In vitro antimicrobial activity of equine mesenchymal stromal cells and platelet lysate against common clinical pathogens

by

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Abstract

Septic arthritis is considered a medical emergency. Following bacterial colonization, significant morbidity and mortality can occur while requiring costly treatment. Antimicrobial properties of regenerative therapies including mesenchymal stromal cells and platelet products have been researched extensively in human medicine and have shown promising results. The purpose of this study was to evaluate bacterial suppression by equine platelet lysate (EPL) and adipose derived mesenchymal stromal cells (ASCs) in vitro. We hypothesized that both products would significantly inhibit the growth of Staphylococcus aureus (Sa) and Escherichia coli (Ec). Pooled blood from 10 horses was used for production of EPL. Mesenchymal stromal cells were isolated from adipose tissue harvested from the gluteal region of 3 horses. The study evaluated three treatment groups; 10X EPL, 1.6 million ASCs, and control using an incomplete unbalanced block design with repeated measurements. Optical density readings and colony forming units/ml were calculated at hours 0, 3, 6, 9, 12, 18, and 24. Decreased bacterial growth was seen at multiple time points for the Sa-ASC and Sa-EPL treatments supporting our hypothesis. Increased bacterial growth was noticed in the Ec-EPL group with no difference in the Ec-ASC treatment which did not support our hypothesis. A clear conclusion of the antimicrobial effects of EPL and ASCs cannot be made from this in vitro study. Although it appears ASCs have a significant effect on decreasing the growth of Sa, further studies are needed to explore these effects particularly in gram-positive bacteria.
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Chapter 1 - Synovial Sepsis in Equine Patients

Bacterial Inoculation

Acute septic synovitis is considered a medical emergency and can lead to mortality despite aggressive medical and surgical treatment (1). Septic synovitis in equine patients develops most commonly through hematogenous or exogenous spread. Studies have shown hematogenous spread secondary to bacteremia is more common in foals than in adults (2,3). Exogenous development is most common secondary to wounds or iatrogenic following intrasynovial injection or surgical procedures (4). Many factors are associated with the establishment of infection. These factors include inoculum size, organism virulence, host defense mechanisms, and local joint factors (3). Distal limbs of horses have little soft tissue and musculature with decreased vascular supply increasing the risk of synovia l infection following injury. A significant inflammatory response ensues following bacterial inoculation of a synovial cavity. The resulting cytokine and inflammatory mediator response can lead to significant cartilage damage and progressive osteoarthritis.

The immature immune system of foals makes them more susceptible to infection and septicemia compared to adults. When incomplete or failure of passive transfer of IgG via colostrum occurs, this increases the risk of hematogenous spread and localization of bacteria in bones and joints (4,5). Studies have shown upwards of 80% of foals with failure of passive transfer develop clinical signs of disease, with septicemia and septic arthritis being leading causes of death in young foals (3). Four types of hematogenous infections articular infections occur in foals. S-type infection occurs only within the synovial membrane and fluid, E-type is
localized to the articular epiphysis, P-type within long bones physis and attachment of the joint capsule, and T-type within tarsal or carpal bones in premature foals (3,4). These four types of infections tend to be seen in different aged foals with various clinical signs such as joint effusion, swelling, pain, and lameness.

Synovial injection in adults is much more common following penetrating trauma or iatrogenic inoculation following surgery or joint injections than hematogenous spread (6–10). Factors associated with increased risk of infection following surgery including draft breeds, tibiotarsal arthroscopy, carpal sheath endoscopy, and removal of 40mm or larger osteochondral fragments (6–8). In one study, preoperative systemic or intraarticular prophylactic antibiotics did not change the incidence of postoperative synovial sepsis (6). Risk factors associated with septic arthritis following synovial injections include experience of the veterinarian, preparation method, use of sterile gloves, and medication used (9,10). In a survey of 241 veterinarians nearly 50% used intrasynovial antibiotics during steroid injections without a significant reduction in joint sepsis (10). Clinical signs of septic arthritis in adults are similar to foals and include lameness, swelling, and joint effusion. Survival to discharge has been reported at rates of 85-90% with fewer (56-81%) returning to previous level of work (11,12).

**Diagnosis**

Obtaining a microbial culture is considered the gold standard for diagnosis of septic arthritis in both foals and adults. Positive synovial culture result rates tend to be low and are described from 25-80% (4,12,13). A recent report of culture results from septic synovitis cases found 66% gram-positive, 38% gram-negative, and 4% anaerobic bacterial cultures (12). In
adult synovial samples, gram positive bacteria isolation occurred in 83% of cases compared to 14% gram-negative bacterial isolation (14). In contrast, in foals gram negative bacterial isolation was more common compared to gram positive, 72% versus 28% (14). *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) were two of the most common gram-positive and gram-negative isolates respectively. Due to high prevalence of *S. aureus* on the skin, it is the most commonly isolated bacterial species from equine wounds (11,13,15). Reports in humans have similar results with both *S. aureus* and *E. coli* being the most common isolates and a much smaller number of anaerobic infections (16). Other common diagnostics that can be utilized for diagnosis and progression of disease are cytologic examination of synovial fluid including total nucleated cell count (TNCC), total protein (TP), and presence of microorganisms. Imaging, such as radiography, computed tomography, magnetic resonance imaging, and ultrasonography has also been described (4).

