Comparison of Platelet Counting Technologies in Equine Platelet Concentrates

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ABSTRACT

Platelet rich plasma (PRP) is a popular autologous biological therapy used for the treatment of various equine ailments, including tendon and ligament injuries, osteoarthritis, and cutaneous wounds. A number of commercial products are available for producing PRP, each generating a slightly different product. Variations in platelet numbers and white blood cell (WBC) counts are believed to be the most critical variables, as they are directly related to concentrations of growth factors and inflammatory cytokines. Accurate documentation of platelet numbers is essential for prospective evaluation of clinical outcomes, but can be problematic in platelet concentrates depending on the counting method employed. The objectives of this study were to compare the performance of four platelet counting technologies in equine platelet concentrates and to evaluate the ability of the Magellan PRP system to concentrate equine platelets. We hypothesized that there would be no differences in platelet counts among the four counting technologies and that the Magellan system would generate platelet concentrations greater than 500,000/μL. Citrated whole blood was collected from 32 horses and platelet, WBC, and red blood cell concentrations were measured using a commercial hematology analyzer (Advia 2120) prior to preparation of PRP using the Magellan system. Platelets were quantified in individual identical aliquots of equine PRP produced by the Magellan system (n=32) using three different technologies: optical scatter (Advia 2120), impedance (CellDyn 3700), and hand count using direct microscopy (Thrombo-TIC). An immunofluorescent counting method was performed on a subset of 15 of

the 32 samples using a mouse monoclonal anti-sheep antibody against integrin alpha $\alpha_{IIb}\beta_3$ (anti-CD41/CD61) and a fluorescent secondary antibody. Measured platelet concentrations were compared using Passing and Bablok regression analyses and mixed model ANOVA. The Magellan PRP system yielded mean ($\pm$ SD) platelet and WBC counts of $893,090 \pm 226,610/\mu L$ and $35,806 \pm 9,971/\mu L$, respectively. Platelet counts generated by optical scatter were consistently higher than those generated by impedance. Systematic and proportional biases were observed between these two automated methods. No bias (systematic or proportional) was observed among any of the other counting methods. Despite the bias detected between the two automated systems, there were no significant differences on average among the four counting methods evaluated, based on the ANOVA. All four platelet counting methods tested are therefore suitable for quantifying platelets in equine PRP for clinical applications. The Magellan PRP system consistently generated desirably high platelet concentrations as well as higher than expected WBC concentrations. The high platelet concentrations served as a good test medium for the study; however, the concurrent high WBC counts may be undesirable for selected orthopedic applications.

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I dedicate this thesis to my parents, James and Kathleen O'Shea, and my sister Colleen, in gratitude for their constant love, support, and prayers, as well as to all of the horses that have inspired me through the years and all of the people in my life who formally and informally guided and nourished my imagination and ambition.

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Introduction

<u>Thesis Organization</u>

This thesis is presented in a format that contains a journal publication as the central portion of the document (Chapter 2). The publication is entitled "Comparison of platelet counting technologies in equine platelet concentrates" and contains its own introduction, materials and methods, results, discussion, and references. The following introduction provides a brief overview of the research topic. The literature review provides a summary of pertinent literature and background information on equine platelet rich plasma and platelet counting methodologies.

<u>Introduction</u>

Since the identification of platelet rich plasma (PRP) as a potent delivery medium for autologous growth factors in the 1990s,[1,2] clinical applications for PRP have expanded to include human dental, orofacial, and cardiothoracic surgery and human and veterinary sports medicine, orthopedics, and wound healing.[3-13] The efficacy of PRP in enhancing soft tissue healing and bone formation has been attributed to the release of growth factors from platelet alpha granules, including platelet derived growth factor (PDGF), transforming growth factor–β (TGF-β), insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF).[14] In equine patients, PRP has been used to enhance wound healing and improve the quality of healed tendon and ligaments.[15-18]

Regeneration of injured tissue is a multifactorial process, relying on the spatial and temporal interaction of cells, growth factors, and matrix scaffold. Both growth factors and

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matrix scaffold, in the form a fibrin clot, are provided by PRP. The goal of regenerative therapies is to restore normal structural architecture and biomechanical function to an injured tissue.[19] Ideally, regenerative therapies yield tissue that is identical to the original tissue, rather than inferior scar tissue that is prone to re-injury. Regenerative therapies rely on enhancing the body's natural healing processes. It follows that because the exact mechanisms of natural healing processes remain elusive, the mechanisms of action of specific regenerative therapies are also still being elucidated.

Interest in regenerative medicine is rapidly growing in human and veterinary medicine, especially in orthopedics and general clinical practice. The use of many regenerative therapies in clinical cases has outstripped supporting research in large part due to the perceived inherent safety of these autologous products. Traditional treatment protocols for tendon and ligament injuries focus on rest, anti-inflammatories, and controlled exercise. Regenerative medicine treatments can easily be combined with these conventional approaches in sports medicine with the hopes of achieving superior results compared to rest and controlled exercise alone.

It is imperative as regenerative medicine moves forward in both research and clinical applications, that what is actually contained in regenerative therapeutic products such as PRP are carefully defined and investigated. Only then can the effects of various treatment protocols be properly compared and evaluated. Although the art of medicine will continue to require patient-specific variations in treatments, standardization of terminology and benchmark values is required for clinicians to make informed decisions in patient care and for researchers to make meaningful comparisons. A previous study in our laboratory comparing various PRP preparation techniques[20] measured lower than anticipated platelet concentrations in equine PRP.

This discrepancy led us to question the accuracy and precision of counting equine platelets in these concentrates and to design a project to further investigate if our counts were reliable.

The Master's project presented here served two purposes. The first was to evaluate several common counting methods for equine platelets in concentrate. Two commercial hematology analyzers were available in the Virginia-Maryland Regional College of Veterinary Medicine Clinical Pathology Laboratory. These machines use different technologies to measure platelets. The commercial machines were also compared to a traditional hand-counting technique and a novel fluorescent-labeled antibody counting method via flow cytometry.

The second purpose of this Master's project was to characterize the PRP product generated by a commercial system that we were interested in using clinically. Because an equine PRP product with consistently high platelet concentrations was required to achieve the first goal of the project, the two objectives proved synergistic. The Magellan Autologous Platelet Separator System is a closed, fully automated PRP production system relying on centrifugation to separate blood components. The Magellan system has previously been reported to consistently yield PRP with high platelet counts in human blood;[21] however, similar data for platelet and white blood cell (WBC) content are not available for equine blood. The equine data generated from this study will be useful to guide treatment protocols for a variety of equine problems, including orthopedic injuries and traumatic wounds.

Chapter 1: Literature Review

The use of PRP in equine sports medicine and surgery has become increasingly popular. In order to develop and enhance therapeutic protocols for diverse ailments ranging from wounds, to tendon and ligament injuries, to osteoarthritis, the complete profile of cellular constituents of individual PRP products used should be quantified and recorded. It is ultimately the growth factor profile of the product that is believed to be responsible for the augmentation of healing. Growth factor levels are in turn intimately related to the platelet and WBC content of the PRP. The following review discusses the dynamic functions of platelets and WBC in healing processes to provide a background for understanding therapeutic theories and the rationale for the study reported in Chapter 2. It also introduces several common methods used for counting platelets and the challenges encountered in accurately counting platelets in concentrates.

Platelets

Platelets, or thrombocytes, are the second most numerous circulating cells in the blood, behind erythrocytes. As more has been learned about platelet structure and function, the term thrombocyte has largely fallen out of favor. This change in nomenclature reflects an acknowledgement that platelets are actually cytoplasmic fragments rather than whole "cytes" or "cells," and that they have many functions in addition to their roles in "thrombosis." The other roles of platelets, including contributions to wound healing and immunologic defense, are important in the clinical use of PRP.

Megakaryocytes, the cells from which platelets originate, represent less than 1% of the cells within the bone marrow. Platelets are formed through a budding process from the

megakaryocytes and are then released into the circulating blood. Alpha granules and dense

bodies contained within the cytoplasm of megakaryocytes are incorporated into the newly

formed platelets, providing them with the machinery necessary to function. Megakaryocytes are

stimulated to release these small (2.5 to 3.5 μm diameter in horses),[22] anucleate cytoplasmic

fragments, or platelets, into the circulation by thrombopoietin.[23,24] Thrombopoietin levels

increase in response to decreases in platelet mass due to normal platelet turnover or platelet

depletion as a result of hemorrhage and clot formation.[23-25] Once in circulation, the life span of

equine platelets is 4 to 6 days.[24,26,27]

Platelets contain a complex network of specialized cell surface receptors and specialized

internal structures that enable them to respond to environmental stimuli and participate in

hemostasis, coagulation, and the maintenance of vascular integrity. In their unstimulated state,

mammalian platelets are discoid in shape; however, upon activation they change shape and

initially become spherical and then they begin to project thin cytoplasmic processes (filopodia)

and become highly irregular in shape.[24] These changes in shape are a result of a reorganization

of the web of microtubules and filaments contained within the cytoplasm.[24] Stimulation of

platelets results in platelet adhesion, activation, aggregation, and secretion of granules.

Specialized receptors located within the plasma membrane receive stimuli causing them to

interact with the cytoskeleton.[28] The platelet cell membrane, cytoskeleton, and cytoplasmic actin

network are designed such that reorganization following stimulus can result in clot retraction.[24,28]

In addition to the traditionally understood functions of hemorrhage control, platelets also

contribute to the less well understood roles of immunologic defenses and wound healing.[23,29] It

is some of these less well understood roles that appear to be critical to the clinical applications of

PRP. Bioactive proteins are released during platelet secretion, or degranulation. They stimulate

wound debridement and healing and attract macrophages, mesenchymal stem cells, and osteoblasts to sites of injury. In addition, these bioactive proteins orchestrate the cell-cell and cell-matrix interactions critical in tissue healing.[30]

Platelets contain three main types of storage granules; dense, alpha, and lysosomal granules.[31] Alpha granules are the most numerous and largest of the storage granules and contain proteins derived from two sources.[28] The majority of proteins within the alpha granules are produced by the parent megakaryocyte and packaged into the alpha granules prior to the platelet budding process.[14,28] Additional proteins encountered as platelets circulate in the blood are added to the alpha granules via endocytosis.[14,28] Proteins are compartmentalized in various regions of the alpha granules, allowing differential release of specific proteins during activation in response to various stimuli.[28] Human alpha granules are known to contain 284 different proteins,[31] including numerous growth factors such as PDGF, TGF-β, EGF, VEGF, IGF-1, bFGF, and hepatocyte growth factor.[14] These are all growth factors demonstrated to promote tissue healing and repair.[14]

Platelet Membrane Receptors

In order to interact with their environment and perform their various functions in hemostasis, wound healing, and immunology, platelets possess antigen receptors on their surface.[29] Cell surface antigens on platelets can be categorized as glycoproteins, glycolipids, or proteins based on their biochemical composition.[32,33] Many of these receptors span the entire platelet membrane, having both intracellular and extracellular domains.[34] This transmembrane property allows receptors to have bidirectional effects. The receptors can interact with coagulation factors and other ligands extracellularly, causing a cascade of intracellular protein-

protein interactions and biochemical events. Similarly, intracellular events can cause conformational changes in the extracellular domains of the receptors, resulting in changes in their affinity for various ligands.[29,34,35]

