Tube centrifugation for processing platelet-rich plasma in the horse

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ABSTRACT

Platelet-rich plasma (PRP) is a popular treatment for equine tendon and ligament injuries; however, commercial PRP systems are expensive. Development of a safe, inexpensive alternative would make PRP therapy more widely available to horse owners. The purpose of this study was to evaluate the quality and bacteriologic safety of PRP produced by three simple, inexpensive tube centrifugation methods and compare the results to a commercial system.

Citrated blood collected from 26 normal horses was processed by four methods: blood collection tubes centrifuged at 1200 and 2000 x g, a 50ml conical tube, and a commercial system. Platelet and cell counts and mean platelet volume (MPV) in whole blood and PRP were determined using an automated hematology analyzer. Results were analyzed using mixed model ANOVA with post-hoc comparisons (MPV and fold change for RBC, WBC, and platelets) and binary logistic generalized estimating equations with horse as a blocking factor (absolute numbers of WBC, and platelets). Aerobic and anaerobic cultures were performed. Significance was set at $p < 0.05$.

Mean platelet concentrations ranged from 1.55 to 2.58 fold. The conical tube method produced the highest number of PRP samples with platelet concentrations of greater than 2.5-fold and within the clinically acceptable range of >250,000 platelets/µl. WBC counts were lowest using the commercial system and unacceptably high using the red top methods. The incidence of bacterial contamination was low (2.1%). Based on these results, the conical tube method may be a suitable alternative to commercial PRP systems in cases with budgetary constraints.

Acknowledgements

I would like to begin by thanking each of my committee members, Dr. Linda Dahlgren, Dr. Nicole Weinstein, and Carolyn Sink for their individual contributions to this project. I would especially like to thank my mentor and advisor, Dr. Dahlgren, for her whole-hearted investment in this project as well as in my surgical training. I wish to thank all of the undergraduate and veterinary student volunteers that went with me on numerous adventures to collect blood from horses all over Montgomery County for this project. Special thanks are owed to Chris Andrews for so many hours of his time spent helping me with blood collection, processing, and data entering.

I thank the technicians in the VMRCVM Clinical Pathology and Microbiology laboratories for their help, patience, and expertise during many weeks of sample processing. Also, I would like to thank the large animal technicians, especially Katie Reuss and Tony Huffman, for their help on this project and each and every day in the clinic. Special thanks are owed to the faculty, staff, and clients that volunteered their horses for this project.

I wish to thank the American College of Veterinary Surgeons for their financial support of this project through the Surgeon-In-Training Grant. Thanks to Vet-Stem, Inc. for their generous donation of the GenesisCS® kits used in this project.

I dedicate this thesis to my husband, Michael Fontenot, and my parents, Arnold and Charlotte Lumbley. Their love, support, and prayers have been the base of support for each and every accomplishment in my life.

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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. The publication is entitled "Simple tube centrifugation for processing platelet-rich plasma in the horse" 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 is an expansion of the introduction to the manuscript and provides a summary of pertinent literature background information.

<u>Introduction</u>

In human and veterinary medicine, there is an increasing demand for regenerative therapies. The goal of regenerative therapies is to restore the normal architecture and functionality of the injured tissue. This is in contrast to the formation of suboptimal scar tissue that results from the natural tissue repair process (1). A wide variety of regenerative therapies are under investigation, and many are currently being used in clinical practice. An example of such a product is platelet-rich plasma (PRP), a blood product made by concentrating platelets in a small volume of plasma. PRP is used extensively in human medicine for a wide variety of surgical and sports medicine applications (2-4). In equine practice, PRP has gained popularity for the treatment of wounds and tendon and ligament injuries (5). The exact mechanism of action of PRP is unknown; however, the release of high concentrations of growth factors from platelet alpha granules as platelets degranulate is widely believed to play a critical role. Alpha granules contain several growth factors known to have beneficial effects on tissue healing,

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including platelet-derived growth factor (PDGF), transforming growth factor β-1, vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF)-I (6).

Although many research questions remain concerning the mechanism of action of PRP, the results of initial clinical investigations are promising. One road block to expanding the clinical use of PRP is the cost of commercial processing systems. Development of a safe, inexpensive alternative to commercial systems would make PRP therapy more widely available to horse owners. The aim of this Master's project was to evaluate the quality and bacteriologic safety of platelet-rich plasma (PRP) produced by three simple, inexpensive tube centrifugation methods and compare the results to those produced by a commercial system

Chapter 1: Literature Review

Platelet-rich plasma (PRP) has become a widely used therapeutic modality in both human and veterinary medicine for a wide range of musculoskeletal applications (7-9). However, the mechanism by which PRP provides a beneficial biologic effect is incompletely understood. In addition, species differences between horses and humans make extrapolation of centrifugation protocols for PRP production from the human to the horse impossible (10, 11). Optimization of the therapeutic use of equine PRP requires an understanding of platelet structure and function, species-specific platelet parameters, and evidence-based information regarding the therapeutic use of PRP.

The Platelet

The study of platelets began with Giulio Bizzozero, an Italian doctor and medical researcher who is credited with their discovery in 1881. In 1906, James Homer Wright, an American pathologist, identified megakaryocytes as the source of platelets when he noted similarities between the granules of platelets and megakaryocytes using his special stain (12, 13). The last century, since these discoveries, has seen a massive expansion of knowledge concerning platelet formation, structure, and function.

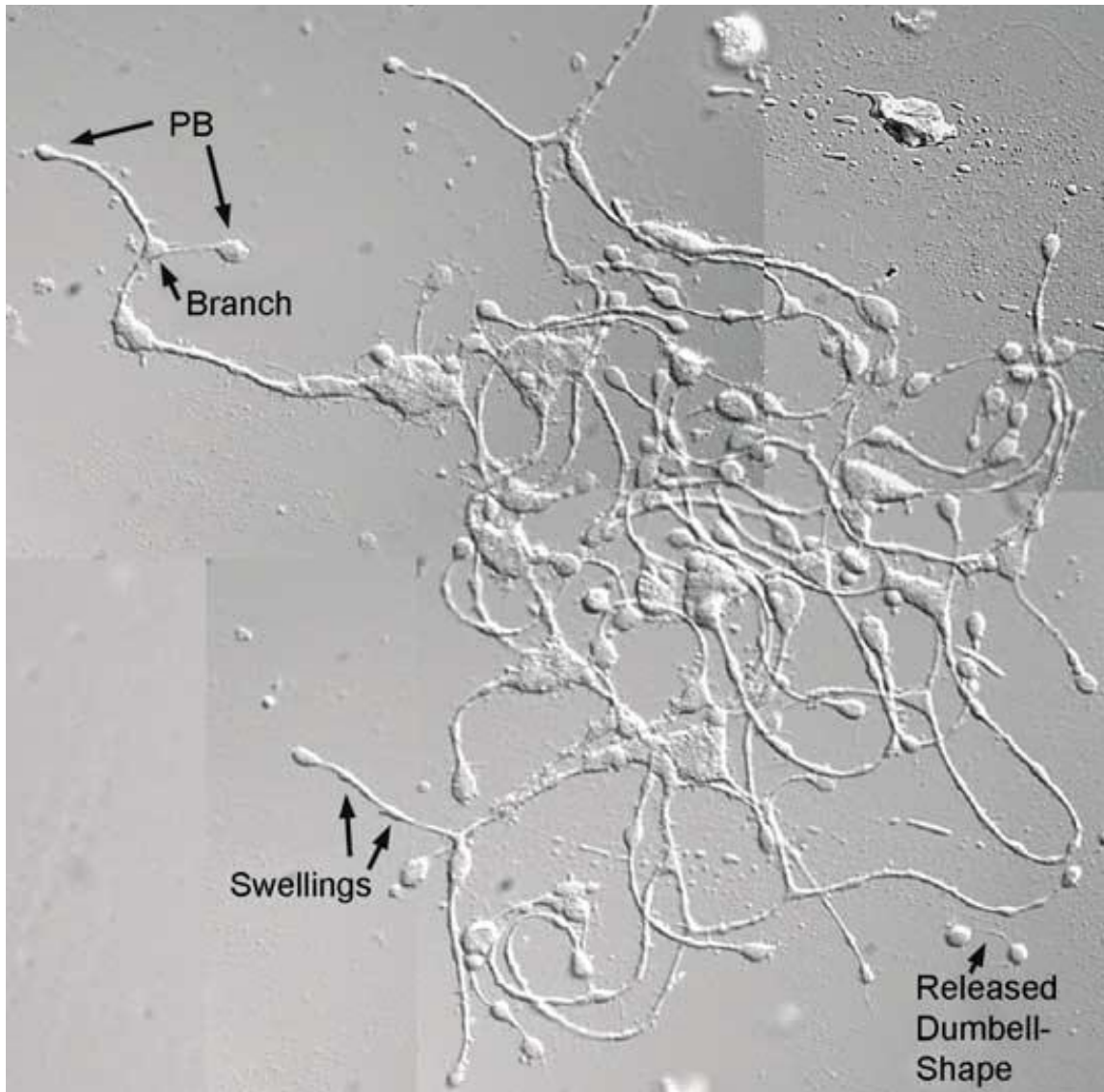### Formation

Platelets are formed from large precursor cells called megakaryocytes. Platelet production and release is mediated by thrombopoietin produced in hepatocytes, renal tubular epithelium, and bone marrow stromal cells (14). The majority of megakaryocytes are found in the bone marrow, although a small percentage are found in pulmonary capillaries and in

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circulation (15). In preparation for platelet formation, megakaryocytes increase their chromosome number through a process called endomitosis, a variant of mitosis that does not include cell division. Following endomitosis, megakaryocytes undergo a stage of rapid cytoplasmic expansion in which the cytoplasm rapidly fills with the proteins, organelles, and membrane components needed to form platelets. Once maturation is complete, the process of platelet formation and release begins. The megakaryocyte converts the majority of its cytoplasm into 10-20 long, thin cytoplasmic processes called proplatelets (Fig 1.1) that extend into the sinusoidal blood vessels of the bone marrow (16). A single microtubule coil at the tip of the proplatelet forms a swelling called a platelet bud. Cytoplasmic elements, including platelet granules, travel up the proplatelets from the megakaryocyte cell body and are packaged in the platelet bud. The process by which the platelet bud separates from the proplatelet is incompletely understood. Current theories suggest that the final maturation from proplatelet to platelet may take place in the bloodstream and be aided by shear forces within vessels. This theory is supported by the fact that proplatelets are consistently isolated from the bloodstream (17). By the above process, each megakaryocyte produces hundreds to thousands of platelets (17-19).

