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Canine Pure Platelet-Rich Plasma for Regenerative Medicine and Platelet Research: Protocol Optimization

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Canine pure platelet-rich plasma for regenerative medicine and platelet research: protocol

optimization

By

Marina Mitie de Souza Monobe

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Canine pure platelet-rich plasma for regenerative medicine and platelet research: protocol

optimization

By

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Platelet-rich plasma (PRP) can be widely used in veterinary medicine in different areas. Studies using PRP frequently use different methodologies making for difficult comparison. The objective of this study was to evaluate the purity and platelet activation of a PRP protocol. A total of 18 blood samples were drawn from six dogs, collected once per week over a total of three weeks. Blood samples were centrifuged six times at 300g for 5 min. Ultra-pure PRP (OP) was obtained by adding PRP a Optiprep 1.063g/mL density barrier and centrifuged at 350g for 15 min. Mean platelet recovery from whole blood was 62.90% in PRP and 45.24% in OP. PRP and OP showed high platelet purity; blood cell contamination <0.01%. Flow cytometry for platelet activation markers was consistent with minimal platelet activation. This study describes the optimization of PRP protocol with high platelet purity, minimal platelet activation, high reliability and reproducibility.

Keywords: Ultra-pure PRP, Protocol, Centrifugation, Dog

DEDICATION

"To Reh, who creates the light from the darkness.

To Geb, who build the beautiful in every living thing.

To Isis, who protects me as her Horus and guide me through the right choices.

To Osiris, who keeps the balance into the Cosmos.

To Horus, who is always close to me in every battle.

To Seckmet, who gives me the strength to fight for my dreams.

To Hathor, who brought me compassion, love, and happiness.

To Thot, whose wisdom is always an inspiration to me.

To the Great Anubis, who measures my heart and gives me the opportunity to correct my mistakes and keep my heart as weightiness as Maat plume."

To my mother, Regina Maria de Souza, who taught me to be an independent and successful woman. Her life is an example to be followed.

To my father, Luis Monobe, who taught me to always do the right and ethical thing, mainly when it is the hardest way.

To my parents, who always supported all my decisions and dreams and who came from a humble family building a life from nowhere to the top, raising me to always give my best no matter where or the situation. 'The best things in life are the hardest ones to accomplish'.

To my grandmother, Hebe Laghi, the first woman to achieve a faculty position in the Biological Science Department at Sao Paulo State University, opening the doors for next generations of women scientists at her time.

To my grandfather, Jorge de Souza, who took great care of me during my childhood and no better person would be able to replace him.

To my great friend, Carol Martins, the big sister, who never let me down and always have the right words I need to hear.

To my best teacher, professor, coach, and friend, Regina Takahira, who always believed in me and brought to my life two things that I love most: running and clinical pathology.

To all my friends, from college and University, who have never been so close even being thousands of miles away, who taught me that family is not always blood-related and we can build greater things when working together.

To my partner, Frank Mosura, who was never a 'character' in my professional life, but I could have never got so far without his support, patience, and understanding. "Behind a great person, there is always a great partner".

For all of you, who never gave up on me, who believed and supported my choices and dreams when I thought I could not go forward. I would not be here if it was not for you all.

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CHAPTER I

INTRODUCTION

Background

Blood platelets are derived from proplatelet cylinders of megakaryocyte

cytoplasm present in hematopoietic organs, such as bone marrow. Platelets are cytoplasm

fragments with no nucleus measuring from 2 to 4 μm in diameter (Harvey, 2012). The

resting platelet structure is divided into three zones, each one with a particular function.

The peripheral zone includes the glycocalyx coat, cytoskeleton, and platelet membrane

and is responsible for platelet adhesion and aggregation. The sol-gel zone is formed

mainly by the canalicular and dense tubular systems and is responsible for platelet

contraction and microtubule system support. The organelle zone is formed by alpha

granules, dense granules, lysosomal granules, and glycogen granules and contains more

than 800 different proteins involved in the major platelet functions (White, 2004; Senzel

et al., 2009).

The major functions of platelets include preventing acute blood loss and inducing

healing of the vascular system and adjacent tissues after injury (Amable et al., 2013).

During vascular injury, collagen is exposed and by contact activates platelets. Once

activated, platelets release stored cytokines, growth factors, coagulation factors, and other

molecules that are responsible for amplifying platelet activation. These factors also have

a paracrine effect in different cell populations, including mesenchymal cells, osteoblasts,

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fibroblasts, and endothelial cells and can lead to cellular proliferation, migration, and angiogenesis, all important for tissue regeneration (Bambace and Holmes, 2011; Amable et al., 2013). Other less known functions include anti-inflammatory, antibiotic, and analgesic effects (Drago et al., 2013; Mazzocca et al., 2013).

Platelet-rich plasma in the veterinary medical practice

Platelet-rich plasma (PRP) is a concentrate of platelets in a relatively small volume of plasma that has been largely used in regenerative medicine. The clinical use of PRP in small animals is relatively recent and, similarly to what happens in human medicine, has shown beneficial results.

Previous studies applying PRP in cutaneous wounds of different sizes and depths in dogs have demonstrated that PRP induces a faster wound closure rate, increased collagen deposition, granulation tissue formation, and better vascularization (Alishahi et al., 2014; Jee et al., 2016). Similar results were also observed in humans(Eppley et al., 2004) and horses (DeRossi et al., 2009). Case reports administering PRP as part of the treatment in multiple or large cutaneous lesions in dogs have suggested decreased healing time and better overall response (Kim et al., 2009; Chung et al., 2015). However, in a few cases, the healing of cutaneous surgical wounds and cutaneous regeneration in dogs is not so clearly affected by the use of PRP, even up to 10 days after injury (Sardari et al., 2011; Karayannopoulou et al., 2015). Contradictory results of the benefit of the adjuvant therapy with PRP in wound healing have also been observed in other species, such as in horses (Monteiro et al., 2009).

Studies on reparative orthopedic surgery, focusing on musculoskeletal disorders (e.g., osteoarthritis and meniscal healing), showed decreased pain and increased articular

function in dogs and horses receiving serial intra-articular PRP injections (Cook et al., 2016; Girolamo et al., 2016 Bosch et al., 2011; Torricelli et al., 2011). In humans, the use of PRP to treat acute and chronic injuries of bone and cartilage is well established (Girolamo et al., 2016; Meheux et al., 2016; Riboh et al., 2016). Dehghan et al. (2015) performed callus distraction after osteotomy in 10 dogs and used two different treatments, application of PRP only and application of PRP associated with bone marrow mesenchymal stem cells (BM-MSC). By radiographic and CT scan evaluations, they observed higher osteogenesis rate and bone healing rate in dogs treated with both PRP and BM-MSC, suggesting a paracrine effect of platelets on the proliferation of mesenchymal stem cells. Similar results were also seen in rabbits (Jiang et al., 2012), sheep (Hernandez-Fernandez et al., 2013Niemeyer et al., 2010), and mini-pigs (Hakimi et al., 2010).

In dogs, the use of PRP caused faster mucosal healing and pulp revascularization after treatment of injured tissue with PRP injections (Carlson and Roach, 2002; Tobita et al., 2013). Simsek et al.,(2012) found that PRP associated with mesenchymal stem cell (but not PRP alone) lead to faster recovery from odontological implant surgery, suggesting that in some cases the association of stem cells with PRP may be required for better results.

Inconsistent results of the benefits of treatment with PRP are reported in human medicine (Battaglia et al., 2013; Filardo and Kon, 2015) and in veterinary medicine (Jensen et al., 2005). However, when comparing the numbers of published scientific publications, it appears that there are fewer studies that found no benefits when using PRP on reparative surgery than those with positive results. Part of this tendency may be

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due to the fact that it is harder to have a manuscript with negative results accepted for publication.

