FIBRINOGEN-DEPLETED PLATELET LYSATE MODULATES EQUINE NEUTROPHIL AND MONOCYTE FUNCTION

by

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(Under the Direction of John Peroni)

ABSTRACT

Equine platelet lysate (ePL) is an acellular platelet derivative that is rich in growth factors and can be manufactured from platelet concentrate. Recently, ePL has been proven to decrease TNF-α production, a pro-inflammatory cytokine, from stimulated monocytes. EPL optimization was explored in order to further modulate monocyte cytokine production. Fibrinogen and immunoglobulin depletion methods from ePL were investigated since they have the potential to initiate an inflammatory response. The depletion method that preserved the most growth factors was used for subsequent leukocyte assays. Monocytes and neutrophils were cultured with fibrinogen depleted platelet lysate (fdePL) to measure cytokine production, and phagocytosis/ROS production, respectively. Although fdePL did not further suppress TNF-α production, there was significant enhancement in neutrophil phagocytosis and ROS production compared to ePL. In conclusion, fdePL comparably suppresses TNF-α, and modulates neutrophil inflammatory reaction.

INDEX WORDS: Platelet Lysate, Fibrinogen, Monocyte, Neutrophils

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CHAPTER 1

INTRODUCTION

Fetal Bovine Serum (FBS) is commonly used for ex-vivo mesenchymal stem cell (MSC) expansion since it contains vital nutrients, growth factors, and hormones which are needed for cell survival and proliferation. However, FBS contains both bovine antigens and endotoxins; potentially altering MSC phenotype and rendering the cells immunogenic. Therefore, culturing equine MSCs in equine platelet lysate (ePL), which would function as a homologous cell culture alternative thus eliminating the concerns associated with FBS use, is an interesting option to investigate. Prior to testing ePL in MSC cultures and in order to test any potential immunogenic effects of ePL, we designed specific experiments to determine the effects of this culture medium alternative in immune system cells such as monocytes. Monocytes are highly reactive leukocytes sensitive to a variety of inflammatory stimuli to which they respond with known inflammatory patterns. The summary of our previously published work is that ePL was able to significantly suppresses TNF-α and IL-1β and enhance IL-10 production from naïve and LPS-stimulated equine monocytes. These interesting results meant that ePL could function as a serum substitute for FBS but, more interestingly, it needed to be further investigated as a potential immune-modulating preparation. The experiments contained in this thesis are therefore designed to optimize the manufacturing process of ePL and to further test its effects on mononuclear cells such as neutrophils. A thorough review of the existing human and veterinary literature, prompted us to hone our efforts on fibrinogen and immunoglobulins, both present in ePL, as potential key mediators of the pro-inflammatory immune response. As a result, our first step was to establish

ePL manufacturing techniques that would result in a final product devoid of fibrinogen and immunoglobulins. We initially employed a standard ePL clotting method that successfully eliminated fibrinogen, unfortunately, this method led to a substantial volume loss and decrease in several key growth factors. Therefore, our methods needed to be perfected in order not to compromise the baseline characteristics of lysate and we tested several strategies to optimally deplete both fibrinogen and immunoglobulins from ePL. Once this was achieved we cultured equine monocytes and neutrophils in optimized ePL to evaluate its immune-modulating capabilities.

Chapter 2 is a literature review which provides background information on fibrinogen, immunoglobulins, monocytes and neutrophils.

Chapter 3 describes our first objective; methodology development of fibrinogen and immunoglobulin depletion in ePL. We discusses the flaws associated with the previous fibrinogen depletion method. Fibrinogen and immunoglobulins removal techniques were explored on both platelet lysate, and its precursor, platelet concentrate. Fibrinogen was successfully depleted from both ePL and platelet concentrate; however, immunoglobulin removal techniques were unsuccessful. The fibrinogen depletion techniques from ePL and platelet concentrate were analyzed for volume recovery and growth factor retention. Optimization of fibrinogen depletion was essential to test the effect of ePL (with and without fibrinogen) on monocytes and neutrophils.

Chapter 4 describes the modulation ability of ePL and fibrinogen depleted ePL (fdePL) on monocytes, and neutrophils. We hypothesized that fdePL would suppress the inflammatory reaction of monocytes and neutrophils when compared to ePL.

CHAPTER 2

LITERATURE REVIEW

Platelet lysate is an a-cellular platelet derivative rich in growth factors, frequently used as a homologous cell culture media. [1-3] A previous study conducted in our laboratory validated manufacturing equine platelet lysate from platelet concentrate following plateletapheresis. [4] Plateletapheresis removes erythrocytes, and reduces the risk of bacterial contamination. Following the collection of platelet concentrate via plateletapheresis, platelets undergo degranulation. [5] Degranulation can be achieved in-vitro by the addition of $CaCl_2$, releasing the platelet contents and creating a growth factor rich acellular product. [4, 5] Even though ePL has shown promise as an immune-modulatory preparation that promotes an anti-inflammatory effect, several proteins are known to be present in ePL that individually have been correlated with a pro-inflammatory response.[6, 7]

Platelets are anucleate cell bodies that play a role in homeostasis. [8, 9] Platelets have three secretory regions (α-granule, dense granule, and lysosomal), with the principle amount of growth factors, chemokines, and coagulation factors localized to the α-granules. [3, 5, 9, 10] The α-granule contents include transforming growth factor-β (TGF-β1), vascular endothelial growth factor (VEGF-A), epidermal growth factor (EGF), platelet derived growth factor- BB (PDGF-BB), coagulation factors, and glycoproteins. [5, 9, 11-13] Compared to humans, equine platelets have smaller dense bodies and larger and more complex α-granules.[12, 14]

Fibrinogen

Fibrinogen consists of two outer D domains connected to a central E domain by a coiled-coil segment.[15] The coil consists of three polypeptide chains; specifically, α, β, and γ chains. The conversion of fibrinogen to fibrin can be triggered in-vitro by calcium ions, which cause thrombin to cleave the fibrinopeptides A and B on the E domain and initiate fibrin polymerization. [16, 17, 6, 18] The peptides released, can act as a chemoattractant to leukocytes and independently modulate inflammatory responses. [18]

While fibrinogen is necessary for coagulation, inflammation, and wound healing, [17] it also causes local and systemic reactions. [19] Fibrinogen is a glycoprotein that can be found in ePL, and has a well-established role in initiating an inflammatory response. [6, 18] Fibrinogen can activate a wide range of immune cells through ligand-receptor interactions.[18] Monocytes and neutrophils have toll like receptors (TLRs) that are capable of binding to fibrinogen, which can lead to a dysregulation of leukocytes resulting in a pro-inflammatory response.[18, 20, 21] Furthermore, fibrinogen can bind to complement receptor 3 (CR3) and complement receptor 4 (CR4), which is also expressed by monocytes and neutrophils. The binding of fibrinogen to CR3 in monocytes results in an active NF-kB pathway, which increases the amount of tumor necrosis factor- α (TNF- α) produced by these cells. The binding of fibrinogen to neutrophils can also increase their phagocytic ability. [18, 22]

Immunoglobulins

Immunoglobulins are a component of platelet lysate, and consist of two identical light chains and two identical heavy chains.[23] [24] Five different immunoglobulin classes have been identified in the horse: IgM, IgA, IgE, IgD and IgG. [25, 26] One functions of immunoglobulins is to activate and regulate the complement system; which is a term used to describe a network of

glycoproteins (C1-C9) produced in the liver. [23, 24, 26] The complement system helps defend against foreign pathogens through various methods, including enhancing phagocytosis. [24, 27] There are three pathways that can lead to complement activation, with the classical pathway being IgM and IgG dependent. [23, 24] After activation, an inflammatory response ensues, causing cell lysis, tissue damage, and neutrophil activation. [23, 28]

Neutrophils

Following an insult to a tissue such that seen in trauma or infection, one of the primary responses is leukocyte recruitment.[25] Neutrophils are multi-lobed cells with a short lifespan, and are the first line of defense during an inflammatory event.[25, 29] Quiescent neutrophils leave the bloodstream via diapedesis and enter the tissue through the vascular endothelial layer guided by an increasing gradient of chemoattracts, and become activated. [30,31] Neutrophils defense systems include, chemotaxis, phagocytosis, Reactive Oxygen Species (ROS) production, and degranulation. [29]

Neutrophils have four granules, primary (azurophilic), secondary (specific granules), tertiary, and secretory granules[32]. The four granules serve as storage facilities for digestive and hydrolytic enzymes that have a role in phagocytosis and/or ROS production. [33] Neutrophils express TLRs receptors capable of recognizing gram-negative and gram-positive bacteria. Specifically, TLR2 recognizes lipoproteins from gram-positive and TLR4 recognizes Lipopolysaccharide (LPS) from gram-negative bacteria, which stimulates phagocytosis. [30] When the pathogen binds to the cell, pseudopods extend, engulf the particle, and the phagosome undergoes maturation.[32] Once phagosomes mature, ROS or degranulation of granules that contain proteases and antimicrobial proteins kill the pathogen. [32] NADPH oxidase generates ROS by transferring electrons to create a superoxide oxygen; which than can further oxidize into

hydrogen peroxide, singlet oxygen, or hydroxyl radicals.[31] For neutrophil phagocytosis to clear certain strains of bacteria, the pathogen needs to be opsonized by immunoglobulins or the complement system.[30] Furthermore, neutrophils express CR3 and CR4, which can bind to complement, and can lead to activation.[22, 34]

Monocytes

Monocytes are derived from the bone marrow and circulate through the body. [35] These peripheral blood mononuclear cells have a variety of functions, including presenting antigens, secreting cytokines, proliferation, and phagocytosis. [35] During inflammation, apoptosis of monocytes is blocked, and the prolonged activation of monocytes can increase an inflammatory reaction.[36] Monocytes migrate to the site of infection and produce inflammatory cytokines (TNF-α & IL-1β, amongst others), and differentiate into macrophages. Monocytes that have an inflammatory role are considered a biomarker for inflammatory disease and present TLR4 on their membrane, which can bind to fibrinogen and also LPS. [23, 37, 38] [36, 39] LPS is a component of gram-negative bacteria that has been shown to activate monocytes and initiate an inflammatory response.[36, 39] Furthermore, monocytes also contain CR3 and CR4 on their membrane, which provides another location for fibrinogen binding.[34] Excess monocyte activation and overproduction of the pro-inflammatory mediators can cause organ failure.[36, 40]

To conclude, ePL has the potential to be used in clinical applications as an immune system suppressant. However, ePL modification was explored to attempt to further suppress monocyte pro-inflammatory cytokine production. To optimize ePL, fibrinogen and immunoglobulin depletion were explored since they can influence an inflammatory reaction. Subsequent to ePL modification, monocytes and neutrophils were cultured with the optimized ePL since they both have important roles in the innate immune system.