**Current Treatment Modalities for Synovial Sepsis**

In equine patients, synovial structures such as a joint, tendon sheath, or bursa can be affected. Treatment following bacterial colonization can be problematic leading to significant morbidity and mortality. These treatments can be costly in the equine patient with standing or surgical joint lavage, and local and systemic antimicrobial therapy to eliminate the bacterial agent while maintaining synovial function.

Early synovial fluid lavage is a key treatment in septic arthritis. High volume lavage of synovial structures with a balanced electrolyte solution facilitates removal of debris, microorganisms, and inflammatory mediators (4). Lavage procedures may be performed in the
standing patient or under general anesthesia. The advantage of performing lavage under anesthesia is the use of endoscopic equipment or large bore needles for removal of large fibrin clots, debridement of devitalized tissues, and evaluation of joint surfaces (4). Economic constraints are common so standing lavage under local or regional anesthesia is often chosen. Repeated lavage treatments are performed based on clinical signs of the patient and cytologic findings of synovial fluid.

Antimicrobial therapy should be immediately implemented and modified as dictated by culture and sensitivity results. Prior to these results, a combination of beta lactam and aminoglycoside antibiotics are commonly used to provide broad spectrum coverage and, if anaerobic infection is suspected metronidazole is added (4). Antimicrobials are often administered via multiple routes including regionally-infused and locally injected therapy. Regional limb perfusions use a proximal tourniquet and infusion of antibiotic into a peripheral vein to provide a high peak concentration relative to the minimum inhibitory concentration. Intraosseous and intraarticular administration provide direct high concentrations of antibiotic to the site of infection. When these techniques are performed, the most common antibiotic used amikacin due to its broad-spectrum, bactericidal, and concentration-dependent properties (11).

Despite early and aggressive intervention 6-14% of septic arthritis patients are euthanized (11,12). Although the majority of patients survive to discharge; many of these will experience long-term complications such as persistent lameness from articular cartilage damage and adhesion formation. Veterinary and human medicine are increasingly concerned about
antimicrobial resistance (AMR). Many causes of resistance have been promoted by excessive or improper use of antimicrobials such as incorrect dosing or duration (17).
Chapter 2 - Properties of Mesenchymal Stromal Cells

What are Mesenchymal Stromal Cells?

Equine and other animal mesenchymal stromal cells (MSCs) are known to be self-renewing and multipotent (18). These cells can be harvested from bone marrow (BMSCs), adipose tissue (ASCs), dental pulp, umbilical cord, peripheral blood, tendon, synovial fluid, and placenta (1,18). In equine medicine BMSCs and ASCs are the most commonly used in a variety of expanded or nonexpanded products (1). Each population of MSCs has a unique differentiation potential and potential phenotype (18). Their regenerative effects are largely accepted to be due to paracrine stimulation through release of growth factors, cytokines, and chemokines (18).

Antimicrobial Mechanisms and Effects of Mesenchymal Stromal Cells

The regenerative potential of MSCs has been researched extensively (1,18–22). In addition, the antimicrobial properties of MSCs have been investigated (18,23–27). The direct antimicrobial properties are attributed to the release of antimicrobial peptides (AMPs) or short string amino acids and growth factors (18,25,27). Many of these AMPs are of particular interest due to their ability to be active against pathogens that are resistant to conventional antibiotics (18). Thousands of AMPs have been discovered and logged in a database. AMPs from the cathelicidin family have been isolated from equine BMSCs, while many others have been found in human MSCs including defensin, hepcidin, and lipocalin peptide families (18,27). These broad AMP families have been studied with varying antimicrobial mechanisms between families and the species in which they are expressed (28). Cathelicidins have been shown to induce
bacterial membrane disruption and cellular lysis (29). Specifically human cathelicidin LL-37 has been linked to direct bacterial killing as well as its capability to reduce cytokine and endotoxin (30). This activity was dependent on and enhanced by the treatment of 1,25 dihydroxy vitamin D₃ (31). The β-defensin AMP family has been connected to paracrine signaling via TLR-4 receptors as well as bacterial lysis secondary to pore formation (32,33). The lipocalin family, specifically lipocalin 2 restricts bacterial growth by iron sequestration (34). When MSCs from horses were stimulated with LPS lipocalin 2 expression was upregulated with endometrial-derived stromal cell expression significantly higher than ASCs or BMSCs (35). Other non-AMP direct mechanisms that have been investigated including indoleamine 2,3-dioxygenase (IDO) expression, direct phagocytosis, nitric oxide secretion, and presence of cysteine protease (28).

