ADVANCES IN TRANSFUSION MEDICINE FOR VETERINARY PATIENTS

A Thesis

by

WHITNEY DANIELLE HINSON

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Chair of Committee,	Kelley Thieman Mankin
Committee Members,	Lisa Howe
	Artem Rogovskyy
	Sara Lawhon
	Gwendolyn Levine
Head of Department,	Jonathan Levine

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ABSTRACT

Red blood cell (RBC) transfusions are common life-saving treatments for hypoxic anemia both for human and veterinary patients. For many years, allogenic blood transfusion (ABT) was the mainstay of transfusion medicine due to efficacy and relative availability, especially perioperatively and in emergency trauma cases. However, ABTs are no longer the preferred treatment for anemia in human and veterinary medicine. There has been a shift away from liberal or unnecessary transfusions due to the inherent risks, and a new focus on more conservative protocols using patient blood management. Similarly, in veterinary medicine, there has been an increase in awareness of risks associated with ABT and adoption of more conservative transfusion principles.

Alternative transfusion methods have been explored to reduce the need for ABT perioperatively. Specifically, autologous blood transfusion (autotransfusion) via cell salvage washing (CSW) is an effective, safe alternative used perioperatively without risk of incompatibility. However, contraindications have limited the use of cell salvage in the past. Leukoreduction filters (LRF) have been investigated to mitigate such contraindications by removing contaminants, specifically bacteria. LRF has proven effective to remove bacteria in human blood. Our proof-of-concept study showed that CSW in combination with LRF reduced bacterial contamination in dog blood. Therefore, CSW and LRF could be used to remove bacterial contamination of blood for intraoperative autotransfusion in veterinary patients.

Despite our best efforts, ABT cannot always be avoided. A major component of ABT that contributes to transfusion-associated complications is storage lesion. Storage lesions and secondary detrimental effects on patient outcome have been well described in the literature.

Microparticles (MPs) derived from blood cells have been identified in stored ABT and are considered a storage lesion. Prestorage leukoreduction has been shown to decrease, not eliminate, the formation of MPs. Prestorage leukoreduction is a standard transfusion protocol in human medicine but not veterinary medicine. Therefore, we investigated the ability of a LRF to remove MPs from stored dog blood prior to transfusion. We concluded that the LRF used in this study did not remove MPs from stored dog blood post-storage. However, based on the limitations, further studies are indicated to validate this conclusion.

DEDICATION

To my mother and father

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NOMENCLATURE

ABT	Allogenic blood transfusion
RBC	Red blood cell
pRBC	Packed red blood cell
CSW	Cell salvage washing
IOCS	Intraoperative cell salvage
LRF	Leukocyte reduction filter/Leukoreduction filtration
MP	Microparticle
EDMP	Erythrocyte-derived microparticle
LDMP	Leukocyte-derived microparticle
PDMP	Platelet-derived microparticle

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

1. INTRODUCTION: THE EVOLUTION OF TRANSFUSION MEDICINE IN VETERINARY PATIENTS

Red blood cell (RBC) transfusions are common life-saving treatments for hypoxic anemia both for human and veterinary patients.[1-6] Examples for indications for RBC transfusions include acute hemorrhage, hemolysis secondary to drugs/toxins, immune-mediated diseases, severe non-regenerative conditions, and neonatal isoerythrolysis. Allogenic blood transfusion (ABT) is defined as transfusion of blood between the same species and are the most common type of transfusions. Improved availability was established via storage with blood banking. For many years, ABT was the mainstay of transfusion medicine due to efficacy and relative availability. However, ABTs are no longer the preferred treatment for anemia in human and veterinary medicine due to increasing concern for transfusion-related complications, disease transmission, decline of availability, and incompatibility. There has been a well-described paradigm shift in human medicine away from liberal or unnecessary transfusions due to the inherent risks of transfusions.[7] Instead, human medicine has a new focus on more conservative transfusion triggers,[8] emphasis on reducing blood loss, and using blood alternatives.[9] Similarly, in veterinary medicine, there has been an increase in awareness of risks associated with ABT.[10,11] Recently, more conservative transfusion principles have been adopted by veterinary practitioners.[12]

In human medicine, transfusion triggers were developed as guidelines to help clinicians make objective decisions on transfusion administration and have been modified over the years to promote more conservative, judicious use of ABT. Liberal transfusion practices have resulted in increased short-term mortality rates and increased adverse postoperative outcomes.[13] Veterinary

medicine has gradually adapted these guidelines into our own conservative transfusion principles. Transfusion triggers have traditionally focused on degree of hemorrhage, hemoglobin concentrations, and hematocrit levels. Some controversy exists on the absolute values for blood loss and hemoglobin concentrations. In addition, acceptable values may differ for individual patients and across species. In general, for patients that are actively bleeding, transfusion is usually not required if blood loss is <15% of patient's blood volume. Transfusion should be considered if blood loss is to 15-30% of the patient's blood volume and is usually necessary if blood loss is 30-40% of the patient's blood volume, and is absolutely indicated if >40%.[14] Critical hemoglobin concentrations, requiring transfusion, have been defined as 2-4 g/dL in humans and 3.3 g/dL in anesthetized dogs[15] with a hematocrit of <12%.[16] Values dropping below critical levels can result in multiple organ failure.[12] In humans, a 10/30 rule for hemoglobin concentration (10 g/dL) and hematocrit level (30%) has been used. Recently, the hemoglobin concentration at which a transfusion is recommended has been lowered to <7 g/dL to favor more restrictive transfusion criteria in humans.[14] Once a transfusion has been given, transfusion targets for hemoglobin concentrations are 7-9 g/dL with a hematocrit of 27% in humans.[10] Despite these objective measures, many clinicians in human and veterinary medicine still make decisions in a patient-dependent manner based on clinical status and comorbidities. These numeric guidelines are supplemental for clinical decision-making when blood transfusions are considered.

Although often initially life-saving, ABT administration comes with significant risks due to potential transfusion reactions. Transfusion reactions can range in severity from no clinical significance to life-threatening and potentially fatal. Donor infectious disease screening and compatibility testing, especially for patients with sensitization from previous transfusions, have been developed in attempts to reduce transfusion reactions. However, transfusion reactions can

occur despite appropriate pre-transfusion screening. The incidence of canine transfusion reactions has been reported between 3.3-40%.[17,18] Therefore, a thorough risk-benefit assessment is indicated prior to considering ABT. Transfusion medicine is currently highly regulated by governmental and professional health care organizations for human patients.[19,20] Advances in veterinary medicine have been made to optimize patient safety. The American College of Veterinary Internal Medicine (ACVIM) and the Association of Veterinary Hematology and Transfusion Medicine (AVHTM) published a consensus statement in 2005 with guidelines for canine and feline donor screening. The consensus statement was updated in 2016.[21] Point-of-care tests are readily available for canine and feline blood typing and cross-matching. These tests are used to try to prevent incompatible RBC transfusions that could result in immune-mediated transfusion reactions. However, despite precautions, pre-transfusion testing does not eliminate the risk of transfusion reactions. For this reason, in veterinary medicine, indications for transfusions still remain controversial.