The most prevalent glycoprotein complex receptor on the surface of platelets is integrin $\alpha_{IIb}\beta_3$ (also known as glycoprotein (GP) IIb/IIIa or CD41/CD61).[35,36] Human platelets express 50,000-80,000 copies of integrin $\alpha_{IIb}\beta_3$ per platelet.[35,36] The receptor was first discovered in the mid-1970s when its deficiency or abnormality was found to cause Glanzmann thrombasthenia, a rare heritable coagulopathy.[35] The GPIIb/IIIa complex is formed by calcium-dependent, non-covalent association of the α_{IIb} chain (CD41) with the β_3 subunit (CD61).[35-37] Mice immunized with human platelets readily produce monoclonal antibodies to GPIIb and GPIIIa and in fact the first reported monoclonal antibody to human platelets was one that specifically bound to GPIIb.[35]

Increased knowledge of the integrin superfamily of cell surface molecules has led to the new nomenclature, integrin $\alpha_{IIb}\beta_3$, describing the α and β chains that assemble as a heterodimer.[38] Integrin $\alpha_{IIb}\beta_3$ is critical for its role in binding fibrinogen, which causes platelet aggregation to form a clot.[28,33,35,36,39] The complex also acts as a receptor for von Willebrand factor, fibronectin, and vitronectin during platelet stimulation.[35-37] Although described and characterized primarily in human platelets,[32,40-42] the major platelet glycoproteins, including integrin $\alpha_{IIb}\beta_3$, are believed to be well conserved among different mammalian species, including the horse.[33,43-45] Integrin $\alpha_{IIb}\beta_3$ is the only integrin expressed uniquely on platelets.[35,36,38] This specificity to platelets enables the utilization of antibodies against the CD41/CD61 subunits (which bind to the α and β chains of integrin $\alpha_{IIb}\beta_3$, respectively) in human hematology to label platelets for counting and characterization.[46,47]

Platelet Rich Plasma (PRP)

The term "platelet rich plasma" or "PRP" refers to blood products used in human and veterinary medicine that contain higher concentrations of platelets than whole blood. Platelet rich plasma delivers a milieu of growth factors at physiologic ratios to the site of application. Growth factors released from platelet alpha granules, especially PDGF, TGF-β, and VEGF, have been shown to play critical roles in angiogenesis, cell proliferation, chemotaxis, and cell differentiation.[21,48] When applied together in PRP, growth factors have synergistic beneficial effects giving them an advantage over individual recombinant growth factor therapies. The specifics of this synergism and its effects are the subject of ongoing investigations. Whether growth factors are released fully by the activation of platelets during treatment (either by exposure to endogenous collagen or by the exogenous addition of thrombin or calcium chloride) is a topic of current research.[49,50] It is generally accepted that higher platelet numbers in PRP correspond to higher levels of available growth factors for enhancement of tissue healing.[1,49,51-56]

There is a significant amount of variation amongst the many autologous equine blood products described as PRP. Platelet concentrations in PRP can be described either in terms of the relative concentration with respect to whole blood baseline (fold change) or as the absolute numbers of platelets per unit volume. Fold change in platelet concentration is useful for comparison of PRP processing machines and methods; however, enumeration of absolute platelet numbers is more important for clinical applications as it better reflects growth factor concentrations. An individual PRP sample could have a high degree of platelet concentration relative to whole blood, but if the baseline platelet concentration in whole blood is low, then the absolute platelet count in PRP could still be low. The most desirable WBC and red blood cell content and their ratios relative to platelet content, along with the threshold numbers or

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concentrations of platelets found within PRP products have been topics of heated debate among clinicians and researchers.[57] The terms "leukocyte and platelet rich plasma" or "L-PRP" and "pure platelet rich plasma" or "P-PRP" have been proposed to acknowledge the contribution of WBC concentration to the products.[58-61]

The currently accepted benchmark for therapeutic PRP in human medicine is a platelet concentration of 1 million/µL, representing a 4 to 5 fold increase from human whole blood baseline.[4,8,49] It is likely however, that the ideal numbers of platelets (and white and red blood cells) in PRP are specific to different clinical applications. These optimal concentrations have yet to be elucidated. Research in human medicine has demonstrated dose-dependent responses to platelet numbers in several commonly used cell lines, including osteoblasts,[1,55] fibroblasts,[53] tenocytes,[54] endothelial cells,[51] and mesenchymal stem cells.[52] An in vivo study investigating the effects of PRP on bone regeneration in rabbits demonstrated the expected dose-dependent positive effects with increasing platelet number up to a certain point.[4] Increasing platelet concentration increased proliferation of osteoblasts up to a platelet concentration of 1 million/µL; however, with increasing platelet concentrations above 1 million/µL, a plateau was reached followed by a decline in osteoblast proliferation.[4] In vitro research on equine tendon explants did not demonstrate a dose-dependent response in expression of tendon matrix molecules to platelet concentration in PRP.[62] Unlike in rabbit osteoblasts, no deleterious or suppressive effects of high platelet concentrations were observed.[62] Much research remains to be done to address the question of optimal platelet numbers in platelet concentrates with attention to the fact that species differences may exist in addition to the specific clinical application. A lack of knowledge as to the optimal concentrations of WBC within PRP is an equally important area of research concentration.

White Blood Cell Content

White blood cells play an active role in tissue healing by releasing cytokines and growth factors and playing a role in stimulating platelet degranulation.[21,63,64] In addition to producing beneficial factors, WBC can also produce mediators such as neutral proteases and acid hydrolases that promote inflammation and tissue catabolism.[65] The neutrophil component of leukocyte-rich PRP may increase local inflammation by releasing pro-inflammatory cytokines and therefore be detrimental to tissue healing.[65] Inflammation may be especially detrimental in the intra-articular environment.[3,21,56,57,62] Products released from WBC during the storage of human allogeneic platelet concentrates have been implicated in transfusion reactions following intravenous administration.[66,67] Although this form of adverse reaction is not applicable to the use of fresh PRP, it may be important to bear in mind when using frozen platelet lysates of leukocyte-rich PRP. In human PRP products, WBC have a strong positive correlation with increased catabolic signaling molecules that can facilitate breakdown of the extracellular matrix.[68] Similarly, catabolic gene expression was positively correlated with WBC concentration in equine tendon and ligament explants treated with PRP in vitro.[56] Therefore, several authors recommend the use of "pure-" or "leukocyte-reduced" PRP produced by removing WBC from PRP via filtration to avoid complications like scar tissue formation due to prolonged inflammation.[56,57,69] A maximum concentration of 3,000 WBC/μL for the treatment of tendon lesions and intra-articular use has been advocated.[21,56]

While in vitro research indicates a catabolic effect of WBC in PRP, in vivo studies and case reports have failed to demonstrate an excessive inflammatory response associated with leukocyte-rich PRP. Some scientists even tout the potential beneficial effects of WBC in PRP

products. For example, there is evidence to support an antimicrobial and immunomodulatory role of WBC in PRP.[21,63,70,71] These infection-fighting effects might be especially beneficial in the treatment of cutaneous wounds where debridement of necrotic and contaminated tissue is desirable. Additionally, significant increases in concentrations of PDGF and VEGF have been documented in leukocyte-rich PRP compared to leukocyte-poor PRP and are attributed to WBC content.[21,64] Increased growth factor concentrations demonstrated in leukocyte-rich PRP are believed to be due to both stimulation of platelets by WBC and production and release of growth factors from the WBC themselves.[21,64]

Although absolute platelet and WBC numbers can be analyzed independently, it may be the ratio of platelets to WBC that optimizes positive effects of PRP treatment. If a low WBC concentration in PRP is indeed desirable, the question arose as to whether higher numbers of platelets compared to WBC could counteract the negative catabolic or inflammatory effects of WBC.[68] If there are enough platelets present to counteract the catabolic processes promoted by WBC, a balance could be achieved. This theory is supported by knowledge that TGF-β has an inhibitory and down-regulatory effect on matrix metalloproteinases and the activity of the catabolic cytokine interleukin–1β is modulated by TGF-β.[56,62,68] In a study examining gene expression in tendon explants, maintenance of an increased ratio of platelets to WBC failed to compensate for the increase in catabolic gene expression in tendon explants caused by elevated WBC numbers.[57]

PRP Processing

The processing method used to produce PRP, concentrating platelets in a small volume of plasma, can have a substantial impact on the cellular content of the product.[20,21,72-77] Numerous

commercial devices and kits and handcrafted protocols are available for separation of platelets to

make PRP.[3,20,77] The devices and kits are largely patient-side, point-of-care methods that

minimize the time from blood collection to PRP injection or application. Commercial systems

can be automated, using a density buoy or infrared light to identify the platelet fraction, or

manual, relying on a human operator to determine the portion of separated blood to be saved as

PRP.[3] Most commercial systems use sterile disposable cartridges into which citrated blood is

collected or transferred for centrifugation to separate out the plasma, buffy coat, and red blood

cell fractions.[3]   Following centrifugation, the highest concentration of platelets is found in and

around the buffy coat;[3] the plasma directly above the buffy coat is termed PRP and the plasma

above the PRP is referred to as platelet poor plasma. Therefore, in an effort to retrieve the

highest number of platelets, especially in horses because of their low normal whole blood

platelet concentration, WBC are often concentrated concurrently.[3]

Magellan Platelet Separator System

The Magellan Platelet Separator System (Fig. 1.1) is an easy-to-use, fully automated,

closed centrifugation system with a sterile double-chambered disposable cartridge. The

Magellan system is designed to provide a relatively compact but highly efficient and

reproducible PRP preparation system [3,21] and offers flexibility in product output through

programming produce different final volumes of PRP. An infrared light is used to differentiate

the various blood fractions and the user can select a desired output volume of PRP ranging from

3mL to 10mL from a 60mL syringe of citrated blood.[3] The 60mL and 10mL syringe pump in the

Magellan system control injection and aspiration of the biologics, respectively as the centrifuge

is running. If larger volumes of PRP are desired, the disposable chamber may be reused for as

many as three cycles with the same patient's blood.[3] This high degree of automation offers improved consistency of product output by minimizing human variation and error in the production process.[78] Some PRP production systems require the biologic sample to be transferred from one syringe or vessel to another, introducing the risk of exposure of the operator to potentially pathogenic material as well as exposing the product to environmental contaminants. While this risk of exposure is more critical in human blood processing,[78] it is avoided in the Magellan system due to the closed design.
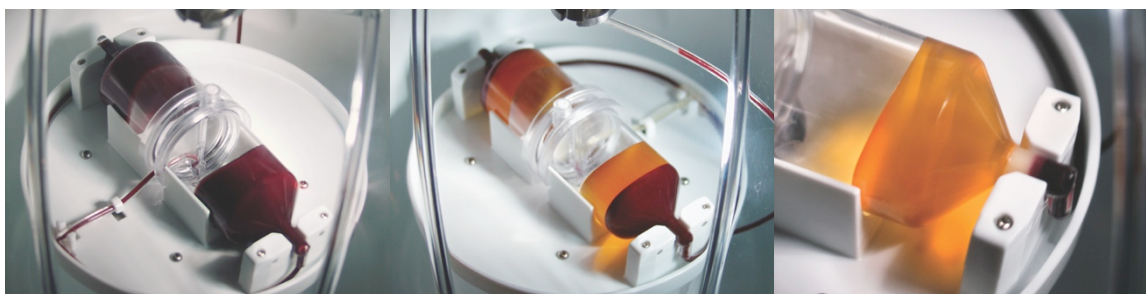
Figure 1.1 Magellan® Platelet Separator System. The closed centrifuge machine is shown in the top image and a time-lapse series of images during PRP processing as the red blood cells are removed is shown at the bottom.   During centrifugation, the red blood cells are aspirated from the sterile double-barrel disposable cartridge and the end-point for aspiration and separation of blood fractions is determined using an infrared sensor at the neck of the cartridge. The amber-colored PRP left in the disposable is then aspirated into a separate syringe.

Top image from Sutter WW: Autologous cell-based therapy for tendon and ligament injuries. *Clin Tech Equine Pract* 2007;6:198-208. Used under fair use.