PRP in research

Many studies have suggested that platelets contribute to cancer progression and metastasis by amplifying cancer-related coagulation, shrouding tumor cells and shielding them from the immune system, and increasing intravasation of cancer cells (Bambace and Holmes, 2011; Labelle et al., 2011). Evidence of the relationship between cancer cells and platelets include the fact that thrombocytosis, thrombotic events, and/or increased coagulation parameters are frequently associated with poor prognosis and high metastatic rate (Gay and Felding-Habermann, 2011). For example, murine models of cancer with impaired platelet function had shown a decreased metastatic rate (Camerer et al., 2004). Also, platelets are able to take up cytokines and growth factors produced by cancer cells and release them upon activation in metastatic sites, helping tumor metastasis (Kerr et al., 2010). More specifically, there appears to be a positive correlation between the concentration of angiogenic markers within platelets and increased aggressiveness in osteosarcoma and mammary tumors in dogs (Selvarajah and Kirpensteijn, 2010).

In cancer research, PRP has been used to verify interactions between platelets and cancer cells. But, despite the clear interaction between platelets and cancer cells, the specific pathways and the difference between species are still largely unknown. Platelets have also been used in the evaluation of cancer prognosis. A study has shown that, in humans, an increased lymphocyte to platelet ratio is associated with a slower cancer progression and decreased numbers of metastases in vivo (Zhang et al., 2016). These

findings were observed in different types of cancer, including breast cancer (Koh et al., 2015) and gastric cancer (Aliustaoglu et al., 2010).

Since platelets can secret pro- and anti-inflammatory cytokines and uptake and release regulatory plasma proteins, they have also been used to study the pathophysiology of several diseases including cardiovascular and infectious diseases. Recently, new aspects of the participation of platelets in the immune system have been described, including the production and secretion of IL-1β and inflammasome assembly (Hottz et al., 2015). Previous studies investigating the link between platelets and the immune system have used malaria (Aggrey et al., 2013), dengue (Suharti et al., 2002; Bozza et al., 2008), toxoplasmosis (Chumpitazi et al., 1998) and different gram-negative bacterias (Yang et al., 2013) to activate platelets and then used proteome, transcriptome, and/or specific secreted cytokines analysis to verify the role of platelets during the progression of the disease. These studies suggest that initial activation of platelets during the disease can be protective inducing an immune response, however, chronic platelet activation is associated with vasculopathy, cardiovascular damage, and even shock (Aggrey et al., 2013; Hottz et al., 2015).

Different protocols, different results

After all, despite some well-established advantages of the use of PRP in animal and human medical practice, there are few studies with conflicting results. One of the possible causes is the use of different protocols to obtain PRP without standardization of platelet concentration, purity, and activation. Unfortunately, the variability of leukocyte contamination, and platelet concentration and activation in PRP, and the different

volumes used in different studies make any effort to interpret the findings nearly impossible (Ehrenfest et al., 2009).

PRP is a generic term, originally used in transfusion medicine, that is used for many types of platelet-plasma products (Araki et al., 2012). The techniques to produce PRP have some common steps: (1) blood is collected with anticoagulant and a first centrifugation is performed to separate the blood into three main layers, including red blood cell (RBC), buffy coat (platelets and leukocytes layer), and acellular plasma or platelet-poor plasma (PPP) (Figure 1); (2) then platelets are collected from the peri-buffy coat area. Those two steps vary greatly among protocols, but in general, all protocols discard RBC and PPP and concentrate platelets.

Due to the recent increased demand for platelet-related research, a classification of platelet concentrates has been suggested (Ehrenfest et al., 2009). This classification is based on fibrin density and leukocyte content, and hopefully, it will increase the degree of standardization of the procedures. It has been suggested that pure PRP (P-PRP), also called leukocyte-poor PRP, does not contain a significant amount of leukocytes (Ehrenfest et al., 2009; Riboh et al., 2016) and leukocyte-rich PRP (L-PRP or P-LRP) would have higher concentrations of leukocytes. However, specific concentration levels of leukocyte used to differentiate P-PRP and L-PRP were not stipulated. While some studies classify P-PRP, as PRPs containing close to 0% leukocyte (Ehrenfest et al., 2009), other studies classified P-PRP as a PRP having any WBC concentration lower than the one in the blood used to obtain the PRP (Riboh et al., 2016). The other classification includes: (1) platelet-rich fibrin (PRF), which can have leukocyte (L-PRF) or not (P-PRF) and is characterized by the presence of fibrin; (2) activated form of PRP, a PRP gel

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characterized by low-density fibrin that can also have leukocyte (L-PRP gel) or not (P-PRP gel). Thus, platelet concentrates can be classified into four main groups (P-PRP, L-PRP, PRP gel, and PRF). This classification is important once many commercial kits are available and offer different final products (Ehrenfest et al., 2009).

Studies involving the optimization of protocols to obtain P-PRP have tested different centrifugation speed, time, and temperature and frequently used only two centrifugation steps. In humans, best platelet recovery was obtained with a relative centrifuge force (RCF) between 200-300g and times ranging from 5 to 12 minutes , while temperature seemed to have minimal influence on platelet retrieval (Amable et al., 2013)(Araki et al. (2012)). Amable et al. (2013) obtained up to 69.5% platelet recovery from whole blood (WB) with less than 0.3% of RBC and leukocyte contamination using a total of 4.5ml of WB, while Araki et al. (2012) obtained more than 80% of platelet recovery with 4.1-5.8% leukocyte contamination using a total of 40-72 mL of WB.

Efforts to standardize a P-PRP protocol specific for dogs have been made, however, most routine PRP protocols were designed based on human protocols. The first described PRP protocol for dogs using two-step centrifugation used ethylenediaminetetraacetic acid (EDTA) as anticoagulant and obtained a final concentration of 1,884,000 platelets/µL with 64,200 WBC/µL, resulting in one leukocyte for every 29 platelets (Jensen et al., 2004). Silva et al. (2011) performed a protocol using the anticoagulant acid citrate dextrose (ACD) and obtained a final concentration of 515,000 platelets/µL with 6,900 WBC/µL. Other studies using the anticoagulant sodium citrate and comparing different RCF values obtained from $4x10^5$ to $1.3x10^6$ platelets/µL (Choi et al., 2005; Ferraz et al., 2007). Unfortunately, without any characterization of

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leukocyte or erythrocyte contamination, it was not clear that these methods were successful.

Another recent study carried out in our laboratory, obtained P-PRP by using a protocol other than two-step centrifugation. This protocol included a single centrifugation step of blood collected in EDTA tubes using an optimized density gradient technique to better separate RBC, buffy coat, and PPP. The result was a final platelet recovery of 51.56% with 99.99% purity (Trichler et al., 2013).

It is important to point out that the type of anticoagulant used might interfere with platelet activation and concentration. The main available anticoagulants include heparin sodium, sodium citrate, ACD, EDTA, and citrate-theophylline-adenosine-dipyridamole (CTAD). Several studies have shown that the use of EDTA as an anticoagulant for the PRP protocols may cause functional, structural, and biochemical alterations in platelets (White and Escolar, 2000; Wilkerson and Shuman, 2001; Silva et al., 2011). Neufeld et al. (1999) compared CTAD and sodium citrate and showed higher platelet activation and aggregation when sodium citrate was used, suggesting that sodium citrate is not the best choice for functional platelet assays. On the other hand, Lei et al. (2009) tested the effects of different anticoagulants on the quality and efficacy of PRP and showed that ACD keeps the best integrity of platelet structure over time preventing spontaneous activation. ACD is the anticoagulant of choice in blood banks, primarily because it maintains platelet viability for up to 6 hours. This longer platelet viability might be explained by the presence of glucose associated with low citrate concentration (1.32%). The low citrate concentration helps to keep the pH from falling and the glucose helps platelet metabolism during in vitro storage keeping their viability (Holme, 1992; Lei et al., 2009).

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Platelet activation analysis by Flow Cytometry

Platelet concentration and activation status are important factors for PRP. It is expected that an adequate PRP contains at least 106 platelets/µL with low activation (Marx, 2001; Amable et al., 2013). Once activated, platelets release a wide range of growth factors, cytokines, and chemokines that are lost during PRP preparation and could be essential for therapeutic and research applications (Amable et al., 2013). For this reason, several studies aiming to optimize PRP preparation have tested different ways to analyze platelet activation (Araki et al., 2012; Amable et al., 2013; Trichler et al., 2013).