In addition to direct mechanisms, MSCs have exhibited the ability to modify the host immune response. Macrophages play a key role in the immune system and can be induced to pro- or anti-inflammatory phenotypes. MSCs have demonstrated the ability to induce both phenotypes with different activation methods (36). With the release of cytokines, neutrophil activation and upregulation of phagocytic abilities occurs (18,25,28). Equine stromal cells isolated from adult peripheral blood had increased expression of IL-17, which is a pro-inflammatory cytokine that links T cell activation to neutrophil mobilization and activation (25). Other cytokines and immunomodulatory genes expressed by MSCs include IL-6, IL-8, INFγ, CCL5, and RANTES (28,35).
The effects of MSCs and antibiotics have also been investigated. It is important to know their interaction and potential synergistic effect. An *in vitro* study of equine BMSC viability and gene expression in the presence of common clinical antibiotics including penicillin, gentamicin, amikacin, ceftiofur, and enrofloxacin was performed (37). Dose dependent effects were seen with both increased and decreased mRNA expression of a variety of cytokines and genes with some antibiotics decreasing cell viability (37).

The antimicrobial effects of human and equine MSCs have been reported against bacteria and yeast such as *E. coli*, *S. aureus*, *Staphylococcus pneumoniae*, *Pseudomonas aeruginosa*, *Candida albicans*, as well as the ability to influence biofilm formation (18,25,27,31,36). Additional studies have indicated their potential use as antiviral and antiparasitic agents (38–40). These studies indicate that there is a potential use for these regenerative therapies in cutaneous wounds and joint infections.

**In Vivo Antimicrobial Studies of Mesenchymal Stromal Cells**

Although *in vitro* work in equine MSCs has been performed; to date there are no *in vivo* studies reported in literature. A canine study using 7 client owned dogs with chronic multidrug resistant (MDR) wound infections was recently reported in which each dog received three intravenous injections at two week intervals of preactivated allogenic ASCs along with antibiotic therapy (36). Following 8 weeks of treatment, 5/7 dogs had cleared the infection with the remaining 2 having clinical improvement but not complete resolution (36). Two *in vivo* studies involving cattle show promising results. A clinical trial involving 48 dairy cows with acute or chronic mastitis was performed comparing intramammary injection of allogenic non-primed
amniotic-derived MSC conditioned media and antibiotics. There was no significant difference in somatic cell counts (SCC), but the rate of recurrence of chronic mastitis was decreased in the conditioned media group, 67%, compared to antibiotics, 100% (41). In experimentally induced mastitis with *S. aureus*, mammary quarters treated with allogenic non-primed ASCs showed reduced bacterial counts in milk with no difference in SCC compared to the untreated cattle (42). No adverse reactions were noted in the bovine studies and the future potential use of MSCs for the treatment of infection in our food producing species.

**Other Uses of Mesenchymal Stromal Cells in Literature**

MSCs have been researched extensively for use in tendon and ligament injuries as well as cartilage damage and osteoarthritis. It is now believed that MSCs assist in the healing of the soft tissues through paracrine signaling. These mechanisms stimulate fibroblasts, modulate inflammation, as well as recruit and activate other progenitor cells (43–46). BMSCs and ASCs have been show to increase vascularization of tendon lesions, improve levels of type I collagen, and fiber organization (47–49). Long term studies have indicated increased return to function with decreased reinjury rates compared to control groups (50,51). *In vitro* studies of BMSCs, ASCs, and umbilical cord derived MSC cultures have shown these cells can differentiate into chondrogenic phenotypes demonstrating multiple MSC types can potentially be used in soft tissue injuries (52–54). Other studies have shown no improvement of cartilage defect scores following MSC implantation on the lateral trochlear ridge of the femur (55). Although there are many studies indicating promising results using MSCs; additional controlled prospective studies are needed to establish protocols for their future use. There are currently no controlled clinical
studies of naturally acquired joint disease to evaluate the efficacy, dose, or route of administration.

The immunomodulatory effects of MSCs have also been explored as therapeutics for inflammatory and immunological disorders in human medicine. MSCS cause suppression of the adaptive immune response by direct inhibition of CD4\(^+\) and CD8\(^+\) T-cells and indirectly by modulation of antigen presentation on dendritic cells (56). The effects on T-cells allow for the potential use of MSCs in individuals with autoimmune disease such as Crohn’s disease, lupus, rheumatoid arthritis, or organ transplant patients (56,57). MSCs have also been explored as vehicles to deliver large doses of cancer biologics due to temporary ability to evade immune response (57). Mouse and rat cancer models have been used to show MSC induced tumor cell death through a variety of mechanisms and cancer types (57).
Chapter 3: Properties of Platelets and Platelet Products

What are Platelets?

Platelets or thrombocytes are a major component of blood produced in bone marrow by megakaryocytes prior to being released into the blood stream (1,58). Platelets are metabolically active, contain intracellular alpha granules with peptides required for coagulation, and surface receptors (1,58). They play a key role in both primary (adhesion to exposed vascular subendothelium) and secondary hemostasis (initiation and propagation of the coagulation cascade).