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CHAPTER 3

OPTIMIZATION OF FIBRINOGEN DEPLETION FROM EQUINE PLATELET CONCENTRATE[1]

[1] Alysha Berezny, Caroline Hawkins, Maria Naskou, John F Peroni. To be submitted to Transfusion Journal.

Abstract

Platelet Lysate has become a promising cell culture alternative and immune suppressor for equine cells due to the ability to suppress monocytes activation in-vitro. However, several proteins that remain in equine platelet lysate after the manufacturing process have the potential to dysregulate the immune system and increase the pro-inflammatory response of leukocytes. In order to augment the immune suppression ability of platelet lysate, our primary focus for this study was to deplete fibrinogen while having a high growth factor yield; with our secondary focus being immunoglobulin depletion. Five different fibrinogen and immunoglobulin depletion methods were tested and analyzed. Growth factors, fibrinogen and immunoglobulin concentrations were tested before and after the depletion methods. Results showed that fibrinogen was successfully depleted using all methods, however, volume recovery and growth factor yield suffered. Immunoglobulins were not successfully depleted following the various methods tested. While, one fibrinogen depletion method was quantitively superior in term of growth factors, we were unable to achieve both a high growth factor retention complemented with a high percentage recovery of the initial volume.

Introduction

Protocols have been established to perform apheresis in donor horses and collect platelet concentrate which is subsequently processed in the laboratory to produce equine platelet lysate (ePL). [1] Once it is manufactured, ePL is an acellular product pooled from multiple equine donors with measurable and promising biological characteristics. [1] Specifically, ePL has been suggested as a viable alternative to fetal bovine serum for the culture of mesenchymal stem cells[2] and it has been found to possess immune-regulatory properties, dampening pro-inflammatory cytokine production such as TNF-α and IL-1β from LPS-stimulated equine monocytes. [2-5]

Platelets play a significant role in maintaining homeostasis, regulating host defenses and inflammation acting as key players in the function of the innate and adaptive immune systems. [6,7-9] Compared to human platelets, equine platelets have been shown to have smaller dense bodies and larger more complex alpha granules attesting to subtle but relevant species differences. [10] Irrespective of the species differences, platelets contain three secretory elements including dense granules, lysosomes and α-granules, the latter of which contain the majority of growth factors, coagulation factors and immunologic factors. [2, 3, 11] Some of the growth factors, and coagulation factors contained in the α-granules and found in platelet lysate, include transforming growth factor-β (TGF-β1), vascular endothelial growth factor (VEGF-A), epidermal growth factor (EGF), platelet derived growth factor- BB (PDGF-BB), fibrinogen, and immunoglobulins. [3, 12] Studying the release kinetics and quantifying these components can be accomplished in the laboratory by activating platelets with a series of freeze-thaw cycles to physically fracture the platelet membrane. Alternatively, platelet activation and degranulation can also be achieved by adding calcium chloride ($CaCl_2$) as a source of Ca++ ions essential to the activation of the coagulation cascade.[2, 3, 6, 13]

Our laboratory has employed these techniques to better define the key components and refine the manufacturing process of platelet lysate.[2, 14] Fibrinogen is one plasma glycoprotein present in lysate that has been the subject of investigation because of its potential to activate a pro-inflammatory response in mononuclear cells [15-17] which is in contrast to the proposed overall dampening effect of lysate on LPS-stimulated equine monocytes. Fibrinogen, in fact, has been shown to bind to CD11b/CD18 receptors present on leukocytes, especially neutrophils, leading to apoptosis, activation of intracellular signaling pathways, and reactive oxygen species production. [18, 19]

In the clinical setting a rise in the plasma value of fibrinogen has been long regarded as a marker for a non-specific acute inflammatory response. [20, 21] After an injury is sustained, the coagulation cascade is activated, resulting in thrombin cleaving fibrinopeptides A and B from fibrinogen, leading to fibrin polymerization. [17] [16] The subsequent release of peptides A and B during fibrin formation are known to act as powerful chemoattractant to leukocytes and independently modulate local inflammatory responses. [17] For the purpose of understanding the role of fibrinogen in lysate, we sought to establish techniques that could effectively eliminate fibrinogen from ePL by promoting its conversion to fibrin, and then removing the clotted proteins from the lysate via centrifugation. [22-24]

Another ingredient of platelet lysate that can initiate an inflammatory reaction are immunoglobulins. Immunoglobulins are found naturally in plasma and play a role in activating the compliment system.[5, 25] The complement system initiates neutrophil phagocytosis to discard pathogens .[26] However, the system can become dysregulated and lead to an increased and undesirable inflammatory response and therefore tissue damage. [25] Due to the rationale stated

above, in order to fully optimize ePL, our primary focus is fibrinogen depletion and our secondary focus on immunoglobulin depletion.

In a previous study conducted in our laboratory, we employed this process to effectively deplete fibrinogen from lysate, however, it unfortunately resulted in a substantial volumetric loss of lysate and in a reduction of key growth factors leading to a concern about a negative impact on the quality and efficacy of ePL as an immune-regulatory preparation.[24] The overall goal of this paper to improve upon our fibrinogen depletion technique in order to minimize loss of lysate and efficiently preserve growth factor concentrations in the final preparation.

Recent evidence suggests that removing fibrinogen from platelet concentrate, the precursor to lysate, may also be a viable alternative to preserve growth factors in the resulting depleted preparation.[25] Therefore, we chose to compare fibrinogen depletion techniques from equine platelet concentrate and lysate by comparing product recovery volume and the concentration of remaining growth factors. We hypothesized that a method could be identified that would effectively eliminate fibrinogen from lysate with minimal volumetric loss and preserve PDGF, TGF-β, and VEGF concentrations similar to those found in un-depleted ePL.

Methods and Materials

Manufacturing platelet concentrate, equine platelet lysate (ePL) and platelet poor plasma

Platelet concentrate was collected via plateletpheresis performed in three mixed breed female equine blood donors as previously described. [1]All horses involved in this study were housed in the University of Georgia research facility for monitoring and acclimation and all of the procedures required to conduct this study were approved by the Institutional Animal Care and Use Committee of the University of Georgia. Study protocol # A2018 01-013-Y1-A2.

After collection, platelet concentrate was aliquoted into 50 mL conical tubes (VWR, Randor, PA), half of which underwent two freeze-thaw cycles and used to generate stock platelet lysate (ePL) pooled from all three horses. (Figure 1).[2] The remaining fresh concentrate was incubated at room temperature for up to five days on a platform shaker (Thermolyne, Duque, Iowa) [26] and then used to test different fibrinogen depletion methods. Platelet poor plasma was produced by centrifuging fresh concentrate at 4410 x g for 10 minutes to remove all platelets [25] and subsequently aliquoted and stored at -80°C. Platelet poor plasma was used as a negative control for the characterization of growth factors found plasma in the absence of platelet derived growth factors.

Manufacturing fibrinogen depleted platelet lysate (fdePL)

Our previously published fibrinogen depletion technique involved adding $CaCl_2$ to ePL to a final concentration of 20 mM to form a fibrin clot that was then removed via centrifugation. Once the fibrinogen was removed, 2IU/mL of heparin were added to the depleted platelet lysate to prevent re-gelling .[24] In the present study we attempted to refine the fibrinogen depletion technique, by titrating $CaCl_2$, heparin concentrations, and heparin molecular weight.
Equine platelet lysate was added to disposable borosilicate glass culture tubes (VWR, West Chester, PA). Calcium chloride ($CaCl_2$) (Sigma, St. Louis, MO) was dissolved in plasma-lyte (Abbott laboratory, Chicago, IL), and varying concentrations were subsequently added to ePL reaching final concentrations of 5mM, 10mM, 15mM, 20mM, 25mM, 30mM, 35mM, and 40mM. Simultaneously in the same tubes, increasing heparin of 0.2 IU, 0.4IU, 0.8IU, 1.0IU, 1.5IU, 2IU, 2.5IU, 3IU, 3.5IU, 3.7IU, or 4IU were added to ePL in triplicates. Each combination contained either 1000IU/1.0mL high molecular weight heparin sodium (McKesson, India) or 12,500 IU/1.0mL low molecular weight heparin sodium (Pfizer, New York, NY). All samples

were then placed into a 37°C incubator to observe clot formation. The samples that formed a clot were removed from the incubator and placed in the 4°C fridge overnight for clot stabilization. The following day, the gels were centrifuged down at 4410 x g for 30 minutes, the fdePL supernatant was collected, and the recovery volume was measured. We attempted to find a balance between the coagulation initiation and the effect of an anti-coagulant to increase volume recovery and growth factor yield. However, we were not able to find a method that satisfied that criteria, even after testing different heparin molecular weights. After trying several combinations, we either were not able to fully deplete fibrinogen, the coagulation process was not initiated, or the volume recovered was still undesirable. After concluding that we were unable to find the appropriate balance between $CaCl_2$ and heparin (data not shown), the need to test other methods became evident. We elected to test three different methods of fibrinogen depletion performed in both platelet concentrate or platelet lysate.