Microstructure

The equine platelet measures approximately 2.5 to 3.5 μm in diameter (20). The plasma membrane is composed of a phospholipids bilayer with a hydrophobic core. A vast array of protein receptors, including integrins, transmembrane protease activated receptors, immunoglobulin based receptors, tetraspanins, serotonin receptors, and thrombopoietin receptors, are located on the plasma membrane and are important for platelet adhesion and

Figure 1.1. Proplatelet formation by a megakaryocyte. Key features: PB, platelet buds; Branch, bifurcation in shaft of proplatelet; Swellings, periodic thickening of proplatelet shaft where granules and organelles have stalled; Dumbbell-shape, many platelets are released as dumbbell-shaped particles (18).
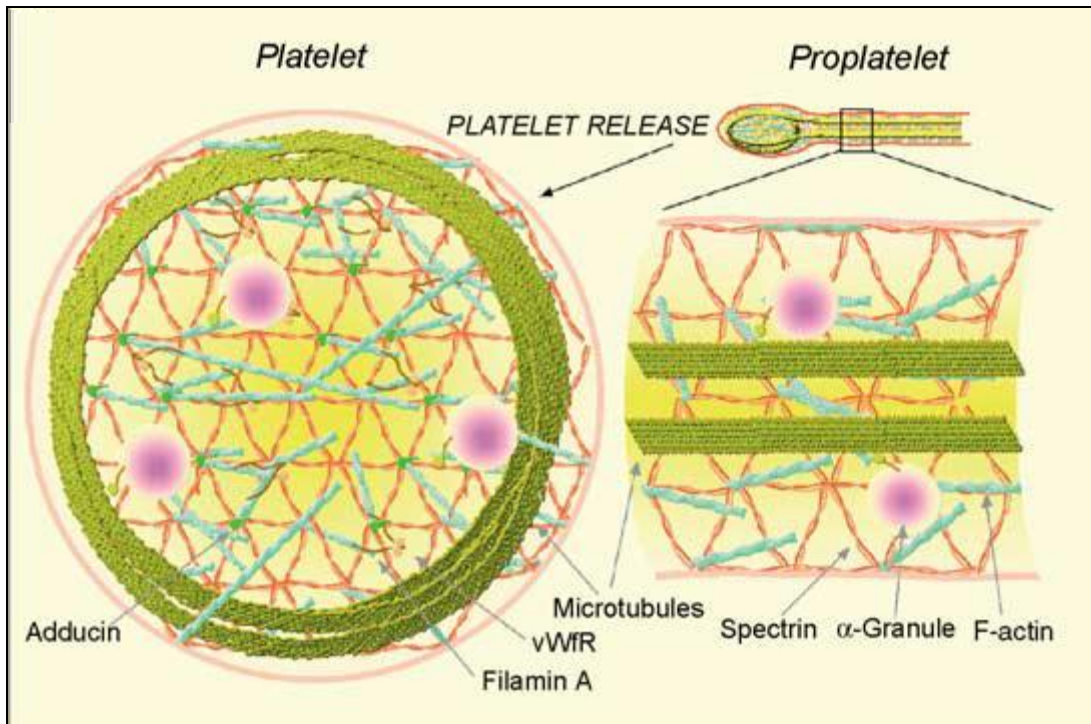
Modified from: Hartwig J and Italiano J, Jr. The birth of the platelet. J Thromb Haemost 2003;1:1580-1586. Used under fair use.

activation (21). The role of these receptors in the mechanism of action of PRP has not been investigated.

Platelets possess a complex cytoskeleton that facilitates necessary conformational changes during the process of platelet activation. A meshwork of spectrin and actin filaments establishes the cytoplasmic skeleton and is anchored to the cytoplasmic membrane (Fig 1.2) (18, 22). Actin filaments are crosslinked by the proteins filamin and α-adducin. Adducin caps the barbed ends of the actin filament, and its removal from actin filaments plays an important role in platelet spreading during platelet activation (23). A single coiled microtubule beneath the plasma membrane is responsible for maintaining the discoid shape of the platelet.

While the basic aspects of platelet structure are conserved, certain species-specific adaptations in platelet structure exist. The platelets of many mammalian species contain a surface-connected maze of plasma membrane invaginations called the open canalicular system (OCS). This system acts as a channel for the release of granules during platelet activation. The same receptors that are found on the plasma membrane are present on the surface of the OCS. During activation, the OCS is everted to the platelet exterior, increasing the number of surface receptors available for ligand binding. Ruminant, equine, and camel platelets do not have an OCS and therefore do not have a mechanism for increasing membrane surface area (Fig 1.3) (10). Platelets from these species release the contents of their granules by fusion of the granules directly to the plasma membrane with subsequent discharge of granule contents (21). This important difference in degranulation may prove important when comparing growth factor release from platelets in human and equine PRP.

Platelets contain three types of secretory granules: alpha granules, dense granules, and lysosomal granules. Alpha granules are the largest and most numerous of the platelet granules.

Figure 1.2 Platelet microstructure. The spectrin-actin meshwork provides structural stability.

The coiled microtubule (shown in green) maintains the platelet's discoid shape (18).

Modified from: Hartwig J and Italiano J, Jr. The birth of the platelet. J Thromb Haemost

2003;1:1580-1586. Used under fair use.

The alpha granules found in equine platelets are larger and more highly organized than the alpha granules of human platelets (10). Human alpha granules are compartmentalized into two regions, which may allow the platelet to differentially release proteins in response to activation (24). It is unknown whether equine alpha granules are compartmentalized in this manner. Alpha granules acquire proteins by both endogenous synthesis and uptake and packaging of plasma proteins by receptor-mediated endocytosis and pinocytosis (25). Human platelet alpha granules contain 284 different proteins, including Von Willebrand factor (vWF), factor V, proteoglycans, thrombospondin, fibronectin, fibrinogen, albumin, immunoglobulin, and numerous growth factors (26). Platelet derived growth factor (PDGF), transforming growth factor (TGF)-β1, TGF-β2, epidermal growth factor, vascular endothelial growth factor, insulin like growth factor-I, and hepatocyte growth factor are found in platelet alpha granules and are believed to promote tissue repair (6).

Dense granules, less numerous and smaller than alpha granules, contain 40 known proteins including cell signaling proteins, molecular chaperones, cytoskeletal proteins, proteins involved in glycolysis, and proteins involved in platelet functions (27). Dense granules also serve as a storage site for calcium and serotonin, important for initial vasoconstriction and formation of the platelet plug (28). The third type of platelet granule, lysosomal granules, contain acid-dependent hydrolases including glycosidases, proteases, and lipases (28). Lysosomal granules are likely vestigial remnants left over from the megakaryocyte cytoplasm and have no known function in platelet function (12).
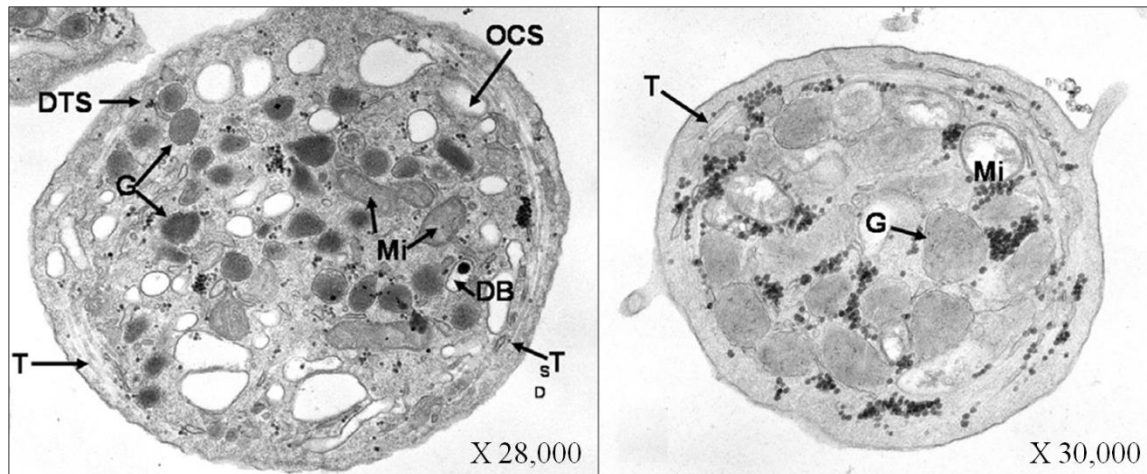
Figure 1.3 Electron micrographs of human (left) **and equine** (right) **platelets.** Key features include mitochondria (Mi), dense bodies (DB), open canalicular system (OCS), alpha granules (G), dense tubular system (DTS), and circumferential microtubule coil (T). Note the larger size of the alpha granules of the equine platelet compared to the human platelet (10).