Bottom three images courtesy of Arteriocyte Medical Systems, Magellan® Platelet Separator System.

The Magellan Platelet Separator System was selected for use in this project because of the perceived benefits of an automated system as well as available evidence in the human literature that the Magellan system produces PRP with consistently high platelet levels.[21] A PRP product with high platelet concentrations was needed to rigorously test and compare the various platelet counting technologies of interest and is desirable for clinical applications. Human data indicate that the Magellan system produces leukocyte-rich PRP, concentrating WBC in the PRP two fold compared to whole blood.[21] When programmed to produce 6mL of PRP from 26mL of whole blood in 4mL acid citrate dextrose-A (ACD-A) the Magellan system demonstrated a high "platelet capture efficiency" of 65.5%, meaning that it could isolate high numbers of platelets from even small initial volumes of whole blood.[21] The Magellan system run according to manufacturer's instructions at 1200g for 17 minutes consistently yielded high platelet concentrations with an average of 780.2 ± 246.5 x 10^3 platelets/μL, representing a 2.8 fold increase over whole blood.[21] These platelet levels correlated with high levels of available PDGF, TGF-β, and VEGF in the PRP.[21] Equine-specific data for characteristics of the PRP produced using the Magellan system were unavailable prior to the completion of this project. Therefore this project served to collect data on equine PRP produced using the Magellan system and make this information available to assess the utility of the Magellan system for equine cases.

Clinical Use of Equine PRP

As is the case in human medicine, PRP has been applied to a wide variety of clinical applications in equine medicine and surgery, with most centering on sports medicine and orthopedics. Anecdotal reports have continued to indicate positive responses to PRP therapy; however, controlled case studies and in vivo research are needed to provide additional data

regarding its efficacy. The majority of equine in vitro studies have focused on tendons and

ligaments, with additional interest in wound healing and intra-articular therapy.

PRP has been shown to have an anabolic effect on equine superficial digital flexor tendon

explants, increasing expression of extracellular matrix molecules with no increase in the

catabolic molecules matrix metalloproteinase–3 and matrix metalloproteinase–13.[62,79] Parallel in

vitro studies were performed using equine suspensory ligament explants, demonstrating similar

effects on gene expression, but less profound anabolic effects compared to tendon explants.[55,78,79]

In vitro a greater response was seen to treatment with acellular bone marrow than to PRP in

suspensory ligament explants.[79] In a clinical setting, however, production of PRP is less invasive

than bone marrow aspirate and it is unknown whether bone marrow aspirate is superior to PRP in

the in vivo treatment of desmitis.

A small clinical case series of 9 Standardbred racehorses combined a single intralesional

PRP injection with a controlled exercise program for midbody suspensory ligament desmitis.[17]

All 9 horses returned to racing, with a median time to return to racing of 32 weeks.[17] A case

series of 5 horses (2 with superficial digital flexor tendon lesions and 3 with proximal desmitis of

the suspensory ligament) showed significant clinical improvement in all cases following PRP

treatment, with all horses returning to their previous levels of performance and no incidence of

re-injury.[18] There was significant improvement in the ultrasonographic appearance of the

superficial digital flexor tendon lesions, but not in the ultrasonographic appearance of the

proximal suspensory ligaments.[18] This difference in resolution of ultrasonographic signs may or

may not be clinically significant and may be due to the chronic nature of the proximal

suspensory ligament changes and the characteristic disruption of fiber pattern rather than the

acute hypoechoic core lesions seen in the tendinopathy cases.[18]

An in vivo experimental study using a single PRP treatment in a mechanically-induced model of superficial digital flexor injury demonstrated that healed tendons treated with PRP had a higher elastic modulus and strength at failure than the control group.[80] Histologically, the PRP-treated tendons demonstrated increased cell density and vascularity compared to placebo-treated controls.[80] These histological improvements, in addition to the finding that DNA, glycosaminoglycan, and collagen levels were higher in PRP-treated tendons, indicated that PRP increased metabolic activity within the tendon lesions through an increase in cell proliferation and migration along with enhanced neovascularization.[80,81] Increased neovascularization in the PRP-treated group was implicated as a possible mechanism for the long lasting effects of a single intratendinous PRP injection.[81]

Although widely reported in human medicine, intra-articular application of PRP in horses has been less popular to date, perhaps due to concern about the WBC content of PRP causing inflammatory joint flares. However, when it is used, PRP has been purported to decrease pain and synovial effusion in arthritic equine joints.[82,83] It has been applied as an intra-articular therapy for osteoarthritis and subchondral cystic lesions in horses.[82-87] Both joint effusion and lameness scores improved following intra-articular PRP treatment of osteoarthritis in a variety of equine clinical cases.[87] Textor et al. compared growth factor and cytokine concentrations synovial fluid cytologic response, and clinical signs following injection of normal fetlock joints with an unactivated, bovine thrombin-activated, and $CaCl_2$-activated PRP and a saline control.[84,85] While PRP activated with bovine thrombin induced an inflammatory cytokine response, PRP activated with $CaCl_2$ and PRP that was not exogenously activated induced only minimal joint inflammation similar to the saline control and was therefore determined to be safe for intra-articular use.[84,85] The platelet rich products used in these two studies were produced by

a gravity filtration system and contained mean platelet concentrations of 542 and 650 x 10^3/μL, and mean WBC concentrations of 13.1 and 14.8 x 10^3/μL.[84,85] It should be noted that the WBC concentrations in these studies were well above the previously recommended maximum cut-off value of 3,000 WBC/μL, yet no adverse effects were observed in these normal joints.[84,85]

By promoting formation of more mature collagen, PRP has been shown to improve healing of chronic, non-healing wounds in human patients.[9] It is believed that wounds treated with PRP are less likely to develop a prolonged inflammatory state associated with chronic non-healing wounds and scarring.[15] Healing of cutaneous wounds, especially on the distal limbs of horses, can be improved following topical application of PRP.[15] More rapid epithelial differentiation and improved organization of dermal collagen orientation was found in PRP-treated wounds compared to controls.[15,16] While PRP application to small granulating equine distal limb wounds promoted excessive development of granulation tissue in one study,[88] its use may be indicated in large wounds with massive tissue loss or chronic wounds that would benefit from a fresh source of growth factors.[88]

Prior to determining optimal concentrations or numbers of platelets for clinical use and therapeutic efficacy in horses, it is critical to first achieve reliable platelet counts. The ability to accurately count platelets merits further investigation because platelet enumeration in general, and in platelet concentrates in particular, is fraught with multiple challenges.

Platelet Enumeration

The counting of blood cells has evolved tremendously in the last century.[89] There are currently four main categories of counting technologies for enumerating platelets: manual microscopy, laser-induced light scatter, electrical impedance, and fluorescent antibody flow

cytometry.[90] Historically, direct microscopic platelet counting methods were used as the gold standard against which automated hematology analyzers were calibrated and gated. However, direct hand counts using a Neubauer hemocytometer (Fig. 1.2) are time-consuming and subject to a great deal of human error. [38,90] Most commercially available hematology analyzers rely on optical scatter, electrical impedance, or a combination of the two technologies. A new reference method, described below, was accepted as the gold standard in 2001 and can now be used to calibrate the automated counting machines.[38,47]

Manual microscopy (Fig. 1.2) is a direct counting method that involves diluting the blood product with ammonium oxalate and counting cells in a hemocytometer. The diluent, 1% ammonium oxalate in distilled water, serves to lyse red blood cells but leave WBC and platelets intact.[91] The removal of the millions of red blood cells from the background of the microscopic view permits easier identification of the less numerous platelets and WBC in the sample. Ammonium oxalate also reduces platelet adhesion and aggregation tendencies.[91] To perform a hand count, a known volume of the sample is loaded into a hemocytometer grid and platelets are counted under a light microscope, preferably phase-contrast as the sample is unstained.[92] This hand-counting method is labor intensive and subject to error due to debris in the hemocytometer chamber and human error in pipetting or discrimination of platelets from other particles such as debris of similar size and refractive quality.[92] Error due to chance distribution of platelets on the grids can be minimized by counting larger numbers (minimum 100), taking the average of the two hemocytometer chambers, and ensuring that the numbers of platelets counted in each area do not differ from one another by more than 10%.[92] However, the number of platelets counted using direct manual microscopy is still drastically lower (multiples of 10 versus multiples of 1000) than that counted with automated methods, and therefore manual counts are less accurate

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and precise.[23] Interobserver coefficient of variations for manual platelet counting are in the

range of 10–25% because coefficient of variations are inversely proportional to the number of
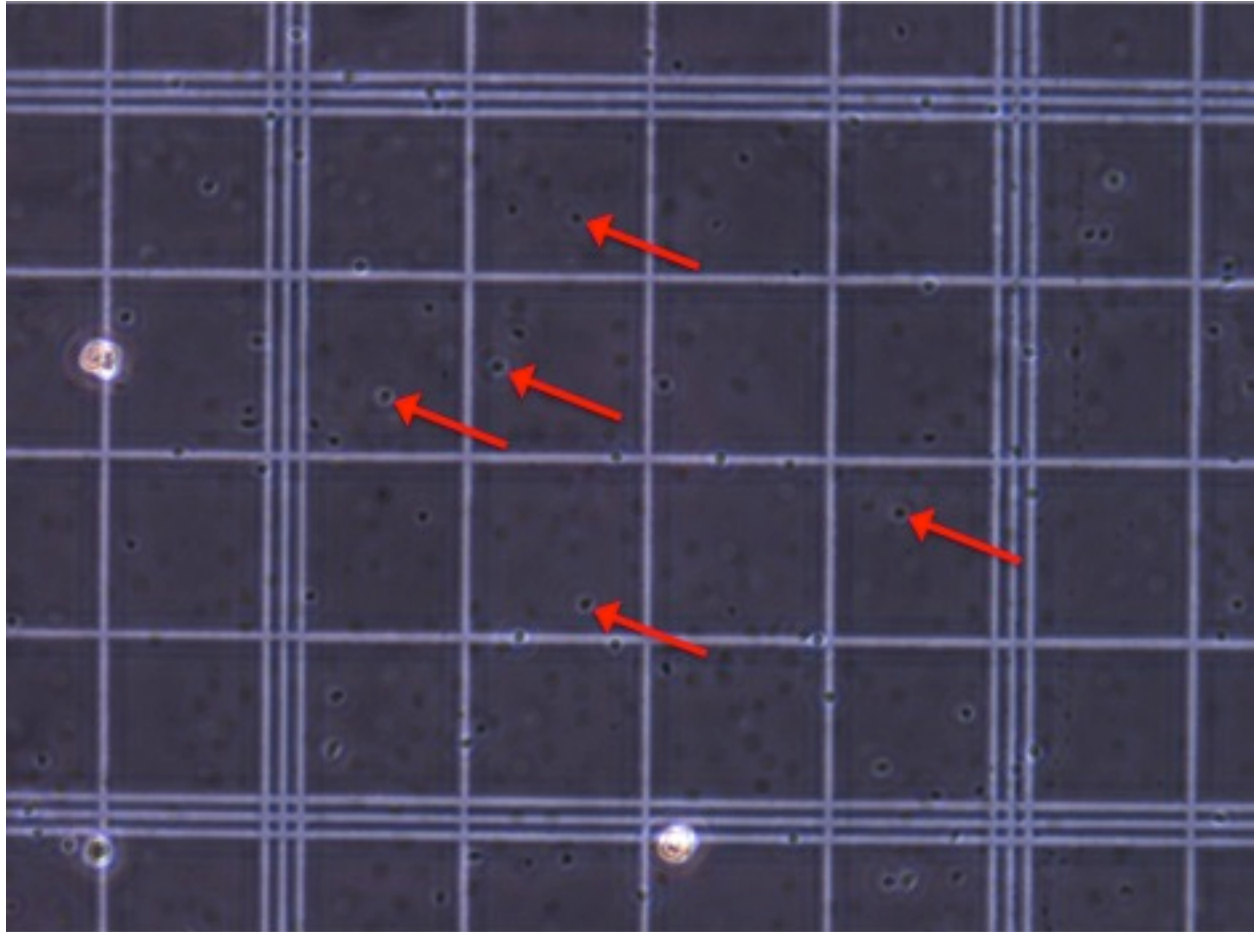
platelets counted.[46]

Figure 1.2 Phase contrast image of Neubauer hemocytometer. The image was taken at 400x

magnification of a portion of the grid from an improved Neubauer hemocytometer showing the

highly refractile platelets (several arrows added for identification) for microscopic hand

counting.