In dogs, flow cytometric analysis to detect platelet activation was first developed to evaluate dogs with suspected thromboembolic disease, allowing identification of prothrombotic state and consequently early medical intervention to prevent thromboembolic cases (Moritz et al., 2003). Flow cytometry assesses canine platelet activation by measuring P-selectin expression, the presence of platelet aggregates and/or platelet microparticles, and expression of phosphatidylserine in the outer cytoplasmic membrane (Wills et al., 2006). Platelet aggregates and microparticles can be identified by a forward-scatter plot (Figure 2) with flow cytometry and confirmed by specific platelet markers (e.g. CD61).

P-selectin, also known as CD62P, is an inflammatory mediator stored in the α-granule membrane of platelets and in Weibel-Palade bodies in endothelial cells. CD62P is expressed to the platelet surface during activation and degranulation by fusion of the α-granules membrane with the cytoplasmic membrane. The detection of P-selectin is performed by using a conjugated anti-CD62P antibody. It is commonly used as a marker

for platelet activation in humans (Koksch et al., 2001) and has been also studied in horses (Segura et al., 2006) and baboons (Michelson et al., 1996).

Upon platelet activation, phosphatidylserine flips out from the inner leaflet of the cytoplasmic membrane to the outer surface of the cytoplasmic membrane adding negative charges (Brooks et al., 2002) and creating nucleation sites for coagulation. Detection of phosphatidylserine can be done using a conjugated Annexin V, a phospholipid-binding protein with high affinity for phosphatidylserine (Thiagarajan and Tait, 1990). Phosphatidylserine is as commonly used as P-selectin, it is well-established, and it has been used for the diagnosis of bleeding disorders in dogs (Brooks et al., 2002) and cell death by apoptosis.

Objective

The objective of the present study was to establish and optimize a method for PRP preparation in dogs. The final goal was to obtain a reliable and practical PRP protocol focusing on high platelet recovery, purity, and low platelet activation.
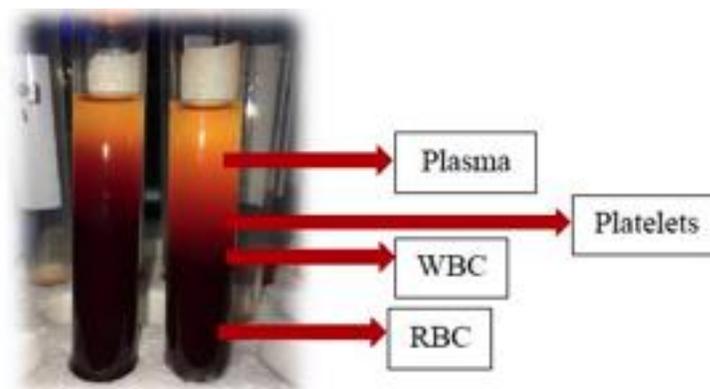
Figure 1.1   Gradient cellular concentration after whole blood centrifugation.
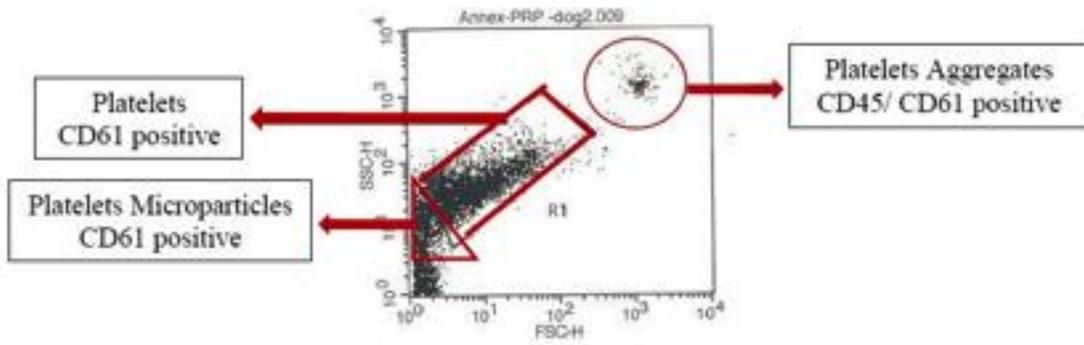
Figure 1.2   Forward and side-angle light scatter plots showing normal platelets, platelet aggregates, and platelets microparticles.
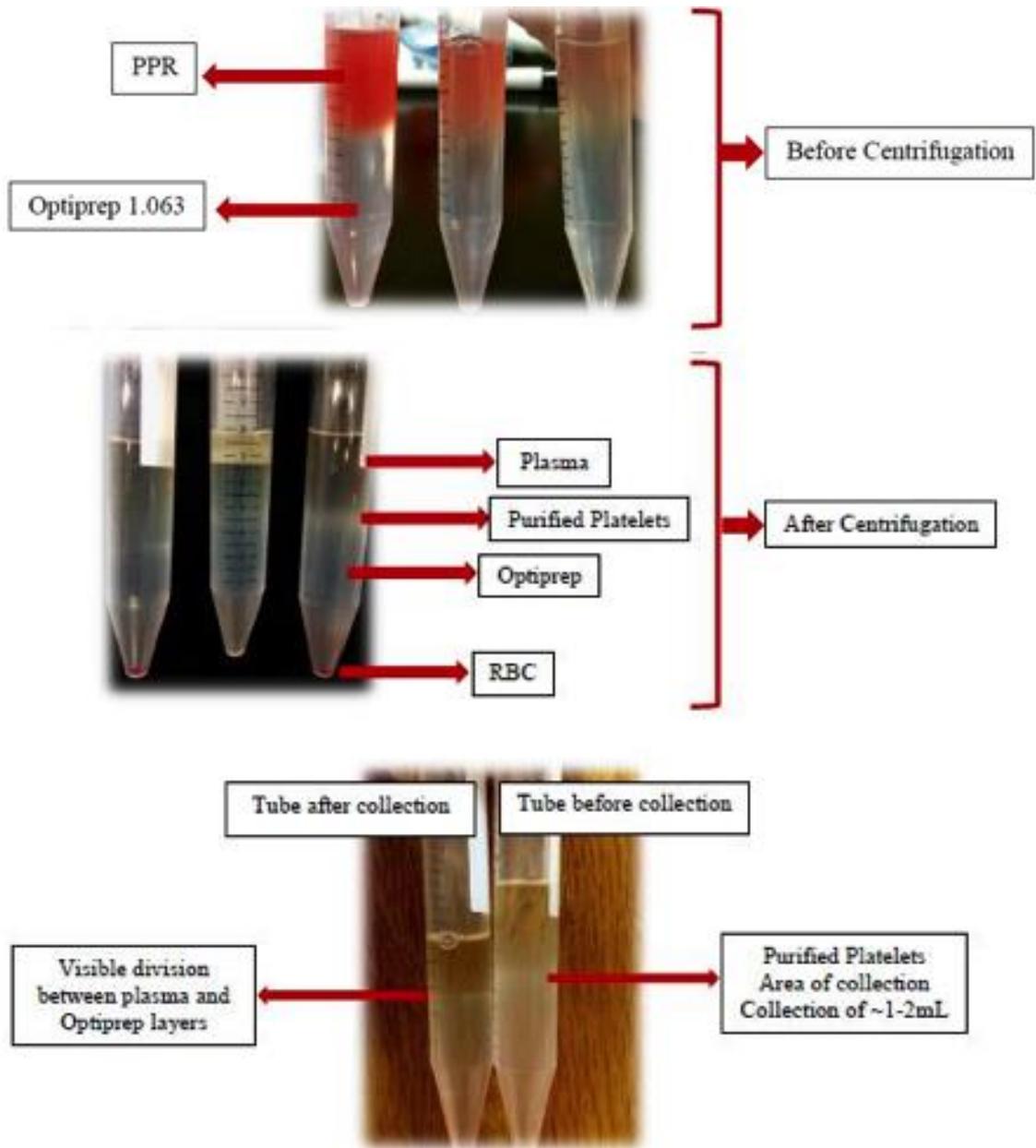
Figure 1.3   Before and after centrifugation of blood using Optiprep 1.063 density barrier.

