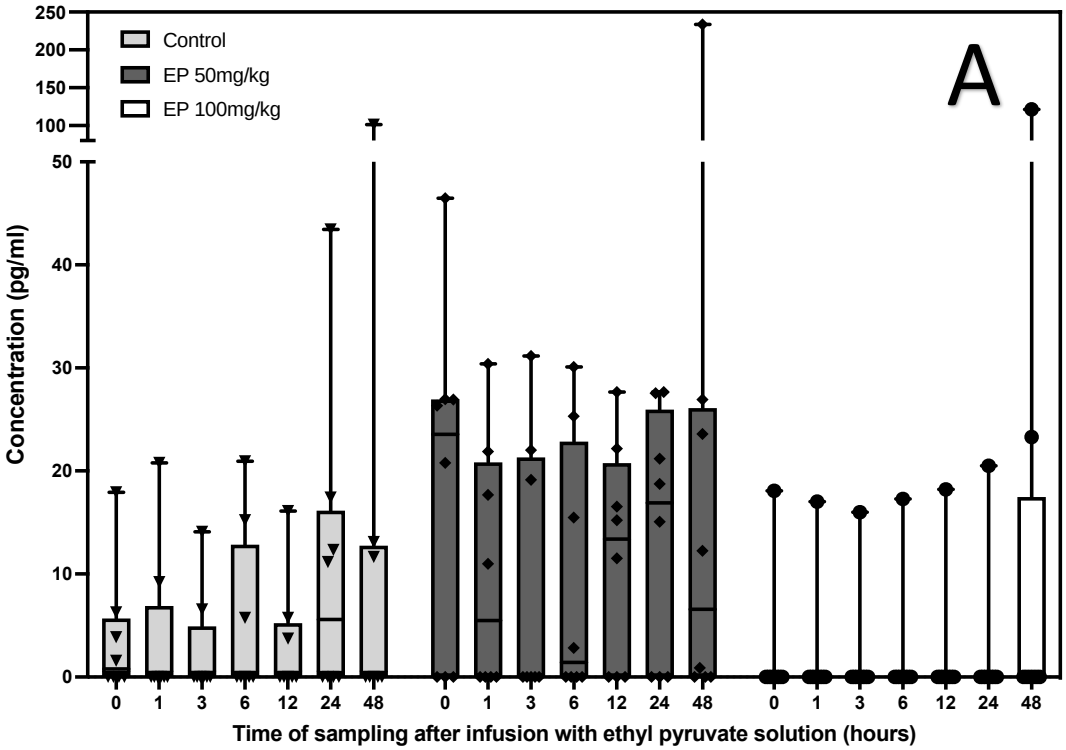

B
Control
EP 50mg/kg
EP 100mg/kg
Concentration (pg/ml)
2500
2000
1500
1000
500
0
0 1 3 6 12 24 48 0 1 3 6 12 24 48 0 1 3 6 12 24 48
Time of sampling after infusion with ethyl pyruvate solution (hours)

Figure 6. Whisker plot of the IL-1β concentration (pg/ml) at each time point for calves infused with ethyl pyruvate at 0 mg/kg, 50 mg/kg, or 100 mg/kg. Time 0 represents pre-infusion sampling. The upper graph (A) displays the concentration of IL-1β in vitro LPS exposure. The lower graph shows the concentration of IL-1β in the same blood sample following exposure to E. coli O55:B5 LPS for 60 minutes as an in vitro inflammatory challenge (B). The range of the data set for each time point is in between bars. The upper bar represents the maximum value, while the lower bar represents the minimum value.


A
Control
EP 50mg/kg
EP 100mg/kg
Concentration (pg/ml)
250
200
150
100
50
40
30
20
10
0
0 1 3 6 12 24 48 0 1 3 6 12 24 48 0 1 3 6 12 24 48
Time of sampling after infusion with ethyl pyruvate solution (hours)

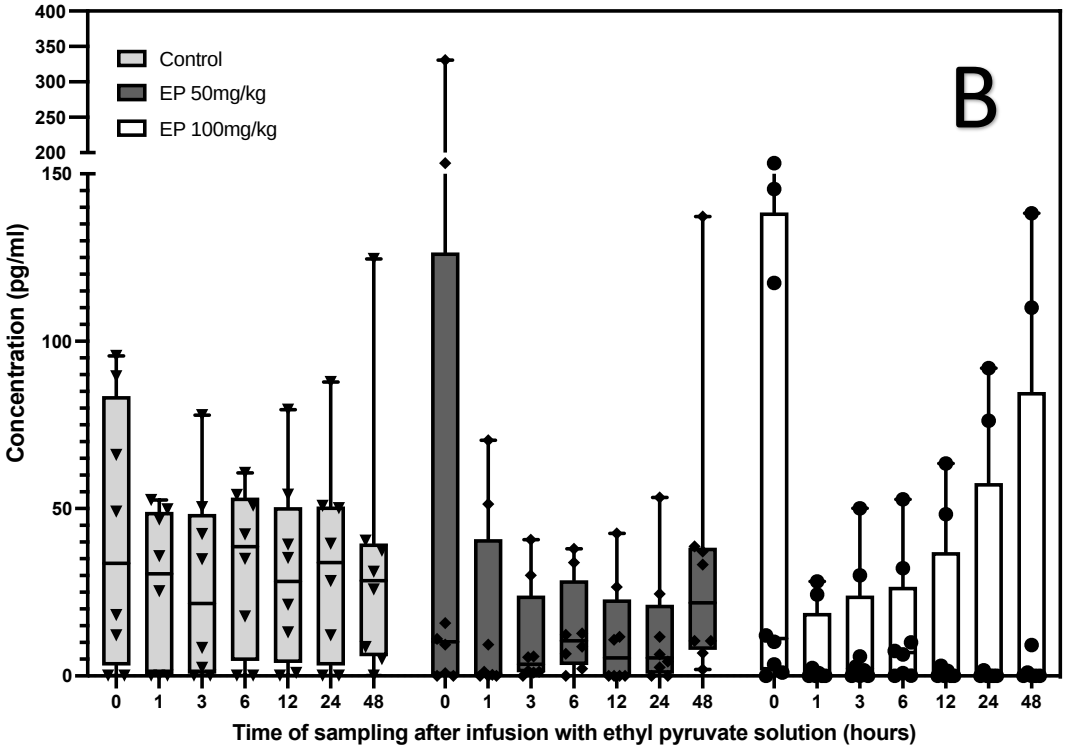
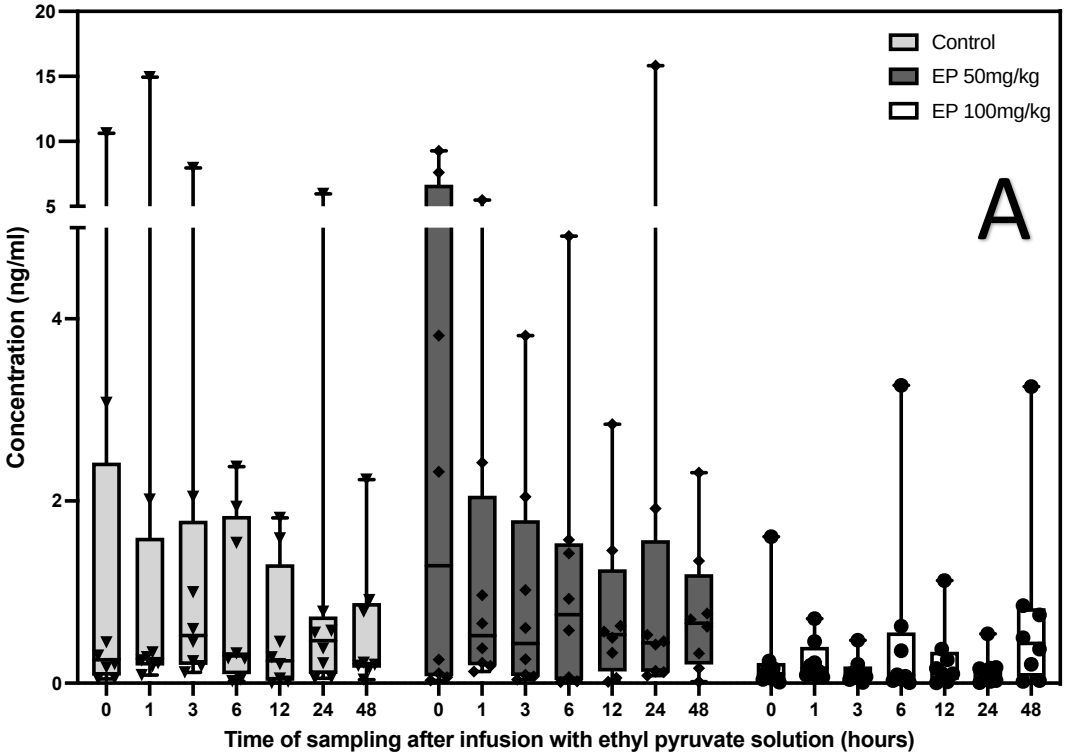
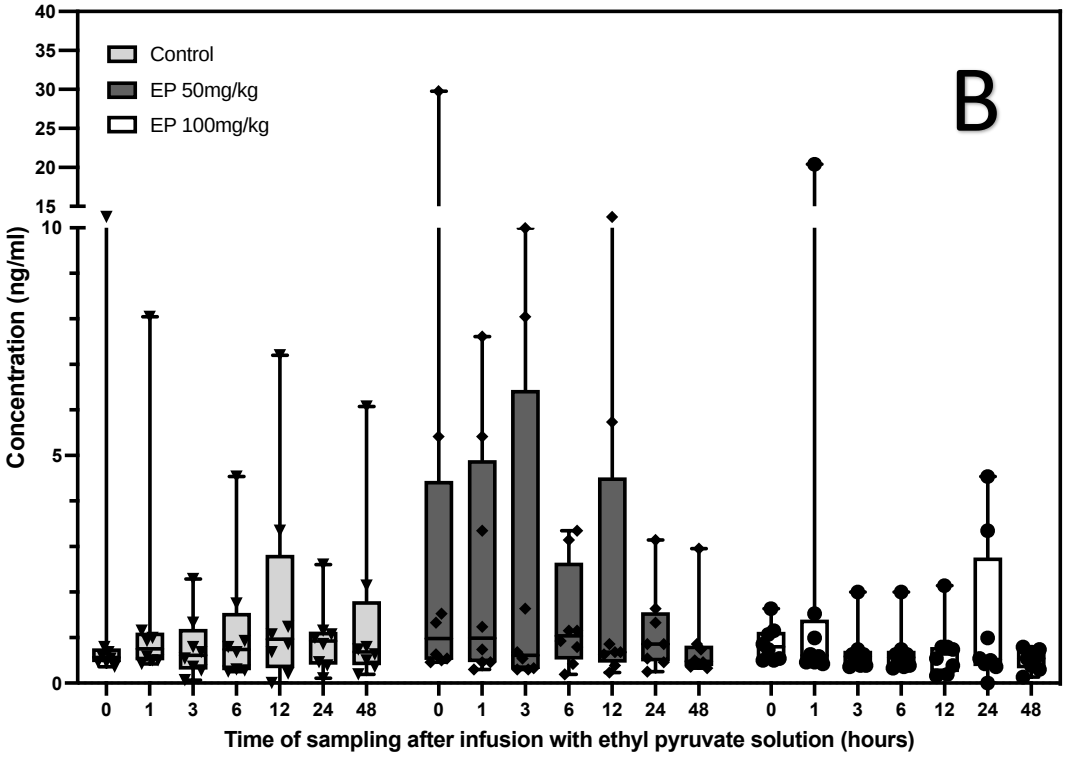