Transfusion reactions are categorized as immune-mediated and nonimmune-mediated and further subdivided into acute (<48 hours) and delayed.[22] Immune-mediated reactions are triggered by a recipient's reaction to donor RBCs, plasma proteins, white blood cells (WBCs), platelet antigens, or antibodies. Acute hemolytic transfusion reactions are the most dangerous classification of immune-mediated transfusion reactions, often due to previous sensitization or pregnancy in dogs and incompatibility in cats. Immune-mediated hemolysis occurs when recipients possess circulating antibodies which bind to epitopes on donor RBCs, targeting them for immediate destruction by the immune system. Subsequently, a systemic inflammatory response, specifically a type II hypersensitivity reaction, occurs that is predominantly mediated by IgG in dogs and IgM in cats.[12] Intravascular hemolysis may lead to disseminated intravascular

coagulation, multiple organ failure, and death.[23] Patients may develop signs similar to anaphylaxis including vomiting, fever, tachycardia, dyspnea, hypotension, and possibly seizures with the addition of hemoglobinemia and hemoglobinuria.[24] In addition, extravascular hemolysis has also been reported as a manifestation in dogs,[25] which could result in milder intravascular signs in addition to jaundice, hyperbilirubinemia, and bilirubinuria. Delayed hemolysis can occur and usually manifests as an unexpected gradual decline in PCV 3-5 days after transfusion in addition to previously mentioned hemolytic signs. Delayed hemolysis has been well documented in humans as a result of previous sensitization[26,27] but not in veterinary medicine.

Acute hypersensitivity reactions are another group of immune-mediated reactions that result in a type I hypersensitivity reaction. Mediated by mast cells and IgE, clinical signs include fever, erythema, pruritus, and urticaria. Due to the rapid onset of type I hypersensitivity reactions, this type of transfusion reaction usually occurs within 45 minutes of starting the transfusion.[28] The reaction can progress to anaphylactic shock and is most commonly associated with plasma rather than RBC transfusions.[28]

Febrile non-hemolytic reactions are immune-mediated reactions defined by an increase in body temperature by more than 1°C without any other explanation. It is the most common adverse reaction reported in veterinary and human medicine that is usually self-limiting with little clinical significance. Febrile, non-hemolytic reactions are likely due to leukocyte and platelet mediated inflammation from cytokines and antibodies in the recipient. Therefore, leukoreduction (filtration to remove leukocytes from donated blood) has the potential to reduce this type of transfusion reaction. Fever usually lasts up to 20 hours. Fever can be an early sign of several types of transfusion reactions. Therefore, any increase in body temperature warrants further investigation to diagnose the underlying cause.

Nonimmune-mediated transfusion reactions typically include hemolysis, bacterial contamination, volume overload, and citrate toxicity. Nonimmune-mediated hemolysis is the final form of acute, non-immunologic transfusion reaction. Nonimmune-mediated hemolysis usually occurs secondary to improper handling or administration of blood products. RBCs will hemolyze if exposed to improper temperatures, transfused using pressure bags or small-bore needles, and co-administered with certain drugs or hypotonic solutions.[29] The blood products are rendered less effective[23] and could cause acute kidney injury.[30]

Bacterial contamination of blood products that results in transfusion-associated sepsis had been considered to be the most common cause of transfusion-related morbidity and mortality in humans until 1991.[31] Contamination of refrigerated blood products most commonly occurs during venipuncture of a donor with subclinical bacteremia. The psychrophilic gram-negative bacteria (e.g., *Yersinia* and *Serratia* spp.) are of particular concern as they can grow at the refrigeration temperature. In contrast, gram-positive bacteria are often implicated in contamination of platelet products stored at room temperature.[31] Despite the fact that some contaminated units may still appear normal, the detection of RBC color change, clots, or hemolysis could be suggestive of bacterial contamination.

Transfusion-associated circulatory overload (TACO) is another form of acute, non-immunologic transfusion reaction. TACO occurs when a normovolemic patient is administered blood products that results in a hypervolemic state. Patients with underlying cardiac or renal disease may be at a higher risk for TACO as with any other volume overload scenario.[29,32] Clinical signs are consistent with those of congestive heart failure and may be confirmed with thoracic radiographs showing evidence of pulmonary edema.

Lastly, citrate toxicity may occur when administering blood products, especially for massive transfusions, and is more likely for whole blood than packed red blood cells (pRBCs).[33] The citrate will chelate the calcium and could result in clinical hypocalcemia. Signs of hypocalcemia can include muscle tremors, arrhythmias, and hypotension.

A few additional acute reactions include dilutional coagulopathy and hyperammonemia. Dilutional coagulopathy is possible secondary to transfusion of whole blood products, especially with massive transfusions, due to depletion of clotting factors and platelets.[29] Hyperammonemia can results in old blood products due to the accumulation of ammonia from RBC metabolism. Patients with liver disease are especially at risk and usually have clinical signs consistent with hepatic encephalopathy such as seizures, head-pressing, or star-gazing.[34]

In addition to the potential morbidity and mortality of transfusion reactions, there are several major disadvantages of ABT that have led to the development of alternative transfusion strategies. The disadvantages of ABT include availability, compatibility, storage lesions, transfusion-related immunomodulation (TRIM), and transfusion-associated inflammation. Availability for blood products depends on the source of donors including specific blood types, resources for blood banking, hospitalized patient demand, and inventory budget for potential expired losses. Each of these factors vary for each hospital and could change daily depending on caseload. Therefore, the availability of blood for an emergency situation can be unpredictable.

Blood compatibility is another disadvantage of ABT especially in an emergency setting. Human and veterinary species have many different blood types. Specifically, there are 8 different DEA blood types identified in dogs.[35] DEA 1.1 and 1.2 are considered the most important in transfusion medicine due to high prevalence and incidence of transfusion reactions.[36] To reduce the risk of hemolytic transfusion reactions, pre-transfusion screening is performed via blood

typing and cross matching, especially for patients with a history of blood transfusions with previous sensitization (alloimmunization). However, as previously discussed, despite this screening process, adverse reactions can still occur. Compatibility testing takes time and contributes to surgical delay,[37] increases costs,[38] and may not guarantee safety.[39]

Storage lesions are another disadvantage of ABT specifically when units are stored for several weeks prior to administration. Stored red blood cells undergo progressive functional and structural changes, due to ongoing cellular metabolism, resulting in loss of adenine nucleotides, drop in pH, glucose, and other metabolites[40] and this constellation of alterations is termed "storage lesion". Studies have shown that transfusion of stored pRBCs over 2 weeks of age has negative effects on patient outcome when compared to fresh RBC transfusion in humans.[41,42] In addition, patients given transfusions of older blood (greater than 20 days of age) after cardiac surgery had an increased risk for postoperative complications and decreased short- and long-term survival.[41] An increased incidence of adverse outcomes in humans has been associated with large volume transfusions or transfusions of blood with longer storage times.[42-45] The adverse events include increased risk of infection, renal failure, respiratory failure, multiple organ failure and death.[42-45] These adverse events are especially noticed in compromised patient populations.[46]

Microparticle (MP) generation is part of storage lesion.[47] Recent studies in dogs have shown MP accumulation is significantly increased after day 7 of blood storage.[48] Microparticles have been found to accumulate in blood products during storage, therefore transfusion of a large volume of blood products or older blood products would result in transfusion of higher numbers of microparticules.[48] While not proven as a direct cause, it is likely that elevated MP levels may play a role in the negative effects that older blood products have on patient outcome.[49,50] Therefore, it is highly suspected that transfused MPs play a role in adverse outcomes.[51]