Antimicrobial Mechanisms and Effects of Platelets

Platelet activation and its role in mediating inflammation has been researched for many years (58). Many platelet concentrates are available and have variable concentrations of platelets and leukocytes depending on the production protocol. Their immunomodulatory properties and stimulation of tissue healing by release of growth factors have been well established (59–61). Several AMPs have been isolated from human platelets. In a study by Krijgsveld, Thrombocidin 1 & 2 were isolated by electrophoresis and sequenced, revealing they are variants of chemokines that are responsible for activation of neutrophils and connective tissue peptides (62). This study demonstrated bactericidal effects against *Bacillus subtilis*, *E. coli*, *Staph aureus*, and *Lactobacillus lactis* as well as being fungicidal for *Cryptococcus neoformans* with 3 and 5 log decreases within 30 minutes (62). Seven additional AMPs from human platelets were later isolated (24). These isolated proteins were then tested in vitro against *E. coli*, *Staph aureus*, *Candida albicans*, and *Cryptococcus neoformans* (24). In this study each protein showed
antimicrobial activity to at least 2 organisms (24). Generally they were more potent against bacteria than fungi, and some were synergistic (24). Another confirmed direct method of killing is generation of reactive oxygen species which bind microbes producing cellular cytotoxicity (63).

In addition to AMPs, indirect mechanisms causing decreased bacterial growth have been found. Platelets express adhesion ligands increasing the ability to bind leukocytes and adhere to endothelial vessels or lymph nodes (64). Intravital microscopy imaging has been used to observe platelets surrounding and neutralizing bacteria while interacting with macrophages in the bloodstream (65). Platelets also express pattern-recognition receptors (PRRs). Some of these PRRs directly recognize LPS which leads to recruitment, activation, degranulation, and enhanced phagocytosis of neutrophils (64).

**In Vivo Antimicrobial Studies of Platelet Products**

Rabbits were the first animal used in surgical site infection and osteomyelitis models. Implant associated infections had significantly fewer bacterial colonies in sites treated with thrombin activated PRP compared to control groups (59,66). Osteomyelitis caused by methicillin resistant and sensitive *S. aureus* was also reduced in rabbits treated with leukocyte rich PRP but to a lower level compared to antibiotic treatment (59,67). Studies performed in animals are limited and the level of bias has been questioned (59). Further clinical studies comparing preparation methods, infection models, species, and spectrum of activity are necessary.
Antimicrobial Clinical Uses of Platelet Products in Human Medicine

Many retrospective case reports have been published in human medicine. Prevalence of incisional and deep sternal infections in 2279 patients following cardiac surgery were evaluated in which, 382 received topical platelet concentrated gel (68). The incidence of both incisional and deep infections were significantly lower (0.03% and 0%) in the platelet group compared to the no platelet (1.5-1.8%) and historic control groups (1.5-1.7%) (68). Other studies in cardiac patients showed shorter ICU and total hospitalization stays, decreased blood loss, and incisional infections in patients treated with platelet products (69). Clinical case reports using platelet products in surgical wound healing can be found in many specialties including oral surgery, orthopedics, and dermatology (70–72). Although many of these studies do not suggest an exact mechanism for the decreased infections, the results are promising.

Objective

Therefore, the purpose of this study is to evaluate the antimicrobial effects of the platelet product, equine lysed platelet rich plasma (EPL) and adipose derived mesenchymal stromal cells (ASCs) on common microbes causing infections in horses. We hypothesize that both EPL and ASCs will significantly inhibit *in vitro* growth of *S. aureus* and *E. coli*. 
Chapter 4 - Materials and Methods

Pooled EPL Production

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC Protocol #4217) at Kansas State University. Ten healthy equine patients were selected for whole blood collection. Five Quarter Horse mares and five Quarter Horse geldings between ages 3-5 were used for the study. Horses were manually restrained and the jugular vein was aseptically prepared for venipuncture using three, 30 second scrubs of chlorhexidine scrub alternating with wipe down of the area with isopropyl alcohol. Sterile venipuncture was performed using an 18-gauge needle and 500 ml of whole blood was collected into 60 ml sterile syringes containing 5-7ml of heparin sodium (West-Ward Pharmaceuticals, Eatontown, NJ, USA).

Following collection, the blood was immediately transported to the laboratory for processing. An aliquot of each whole blood sample was set aside for manual platelet counts. The whole blood was then transferred into 50ml conical tubes and spun for 10 minutes at 150 x g. The plasma was then transferred to a new centrifuge tube and spun at 400 x g for 15 minutes. The platelet pellet was then resuspended to 10X whole blood platelet concentration using platelet poor plasma collected during the second centrifugation step. The samples were then subjected to two freeze thaw cycles at -80C. At this point, the samples from the 10 horses were pooled, aliquoted in 1ml vials and frozen at -80C until future use. Prior to bacterial assays the samples were thawed and centrifuged for 30 minutes at 1000 x g for 30 minutes at 4C and filtered through a sterile 0.22 µm filter. All of the above procedures were performed aseptically.
Figure 1: Production of Pooled 10X EPL beginning with single horse EPL production and pooling of five horses following freeze/thaw cycles.