Modified from: Gader AG, Ghumlas AK, Hussain MF, Haidari AA, and White JG. The ultrastructure of camel blood platelets: A comparative study with human, bovine, and equine cells. Platelets 2008;19:51-58. Used under fair use.

Activation

Platelet activation is an essential component of hemostasis. Platelet activation is stimulated by exposure to collagen, adenosine 5'-diphosphate (ADP), proteolytic enzymes (e.g., thrombin, trypsin), antigen-antibody complexes, and platelet activating factor secreted by leukocytes (29). Regardless of the stimulus platelets undergo a specific sequence of events following activation that includes adhesion, aggregation, and the release reaction where granule contents are released to the extracellular environment. *In vivo*, platelets adhere to exposed collagen fibrils via membrane glycoprotein receptors (30). These receptors bind to the plasma glycoprotein vWF that in turn binds collagen. Following adhesion to the extracellular matrix, platelets adhere to one another through the interaction of fibrinogen with surface receptors (IIb and IIIa) (31). Platelet activation triggers cytoskeletal reorganization, changing their shape from discoid to spherical. Next, finger-like projections extend from the periphery of the platelet and the platelet flattens, concentrating its granules in the center and giving it a "fried egg" appearance (Fig 1.4) (22). This change in shape places platelet granules in close proximity to the OCS (or plasma membrane in those species without an OCS) in preparation for the release reaction. Membrane fusion occurs between the granule and platelet membrane, and the granule contents are exocytosed. Coagulation factors within alpha granules ensure fibrin formation and promote platelet plug stabilization. Calcium and ADP from dense granules recruit additional platelets to the aggregate, forming the platelet plug (31, 32). Following formation of the platelet plug, clot retraction occurs via platelet myosin filament contraction (22). Initially clot retraction stabilizes the platelet plug, with further retraction serving as the primary step in thrombus removal.

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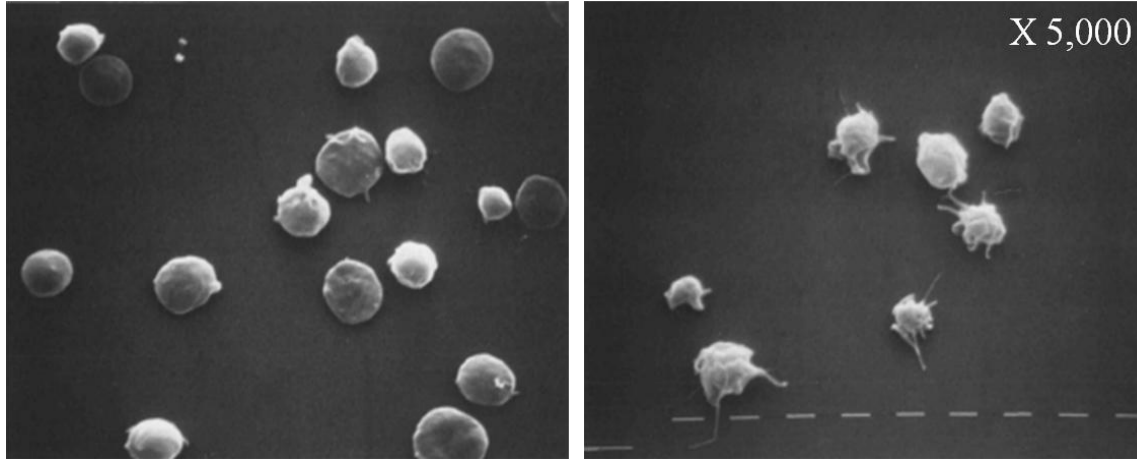
Figure 1.4 Electron micrographs of resting (left) **and activated** (right) **platelets.** The resting

platelets have a discoid shape. ;Note the thin filopodial extensions and irregular shape of the

activated platelets (29).

Modified from: Zucker MB and Nachmias VT. Platelet activation. Arteriosclerosis 1985;5:2-18.

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Platelet-Rich Plasma

The term "platelet-rich plasma" is a nonspecific term often used to refer to a heterogenous group of blood products more correctly referred to as "platelet concentrates". Platelet concentrates created using different techniques contain variable numbers of platelets and leukocytes and may be classified as leukocyte- and platelet-rich plasma (L-PRP) or "pure" PRP (P-PRP) (33). In L-PRP, both platelets and leukocytes are in higher concentration than that found in whole blood. Producing P-PRP involves an additional step of filtering to remove leukocytes or a technique that allows harvest of platelets without the white blood cells (WBC) (34). In the human and equine PRP literature, platelet and WBC numbers for a specific product used are often not reported, making comparisons between "PRP" products difficult when assessing efficacy.

For most applications, anti-coagulated blood is collected, processed into PRP, and injected intra-lesionally (tendon or ligament injuries) or applied topically (wounds or surgical sites). PRP may also be used intra-articularly for the treatment of osteoarthritis (8). Clinicians may choose to utilize the PRP in an inactivated state and rely on exposure of platelets to damaged tissue or collagen to stimulate platelet activation and granule release. Theoretically, this may result in a "sustained release" of growth factors from platelets. Some *in vitro* studies suggest that relying on endogenous activation results in incomplete degranulation and growth factor release (35-37). Alternatively, platelets can be activated prior to use by exposure to substances such as bovine thrombin or calcium chloride (38). Almost 100% of stored growth factors are released within one hour of exogenous activation (39).

The versatility and ready availability of PRP makes it a promising therapy for a wide range of applications in human and equine medicine. Further investigation into its mechanism of

action will make it possible to optimize preparation protocols and identify those disease conditions that will benefit most from its application.

<u>In Human Medicine</u>

Autologous PRP has been used extensively in human medicine for over 20 years. The fields of dentistry and orofacial surgery quickly adopted this therapeutic as a way to enhance bone formation and quality at dental extraction (40) and implant sites (41, 42), and improve success rates in difficult cases such as severe periodontal disease (40) or sinus floor reconstructions (43-45).

The use of platelet concentrates has shown beneficial effects on human wound healing. The use of platelet-fibrin glues in cosmetic surgeries resulted in decreased drain usage, post-operative pain and swelling, and operating time (46, 47). Donor sites for split-thickness skin grafts treated with PRP demonstrated more rapid epithelialization and less scarring and depigmentation than control donor sites and demonstrated increased epithelial budding and a more mature dermis histologically (48). In patients with chronic, non-healing wounds of the distal extremities, twice daily application of platelet concentrate resulted in epithelialization in 93% of wounds (49) and a 78% rate of limb salvage in patients for which amputation had previously been recommended (50).

Randomized, controlled clinical trials on the effects of PRP for orthopedic applications are largely lacking; however, smaller case series support a positive clinical effect. An increased rate of fracture union has been demonstrated when platelet concentrates were applied at the time of fracture revision or injected percutaneously into the fracture gap (51, 52). In total knee arthroplasty patients, intra-operative application of PRP to bone ends and exposed tissues

resulted in less post-operative blood loss and pain, greater range of motion, and a shorter hospital stay than patients that did not receive PRP (53). In diabetic patients, PRP application resulted in improved healing and fewer complications following ankle fusion (53).

PRP therapy in human sports medicine most closely parallels the use of PRP in the equine patient. In a large, randomized, controlled trial of patients with lateral epicondylitis, more patients who received PRP injections had a positive outcome compared with corticosteroid-treated controls (73% versus 50%) (54). Patients in the corticosteroid group improve initially and then declined, while patients in the PRP group continued to improve (54). Other smaller trials have found similar results when treating lateral epicondylitis with PRP (55, 56). A small study of PRP injection for chronic plantar fasciitis found that 77.9% of patients were symptom free one year following injection (57). It should be noted that this trial had a very small sample size and did not include controls (57). The results of pilot studies using PRP for chronic patellar tendonitis have been encouraging (58, 59).

In Equine Medicine

Tendon and ligament injuries are a common cause of morbidity in horses of all disciplines and represent the most commonly used application for platelet therapies in the horse. Healing of tendons and ligaments is notoriously slow, and the resulting scar is biomechanically inferior, making the tissues prone to reinjury (60). PRP is one of several regenerative therapies investigated to promote tendon and ligament healing. In a study using equine superficial digital flexor (SDF) tendon explants, PRP significantly increased expression of the tendon matrix genes collagens type I and III and cartilage oligomeric matrix protein without an increase in catabolic cytokines when compared to controls (61). Other studies have demonstrated a similar anabolic

effect (35, 62). A less profound effect was observed in suspensory ligament explants, potentially due to differences in matrix molecule expression between the two tissue types, and the authors suggest that PRP therapy may be most beneficial in specific types of injuries (63).

Bosch et al. evaluated the effect of a single PRP treatment in horses using a bilateral mechanically-induced model of forelimb SDF tendonitis (7, 64). Collagen, glycosaminoglycan, and DNA content were higher in the PRP group (7), further supporting the existence of an anabolic effect. PRP-treated tendons had a higher strength at failure and elastic modulus than controls (7), suggesting a stronger repair. Neovascularization was higher in the PRP-treated group (64), and may be one of the mechanisms by which PRP contributes to tendon and ligament healing. Although the mechanical model results in gross and ultrasonographic lesions similar in appearance to clinical core lesions, differences in the underlying pathophysiology of the disease do exist. Controlled clinical trials are needed to more completely assess the efficacy of PRP in clinical cases of flexor tendonitis.