Transfusion-related immunomodulation (TRIM) is a well described disadvantage of ABT in human medicine and animal models. Most commonly associated with anemic cancer patients, TRIM refers to down-regulation of the immune system following ABT and subsequent cancer recurrence and bacterial infection. TRIM is a multifactorial phenomenon documented in many cancer patients receiving ABT and may be mediated by donor leukocytes, soluble inflammatory mediators, microparticles, and growth factors. Therefore, TRIM effects can be immunosuppressive, pro-inflammatory, and anti-inflammatory. Blood storage likely plays a direct role due to storage lesions, especially microparticles, and often leads to more deleterious effects.[52,53] The role of microparticles will be further discussed in Chapter 3. Human and animal studies have shown that ABT, in contrast to autotransfusions, negatively influences innate and cellular immunity.[54,55] Perioperative RBC transfusions and secondary TRIM has been associated with increased mortality, recurrence of solid tumors, and distant metastatic rate, specifically of the lung, gastrointestinal tract, and hepatobiliary system.[56-58] Studies in veterinary patients have shown an association between ABT and increased morbidity, specifically postoperative lung injury[59] and surgical site dehiscence.[60]

Transfusion-related acute lung injury (TRALI) is a common manifestation of TRIM and secondary inflammation reported in humans and animals. TRALI is now the leading cause of transfusion-associated death.[61] The pathophysiology is not fully understood but research using animal models shows immune and nonimmune mechanisms.[62-64] There could be reactions between donor leukocyte antibodies and recipient neutrophils. In addition, there is likely direct damage to pulmonary vascular endothelium resulting in vascular leakage and edema formation. One study using a murine model showed MPs as potential mediators of transfusion-related acute lung injury (TRALI) following transfusion of stored pRBCs.[65] Initially, TRALI can present

similarly to acute respiratory distress syndrome (ARDS) but usually occurs within 4-6 hours after transfusion. TRALI is thought to be a major cause of mortality in humans receiving transfusions and is likely under-recognized and subsequently underreported. TRALI is usually self-limiting, and human patients often recover within 96 hours with supportive care; however, the reported mortality rate ranges from 5-10%.[66]

Pre-storage leukoreduction of RBC transfusion can potentially mitigate some of the adverse immunomodulatory effects caused by donor leukocytes as seen in experimental animal models and human clinical studies. There has been evidence that leukoreduction of perioperative transfusions significantly reduced morbidity and mortality rates.[67] However, some data suggests that leukoreduction does not seem to influence survival or recurrence for cancer patients.[68-70] Leukoreduction will be further discussed in Chapter 2.

New practical emphasis on alternative transfusion strategies to promote restrictive transfusion practices led to the concept and implementation of patient blood management (PBM). PBM was first applied in human patients that were not candidates for transfusions due to religious beliefs or documented presence of alloantibodies but has evolved for broader application to improve patient outcomes. PBM limits or avoids the need for blood product administration by mitigating predisposing risk factors for ABT. Prior to considering alternative methods of transfusion, mitigating strategies used in human patients that can be applied to veterinary patients include optimizing hematopoiesis, optimizing response to anemia, and minimizing blood loss.[71] Since preoperative anemia is a major risk factor for transfusion in people, the Network for Advancement of Transfusion Alternatives (NATA) suggests postponing elective procedures that may result in large blood loss until the anemia can be resolved.[72,73] We are not always able to delay surgeries in the emergency setting, but could consider this protocol

for anemic veterinary patients undergoing elective procedures. Optimizing the body's normal physiologic response to anemia can be exploited by providing oxygen supplementation, maintaining normovolemia and adequate perfusion, and avoiding increases in metabolic oxygen demand as seen with tachycardia and hyperthermia. Minimizing blood loss starts with identifying underlying patient factors that could contribute to hemorrhage such as medications or coagulopathies. In addition, limiting iatrogenic and surgical blood loss is crucial to reduce the necessity for blood transfusions. It is important to be cognizant of the blood volume collected for laboratory testing, especially if serial samples are obtained in an anemic patient. Using pediatric-sized tubes can help limit iatrogenic blood loss.[72] Strategies to limit surgical blood loss should be implemented routinely as part of good surgical technique. These strategies include application of minimally-invasive techniques when possible, reducing surgical times, use of tourniquets and regional anesthesia if indicated, controlled hypotension, proper patient positioning, and use of traditional methods of hemostasis such as hemoclips, electrocautery, ligatures, and manual pressure. In some cases, these conventional methods are not effective for adequate hemostasis. Topical and systemic hemostatic agents are available adjuncts to provide additional hemostasis. Topical dressings, sealants, or adhesives can be used to provide mechanical blockage, promote thrombosis, or inhibit fibrinolysis. Typically, these products are composed of collagen, gelatin, or cellulose. Older generation agents rely on the patient's functional coagulation system to be effective. Newer generation agents contain thrombin and therefore do not rely on the patient's coagulation system since they can function independently by mimicking the last stages of the coagulation cascade.[74] Studies using these products in veterinary medicine show that they are safe and effective.[75] However, there is no data at this time that proves that the use of thrombin containing hemostatic agents reduces ABT. Systemic hemostatic agents, such as antifibrinolytics

and desmopressin, should be considered if patients have underlying coagulopathies or potential for excessive perioperative hemorrhage. Specifically, at-risk breeds such as Greyhounds may benefit from perioperative administration of aminocaproic acid to reduce postoperative hemorrhage.[76,77] Although there is no evidence yet to say these drugs reduce administration of ABT, this is likely due to the limited data available so far. Desmopressin is commonly used preoperatively for coagulation prophylaxis in dogs with type 1 von Willebrand disease. Its use is very limited as it is unlikely to impact transfusion requirements in patients without coagulopathies.

Despite the application of PBM to restrict transfusion administration, transfusions are still necessary, most often perioperatively and in emergency trauma cases. Although there are no transfusion methods that eliminate all potential risks, alternative transfusion methods have been used to further reduce the need for ABT perioperatively. For patients undergoing surgical procedures in which there is possibility of significant blood loss, autologous transfusion (autotransfusion) is an effective, safe alternative to ABT without risk of incompatibility. There are several different methods of autologous transfusion. Preoperative autologous donation (PAD) is a method by which blood is collected from a patient several weeks in advance then stored for use if/when needed. The disadvantages of this method include inconvenience, cost, storage lesion, potential contamination, and risk of administration error.[72] One study in the veterinary literature reports use of PAD but the potential disadvantages have made it an unfavorable option.[78] Acute normovolemic hemodilution (ANH) is a method in which blood is collected immediately before surgery followed by administration of crystalloids or colloids until normovolemia is achieved. This method is simple, low cost, and reduces the chances for storage lesions compared to PAD as the blood is stored for a very short period of time. The

consequences of hemodilution are unknown, but could result in cardiac injury.[14] Both PAD and ANH can result in wasted blood if transfusion is not deemed necessary intra-operatively. Although the use of ANH has not been reported in veterinary medicine, its use could be adapted from protocols in human medicine. Intraoperative cell salvage (IOCS) is an additional autologous transfusion method available to limit the need for ABT intraoperatively and postoperatively. IOCS involves recycling a patient's own shed blood. This method requires specialized equipment for collection, washing, and filtering of the blood prior to autotransfusion.[14,79] Blood that would otherwise be lost can be collected and administered to reduce the requirement for ABT.[79] Minimizing blood waste is a major advantage over the other methods of autologous transfusion. In addition, the blood is at minimal to no risk for storage lesions. A limitation is that the cell salvaged blood should be used within 24 hours of collection.[80] Certain contraindications, specifically bacterial contamination and presence of neoplastic cells, made administration of cell salvaged blood controversial. However, recent advances in leukoreduction filtration as an adjunct to IOCS have started to mitigate these contraindications.[81-83] This will be discussed in further detail in Chapter 2.