1. Equine Blood + Heparin
   - Spin 10 min 150xg

2. Transfer supernatant PRP to new tube.
   - Count platelets in PRP
   - Spin 15 min 400xg

3. Transfer supernatant PPP to new tube.
   - Concentrate platelets to 10X
   - 2x Freeze/thaw at -80°C

4. Add thawed PPP to EPL to get desired platelet concentration Pool EPLs to use (n=5)

5. Spin 30 min 1000xg 4°C

6. Sterile filter EPL (0.22μm)
Isolation and Culture of Adipose Mesenchymal Stromal Cells

Equine adipose mesenchymal stromal cells used in this study were isolated from three donor horses using aseptic technique at Kansas State University College of Veterinary Medicine, Manhattan, USA. The fat was collected in sterile 1X DPBS with 1% antibiotic-antimycotic (penicillin-streptomycin mixture with amphotericin B) and processed within 24 after collection. The tissue was initially transferred to petri plates to mechanically separate muscle and fascia. The tissue was weighed in a centrifuge tube and then minced into manageable pieces using sterile scissors under a biosafety cabinet. The tissue was digested using 0.1% collagenase solution for 30-40 minute at 37°C in a water bath with occasional shaking. After the fat tissue was digested to obtain a homogenous mix, excess digestion was neutralized using 10 ml of 20% EPL culture media. The digested fat tissue was centrifuged at 720 x g for 10 minute and the supernatant was discarded. The cell suspension was washed twice with DPBS and strained through 100 µm cell strainers. Cell counts for the final stromal vascular fraction were obtained by using the LUNA-STEM™ (Logos Biosystem, South Korea) with Acridine Orange/Propidium Iodide staining. The cells were plated at 250,000/cm² density in low glucose DMEM containing 1% antibiotic-antimycotic and 20% pooled-equine platelet lysate. Cells were passaged by 0.1 % trypsin EDTA when they reached 80%-85% confluence. Manual cell counts were obtained using a Hemocytometer with trypan blue staining and plated at 10,000 cells/cm². The cells from passage 4-6 were used in this study.
**Standard Curve**

Bacterial stock of *Escherichia coli* ATCC 25933™ (Ec) [ATCC, Manassas, VA, USA] was inoculated into 10ml of Mueller Hinton II broth (MHII) and incubated for 24 hours. Absorbance readings at OD 600 with 2-fold serial dilutions and plating in duplicate were performed to determine colony forming units per milliliter (cfu/ml) at absorbance readings between 0.900-0.001. The same procedure was performed using *Staphylococcus aureus* ATCC 29213™ (Sa) [ATCC]. Absorbance vs. cfu/ml graphs were made and fit to a logarithmic scale and used for the remainder of the study to determine cfu/ml for the bacterial assays.

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**Figure 2: Standard Curve 2-fold Serial Dilution Methods for determination of CFU/mL of specific OD₆₀₀ readings from as close to 0.001-1 of *Escherichia coli* and *Staphylococcus aureus*, dilutions performed from 1:10 to 1:1,000,000**
Figure 3: *Escherichia coli* Standard Curve (logarithmic scale), OD vs. CFU/mL, and associated $R^2$ value performed using bacterial stock and series of 2-fold dilutions for OD$_{600}$ readings between 0.002-0.865.

Figure 4: *Staphylococcus aureus* Standard Curve (logarithmic scale) OD vs. CFU/mL, and associated $R^2$ value performed using bacterial stock and series of 2-fold dilutions for OD$_{600}$ readings between 0.005-0.860.
Bacterial Assays

The bacterial stock used for the standard curves of Ec and Sa was used for the entirety of the study. A single loop of individual stock was used and four-quadrant streaking was performed on new blood agar plates (BAP) and incubated overnight at 37°C. The following day, multiple isolated colonies were placed in 10ml of MHII broth to achieve a 0.5 McFarland standard bacterial turbidity. A 1:10 dilution was then performed and 100uL of the bacterial dilution was then inoculated into each of the experimental tubes with 9ml MHII broth and 1ml volume of treatment, 10X EPL or 1.6 million ASCs. The volumes were chosen to simulate the treatment volumes typically used for adult equine lower limb joints (15). Each assay was performed with both Ec and Sa in duplicate and repeated for 3 replicates. After bacterial inoculation, optical density readings at wavelength 600nm were performed and tubes were incubated at 37°C with readings at 0, 3, 6, 12, 18, and 24 hours. Serial dilutions and plating of BAPs were performed in triplicate for cfu/ml counts at each timepoint during the first replicate to ensure optical density readings correlated with the standard curve for each bacterium used.
Figure 5: Experimental Design & Bacterial Assays- Positive Controls (E. coli and S. aureus), Negative Controls (Broth only, ASC only, EPL only), Treatment groups in duplicate (EPL + Bacteria, ASCs + bacteria)
**Experimental Design**

The study evaluated three treatment groups including 10X EPL (1ml volume) and 1.6 million ASCs (1ml volume) with control groups of Sa, Ec, 10X EPL only, and ASCs only. An incomplete unbalanced block design with repeated measurements was implemented.