Small clinical case reports have shown promise for the successful treatment of tendon and ligament injuries with PRP. Intralesional injection of PRP in Standardbreds with severe midbody suspensory ligament desmitis enabled return to racing when combined with a controlled exercise program (5). Ultrasonographic improvement and decrease in lameness scores have also been documented in horses with SDF tendinopathy and suspensory ligament desmitis (65).

Chronic wounds are commonly encountered in equine medicine, and wounds on the distal limb are often slow to heal. Therapies that promote faster, more cosmetic wound healing result in decreased economic burden to the client and a faster return to use for the horse. Biopsies of surgical wounds treated with PRP combined with ascorbic acid contained more densely arranged collagen bundles (66) and more rapid epithelial differentiation (67) than

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controls. PRP application to small granulating surgical distal limb wounds favored granulation

tissue formation, and slowed healing during the first three weeks, although total healing time was

not significantly different from controls (9). Faster time to wound healing has not been

documented in surgical wounds; however, PRP may provide a benefit in large or chronic, non-

healing wounds, as seen in human medicine (49), by promoting formation of more mature

collagen and potentially decreasing scar tissue formation.

Intra-articular use of platelet concentrates is becoming increasingly popular. Potential

applications include the treatment of osteoarthritis and as an adjunct source of growth factors in

the treatment of subchondral cystic lesions. Lameness and joint effusion scores improved, and

no adverse effects were observed when PRP was administered intra-articularly for the treatment

of osteoarthritis (8). PRP has been used successfully in conjunction with bone marrow aspirate

concentrate and bone substitutes to fill medial femoral condyle cysts in a small number of horses

(68).

Methods of Preparing Platelet-rich Plasma

Prior to the widespread availability of commercial systems for platelet concentration,

blood banking apheresis units were used to make PRP for clinical use. For equine applications,

the term "apheresis" is somewhat of a misnomer, as apheresis technically refers to "the process

of removing a specific component from blood and returning the remaining components to the

donor" (69). Due to the large blood volume of the horse, an adequate volume of blood can be

collected safely without readministering the remaining blood components to the horse. The

major disadvantage of apheresis units is their cost, limited availability, longer set up and

processing times, and the expertise required to operate the equipment. Advantages include high platelet yields and the closed nature of the processing system (70).

As the popularity of PRP use has increased, commercial systems have been developed to make in-clinic preparation of PRP more practical and available. Commercial systems can be divided into automated/semi-automated systems and manual systems. Manual systems depend on the operator to determine what portion of the centrifuged blood is collected as PRP, while automated systems use infrared light, a density buoy, or shelf to sequester the platelet fraction (71). Many commercial systems require special centrifuges designed to accommodate the disposable.

One of the first semi-automated systems to be reported in the equine literature was the SmartPReP2® system (Harvest Technologies, Plymouth, MA) (Fig 3.1) (71). The system consists of an automated centrifuge with decanting capability and a dual chamber sterile disposable. The system uses an initial spin to separate the plasma from the majority of the red blood cells and then the plasma is automatically decanted into a separate chamber. A second spin serves to concentrate the platelets within the plasma fraction. Studies utilizing this system to concentrate equine platelets have reported platelet concentrations 3.77 to 5.0 times that of whole blood (35, 61, 63). In one report, the SmartPReP2® produced PRP with a 1.85 fold decrease in mononuclear cells compared to whole blood (61).

A relatively new automated system being used in equine practice (Magellan® Platelet Separator System, Arteriocyte Medical Systems, Cleveland, OH) (Fig 3.2) is designed to provide the sophisticated centrifugation technology available in blood banking equipment in a compact, less expensive, and more operator friendly system. If larger volumes of PRP are needed, the disposable kit may be used for up to three centrifugation cycles in the same patient. The starting

blood volume and final PRP volume (and therefore the degree of platelet concentration) can be customized for each patient. Syringe pumps on the unit control PRP aspiration, allowing for very minimal operator handling of the sample. When 60ml of whole blood is used to make 3ml of PRP, platelet counts over one million per microliter can be consistently achieved and are significantly higher than those reported for the SmartPReP2® system (71).

Manual commercial systems for preparing PRP are also available. Most are available in kit form. These systems provide a closed means of processing; however, they require a greater degree of sample handling than the Magellan system. Some systems have the added advantage of being less expensive than the disposables required for the automated systems. Purchase of a specialized centrifuge may or may not be required, depending on the system. Most systems are variations on a common theme: a tube for centrifugation equipped with a proprietary method for directed aspiration of PRP and platelet-poor plasma (PPP). Two examples are the GPS III® (Biomet Biologics, Warsaw, IN) and the GenesisCS® (Vet-Stem, Inc, Poway, CA) systems (Fig 3.3). Neither system has been reported in the equine literature; however, both systems are marketed to the veterinary community. Both systems utilize a single centrifugation cycle to remove the red blood cells and concentrate the platelets in a small plasma volume just above the packed red cells. The GPS III® system has a fixed, slanted dual density buoy designed to separate the platelet-rich layer from the platelet-poor layer of plasma. PRP and PPP are aspirated from separate ports on the system through tubing designed to access the desired fraction specifically. Although the manufacturer classifies the system as automated, the system requires the operator to execute multiple steps, including the resuspension of platelets. The manufacturer claims a 90% platelet recovery using human blood.

Figure 1.5 Harvest SmartPReP2® system. Disposable prior to centrifugation (left), centrifuge

(center), and disposable after centrifugation (right) showing separated blood components (72).

Available from http://www.harvesttech.com/products/whatisprp.html. Reprinted with

permission.

Figure 1.6 Magellan® Platelet Separator System shown empty (left) and loaded with separation kit (right) (71, 73).

Image on left modified from Sutter W. Autologous Cell-Based Therapy for Tendon and Ligament Injuries. Clin Tech Equine Pract 2007;6:198-208. Used under fair use.

Image on right modified from McCurdy B. Top ten innovations in podiatric care. Pod Today 2010;23:32-38. Used under fair use.

A similar but simplified manual system is the GenesisCS® system. The separation tube has one aspiration port for removing both PPP and PRP that is connected to a concave aspiration disc by a length of tubing. The aspiration disc does not have a function in separating the PRP layer and remains on top of the plasma to aid smooth aspiration of the blood components.

The cost and need for special equipment to produce PRP can be avoided by centrifuging blood collection tubes using a variety of protocols (36, 74). Blood is collected into syringes containing anticoagulant and transferred to empty blood collection tubes (74) or collected directly into evacuated tubes containing sodium citrate (8). Single, double, and triple centrifugation protocols have been reported (36, 65). These methods require the removal of caps and stoppers from the tubes to collect PRP, exposing the product to the environment and possible contamination, and are therefore considered "open" techniques. However, a recent report concluded that sterile platelet concentrates could be prepared using tube centrifugation as long as processing took place in a clean laboratory environment (76). Except for a single report (36), platelet numbers obtained with tube centrifugation methods are typically lower than those from commercial methods (74).

Quality Assessment Parameters

Platelet Numbers

Of particular clinical importance is the question of how many platelets are needed to produce a beneficial treatment effect. Several studies have investigated this question. Some authors recommend maximal platelet concentration to achieve PRP with very high platelet numbers (6, 48). An early *in vitro* study demonstrated that PRP, at platelet concentrations greater than four times baseline, enhanced proliferation and differentiation of adult human

Figure 1.7 Examples of manual PRP systems. The GPS III® (left) with slanted density buoy
(inset) and the GenesisCS® (right) with attached collection syringes (71, 75).

Image on left modified from GPS III Gravitational Platelet System Brochure. Warsaw, Indiana.
Used under fair use.

Image on right modified from Sutter W. Autologous Cell-Based Therapy for Tendon and
Ligament Injuries. Clin Tech Equine Pract 2007;6:198-208. Used under fair use.

mesenchymal stem cells in a dose-dependent manner (77). Extrapolation from this study led to

the commonly recommended target of 1 million platelets (equal to four to five times the average

human platelet count of 200,000 ± 75,000/µl) (2, 48).

Not all studies support the idea that very high platelet numbers are desirable in PRP.

Fibroblasts demonstrated a dose-dependent increase in type I collagen production when exposed

to platelet lysate; however, there was an inhibitory effect observed at the highest concentrations

(78). PRP with a platelet concentration 2.5 times that of baseline promoted proliferation of

cultured human osteoblasts and fibroblasts; however, PRP with higher platelet concentrations (5

times that of baseline) had an inhibitory effect (79). A similar study of cultured canine

osteoblasts demonstrated a marked cytotoxic effect at high PRP concentrations (80). The results

of these *in vitro* experiments are compelling, and several *in vivo* studies have demonstrated

similar findings. In an *in vivo* porcine model of anterior cruciate ligament repair, there was no

difference in biomechanical properties when platelets were concentrated at 3X and 5X baseline

(81). Bursting strengths of rat jejunal anastomoses were significantly higher than controls when

platelets were concentrated at 2X baseline; but PRP concentrated at 5X baseline resulted in a

significant decrease in the strength of the repair (82).

Several possible explanations exist for the inhibitory effect of platelets at high

concentrations. Increasing the platelet concentration of platelet lysates leads to an increase in pH

that is suboptimal for wound healing (78). Along with beneficial growth factors, PRP may also

contain inhibitory substances that have a negative effect on healing in high concentrations.

Thrombospondin-1 (TSP-1), a large extracellular matrix protein, inhibits cell adhesion,

proliferation, and neovascularization (83). TSP-1 is found in higher levels in concentrated PRP

and inhibits cell proliferation in a dose-dependent manner (84). High concentrations of growth factors may have cytotoxic or anti-mitogenic effects (41).