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2. REMOVAL OF BACTERIA FROM WHOLE DOG BLOOD USING A CELL SALVAGE WASHING SYSTEM AND LEUKOCYTE REDUCTION FILTER

2.1 Introduction

Autologous blood transfusion following red blood cell salvage washing (CSW) has become a preferred alternative to allogeneic blood transfusion (ABT) in human patients with either anticipated or unexpected intraoperative.[1-4] Cell salvage was shown to reduce ABT administration, which resulted in fewer transfusion-associated complications and postoperative morbidity.[5] In addition, cell salvage blood can be used up to 24 hours after collection for transfusion.[5] Evaluation of cell salvage in clinical veterinary patients is limited, but studies so far suggest that it is safe and effective for treating perioperative hemorrhage in dogs.[6-8] The use of cell salvage is feasible even with relatively low volume hemorrhage[9] and eliminates the risks of transfusion incompatibility.[10,11] Potential blood work abnormalities associated with autologous blood transfusion in dogs include hypocalcemia,[8] prolonged coagulation times, and hemolysis.[12] Reportedly, these blood work abnormalities are not clinically significant.[8,13]

Prior to autotransfusion, salvaged blood is routinely washed and filtered to remove cellular waste products, plasma proteins, and gross contaminants.[14] This process leaves behind a significant proportion of inflammatory cells and microscopic contaminants, including bacteria.[13] Bacterial contamination of autologous blood transfusions is common[15,16] with over 30% of sampled blood collected by the cell salvage system and readministered to human patients being culture-positive.[15] Sources of bacterial contamination include skin commensals, penetrating foreign bodies, gastrointestinal/hepatobiliary/genitourinary microbiota, bacteremia, visceral or cavitary infection/abscessation, and operative room contaminants.

Bacterial contamination is a relative contraindication for autotransfusion of cell-salvaged blood.[10] One experimental animal study showed that dogs with severe hemorrhagic shock (40% estimated blood volume loss) were at risk for sepsis when transfused with blood containing fecal contamination.[17] Most research on autotransfusion has focused on approaches that would reduce bacterial contamination to levels comparable to that in asymptomatic bacteremia. However, one study showed similar polymicrobial bacterial loads between asymptomatic and symptomatic human patients receiving contaminated transfusions of stored platelets.[18] Thus, the type of bacteria may have a significant influence on the likelihood of symptomatic illness following autotransfusion of blood products with bacterial contamination. Despite the research indicating that autotransfusion of culture-positive blood is appropriate in some scenarios, clinicians hesitate to autotransfuse blood products with known bacterial contamination due to the potential for transfusion-associated sepsis.[19,20]

Leukocyte reduction filters (LRFs) have been used to remove leukocytes and contaminants from allogeneic and autologous blood transfusions in human patients.[21,22] Leukoreduction by use of a LRF has been shown to correlate with an approximate 50% reduction in postoperative infection rates in surgical patients given perioperative transfusions.[23] However, the literature regarding the use of LRFs for veterinary patients is limited.[24] McMichael *et al*[25] demonstrated that leukoreduction of packed red blood cells via LRF (Sepacell RS2000, Baxter Healthcare Corp) eliminated transfusion reactions and the inflammatory response after transfusion in clinically healthy dogs.

LRFs are designed to remove white blood cells and debris while allowing red blood cells to pass.[26,27] The exact mechanism of filtration by LRFs is not completely understood, but both screen filtration and adsorption are likely involved.[27] Removal by adsorption relies on the

electrical charge of a particle rather than its size.[27] The use of LRFs to remove bacteria from blood seems counterintuitive because bacteria (2 μm on average) are much smaller than the red blood cells (7-8 μm) that pass through the filter (40 μm). The ability of LRFs to remove bacterial contamination and bowel contents from autologous blood transfusions has been investigated in human patients.[28] Proposed mechanisms of bacterial removal via LRF include phagocytosis by leukocytes trapped within the filter, adhesion to filtered leukocytes, direct filter adhesion, and complement-mediated cell death.[21,22] Waters *et al*[28] showed that a commercial LRF removed 97.6% to 100% of various bacterial contaminants from inoculated units of human packed red blood cells, significantly more than cell salvage processing alone. To date, the ability of LRFs to reduce bacterial contamination in whole dog blood has not been examined.

We examined whether CSW and LRF would reduce bacterial contamination in whole dog blood. We hypothesized that the combination of CSW and a LRF would reduce bacterial load of whole dog blood by at least 99% and that a second LRF would further reduce the bacteria count. We additionally hypothesized that CSW and LRF would remove different bacterial species with similar effectiveness.

2.2 Materials and Methods

A power analysis was performed to generate the appropriate sample size for an outcome of at least 80% power with a 95% confidence interval. This calculation resulted in a need of at least 8 samples per treatment group/bacteria. Therefore, 33 units of whole dog blood (1 unit = 250 mLs) were purchased from Animal Blood Resources International (ABRI, Dixon, CA, USA). Eleven units were received and processed on each of 3 days. The units of blood were collected from the donors the day prior and shipped overnight on ice.

A randomized block design was used to control for potential experimental bias across

treatment days. Each day, the units were randomly assigned for inoculation with 1 of 3 types of bacteria or as a control. One control unit was assigned each day. The remaining 10 units were randomly assigned to a type of bacteria using a randomization software. The experimental units were inoculated with one of the following strains of bacteria from American Type Culture Collections (ATCC): *Escherichia coli* (ATCC® 25922™), *Staphylococcus pseudintermedius* (Quality control species, Texas A&M Microbiology Laboratory) or *Pseudomonas aeruginosa* (ATCC® 27853™).

For each species of bacteria, a purified bacterial colony was inoculated into 10 mLs of lysogeny broth (LB). The broth was incubated in a tissue culture rotator (1640Q Cel-Gro Tissue Culture Rotator, Thermo Fisher, Waltham, MA) at 42 rpm for 18 hours at 37°C under 6.1% CO_2. Then, the culture was resuspended in 2.8 mL of sterile saline (0.45%) to reach a turbidity of 0.5 McFarland unit, correlating to an estimated bacterial concentration of approximately 1.5×10^8 colony forming units (cfu)/mL. Then, the bacterial suspension was diluted with sterile saline at 1:100. Thus, each inoculum contained approximately 1.5×10^6 CFU/mL.

Prior to inoculation, 1 mL of blood was collected aseptically from each blood unit and 100 µl was plated on LB agar to ensure that the blood was sterile (pre-inoculation sample). Each unit of blood was inoculated with 800 µl of inoculum. Mean inoculum concentrations were $7.05 \times 10^7 \pm 2.3 \times 10^7$ (CFU/mL), $2.81 \times 10^7 \pm 1.03 \times 10^7$ (CFU/mL), and $3.37 \times 10^7 \pm 2.37 \times 10^7$ (CFU/mL) for *E. coli*, *S. pseudintermedius,* and *P. aeruginosa*, respectively. Each control unit was inoculated with 800 µl of sterile saline and treated in the same fashion as the inoculated units. The units were then immediately rocked on a rocking platform (VWR 100 Rocking Platform Shaker, Marshall Scientific, Hampton, NH) at 60 tilts per minute for 1 hour at room temperature. Prior to cell salvage processing, inoculated blood samples (1 mL aliquots) were aseptically

collected and plated on LB agar in order to identify initial bacterial loads in all pre-wash samples.