**Statistical Analysis**

Trial served as the blocking factor. Two trials contained EPL and PC, 2 trials contained ASC and PC, and 1 trial contained EPL, ASC and PC. Treatment was applied to bacterial stock (Ec and Sa) at 0 hr. Bacterial counts were measured at 0, 3, 6, 12, 18 and 24 hours post treatment. Duplicates followed by the trials were averaged and log transformations performed for analysis. The log10 counts of Ec and Sa were analyzed separately using the linear mixed model. Fixed effects of the model include trial, treatment, time and treatment-by-time interaction. Random effect of the model is the trial-by-treatment interaction (i.e. the vector of error term at various time points). According to the model fitting criteria, variance-covariance matrix of the error term vector was taken as unstructured for Ec and heterogenous compound symmetry for Sa. For Ec, counts at 0 hours were excluded from the model due to lack of variability. The least square means (LS Means) and its standard error (SE) are reported for each treatment group at every time point. Comparisons of EPL and ASC with PC at a given time were performed based on the two-sided test for non-zero difference with Dunnett’s adjustment for multiplicity. The level of significance was set at p < 0.05. Statistical analysis was executed via Statistical Analysis Software (SAS Version 9.4; Cary, NC, USA) PROC MIXED with option DDFM = KR.
Chapter 5 - Results

PRP Collection

The mean platelet count from the ten donor horses was $2.57 \times 10^5/\mu L$ with a range of $2.34-2.80 \times 10^5/\mu L$. Following EPL production, the samples were pooled and analyzed with a final concentration of platelets of $2.57 \times 10^6/\mu L$ and an individual horse range from $2.34-2.80 \times 10^6/\mu L$. The final EPL preparation was a ten-fold enrichment compared to the whole blood platelet count. Platelet counts are summarized in table 4.1.

<table>
<thead>
<tr>
<th>Horse #</th>
<th>Count 1</th>
<th>Count 2</th>
<th>Count $10^5/\mu L$</th>
<th>10x Conc ($10^6/\mu L$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>280</td>
<td>262</td>
<td>2.71</td>
<td>2.71</td>
</tr>
<tr>
<td>2</td>
<td>261</td>
<td>259</td>
<td>2.60</td>
<td>2.60</td>
</tr>
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<td>3</td>
<td>242</td>
<td>257</td>
<td>2.50</td>
<td>2.50</td>
</tr>
<tr>
<td>4</td>
<td>257</td>
<td>239</td>
<td>2.48</td>
<td>2.48</td>
</tr>
<tr>
<td>5</td>
<td>263</td>
<td>271</td>
<td>2.67</td>
<td>2.67</td>
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<td>6</td>
<td>235</td>
<td>234</td>
<td>2.35</td>
<td>2.35</td>
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<tr>
<td>7</td>
<td>251</td>
<td>254</td>
<td>2.53</td>
<td>2.53</td>
</tr>
<tr>
<td>8</td>
<td>280</td>
<td>280</td>
<td>2.80</td>
<td>2.80</td>
</tr>
<tr>
<td>9</td>
<td>262</td>
<td>265</td>
<td>2.64</td>
<td>2.64</td>
</tr>
<tr>
<td>10</td>
<td>235</td>
<td>251</td>
<td>2.43</td>
<td>2.43</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>2.57</td>
<td>2.57</td>
</tr>
</tbody>
</table>

Table 0.1: Individual horse platelet counts following jugular venipuncture. Two manual platelet counts were performed and averaged. Average count determined individual and pooled 10X platelet concentrations used.
Bacterial Assays

Starting bacterial concentrations ranged from $2.31 \times 10^7$ to $2.32 \times 10^7$ CFU/ml for the EcPC, Ec-ASC, and Ec-EPL groups with the log10 mean at 7.36 and SE of 0.0. The SaPC, Sa-ASC, and Sa-EPL test groups starting concentrations were $7.55 \times 10^6$ to $1.64 \times 10^7$ CFU/ml with the log 10 mean and SE at 6.93 +/- 0.14, 7.01 +/- 0.18, and 7.01 +/- 0.18 respectively. Although a 0.5 McFarland standard suspension turbidity was used for initial bacterial concentrations, our starting concentrations were below the typical equivalent number of $1.5 \times 10^8$ CFU/ml.