The introduction of automated hematology analyzers improved the precision of platelet counting, with typical coefficient of variations of <3% for machines utilizing the Coulter principle. The decreased coefficient of variation was the result of many more platelets counted per sample than would be possible in manual microscopy.[93] The Coulter principle was patented in the United States in 1953[90] and is based on the fact that platelets (and cells) are poor conductors of electricity. When platelets suspended in solution are passed through an aperture between two electrodes with a continuous current, a change in conductance occurs as the platelets interrupt the current. The degree of change in conductance is proportional to the interfering particle's size, thereby allowing differentiation between platelets, WBC, and red blood cells.[89] The CellDyn 3700 hematology analyzer uses impedance technology (the Coulter principle) to measure platelet numbers.

While it uses impedance only to count platelets, the CellDyn 3700 instrument uses a combination of light scatter and impedance to count WBC. Optical light scatter technology is used by the Advia 2120 hematology analyzer to enumerate platelets. One dimensional or multi-dimensional light scatter patterns can be analyzed.[89,90] As cells suspended in solution pass through a beam of light they cause diffraction (bending), refraction (change in direction), and reflection (bouncing back) of the light as the light interacts with the change in density at the cell surface.[89] The scattered light pattern can be detected and recorded for analysis.[89] Forward low-angle (1-3°) light scatter, forward high-angle light scatter (4-9°), right angle scatter (90°), and extinction (0-0.5°) offer information about cell volume, granularity, cell surface and lobularity, and cell volume, respectively.[89] More succinctly, forward light scatter indicates volume and side scatter indicates internal structure or refractive index, such as the density and character of cytoplasmic granules.[78,90] Optical scatter platelet counting methods detect and record the

scattered light patterns, analyzing them to determine the concentrations of platelets and other cells in the sample.[89,90] The Advia 2120 machine uses two dimensional analysis of low-angle light scatter (2–3°) and high-angle light scatter (5–15°) to measure platelets.[89] The high and low angle scatter values for a sample can be plotted against each other to produce a platelet scatter cytogram to differentiate platelets from large platelets, red blood cell fragments, and red blood cell ghosts (Fig. 1.3).[89] Measurement of more than one angle or dimension of light scatter increases the specificity of the counting method for identification of platelets and exclusion of debris and other blood cells from the count by providing more information about the character of particles in the solution.[90]
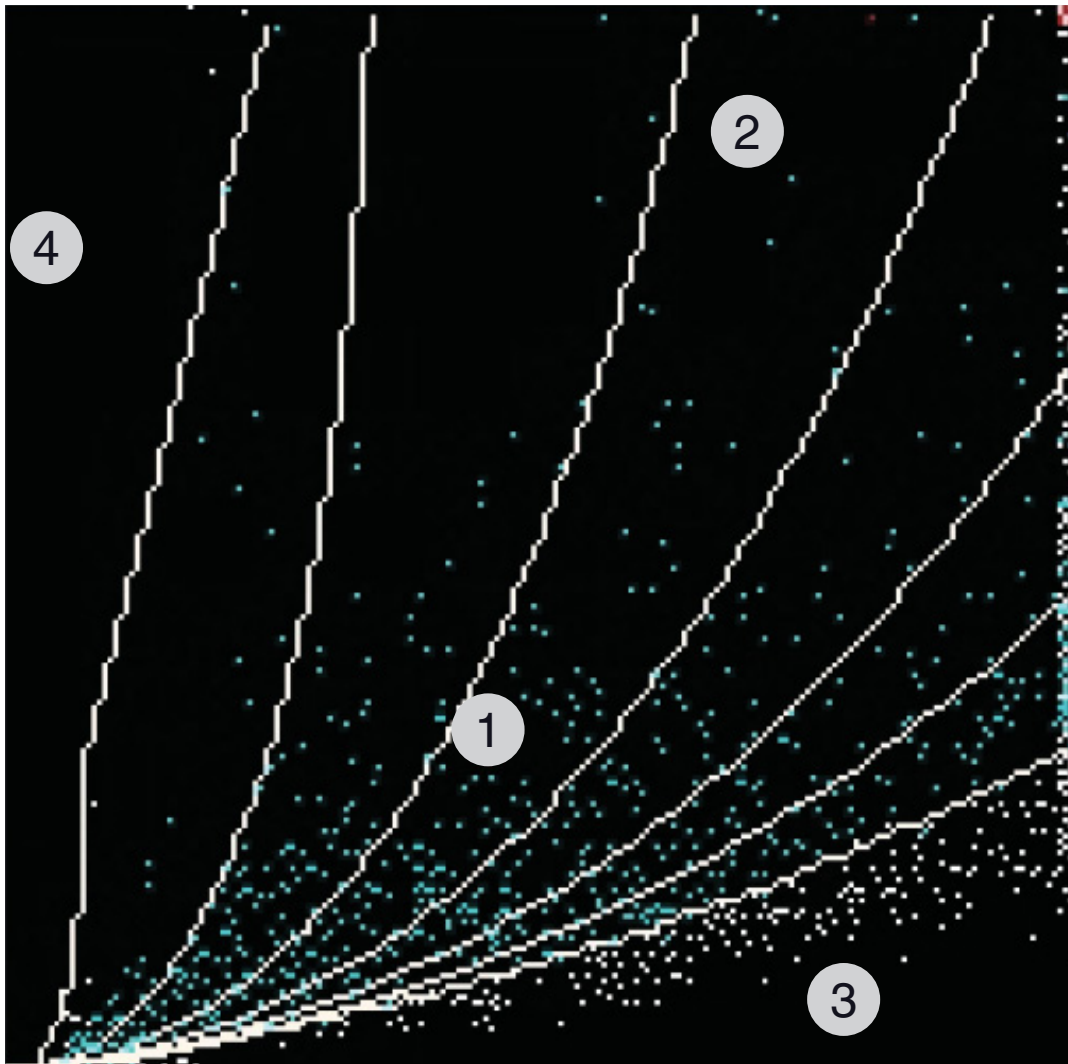
Figure 1.3 Platelet scatter cytogram. Whole blood from a healthy horse was analyzed using the Advia 2120 hematology analyzer. The high-angle (5–15 °) light scatter is plotted on the x-axis, and the low-angle (2–3 °) light scatter is plotted on the y-axis. 1= platelets; 2= large platelets; 3= red blood cell fragments; 4= red blood cell ghosts.

Image from Moritz A, Becker M: Automated Hematology Systems in Weiss DJ (ed): Schalm's Veterinary Hematology, 6th ed. (ed 6), Vol. Ames, Iowa, Blackwell, 2010, pp 1054-1066. Used under fair use.

Commercially available hematology analyzers are technically examples of flow cytometers.[94] Flow cytometry simply refers to suspending particles such as cells in a solution and passing them in single file through an electronic detection apparatus.[94] Colloquially, however, "flow cytometry" has come to mean those machines that measure fluorescence as well as light scatter. The monoclonal antibody counting technique described in Chapter 2 is an example of fluorescence flow cytometry. As previously mentioned, platelets have unique surface markers that can be used to identify them. Indirect counting via a red blood cell to CD41/CD61-postitive platelet ratio has recently been accepted by the International Council for Standardization in Haematology and the International Society of Laboratory Hematology as the gold standard method for counting platelets in human whole blood samples.[38,47] This reference method utilizes fluorescent-labeled antibodies to platelet cell membrane markers (CD41/CD61) to identify platelets within a sample.[47] The number of fluorescent platelet events counted is then compared to the number of red blood cell events and the platelet concentration is calculated using the known number of red blood cells as determined using an impedance counter.[38,47] New fully automated hematology analyzers are being developed to utilize this immunofluorescent technology for platelet counting in human blood,[90] but analogous products for veterinary use are not yet available.

Challenges in Platelet Counting

Due to their small size and propensity for adherence, platelets have been one of the most challenging blood cells to count. Coincidence events, platelet clumping, and particulate debris complicate the development of accurate and precise counting methods. Interference from the much more numerous red blood cells in whole blood samples is also problematic because

platelets constitute a relatively low percentage of the cells in whole blood. The problem of interference is compounded in equine whole blood because the normal equine platelet concentrations are lower than most other mammals. Platelet counts in horses are normally in the range of 150,000-250,000 platelets/μL.[95,96] This means that platelets constitute a relatively lower percentage of the total cells in equine whole blood than in other mammalian species[25] and are therefore subject to more counting error from interference.

Artifactual or spurious platelet counts can be obtained in blood samples for a variety of reasons. Falsely high platelet counts are referred to as pseudothrombocytosis and falsely low platelet counts are referred to as pseudothrombocytopenia. Platelet counts in PRP may be even more susceptible to error than whole blood samples. Platelet concentrate products such as PRP eliminate the majority of red blood cells that are known to cause background interference with counting in whole blood, but PRP processing may stress the platelet population, causing activation and shape changes. Because each counting method is equipped to face different challenges, many currently available hematology machines employ a combination of counting methodologies for enumeration and characterization of platelets and blood cells. Generally, thrombocytopenia is more clinically important than thrombocytosis, as platelet counts in thrombocytopenic patients are used to determine when a platelet or whole blood transfusion is indicated.[23] Therefore, commercial hematology analyzers are calibrated more closely for lower platelet concentrations than for the high concentrations encountered in PRP. Samples of PRP may need to be diluted to allow for platelet counting by certain methods; however, this dilution adds an additional level of potential operator error to the process.

Due to their physiologic functions in hemostasis, forming platelet aggregates and facilitating stabilization of clots to prevent blood loss, platelets have a tendency to adhere and

aggregate, clumping together. Platelet clumping is the most common reason for falsely low platelet concentrations measured by automated methods.[97,98] This pseudothrombocytopenia is due to the machines' inability to distinguish platelet clumps from red blood cells based on size.[97] Clumping can result when platelets are activated during blood collection and handling, due to delayed time to exposure to anticoagulant, improper mixing, and agitation during venipuncture or transfer to tubes.[23,99] The processing methods to produce PRP may also cause varying degrees of platelet activation and aggregation.[21] This not only results in artificially low platelet counts in PRP samples, but also can lead to degranulation and premature release of growth factors.[21,49,100-102] Impedance hematology analyzers have been shown to be particularly affected by platelet clumping.[103] The shape change from discoid to spherical that occurs following activation[23] can affect light scatter measurements and platelet parameters.

The presence of abnormally large platelets and coincidence events can falsely lower automated platelet counts in a manner similar to clumping,.[104] Large platelets and those adhered to themselves or other larger cells are excluded from the platelet count based on size, artificially lowering the platelet count.[104] The machine cannot distinguish between one large platelet, two platelets stuck together, or a platelet adhered to a WBC.[104] Coincidence events refer to instances when more than one particle passes through the aperture of the machine at the same time and all coincident particles are counted as one.