B
Control
EP 50mg/kg
EP 100mg/kg
Concentration (pg/ml)
400
350
300
250
200
150
100
50
0
0 1 3 6 12 24 48 0 1 3 6 12 24 48 0 1 3 6 12 24 48
Time of sampling after infusion with ethyl pyruvate solution (hours)

Figure 7. Whisker plot of the TNF-α concentration (ng/ml) concentration at each time point for calves infused with ethyl pyruvate at 0 mg/kg, 50 mg/kg, or 100 mg/kg. Time 0 represents pre-infusion sampling. The upper graph (A) displays the concentration of TNF-α without in vitro LPS exposure. The lower graph shows the concentration of TNF-α in the same blood sample following exposure to E. coli O55:B5 LPS for 60 minutes as an in vitro inflammatory challenge (B). The range of the data set for each time point is in between bars. The upper bar represents the maximum value, while the lower bar represents the minimum value.


Control
EP 50mg/kg
EP 100mg/kg
A
Concentration (ng/ml)
20
15
10
5
4
2
0
0 1 3 6 12 24 48 0 1 3 6 12 24 48 0 1 3 6 12 24 48
Time of sampling after infusion with ethyl pyruvate solution (hours)


Control
EP 50mg/kg
EP 100mg/kg
B
Concentration (ng/ml)
40
35
30
25
20
15
10
5
0
0 1 3 6 12 24 48 0 1 3 6 12 24 48 0 1 3 6 12 24 48
Time of sampling after infusion with ethyl pyruvate solution (hours)

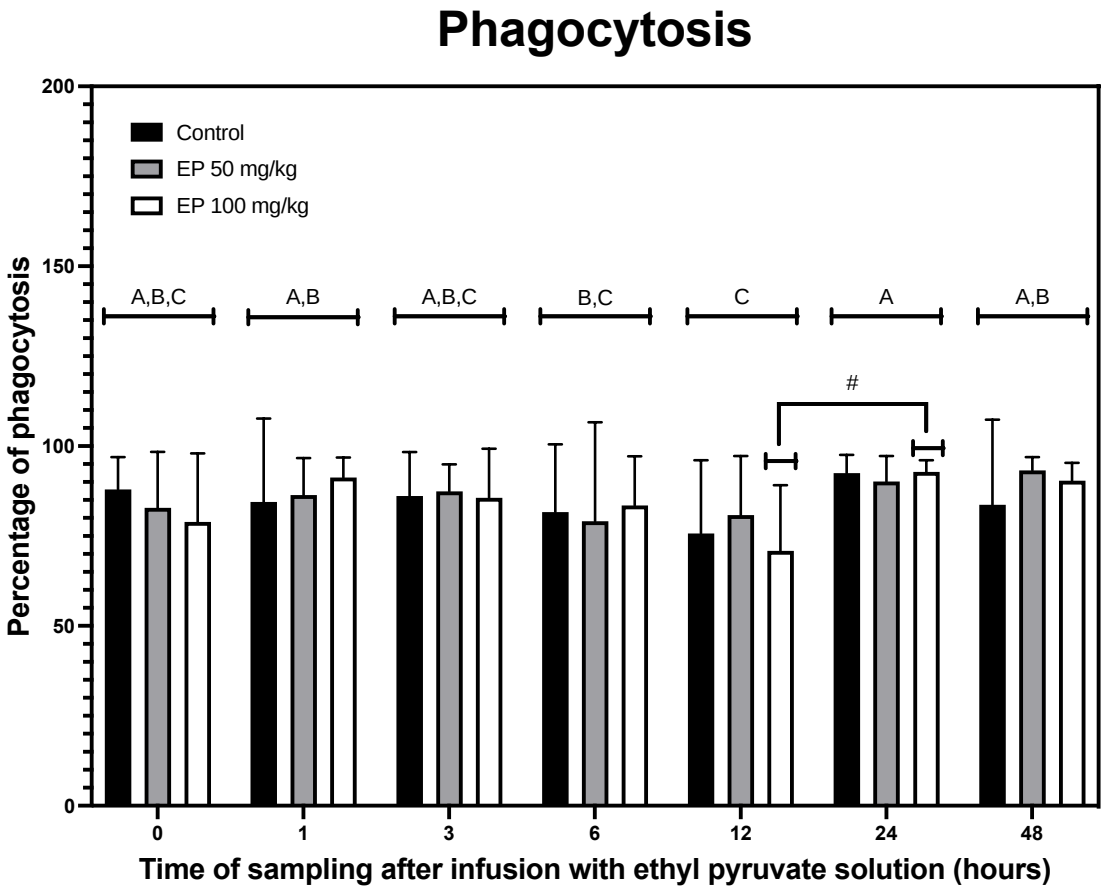


Figure 8. Phagocytosis percentage at 37°C at each time point following infusion with ethyl pyruvate at 0 mg/kg, 50 mg/kg, and 100 mg/kg. $^{A-C}$ Time group without a common letter differ significantly between time group (P < 0.01). # Significantly different between experimental time points in the same treatment group.

CHAPTER II
IN VITRO STIMULATION OF CALVES' WHOLE BLOOD WITH ETHYL PYRUVATE

A version of this chapter will be submitted to the Journal of Dairy Science.

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Abstract

Sepsis, a potentially fatal clinical syndrome, is mediated by an early and late pro-inflammatory cytokine response to infection. Neonatal diarrhea is a common disease seen on farms that could lead to sepsis. Ethyl pyruvate was identified as a promising therapeutic to reduce early and especially late mediators of inflammation, leading to improved survival in different models of sepsis and endotoxemia. As ethyl pyruvate is safe for human consumption, it may be an attractive candidate for therapeutic use in bovine medicine. Our study's objective was to evaluate the effect of ethyl pyruvate on specific pro-

inflammatory cytokines in vitro using the whole blood of neonatal dairy calves at different dosages with or without the addition of LPS. Ethyl pyruvate was initially evaluated at 5 and 10 mM doses within endotoxemia challenge with *E. coli O55:B5* LPS at doses of 0.1 and 1 ng/ml. A similar experiment was then performed with the same ethyl pyruvate doses but with a more extended incubation time before ethyl pyruvate addition to the solution (0, 1, and 6 hours). To further evaluate the effects of ethyl pyruvate dosage, decreasing doses of ethyl pyruvate (0 mM, 0.1 mM, 0.001 mM, 0.0001 mM, 0.0001 mM, 0.00001 mM) were evaluated with and without LPS stimulation at one ng/ml. The overall result showed that ethyl pyruvate at 5- and 10-mM doses increases IL-6 and Il-1β concentration while lower dosages don't. Rescue treatment with ethyl pyruvate 6 hours post-incubation LPS reduces the production of Il-1β at a dosage of 5 mM and IL-6 and IL-1β at a dosage of 10 mM. Results of this study suggest that the ethyl pyruvate effect is dose-dependent and could lead to increased cell destruction, leading to increased production of divers' cytokines at higher dosages. The ability of ethyl pyruvate to effectively decrease the production of potent pro-inflammatory mediators *in vitro* warrants further investigations.

Keywords: ethyl pyruvate, high mobility box group 1, lipopolysaccharide, endotoxemia, whole blood

Introduction

Neonatal calf diarrhea is the leading cause of death in pre-weaned dairy heifer (Urie et al., 2018). Bacteremia, endotoxemia and septicemia are commonly associated with diarrhea trigged by *Escherichia coli* lipopolysaccharides (LPS) (Fecteau et al., 2009). Nonsteroidal anti-inflammatory drugs, such as flunixin meglumine or meloxicam, are recommended as part of a comprehensive treatment for sepsis in calves due to their beneficial analgesic, anti-inflammatory, antipyretic and antisecretory properties. However, the use of NSAIDs in these cases is often complicated by a narrow safety window in severely dehydrated patients. Current best practices suggest limiting therapy to 3 days or less and only in well-hydrated calves to avoid renal and gastric mucosal injury. Moreover, while the use of an NSAID for sepsis is logical, evidence to support their efficacy is equivocal (Barnett et al., 2003, Constable, 2009). Therefore, there is a need to continue identifying new therapies with improved efficacy and safety profiles to improve clinical outcomes in septic calves.

Ethyl pyruvate is a natural derivative of pyruvic acid and is commonly used in the food industry as a flavour enhancement and stabilizer. Over the past twenty years, this molecule has been studied in human and veterinary medicine for its anti-inflammatory effect, especially its ability to reduce the expression of pro-inflammatory mediators (Vyawahare et al., 2012, Koprivica et al., 2022). Interest in its anti-inflammatory properties stems from early investigations into its parent

compound, pyruvate. Therefore, it has been broadly investigated in critical inflammatory disease models ranging from the treatment of sepsis, systemic inflammatory response syndrome, acute lung injury, pancreatitis, myocardial injury and stroke (Fink, 2008). A brief synopsis of work in veterinary patients reveals that ethyl pyruvate is safe and well tolerated in dogs, sheep, and horses and affect directly production and gene expression of TNF-α and IL-6 (Su et al., 2007, Yu et al., 2010, Jacobs et al., 2013). Corl et al. reported significantly reduced gene expression of IL-6, IL-8, and intercellular adhesion molecule o, me as well as expression of eicosanoid producing enzymes, including cyclooxygenase two and 15-lipoxygenase 1, in bovine mammary endothelial cells after treatment with LPS as a model of coliform mastitis (Corl et al., 2010). Considering the positive results in previous animal models and the fact that ethyl pyruvate is natural and safe for human consumption, this molecule could be regarded as a therapeutic agent to reduce the systemic effects of endotoxemia on neonatal calves.

Our study's objective was to evaluate the effect of ethyl pyruvate on specific pro-inflammatory cytokines in vitro using the whole blood of neonatal dairy calves at different dosages with or without the addition of LPS. The hypothesis was that ethyl pyruvate is biologically active in healthy neonatal calves and can reduce the concentration of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) following in vitro stimulation of whole blood with LPS in different experiment settings.