CHAPTER II

MATERIALS AND METHODS

Animal care and use

All procedures were approved by the Mississippi State University Institutional

Animal Care and Use Committee under the approval number #15-103 and were in

compliance with the requirements at a facility accredited by the American Association for

Accreditation of Laboratory Care.

Sample collection

Six healthy female intact Walker hound dogs were used in this study. The dogs

were not in estrus throughout the experiment or exposed to any medications or vaccines

for at least two months before starting this study. Whole blood samples were collected by

jugular venipuncture with a 20-gauge needle directly into a glass vacutainer tube

containing 1.5 mL of ACD and approximately 8.5 mL of blood. ACD was composed by

0.48% (w/v) citric acid, 1.32% (w/v) sodium citrate and 1.47% (w/v) glucose. Each dog

was collected once a week during three consecutive weeks to analyze the reproducibility

of the method. All samples were processed within an hour after collection.

Hematological Analysis

Total blood cell count was determined in whole blood and after each

centrifugation. The blood cell counts were performed manually with a hemocytometer

(Hausser Scientific Co, Harsham, PA 19044) using standard phosphate-buffered saline (PBS) to dilute the samples when necessary. Blood smears were performed to analyze cellular morphology, the presence of activated platelets, and platelet aggregates. Whole blood samples presenting visible fibrin formation or platelet aggregates were excluded.

PRP preparation

After blood collection and hematological analysis, whole blood was centrifuged at $300 \times g$ for 5 minutes at 12°C in a swinging bucket rotor with the brake turned off (Eppendorf® Centrifuge 5804R). After centrifugation, samples were divided into three visible layers (i.e. RBC, buffy coat, and PPP). The entire opaque platelet fraction between the dense buffy coat (composed mainly by leukocytes) and the clear PPP layer was collected and the fraction, called PRP-1, was transferred to a new plastic tube. Without homogenization, the remaining blood sample was centrifuged for five more times and five subsequent fractions PRP-2, PRP-3, PRP-4, PRP-5 and PRP-6 were collected as described for PRP-1. Total platelet count was assessed in each PRP fraction before combining all fractions (pooled PRP) for further procedures. Summary of procedures are detailed in Figure 2.1.

Low erythrocytes PRP preparation

Approximately 3 mL of the pooled PRP was carefully layered over 5 mL of 1.063g/ mL density barrier (OptiPrep™, Sigma-Aldrich®, St. Louis, MO, USA). The sample was centrifuged at $350 \times g$ for 15 minutes at 20°C. Approximately 1-2 mL of the platelet layer, localized close to the 4.5 mL mark of the tube, was collected and transferred to a new plastic tube labeled PRP-Opt (Figure 2.1).

Flow Cytometry Analysis

A total of 12 samples, two samples from each dog (pooled PRP and PRP-Opt) were used to evaluate the platelet activation status. The analysis was performed using the FACScalibur flow cytometer and the Cell Quest Pro Software (BD Biosciences, San Jose, CA, USA). Approximately 1×10^7 platelets were spun down and incubated either with FITC-labelled Annexin V (BD Biosciences) or RPE-labelled mouse anti-human CD62P (AbD Serotec) according to the manufacturer's protocol. After incubation, samples were washed and resuspended in PBS for analysis. As a positive control, platelets were activated with thrombin (Chrono-log©, Havertown, PA 19083, ref #386) and collagen (Chrono-log©, Havertown, PA 19083, ref #385) following the manufacturer's protocol. As negative controls, isotype-matched control for P-selectin (Mouse IgG1 Negative Control RPE, MCA928A405, BIO-RAD) was used and annexin-binding buffer for Annexin V. Platelets were displayed on log forward-angle versus log side-angle light scatter plots. The platelets were gated and 10,000 events were recorded for each labeling. The expression of those markers on platelets was quantified using the percentage of positive events. The presence of potential microparticles and platelet aggregates was recorded.