Pseudothrombosis can occur when cellular debris or small, fragmented, or hemolyzed red blood cells or fragmented WBC mimic the size and irregular shape of platelets, causing them to be included in the platelet count.[105] This artifact could occur with any of the automated counting methods discussed above.[105]

Verification of blood count results is an ongoing challenge, regardless of the counting technology used. Microscopic examination of blood smears allows evaluation of platelet morphology and size as well as identification of samples with platelet clumping or platelets adhering to WBC.[23,24] As pseudothrombocytopenia is a much more common complication of automated platelet counting, any flagged samples or those with decreased platelet counts should have a blood smear examined microscopically.[23] Manual platelet counts are advised when platelet counts fall outside the upper or lower limits of linearity of hematology analyzers in order to correct for these potential artifacts.[23] It should be noted however, that accuracy and precision of manual platelet counts is largely dependent on operator experience and can be maximized when performed by those who do them regularly.[23] Many commercially available hematology analyzers display histograms for the platelet data obtained in a count.[89] Most machines flag samples, displaying an error message, with potentially erroneous platelet counts based on these distribution plots differing from a normal or expected result.[89,106] The lack of a warning flag or error message is not completely reliable, as many samples with inaccurate results go undetected, while some accurate results are marked as suspicious.[89,106]

In addition to mechanical difficulties in counting, the type of anticoagulant used when collecting blood can affect platelet parameters including shape, mean platelet volume, mean platelet component concentration, and platelet component concentration distribution, indirectly affecting the platelet count.[23,107] Heparin is not used commonly as an anticoagulant because it does not prevent platelet clumping.[99] The anticoagulant of choice used in hematology laboratories for whole blood cell counts by commercial machines is generally ethylenediaminetetraacetic acid (EDTA). It preserves cells well[108] and has been shown to reversibly "spherize" platelets, causing a more uniform shape. The more uniform shape of the

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platelets following collection in EDTA makes counting by optical scatter and impedance technologies more accurate.[107]

While useful for counting, the shape change or spherization due to EDTA is also associated with a degree of platelet activation that may be undesirable in PRP for clinical use. The anticoagulant ACD-A is most commonly used in the preparation of human and veterinary PRP products because it supports platelet viability and metabolism,[15,88,109,110] and the dextrose in ACD-A helps nourish platelets during processing.[111,112] Acid citrate dextrose-A does not, however, create the uniformity of shape created by EDTA. In one study comparing platelet counts in human PRP anticoagulated with EDTA and citrated anticoagulants, including ACD-A, lower platelet counts were demonstrated in the citrated samples.[107] This discrepancy in counts was corrected by the addition of EDTA to citrated samples; however, it was also associated with an increase in the mean platelet volume (MPV), indicative of some degree of activation.[107] The necessity or benefit of addition of EDTA to PRP samples prior to counting has not been supported by other studies.[112] Interestingly, in whole blood samples, EDTA is known to be a cause of pseudothrombocytopenia, by inducing platelet clumping.[23,113,114]

Conclusion

Supporting evidence for the use of PRP to date has been made up largely of anecdotal case reports and small case series along with theoretical knowledge. The PRP used in these reported cases was obtained using a variety of preparation methods and the actual content of the PRP product was analyzed in only very few cases. It is our hope that the project presented in this Master's thesis will serve to encourage clinicians and researchers to evaluate and record the PRP content used, in order to help characterize platelet concentrate products and make meaningful

comparisons of treatment outcomes in the future. While measurement of specific growth factor

levels in PRP may be impractical in a clinical setting, enumeration of platelets and WBC is

reasonably achievable and prudent.

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Chapter 2

Comparison of Platelet Counting Technologies in Equine Platelet Concentrates

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Abstract

Objective: Compare the performance of four platelet counting technologies in equine platelet concentrates and to evaluate the ability of the Magellan platelet rich plasma (PRP) system to concentrate equine platelets.

Study Design: Experimental study to assess method agreement.

Animals: 32 adult mixed breed horses.

Methods: Acid citrate dextrose-A anti-coagulated whole blood was collected and PRP produced using the Magellan system according to the manufacturer's instructions. Platelets were quantified using four counting methods: optical scatter (Advia 2120), impedance (CellDyn 3700), hand counting, and fluorescent antibody flow cytometry. Platelet concentrations were compared using Passing and Bablok regression analyses and mixed model ANOVA. Significance was set at $P < 0.05$.

Results: Platelet concentrations measured in identical PRP samples were consistently higher for the Advia 2120 than the CellDyn 3700. Systematic and proportional biases were observed between these two automated methods when analyzed by regression analysis of the larger sample size. No bias (systematic or proportional) was observed among any of the other counting methods. Despite the bias detected between the two automated systems, there were no significant differences on average among the four counting methods evaluated, based on the ANOVA. The Magellan system consistently generated high platelet concentrations as well as higher than expected WBC concentrations.

Conclusions: The Magellan system delivered desirably high platelet concentrations; however, WBC concentrations may be unacceptably high for some orthopedic applications. All four platelet counting methods tested were equivalent on average and therefore suitable for quantifying platelets in equine PRP used for clinical applications.

INTRODUCTION

Since platelet rich plasma (PRP) was first proposed as a potent delivery medium for growth factors in the 1990s,[1,2] applications for its use have expanded dramatically to include human dental and orofacial surgery, human and veterinary sports medicine, orthopedics, and cutaneous wound healing.[3-8] The efficacy of PRP in enhancing soft tissue healing and bone formation has been attributed to the release of growth factors including platelet-derived growth factor, transforming growth factor-β1, insulin like growth factor-1, and vascular endothelial growth factor from platelet alpha granules in what are assumed to be biologically relevant concentrations and ratios.[4] In equine patients, PRP has been used to enhance wound healing and improve the quality of healing in tendons and ligaments.[3,6,9-11]

The term "platelet rich plasma" commonly refers to blood products that contain higher concentrations of platelets than those found in whole blood. Platelet rich plasma is a suspension of platelets, white blood cells (WBC), and red blood cells (RBC) in a small volume of plasma. The optimal concentration of platelets and WBC in PRP products is as yet unknown and is the topic of heated debate and ongoing research among clinicians and researchers.[5,11-19] The currently accepted benchmark for "therapeutic PRP" in human medicine is a platelet concentration of 1,000,000 platelets/μL, representing a 4 to 5 fold increase over whole blood.[5,20] Due to the lower baseline platelet content of equine whole blood, a benchmark of 500,000/μL has been commonly used for equine PRP.[19,21,22] Whether fold change (concentration compared to whole blood platelet count) or absolute number of platelets is more important in clinical applications is unknown.[19] The actual concentrations of growth factors released by the platelets may determine efficacy in enhancing tissue regeneration.[23-27] Higher platelet numbers in PRP correspond to higher levels of available growth factors.[3,24,26,27] Optimal concentrations of

platelets and WBC are likely to be specific to the different clinical applications and are the topics of ongoing research.

A number of commercial systems are available for producing PRP in horses. Each results in a unique product with respect to platelet, WBC, and RBC content. The Magellan Autologous Platelet Separator System (Magellan system) is a closed, automated centrifugation system that has been shown to generate average platelet counts of greater than 780,000/µL and average WBC counts of 11,000/µL in human PRP.[28] Equivalent values are not available for equine PRP generated using the Magellan system, but are critical in selecting a PRP system for clinical applications. The advantages offered by the closed and automated features of the Magellan system merited assessment of the PRP produced with an eye toward incorporation into our regenerative medicine service.

The ability to accurately enumerate platelets within prepared products must first be established in order to make useful comparisons between PRP products and their clinical efficacy. Previous studies have documented significant variability in the concentrating capabilities of some PRP preparation methods.[19] Reports of inconsistent platelet and WBC content of PRP produced by devices led to questions regarding the accuracy and precision of various methods used to count equine platelets in concentrated samples. Commonly used hematology analyzers are calibrated for lower platelet concentrations so that the range includes both normal and very low levels since thrombocytopenia in patients is of greatest clinical concern. The high concentrations of platelets found in PRP pose an unusual challenge for these counting technologies.[29-32]

In order to count a particular cell type, in this case platelets, each cell population must first be identified and distinguishable from other cell types in the suspension. The intrinsic

structural parameters of size, shape, and granularity can easily be used to differentiate and

therefore count cell types. However, biologic variation in size and the presence of debris and

fragmented cells pose significant challenges in accurately identifying what should be counted as

a platelet. Because they are significantly larger than platelets, WBC and RBC are less

susceptible to confusion with cellular debris. The small size (2.5 to 3.5 μm diameter in horses)[33]

and adherent potential of platelets make them one of the most difficult blood cells to count.[31]

Challenges encountered in developing accurate and precise counting methods include

coincidence events (in which more than one particle passes through the aperture at the same time

and are counted as one) and debris from other cells that mimics the size and irregular shape of

platelets. Platelet counts are lower in horses than in most other mammals, resulting in a

relatively lower percentage of the total cells in whole blood.[21] The more numerous cell types

(WBC and RBC) can interfere with accurate counting by adhering to platelets and/or creating

coincidence events as the cells pass through the detector. To some extent this problem is

alleviated through the reduction in RBC numbers in PRP. However, stress during processing can

cause platelet activation and further shape changes that can make counting platelets in PRP even

more challenging than in whole blood.[23,34]

Four main categories of technologies are available for counting platelets: manual

microscopy, optical scatter, electrical impedance, and fluorescent antibody flow cytometry.[35]

Optical scatter technology utilizes light diffraction, refraction, and reflection related to cell

shape, volume, and content to differentiate and enumerate cells. Electrical impedance relies on

changes in electrical conductance caused by cells passing between two electrodes. The change

in impedance is directly proportional to the size of interfering cells.[35] Historically, direct platelet

counting methods were used as the gold standard against which automated hematology analyzers

were calibrated and gated. However, direct hand counts using Neubauer hemocytometers are time-consuming and subject to a great deal of human error.[30,36] Indirect counting via an RBC to CD41/61+ platelet ratio has recently been accepted by the International Council for Standardization in Hematology and the International Society of Laboratory Hematology as the standard method for counting platelets in human whole blood samples.[36,37] This reference method utilizes fluorescent-labeled antibodies to a marker on the platelet cell membrane (CD41/CD61) to quantify platelets within a sample. This method has not yet been described in horses. In this study, a novel immunofluorescent platelet counting method using indirect flow cytometry was extrapolated from the current reference method for human blood and developed for use as a possible gold standard in horses.

The objective of this study was to compare the performance of four platelet counting technologies in quantifying platelets in equine platelet concentrates. In addition, this study served to characterize the platelet and WBC concentrations in equine PRP produced using the Magellan system. We hypothesized that there would be no differences on average in platelet counts among the four counting technologies and that the Magellan system would generate platelet concentrations greater than 500,000/μL.

MATERIALS AND METHODS

This study was approved by and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee. Thirty-two horses of various light breeds, with no clinical signs or recent history of disease, were used as blood donors. All horses were between 3 and 12 years of age (mean = 10.5 years).

Blood Collection and PRP Preparation

The left or right jugular vein was randomly selected and prepared for venipuncture using standard aseptic technique. Whole blood (52 ml) was collected from each horse into a 60 ml syringe containing 8 ml of acid citrate dextrose-A (ACD-A) anticoagulant using an 18 gauge 1.5 inch hypodermic needle and an extension set. Blood was drawn over 1 minute and the syringe was gently rocked during collection to ensure proper mixing. Prior to PRP processing, a 1 ml aliquot of whole blood was saved for analysis of baseline values. Platelet rich plasma was prepared using an automated commercial system (Magellan Autologous Platelet Separator System, Arteriocyte Medical Systems, Inc., Hopkinton, MA) according to manufacturer's instructions. The system was programmed to yield 6 ml of platelet concentrate from each 60 ml syringe sample. All PRP samples were placed on an oscillating blood mixer for no less than 5 minutes and no more than 20 minutes[31] to ensure adequate and even suspension of the platelets within the plasma at the time aliquots were divided for counting. One 3 ml aliquot was transferred to a polypropylene tube for submission to the Clinical Pathology Laboratory. One 2 ml PRP aliquot was saved for flow cytometry processing. The final 1 ml PRP sample was saved for hand counting as described below.