Each unit of blood was processed at room temperature via a cell salvage machine (Fresenius C.A.T.S. Continuous Autotransfusion System, Fresenius Kabi AG, Ban Homburg, Germany). The machine was primed using the routine protocol with isotonic saline according to the manufacturer's instructions. Approximately 200 mLs of heparinized saline (30 USB units/mL) was run through the cardiotomy chamber as would be performed clinically to prevent clotting of blood within the cardiotomy filter. The unit of blood was then emptied into the cardiotomy chamber via suction. The entire volume of heparinized saline and blood was processed at a wash rate of 300 mLs/min (High Quality Wash setting), lasting approximately 20 minutes. After cell salvage processing, the red blood cell concentrate was filtered through two leukocyte reduction filters (40 μm RS Leukocyte Reduction Filter for Intraoperatively Salvaged Washed Blood, Haemonetics, Braintree, MA). One-milliliter samples were collected after washing (post-wash), first filtration (post-LRF1), and second filtration (post-LRF2) (Figure 1). All samples (inoculum, pre-wash, post-wash, post-LRF1, and post-LRF2) were immediately processed for bacterial enumeration. Specifically, the samples were serially diluted using phosphate buffered saline (PBS). The dilutions were vortexed at 2,800 rpm for 5 seconds, and 50 μl were plated on LB agar in triplicate. The plates were incubated for 24 hours at 37°C under 6.1% CO_2, and then bacterial colonies were quantified (CFU/mL).

2.2.1 Statistical Analysis

Bacteria counts at each testing point for each unit were recorded as the mean ± standard deviation (SD) CFU/mL per plated triplicate. Because no bacteria were found at the post-filtration stages other than in one sample after the first filtration, the statistical analysis focused

on bacteria reduction between the pre-wash and post-wash samples. A one-way ANOVA was performed to compare the reduction in bacteria between pre-wash and post-wash samples. The response variable was recorded as the mean log reduction ± SD. The variables of interest were the treatment day, type of bacteria, and log of pre-wash bacteria count. The treatment day was included as a variable to confirm there was no experimental bias across days. The type of bacteria was included as a variable to determine if there was a difference in treatment effectiveness between bacteria types. The log of the pre-wash bacteria count was included as a variable because it is highly correlated with bacterial reduction. A Wilcoxon Sign Rank test was used to compare bacterial reduction between stages. Statistical significance was set at $P < 0.05$.

2.3 Results

The mean wash duration was 18.0 ± 3.1 minutes (Table 1). The mean packed cell volume (PCV) was increased from 37.2 ± 2.4% before treatment to 66.0 ± 4.5% after treatment. The mean red blood cell concentrate volume after washing was 114.6 ± 16.3 mLs, compared to the starting volume of 250 mLs. The CSW alone reduced bacterial levels, on average, by 85.2%, 91.5%, and 93.9% for *E. coli, S. pseudintermedius,* and *P. aeruginosa*, respectively ($P < 0.0001$). Following the first filtration, there was a 99.9%, 100%, and 100% reduction in bacteria, respectively ($P < 0.0001$). Finally, after the second LRF, no bacteria were recovered ($P < 0.0001$).

There was no statistical difference between post-LRF1 and post-LRF2 samples ($P = 0.33$). None of the negative controls (pre-inoculation samples) had any growth at any testing points. The mean bacteria counts (CFU/mL) for the pre-wash, post-wash, post-first filtration (post-LRF1), and post-second filtration (post-LRF2) samples are summarized in Table 3. After

accounting for the pre-wash bacteria count ($P < 0.0001$), neither the treatment day ($P = 0.14$) nor the type of bacteria ($P = 0.29$) influenced the bacterial reductions.

2.4 Discussion

The results of this study indicate that a combination of cell salvage washing and leukoreduction filtration effectively remove bacteria from dog blood for autotransfusion. Autotransfusion is a safe and effective alternative to allogenic blood transfusions that can be used intraoperatively to take advantage of a patient's viable extravasated RBCs and minimize the risk of transfusion reactions, especially in an emergency setting[1,2,6,7,11,12,29,30]. Bacterial contamination has been a listed contraindication for autotransfusion of cell salvaged blood in humans due to the concern for transfusion-associated sepsis.

Patients requiring blood transfusions have been shown to have increased morbidity and mortality.[31-35] This is most likely due to potential life-threatening reactions caused by administration of allogenic blood transfusions. Critically-ill patients usually receive the highest volume of blood products, and therefore are most susceptible to the adverse effects and worse prognosis due to ABT.[36] Autotransfusions have been shown to reduce the need for allogenic blood transfusions and thus the potential for adverse events.[5,37] In addition, the patient's own RBCs can be recycled rather than discarded, eliminating the risk of incompatibility and delay for blood screening. This study further supports the use of LRF to increase safety of autotranfusions, decreasing the need for ABT, and as a result, possibly improving patient outcome.

Removal of bacterial contamination is important to minimize the risk of transfusion-associated sepsis and maximize the benefits of autotransfusion. Bacterial contamination of blood suctioned during surgery is common and occurs secondary to a variety of occurrences including: visceral damage, penetrating abdominal wounds, and inadvertent surgical site contamination.

Intracavitary blood that originates from trauma or neoplasia of visceral organs (e.g., gastrointestinal and hepatobiliary tracts) is likely contaminated.[38] Not only do bacteria enter the surgical site from the viscera, but also from intraoperative contamination of suction tips, even in clean procedures,[39] with contamination rates as high as 92%.[40] As suction is a necessary component of the CSW process, suction tip contamination is expected with cell salvage processing due to constant suction of room air, skin contaminants, and visceral contamination. Therefore, consideration should be given to leukocyte reduction filters as adjuncts to intraoperative cell salvage due to the high likelihood of bacterial contamination and the ability of the LRF to remove bacteria from blood *ex vivo*.

Cell salvage washing involves suctioning cavitary hemorrhage through a centrifuge to concentrate the RBCs and to remove contaminants. Since our goal was to create an *ex vivo* model for a septic hemoabdomen, we measured pre- and post-treatment PCVs to assess concentrating abilities of our system with reported *in vivo* comparisons. The pre-treatment PCV was 37.2 ± 2.4%. Similarly, Hirst *et al*[6] reported abdominocentesis and peripheral blood PCVs of 28-33% for cases of hemoabdomen. Therefore, the pre-treatment PCV is comparable to an *in vivo* hemorrhage scenario. The post-treatment PCV was 66 ± 4.5%, which is within the range of previously reported processed RBC hematocrit values.[7,29] Based on these comparisons, it seems that our model was successful in mimicking an *in vivo* scenario of cavitary hemorrhage.

In the present study, cell salvage washing alone reduced contamination by an average of 90.2% for all bacteria species tested and LRF resulted in elimination of remaining bacterial contamination. These results are similar to those previously reported.[13,38] Cell salvage washing alone was previously shown to remove 77-95% of bacterial contamination.[13] Our results from CSW alone showed a high percentage of bacterial reduction within this range, likely due to the

cell salvage machine settings applied in the present study. First, we used a "high quality wash" setting that washes more slowly, probably resulting in more dilution and subsequent decontamination. Second, we suctioned an additional liter of sterile saline through the cardiotomy filter to remove trapped red blood cells and capture remaining RBCs.

The first of two LRFs reduced bacteria to an undetectable level for all samples except one. The second LRF was able to subsequently reduce the bacteria to an undetectable level for that sample. Therefore, we recommend as thorough a washing of RBCs as possible given any time constraints. If a lower quality CSW is performed, fewer bacteria may be removed, possibly impacting the ability of CSW and LRF combination to sufficiently remove bacteria and necessitating multiple LRFs. The exact mechanism for removal of bacteria by LRF is not completely understood. However, similar to a human clinical study,[38] the addition of the LRF in combination with CSW was able to further reduce any remaining contamination left behind by CSW.