Statistical Analysis

Results were expressed as mean ± standard deviation. Statistical significance was assessed by single-factor analysis of variance (ANOVA) in conjunction with a multiple comparison test (i.e., Tukey's test) to test the differences among the PRP samples. $P < 0.005$ was considered statistically significant.
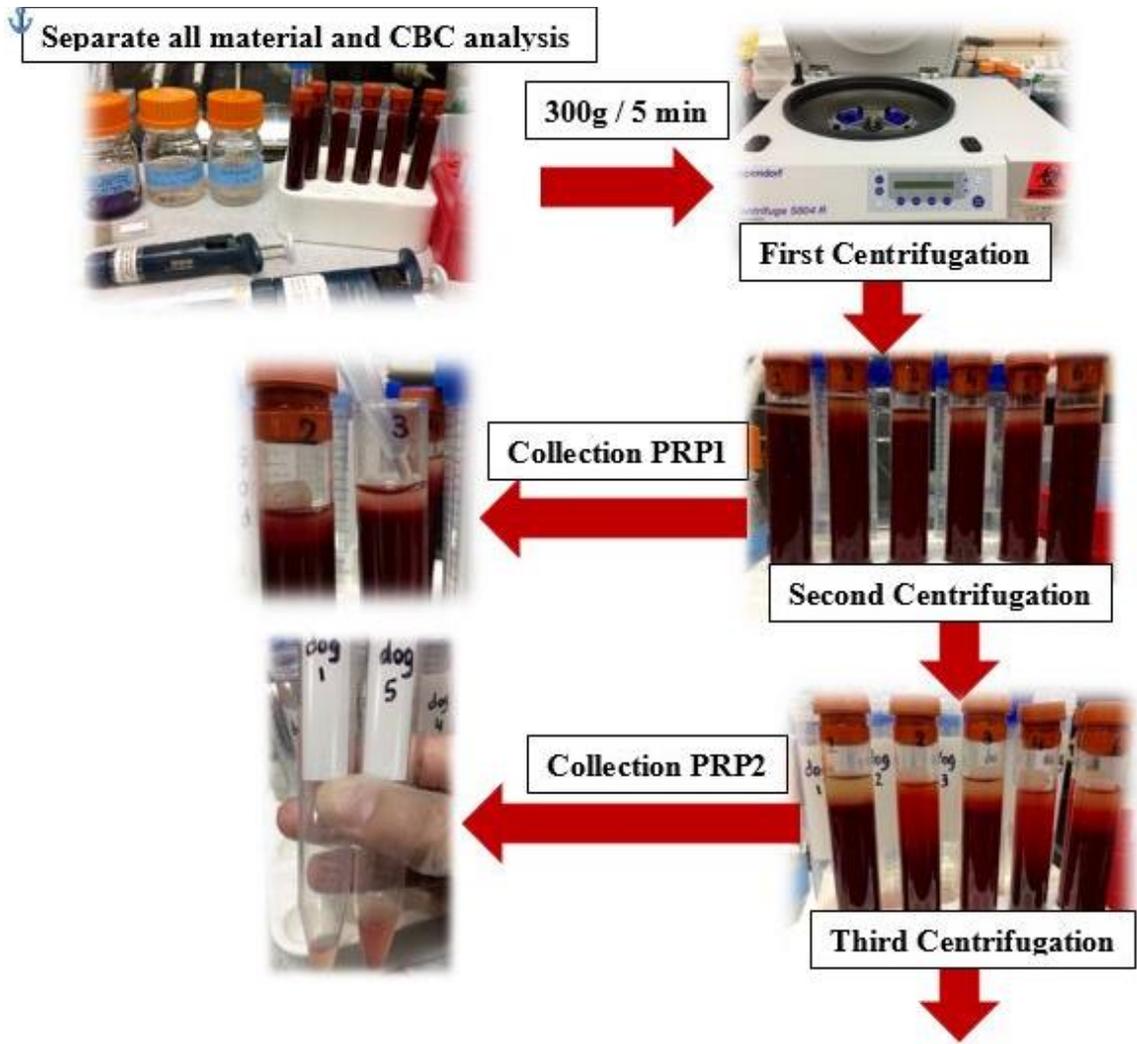
Figure 2.1   Materials and methods.
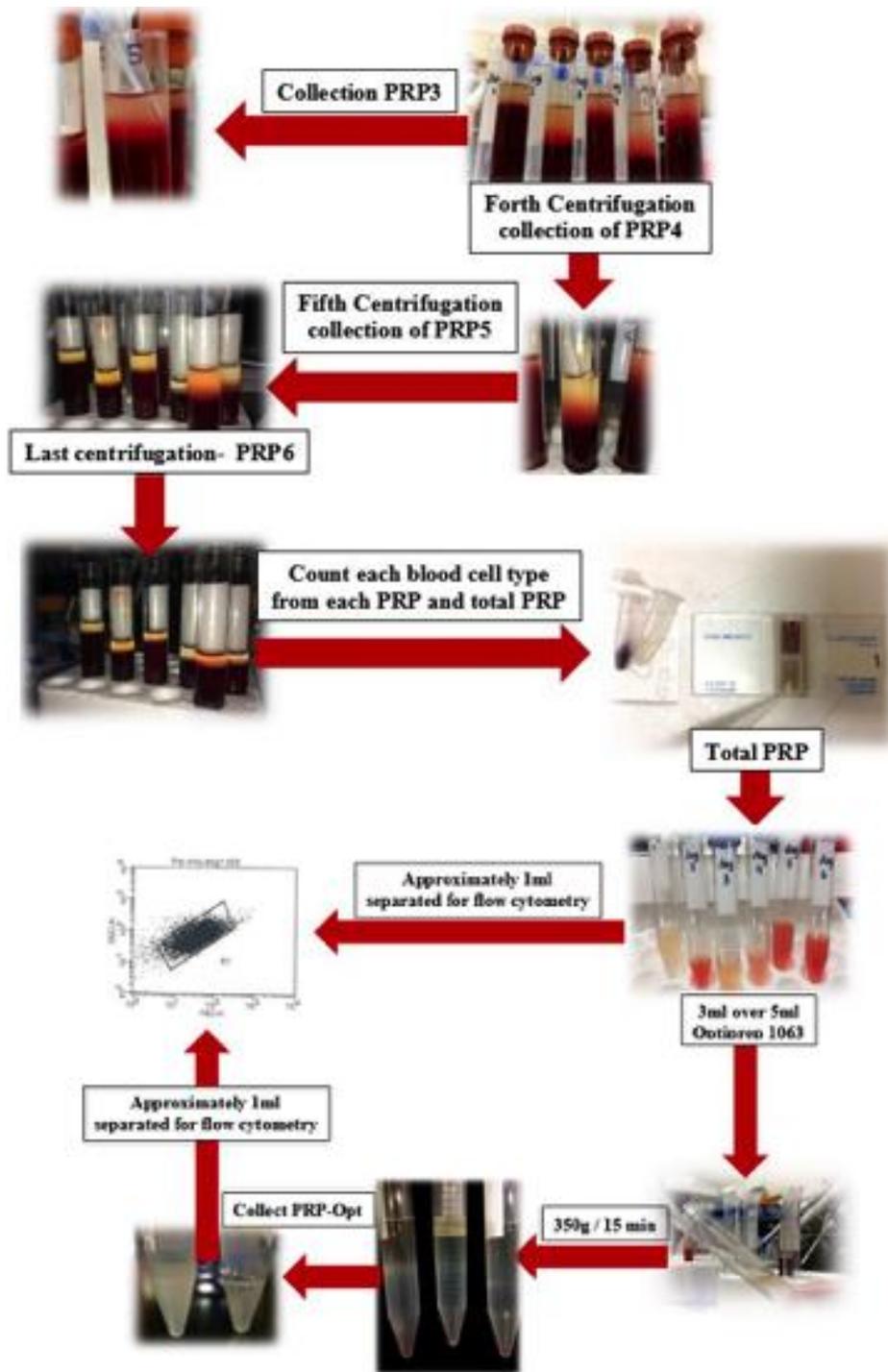
Figure 2.1 (continued)

CHAPTER III

RESULTS AND DISCUSSION

The goal of the present study was to optimize a PRP protocol to increase platelet recovery and reduce other blood cell contamination. The reasoning for the use of multiple centrifugation steps was that it would allow the recovery of the highest amount of the platelet layer at each centrifugation without collecting the buffy coat (leukocytes) or the PPP layer. It also could avoid the loss of all platelets from a sample if platelet activation was to happen during the later stages of the protocol. This protocol used a fixed RCF, 300 × g, which was shown to give the best centrifugation results from other studies (Silva et al., 2011; Araki et al., 2012; Amable et al., 2013).

First centrifugation step

Most of the PRP protocols include a first centrifugation step in which the plasma layer above the buffy coat is collected and separated into a different tube. Then, the whole plasma is centrifuged again at a higher RCF so that platelets can form a concentrated pellet. In our experience using one step with high RCF is usually associated with high platelet recovery but and increases the chance of platelet activation. Also, the use of a low RCF (to avoid activation) with single centrifugation frequently cause loss of platelets due to entrapment within the RBC layer.

The first centrifugation step resulted in a mean total concentration of 1.85×10^8 platelets (minimum of 1.91×10^7 platelets and maximum of 4.17×10^8 platelets) (Table 2.1).

The mean WBC contamination concentration in our PRP-1 was 733 cells/µL, lower than any other canine PRP techniques described. In most of these other studies, optimization of the PRP protocol was not the objective, as they were testing the benefits of PRP use in small animals clinical settings. The lack of information related to the PRP volume and total platelet recovery from all reviewed references precludes a more detailed comparison between our results and theirs.

From second to the final centrifugation step

The combination of all centrifugation steps (PRP1 to PRP6) allowed a final platelet recovery of 66.45% ± 32.15%. RBC contamination ranged from 0.14 to 9.06 cells for every 10^4 platelets counted, while WBC ranged from 0.54 to 4.04 cells for every 10^9 platelets.

A significant increase in the mean total recovered platelet from the first to second and third centrifugation step was observed. PRP-2 and PRP-3 had a significantly higher ($p<0.005$) mean total platelet recovery than any other steps (Figure 3.2). No significant difference in mean platelet recovery was observed between PRP-2 and PRP-3, or between PRP-4, PRP-5, and PRP-6. However, a pattern of steady decrease in the number of retrieved platelets was observed among sequential steps (Figure 3.1).

There was a significant variation of platelet recovery among different dogs ($p=0.0045$). Table 2.2 shows the mean platelet recovery from individual dogs at each centrifugation step.

Table 3.1   Mean volume and platelet recovery after each centrifugation.

Mean Values	Blood	PRP-1	PRP-2	PRP-3	PRP-4	PRP-5	PRP-6
Volume (µL)	8,500	517.4	819.5	745.8	516.3	429	402
Platelets	2.17E+09	1.85E+08	4.70E+08	3.77E+08	2.22E+08	1.65E+08	1.10E+08

Table 3.2   Mean platelet recovery in each dog after each centrifugation.