Extrapolation of an ideal platelet number for equine PRP from the human literature is difficult because of the lower average equine platelet count (142,000/µl) compared to humans (85). It is possible that species-specific differences in platelet physiology, such as mechanism of degranulation (10), or growth factor content of platelet alpha granules may influence the number of platelets needed in a PRP sample. Also, different applications (tendon and ligament injuries versus wounds) or different stages of the healing process (86, 87) may have differing requirements for platelet numbers and growth factor levels. *In vitro* and *in vivo* equine studies are needed to determine the optimal "dose" of platelets in equine PRP that are needed to achieve a positive tissue response.

Growth Factor Levels

The positive effects of PRP are believed to be due to the release of growth factors from platelet alpha granules (2, 88). Growth factors enhance healing by recruitment of cells to the wounded area, stimulation of cell proliferation, and enhancement of matrix synthesis (89). Some investigators suggest that the levels of growth factors present in PRP may be more important than platelet numbers (90). Several growth factors demonstrate a dose-response curve that reaches a point of diminishing returns as cell surface receptors for that growth factor are completely occupied (89, 91). Some growth factors have an inhibitory effect on cell functions once a high enough concentration is reached (92). The dose-response relationship is both growth factor and cell type dependent.

There are several potential problems with using growth factor levels to assess the quality of a platelet concentrate. Therapeutic concentrations of growth factors for specific equine applications have not been thoroughly defined (93). The commercial ELISAs that are used for quantifying growth factors are designed for quantifying human, not equine, growth factors and are sensitive to red blood cell contamination (62). Substances such as fibrin monomers, thrombospondins, and other proteins within the PRP sample itself may bind or interact with growth factors and prevent accurate measurement (94). Experimental sample handling, such as freezing or storing, alter *in vitro* growth factor concentrations, making extrapolation to clinical applications difficult (95). Additionally, growth factor levels in platelet concentrates vary greatly among individuals and can vary among different samples from the same patient (96-98).

Measuring growth factor levels in the clinical setting is impractical, and several studies have examined correlations with other parameters that are more easily measured, such as platelet and WBC numbers (70). An overall positive correlation between equine TGF-β1 and platelet numbers has been demonstrated (35, 70), but the same association was not found for IGF-1 (70). Other studies have failed to demonstrate a correlation between platelet numbers and growth factor levels, suggesting that platelet numbers may not provide an accurate estimate of growth factor levels within a sample (8, 74, 98, 99, 100).

White Blood Cell Numbers

Different methods of PRP preparation vary widely in the degree to which they concentrate WBCs. The role of WBCs in PRP remains unclear. Arguments have been made for both beneficial and harmful effects of WBCs. Some authors recommend the elimination of WBCs from PRP because of the pro-inflammatory cytokines they contain (35, 61, 101).

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Cytokine accumulation has been associated with the presence of WBCs in stored human allogeneic platelet concentrates intended for systemic administration (102, 103). White blood cells were positively correlated with expression of catabolic cytokines and negatively correlated with matrix gene expression in human PRP products (104) and in equine tendon and ligament explants treated with PRP (35). For this reason, some authors suggest the use of P-PRP (<3,000 WBC/µl) for specific applications such as intra-articular use or treatment of tendon and ligament injuries (35, 105).

Other authors propose several potential beneficial roles of WBCs in PRP. White blood cells play a vital role in tissue repair by modulating the immune response by secreting cytokines that mediate inflammation (interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6) and promote cell proliferation and differentiation (IL-4, VEGF) (106). White blood cells release VEGF and PDGF and stimulate platelets to release growth factors, enhancing total growth factor concentration over P-PRP (105, 107, 108). White blood cells are also likely responsible for the antimicrobial properties of PRP (88, 109). PRP inhibits the *in vitro* growth of *Staphylococcus aureus* and *Escherichia coli*. This suggests that L-PRP may be a useful tool in treating infections involving these organisms. When used during coronary artery surgery, PRP significantly decreased the occurrence of chest wound infection, chest wound drainage, and leg wound drainage (110).

Leukocyte- and platelet-rich plasma has been used clinically for a variety of applications in humans and horses (8, 110, 111). It has been used extensively to treat chronic tendonitis (5, 55), fracture nonunions (52), and chronic wounds (50) in humans and intra-articularly in both horses and humans without adverse effects (8, 111). There have been no clinical reports of an uncontrolled inflammatory response following L-PRP use. On the contrary, the use of L-PRP

has been reported to decrease pain and inflammation at treated sites (109, 112). Further *in vivo* studies comparing L-PRP and P-PRP are needed to determine the role of WBCs in PRP.

Platelet Counting and Activation

The ability to accurately count platelets in a PRP sample is an important consideration for quality assessment or comparison of PRP systems. Several factors should be considered when performing platelet counts. Platelet aggregation may occur during blood collection or processing of PRP and is of major significance when counting platelets in whole blood or platelet concentrates (12). Aggregation in PRP samples can lead to platelet counts that are falsely low because the analyzer counts these aggregates as a single platelet (113). Platelet aggregation may be influenced by the type of anticoagulant used. Lower platelet counts were demonstrated in human PRP collected in citrated anticoagulants compared to those collected in EDTA (114) However, this finding was not confirmed in another study (115). Most human and equine PRP studies have used citrate as the anticoagulant because it supports platelet metabolism (9, 66, 74, 116). The feathered edge of stained smears may be examined for platelet clumping in an effort to verify the accuracy of platelet counts. Hematology analyzers should be gaited for species-specific platelet and cell sizes to obtain accurate counts and caution should be exercised when comparing platelet counts from studies using different analyzers.

Another important parameter for assessing the quality of a PRP product is the degree of platelet activation that occurs during processing. Platelet activation may occur during blood collection, handling, and processing and can lead to platelet degranulation and loss of growth factors into the discarded PPP (36, 37, 94). Mean platelet volume (MPV) is an estimate of platelet size and increases with platelet activation as platelets change from a discoid to spherical

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shape (117). Measurement of MPV is possible using many automated hematology analyzers and may provide a rough clinical estimation of platelet activation in a PRP sample (117). Prolonged exposure to EDTA, prolonged storage at room temperature, refrigeration, and delayed exposure to anticoagulant may cause increases in MPV (118). Minimal changes in MPV are observed when blood is stored at 37°C, and increases in MPV associated with cooling are reversible (119). MPV ranges from 4.3 to 5.6fL in normal adult horses (85). Most hematology analyzers are capable of measuring MPV, making this a clinically useful method for estimating platelet activation in a PRP sample.

Conclusions

Platelet-rich plasma has been used for a wide range of applications in human and veterinary medicine. Although large, controlled, clinical studies are needed, especially in horses, the body of literature does support a positive effect. PRP has several advantages over other therapies. It is a regenerative therapy and therefore promotes restoration of normal tissue architecture and function. Platelet-rich plasma is used in an autologous manner in equine medicine; therefore, the risk of disease transmission or immune rejection is avoided. Platelet-rich plasma treatments are applied in a point-of-care manner, avoiding the hospitalization costs and processing delays associated with other regenerative therapies.

Many questions remain regarding the mechanism by which PRP may support healing. Determination of optimal platelet and WBC numbers in PRP for different applications is needed. Equine processing protocols must take into account the unique aspects of equine blood and platelets in order to reproducibly produce PRP products for specific clinical applications. Some clients are unable to afford PRP produced with expensive commercial systems. Development of

an inexpensive, safe, method of PRP preparation would make this therapy more widely available. An experiment was designed to compare three simple tube centrifugation methods with a commercially available manual method of PRP preparation and the study design and details are outlined in Chapter 2 of this thesis.

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Objectives and Hypothesis

Objectives

To evaluate the quality and bacteriologic safety of platelet-rich plasma (PRP) produced by three simple, inexpensive tube centrifugation methods and compare the results to those produced by a commercial system.

Hypothesis

At least one of the three tube methods would concentrate platelets within a clinically useful range with acceptable white blood cell concentration.

Chapter 2

Simple tube centrifugation for processing platelet-rich plasma in the horse

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Abstract

The purpose of this study was to evaluate the quality and bacteriologic safety of platelet-rich plasma (PRP) produced by three simple, inexpensive tube centrifugation methods and compare the results to a commercial system. Citrated equine blood collected from 26 normal horses was processed by four methods: blood collection tubes centrifuged at 1200 and 2000xg, 50ml conical tube, and commercial system. WBC, RBC, and platelet counts and mean platelet volume (MPV) were analyzed for whole blood and PRP, and aerobic and anaerobic cultures were performed. Mean platelet concentrations ranged from 1.55 to 2.58 fold. The conical method yielded the most samples with platelet concentrations greater than 2.5-fold and within the clinically acceptable range of >250,000 platelets/μl. WBC counts were lowest using the commercial system and unacceptably high using the red top methods. The conical tube method

may offer an economically feasible and comparatively safe therapeutic alternative to commercial

PRP systems.

Introduction

Platelet-rich plasma (PRP) is a blood product made by concentrating platelets in a small volume of plasma and is used extensively in human dentistry, orofacial surgery, and sports medicine for a wide variety of applications (1-3). In equine practice, PRP has gained popularity for the treatment of wounds and tendon and ligament injuries (4-7). The exact mechanism of action of PRP is unknown; however, the release of high concentrations of growth factors from platelet alpha granules as platelets degranulate is widely believed to play a critical role (8, 9). Alpha granules contain several growth factors known to have beneficial effects on tissue healing, including platelet-derived growth factor (PDGF), transforming growth factor β-1, vascular endothelial growth factor (VEGF), and insulin-like growth factor-I (4, 10, 11).