In an emergency situation necessitating a blood transfusion, the method of transfusion needs to be time efficient, as this could impact patient survival. The protocol for CSW used in this study included two processing modifications in attempt to minimize RBC waste through our system. The combination of these processing modifications increased the total run time for CSW to an average of 18.0 ± 3.1 minutes compared to 5 minutes reported by Kellett-Gregory *et al*.[7] In general, the processing time for CSW may be slightly longer than administering ABT. However, some may consider the time difference to be negligible due to the time necessary for compatibility testing and equipment setup for allogenic transfusions. In any case, if a patient's blood loss is approaching a critical volume, the clinician should prioritize using the most time efficient protocol to minimize time to transfusion.

One of the limitations of our study is a limited spectrum of bacterial pathogens tested. We assessed removal of three facultative anaerobes commonly encountered in veterinary medicine, especially in surgical patients. These bacteria are considered to be pathogenic and routinely isolated from animal infections. *E. coli* is one of the most common bacterial isolates in septic peritonitis and trauma to the hepatobiliary or gastrointestinal tracts.[41-45] *P. aeruginosa* is associated with various surgical site infections[46] and abdominal evisceration injuries.[45] *S. pseudintermedius* is part of commensal microflora on dog skin[47] and therefore is often a common contaminant of wounds.[40,48] Importantly, the three bacteria are consistently cultured from suction tips and constitute the predominant sources of intraoperative contamination in dogs and cats.[40,49] Bacterial decontamination with CSW and LRF did not differ significantly between the three bacterial species tested here, suggesting that the overall procedure might be equally efficient at removing other species of bacteria.

Previously, Waters *et al*[28] showed that, in human blood, numbers of *Bacteroides fragilis* could be reduced by approximately one log via LRF and that the combination of CSW and LRF could remove bacterial concentrations of approximately 10^3 CFU/mL in the presence of gross fecal contamination. We tested the efficacy of CSW and LRF to reduce bacterial concentrations of up to 2600 CFU/mL. However, our *ex vivo* model did not test a mixed population of bacteria. Therefore, the data presented here is likely not representative of a septic abdomen from gross fecal contamination. As a result, we cannot comment on the ability of CSW and LRF to remove polymicrobial contamination at high concentrations. We expect that our data is consistent with intraoperative contamination during hemoabdomen, or traumatic hemoabdomen due to puncture or visceral trauma. Liang *et al*[38] indicated that LRF is successful at removing bacteria in human clinical cases of liver transplantation with polymicrobial contamination.

A previous study suggested that the efficacy of filtration varied for different bacteria.[18] This is in contrast with the results of our study in which we did not find a difference in filtration between bacterial species. The disagreement in findings could be due to patient species, bacterial species, and/or the *ex vivo* nature of our study. Further studies are needed in veterinary patients to assess the clinical success of LRF. However, data from human studies and our *ex vivo* study shows that CSW and LRF have the ability to remove bacteria from blood.

In conclusion, cell salvage washing and leukoreduction filtration proved to be effective in removing bacteria from whole dog blood. The leukoreduction filter is an easy, inexpensive addition to cell salvage washing that, in combination with CSW, has the ability to remove bacteria from blood. This approach could be applied to intraoperative autotransfusion of blood in veterinary patients, even for clean procedures. Future clinical trials in client-owned patients are warranted to determine whether this autotransfusion process improves patient outcome. The outcome should be compared with those of current standard protocols (allogeneic blood transfusions) in dogs with intracavity hemorrhage with bacterial contamination.

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3. QUANTIFICATION AND PHENOTYPING OF MICROPARTICLES IN STORED DOG BLOOD PRE- AND POST-FILTRATION WITH A LEUKOCYTE REDUCTION FILTER

3.1 Introduction

In the United States, approximately 15 million red blood cell (RBC) transfusions are administered to humans every year.[1] In 2014, Pet Blood Bank UK stated that it supplied over 3,000 units of canine blood products to veterinary professionals with a national blood bank reporting over 35,000 canine units every year.[2] Over the last 30 years, the demand for blood products in veterinary medicine has climbed dramatically. There has also been a shift from whole blood to selective blood products with an average increase in demand of 25% each year.[2] This shift is in response to advances in knowledge about the potential harmful effects of blood transfusions. As in human medicine, veterinary medicine is making advances in patient blood management with the goal of providing life-saving treatments to patients without causing unnecessary harm.

With the increase in demand for blood products, blood banking has become a necessity for many veterinary practices in which blood is subsequently stored for use in an emergency setting. Studies have shown that transfusion of stored pRBCs over 2 weeks of age has negative effects on patient outcome when compared to fresh RBC transfusion in humans.[3,4] An increased incidence of adverse outcomes in humans has been associated with large volume transfusions or transfusions of blood with longer storage times.[4-7] The adverse events include increased risk of infection, renal failure, respiratory failure, multiple organ failure and death[4-7] and are especially reported in critical patient populations.[8] Patients given transfusions of older blood (greater than

20 days of age) after cardiac surgery had an increased risk for postoperative complications and decreased short- and long-term survival.[3]

Although the mechanism linking adverse outcomes with transfusions and increased duration of blood storage is unclear, several factors likely contribute. When blood is stored for blood transfusions, certain substances accumulate over time, this constellation of biochemical abnormalities is termed storage lesions. Stored red blood cells undergo progressive functional and structural changes, due to ongoing cellular metabolism, resulting in loss of adenine nucleotides, drop in pH, glucose, and other metabolites.[9] MPs have been found to accumulate in blood products during storage,[5,10-15] therefore transfusion of a large volume of blood products or older blood products would result in transfusion of higher numbers of MPs.[16] A recent study showed MP accumulation in dog blood is significantly increased after day 7 of blood storage.[16] While not proven as a direct cause, it is likely that elevated MP levels, as part of storage lesion, may play a role in the negative effects that older blood products have on both human and canine patient outcomes.[10,12,16,17]

MPs are biologically active, nanovesicles derived from the cell membrane of white blood cells, red blood cells, platelets and endothelial cells *in vivo* and are considered a significant storage lesion *ex vivo.*[16,18,19] *In vivo* cell-derived MPs are detected in circulation in healthy humans[20-22] and dogs.[23] At normal physiologic levels, MPs function to support low-grade thrombin production, which activates protein C, thereby providing an anticoagulant function within the vasculature.[24] Concentrations above physiologic levels have been evaluated in a variety of human[22,25-27] and veterinary[16,28] systemic diseases, including neoplasia.[15,29] Despite the necessity for MPs to be present for hemostasis, concentrations above normal physiologic levels may be detrimental as MPs demonstrate pro-coagulant activity from 50 to 100-times higher than

platelets.[5,30] The pro-coagulant activity is in large part due to exposure of the anionic phospholipid phosphatidylserine which allows assembly of coagulation factors into active complexes for thrombin generation.[5,16,24] Due to their potent procoagulant and immunomodulatory properties, MPs have been shown to contribute to transfusion reactions[11,12] and hemostatic disorders,[20,22] often resulting in increased morbidity and mortality.[12] Specifically, erythrocyte, leukocyte, and platelet-derived MPs have been found at elevated levels in humans with sepsis and pathologic coagulopathies.[17,26] Recent studies in human medicine have also suggested that MPs play a significant role in the development and metastasis of cancer and multi-drug resistance to chemotherapeutics.[29,31] Due to these findings in the recent literature, we suspect that the number of MPs administered to our veterinary patients should be minimized, especially in patients that are critically ill and may be more susceptible to adverse effects due to higher numbers of circulating MPs *in vivo* prior to transfusion.[17,26]