Animal	Blood	PRP-1	PRP-2	PRP-3	PRP-4	PRP-5	PRP-6
1	2.35E+09	7.20E+07	2.79E+08	3.16E+08	3.23E+08	3.32E+08	1.92E+08
2	1.78E+09	1.90E+07	1.46E+08	1.31E+08	1.61E+08	1.07E+08	1.58E+08
3	2.06E+09	2.67E+08	6.66E+08	5.27E+08	2.51E+08	1.10E+08	7.60E+07
4	2.05E+09	1.61E+08	4.29E+08	4.75E+08	2.15E+08	2.09E+08	7.50E+07
5	2.11E+09	4.17E+08	8.42E+08	4.57E+08	9.80E+07	3.20E+07	1.80E+07
6	2.67E+09	1.75E+08	4.57E+08	3.58E+08	2.84E+08	2.02E+08	1.41E+08
Mean	2.17E+09	1.85E+08	4.70E+08	3.77E+08	2.22E+08	1.65E+08	1.10E+08

Ultra-pure PRP procedure

After centrifugation of 3 mL of PRP with 5 mL of Optiprep 1.063g/mL, the final platelet recovery from blood was 46.01%± 24.78%. RBC decreased from 9,350 RBC / 10^7 platelets to 0.0164 RBC / 10^7 platelets and WBC varied from 0.0126 WBC / 10^7 platelets to 0.0158 WBC / 10^7 platelets. It is possible that there was a decrease in leukocytes contamination that was masked by the sensitivity of the test used to count leukocytes. The minimum detection limit of leukocytes using hemocytometer and manual counting is 1.1 WBC/uL and several counts were already at this level at the PRP total step. The average number of total platelets harvested was $1.03 \times 10^9 \pm 0.49 \times 10^9$ platelets.

Blood components have different densities. Erythrocytes are the densest cells with a specific gravity of approximately 1.095 kg/m³, followed by WBC, which specific

gravity ranges from 1.063 to 1.085 kg/m^3, and platelets with a specific gravity of approximately 1.032 kg/m^3. The plasma specific gravity varies from 1.025 to 1.029 kg/m^3. Because of these characteristics, centrifugation is able to separate different blood components in separate layers. Contamination of PRP usually occurs in part due to the slightly overlapping specific gravities among other blood components (Araki et al., 2012) and in part due to cell-cell interactions. The use of density gradient centrifugation has shown to improve purity and quality of PRP by improving separation of its different layers after centrifugation (Birschmann et al., 2008; Trichler et al., 2013).

Tables 2.3 and 2.4 show the mean percentage of platelet recovered from the blood samples at the end of the six centrifugation steps (polled PRP) and after Optiprep (PRP-Opt) centrifugation, along with the number of RBC and WBC counted for every 10^7 platelets. Overall the use of Optiprep did not cause statistically significant loss of platelets. Interestingly, it was possible to observe that the 'dog 2' had the lowest percentage of platelet recovery after Optiprep centrifugation 31.57% (Table 2.4), and this finding was observed in all the three repetitions for this animal, but not for the others. This suggests an individual factor, such as hydration status, high hematocrit, lipemia, circadian rhythms in platelet numbers (Boswell et al., 2012; Smith et al., 2012), or higher overlap of specific gravities.

An association between hematocrit, platelet aggregation, and platelet recovery has been described in humans (Kelton et al., 1980; Boswell et al., 2012). Platelet aggregates have higher sedimentation rate than the individualized platelets, and after centrifugation, aggregates tend to stay closer to the RBC layer (Rinnovati et al., 2016).

Kelton et al. (1980) showed that women have lower hematocrit and higher platelet aggregation when using citrate, 9v:1v proportion, to collect the blood. Adjusting of the citrate concentration to the hematocrit values of individual persons decreased the differences. No evident difference in platelet concentration between female and male dogs was found (Bauer et al., 2009). However, Smith et al. (2012) showed the association between hematocrit and platelet recovery in PRP from dogs. They evaluated coagulation parameters in 127 dogs and observed that RBC acts as functional diluent of blood, so high hematocrit limits plasma volume, reducing recovery of platelets. The same negative association between hematocrit and platelet yield was also observed in rabbits (Andrade et al., 2008). In horses, hydration status appears to interfere with platelet retrieval (Rinnovati et al., 2016). It is expected that dehydrated animals, with higher hematocrit and decreased plasma volume, will decrease platelets recovery for the same reasons seen in animals presenting higher hematocrit without dehydration. We did not evaluate hematocrit at the days of the experiment, but during routine evaluations 'dog 2' often present a hematocrit that was higher (up to 8%) than the other animals.

Table 3.3   Mean percentage of platelets recovered in the pooled PRP.

Animal	Platelet recovery (%)	WBC/ 10^7 platelets	RBC/ 10^7 platelets
1	54.67%	0.0085	140
2	42.57%	0.0404	8,790
3	74.16%	0.0072	150
4	73.75%	0.0072	7,810
5	95.87%	0.0054	3,130
6	49.66%	0.0083	9,060
Mean	**66.45%**	**0.0126**	**9,350**

Mean percentage of platelets recovered in the pooled PRP in each dog and numbers of white blood cells (WBC) and red blood cells (RBC) counted for every 107 platelets. Platelet recovery was compared to the total number of platelets counted in the blood.

Table 3.4   Mean percentage of platelets recovered in the PRP-Opt.

Animal	% Platelet recovery from total blood	% Platelet recovery from PRP	WBC/ 10^7 platelets	RBC/ 10^7 platelets
1	40.87%	74.29%	0.0119	0.0119
2	31.57%	59.45%	0.0221	0.0221
3	66.87%	91.06%	0.0080	0.0104
4	41.19%	57.07%	0.0126	0.0126
5	70.20%	69.73%	0.0077	0.0077
6	41.34%	86.25%	0.0097	0.0097
Mean	**46.01%**	**72.04%**	**0.0158**	**0.0164**

Mean percentage of platelets recovered in the PRP-Opt in each dog and numbers of white blood cells (WBC) and red blood cells (RBC) counted for every 107 platelets. Platelet recovery was compared to the total number of platelets counted in blood and poll.

Method Reproducibility

The experiment was carried out three times for each dog in three different weeks. Within the same dog, there was no evident differences in platelet recovery between collections (p=0.6114) or between centrifugations (p=0.2675), what suggests high reproducibility of the protocol.

Flow Cytometric Analysis

A significant difference (p=0.0032) in the percentage of Annexin V binding and platelet aggregates were observed between thrombin-activated PRP and Optiprep and between collagen-activated PRP and Optiprep, showing that platelet function was maintained during the protocol. Figure 3.3 indicates that PRP maintained a low mean cell fluorescence percentage (3.29 % ± 1.04 %) and that there was no significant difference in fluorescence when compared to Optiprep samples (3.48 % ± 1.74 %). These results suggest that no significant activation occurred across multiple centrifugation steps or Optiprep procedure. The highest platelet activation rate in our positive controls was observed using collagen and PRP (33.53 % ± 8.51 %). Despite the low platelet activation

23

percentage using the standardized protocol, individual variations were observed. Possible causes include handling, centrifugation, and individual physiological factors. Our results were similar than those observed in other studies involving dogs (Brooks et al., 2002; Wills et al., 2006). Wills et al. (2006) observed higher variability in their results, in which non-activated samples expressed 1.1-7.3% mean cell fluorescence, while activated samples ranged from 7 to 47.3%. These variabilities led the authors to conclude that Annexin V should not be the first option for platelet activation studies and clinical settings (Wills et al., 2006). However, in this study, little overlap was detected between PRP samples and controls, suggesting that it was a good marker for platelet activation.

Differently from Annexin V, P-selectin labeling did not show a significant difference (p=0.083) in the percentage of mean platelet fluorescence between pooled PRP and Optiprep, thrombin-activated PRP and Optiprep, and collagen-activated PRP and Optiprep (Figure 3.4). Our positive controls showed the lower mean percentage of cell fluorescence when compared with other studies using dogs, in which the positive control ranged from approximately 18% to 30% fluorescence (Moritz et al., 2003,2005; Trichler et al., 2013). In this study, our activated polled PRP expressed 11.98% fluorescence for thrombin and 7.61% for collagen and the activated PRP-Opt expressed 5.01% fluorescence for collagen and 7.97% for thrombin. Similarly, Wills et al. (2006) showed fluorescence range from 0.4 to 6.85% in activated samples, suggesting that low intensity labeling with p-selectin is not uncommon.