Materials and Methods

Animals

This study used seven healthy Holstein female calves between 7 and 21 days old. Four were used for the first two experiments and three for the others. Calves were used for blood sampling only and did not receive any drug. The calves were part of the East Tennessee Research and Education Center – Little River Dairy Unit dairy herd. According to farm protocol, they were placed in individuals' hutchs and fed. Calves were only sampled once in the jugular vein on the day of the experiment.

Sample collection

On the day of the experiment, calves were manually restrained, and 10 ml of whole blood was collected through the jugular vein using a 10cc luer lock syringe and an 18 GA x 1 1/2" needle. The site of venipuncture was previously cleaned using an alcohol swab. Whole blood was transferred to EDTA tubes and put on ice until manipulation at the lab.

In vitro stimulation with ethyl pyruvate

Experiment 1

This study was designed to evaluate the effect of different dosages of ethyl pyruvate on the production of IL-6 and IL-1β while co-incubating with various levels of LPS. In this study, four calves' whole blood samples were used.

Whole blood in EDTA samples were divided into 2 ml aliquots and placed in 10 ml disposable glass tubes. Lipopolysaccharide (E. coli O55:B5 LPS) was reconstituted in RPMI-1640 to 1 mg/ml and stored at -20°C. Before use, LPS was diluted in RPMI-1640 in a 50 ml canonical tube and mixed by vortexing to obtain a concentration of 0.1 ng/ml or 1 ng/ml. Each blood sample was subject to three concentrations of ethyl pyruvate (0 mM, 5 mM, 10 mM) and three concentrations of LPS (absence: 0 ng/ml, Low dose (LD): 0.1 ng/ml, High dose (HD): 1 ng/ml) and was run in duplicate. Ethyl pyruvate was diluted in RPMI-1640 to obtain the two different concentrations. Negative control for LPS and ethyl pyruvate was obtained by adding an equal volume of RMPI-1640 to the solution. The final volume of each solution was 4 ml.

As described above, once mixed with the desired ethyl pyruvate concentration, a dose of 0, 0.1 or 1 ng/ml was added to the calf whole blood. The same volume of RPMI was added to control samples. Stimulated blood was incubated for 60 minutes in a water bath at 37°C. All samples were run in duplicate.

Following stimulation with LPS, each tube was centrifugated at 2,500 g for 10 minutes at 4°C for plasma extraction. The resulting plasma was collected and transferred to a clean polypropylene tube using a pipette. Samples were maintained at 2–8°C while handling. The plasma was apportioned into 2 ml

aliquots, stored, and placed in a freezer at -20 °C until all samples were collected. All samples were thawed and maintained between 2-8°C on ice when ready. Samples were then used to run testing for pro-inflammatory cytokines (IL-6 and IL-1β) using pre-validated bovine-specific ELISA protocol.

Experiment 2

The second experiment was designed to see the effect of two delayed doses of ethyl pyruvate on some pro-inflammatory cytokines. For that experiment, whole blood samples from three calves were used. As described previously, whole blood in EDTA samples were divided into 2 ml aliquots and placed in 10 ml disposable glass tubes. Lipopolysaccharide (E. coli O55:B5 LPS) was reconstituted in RPMI-1640 to 1 mg/ml and stored at -20°C. Before use, LPS was diluted in RPMI-1640 in a 50 ml canonical tube and mixed by vortexing to obtain a 0.1 ng/ml concentration.

Each blood sample was subject to three concentrations of ethyl pyruvate (0 mM, 5 mM, 10 mM) and one concentration of LPS (0.1 ng/ml), except for the negative control (LPS concentration of 0 ng/ml). Each sample was run in duplicate. Ethyl pyruvate was diluted in RPMI-1640 to obtain the two different concentrations. Negative control for LPS and ethyl pyruvate was obtained by adding an equal volume of RMPI-1640 to the solution. The final volume of each solution was 4 ml.

Once mixed with the desired LPS concentration, the samples were incubated for 0, 1 or 6 hours in a water bath at 37°C before adding ethyl pyruvate concentration of 5 mM or 10 mM. One negative sample contained no LPS or ethyl pyruvate and was incubated for 1 hour. Once ethyl pyruvate was added, stimulated blood was set for 60 minutes in a water bath at 37°C.

Following stimulation co-incubation with ethyl pyruvate, each tube was centrifugated at 2,500 g for 10 minutes at 4C for plasma extraction. The resulting plasma was collected and transferred to a clean polypropylene tube using a pipette. Samples were maintained at 2–8°C while handling. The plasma was apportioned into 2 ml aliquots, stored, and placed in a freezer at -20 °C until all samples were collected. When ready, all samples were thawed and maintained between 2-8°C. Samples were then used to run testing for pro-inflammatory cytokines (IL-6 and IL-1β) using pre-validated bovine-specific ELISA.

Experiment 3

This experiment was designed to compare the production of IL-6, IL-1β and TNF-α concentrations following stimulation during one hour at 37°C with LPS. Blood samples were mixed with decreasing doses of ethyl pyruvate (0 mM, 0.1 mM, 0.001 mM, 0.0001 mM, 0.0001 mM, 0.00001 mM) simultaneously. Four whole blood samples from calves were used for that experiment. The protocol was like the first experiment except that only the concentration of 0 or 1 ng/ml of LPS was used. Whole blood, ethyl pyruvate and LPS were mixed simultaneously

and incubated at 37°C in a water bath for 1 hour. Samples were all run in duplicate.

Following stimulation with LPS, each tube was centrifugated as previously described, and plasma was extracted for pro-inflammatory cytokines testing using pre-validated bovine-specific ELISA for IL-6, IL-1β and TNF-α.

Experiment 4

In this experiment, blood sample was mixed with decreasing dose of ethyl pyruvate (same concentration as experiment 3) to see if ethyl pyruvate stimulates interleukins production by comparing IL-6, IL-1B and TNF-a concentrations following stimulation during one hour at 37°C with only decreasing doses of ethyl pyruvate (0 mM, 0.1 mM, 0.001 mM, 0.0001 mM, 0.0001 mM, 0.00001 mM). Only one whole blood sample from the calf was used for the last experiment. Whole blood in an EDTA sample was used. As previously described, whole blood sample and ethyl pyruvate were incubated at 37°C in a water bath for 1 hour. Samples were all run in duplicate. Following stimulation with ethyl pyruvate, each tube was centrifugated as previously described, and plasma was extracted for pro-inflammatory cytokines testing using pre-validated bovine-specific ELISA for IL-6, IL-1β and TNF-α.

Pro-inflammatory cytokines

Pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and HMGB1 were measured in plasma in duplicate from LPS-challenged and sham-challenged aliquots. Samples were thawed rapidly at room temperature and were kept on ice until analyzed. For each sample, 100μL of plasma was used per experiment. The day before analysis, ELISA plates were prepared to detect IL-1β and IL-6. Plates were prepared using 8-Well Polystyrene Strip Plates (Pierce™ 8-Well Polystyrene Strip Plates, Corner Notch, No. 15031, Thermofisher™). All reagents and buffers were to equilibrate to room temperature (22-25°C) before use. The coating antibody for IL-1β and IL-6 was diluted at 1:100 in carbonate-bicarbonate buffer by adding 110μL coating antibody to 10.89mL of carbonate-bicarbonate buffer solution (Carbonate/Bicarbonate Buffer, No. 28382, Thermofisher™). A volume of 100μL of diluted Coating Antibody was added to each well using a multichannel micropipette. The plates were covered with plate sealer (Sealing tape for 96-Well plates, No. 15036, Thermofisher™) and incubated overnight at room temperature. The next day, the coating antibody solution was aspirated with the multichannel pipette and 300μL of blocking buffer (ELISA Blocker Blocking Buffer, No. N502, Thermofisher™) was added to each well. Plates were once more covered with a plate sealer and incubated for 1 hour at room temperature. The blocking buffer was aspirated, and the assay was run. ELISA testing was performed according to the manufacturer's instructions (IL-6 Bovine Uncoated ELISA Kit, Invitrogen, ThermoFisher Scientific; IL-1 beta Bovine

Uncoated ELISA Kit Invitrogen, ThermoFisher Scientific; TNF alpha Bovine ELISA Kit, Invitrogen, ThermoFisher Scientific; Bovine high-mobility group box 1 ELISA Kit, Mybiosource). For IL-6 and IL-1beta, ELISA assays were run using the premade plate. Standard was reconstituted with reagent diluent at 10,000 pg/ml or IL-6 and 4,000 pg/ml for Il-1β. Standards were diluted 1:2 and 100μL of standards and samples were added to each well in duplicate. The plate was covered and incubated for 1 hour at room temperature. The wash procedure was performed three times following aspiration of the well content. 300μL of wash buffer was used per well at each time. The plate was dried on an absorbent paper towel before the next step. The detection antibody was diluted in reagent diluent by adding 110μL of detection antibody to 10,89ml of reagent diluent (1:100). 100μL of detection antibody solution was added to each well. The plate was covered with a plate sealer and incubated for another 1 hour at room temperature. Another cycle of plate wash was performed as previously described. Streptavidin-HRP was diluted into reagent diluent by adding 30μL of Streptavidin-HRP to 12 ml of reagent diluent (1:400). 100μL of diluted streptavidin-DRP was added to each well. The plate was covered with a plate sealer and incubated for another 30 minutes at room temperature. Another cycle of plate wash was performed as previously described. 100μL of substrate solution was added to each well. The plate was covered with a plate sealer and incubated for another 20 minutes at room temperature in the dark. 100μL of stop solution was added to each well to stop the reaction. Absorbance was measured

using a plate reader. For TNF- α, a similar process was used. ELISA plate provided with the kit was used. Standard were reconstituted with reagent diluent at 30 ng/ml concentration. Standards were diluted 1:2 (0, 0.123, 0.307, 0.768, 1.92, 4.8, 12 and 30 ng/ml), and 100μL standards and experimental samples were added to each well in duplicate. The plate was covered and incubated for 2.5 hours at room temperature with gentle shaking. The wash procedure was performed four times following aspiration of the well content. 300μL of wash buffer was used per well at each time. The plate was dried on an absorbent paper towel after the last wash. Biotin conjugate 80-fold with assay diluent B. 100μL of prepared biotin conjugate solution was added to each well. The plate was covered with a plate sealer and incubated for another 1 hour at room temperature with gentle shaking. Another cycle of plate wash was performed as previously described. Streptavidin-HRP was diluted 600-fold by adding assay diluent B. 100μL of diluted streptavidin-DRP solution was added to each well. The plate was covered with a plate sealer and incubated for another 45 minutes at room temperature with gentle shaking. The solution was discarded, and another cycle of plate washing was performed as previously described. 100μL of TMS substrate solution was added to each well. The plate was covered with a plate sealer and incubated for another 30 minutes at room temperature in the dark with gentle shaking. 50μL of stop solution was added to each well to stop the reaction. Absorbance was measured using a plate reader. The intensity of the reaction signal was read with an ELISA plate reader at 450nm and 550nm 30

minutes after stopping the reaction. It was correlated to the standard curve to determine the sample concentration. For TNF- α, the signal was only read at 450nm. Standard curves for each plate were made using Prism 10 (GraphPad Software, Boston, MA) (see Appendix at the end of the dissertation). Minimal limit detection according to the company pamphlet for HMGB1, IL-6, IL1-β and TNF-α ELISA assay are 1.0 ng/ml, 78.1 pg/ml, 31.3 pg/ml and 0.1 ng/ml respectively. Data below the detection limit were considered as a zero value for analysis.