Hematologic Analysis

Whole blood and PRP samples for all horses were submitted to the Clinical Pathology

Laboratory for automated quantification of platelet, WBC, and RBC counts. Platelet

concentrations in PRP samples were determined using two commercial hematology analyzers

that utilize two different counting technologies: optical scatter (Advia 2120, Siemens Healthcare

Diagnostics Inc., Tarrytown, NY) and electrical impedance (CellDyn 3700, Abbott Laboratories,

Abbott Park, IL). Whole blood was analyzed using the Advia 2120. White blood cell and RBC

concentrations in PRP samples measured with the Advia 2120 were used for comparison to

whole blood values to assess the product of the Magellan system. All samples were placed on an

oscillating rocker for 10 minutes prior to analysis to ensure adequate mixing.[31]

Hand Counting

Hand counts of platelet numbers were performed on all PRP samples using a commercial

kit (Thrombo-TIC, Bioanalytic GmbH, Germany). The same individual performed all counts. A

1:100 dilution of PRP was made using 1% ammonium oxalate solution. The samples were

mixed thoroughly and allowed to react for at least 5 minutes but not more than 6 hours, in

accordance with manufacturer's instructions, to ensure complete RBC lysis and disaggregation

of platelets. Prior to loading the improved Neubauer hemocytometer, the sample was again

mixed to ensure even suspension. Once the chambers were loaded, the cells were allowed to

sediment for 20 to 30 minutes in a humidified chamber. Platelets were counted using phase-

contrast microscopy (CKX-41, Olympus, Center Valley, PA). The average of both counting

chambers was utilized for statistical analysis.

Flow Cytometry

An immunofluorescent counting method was performed on 15 of the 32 samples using a

mouse monoclonal anti-sheep antibody against integrin alpha 2b + beta 3 (clone CO.35E4) (anti-

CD41/CD61) (Abcam Inc., Cambridge, MA).[38-41] The optimal concentration of primary and

secondary antibodies to demonstrate specific receptor binding was determined by titration. The

zero dilution for the titrations (secondary antibody alone) served as a negative control. Positive

controls included human and ovine whole blood. Platelet rich plasma was diluted 1:20 in

Tyrode's Albumin Buffer without $CaCl_2$ (Table 1) and incubated with the primary antibody (at a

dilution of 1:20) for 30 minutes at room temperature. The secondary phycoerythrin (PE)-

conjugated polyclonal goat anti-mouse IgG1 antibody (Abcam Inc.) (at a dilution of 1:2) was

then added and samples were incubated for an additional 30 minutes in the dark at room

temperature. Samples were diluted to obtain an optimal flow rate of approximately 100 events

per second to minimize confounding coincident events. A calibrated suspension (50,000 beads)

of fluorescent microspheres (CountBright Absolute Counting Beads, Invitrogen, Eugene, OR)

was added to each sample prior to submission. All samples were processed in the flow

cytometry laboratory by the same individual on the FACSAria cell sorter (BD Biosciences,

Becton, Dickinson and Company, Franklin Lakes, NJ). Samples were run to obtain a total of

1,000 bead events and the ratio of bead events to platelet events was used to calculate the

concentration of platelets within PRP samples. FACS analysis was carried out using commercial

software (FlowJo Version 7, Tree Star, Inc., Ashland, OR).

Statistical Analysis

Data were separated into two sets; one comparing all four counting methods (n=15) and one comparing three counting methods (Advia 2120, CellDyn 3700, hand counts; n=32). Descriptive statistics were calculated on the counts obtained for whole blood and PRP using the Advia 2120 hemocytology analyzer and are presented as mean ± SD. Data were log transformed and compared using mixed model ANOVA (SAS Version 9.2, SAS Institute Inc., Cary, NC, USA) and Passing and Bablok regression analyses (MedCalc, Version 12.7.0, MedCalc Software, Ostend, Belgium). The data for each of the counting methods were plotted against each other, yielding 3 Passing and Bablok regression lines for the data set with 32 samples and 6 for the data set with 15 samples. Significance was set at $P < 0.05$.

Table 2-1 Formula for Tyrode's Albumin Buffer without $CaCl_2$ (pH 7.4)

134 mM NaCl
12 mM $NaHCO_3$
2.9 mM KCl
0.34 mM Na_2HPO_4
1 mM $MgCl_2$
10 mM HEPES pH 7.4

RESULTS

The Magellan system yielded PRP samples with high platelet concentrations (893,090 ± 226,610/µL). Greater than 75% of all PRP samples had concentrations greater than 700,000 platelets/µL (Fig 1). These values represent a mean 8.0 ± 2.9 fold increase in platelet concentration compared to whole blood (range 3.5 to 16.6) (Fig 2).

The WBC concentration in PRP samples produced by the Magellan system was 35,806 ± 9,971/µL. Fewer than 16% of the PRP samples had WBC concentrations less than 25,000/µL (Fig 1). These data represent a mean 6.1 (±1.7) fold increase in WBC concentration compared to whole blood (range 3.9 to 12.4) (Fig 3). Red blood cell concentrations in PRP decreased compared to whole blood (0.5 ± 0.2 times baseline; range 0.2 - 1.0) (Fig 3). The mean RBC concentration in PRP samples was 3,280 ± 809/µL.

There were no significant differences on average among the four counting methods evaluated, based on the ANOVA for the 3 methods ($P = 0.45$) or all 4 methods ($P = 0.09$) (Fig 2). Platelet concentrations measured in identical PRP samples were higher for the Advia 2120 than the CellDyn 3700 in 31 of the 32 cases. Systematic and proportional biases were observed between these two automated methods when analyzed by regression analysis of all 32 cases ($P < 0.05$) (Fig 4, Table 2). However, no bias (systematic or proportional) was observed between these methods on regression analysis using the smaller data set of 15 horses in which flow cytometric immunofluorescent counting was performed (Fig 5, Table 3). No bias (systematic or proportional) was observed among any of the other counting methods during analysis of either data set (n=32 or n=15) (Figs 4 and 5, Tables 2 and 3). Systematic bias between counting methods was identified if the 95% confidence interval of the intercept excluded zero. Proportional bias was identified if the 95% confidence interval of the slope excluded one. Bias

measures the agreement between methods. A lack of systematic and proportional bias means

that two methods generate essentially the same result. The presence of systematic bias means

that two methods generate results that differ from each other by a constant value regardless of the

magnitude of the resulting count. The presence of proportional bias means that two methods

generate results that differ by an amount that varies depending on the magnitude of the count.[42]

Flow cytometric analysis using the CD41/CD61 antibody with fluorescent label produced

data that were statistically equivalent to the other 3 methods (Fig 5). The fluorescent-labeled

platelet scatter plots and the forward and side scatter dot plots obtained from the

immunofluorescent flow cytometry counts displayed good agreement with what would normally

be gated for a platelet population based on light scattering parameters (Fig 6).

Table 2-2 Results of Passing and Bablok Regression for three counting methods (n = 32)

Comparison	Intercept (95% CI)	Systematic Bias	Slope (95% CI)	Proportional Bias
CellDyn vs. Hand Count	0.5 (-1.2 to 2.0)	No	0.9 (0.7 to 1.2)	No
Advia vs. CellDyn	-1.8 (-3.0 to -0.8)	Yes	1.2 (1.1 to 1.4)	Yes
Advia vs. Hand Count	-1.3 (-3.8 to 0.8)	No	1.2 (0.9 to 1.6)	No

CI = Confidence Interval

**Table 2-3 Results of Passing and Bablok Regression for four counting methods (n = 15)**

| Comparison	Intercept (95% CI)	Systematic Bias	Slope (95% CI)	Proportional Bias
CellDyn vs. Hand Count	-1.2 (-5.9 to 1.2)	No	1.2 (0.8 to 1.9)	No
Advia vs. CellDyn	-1.7 (-3.8 to -0.3)	No	1.2 (1.0 to 1.5)	No
Advia vs. Hand Count	-1.8 (-11.8 to 0.5)	No	1.3 (0.9 to 2.7)	No
Immuno Count vs. Hand Count	-0.7 (-4.4 to 1.7)	No	1.1 (0.7 to 1.7)	No
Immuno Count vs. Advia	1.7 (-1.9 to 3.6)	No	0.8 (0.5 to 1.3)	No
Immuno Count vs. CellDyn	0.6 (-2.6 to 1.6)	No	0.9 (0.8 to 1.4)	No