Leukoreduction has been used to remove white blood cells, cell salvage debris, neoplastic cells,[32] and bacteria[33] from blood products. Prestorage leukoreduction involves filtering the blood products through a leukocyte reduction filter prior to storage to remove white blood cells and is standard protocol in human transfusion medicine. Prestorage leukoreduction of human whole blood has been shown to significantly decrease MP formation during blood storage[14,34] and mitigate the deleterious effects of blood aging.[35,36] Prestorage leukoreduction of units of dog packed red blood cells (pRBCs) has been shown successful in eliminating transfusion-induced inflammation and preserving red blood cell viability.[37,38] Despite these findings, prestorage leukoreduction is not standard protocol in veterinary medicine, and although it decreases the number of MPs formed during storage, it does not eliminate them. Further accumulation of erythrocyte-derived MPs occurs between the time of blood collection and blood administration

regardless of whether prestorage leukoreduction is performed or not. If MPs could be removed post-storage at the time of transfusion administration, then dogs receiving blood transfusions may be spared from the detrimental effects of MPs found in stored dog blood.

The objectives of this study were to quantify and phenotype microparticles in stored dog packed red blood cells and to assess the ability of a leukocyte reduction filter to remove microparticles from the blood at the time of transfusion. We hypothesized that the majority of microparticles in stored dog packed red blood cells would be erythrocyte-derived. We further hypothesized that a commercial leukocyte reduction filter would reduce the quantity of microparticles in stored dog blood.

3.2 Materials and Methods

3.2.1 Flow cytometer calibration

Since the resolution of the flow cytometer would directly affect our ability to detect MPs for enumeration and differentiation, we validated the resolution of our flow cytometer (BD LSRFortessa, Cell Analyzer, BD Biosciences) by performing a preliminary calibration study. In order to properly calibrate the flow cytometer to detect submicron size particles (ie MPs), a SPHERO Nano Fluorescent Size Standard Kit (Flow Cytometry grade, yellow, IE6/mL, Spherotech, Lak Forest, IL) was used to verify the instrument's performance in nano cytometry fluorescence, side scatter, and width parameters. Since MPs range in size between 0.1 micrometers to 1 micrometer, we used a mixture of nanobead sizes including 0.1-0.3 μm, 0.4-0.6 μm, 0.7-0.9 μm, and 1.0-1.9 μm. Our flow cytometer was successfully able to detect particle sizes in these ranges.

3.2.2 Sample preparation for MP processing

Eight units of expired canine packed red blood cells were acquired from the Texas A&M University Veterinary Medical Teaching Hospital blood bank. The units were cultured to confirm absence of bacterial contamination since bacteria can produce their own microparticles[39,40] and would interfere with our quantification. Three milliliter aliquots from each blood unit were collected prior to leukoreduction filtration (pre-LRF sample). The units were then filtered with a commercial leukocyte reduction filter (40 μm RS Leukocyte Reduction Filter for Intraoperatively Salvaged Washed Blood, Haemonetics, Braintree, MA). After approximately half of the unit volume was filtered, a second 3 mL aliquot of RBC concentrate was collected (post-LRF sample). The pre-LRF and post-LRF samples were centrifuged at 2500 x g for 10 minutes at 20°C to obtain platelet poor plasma (PPP) as previously described[41] for MP quantification and phenotyping.

3.2.3 Flow cytometric quantification and phenotyping of MPs

Flow cytometry is the current gold standard for MP quantification. Thus far in the literature, MPs are quantified based on size and fluorescence. The MPs were defined by their small size (log forward scatter) and phenotyped via labeling with multiple monoclonal antibody staining and cross-reactive fluorescence. Monoclonal antibodies were chosen as follows based on reported markers for cell-derived MPs[42]: Mouse anti Pig CD61-APC antibody (clone JM2E5) (CD61/Integrin beta 3 Monoclonal Antibody, APC, eBioscience, ThermoFisher Scientific, Waltham, MA), Rat anti Dog CD45-eFluor450/BV421 antibody (clone YKIX716.13) (CD45 Monoclonal Antibody, eFluor 450, eBioscience, ThermoFisher Scientific, Waltham, MA), and annexin V-FITC (ApoScreen Annexin V, SouthernBiotech, Birmingham, AL). CD61 is a specific surface protein for cells of thrombocytic origin directed against the platelet membrane

antigen GpIIIa and has been used to label platelet-derived MPs in equine plasma.[41] CD45 is present in all leukocytic cell membranes and has been reported for labeling leukocyte-derived MPs in the human literature.[14] Annexin-V stains exposed phosphatidylserine (PS) on the outer cell membrane and has been used to label MPs with PS exposure in the veterinary literature.[16,41] Quantification was performed by simultaneous analysis of 5.1 micrometer fluorescent counting beads (AccuCount Fluorescent Particles, Spherotech, Lake Forest, IL) on PPP. For analyses, 5 microliters of PPP were added to annexin-binding buffer to a final reaction volume of 100 microliters. The samples were then incubated with annexin V-FITC (1 microliter; 1:100 concentration), CD-61-APC (1:100 concentration) and CD-45-eFluor450/BV421 (1:100 concentration) in a dark room at room temperature for 30 minutes. A negative control consisted of PPP incubated in annexin-binding buffer (Annexin Binding Buffer, Invitrogen, ThermoFisher Scientific, Waltham, MA). The reaction was quenched with 400 µL of annexin-binding buffer. In order to quantify MP events (per µL), 50 µL of fluorescent counting beads were added to each sample after vigorous vortexing to disperse bead aggregates. Positive controls of antibody compensation beads (AbC Total Antibody Compensation Bead Kit, Spherotech, Lake Forest, IL) were simultaneously run with each sample according to the manufacturer's instructions.

Flow cytometric analysis was performed on a flow cytometer with manufacturer's software package and standard settings as follows: side scatter, forward scatter, and fluorescence were performed on log mode. Voltage for annexin V-FITC was 396 V; CD-61-APC was 605 V; CD-45-eFluor/BV421 was 363 V; forward scatter threshold was 148 V; side scatter threshold was 126 V; parameter threshold for side scatter was 200 V. MP gates were created based on forward scatter and compensated FITC fluorescence. Dual florescence quadrant plots where then derived for FITC positive and negative populations based on compensated APC and BV421

fluorescences. Representative dot plots are shown in Figure 2 for sample unit 4. Platelet-derived microparticles (PDMPs) were defined as those events that were double positive for annexin-V and CD-61 and negative for CD-45. Leukocyte-derived microparticles (LDMPs) were defined as those events that were double positive for annexin-V and CD-45 and negative for CD-61. Erythrocyte-derived microparticles (EDMPs) were defined as those events that were annexin-V positive and double negative for CD-61 and CD-45. Events were quantified using FlowJo data analysis software (FlowJo, LLC). Conversion of events to MPs/μL was calculated by the following formula:

(number of events in test sample/number of bead events) x (number of beads per sample/volume of test sample initially used)

Events were collected under high flow rates, and acquisition ceased when 2,500 bead events were counted in the fluorescent bead gate. There were 2,500 bead events and 49,655 beads/50 microliters for the lot number used in this study.