Fibrinogen depletion of platelet concentrate using $CaCl_2$ plus glass beads (fdePL method 1)

After incubating for 5 days on a platform shaker, platelet concentrate from 3 horses was pooled and aliquoted into 50 mL conical tubes. Glass beads (3mm diameter, 0.5g/mL; Millipore, Darmstadt, Germany), and 20 mM $CaCl_2$ were added to all tubes, and then placed on a platform shaker at room temperature for 12-15 minutes or until fully clotted. Samples were then shaken vigorously to break up the clot and centrifuged at 4410 x g for 30 minutes. [25] The resulting supernatant (now fdePL 1) was filtered through a 40 μm Falcon nylon cell strainer (Corning, Inc), aliquoted and stored at -80°C.

Fibrinogen depletion of platelet concentrate using $CaCl_2$ plus glass beads and a water bath (fdePL method 2)

The other half of the fdePL 1 was fully submerged in a water bath at 56° C for 30 minutes, in an attempt to deplete immunoglobulins in addition to fibrinogen. [15] After the 30 minutes, the fdePL 2 was frozen at -80°C.

Fibrinogen depletion of platelet lysate using $CaCl_2$ (fdePL method 3)

Frozen ePL aliquots were thawed overnight at 4°C then transferred to a glass Erlenmeyer flask. $CaCl_2$ (final concentration 20mM) was added to the ePL and the flask was placed in a 37°C incubator for approximately 11 minutes, or until gel formation was initiated. Samples were then placed at 4°C overnight to allow clot stabilization. The following day, the fibrin clot was transferred to 50 mL conical tubes and centrifuged at 4410 x g for 30 minutes. The resulting supernatant (now fdePL 2) was collected, and 2 IU/mL of high molecular weight heparin was added to prevent re-gelling .[24] Aliquots were stored at -80°C.

Fibrinogen depletion of platelet lysate using $CaCl_2$ plus glass beads (fdePL method 4)

Frozen pooled ePL aliquots were thawed overnight at 4°C. The following day, 20 mM $CaCl_2$ were added, together with 0.5g/mL 3mm glass beads (Millipore, Darmstadt, Germany), and incubated at room temperature for 30 minutes, or until a clot formed. The tubes were shaken vigorously to break up the clot, then centrifuged at 4410 x g for 30 minutes. [15] The supernatant (now fdePL 3) was collected and filtered through 40 μm Falcon nylon strainer (Corning, Inc). Aliquots were stored at -80°C.

Fibrinogen depletion of platelet lysate using $CaCl_2$ plus glass beads and a water bath (fdePL method 5)

Half of the fdePL 4 was submerged in a 56° C water bath for 30 minutes in an attempt to remove immunoglobulins subsequently after removing fibrinogen. [15] After the 30 minutes in the water bath, the fdePL 5 was frozen at -80°C.

Fibrinogen and platelet concentrations

A commercially available VSpro fibrinogen test (Abaxis, Union City, CA) was used to measure fibrinogen concentration in the various platelet-derived products, according to the manufacturer's instructions.

Growth factor and cytokine quantification

The concentrations of the growth factors platelet-derived growth factor (PDGF-BB), and transforming growth factor beta 1 (TGF-β1) were determined by performing ELISAs according to the manufacturer's instructions (human duo set ELISA, R&D Systems, Minneapolis, MN). All samples were tested in triplicates and standards were diluted according to protocol on each individual kit. [1, 4, 24] The concentration of vascular endothelial growth factor (Equine VEGF-A ELISA vetset, 2 Plates, Saint Paul, MN) was determined following manufacturing protocol. An ELISA accessory pack was ordered separately, and is needed for the VEGF-A ELISA to function appropriately (ELISA Accessory Pack: TMB Substrate and Stop Solution, Saint Paul, MN).

Statistical Analysis

Statistical analysis on growth factor concentration could not be performed because the ePL was pooled (n = 1). Results were reported as mean +/- standard deviation (s.d).

Results

Growth factor concentrations in undepleted ePL and platelet poor plasma

The growth factor concentrations determined in the stock ePL pooled from the three donor horses acted as the baseline concentration for growth factors against which all of our other preparations were compared. In ePL, the concentration of VEGF was 3952 pg/mL ± 197.59 (Figure 2), PDGF was 3739 ± 386 pg/mL (Figure 2), and TGF-β was 12269 ± 474 pg/mL (Figure 2). Platelet poor plasma was evaluated to measure any residual growth factor concentrations in plasma without platelets. In platelet poor plasma the concentration of VEGF was 3144 ± 278 pg/mL (Figure 2), PDGF was 418 ± 42 pg/mL (Figure 2), and TGF-β was 2821 ± 38 pg/mL (Figure 2).

Fibrinogen depletion of platelet concentrate using $CaCl_2$ plus glass beads (fdePL method 1)

It is important to note that platelet concentrate is the precursor to ePL, which means platelets are intact at the start of the fibrinogen depletion process. We chose this method because treating platelet concentrate with a combination of glass beads and $CaCl_2$ has been shown to stimulate fibrin clot formation, platelet degranulation, and deplete fibrinogen in a single step. [25] With this technique we were able to achieve full clotting after approximately 50 minutes at 37°C. After centrifugation, 90% of the original volume was recovered and only 0.13 g/dL fibrinogen remained (Table 1). This fibrinogen depletion method yielded lower growth factor concentrations compared to ePL: the amount of VEGF was 2501 ± 67.41 pg/mL in fdePL 1 compared to 3952 pg/mL ± 197.59 in ePL (Figure 2); PDGF was 1496 pg/mL ± 66 compared to 3739± 386 pg/mL in ePL (Figure 2); and TGF-β was only 4058 ± 61 pg/mL compared to 12269± 474 pg/mL in ePL (Figure 2). In summary, this first method effectively depleted fibrinogen with

a 90% recovery volume but resulted in a substantial decline in the three key growth factors measured.

Fibrinogen depletion of platelet concentrate using $CaCl_2$ plus glass beads and water bath (fdePL method 2)

Since immunoglobulins have potential to influence an inflammatory response, fdePL1 was placed into a water bath to attempt to heat inactivate immunoglobulins as well. [15] Following the submersion in the water bath, the fibrinogen, immunoglobulins, growth factors, and percent recovery was re-assessed to determine if the water bath had an effect on the other proteins besides immunoglobulins. After submersion, the volume remaining was 88% and the fibrinogen concentration was 0.0g/L (Table 1). The amount of VEGF was 2943 ± 284.24 pg/mL in fdePL 2 compared to 3952 pg/mL ± 197.59 in ePL (Figure 2); PDGF was 3739± 386 pg/mL compared to 3739± 386 pg/mL in ePL (Figure 2); and TGF-β was only 4124± 274 pg/mL compared to 12269± 474 pg/mL in ePL (Figure 2). The IgG concentration before being placed in the water bath was 1620 mg/dL and the concentration after the water bath was 1543 mg/dL (Table 2). The IgM concentration before the water bath was 59 mg/dL, and 59 mg/dL after the water bath (Table 2).

Fibrinogen depletion of platelet lysate using $CaCl_2$ (fdePL method 3)

The addition of $CaCl_2$ (final concentration 20mM) to ePL led to complete clot formation after 15 minutes of incubation. After clot removal, volume was only 49% of the starting ePL, which is less than was previously reported using the same technique (Table 1).[24] Growth factor concentrations were lower compared to the starting stock ePL product, but not as low as for the first depletion method from platelet concentrate. The concentration of VEGF in was 3149 ± 80 pg/mL compared to 3952 ± 197 pg/mL in ePL (Figure 2); PDGF was 2011 ± 42 pg/mL

compared to 3739 ± 386 pg/mL in ePL (Figure 2); and TGF-β was 6918 ± 1014 pg/mL compared to 12269 ± 474 pg/mL in ePL (Figure 2).

Fibrinogen depletion of platelet lysate using $CaCl_2$ plus glass beads (fdePL method 4)

This method mimicked the previous except for the addition of glass beads which were used as a physical catalyst to speed up the conversion of fibrinogen to fibrin and according to the literature, would not require the addition of heparin to prevent further clotting. [15] The addition of $CaCl_2$ and glass beads to ePL led to full clotting after 45 minutes at room temperature. This method of fibrinogen depletion resulted in a volume recovery of 59%, which is slightly improved compared to fdePL 3, with a final fibrinogen concentration of only 0.03 g/L, (Table 2). Using this method, the concentration of VEGF in fdePL 3 was 2842 ± 164.20 pg/mL compared to 3952 ± 197.59 pg/mL in ePL (Figure 2); PDGF was 2067 ± 369 pg/mL compared to 3739 ± 386 pg/mL in ePL (Figure 2); and TGF-β was 9094 ± 445 pg/mL compared to 12269 ± 474 pg/mL in ePL (Figure 2). Thus, as reported for fdePL 3, growth factor concentrations in fdePL 4 were lower compared to the starting ePL product, but not as low as for fdePL 1.

Fibrinogen depletion of platelet lysate using $CaCl_2$ plus glass beads and water bath (fdePL method 5)

Half of fdePL 4 was fully submerged in a water bath and the effect on immunoglobulins, fibrinogen, growth factors and initial volume was measured.[15] The volume recovered was 56% with .03 g/L of fibrinogen remaining (Table 1). Using this method, the concentration of VEGF in fdePL 5 was 2444± 242.52 pg/mL compared to 3952 ± 197.59 pg/mL in ePL (Figure 2); PDGF was 1652 ± 86 pg/mL compared to 3739 ± 386 pg/mL in ePL (Figure 2); and TGF-β was 7963.78± 898 pg/mL compared to 12269 ± 474 pg/mL in ePL (Figure 2). The IgG concentration before being placed in the water bath was 1960 mg/dL and the concentration after the water bath

was 1602 mg/dL (Table 2). The IgM concentration before the water bath was 59 mg/dL, and 63 mg/dL after the water bath (Table 2).