Statistical and data analysis

Pro-inflammatory cytokines concentration data were analyzed using Proc Glimmix in SAS. The effects of LPS, ethyl pyruvate and time on plasma cytokine concentration were examined using mixed model analysis with individual subjects as the random block factor. Rank data transformation was applied when diagnostic analysis on residuals violated normality and equal variance assumptions using the Shapiro–Wilk test and Levene's test. Post hoc multiple comparisons were performed with Tukey's adjustment. Statistical significance was identified at $p<0.05$. Analyses were conducted in SAS 9.4 TS1M8 (SAS Institute Inc., Cary, NC).

Results

Experiment 1

The concentration of IL-6 was significantly higher in association with a greater concentration of ethyl pyruvate in the sample. A concentration of 10mM showed a substantially higher increase in IL-6 in the sample by blood cells compared to a concentration of 5mM or 0mM. Similarly, a concentration of 5mM showed a higher increase in IL-6 concentration than 0mM. Adding LPS to the solution was not considered significant, even if the concentration was more remarkable. All results are shown in figure 9.

Compared with IL-6, LPS treatment and ethyl pyruvate treatment were significantly different while looking at IL-1β concentration). An increase in concentration of ethyl pyruvate increases the IL-1β concentration significantly showing a higher increase with a concentration of 10mM vs 5mM vs 0mM. Adding 1 ng/ml of LPS showed an increased concentration of IL-1βbut not 0.1 ng/ml). All results are shown in figure 10.

Experiment 2

The analysis showed a significant effect of ethyl pyruvate, time, and interaction between time and ethyl pyruvate for IL-6 concentration. The concentration of IL-6 was significantly higher in association with a greater

concentration of ethyl pyruvate in the sample. Concentrations of 10mM and 5mM showed a significantly higher increase in IL-6 in the sample by blood cells compared to a concentration of 0mM. There was no difference between those two dosages, but overall, we can see a reduction in IL-6 concentration at 6 hours for both concentrations of ethyl pyruvate. Similarly, there was a significant difference due to incubation time with increased concentration of IL-6 after 1 hour and 6 hours of incubation with LPS. A greater incubation time, above 1 hour, did not significantly affect the concentration of IL-6. Finally, adding ethyl pyruvate and a longer incubation time together considerably increased the concentration of IL-6. Still, there was no difference between treatment concentration and time of incubation together. All results are shown in figure 11.

Similarly, ethyl pyruvate treatment, time and interaction between time ethyl pyruvate treatment were all significant regarding IL-1β concentration. The concentration of ethyl pyruvate increases the concentration of IL-1β at time 0 while adding ethyl pyruvate and LPS to the whole blood. Similarly, a higher concentration of ethyl pyruvate increases IL-1β at time 1 was seen with the addition of ethyl pyruvate and LPS to the blood. Overall, treatment with either 5mM or 10mM of ethyl pyruvate showed a significant increase in IL-1β concentration compared to no ethyl pyruvate. No difference was noted between the two dosages. The incubation time before adding ethyl pyruvate also showed an increase in IL-1β concentration but was similar between the 1 and 6 hours of

incubation overall. Interestingly, the concentration of IL-1β at time 6 was higher in the non-treated group compared to the 5mM ethyl pyruvate concentration. All results are shown in figure 12.

Experiment 3

The concentration of IL-6 was not affected by the addition of ethyl pyruvate at any concentration. The only significant data was that IL-6 concentration was significantly higher in the sample when LPS was added at 1 ng/ml. Means concentration of IL-6 were slightly higher at the doses of 0.00001mM, 0.0001 mM and 0.001mM, but did not show a statistical difference with the other IL-6 value in the 1 ng/ml of the LPS group.

The result of that experiment showed no effect of ethyl pyruvate concentration at 0mM, 0.1mM, 0.001mM, 0.0001mM, 0.0001mM or 0.00001mM while incubated for one hour in a water bath with whole blood as all concentration for IL-1β and TNF-α. Values were below the detection limit for IL-1β and TNF-α in more than 80% of the sample for all concentrations.

Experiment 4

The result of that experiment showed no effect of ethyl pyruvate concentration at 0mM, 0.1mM, 0.001mM, 0.0001mM, 0.0001mM or 0.00001mM

while incubated for one hour in a water bath with whole blood as all concentrations value were below the detection limit for IL-6, IL-1β and TNF-α.

Discussion

Using bovine mammary endothelial cells, Corl et al. 2010 demonstrate that ethyl pyruvate at 5 and 10mM doses inhibited 1L-1β, IL-6, and IL-8 mRNA expression significantly. Treatment of endotoxin-stimulated BMEC with 5 and 10mM ethyl pyruvate resulted in 70.4 and 84.8% reductions for IL-6 compared with endotoxin-stimulated BMEC. Treatment with ethyl pyruvate did decrease endotoxin-induced IL-1β gene expression, but the change in expression was not significantly different compared with the peak expression observed in the endotoxin-stimulated group. The bovine mammary endothelial cells were unstimulated/untreated or stimulated with 50 ng/mL endotoxin for 1 hour and treated with 0, 5, or 1mM EP for an additional 1 hour before isolation of RNA. Compared with our study, they used a much higher dose of LPS and a different *Escherichia coli* endotoxin (*E. coli* O111:B4).

Interestingly, in our study, the production of IL-1β IL-6 was higher than the blood only stimulated with LPS at both concentrations described in the Corl et al. 2010 study. This difference could result from using whole blood assay compared to individual cell lines. The difference may come from the interaction with the red blood cells, as seen in hemolytic diseases (Sesti-Costa et al., 2021). Red blood cell signalling would be due to a stress response from the damaged red blood

cells. For this reason, to further assess ethyl pyruvate's effect on monocytes or neutrophils in the future, those cells should be isolated before the LPS challenge.

The effect of ethyl pyruvate at 5 and 10mM was surprising compared to known data. The incubation with whole blood led to a change in the colour of the solution from red to dark brown. It was interesting to see that it was only seen with a higher molecule concentration. While doing the third experiment with lower doses of ethyl pyruvate, such a change was not noticed. It is possible, due to its nature as a volatile agent and having a flash point at 46°C that the solution with a higher dosage of ethyl pyruvate was very close to the flash point due to fewer dilutions of ethyl pyruvate while compared with the lowest concentration. This observation could also explain why adding ethyl pyruvate at a higher dose increases stress on whole blood cells and could increase IL-6 and IL-1β if the cells are close to the drug flash point).

In vitro, cytotoxicity of ethyl pyruvate has been assessed by monitoring in vitro bovine mammary endothelial cell viability (Corl et al., 2010). A dosage from 0 to 80mM ethyl pyruvate was used. In this paper, cell viability using trypan blue was over 87% up to 4 hours of incubation but showed viability decreased to 75% and 15% at the 20 and 40mM doses, respectively. Treatment with 80mM ethyl pyruvate after 4 and 6 hours of exposure decreased viability below the detection threshold. Similar findings were found with the measurement of ATP production

by the cell to correlate metabolic activity with cell viability. There was a decrease in cellular ATP with 20 and 40mM ethyl pyruvate at 4 hours of incubation. No significant reduction in bovine mammary endothelial cell viability was observed with 5 or 10mM ethyl pyruvate at any time points over the study period. This paper concluded that 10 mM ethyl pyruvate was the maximum tolerable concentration in vitro for that cell type (Corl et al., 2010).

Similarly, 0, 1,5, and 10mM ethyl pyruvate dosages were used to stimulate equine peripheral monocytes for four hours without affecting monocyte viability (Cook et al., 2011). A dose of 50mM was killing all the cells. Similar findings were found by Ulloa et al. using murine macrophages (RAW264.7) (Ulloa et al., 2002). So, it is still unclear why our cells acted differently with the exact ethyl pyruvate dosage.

The timing for the second experiment was decided while comparing studies performed in rats and horses (Venkataraman et al., 2002, Schroeder et al., 2011). In those studies, a significant difference was noted 6 hours after the beginning of endotoxemia. In the rat study, the level of IL-6 increased throughout the experiment and was higher at 6 hours than at 3 hours. Overall, a reduction in IL-6 was noted compared to the non-treated group at both times. These results are consistent with the positive effects of ethyl pyruvate observed in mice when the treatment is delayed until 24 h after the onset of sepsis (Ulloa et al., 2002). In

our study, we see a drop in IL-1β concentration at 6 hours with both dosages of ethyl pyruvate), with the value being lower than that of the non-treated cell.

Similarly, IL-6 concentration drops from time 1 to 6 in the 10 mM group. Those values were not significant, but they warrant further investigation. A report from a recent study showed that IL-1β concentration peaks at 2 hours post LPS infusion in beef steers (Smock et al., 2023). A drop at 6 hours could eventually be expected for this cytokine. Ethyl pyruvate is one of the first compounds to show beneficial effects in a post-treatment rescue model in many other species, making it potentially attractive for use in clinical cases of endotoxemia and septicemia in calves.

Conclusion

Ethyl pyruvate is a potent anti-inflammatory in various species. In bovine whole blood, we found that ethyl pyruvate concentration at higher concentrations could increase cytokine production by immune cells. As ethyl pyruvate at lower dosages did not alter the production of inflammatory cytokines following incubation with whole calves' blood, it seems that the ethyl pyruvate effect is dose-dependent and could lead to increased cell destruction, leading to increased production of divers' cytokines at a higher dosage. Those data are essential for future evaluation of this molecule in the bovine species for in vitro and in vivo experiments to select a dosage that won't affect cell survival.

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Conflict of interests

The authors have no competing interests to disclose.