Alpha-thrombin (Chrono-log©, Havertown, PA 19083, ref #386) was used to activate platelets, leading to a clot formation. Despite the attempt to mechanically break the clot, there was a clear decrease in the numbers of platelets when we used thrombin to

activate platelets. Figure 3.5 show the decreased in platelet numbers (gated population) on scatter plot in comparison with a non-activated sample. There is also an increase in debris, possibly in part, due to an increased in the number of microparticles. The loss of platelets by entrapment in fibrin clot could have been avoided by the use of gamma-thrombin. Gamma-thrombin is a form of thrombin that activates platelets but has a weak effect in fibrin formation.
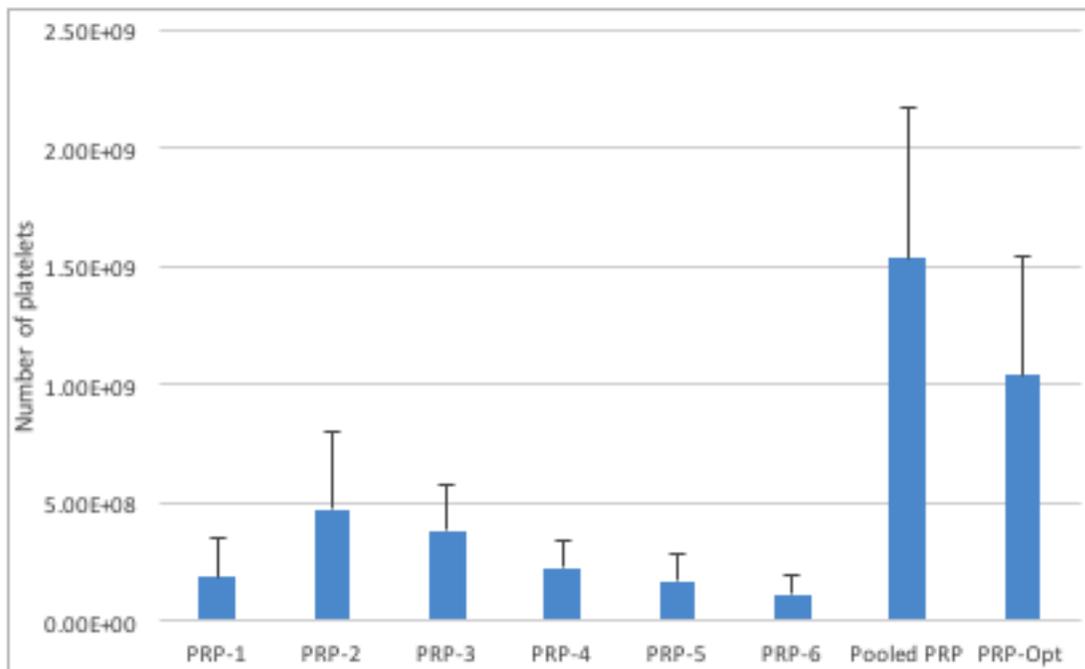
Figure 3.1   Mean platelet recovery.

Mean platelet recovery after each centrifugation (PRP-1 – PRP-6), polled PRP, and after centrifugation on a 1.063 g/ml iodixanol density barrier (PRP-Opt).

Figure 3.2   Multiple comparison test using mean total platelet recovery and t-grouping.

Multiple comparison test (ANOVA, Turkey's test, α=0.05) based on each mean total platelet across different centrifugation steps (PRP1 to PRP6). Means with same letter are not significant different.

(A)

(B)

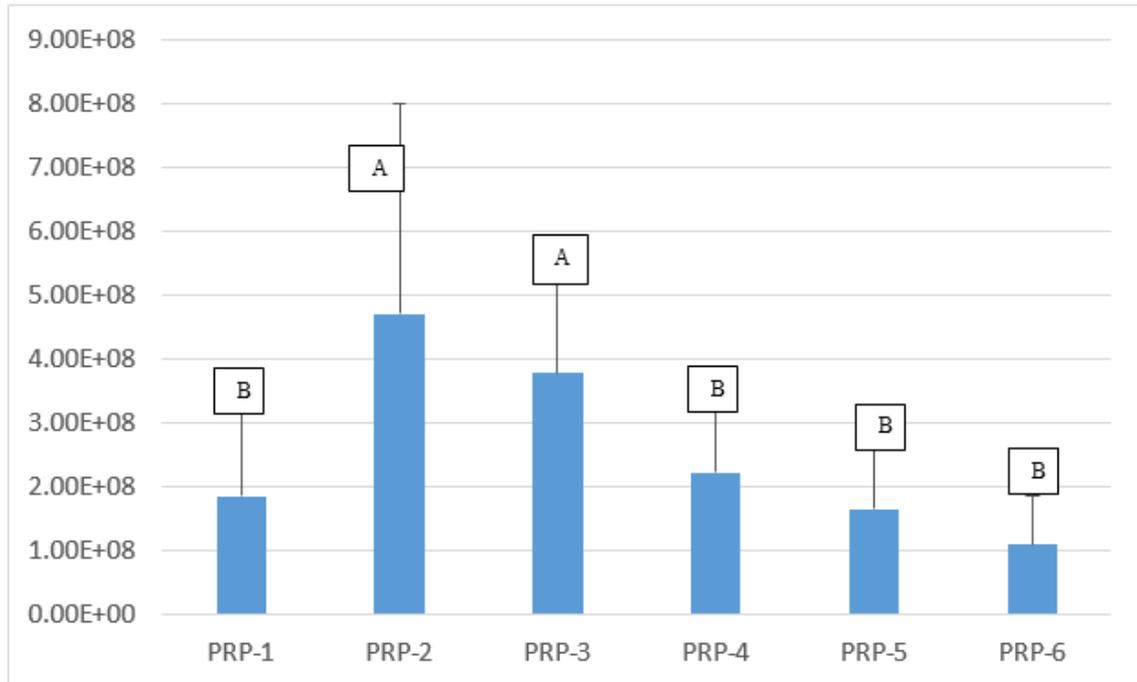Figure 3.3   Platelet activation status using Annexin V.

Platelet activation status was evaluated using Annexin V. (A) Pooled PRP and PRP-Opt.
(B) Pooled PRP + collagen, Pooled PRP + thrombin, PRP-Opt + collagen, and PRP-Opt
+ thrombin (collagen and thrombin were used to activate platelets and served as positive
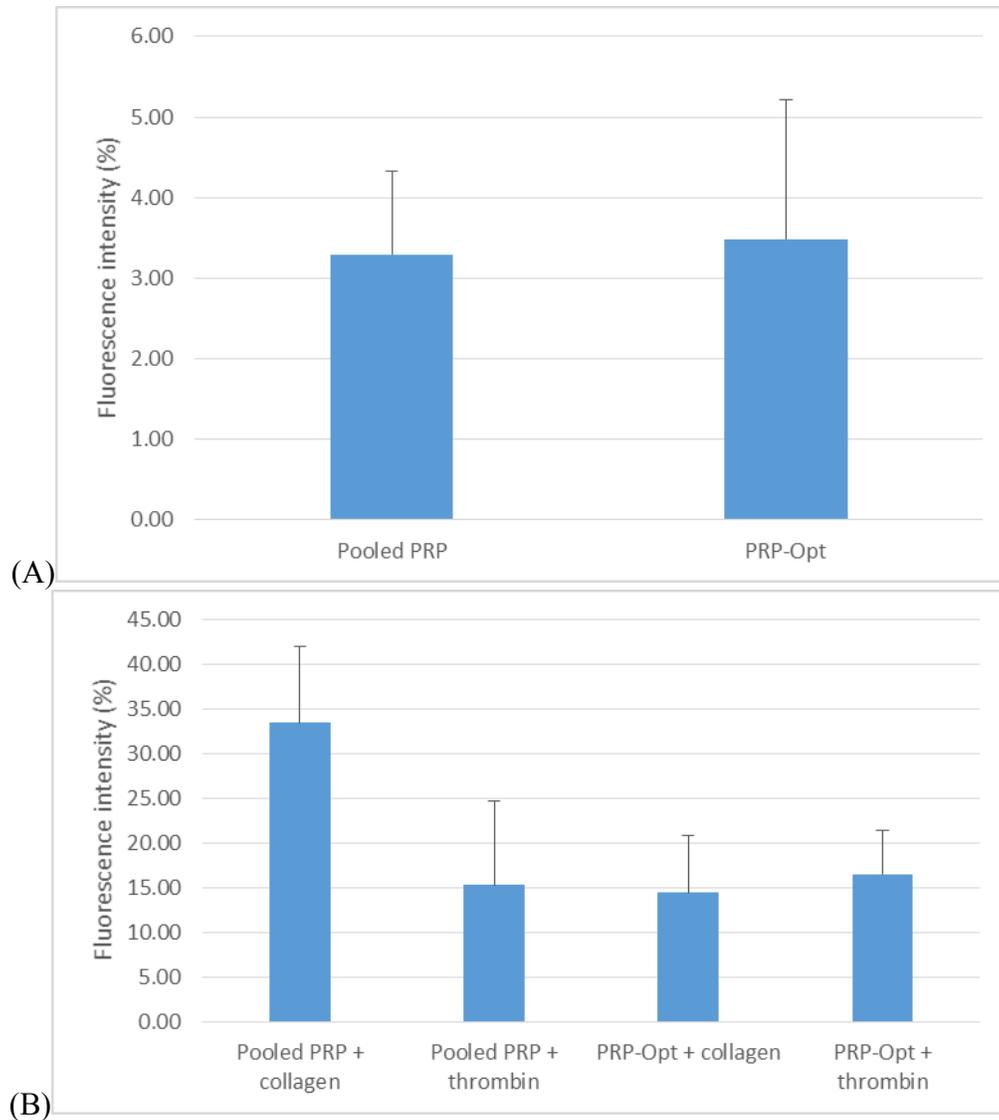controls in the flow cytometry protocol).