Discussion

In this study we found that depleting fibrinogen from ePL was easily achieved regardless of the technique used but not without a compromise between volume of lysate recovered and a decreased in the relative concentrations of the growth factors we measured.

In the present study, we compared five different methods to deplete fibrinogen and immunoglobulins from ePL and platelet concentrate, adopting techniques that have been previously described. [5, 15, 25] We had previously shown that $CaCl_2$ at 20 mM concentration was capable of activating the coagulation cascade in ePL and convert fibrinogen to fibrin allowing its removal [24]. As a result, we chose to treat platelet concentrate with a similar concentration $CaCl_2$ able to create fibrin polymers in ePL, we used this concentration with the in order to standardize the process. Since ePL manufacturing requires two freeze-thaw cycles in order to degranulate the platelets, depletion via glass beads from platelet concentrate seemed like a promising alternative, since avoiding the freeze-thaw cycles should allow for the preservation of more proteins including growth factors.[25] The treatment of platelet concentrate with both glass beads and $CaCl_2$ was sufficient to cause degranulation while bypassing the freeze-thaw technique. Glass beads were added in addition to $CaCl_2$ to act as a physical catalyst and assist in the physical breakup of the fibrin clot. [25,15] We did not test platelet concentrate + $CaCl_2$ because it has been shown that platelets treated with $CaCl_2$ alone leads to incomplete platelet activation and inconsistent with growth factor release .[27, 28]

Even though it was suggested that a 56°C water bath would result in an elimination of immunoglobulins in the plasma such as IgG and IgM, this was not the case according to our

results. In previous studies which used heat inactivation to decrease the concentration of the compliment proteins in human plasma, they did not perform assays to conclude that the concentration of immunoglobulins was diminished.[15, 27] In order to accurately determine if the compliment was effectively removed, we tested the concentration of IgG and IgM before and after the water bath. We concluded that the immunoglobulin inactivation via water bath technique process used in humans is not adequate for equine serum.[15, 27] Additional, it has been previously shown that heat inactivation can have a negative effect on growth factor content in a sample, which could be the case for equine serum. [33, 34]

Of the depletion methods tested in the present study, fdePL 3 is most comparable to our previous paper. [24] Importantly, possibly due to the presence of additional coagulation plasmatic factors, it requires the addition of 2 IU/mL heparin to prevent future clot formation. This has the drawback of being xenogeneic, of porcine origin. [25] However, fdePL 4, generated using glass beads and $CaCl_2$, does not require the addition of heparin after depletion. Additionally, even though the techniques are identical, our volume recovery and growth factor retention findings for fdePL were less compared to the numbers reported in our previous paper.[24]This is most likely due to the fact that, due to supply limitations, different batches of pooled lysate that were used in the two studies. The technique still acted as an important comparison since the lysate batches are not identical.

In summary, our findings indicate that all of the methods tested here resulted in essentially complete fibrinogen depletion from both platelet concentrate and ePL starting products. However, none of the methods resulted in the ideal combination of high-volume recovery and complete growth factor retention. More specifically, depleting fibrinogen from PC resulted in the highest recovery volume of the three methods tested, but also gave the lowest

yield of growth factors. Unfortunately, the baseline growth factor concentration of PC cannot be quantified due to the fact that the growth factors are contained within the platelets. There appeared to be little difference between fdePL 3 and fdePL 4 in terms of volume recovery and growth factor concentration, however, we selected fdePL 4 as the optimal method due to the fact that it does not require the addition of heparin to prevent further clotting. The poor growth factor retention following fibrinogen depletion could be attributed to the rapid activation and clotting process, which has been correlated with a decrease in the total amount of growth factors present.[29]

More data need to be produced before deciding the importance or unimportance of fibrinogen depletion. Therefore, subsequent cell inhibition assay needs to be performed, culturing cells in fdePL and investigating the effect. Functional testing on monocytes and neutrophils since they are a part of the innate immune system.

Conclusion

In conclusion, we developed a fibrinogen depletion method for equine platelet lysate that successfully removes potentially harmful fibrinogen while preserving adequate levels of valuable growth factors, and does not require the addition of heparin to prevent clotting.

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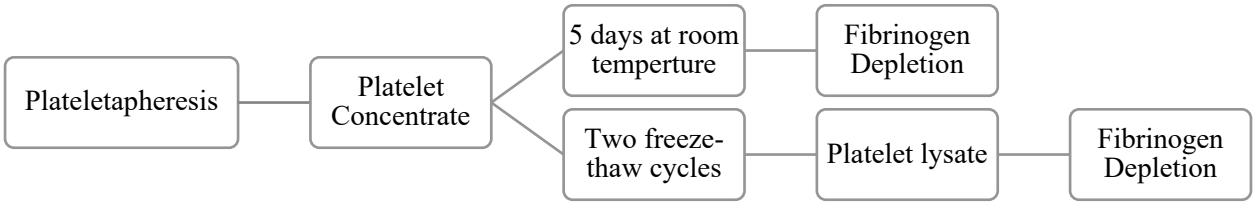

Figure 1. Overview of the fibrinogen depletion process from ePL and platelet concentrate. Plateletapheresis is performed and platelet concentrate is collected. Half of the concentrate remained at room temperature for 5 days, and the other half underwent immediate processing to create platelet lysate and degranulate platelets. Fibrinogen depletion occurred at both ePL and platelet concentrate.

Table 1. Fibrinogen concentration, volume recovery, and platelet characterization on ePL and platelet concentrate before depletion methods were tested, and after testing the five various methods. FdePL 1 and 2 are manufactured from platelet concentrate. FdePL 3, 4 and 5 are manufactured from ePL.

	Starting Fibrinogen Concentration (g/dL)	Volume recovered	Final Fibrinogen Concentration (g/dL)
PC	2.6	N/A	N/A
ePL	2.3	N/A	N/A
fdePL 1 (PC)	2.6	90%	0.13
fdePL 2 (PC)	2.6	88%	0
fdePL 3 (ePL)	2.3	49%	0
fdePL 4 (ePL)	2.3	59%	0.03
fdePL 5 (ePL)	2.3	56%	0.03

Table 2. Immunoglobulin (IgG & IgM) concentration before and after the 30 minute 56°C water bath.

	IgG (mg/dL)	IgM (mg/dL)
fdePL 1 (PC-before)	1960	59
fdePL 2 (PC-after)	1602	63
fdePL 4 (ePL-before)	1620	59
fdePL 5 (ePL- after)	1543	59

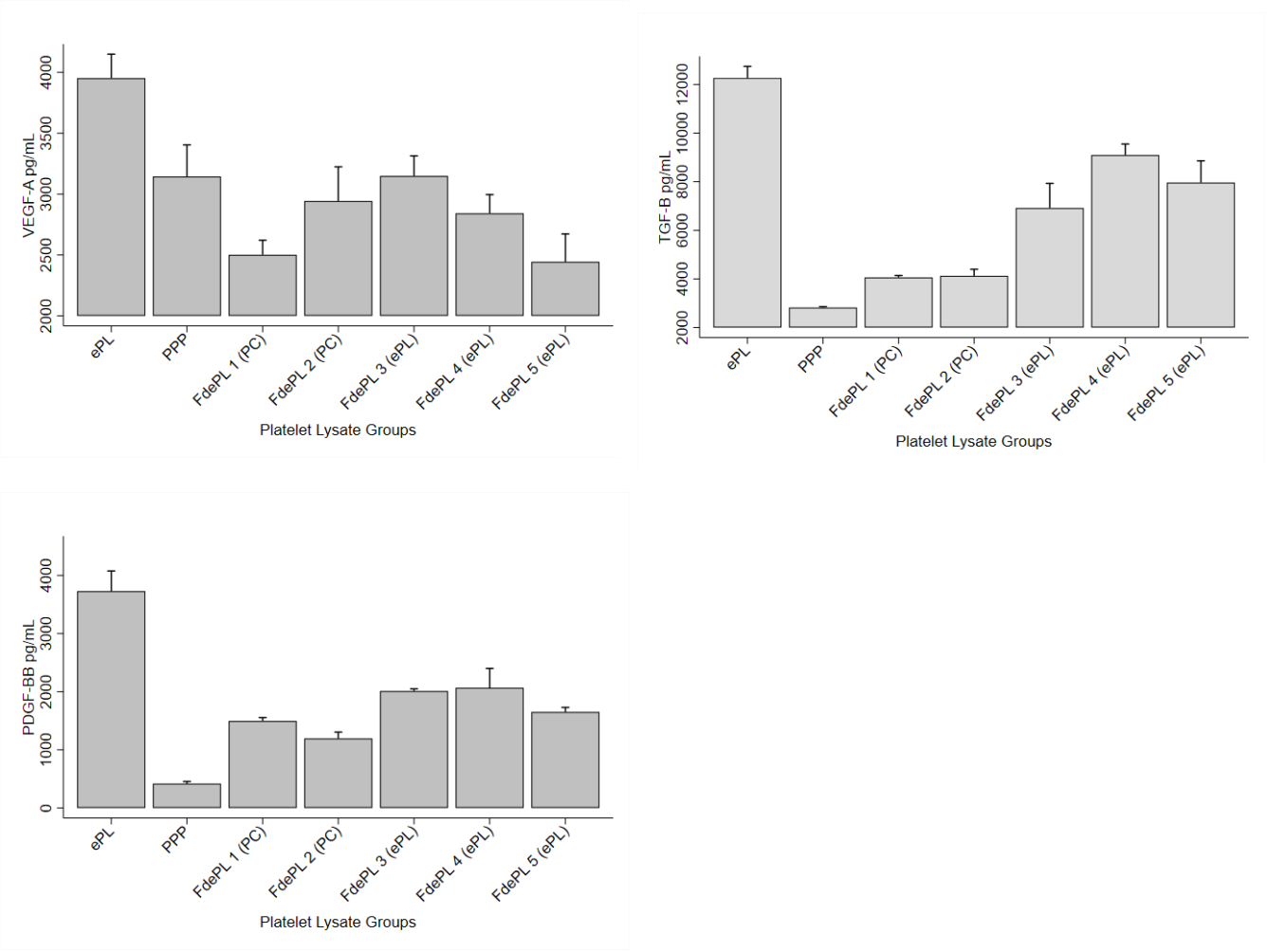

Figure 2: Comparison of growth factor concentration before and after fibrinogen and immunoglobulin depletion techniques; including, VEGF-A, PDGF-BB, and TGF-β.