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Appendix

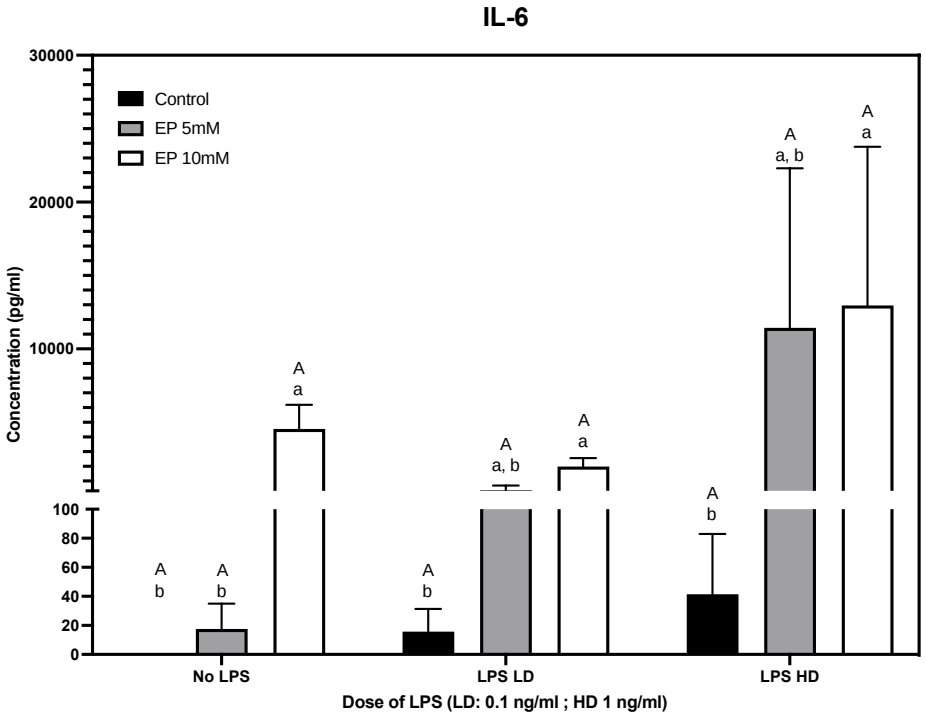
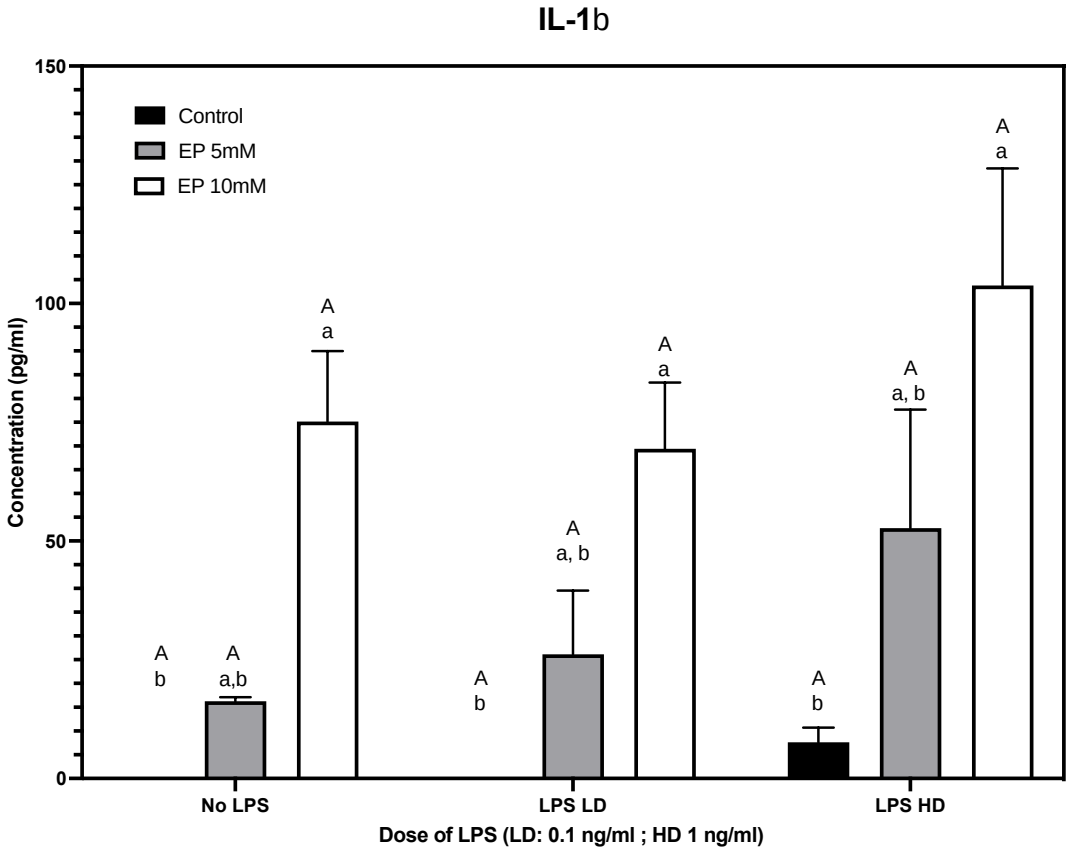


Figure 9. The ability of ethyl pyruvate (EP) to affect endotoxin-induced IL-6 protein excretion in neonatal bovine whole blood. The blood samples were unstimulated/untreated or stimulated with 0.1 ng/mL (LD) or 1 ng/ml (HD) endotoxin for 1 hour and treated with 0-, 5-, or 10-mM EP for an additional 1 hour before plasma isolation. Enzyme-linked immunosorbent assay evaluated the production of IL-6. Data are expressed as concentration values following correlation with a standard curve. A-C Means without a common letter differ significantly (P < 0.001) between treatment groups. a–c Means without a common letter differ significantly (P < 0.05) within the LPS group. Error bars represent standard deviation.

Figure 80. The ability of ethyl pyruvate (EP) to affect endotoxin-induced IL-1β protein excretion in neonatal bovine whole blood. The blood samples were unstimulated/untreated or stimulated with 0.1 ng/mL (LD) or 1 ng/ml (HD) endotoxin for 1 hour and treated with 0-, 5-, or 10-mM EP for an additional 1 hour before plasma isolation. Enzyme-linked immunosorbent assay evaluated the production of IL-1β. Data are expressed as concentration values following correlation with a standard curve. A-C Means without a common letter differ significantly (P < 0.001) between treatment groups. a–c Means without a common letter differ significantly (P < 0.05) within the LPS group. Between treatment groups. a–c Means without a common letter differ significantly (P < 0.05) within the LPS group. Error bars represent standard deviation.


IL-1b
150
100
50
0
Concentration (pg/ml)
Control
EP 5mM
EP 10mM
A
b
A
a,b
A
a
A
b
A
a, b
A
a
A
b
A
a, b
A
a
No LPS
LPS LD
LPS HD
Dose of LPS (LD: 0.1 ng/ml ; HD 1 ng/ml)

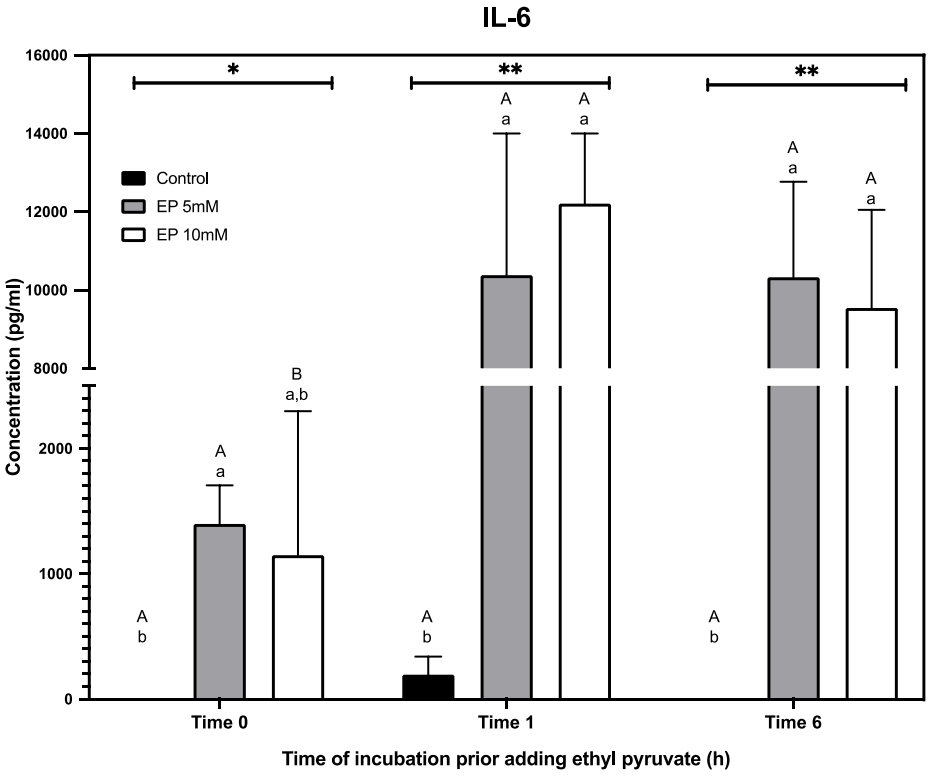


Figure 9. Ethyl pyruvate effect on endotoxin-induced IL-6 production. IL-6 protein concentration was measured in whole blood following stimulation with 1 ng/ml endotoxin for 1 hour. Ethyl pyruvate, 0, 5, or 10 mM, was added at 0 before incubation or 1- or 6 hours following stimulation with LPS. The solution was incubated for an additional 1 hour before plasma isolation. A-C Means without a common letter differs significantly (P < 0.001) within the treatment group. a–c Means without a common letter differ significantly (P < 0.05) within the Time group. *, **,* Means without a common sign differ significantly (P < 0.001) between overall Time treatments group. Error bars represent standard deviation.**

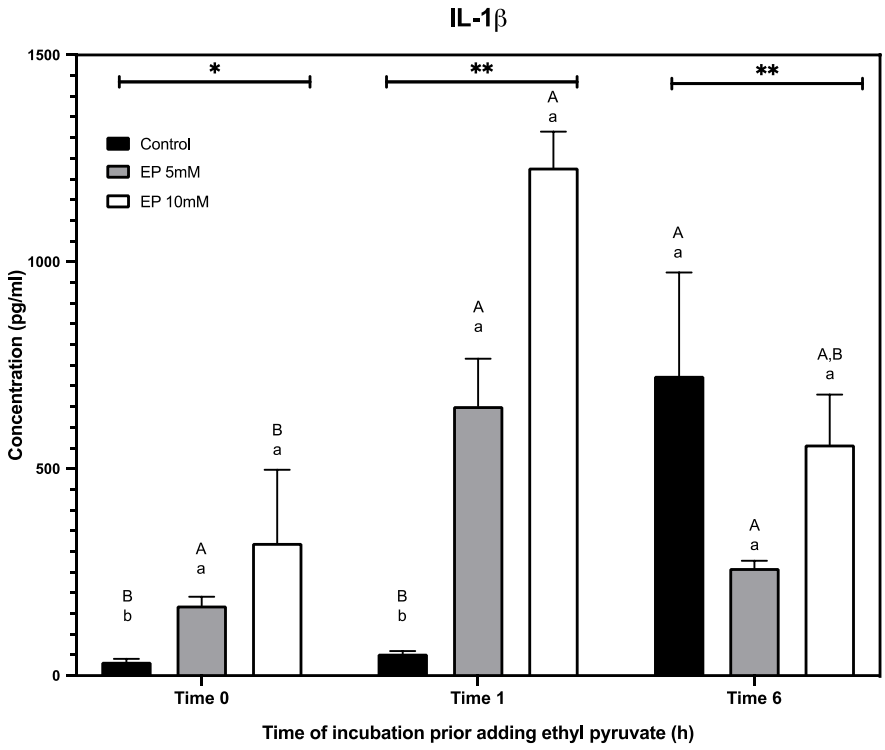


Figure 10. Ethyl pyruvate effect on endotoxin-induced IL-1β production. IL-1β protein concentration was measured in whole blood following stimulation with 1 ng/ml endotoxin for 1 hour. Ethyl pyruvate, 0, 5, or 10 mM, was added at 0 before incubation or 1- or 6 hours following stimulation with LPS. The solution was incubated for an additional 1 hour before plasma isolation. A-C Means without a common letter differ significantly (P < 0.001) within the ethyl pyruvate treatment group. a–c Means without a common letter differ significantly (P < 0.05) within the Time group. *, **,* Means without a common sign differ significantly (P < 0.001) between overall Time treatments group. Error bars represent standard deviation.**