(A)

(B)
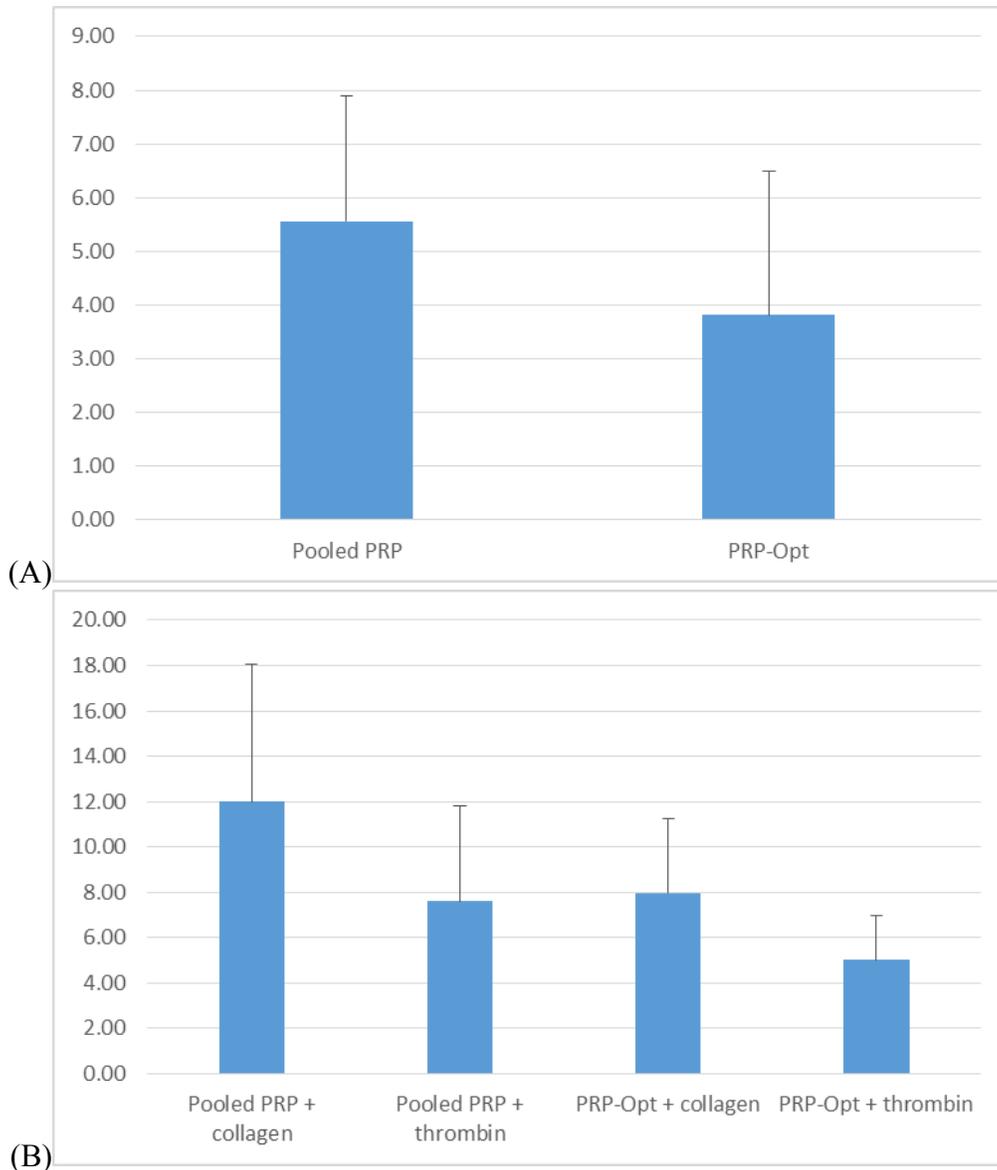
Figure 3.4   Platelet activation status using P-selectin.

Platelet activation status was evaluated using P-selectin. (A) Polled PRP and PRP-Opt.
(B) Pooled PRP + collagen, Pooled PRP + thrombin, PRP-Opt + collagen, and PRP-Opt
+ thrombin (collagen and thrombin were used to activate platelets and served as positive
controls in the flow cytometry protocol).

Figure 3.5   Platelet populations identified by flow cytometry.
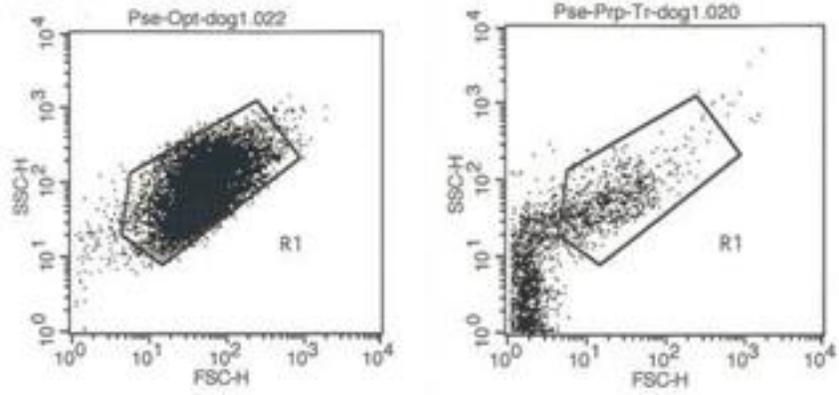
Platelet populations identified by flow cytometric display in a forward-angle versus side-angle light scatter plots. On the left, non-activated Thrombin sample – Optiprep PRP. On the right, PRP activated sample by Thrombin.

CHAPTER IV

CONCLUSION

The first step of the objective of this study was to develop and optimize a procedure for harvesting PRP from dogs. An average of 1.40x 10^9 ± 0.63x 10^9 platelets was recovered from 8.5 mL of WB with minor WBC and RBC contamination. The second step was to generate an ultra-pure PRP, that was obtained using Optiprep 1.063. The ultra-pure PRP generated had an average of 0.0164 RBC / 10^7 platelets and 0.0158 WBC / 10^7 platelets. The ultra-low numbers of RBC and WBC contamination are desirable for several applications, including proteomics and transcriptomics analysis. It was evident by Annexin V labeling that the harvested platelets were functional and that activation due to the protocol was minimal to nonexistent. Thus, loss of platelet content due to degranulation was unlikely.

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