CHAPTER 4

THE EFFECT OF FIBRINOGEN DEPLETED EQUINE PLATELET LYSATE ON LEUKOCYTE ACTIVATION[2]

[2] Alysha Berezny, Kate Birdwhistell, Caroline Hawkins, John F Peroni. To be submitted to Transfusion Journal.

Abstract

Fibrinogen is a glycoprotein found naturally in plasma which is known to activate the pro-inflammatory response of leukocytes. An equine fibrinogen depleted platelet lysate (fdePL) has been recently manufactured and characterized and our laboratory has designed experiments to culture monocytes and neutrophils in the regular ePL that contains fibrinogen, fdePL, and the standard culture media, and examine if cell activation occurs.

We cultured LPS stimulated monocytes and unstimulated monocytes in 10% of the media preparations including ePL, fdePL and FBS and examined tumor necrosis factor-α (TNF-α) and interlukein-10 (IL-10) cytokine production. There was no statistical difference between TNF-α and IL-10 production from LPS stimulated monocytes and unstimulated monocytes.

Neutrophils were cultured in 10, 25, 50, 75, 100% of the various media preparations to measure reactive oxygen species (ROS) production by a fluorescence assay and flow cytometry and phagocytosis via flow cytometry. The neutrophils were cultured with PMA and DHR to measure ROS, and *E.coli* and *S.aureus* to measure phagocytosis. An increase in percentage of media concentration resulted in a statistically significant linear decline of ROS produced. Neutrophils exposed to ePL and fdePL were able to improve their ability to phagocytose *S.aureus* at all media concentrations. However, neutrophils exposed to *E.coli* did not have a statistically significant increase of phagocytosis until 75% of fdePL was reached.

Introduction

Equine platelet lysate (ePL) is a growth factor rich platelet derived product that has been investigated for therapeutic applications, immune system suppression, and as a cell culture alternative. [1-5] This acellular product is manufactured from platelet concentrate by performing two-freeze-thaw cycles, which lead to platelet degranulation and the release of the contents. [1-3] We have shown that ePL can decrease pro-inflammatory cytokine production from LPS-stimulated monocytes, however, cytokine production does not cease completely. [2, 3] In an attempt to further suppress cytokine production from monocytes, we began to investigate ways to optimize the ePL product. [6] Fibrinogen is a significant component of ePL and may be associated with a stimulatory effect of the inflammatory activation of leukocytes. [6] We therefore chose to remove fibrinogen from our ePL product. The resulting fibrinogen depleted platelet lysate (fdePL) has been shown to retain transforming growth factor-β (TGF-β1), vascular endothelial growth factor (VEGF-A), and platelet derived growth factor- BB (PDGF-BB). [1, 7] However, the effect of fdePL vs ePL on monocyte and neutrophil activation has not been assessed.

Fibrinogen is a glycoprotein that has the ability to activate immune cells through ligand-receptor interactions. [8, 9] Fibrinogen can activate monocytes and neutrophils through TLR transmembrane activation, which signals an upregulation of complement receptors. [8, 10-13] When fibrinogen binds to, and thus activates, TLR4, CR3 or CR4 are translocated from the intracellular location to the membrane; fibrinogen can also bind to complement receptor 3 (CR3) and complement receptor 4 (CR4). [9, 14, 10, 15] Binding of fibrinogen to monocytes and neutrophils can lead to NF-kB activation and tumor necrosis factor- α (TNF- α) production, and ROS production, respectively. [8, 9, 14, 16, 17] It can also cause activation of NADPH oxidase chain in neutrophils, which is responsible for ROS production.[18]

Neutrophils are polymorphonuclear leukocytes that represent the first line of defense, and eliminate pathogens via reactive oxygen species (ROS) production and phagocytosis.[19] After bacteria are phagocytosed, neutrophils kill the pathogen with antibacterial proteins or reducing NADPH oxidase, which in turn creates superoxide anions, hydrogen peroxide, and hydroxyl radicals.[20] Exposure of neutrophils to fibrinogen can lead to functional changes, including apoptosis and degranulation.[14, 21] Neutrophil overactivation has been correlated with numerous illnesses in horses, including laminitis and sepsis.[22, 23]

Monocytes are the precursor of macrophages and pattern recognition signaling to detect invaders. Once stimulated, monocytes are able to phagocytose, secrete cytokines, proliferate, and differentiate.[24] Monocytes can be activated via TLR4 binding, which induces an inflammatory reaction and the production of cytokines. [13, 25-27]An overproduction of pro-inflammatory cytokines leads to an imbalance in the immune system.[28] To understand the immunosuppressive ability of platelet lysate on cytokine production, monocytes were cultured in either ePL or fdePL with and without lipopolysaccharide (LPS), a component of gram-negative bacteria that binds to TLR4 on equine monocytes and triggers a pro-inflammatory reaction. [12, 13, 29, 30]

A previous study from our laboratory explored the possibility of using ePL and fdePL as an immune suppressant.[31] More specifically, we wanted to know whether fdePL was better able to suppress pro-inflammatory cytokine production from LPS-stimulated monocytes than ePL. In those studies, we found no significant difference between fdePL and ePL on TNF-α production from monocytes. It is possible that the fibrinogen depletion methodology lead to a poor growth factor yield and/or the reduced recovery volume associated with the depletion method.[31] We recently reported an improved fibrinogen depletion technique, but the effect on monocyte and neutrophil activation still needs to be evaluated. [6] The objective of the current study is to

compare the effect of fdePL versus ePL on monocyte and neutrophil activation. We hypothesize that fdePL will suppress ROS production from neutrophils and modulate cytokine production from stimulated monocytes more effectively than ePL.

Methods and Materials

Isolation/preparation of monocytes

PBMCs were collected from 6 adult horses and monocytes were isolated as previously described[2, 32-34]. Briefly, leukocyte rich plasma (LRP) was collected from 60 mL of blood with 1.5 mL 100 uM EDTA and layered over lymphocyte separation media (LSM) (Corning Cellgro®, Inc. Manassas, VA) and centrifuged. [2, 34] The resulting PBMCs were resuspended at a final concentration of $4x10^6$ in RPMI 1640 with L Glutamine (Corning Cellgro®, Inc. Manassas, VA) + 10% Donor Horse Serum (DHS) (Corning Cellgro®, Inc. Manassas, VA) and plated onto a 24-well plate at a density of $2x10^6$ per well. After a 2-hour incubation at 37°C in 5% CO_2, the media along with non-adherent cells was removed and replaced with either10% ePL, 10% fdePL, or fresh 10% DHS. To examine the effect of ePL and fdePL as an immune suppressant, 50 ng/mL *E. coli* 0111:B4 LPS (List Biologicals Inc., Campbell, CA) was added to half of the wells. RPMI media supplemented with 10% DHS, ePL or fdePL served as a positive control, and media only (without cells) served as a negative control. Supernatant samples were collected after 6 hours to analyze TNF-α and 18 hours to analyze IL-10, and stored at -80°C.[2, 3]

Isolation/preparation of neutrophils

Whole blood was collected from 6 adult horses and neutrophils were isolated as previously described.[35] Briefly, LRP was layered over LSM and centrifuged. Contaminating erythrocytes were removed via hypotonic lysis and the resulting neutrophils were re-suspended in the appropriate culture media. To assess the effect of depleted and undepleted platelet lysate

on stimulated and unstimulated neutrophil reactive oxygen species (ROS) production, a fluorometric assay was performed; The cells were then resuspended in either 10 % FBS or 10 20, 30, 40, or 50% of ePL or fdePL. 600,000 cells from each media were plated onto a 96-well flat bottom tissue culture plate.

ROS assay

Neutrophils were isolated as described above and resuspended in ROS media (RPMI-1640 without phenol red (ThermoFisher Scientific, Eugene, OR), 10% heat inactivated fetal bovine serum, and 2mM L-Glutamine) at a density of $6x10^6$ cells/mL.

Reactive oxygen species production measured via flow cytometry

The cells were exposed to 5 μm dihydroergotamine 123 (DHR-123) (ThermoFisher Scientific, Eugene, OR) for 15 minutes then centrifuged, and resuspended in 10%, 25%, 50%, 75%, or 100% FBS, ePL, or fdePL or 0.1%, 0.3%, 1%, 3%, or 5% lipid cholesterol (Sigma, St. Louis, MO) and phorbol myristate acetate (PMA, 10^{-7}; Molecular Probes, Eugene, OR) for 30 minutes. Finally, the cells were centrifuged and resuspended in FACS buffer with propidium iodine (PI) (ThermoFisher Scientific, Eugene, OR) [36] then analyzed immediately via flow cytometry. The resulting data underwent log transformation for statistical analysis.

Reactive oxygen species production measured via plate reader

To measure reactive oxygen species production, the cells were resuspended in 10 % FBS, ePL, or fdePL, and cultured in a black 96 well plate with a clear bottom (Corning Cellgro®, Kennebunk, ME) with the same concentration of heat inactivated *S. aureus* and *E. coli* that is used in the phagocytosis assay, with and without DHR. Cells in ePL, fdePL, and FBS, were also cultured with 10^{-7} PMA and 5 μm DHR. ROS concentration was measured every 10 minutes for 2 hours.