In vitro and *in vivo* studies investigating the effects of PRP on healing collagenous tissues in horses are limited (4, 7, 12-14). Treatment of equine distal limb wounds with PRP have shown variable results from improved healing through the promotion of cell differentiation and the formation of organized collagen (7, 12) to the stimulation of excessive granulation tissue and delayed wound healing (13). Treatment of superficial digital flexor tendon explants with PRP showed enhanced tendon matrix gene expression (4). In surgically-created superficial digital flexor tendon lesions, PRP resulted in higher strength at failure and improved collagen fiber organization and biochemical properties (6). Two small clinical case series treated with PRP show promising results for treating tendon and ligament injuries (5, 15).

Use of PRP in equine practice is becoming increasingly popular. A number of commercial systems are available that result in platelet products with a wide range of platelet and WBC concentrations. There is limited information available regarding the optimal platelet and WBC content necessary to achieve a desired biologic effect and it may be that specific products

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are better for certain applications (10, 16-18). Both fold change in platelet numbers compared to whole blood and absolute platelet numbers are used for determining adequacy of platelet concentration (9). Fold change is most commonly used for describing a particular PRP product; however, it may be that absolute platelet number is a more valid point of comparison than simply the relative degree of concentration.   Without actually measuring growth factor levels in individual samples, platelet numbers are the best estimate of the growth factor levels expected within a tissue following platelet degranulation (19-21).

There is no single definition of PRP in the human or veterinary literature and "PRP" products include wide ranges of platelet and WBC concentrations. These ranges reflect the various separation methods and the lack of consensus on specific composition of the final product. Obviously platelet numbers are the primary concern; however, optimal WBC concentrations in PRP is a topic of discussion (17, 18, 22). The presence of WBCs in stored human platelet concentrates has been associated with posttransfusion complications such as febrile nonhemolytic transfusion reactions due to the accumulation of inflammatory cytokines (23, 24). Prestorage leukocyte removal prevents increasing cytokine levels (25, 26). How this information may apply to equine applications of PRP is not known; however, consideration must be given to avoiding excessive WBC concentrations in PRP for clinical applications.

Platelet concentrations in PRP in the literature commonly range from a three to five fold increase over whole blood or a minimum concentration of 300,000 to 1,000,000 platelets/μl (8, 27-29). These values proposed for human PRP are not based, however, on controlled studies but rather educated estimations from the limited data available *in vitro* and *in vivo* (9, 30). More recently, some studies suggest that high platelet numbers are no better than moderate numbers or may actually have detrimental effects (31, 32). Proposed minimum platelet numbers for equine

PRP have been extrapolated from the human literature rather than controlled studies either *in vitro* or *in vivo* in the horse. Key species differences in average platelet counts and platelet physiology between humans and horses precludes an accurate extrapolation from the human literature (33, 34). Equine platelet counts are amongst the lowest reported for mammals (33). The average platelet count for humans is 200,000/µl (9) compared to 142,000/µl for horses (33). Because of these species differences, there are no established cut-off values for platelet numbers in equine PRP, merely suggested values.

The final platelet and WBC concentration in PRP is dependent on the method of preparation used and the original platelet number in whole blood. Methods of PRP preparation include apheresis, fully automated systems, and centrifugation using either commercial systems or blood collection tubes (10, 35, 36). Apheresis and automated methods are relatively closed systems that minimize operator error, have high repeatability, yield consistently high platelet concentrations, and have a low risk of contamination (4, 10, 18); however, they require expensive specialized equipment and disposables. Commercial tube centrifugation systems provide a means of processing PRP with minimal manipulation and a relatively low risk of contamination; however, the equipment and disposables are also expensive and can achieve inconsistent platelet yields as a result of operator error (18).

Techniques using blood collection tubes lack the need for special equipment and are inexpensive; however, removal of PRP manually is prone to greater operator error and greater potential for bacterial contamination because of the open nature of the final PRP harvest. The potential for the introduction of environmental contaminants is of particular concern when working with a blood product. Development of an inexpensive point-of-care alternative to

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commercial systems capable of producing a product free of bacterial contamination would make PRP therapy more widely available to horse owners of all economic means.

The purpose of this study was to evaluate the quality and bacteriologic safety of platelet-rich plasma (PRP) produced by three simple, inexpensive tube centrifugation methods and compare the results to those produced by a commercial system. We hypothesized that one of the three tube methods would concentrate platelets within a clinically useful range with acceptable WBC concentration.

Materials and Methods

Experimental animals – This project was approved and performed according to the guidelines of the Virginia Tech Institutional Animal Care and Use Committee. Informed consent was obtained from the owner for each horse included in the study. Twenty-six healthy horses without clinical signs of disease were used for the study (7 mares, 19 geldings; 2 – 12 years (mean 6.4 ± 2.2 years)). Breeds included Quarter Horse (2), American Paint Horse (4), Tennessee Walking Horse (5), Thoroughbred (5), Standardbred (1), and mixed breed (9). Horses used for the study were owned by clients and staff of the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM).

Blood collection and laboratory set-up – Blood was collected from either the left or right jugular vein. The venipuncture site was prepared using standard aseptic technique. Sterile technique was maintained throughout the collection process. Blood (180 ml) was collected into three 60 ml syringes containing 8 ml of acid citrate dextrose-A anticoagulant each using an 18 gauge 1.5 inch hypodermic needle and a sterile extension set. Syringes were gently rocked during collection and transport to the lab to ensure proper mixing. Platelet-rich plasma was processed using four different techniques: two blood tube centrifugation methods, a conical tube method, and a commercial method (GenesisCS, Vet-Stem, Inc., Poway, CA, USA). Samples were processed using sterile technique on a laboratory bench top cleaned with 70% ethanol. Prior to harvest of PRP using the various techniques described, the cap of the blood collection tube was disinfected with alcohol, allowed to dry and removed. Centrifuges were calibrated prior to starting the study. Prior to aliquotting blood for sample processing, a 3ml sample of whole blood was saved for complete blood count and culture.

Blood tube centrifugation methods for processing PRP – The protocols utilized for the blood

tube centrifugation method were selected based on the available equine literature and preliminary

studies using this method. For each of the blood tube centrifugation methods, three glass blood

tubes (red top, BD Vacutainer Serum Tubes, Franklin Lakes, NJ, USA) were filled with 10ml of

blood each. The amount of blood in each tube was standardized by weight. The tubes were

centrifuged in a table top centrifuge (Model CR4-22 Juan, Inc., Winchester, VA, USA) for 3

minutes at either 1200 x g (red top 1200g) or 2000 x g (red top 2000g) depending on method.

The platelet poor plasma (PPP) was removed from the top of the tube using a sterile 14 gauge

pipetting needle (blunt end pipetting needle, Poppers and Sons, Inc., New Hyde Park, NY, USA)

and a 6ml syringe, leaving a standardized 1 ml volume of PRP. The remaining plasma, buffy

coat, and top 1mm of the packed RBCs was collected for a total of 1ml of PRP from each tube.

Platelet-rich plasma samples from the three tubes were pooled, yielding a total of 3ml of PRP for

each tube centrifugation method.

Conical tube method for processing PRP – Thirty milliliters of blood was aliquotted into a

sterile, skirted, polypropylene, 50ml conical centrifuge tube (Corning Life Sciences, Lowell,

MA). The tubes were centrifuged for 15 minutes at 720 x g (conical 720g). The PPP was

removed using a sterile 14 gauge pipetting needle (as described above) and a 20ml syringe,

leaving a standardized 3 ml volume of PRP. The remaining plasma, buffy coat, and top 1mm of

the packed RBCs were collected for a total of 3ml of PRP.

Commercial method for processing PRP – The sterile disposable (GenesisCS) was filled with

30ml of blood and centrifuged for 15 minutes at 720 x g (Genesis 720g) in a specialized

centrifuge (Hermle Z300, Labnet, Woodridge, NJ, USA) designed to accommodate the

disposable and according to manufacturer's instructions. A three-way stopcock with attached

20ml and 6ml syringes was connected to the access port on the disposable. A line drawn 3mm above the packed RBCs was used to standardize the amount of PPP removed. The 20ml syringe was used to aspirate the PPP until the aspiration disc reached the line drawn on the tube, the stopcock was turned, and the remaining plasma, buffy coat, and top 1mm of the packed RBCs was collected for a total of 3ml of PRP.

Sample Analysis – Whole blood and PRP samples for all horses were submitted to the VMRCVM Clinical Pathology Laboratory for automated quantification of platelet, white blood cell (WBC) and RBC counts, and mean platelet volume (MPV) (Advia 2120 Hematology Analyzer, Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA). All samples were placed on a rocker for 10 minutes prior to analysis to ensure adequate mixing.

Bacteriologic culture of PRP samples – Samples of whole blood and PRP from each horse were reserved for aerobic and anaerobic culture. A 250μl aliquot of each sample was transferred to a 5% sheep blood agar plate and a pre-reduced anaerobic plate (Anaerobe Systems, Morgan Hill, CA, USA). All plates were incubated at 37˚C; blood agar plates were maintained in an atmosphere of 7% CO_2. Cultures were checked daily for growth and reported as negative if there was no visible growth by day 7. Positive cultures were submitted to the VMRCVM Microbiology Laboratory for identification.