When compared to the control group, there were no significant differences in growth patterns in the Ec-ASC treatment groups at any time point up to 24 hours. Contrary to our hypothesis, Ec-EPL treatment groups had significantly more bacterial growth at hours 12, 18, and 24 (P <0.001) compared to the control group. The Sa-ASC showed a significant decrease in bacterial growth at hours 18 (P=0.034) and 24 (P=0.008) with the Sa-EPL showing a significant decrease only at hour 6 (P=0.006). Table 4.2 and 4.3 summarize the growth patterns of Ec and Sa treatment groups.
Table 0.2: Mean *Escherichia coli* CFU levels, calculated from OD readings and a standardized growth curve, over a 24-hour time period in broth cultures treated with equine origin platelet lysate (EPL) and adipose derived mesenchymal stromal cells (ASC) compared to *E. coli* alone (positive control, PC). † indicates statistically significant increase in bacterial growth compared to PC.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Ec-EPL</th>
<th></th>
<th>Ec-ASC</th>
<th></th>
<th>PC</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Log&lt;sub&gt;10&lt;/sub&gt;(CFU/ml)</td>
<td>LS Mean +/- S.E.</td>
<td>Mean CFU/ml</td>
<td>Log&lt;sub&gt;10&lt;/sub&gt;(CFU/ml)</td>
<td>LS Mean +/- S.E.</td>
<td>Mean CFU/ml</td>
</tr>
<tr>
<td>0</td>
<td>7.36 +/- 0.00</td>
<td>2.31E+07</td>
<td></td>
<td>7.36 +/- 0.00</td>
<td>2.31E+07</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>7.40 +/- 0.03</td>
<td>2.33E+07</td>
<td></td>
<td>7.35 +/- 0.03</td>
<td>2.32E+07</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>8.41 +/- 0.20</td>
<td>1.88E+08</td>
<td></td>
<td>8.37 +/- 0.20</td>
<td>2.45E+08</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>8.85 +/- 0.03†</td>
<td>6.66E+08†</td>
<td></td>
<td>8.49 +/- 0.03</td>
<td>3.35E+08</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>8.88 +/- 0.05†</td>
<td>7.27E+08†</td>
<td></td>
<td>8.50 +/- 0.04</td>
<td>3.38E+08</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>8.90 +/- 0.03†</td>
<td>7.39E+08†</td>
<td></td>
<td>8.56 +/- 0.03</td>
<td>3.82E+08</td>
<td></td>
</tr>
</tbody>
</table>

Table 0.3: Mean *Staphylococcus aureus* CFU levels, calculated from OD readings and a standardized growth curve, over a 24-hour time period in broth cultures treated with equine origin platelet lysate (EPL) and adipose derived mesenchymal stromal cells (ASC) compared to *E. coli* alone (positive control, PC). * indicates statistically significant decrease in bacterial growth compared to PC.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Sa-EPL</th>
<th></th>
<th>Sa-ASC</th>
<th></th>
<th>PC</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log&lt;sub&gt;10&lt;/sub&gt;(CFU/ml)</td>
<td>LS Mean +/- S.E.</td>
<td>Mean CFU/ml</td>
<td>Log&lt;sub&gt;10&lt;/sub&gt;(CFU/ml)</td>
<td>LS Mean +/- S.E.</td>
<td>Mean CFU/ml</td>
</tr>
<tr>
<td>0</td>
<td>6.97 +/- 0.00</td>
<td>1.08E+07</td>
<td></td>
<td>6.97 +/- 0.00</td>
<td>1.08E+07</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>6.89 +/- 0.08</td>
<td>8.53E+06</td>
<td></td>
<td>6.85 +/- 0.08</td>
<td>7.76E+06</td>
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</tr>
<tr>
<td>6</td>
<td>6.90 +/- 0.21</td>
<td>1.33E+07*</td>
<td></td>
<td>7.42 +/- 0.21</td>
<td>3.37E+07</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>8.64 +/- 0.15</td>
<td>5.17E+08</td>
<td></td>
<td>8.28 +/- 0.15</td>
<td>2.93E+08</td>
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<tr>
<td>18</td>
<td>8.69 +/- 0.11</td>
<td>5.92E+08</td>
<td></td>
<td>8.32 +/- 0.11</td>
<td>3.10E+08</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>8.72 +/- 0.10</td>
<td>5.97E+08</td>
<td></td>
<td>8.33 +/- 0.10</td>
<td>3.16E+08</td>
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</tbody>
</table>
Chapter 6 - Discussion