Phagocytosis assay

Phagocytosis functionality of neutrophils was assessed using flow cytometry. Neutrophils were isolated from 6 adult horses as described above and resuspended at a density of $3x10^6$ cells/mL before being exposed to bacteria, as previously described. [33, 35] The cells were resuspended in 10, 25, 50, 75, or 100% FBS, ePL, or fdePL and plated onto a 96-well round-bottom plate. Biodipy labeled, heat-inactivated *Escherichia coli* (*E. coli*) or *Staphylococcus aureus* (*S. aureus*) (Invitrogen, Carlsbad, CA) was added, and the cells were incubated for 60 minutes. After this, FACS fixative was added, then the cells were exposed to 0.4% trypan blue, washed with FACS buffer, and subjected to flow cytometry.

Growth factor and cytokine analysis

Growth factor concentration was determined by performing ELISAs according to the manufacturer's instructions. The growth factors examined were interleukin-10 (IL-10) (IL-10 equine duo set ELISA, R&D systems, Minneapolis, MN). TNF-α cytokine was measured according to an established protocol[37].

Statistics

To determine equality of variance, Levene's test was used. Monocyte data were analyzed using repeated measure ANOVA (Stata version 15.1, StataCorp LP, College Station, TX®), with post-hoc comparison via Tukey-Kramer analysis. Since the neutrophil ROS data did not display equality of variance, the data underwent log transformation. Neutrophil ROS and phagocytosis data were analyzed using a linear mixed model (Stata version 15.1, StataCorp LP, College Station, TX®), specifically the Sidak test. Numbers are reported as average ± standard deviation.

Results

Decreased TNF-α production in monocytes cultured with ePL or fdePL

To determine whether fibrinogen depletion affected the pro-inflammatory response of monocytes, levels of TNF-α production were measured in cells cultured in either 10% fdePL, 10% ePL, or 10% DHS. TNF-α production from LPS-stimulated monocytes cultured in fdePL and ePL was significantly lower ($P < 0.05$) compared to stimulated monocytes cultured in DHS. Monocytes that were cultured in 10% DHS produced 876 ± 358 pg/mL of TNF-α; those cultured in 10% ePL produced 551± 260 pg/mL; and those cultured in 10% fdePL produced 533± 272 pg/mL. However, there was no statistical difference between LPS-stimulated monocytes cultured in the 10% ePL versus 10% fdePL (Figure 1). For unstimulated monocytes, TNF-α production was also lower in cells cultured in 10% fdePL or 10% ePL compared to those cultured in DHS ($P<0.05$). Monocytes that were cultured in 10% DHS produced 182 ± 69 pg/mL; those cultured in 10% ePL produced 79± 34; and those cultured in 10% fdePL produced 56± 53 pg/mL. However, there was no statistical difference between unstimulated monocytes cultured in 10% ePL versus 10% fdePL (Figure 3). These results suggest that the presence of ePL or fdePL decreases the production of pro-inflammatory cytokines by monocytes, but this was not further decreased following fibrinogen depletion of the ePL.

Increased IL-10 production in monocytes cultured with ePL or fdePL

To determine whether fibrinogen depletion affected the anti-inflammatory response of monocytes, levels of IL-10 production were measured in cells cultured in either 10% fdePL, 10% ePL, or 10% DHS. Levels of IL-10 were significantly higher ($P < 0.05$) in LPS-stimulated monocytes cultured in fdePL or ePL compared to those cultured in DHS. LPS-monocytes that were cultured in 10% DHS produced 566 ± 424 pg/mL of IL-10; those cultured in 10% ePL

produced 2951± 168 pg/mL; and those cultured in 10% fdePL produced 2637± 107 pg/mL. However, there was no statistical difference in IL-10 production between the 10% ePL- and 10% fdePL-cultured LPS-stimulated monocytes (Figure 2). Levels of IL-10 were also measured in supernatants obtained from unstimulated monocytes cultured in 10% fdePL, 10% ePL, or 10% DHS. There was significantly more IL-10 produced from unstimulated monocytes cultured in 10% fdePL or 10% ePL compared to those cultured in DHS ($P < 0.05$). Unstimulated monocytes cultured in 10% DHS produced 245 ± 41 pg/mL of IL-10; those cultured in 10% ePL produced 3080 ± 266 pg/mL; and those cultured in 10% fdePL produced 2882 ± 323 pg/mL. However, there was no statistical difference in IL-10 production between the 10% ePL- and 10% fdePL-cultured unstimulated monocytes (Figure 4). These results suggest that the presence of ePL or fdePL increases the production of anti-inflammatory cytokines by monocytes, but that this was not further increased following fibrinogen depletion of ePL.

ROS production via flow cytometry was not affected by ePL or fdePL

To determine the effect of fibrinogen depletion on neutrophil activation, we cultured the cells in increasing concentrations of ePL, fdePL, or FBS and used flow cytometry to measure mean fluorescence intensity (MFI). Neutrophils were cultured in 0.1%, 0.3% 1%, 3%, 5% lipids or 10%, 25%, 50%, 75%, 100% ePL, fdePL or FBS. Lipids are an ingredient of serum, therefore, we were interested if the high concentration of lipids alone would effect reactive oxygen species production.[7]. There was no statistical significance reported ($P < 0.05$) when statistics were performed comparing fdePL and ePL to the same media concentration of FBS or the correlating percentage of lipids. Additionally, there was no statistical significance reported ($P < 0.05$) between fdePL and ePL at any of the concentrations. The reactive oxygen species production results are listed in Table 3. These results suggest that there is no difference in reactive oxygen

species production when neutrophils are cultured in different medium at an increasing concentration. However, due to the increase of proteins at higher percentages, fluorescence quenching may play a contributing role.[40] Therefore, we wanted to explore ROS using a different methodology.

Increased phagocytosis in neutrophils cultured in ePL or fdePL

To determine whether ePL or fdePL has an effect on neutrophil function, a phagocytosis assay was performed as previously described. [33] Neutrophils were cultured in 10%, 25%, 50%, 75%, 100% ePL, fdePL or DHS. EPL and fdePL statistically enhanced ($P < 0.05$) phagocytosis of *S. aureus* compared to FBS at all concentrations. However, there was no difference between ePL and fdePL on the phagocytosis ability of neutrophils. FdePL and FBS statistically enhanced ($P < 0.05$) phagocytosis of *E. coli* compared to ePL at 75% and 100% concentrations.

Initially, the phagocytosis assay was performed without exploring a potential correlation with ROS production. Thus, in order to compare ROS and phagocytosis in the same cells, the phagocytosis assay was repeated. The initial phagocytosis assay was executed in December, and then was repeated in March. We expected to observe the same data trend, but instead saw some variation in our results. In March, we found that fdePL statistically enhanced phagocytosis of *S.aureus* at all concentrations compared to FBS. However, ePL only enhanced phagocytosis of *S.aureus* at 10% and 25% concentration compared to FBS. Additionally, fdePL statistically enhanced phagocytosis compared to ePL at 50%, 75%, and 100% (Figure 6). For *E. coli*, fdePL statistically enhanced phagocytosis at all concentrations compared to FBS and ePL. However, there was no significance between ePL and FBS at any concentrations (Figure 5).

Neutrophil functionally is known to vary with seasons. [38] However, our results suggest that when neutrophils are cultured in fdePL compared to ePL, the increase in phagocytosis ability was consistent between December and March.

Correlation between ROS production and phagocytosis in neutrophils cultured in ePL or fdePL

To determine if there is a correlation between phagocytosis and ROS production, a ROS kinetics assay was performed using a plate reader (BioTek, Winooski, VT). Neutrophils were cultured in 10% ePL, fdePL, or FBS. When neutrophils were cultured in 10% ePL, fdePL or FBS, with *S .aureus* or *E. coli*, fdePL and ePL produced significantly more reactive oxygen species compared to FBS for both bacteria ($P<0.05$). The results of the ROS kinetics study are shown in Table 4; statistics were performed only for the 1-hour timepoint to compare ROS production and phagocytosis. Together, these results confirm that the increased ability to phagocytose bacteria does correlate with an increase in ROS production in 10% concentrations of all media.

Discussion

After optimizing fibrinogen depleted platelet lysate, the effect of fdePL on monocytes and neutrophils was assessed. The aim of the present study was to determine if fdePL can modulate monocytes and neutrophils more effectively than ePL.

Monocytes are sensitive cells that have the potential to create an immune system imbalance.[2, 3, 12, 28] Monocytes were cultured with and without LPS to determine the effect of fdePL on naïve and activated monocytes. LPS contains Lipid A, which binds to TLR4 and creates a pro-inflammatory response. [12] There was no statistically significant difference in IL-10 and TNF-α production between ePL and fdePL when monocytes were cultured with and without

LPS. This could be attributable to other proteins in the ePL that are responsible for producing TNF-α. Although there was no improvement of fdePL compared to ePL, it is important to know the presence of fibrinogen does not increase TNF-α production. Fibrinogen is a common ingredient in many plasma products administered in a clinical application.[10] Even though the effect of fibrinogen on human monocytes is well established, this is the first study in veterinary medicine that addressed the effect of fibrinogen on equine monocytes.

Next, we tested the effect of fdePL and ePL on neutrophils, which are the first responders of the immune system,[39] by measuring ROS production and phagocytosis. Initially, we measured ROS production and phagocytosis under separate conditions. Our ROS data obtained via flow cytometry appeared to indicate that FBS is able to suppress ROS production at increasing concentrations. We were uncertain if an increasing concentration of FBS should create a linear suppression of ROS production. Therefore, in order to validate our data and confirm that it was or was not a fluorescence quenching effect, we explored other assays to measure ROS production. [40]

After further consideration, we decided to investigate the correlation between phagocytosis and ROS production, as has been reported in the literature. [41] To this end, we repeated the phagocytosis assay, and used a subpopulation of the same cells to simultaneously perform the ROS analysis. For these experiments, we used only 10% ePL, fdePL, or FBS to eliminate the issue of fluorescence quenching. Additionally, due to the logistics of measuring phagocytosis and ROS production on the same cells, ROS production was measured using a plate reader. When the phagocytosis assay was repeated in March, we expected to see a similar trend to the one we observed in December. But what we observed was that fdePL was now more consistent at enhancing phagocytosis ability compared to ePL, indicating some seasonal variation

in our results. This has been reported in human neutrophils, which undergo functional changes with the seasons. In human neutrophils, there is an upregulation in receptor expression, an increase in adhesion ability, and an increase in ROS production in the spring compared to the winter. [38] We suspect that equine neutrophils exhibit similar differences in functionality according to the season.