CI = Confidence Interval
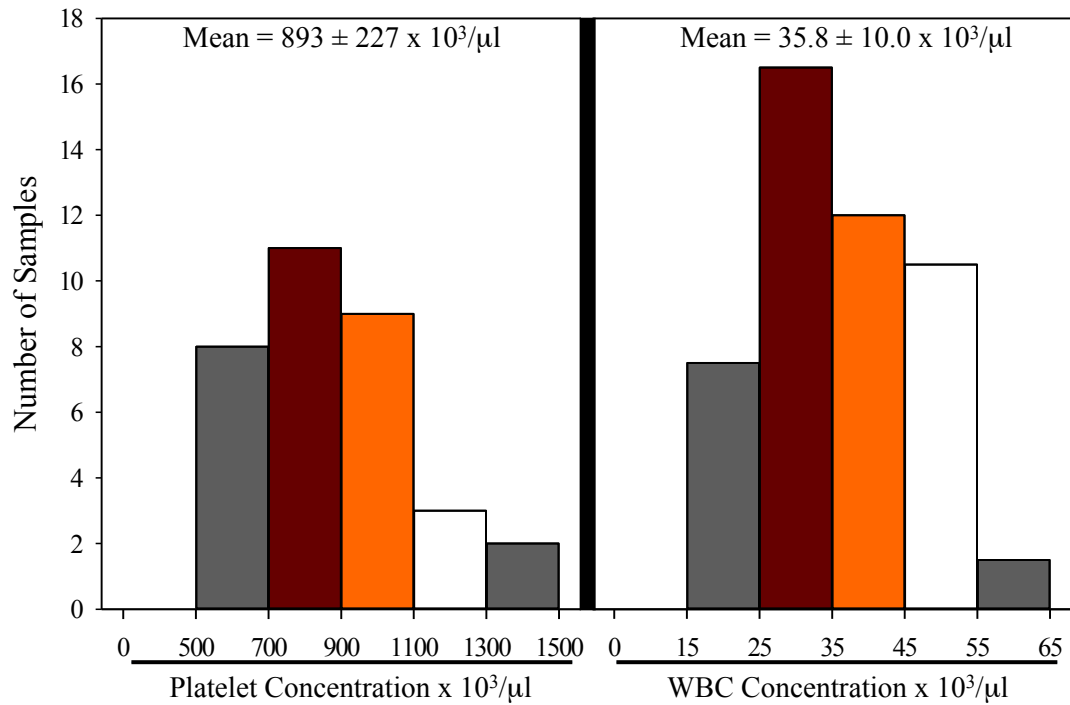
Figure 2-1 Numbers of platelet rich plasma (PRP) samples produced by the Magellan system grouped by clinically relevant ranges of platelet and white blood cell (WBC) concentrations. All platelet concentrations were greater than 500,000/µl and concentrations of WBC were greater than 15,000/ µl as measured by the Advia 2120 hematology analyzer.
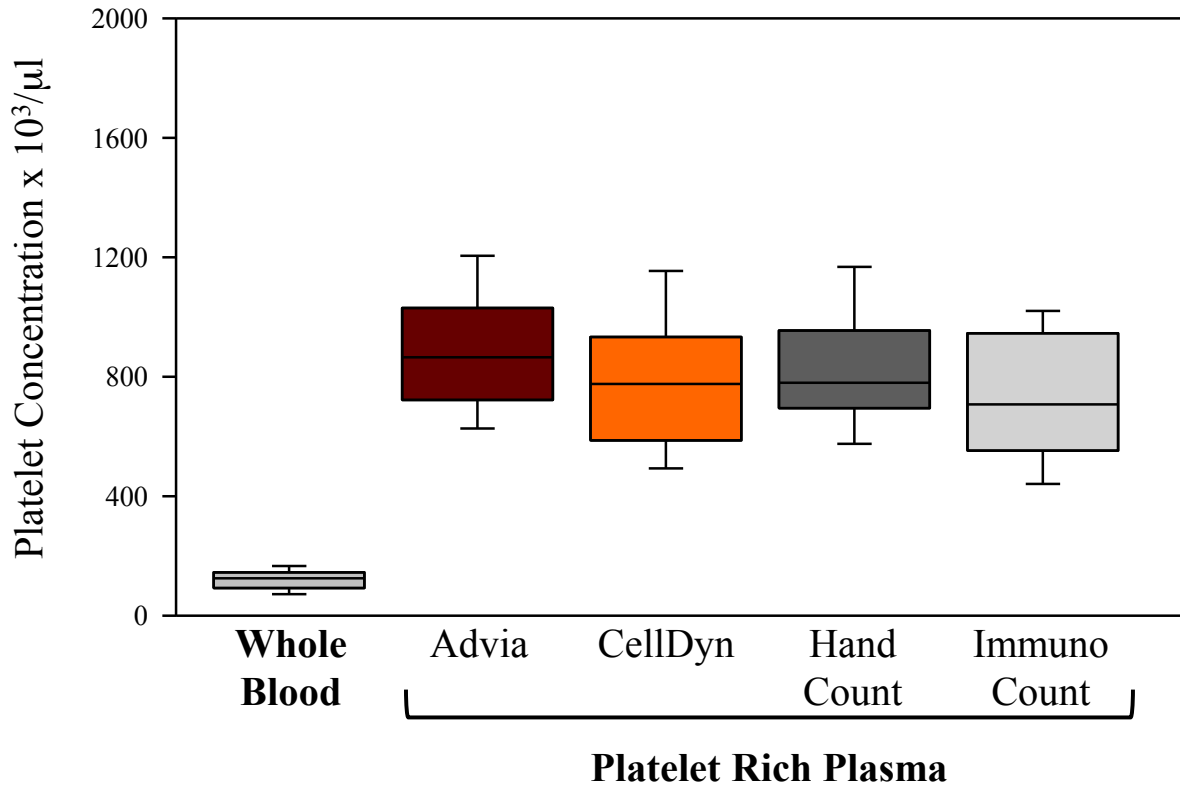
Figure 2-2 Comparative distribution of platelet concentrations in equine whole blood and platelet rich plasma as measured by four counting methods: optical scatter (Advia), electrical impedance (CellDyn), Hand Count, and immunofluorescent flow cytometry (Immuno Count). On average, based on the ANOVA, there were no significant differences between counting techniques.
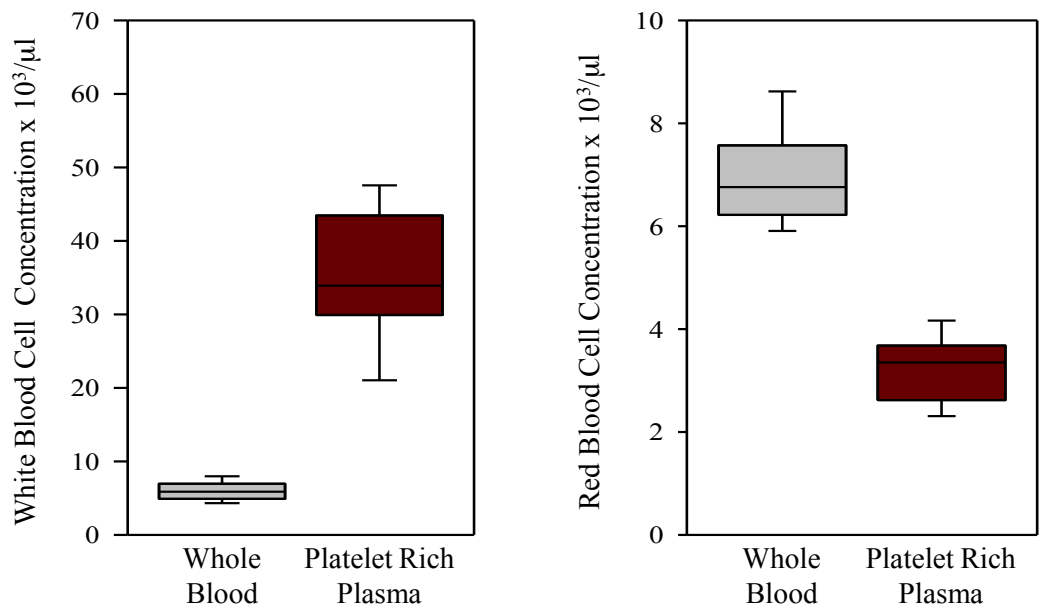
Figure 2-3 Comparative distribution of white and red blood cell concentrations between equine whole blood and platelet rich plasma.
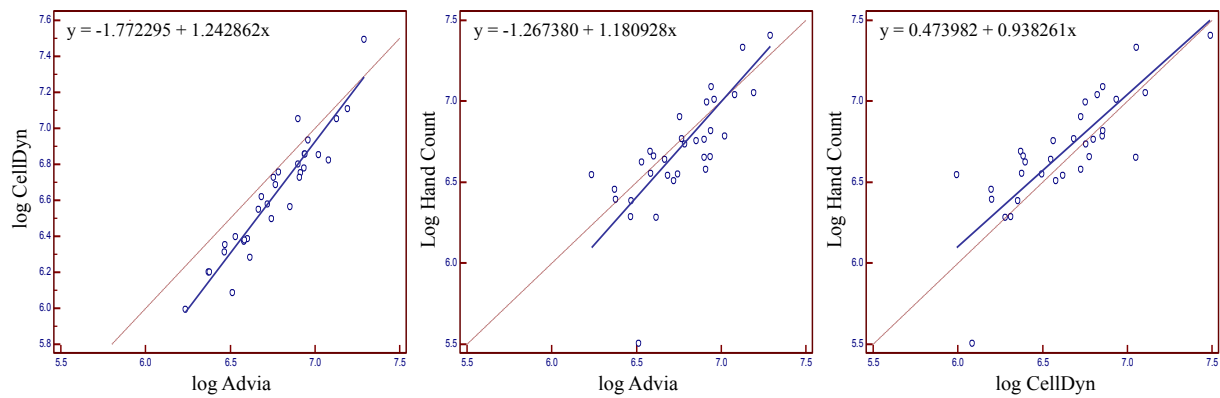
Figure 2-4 Passing and Bablok Regression scatterplots comparing the three counting methods used on the larger sample size (Advia, CellDyn, and Hand Count; n=32). The thicker solid lines indicate the regression lines; corresponding equations are listed in the upper left corners. The thinner dotted lines show the lines of identity (complete agreement). The plot on the left depicts the systematic and proportional bias observed between Advia and CellDyn counts, indicated by the divergence of the regression line from the line of identity. No bias (systematic or proportional) was observed in the other comparisons.
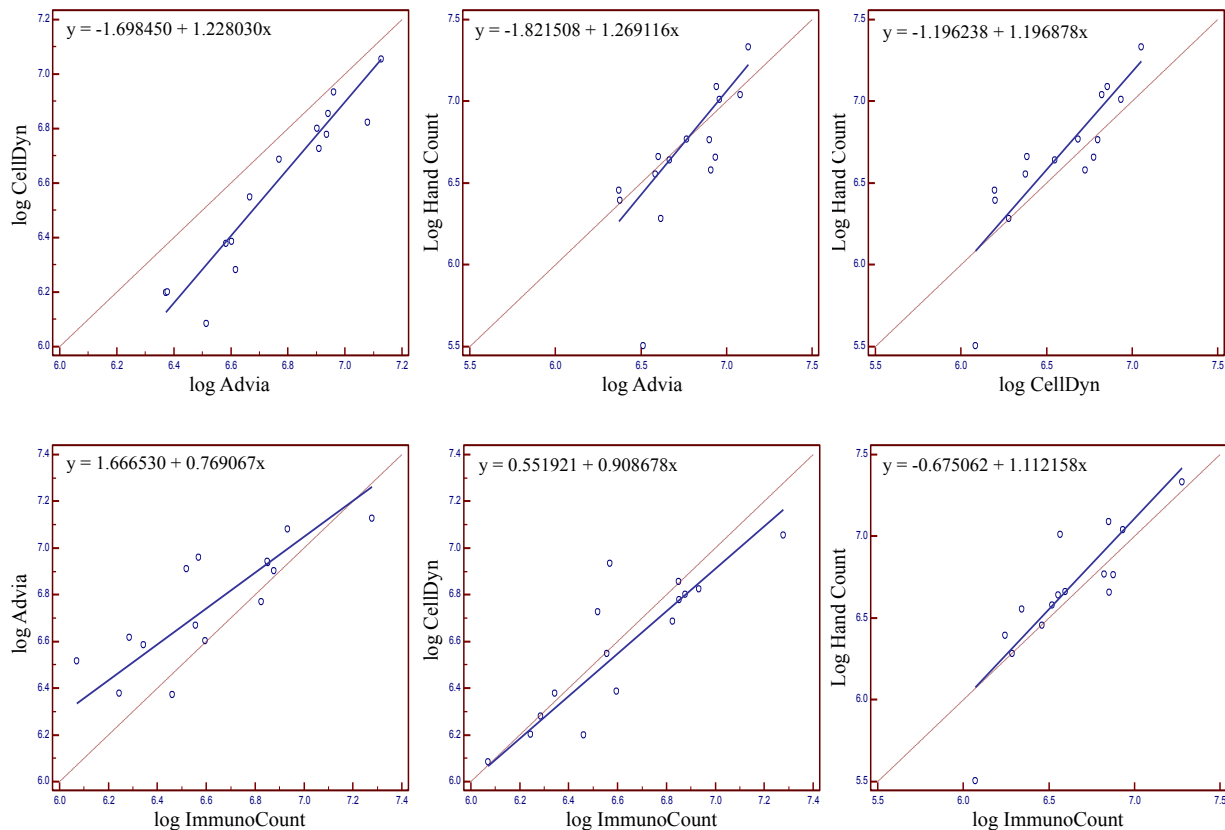
Figure 2-5 Passing and Bablok Regression scatterplots comparing all four counting methods used on the smaller subset of samples (Advia, CellDyn, Hand Count, and Immuno Count; n=15). The thicker solid lines indicate the regression lines; corresponding equations are listed in the upper left corners. The thinner dotted lines show the lines of identity (complete agreement). No bias (systematic or proportional) was observed in any of these comparisons.
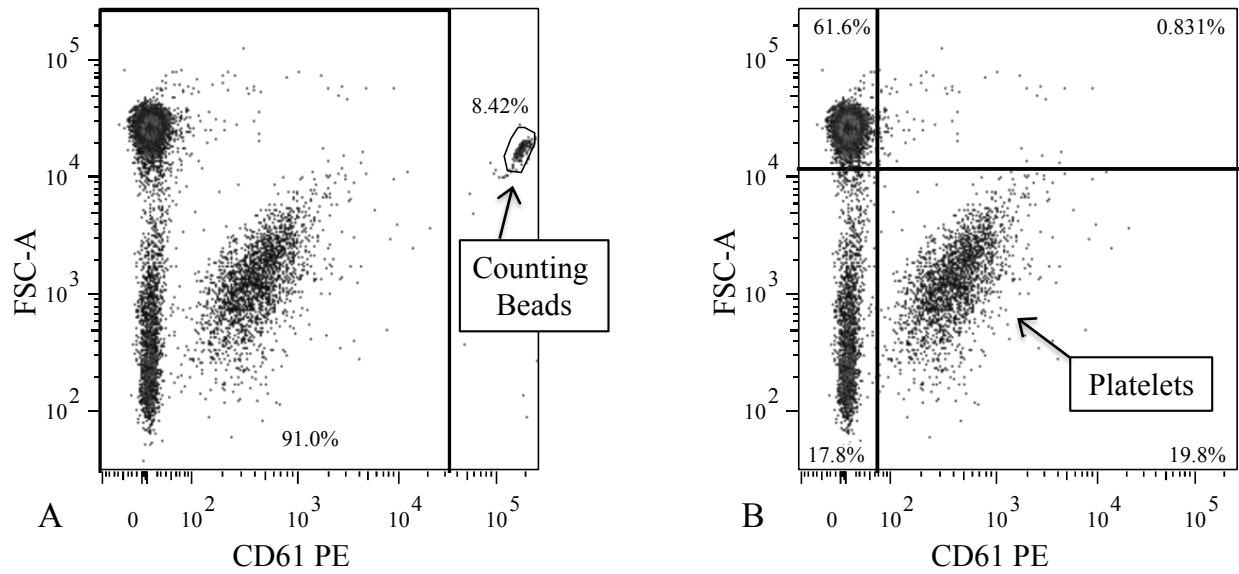
Figure 2-6 Representative dot plots from immunocounting method depicting flow cytometry gates and threshold boundaries for characterizing platelets in equine platelet rich plasma (PRP) samples prepared by the Magellan system. (A) Representative PRP sample containing fluorescent counting beads. Data were acquired for each sample to obtain 1,000 events within the counting bead gate. (B) The same representative PRP sample depicting the platelet gate. The forward scatter of the RBC population defines the platelet gate upper y-axis boundary and fluorescence of a CD41/61-phycoetherin (PE)–conjugated antibody (CD41/61-PE) defines the x-axis boundary.