CONCLUSION

In conclusion, we demonstrate that ethyl pyruvate can be administered with low side effects while diluting in a 1-liter solution of LRS over 30 to 60 minutes of infusion time. Infusing the drug slowly is a crucial factor in reducing any side effects, including respiratory arrest. For this reason, ethyl pyruvate infusion should not be used in the field but only in research settings with close monitoring to avoid side effects. We found that ethyl pyruvate does not reduce the release of proinflammatory cytokines 1-hour post-infusion with LPS. Considering cytokines' release peak, the LPS challenge could need a more extended incubation period to see a significant reduction. We found that ethyl pyruvate *in vitro* at higher concentrations could increase cytokine production by immune cells and show a dose-dependent effect on the production of proinflammatory cytokines. The efficacy of ethyl pyruvate in an *in vivo* endotoxemic challenge still needs to be assessed. The result of this dissertation does not support the hypothesis that ethyl pyruvate could alleviate the release of proinflammatory cytokines following an *in vitro* endotoxemia challenge in dairy calves. For this reason and due to possible safety concerns, this molecule should not be used clinically in neonatal dairy calves at this point.

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APPENDIX

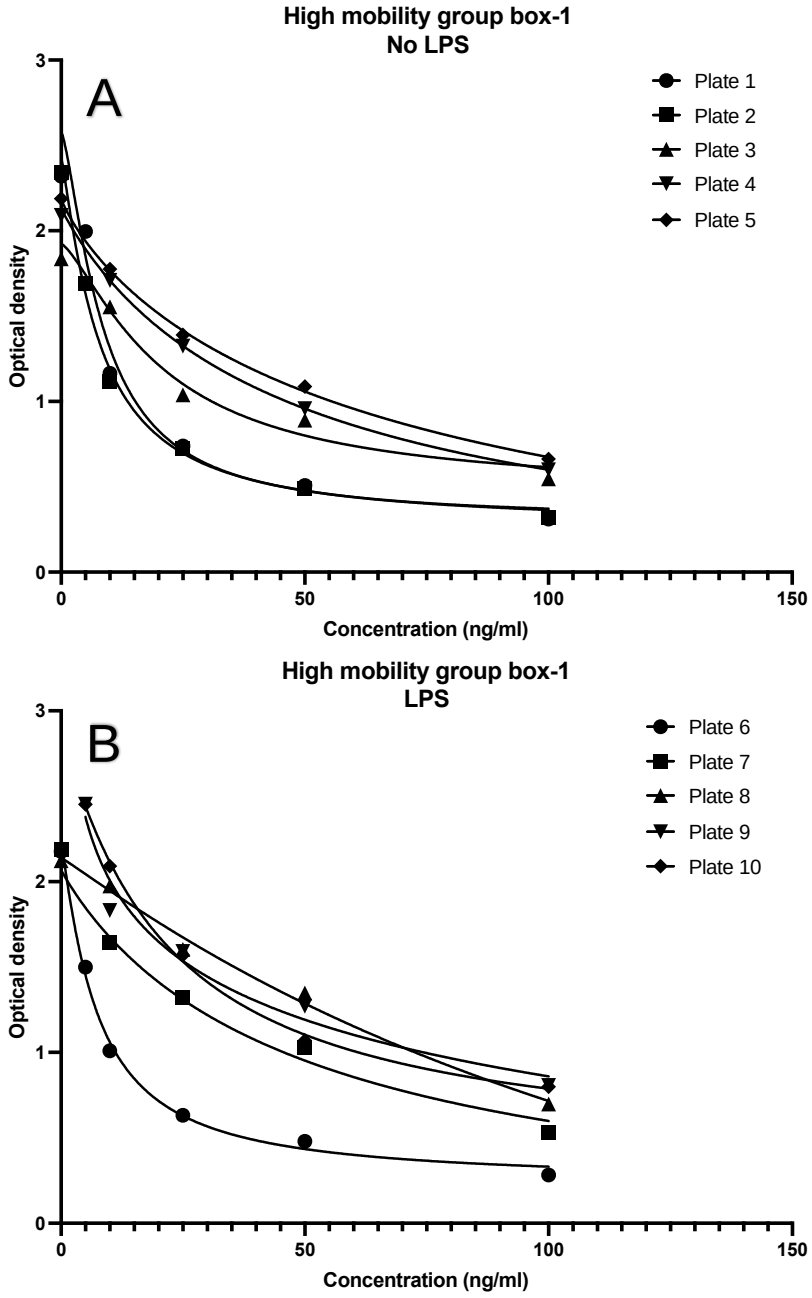


Figure 11. Standard curves used to extrapolate High mobility group box-1 concentration for analysis with (A) and without LPS stimulation (B) for Chapter 1. Concentrations are in ng/ml.

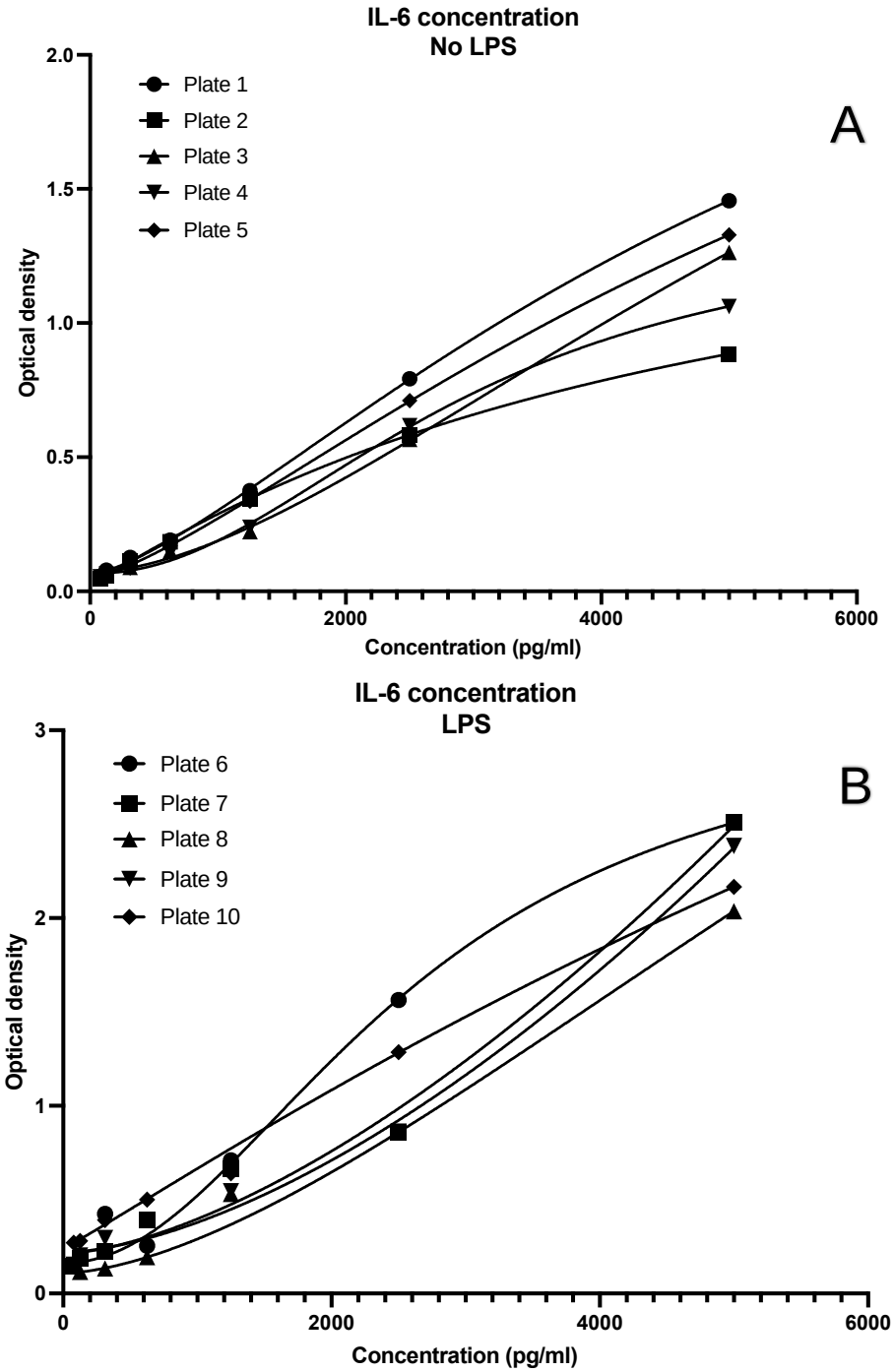


Figure 12. Standard curves used to extrapolate Il-6 concentration for analysis with (A) and without LPS stimulation (B) for Chapter 1. Concentrations are in pg/ml.