Interestingly, we observed a difference in phagocytosis between *S. aureus* and *E. coli*, which may be attributable to a gram-positive versus a gram-negative effect. The peptidoglycans from gram-positive bacteria bind to TLR2, and gram-negative bacteria bind to TLR4 on the membrane, both of which activate of MyD88. [12] However, the binding of TLR4 versus TLR2 can affect gene upregulation. For example, TLR2 causes a reduction in CXCR2, while binding via TLR4 does not.[42]Although both TLRs lead to the engagement of MyD88, there may be a signaling pathway difference that effects the ability of neutrophils to phagocytose. It is also possible that while the presence of fibrinogen has been shown to increase phagocytosis in human neutrophils, the same may not be true for equine neutrophils.[43]

Conclusion

Based on the immune cells we tested, depleting fibrinogen from platelet lysate did not affect monocyte or neutrophil function in vitro, and therefore removing fibrinogen is not necessary to improve the immunosuppressive abilities of lysate. Further investigation will be needed to identify the impact of other plasma components on immune cell function.

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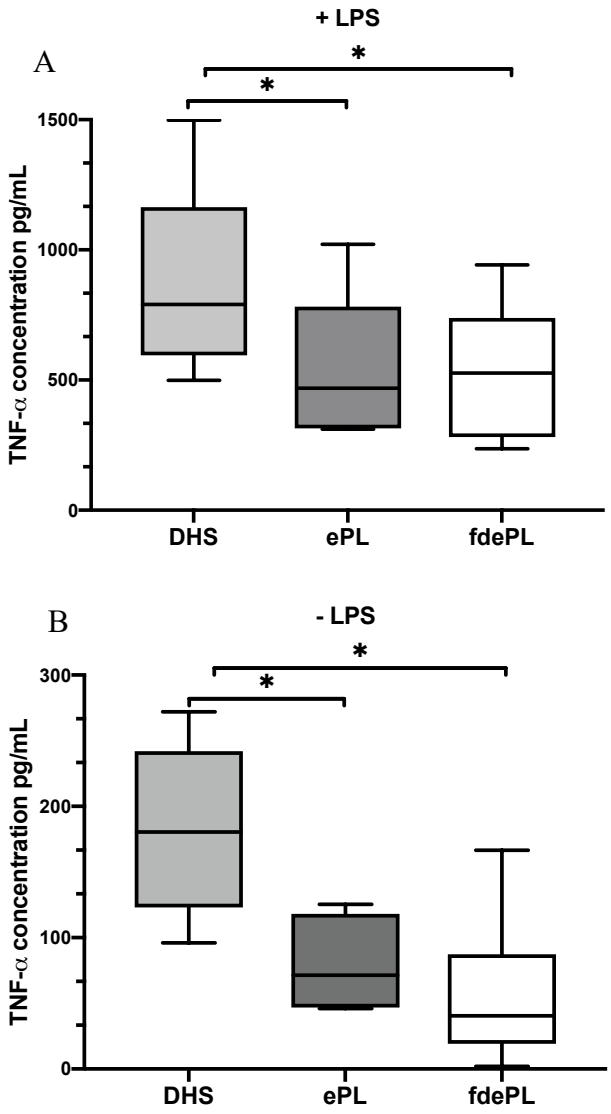

Figure 3. A) TNF-α production from monocytes cultured in DHS, ePL or fdePL at six hours post LPS stimulation (n=6) and B) TNF-α production from unstimulated monocytes cultured in DHS, ePL, or fdePL (n=6). *Statistical Significance: $P<0.05$.

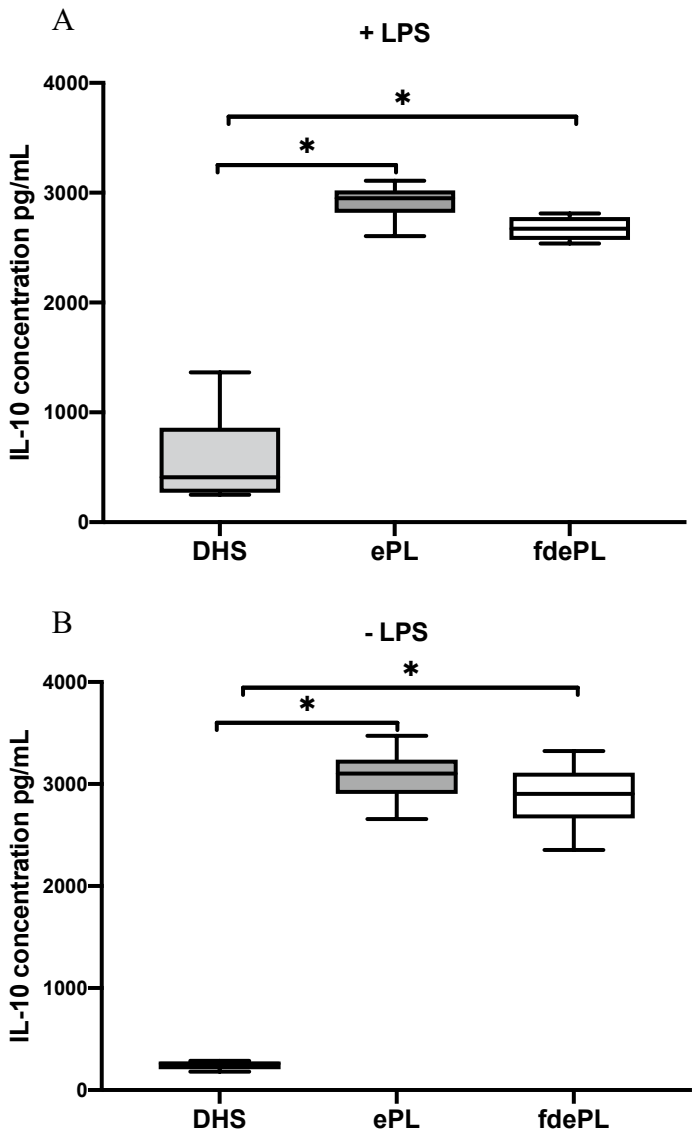

Figure 4. A) IL-10 production from monocytes cultured in DHS, ePL or fdePL at six hours post LPS stimulation (n=6) and B) IL-10 production from unstimulated monocytes cultured in DHS, ePL, or fdePL (n=6). *Statistical Significance: $P<0.05$.

Table 3. Flow cytometry was performed to find the mean fluorescence intensity produced by neutrophils when exposed to 0.1, 0.3, 1, 3, 5% lipid, 10, 25, 50, 75, 100% FBS, ePL or fdePL. *Statistical significance ($P<0.05$) is represented and was determined when comparing ePL or fdePL to same percentage as FBS only.

Media	Mean Fluorescence Intensity (MFI) ± s.d
10% FBS	5.07±0.49
10% ePL	5.07±0.48
0.1% Lipid	5.09±0.51
10% fdePL	5.10±0.58
25% FBS	4.90± 0.51
25% ePL	4.90±0.51
0.3% Lipid	5.0±0.52
25% fdePL	4.95±0.50
50% FBS	4.71±0.54
1% Lipid	4.89±0.52
50% ePL	4.67±0.52
50% fdePL	4.89 ±0.52
75% FBS	4.48±0.56
3% Lipid	4.78±0.54
75% ePL	4.51±0.54
75% fdePL	4.71±0.56
100% FBS	4.43±0.62
5% Lipid	4.63±0.57
100% ePL	4.44±0.61
100% fdePL	4.63±0.59