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DISCUSSION

  The equine PRP product produced using the Magellan system served as a good test medium for this study as all samples contained platelet concentrations within the range of 500,000 – 1,500,000/μL, which is well above currently accepted standards for equine practice.[20,22] Without a gold standard counting method or known reference sample, it was not possible to determine a definitively superior method of platelet quantification. All four platelet counting technologies evaluated resulted in similar platelet counts in equine platelet concentrates. The data presented can serve as preliminary data for validating immunocounting of platelets in horses with the addition of more horses to create a larger sample size. Following additional validation, the described flow cytometric immunofluorescent counting method may become a gold standard method in equine platelet counting and serve as a useful reference method for future standardization of equine PRP products.

  There was little inter-individual variation in the platelet concentrating ability of the Magellan system based on fold change compared to whole blood samples. A four-fold increase in platelet concentration over whole blood baseline is purported to be therapeutically relevant in human applications[20,43] however, the optimal platelet concentration for specific indications remains unknown. Published values for PRP harvested using the Magellan system were not available for equine blood prior to this study and this information was deemed valuable for comparison of the various PRP systems commercially available. The Magellan system consistently and reliably surpassed the four-fold benchmark in our equine samples, resulting in an average 8.0 fold increase in platelet number and a mean concentration of 893,100 platelets/μL. These values for platelet numbers are consistent with those published using the same system in humans where PRP contained an average of $780,200 \pm 246,500$ platelets/μL.

The relative concentrating ability was greater in equine blood compared to the 2.8 fold increase reported in human blood.[28]

Human PRP harvested using the Magellan system contained $11,000 \pm 8,200$ WBC/μL, representing a 2-fold increase in WBC concentration compared to whole blood.[28] Equine PRP produced in this study by the Magellan system contained an approximately 3 times higher WBC concentration compared to the human product. This represents a higher mean fold change in equine PRP samples compared to available human data. In both this equine study and the reported human data, the Magellan system was set for an output of 6 ml of PRP from each 60 ml of whole blood sample. Further work could be pursued to determine if varying the setting for output volume on the Magellan system would change the relative concentrating ability for platelets versus WBC. The term "platelet-leukocyte rich plasma" (P-LRP) has been proposed to more accurately describe PRP products with moderate to high WBC concentrations compared to pure PRP (WBC-depleted).[44,45] Both the reported human data and the equine data in this study for the Magellan system would be classified as P-LRP.[28] The clinical implications of elevated WBC concentrations in PRP used for various treatment regimens have yet to be fully explored and remain controversial. Immunomodulatory and antimicrobial effects resulting from high WBC numbers in PRP products may be beneficial for specific applications such as wound healing.[19,46] However, the pro-inflammatory effects of high WBC concentrations in P-LRP may be deleterious for orthopedic applications. Increased expression of inflammatory cytokines was reported in equine tendon explants cultured in concentrated-leukocyte PRP.[11]

Commercially available hematology analyzers have been designed to enumerate blood cells and particles within the ranges encountered in normal human whole blood samples. Many machines are also equipped with veterinary software to adjust and account for species

differences in the size, granularity, and shape of blood cells.[21] However, when the

concentrations of blood cells in samples fall far outside the clinically normal ranges, accuracy of

counts may be compromised due to difficulty in gating expected cell populations. Most

clinically important decisions must be made in samples that fall outside normal ranges. For

example, in human medicine, the challenge of counting platelets in thrombocytopenic patients is

paramount to determining a cut off value below which blood transfusion should be

recommended. In this study, the opposite challenge is posed by analyzing PRP samples where

the platelet concentrations are several fold higher than the normal range. An analyzer must

either be calibrated to count platelets across a wider range of concentrations that includes those

typical for platelet concentrates or the sample must be diluted to bring the sample within the

range of calibration of a given machine.

Platelet rich plasma poses some additional unique challenges and sources of error for

counting methodologies compared to whole blood samples. Coincidence error, in which

multiple platelets are counted as one event, is more likely to occur when cell counts are very

high, as in PRP samples, and result in lower reported platelet concentrations. This problem can

be compounded due to platelets' tendency to adhere to themselves or to other larger cells.[47]

Inadequate mixing of the PRP product, resulting in in homogenous resuspension of blood

components in the plasma, can be a significant source of error in platelet counts.[31] Without the

presence of adequate numbers of RBC to increase the viscosity of the PRP suspension, platelets

settle to the bottom of the sample more rapidly than in whole blood.[31] The result may be an

artificially low or high platelet count due to platelet sedimentation and whether the sample was

collected from the top or bottom of the tube. Additionally, when PRP is first processed, many of

the platelets may be clumped leading to false counts if at least 5 minutes is not provided to allow

time for platelet clumps to relax and separate. Previous studies have demonstrated that agitation on a rocker for 5 – 20 minutes prior to counting was necessary and adequate to uniformly resuspend the platelets in PRP samples.[31] For these reasons, in this study care was taken to provide adequate mixing of our samples and avoid inaccuracies in our counts.

A potential source of error in platelet counts from automated blood counters using optical scatter (such as Advia 2120) or electrical impedance (such as CellDyn 3700) technology is interference by similarly-sized particles, such as small or fragmented RBC. Red blood cell fragmentation could occur during PRP processing by centrifugation and transfer and these RBC particles could erroneously be counted as platelets by an automated counter, resulting in an artificially high count.[48] Also, large platelets may be excluded from the count on the basis of their size, because of the difficulty in distinguishing them from RBC.[35]

Platelet enumeration is susceptible to errors precipitated by variation in cell shape and size. Anticoagulants can affect platelet parameters including mean platelet volume and shape.[49] Ethylenediaminetetraacetic acid is generally the anticoagulant of choice used in hematology labs for whole blood cell counts by commercial machines. It preserves cells well[50] and has been shown to reversibly "spherize" platelets, causing a more uniform shape. The more uniform shape of the platelets following collection in EDTA makes counting by optical scatter and impedance technologies more accurate.[49] However, this shape change is also associated with a degree of platelet activation that may be undesirable in PRP for clinical use. The anticoagulant ACD-A is most commonly used in the preparation of human and veterinary PRP products because it supports platelet viability and metabolism[6,51-53] and the dextrose in ACD-A helps nourish platelets during processing.[31,54] Acid citrate dextrose-A does not, however, create the uniformity of shape created by EDTA. Our samples were collected in ACD-A as directed in the

manufacturer's instructions for the Magellan system and as is the convention in the industry for PRP preparation.

Platelet counts obtained using automated hematology analyzers are generally more precise than manual platelet counts, because they count approximately 100 times greater numbers of cells and therefore result in a smaller coefficient of variation.[47] The hand counts in this study displayed more outlying data points than the other three counting methods. This is consistent with the statistical assumption that hand counts would have a higher coefficient of variation due to a lower total number of platelets counted. It may also be due in part to the samples being outside the validated reference range for the Thrombo-TIC system (100,000 – 440,000 cells/μL). Our PRP samples were diluted in ammonium oxalate to lyse any remaining RBC according to manufacturer's instructions based on values assumed for whole blood. Using a greater dilution of PRP in the ammonium oxalate solution could potentially make the hand counts more precise; however, this does not appear to be necessary clinically as no bias was observed with the hand counts in either data set in this study.

Both optical scatter and electrical impedance hematology analyzers are specialized types of flow cytometers. The monoclonal antibody counting technique described in this project is a more specialized type of fluorescence flow cytometry that enables greater selectivity in counting specific cell types.[55,56] Counting by immunofluorescent flow cytometry was selected for evaluation because it is used as the gold standard platelet counting method in human whole blood.[37] To the authors' knowledge its use has not been described in equine PRP samples. In human whole blood, a ratio of RBC to fluorescent-labeled platelets is used to calculate platelet concentrations. Because the RBC concentration in PRP was anticipated to be negligible,

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fluorescent calibration beads were used in place of RBC in this study to create the necessary ratio.

The selected antibody clone against integrin alpha 2b (CD41) and beta 3 (CD61) has previously been validated for use with equine platelets[38-41] and was therefore selected for use in this study. Integrins are a family of heterodimeric transmembrane glycoprotein complexes, consisting of alpha and beta subunits that mediate cell adhesions and signal transduction. The platelet membrane protein against which this antibody is made is constitutively expressed on platelets, regardless of activation status.[57] The use of this platelet-specific antibody ensured that no platelets were lost to counting among debris or larger cells.[55,56] Future research using additional and alternative antibodies could be pursued to evaluate the activation status of platelets following PRP processing in different production devices. Platelet activation, leading to degranulation and loss of growth factors, may occur during PRP processing and affect the quality and efficacy of the product.[23,34]

The Magellan system consistently generated PRP with very high platelet concentrations and therefore served as a good test medium for counting equine platelets in concentrates. Moderate to high WBC concentrations in the resulting PRP samples may, however, be unacceptably high for certain orthopedic applications, due to the potential for upregulation of pro-inflammatory cytokines.[11] Further research is also necessary to determine the clinical relevance of platelet and WBC concentrations for the various equine applications of PRP. Based on our results, all four platelet counting methods tested are acceptable for use on equine platelet concentrates ranging from 500,000 to 1,500,000/μL and therefore are suitable methods of quantifying platelets in equine PRP used for clinical applications. Standardization of equine PRP

products and the accurate reporting of platelet and WBC numbers are essential for the direct comparison of future clinical studies in the horse.

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