Statistical Analysis – Based on the lack of a consensus in the literature regarding validity of using either fold change (concentration compared to whole blood) or absolute numbers, data for platelets and WBCs were analyzed by two different statistical methods. Normal probability plots were generated to verify that MPV and fold change for RBC, WBC, and platelets, and the ratio of fold changes for WBC and platelets (WBC:Platelet) followed an approximately normal distribution. The effect of method on these outcomes (MPV and fold change for RBC, WBC,

and platelets, and WBC:Platelet) was then assessed using mixed model ANOVA followed by Tukey's procedure for multiple comparisons (Proc MIXED, SAS version 9.2, SAS Institute, Inc., Cary, NC, USA). The linear model specified method as a fixed effect, horse as a random effect and Kenward-Roger denominator degrees of freedom. For each model, residual plots were inspected to verify model adequacy (i.e., that the errors followed a normal distribution with constant variance).

Estimates of what might be considered clinically useful products were extrapolated from the available literature (6, 8, 27-31, 33, 37). In the human and equine literature, optimal numbers of platelets and WBCs are at best speculative and based on limited data. Most products produced by point of care devices used commonly in equine and human practice are leukocyte rich and were therefore deemed a relevant comparison for this study (6, 38-41). Absolute numbers of platelets and WBC were categorized as either clinically "acceptable" or "unacceptable" using a clinically relevant cutoff. The cutoff point for clinically acceptable platelet and WBC values were > 250,000 platelets/μl and < 30,000 WBC/μl. Subsequently, the number of "acceptable" samples (separately for platelets or WBCs) was compared between the methods using binary logistic generalized estimating equations (GEE) (Proc GENMOD, SAS version 9.2, SAS Institute, Inc., Cary, NC, USA). The model specified sample classification ("acceptable" vs. "unacceptable") as outcome, method as a predictor, logit as the link function, binomial as the distribution, and horse as a blocking factor (with an independent working correlation matrix). P-values were based on type 3 Wald statistics. Statistical significance for all analyses was set at $P < 0.05$.

Results

Mean platelet fold change was greatest for the conical method (2.58x); however, this difference only reached statistical significance in comparison to 1200g (P<0.0001; figure 1). Mean WBC fold change for the Genesis method was significantly lower than the other 3 methods (P<0.0001; figure 1). Mean WBC fold change for the conical method was significantly lower than the means of either red top 1200g or 2000g. Mean WBC:Platelet ratios were significantly different between each of the groups (P<0.0001; figure 2). Red top 1200g and 2000g were significantly greater than either conical or Genesis methods and the conical method was significantly greater than Genesis.

Mean total platelet counts were significantly different between groups (P<0.0001; figure 3). Platelet counts for red top 1200g were significantly less than red top 2000g and conical 720g. Because the mean platelet count for a group may not accurately represent the number of samples containing platelets at clinically relevant concentrations (> 250,000/µl), the frequency of samples considered "acceptable" versus "unacceptable" was analyzed and compared in pairwise fashion to determine an Odds Ratio (OR) (Table 1). Odds Ratios for all pairwise comparisons are reported; however, only that comparing the conical and Genesis methods is considered clinically relevant because of the unacceptably high WBC numbers in the two red top tube methods. The conical method resulted in a significantly greater number of "acceptable" samples (92.3%) compared to the Genesis method (69.2%) when considering platelet number alone (OR=3.67, P=0.04).

White blood cell counts for red top 1200g and 2000g were significantly greater than for either conical or Genesis methods and the conical method was significantly greater than Genesis. The Genesis system had more samples with WBC within the "acceptable" range (96.1%) than

the other three methods and the conical method had significantly more WBC within the "acceptable" range (69.2%) than red top 1200g or 2000g (26.9% each) based on the GEE analysis ($P<0.0001$; Table 1).

Mean MPV for the red top tube methods was not significantly different from whole blood, and mean MPV for the conical and Genesis methods were significantly lower than whole blood (Table 2; $P=0.0001$). Mean RBC fold change for Genesis was significantly lower than the other 3 groups (Table 2; $P<0.0001$). There was no bacterial growth on any plates up to day 6. A single small colony was present on 6/280 samples (2.1%) on day 7: Whole blood, red top 1200g, and Genesis (2 each). Isolated organisms included *Kocuria rosea* (3), *Micrococcus species* (2), and *Propionibacterium sp* (1).
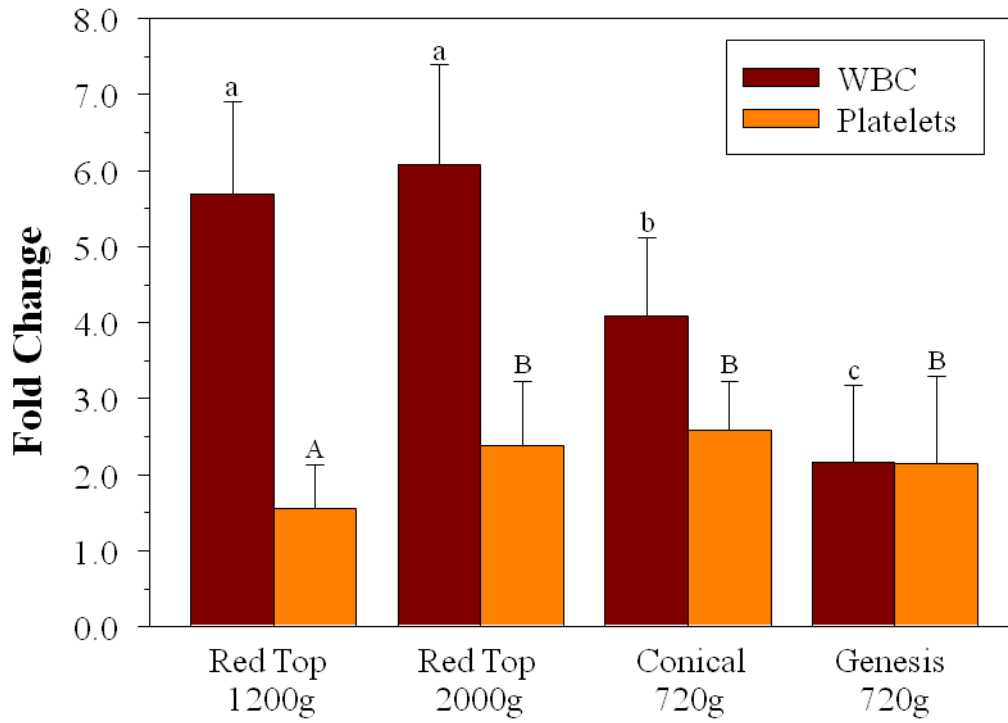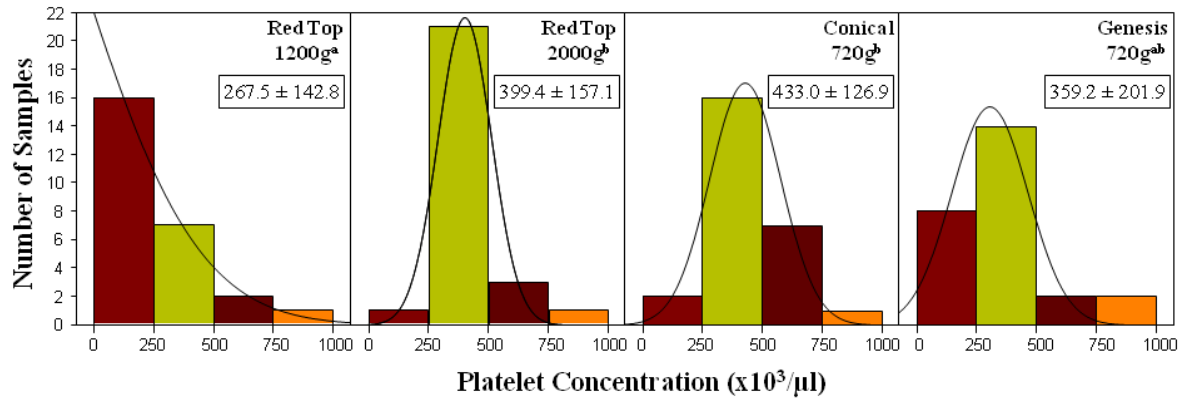
Figure 2.1 Fold change of white blood cells (WBC) and platelets for four methods of preparing platelet-rich plasma. Mean ± SD. Superscript letters indicate significant differences between groups.
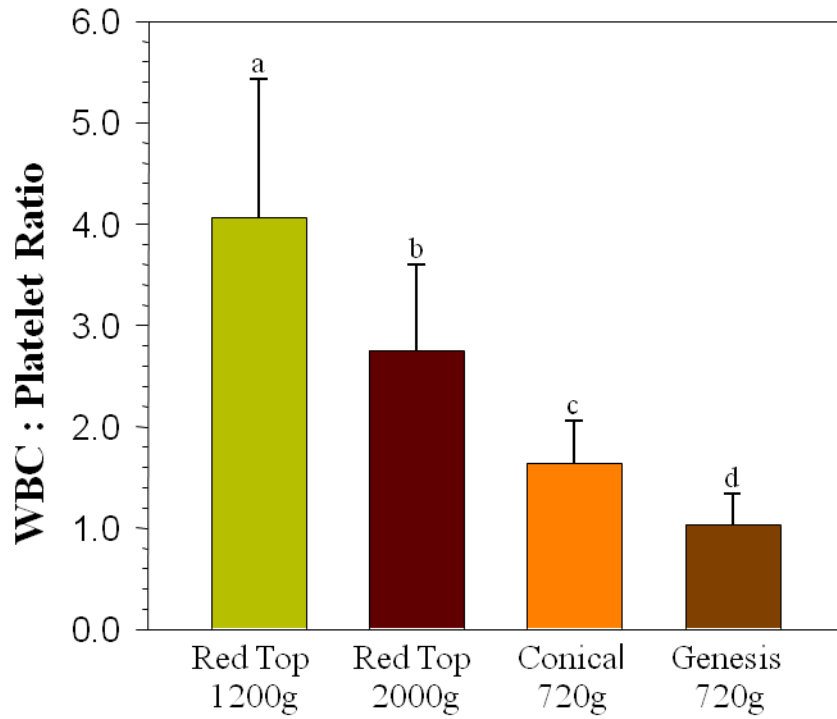
Figure 2.2 White blood cell (WBC):Platelet ratio for four methods of preparing platelet-rich plasma. Mean ± SD. Superscript letters indicate significant differences between groups.