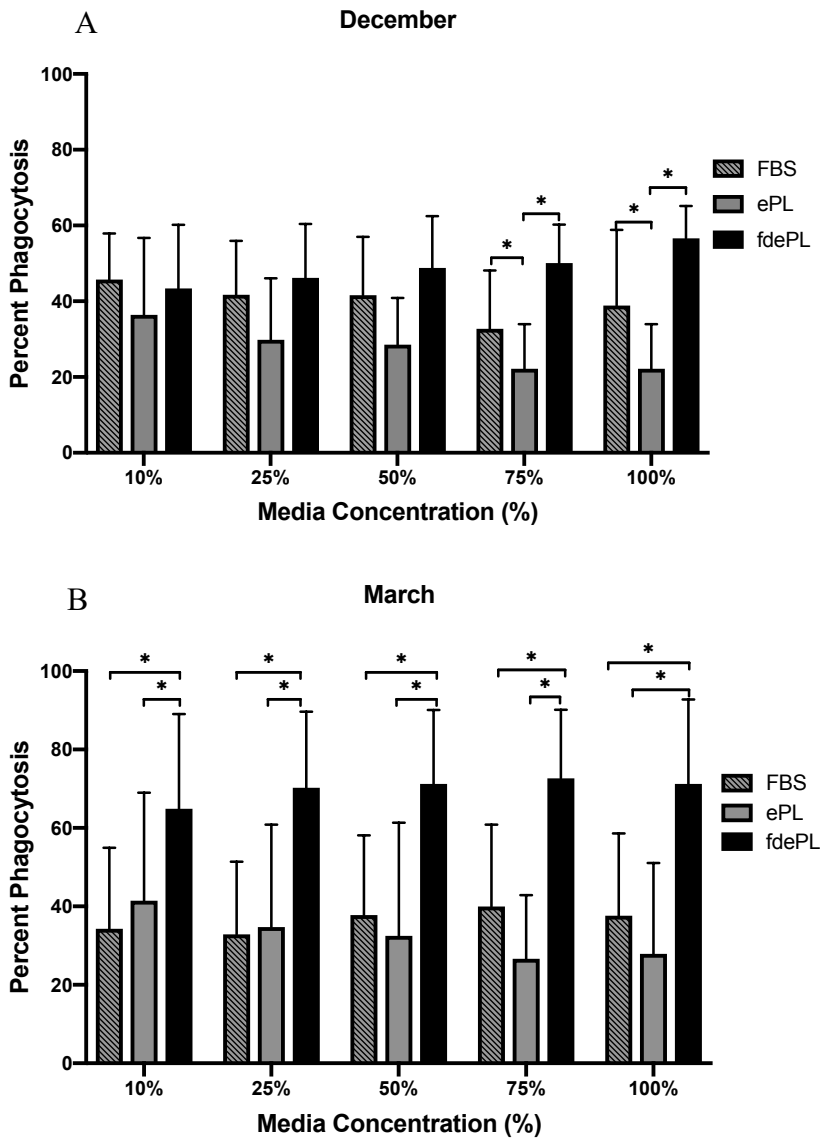

Figure 5. The percentage of *Escherichia Coli* phagocytosed per neutrophil when exposed to varying concentrations of FBS, ePL, and fdePL. Identical media additive percentages (ex:10) were compared with each other to determine statistical significance ($P<0.05$). A) The percentage phagocytosis data collected in December. Both FBS and FdePL had statistical significance at 75% and 100%, when compared to 75% and 100% of ePL. B) The percent phagocytosis data collected in March. FdePL was statistically significant at every concentration when compared to FBS and ePL at the identical percentage.

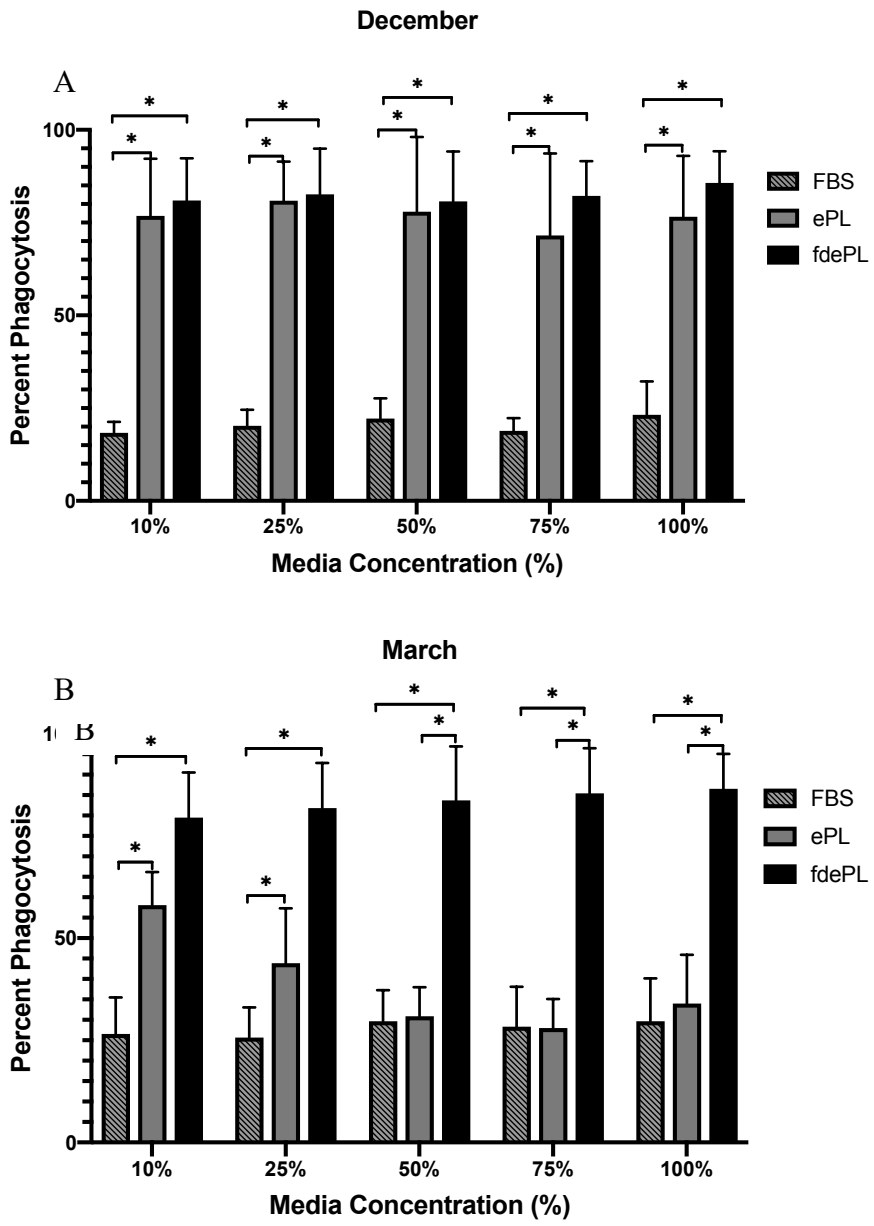

Figure 6. The percentage of *Staphylococcus Aureus* phagocytosed per neutrophil when exposed to varying concentrations of FBS, ePL, and fdePL in December and March. Identical media additive percentages (ex:10) were compared with each other to determine statistical significance ($P<0.05$). A) The percentage phagocytosis data collected in December. Both ePL and FdePL had statistical significance at every percentage of media concentration, when compared to FBS at the identical percentage. However, there was no statistically significant difference between ePL and

FdePL in any of the groups. B) The percent phagocytosis data collected in March. Both ePL and FdePL had statistical significance at 10% and 25% when compared to FBS at the identical percentage. However, at 50, 75, and 100% media concentration, fdePL was statistically significant compared to FBS and ePL.

Table 4. ROS production from neutrophils cultured in 10% ePL, fdePL, and FBS. *Statistical significance (P<0.05). 1:00 hour ROS production correlates with how long the cells phagocytosed before fixation. Numbers are represented at Average Fluorescent Intensity with corrected AFU. Statistical significance was determine when comparing ePL + staph and fdePL + staph to FBS + Staph, and the same concept for *E.coli*.

	10% FBS STAPH	10% FBS COLI	10% ePL STAPH	10% ePL COLI	10% fdePL STAPH	10% fdePL COLI
0:00:00	3.53±1.88	3.39±1.74	2.42±0.88	2.31±0.88	2.91±0.95	2.68±0.92
0:10:00	4.54±2.27	4.52±2.14	3.17±1.29	3.82±1.74	6.25±4.27	4.85±2.03
0:20:00	5.72±3.27	6.00±3.21	5.10±2.74	7.35±4.00	14.58±13.50	10.43±6.07
0:30:00	7.29±4.56	8.25±5.18	9.16±4.71	13.48±7.90	24.48±20.86	17.41±10.29
0:40:00	8.59±5.57	11.95±7.75	13.55±5.60	19.52±12.23	32.77±26.00	23.93±13.81
0:50:00	9.77±6.20	14.77±9.22	17.70±5.41	25.20±15.51	39.44±29.38	28.74±16.53
1:00:00	10.84±6.57	16.83±10.55	20.97±6.00*	29.83±18.3*	45.74±33.9*	34.11±19.9*
1:10:00	11.86±7.00	18.07±10.87	23.88±6.21	33.61±20.68	50.06±35.28	38.46±22.80
1:20:00	12.50±6.88	19.70±11.92	26.16±6.98	36.31±22.26	53.65±37.52	41.05±24.38
1:30:00	13.50±7.67	20.64±12.22	27.78±7.00	38.74±24.04	55.77±37.69	42.78±25.26
1:40:00	14.00±7.50	21.96±12.80	28.97±7.23	40.66±25.33	57.56±37.91	43.81±25.12
1:50:00	15.00±8.15	22.17±12.40	30.12±7.63	41.59±25.45	58.73±38.17	45.25±26.01
2:00:00	15.58±8.33	23.56±13.19	31.12±7.73	42.52±25.69	59.32±38.56	45.76±26.17

CHAPTER 5

CONCLUSIONS

In recent studies, platelet lysate has been explored as a homologous cell culture alternative when culturing equine monocytes. Platelet lysate is an acellular platelet derived product that can be manufactured via plateletapheresis. The overall objective of this research was to optimize platelet lysate and then examine the ability to modulate monocyte and neutrophil activation.

The purpose of Chapter 3 was to manufacture a fibrinogen and immunoglobulin depleted equine platelet lysate that had an improved recovery volume, and growth factor concentration similar to ePL. While we were able to deplete fibrinogen, our methods did not successfully deplete immunoglobulins. These set of experiments highlighted that none of the depletion techniques tested, regardless if from ePL or PC, had the same number of GFs compared to baseline. Determining which method best fit our criteria was necessary so we could subsequently determine if there was an improved TNF-α modulation compared to the previous study.

In Chapter 4, we tested the newly improved fibrinogen depleted platelet lysate on monocytes and neutrophils. We wanted to evaluate neutrophils as well as monocytes, since they are the first responders of the immune system. Although we did not observe that fdePL further suppressed TNF-α production from monocytes with and without LPS, we are still pleased with the results since we can conclude that the presence of fibrinogen does not enhance TNF-α production. Furthermore, when reactive oxygen species production was analyzed using an increasing concentration of ePL, fdePL, and FBS via flow cytometry, we did not observe

statistical significance. Multiple reactive oxygen species assays were performed to determine if fluorescence quenching was affecting the results, trouble-shooting was required. Additionally, we were able to confirm that there is a correlation between phagocytosis and reactive oxygen species production. Interestingly, we did observe a difference in the ability of neutrophils to phagocytose gram-positive versus gram-negative bacteria. This discovery now opens up numerous questions about the ability of ePL and fdePL to effect gram-negative and gram-positive bacteria in other assays. There was also a seasonal variation affect that effected our results. We made some interesting discoveries about ePL and fdePL that need to be addressed further in the future.