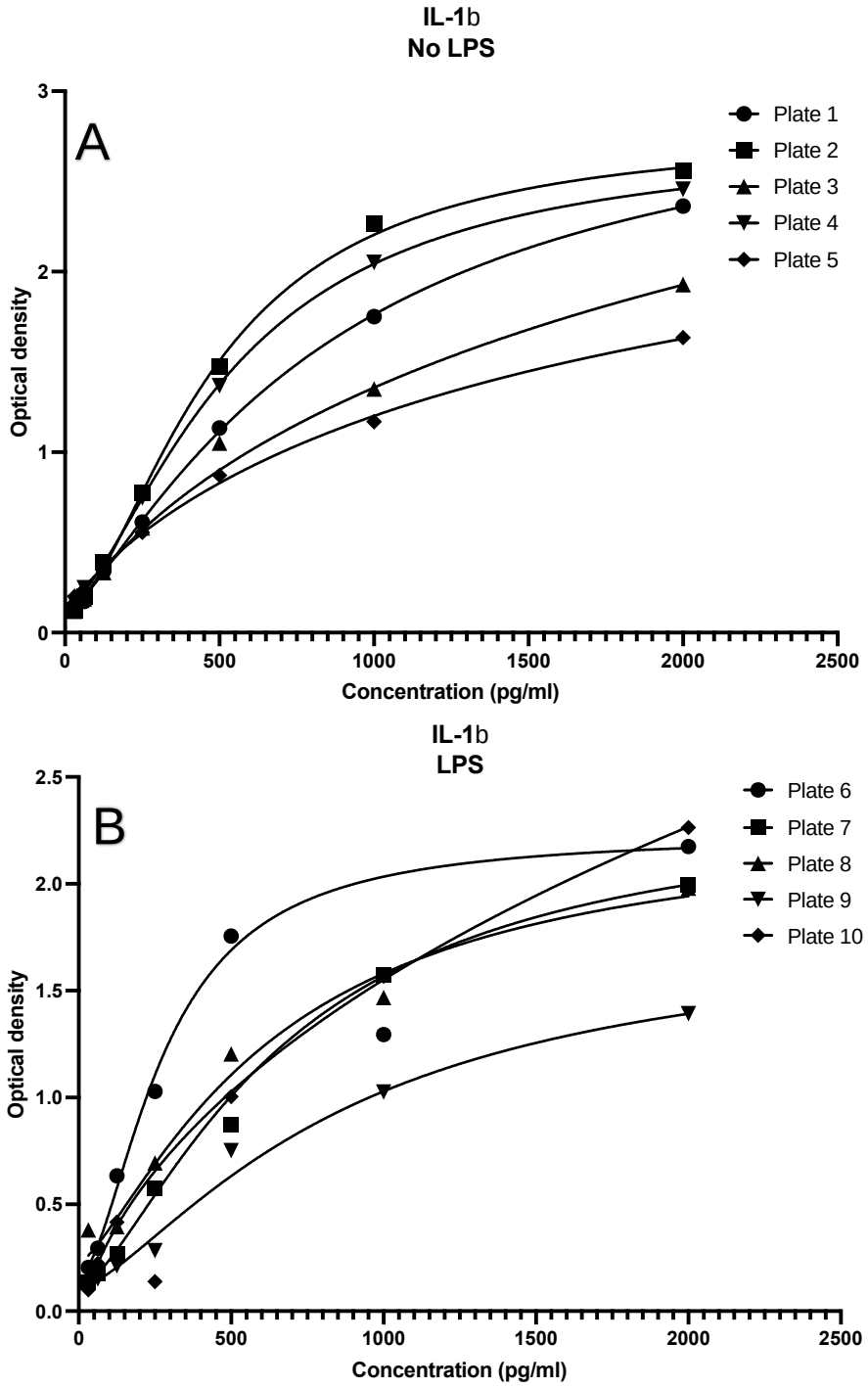


Figure 13. Standard curves used to extrapolate IL-1β concentration for analysis with (A) and without LPS stimulation (B) for Chapter 1. Concentrations are in pg/ml.

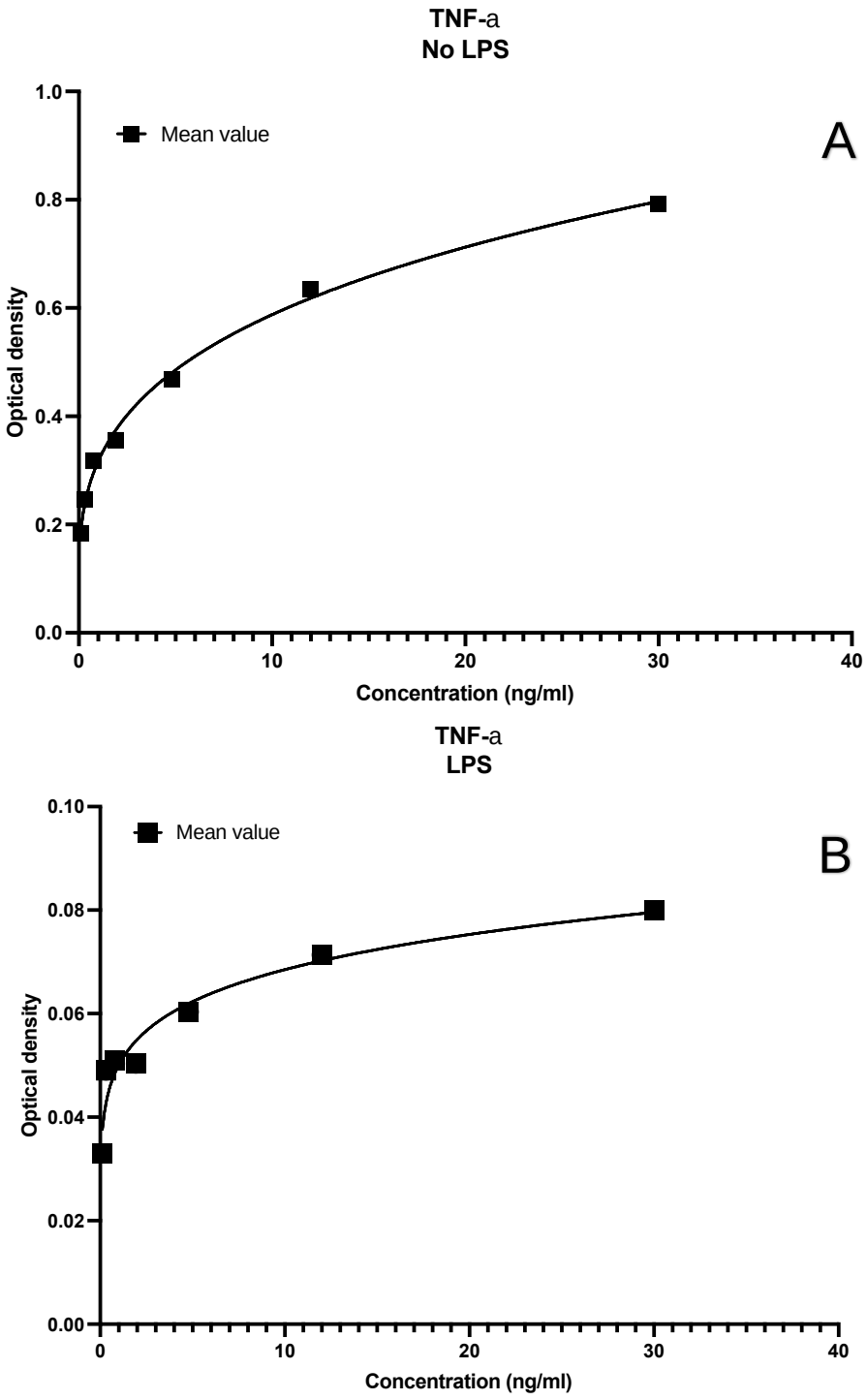


Figure 14. Standard curves used to extrapolate TNF-α concentration for analysis with (A) or without LPS stimulation (B) for Chapter 1. Concentrations are in ng/ml.

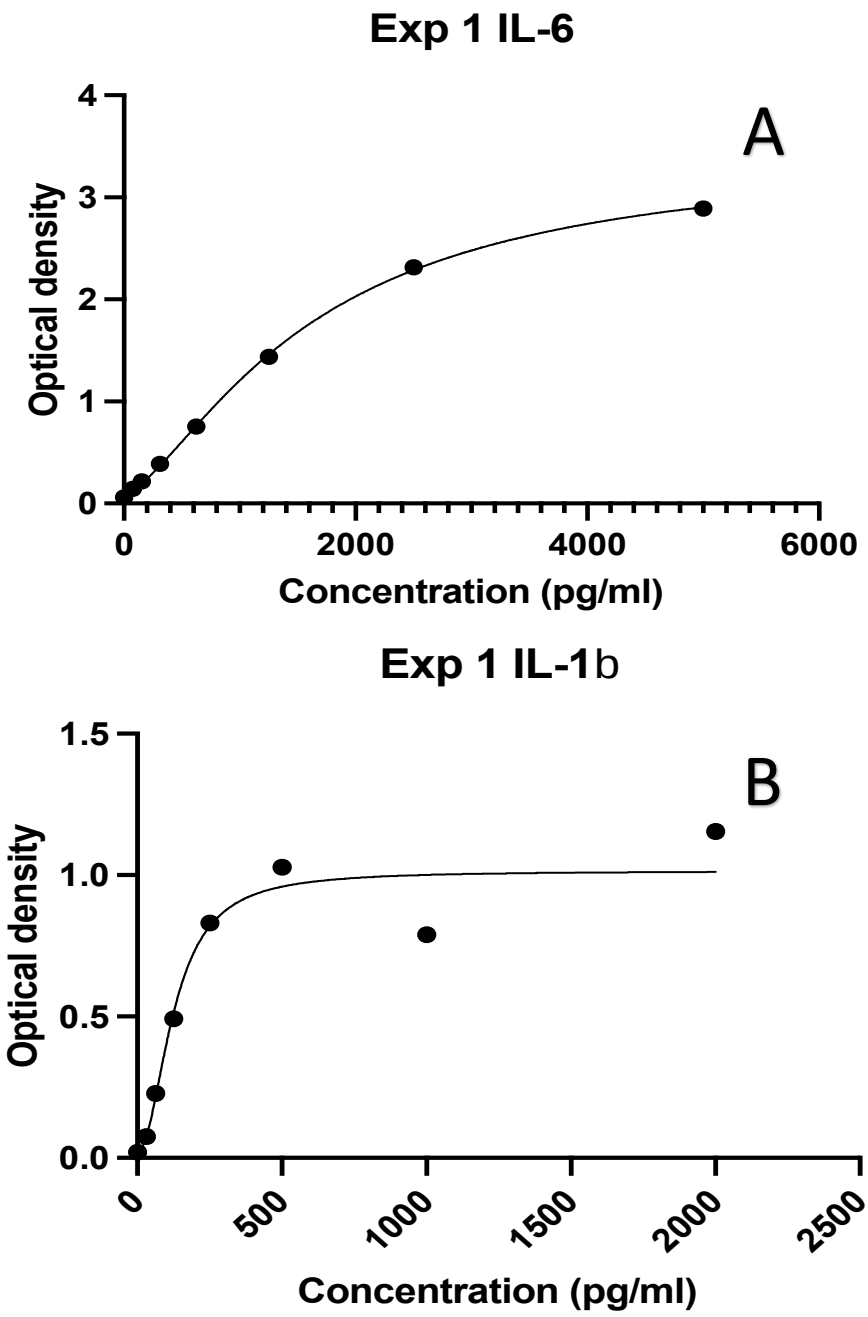


Figure 15. Standard curves used to extrapolate IL-6 (A) and IL-1β (B) concentration for analysis for Chapter 2 Experiment 1. Concentrations are in pg/ml.