Figure 2.3 Number of total samples for each of four methods of preparing platelet-rich plasma achieving relevant ranges for platelet concentration and associated Gaussian curves demonstrating distribution of samples. Superscript letters indicate significant differences between groups. Small boxes: Mean platelet count ± SD (x 10^3/µl)

Table 2.1 Odds ratios (OR) and 95% confidence intervals for platelet and white blood cell numbers based on pairwise comparisons between groups. An OR greater than 1.00 indicates that the first member of the pair has a greater number of "acceptable" samples. An OR less than 1.00 indicates that the second member of the pair has a greater number of "acceptable" samples.

Pairwise Comparison	Platelet Numbers		WBC Numbers	
	Odds Ratio (95% CI)	P value	Odds Ratio (95% CI)	P value
Red Top 1200g vs. Genesis 720g	0.27 (0.08–0.93)	0.04	0.003 (0.001–0.014)	<0.001
Red Top 2000g vs. Genesis 720g	2.44 (0.89–6.69)	0.08	0.003 (0.001–0.011)	<0.001
Conical 720g vs. Genesis 720g	3.67 (1.07–12.63)	0.04	0.049 (0.013–0.189)	<0.001
Red Top 2000g vs. Red Top 1200g	8.88 (3.49–22.60)	<0.001	0.85 (0.50–1.47)	0.57
Conical 720g vs. Red Top 1200g	13.37 (4.42–40.43)	<0.001	15.13 (3.41–67.01)	0.001
Conical 720g vs. Red Top 2000g	1.50 (0.60–3.78)	0.38	17.70 (3.83–81.75)	0.001

Table 2.2 Mean platelet volume (MPV) and red blood cell (RBC) fold change for whole blood and four methods of preparing platelet-rich plasma Mean ± SD. Superscript letters indicate significant differences between groups.

Preparation Method	MPV (fL)	RBC Fold Change
Whole Blood	9.63 ± 1.29^a	N/A
Red Top 1200g	9.20 ± 1.05^{ab}	0.79 ± 0.23^a
Red Top 2000g	9.11 ± 1.29^{ab}	0.84 ± 0.19^a
Conical 720g	8.33 ± 1.38^c	1.06 ± 0.31^b
Genesis 720g	8.56 ± 0.98^{bc}	0.63 ± 0.27^c

Discussion

        Three methods of concentrating platelets by tube centrifugation were evaluated and compared to a commercial system. The goal was to identify a simple, inexpensive means of producing bacteria-free PRP for clinical applications in the horse that would make this regenerative medicine therapy more widely available. We specifically chose only protocols involving a single centrifugation rather than double centrifugation protocol to minimize the potential for bacterial contamination and to keep the processing as simple as possible for application in clinical practice. The two methods investigated utilizing blood collection tubes were capable of marginal platelet concentration but were deemed clinically unacceptable due to excessive concentration of WBCs. The conical tube method demonstrated adequate ability to concentrate platelets without undue WBC concentration and may be a suitable alternative to commercial systems for selected equine applications.

        Despite increasing numbers of PRP studies in the veterinary literature, equine-specific parameters for platelet and WBC counts for clinical applications of PRP are lacking. Although fold change is commonly used to evaluate the quality of platelet concentrates, absolute numbers of platelets may provide a more accurate standard of quality. Statistical analyses performed in this study yielded comparable results for both fold change and raw platelet numbers as a means of comparison. Target values for platelet concentrations in PRP described in the equine literature are extrapolated from the human literature and do not take into account potentially important species differences in platelet counts or physiology such as mechanism of degranulation or growth factor contents per platelet (33, 34). Neither human nor equine values suggested in the literature have a basis in controlled scientific studies and are instead inferred from *in vitro* studies (28, 30), suggesting that further work be done to identify optimal doses of

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platelets for specific applications. Studies involving *in vivo* use of PRP in horses are limited and reported platelet numbers are highly variable (5-7, 13). Additional equine-specific research is needed to define the minimum platelet concentration necessary to achieve beneficial biologic effects.

Recent studies in pigs and humans suggest that lower platelet concentrations may be equally or more effective than higher concentrations and that high platelet concentrations may even have detrimental effects (31, 32). There was no significant difference in the mechanical properties in a porcine model of anterior cruciate ligament repair between 3x and 5x platelet concentration (32). Human osteoblasts and fibroblasts demonstrated maximal cell proliferation at 2.5x platelet concentration compared to 3.5x and 4.2-5.5x (31). The mean platelet concentrations achieved using the conical method was within the range of those described in these studies and another equine study (4).

The importance of low WBC numbers in autologous platelet concentrates is controversial; the use of leukocyte reduced PRP (<3,000 WBC/μl) has been suggested for use in specific applications (16-18, 21, 37). White blood cells were positively correlated with expression of catabolic cytokines and negatively correlated with matrix gene expression in human PRP products (19) and in equine tendon and ligament explants treated with PRP (21). Although this *in vitro* work is compelling, the clinical relevance is unknown because of the lack of direct clinical comparison between leukocyte rich and leukocyte depleted products *in vivo* (18).

White blood cells do play a beneficial role in enhancing tissue repair as a result of their immunomodulatory and antimicrobial effects. Myeloperoxidase contained within neutrophils and monocytes contributes to the antimicrobial activity of PRP (42-45). Leukocyte rich PRP

reduced wound drainage when applied following coronary artery bypass surgery (40) and has

shown beneficial effects in human and equine studies, including application during open

subacromial decompression (39), chronic elbow tendinosis (41), and delayed bone union (38) in

human patients and mechanically-induced tendon lesions in horses (6). Human patients reported

diminished pain and inflammation at the treated sites (39, 41). In addition, leukocytes release

VEGF and PDGF themselves and stimulate platelets to release growth factors, enhancing the

growth factor concentration over leukocyte reduced PRP (17, 20, 46).

MPV is a measure of average platelet size in a sample and has been shown to increase as

platelets become activated and change from a discoid to spherical shape (47). Platelet activation

may be undesirable for applications where slow release of growth factors from gradual platelet

degranulation is considered beneficial. A PRP sample with an MPV similar to whole blood

implies that processing did not cause platelet activation. MPV in our PRP samples was not

significantly higher than those from whole blood for any of the processing methods, suggesting

lack of platelet activation.

Despite using clean, but not strictly aseptic techniques (i.e. processing the samples in a

laminar flow hood), only 2.1% of samples were positive for bacterial growth. Those that were

positive were identified as species that were most likely environmental contaminants associated

with the culture process itself. The ability to process PRP samples without contamination is

critical due to the inability to filter sterilize the end product prior to injection. Use of a laminar

flow hood for processing may be ideal; however, in most practice situations this may not be a

realistic option. Based on our results, the methods described in this study appear to be adequate,

when strictly adhered to, to produce a clinically safe product free from bacterial contamination.

These results are consistent with previous reports (48).

The repeatability of a technique should be considered when selecting a method for clinical applications. Evaluation of platelet and WBC counts in whole blood and platelet concentrates should be performed on each sample to more accurately detail the quality of the product used, regardless of the method of concentration. The conical tube method described in this study achieved the highest number of samples with platelet numbers exceeding $250,000/\mu l$ and platelet fold change greater than 2.5. Recent studies suggest that these values, although lower than what some authors propose as the therapeutic range, may be clinically relevant (31, 32). The WBC numbers achieved using this method may also be within a clinically acceptable range based on available information (6, 38). The supplies needed for the conical tube method cost less than $20 US dollars per sample and are readily available, including the centrifuge. Based on the results of this study, the conical tube method of PRP processing is bacteriologically safe when performed under controlled aseptic conditions and may be a suitable clinical alternative to commercial systems in low budget cases.

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Final Comments

This thesis explores numerous aspects of processing PRP from whole blood and evaluating the quality of the final product. The intent was to identify a simple, inexpensive method of processing PRP that would be comparable to more expensive commercial methods. One of the simplified methods in this study resulted in a PRP product comparable to the one produced by the commercial system in this study. All methods, including the commercial system, resulted in highly variable platelet and WBC concentration, even when multiple samples were processed from the same blood sample. This variability is likely an inherent feature of manual PRP processing and should be considered when choosing a processing protocol. Further investigation is needed identify protocols that reliably produce a more uniform PRP product, and quality assessment should be performed on platelet concentrates that are used clinically.

All four centrifugation protocols used in this study produced PRP with a WBC concentration higher than that of whole blood, and the degree of WBC concentration varied among methods. As discussed previously, the clinical implication of high WBC numbers in PRP is unclear. Evaluation of additional centrifugation protocols and different types of centrifuge tubes might allow further manipulation of this variable.

The clinical use of PRP is widespread, but many research questions remain concerning the use of this promising regenerative therapy. This study highlights important technical considerations when processing and evaluating PRP; however, further studies are required to further characterize the PRP products outlined in this investigation. More targeted investigations are needed to compare the growth factor content, degree of platelet activation, *in vitro* effects, and clinical efficacy of the PRP products produced by these protocols.