This study showed that EPL and ASCs had in vitro antimicrobial activity against *S. aureus* but not *E. coli*. The results of the current study could differ from other antimicrobial studies due to multiple factors including, pH of the assay, dose used, use of non-fractionated EPL, and the experimental design used in this study. Isolated human platelet AMPs have been shown to be generally most potent at an acidic pH and have dose dependent antimicrobial effects (59). Studies have suggested that platelet AMPs in an acidic environment may potentiate the antimicrobial effect of leukocytes through nonoxidative mechanisms (63,73). The pH of the treatment assays was not measured or standardized during this study and only one concentration of both EPL and ASCs was used. During the pilot stage, EPL was used to find minimum inhibitory concentrations (MIC) for both *E. coli* and *S. aureus*. Our experimental results mirrored the pilot study that EPL had a bacteriostatic effect against gram positive Sa and the Ec-EPL group showing no growth inhibition. Bacteriostatic effects of human platelet products have been shown in many reports (24,74). The use of non-fractionated EPL product could also have an effect, both AMPs as well as peptides favoring bacterial growth could be present in the assays. Purified human platelet proteins have been shown to have a range a bactericidal effects with thrombocidin-1 and thrombocidin-2 eliminating growth of *S. aureus* and *Bacillus subtilis* while *E. coli* was less susceptible (62). This same study investigated other platelet proteins that did not have a bactericidal effect. It has been shown that at least seven human platelet AMPs had antimicrobial effects of varying degrees against not only bacteria but also fungi (24). Further investigation into equine platelet proteins by liquid chromatography-mass spectrometry, their concentrations in the EPL preparation, and individual microbial effects would be of interest.
Similarly, human MSCs from various sources including adipose tissue, bone marrow, umbilical cord, and placenta have been shown to express four distinct AMPs; cathelicidin LL-37, human b-defensin-2, hepcidin, and lipocalin-2 (18). Using reverse transcriptase-polymerase chain reaction increased concentrations of AMPs cathelicidin, cystatin C, elafin, and lipocalin-2 were seen in peripheral blood MSCs and when challenged with anti-AMP antibodies (25). *E. coli* and *S. aureus* growth was increased when the stomal cells were preconditions with anti-AMP antibodies (25). In the same study, additional bioactive factors affecting bacterial membrane depolarization were found with varying effects on *E. coli* and *S. aureus* (25). Many variables can impact the phenotype of MSCs including tissue origin and preconditioning (activation or priming) of the cells prior to use (28,31,36). Previous research indicates that preconditioning is an effective way to induce a stronger antimicrobial phenotype (28). MSC antimicrobial mechanisms appear to be dependent on AMP secretion, stimulation of the host immune system, and direct phagocytosis. These factors need further investigation to know the impact on both the direct and indirect mechanisms (28). Although the mode of bacterial inhibition was not investigated in this study, we were able to confirm that ASCs inhibit the growth of *S. aureus* at multiple time points.

This study was performed in vitro and may not represent clinical results. In the current study, growth curves and OD reading were measured as opposed to absolute CFU/ml counts. The OD readings are measurements of growth inhibition and include replicating and dead bacteria. Further investigations in our laboratory have shown a significant bacterial growth suppression in *E. coli* and 3 other bacteria by using absolute CFU counts (results not shown).
Other studies including additional concentrations of EPL and ASCs with a variety of other gram-positive and gram-negative bacteria are warranted. Investigating concentrations of AMPs derived from both EPL and ASCs would be beneficial in future studies. Many studies involving platelet products and MSCs have indicated their potential for use in treating infections. Further research in both human and veterinary medicine is necessary prior to the replacement of conventional antibiotics or combination use with antibiotics. Expansion of focus to additional veterinary species, including small animals and livestock is justified. Efforts to decrease overall antibiotic use and AMR would be a benefit for not only veterinary medicine but human-animal interactions under the “One Health” initiative.
References


39. Khatri M, Richardson LA, Meulia T. Mesenchymal stem cell-derived extracellular


Available from:
https://link.gale.com/apps/doc/A187563297/AONE?u=ksu&sid=AONE&xid=62a04a02


Appendix A- Additional Assay Graphs

Figure 6: Combined mean *Escherichia coli* CFU/ml levels illustrated as a line graph, calculated from OD<sub>600</sub> readings and a standardized growth curve, over a 24-hour time period in broth cultures treated with equine origin platelet lysate (EPL) and adipose derived mesenchymal stromal cells (ASC) compared to E. coli alone (positive control, PC). † indicates significantly increased bacterial growth compared to controls.
Figure 7: Combined mean *Escherichia coli* CFU/ml levels illustrated as a bar graph, calculated from OD<sub>600</sub> readings and a standardized growth curve, over a 24-hour time period in broth cultures treated with equine origin platelet lysate (EPL) and adipose derived mesenchymal stromal cells (ASC) compared to E. coli alone (positive control, PC). † indicates significantly increased bacterial growth compared to controls.
Figure 8: Combined mean *Staphylococcus aureus* CFU/ml levels illustrated as a line graph, calculated from OD$_{600}$ readings and a standardized growth curve, over a 24-hour time period in broth cultures treated with equine origin platelet lysate (EPL) and adipose derived mesenchymal stromal cells (ASC) compared to E. coli alone (positive control, PC). * indicates significantly decreased bacterial growth ASC, + indicates significantly decreased bacterial growth EPL compared to controls.
Figure 9: Combined mean *Staphylococcus aureus* CFU/ml levels illustrated as a bar graph, calculated from OD$_{600}$ readings and a standardized growth curve, over a 24-hour time period in broth cultures treated with equine origin platelet lysate (EPL) and adipose derived mesenchymal stromal cells (ASC) compared to E. coli alone (positive control, PC). * indicates significantly decreased bacterial growth ASC, + indicates significantly decreased bacterial growth EPL compared to controls.