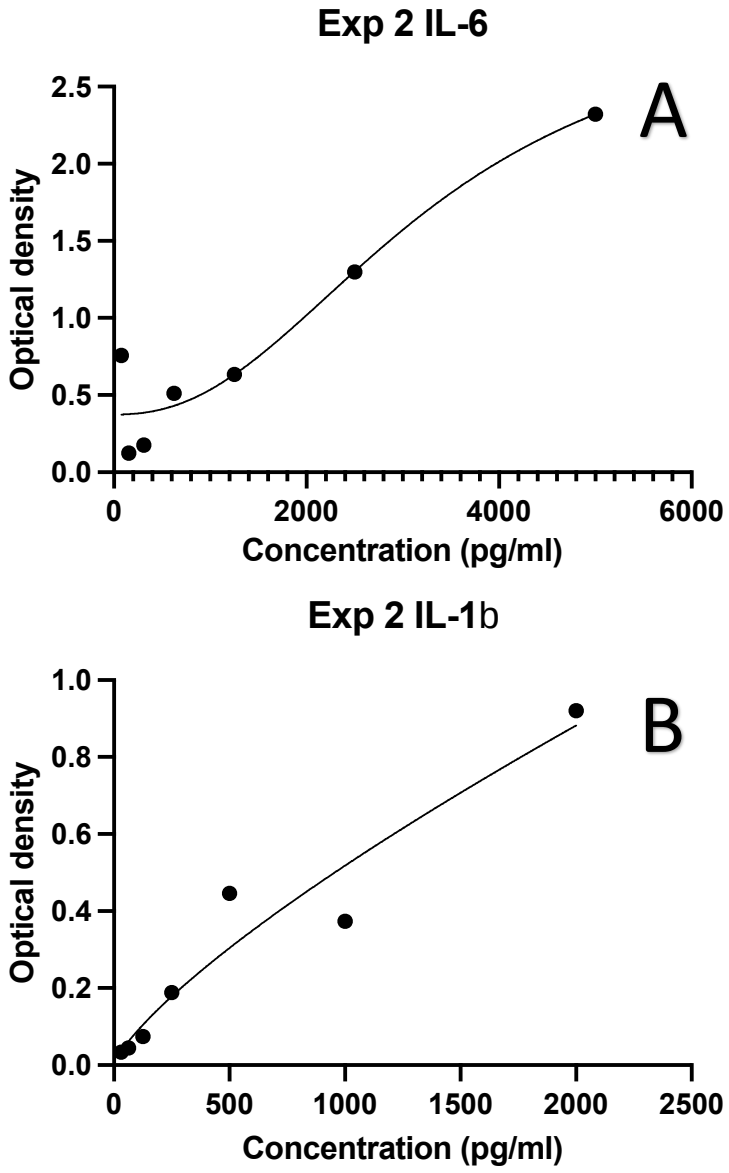
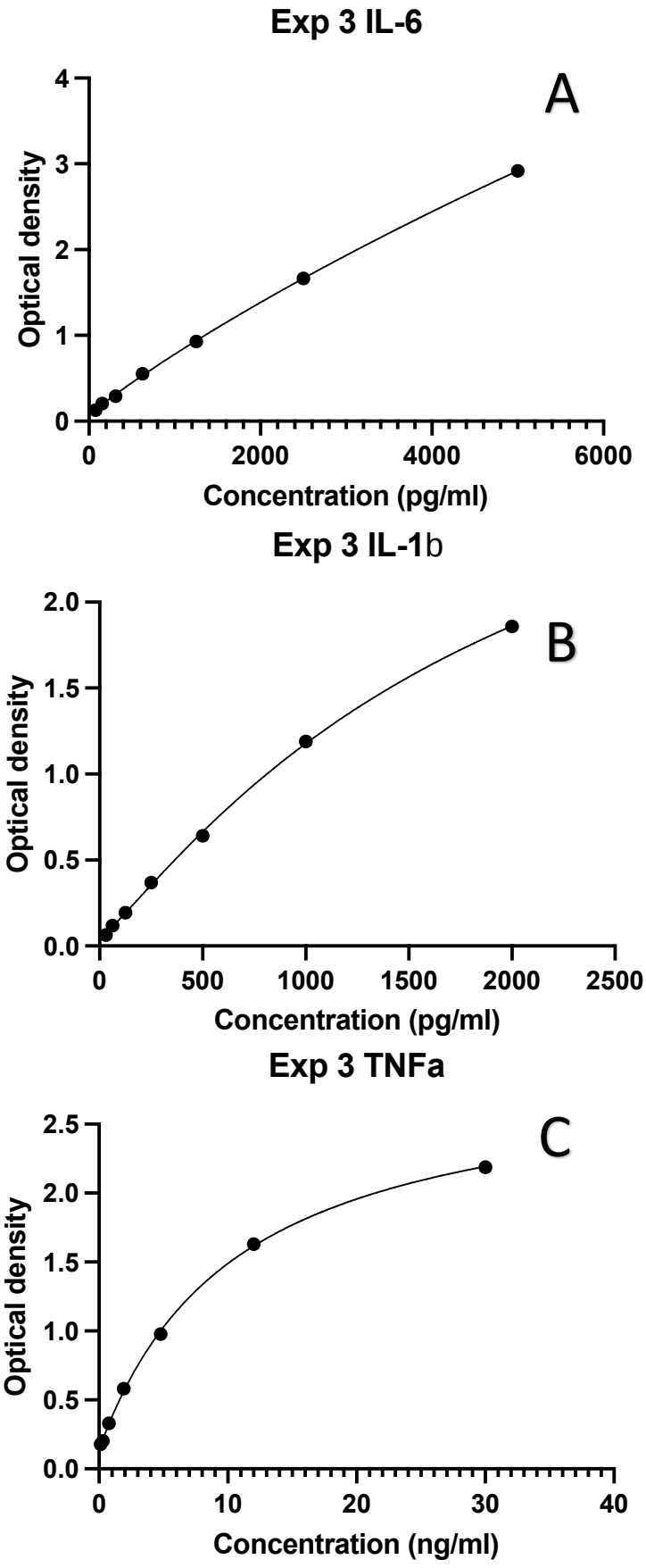


Figure 16. Standard curves used to extrapolate IL-6 (A) and IL-1β (B) concentration for analysis for Chapter 2 Experiment 2. Concentrations are in pg/ml.

Figure 17. Standard curves used to extrapolate IL-6 (A), IL-1β (B) and TNF-α (C) concentration for analysis for Chapter 2 Experiment 3. Concentrations are in pg/ml for IL- IL-1β and in ng/ml for TNF-α.


Exp 3 IL-6
A
Optical density
Concentration (pg/ml)
Exp 3 IL-1b
B
Optical density
Concentration (pg/ml)
Exp 3 TNFa
C
Optical density
Concentration (ng/ml)

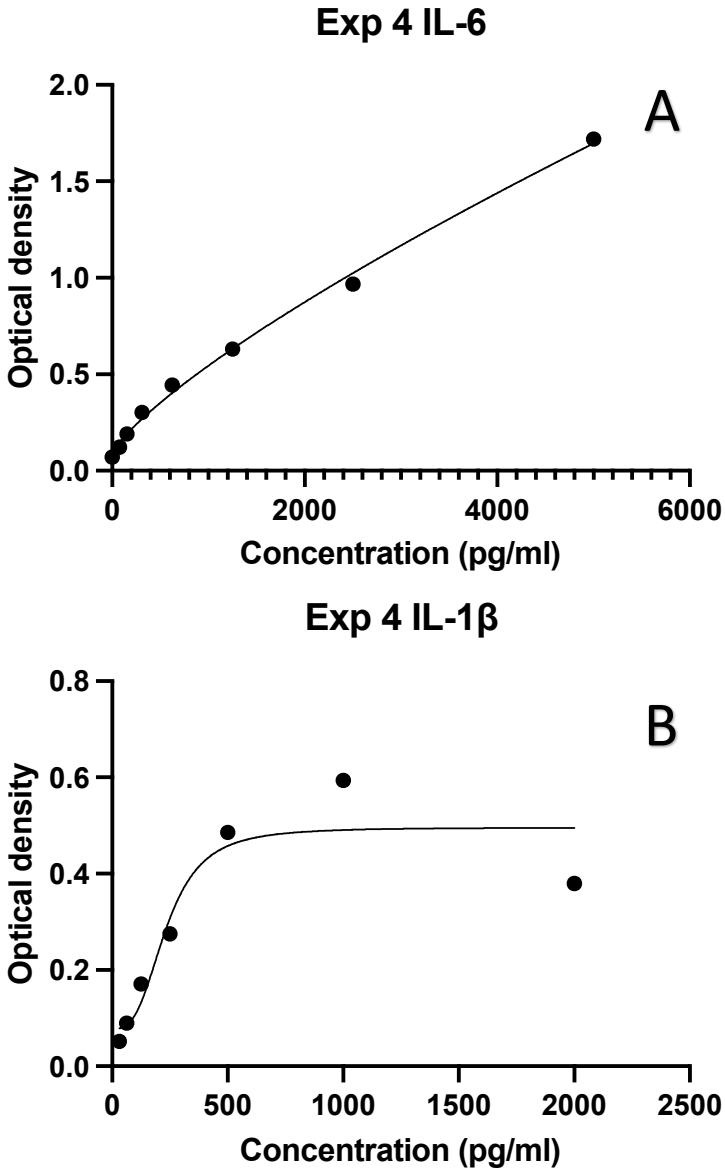


Figure 20. Standard curves used to extrapolate IL-6 (A) and IL-1β (B) concentration for analysis for Chapter 2 Experiment 4. Concentrations are in pg/ml.

VITA

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Dr. Vincent Doré holds a DVM from the Faculty of Veterinary Medicine of Université de Montréal. He followed his DVM with a rotating internship in bovine medicine and surgery at the Farm Animal Field Service unit of Université de Montréal. He then completed a master's degree in veterinary sciences at the same institution, focusing on dairy goats' metabolic disorders, emphasizing pregnancy toxemia while working as a clinical instructor in the Farm Animal Field Service unit. He pursued his training by completing a large animal internal medicine residency in ruminant health management at North Carolina State University College of Veterinary Medicine. Dr. Doré joined the Farm Animal Hospital Service team at the University of Tennessee and pursued a Ph.D. in immunology and infectious disease in Dr. Caldwell's lab. While finishing his doctorate, he moved back to Canada. He worked as a clinical instructor at the Farm Animal Hospital of the Faculty of Veterinary Medicine of Université de Montréal. His research focuses on neonatology, including sepsis, immunomodulation, and ruminant metabolic disorders.