3.2.4 Statistical Analysis

Annexin-V-positive MP concentrations from PPP were analyzed using the nonparametric Kruskal-Wallis one-way ANOVA test for comparison of means between pre- and post-LRF groups. Comparison of means between phenotype were further analyzed using the Wilcoxon method for nonparametric comparisons for variance between phenotypes for pre- and post-LRF groups. Nonparametric linear regression was used to evaluate for an effect of group (pre- vs post-LRF), microparticle phenotype (PDMP, LDMP, EDMP), and storage duration (days) on MP concentrations. Microparticle counts and storage duration were reported as mean ± SD. Significance was defined as $P < 0.05$. Statistical analysis was performed with the aid of commercially available software (JMP Statistical Analysis Software, SAS, Cary, NC).

3.3 Results

Annexin-V-FITC-positive MPs were detected in PPP from all expired packed RBC units. The mean pre- and post-LRF MP concentrations by phenotype are summarized in Table 2. Mean MP concentrations were significantly different among MP phenotypes for pre- ($P = 0.003$) and post-LRF ($P < 0.0001$). For pre-LRF MP concentrations, there was a significant difference between PDMP and LDMP ($P = 0.0027$) and PDMP and EDMP ($P = 0.0009$) concentrations, but not between LDMP and EDMP ($P = 0.0520$) concentrations. For post-LRF MP concentrations, there was a significant difference between LDMP and EDMP ($P = 0.0086$), PDMP and EDMP ($P = 0.0009$), and PDMP and LDMP ($P = 0.0009$) concentrations. The mean absolute pre-MP concentration was 1,169.87 ± 4,388.79 MPs/μL. The mean absolute post-MP concentration was 683.75 ± 1,940.72 MPs/μL. Despite a decrease in absolute mean MP concentrations, the difference between the pre- and post-LRF concentrations were not significant ($P = 0.6221$). MP phenotype had a significant effect on absolute MP concentrations ($P = 0.0265$). The mean storage duration of the expired units of packed RBCs was 52.25 ± 11.41 days at the time of processing. Storage duration had a significant effect on MP concentration alone ($P = 0.0095$) and when accounting for MP phenotype ($P = 0.0010$). Specifically, storage duration had a significant effect on EDMP ($P = 0.0002$) and LDMP ($P = 0.0389$) concentrations. There was no bacterial growth on pre-LRF aerobic cultures for any sample.

3.4 Discussion

This study demonstrated that there is a mixed population of MPs in pRBCs, corresponding to the parent population of blood cells, with EDMPs being the majority. Furthermore, we found that storage duration had a positive effect on MP concentrations, specifically EDMPs. Our findings

are consistent with previous reports in human and veterinary literature, as packed red blood cells are the predominant cell type present and most susceptible to oxidative changes.[10,12,16,43] Studies have shown that transfusion of stored pRBCs over 2 weeks of age has negative effects on patient outcome when compared to fresh RBC transfusion in humans.[3,4] Although the mechanism linking adverse outcomes with transfusions and increased duration of blood storage is unclear, MPs as a part of storage lesion may play a role. One study using a murine model showed EDMPs as potential mediators of transfusion-related acute lung injury (TRALI) following transfusion of stored pRBCs.[44] The procoagulant activity of MPs, especially PDMP and EDMPs, in stored blood is thought to be the main contributor. However, a recent study found that MP depletion of pRBC did not result in decreased clotting times, suggesting that other factors smaller than 0.22 micrometers that increased with storage were responsible for the procoagulant activity.[10]

We rejected the hypothesis that LRF would significantly reduce the absolute MP concentrations despite the decrease in post-LRF MP concentrations. These findings suggest this filter may not be effective at reducing post-storage MPs of canine pRBCs. The mechanism for removal of microscopic contaminants, such as bacteria, via LRF has been hypothesized to include phagocytosis via leukocytes, direct removal by filter media, adhesion to leukocyte aggregates, and complement-mediated cell death.[45,46] Based on our findings, these mechanisms do not seem to be involved in MP removal *ex vivo*. This may be due to the fact that MPs originate from host cells, and therefore immune mediated mechanisms for removal *ex vivo* are null. Although we thought the procoagulant characteristics of MPs may contribute to filtration via adhesion, we do not have evidence to support this theory. There may be receptor-mediated adhesion to other cells passing through the filter such as erythrocytes and platelets. Further

microscopic imaging studies are needed to determine the passage mechanism of microparticles through the LRF. Herring *et al*[16] previously hypothesized that LRF was not able to remove MPs using a similar third generation leukocyte reduction filter in order to leukoreduce dog blood at the time of donation. They compared annexin-V-positive MP concentrations at time zero (time of blood donation) between leukoreduced and non-leukoreduced blood and did not find a difference, leading them to suspect that prestorage leukoreduction is not highly effective at removing annexin-V-positive MPs. However, prestorage leukoreduction did decrease the overall number of MPs at the end of the storage period.

The commercial leukocyte filter used in this study has a pore size of 40 μm. This has shown to be effective at removing bacteria.[33,47] Since MPs are similar in size to the bacteria tested, we predicted similar ability and mechanism of removal for MPs. Bacteria and MPs are too small to be sieved by a leukocytic filter pore. This is intuitive as the red blood cells and platelets are intended to pass through the filter, and MPs and bacteria are smaller than red blood cells and platelets. We postulated that the bacteria, and possibly the MPs, are removed via adhesion to the filter, plastic tubing, as well as through obstruction of the filter due to microaggregates of leukocytes. Also, MPs are known for their procoagulant properties and may have a higher likelihood of adhesion to the filter and other cells.[48] It seems from our results and those of previous studies that leukocyte reduction filters may not be effective at removing MPs. Aung *et al*[10] showed that a 0.22 micrometer filter would remove MPs from pRBCs. However, leukoreduction did not seem to affect procoagulant phospholipid concentrations. Chou *et al*[49] showed that nanofiltration with 75-nm filters was effective at removing MPs from leukoreduced plasma. However, this filter is not likely to be applicable in a clinical setting.

Although LRF may not be able to significantly remove MPs or prevent the formation in stored blood products, it is important to note that the LRF does not seem to increase MP concentrations. Shear stress is considered a stimulus for microvesiculation[50] and could occur as blood cells are passing through the leukoreduction filter. However, this does not seem to occur based on the results of our study. Therefore, LRF should not be implicated as a source of MP formation when used in a transfusion setting. In fact, LRF should still be used in stored canine blood products immediately prior to transfusion due to the lack of prestorage protocols in veterinary medicine. We know that leukoreduction has the ability to decrease transfusion reactions by removing donor leukocytes[37] and can reduce the magnitude of MP formation in canine blood.[16]

MPs originate from a variety of cells including white blood cells, red blood cells, platelets, and endothelial cells.[18,20,25] Hemostatic modulation by MPs is the result of conserved surface proteins from the cells of origin. Because MPs share surface proteins with their cell of origin, these proteins can be used as markers to identify cellular origin of MPs within a biological solution. Studies in the veterinary literature have only quantified MPs based on annexin V and CD61 labeling. Therefore, this is the first study to phenotype MPs in canine pRBCs. In addition, this is the first study to use a multilabel flow cytometry protocol to identify LDMPs and EDMPs in canine pRBCs. Multilabel flow cytometry protocols have been used to quantify and phenotype MPs in human literature.[10,51] Unfortunately for canine species, there is no available antibody label that is cross-reactive with canine glycophorin-A for identification of EDMPs. Therefore, we adopted our own multilabel approach using process of elimination to classify EDMPs based on their CD-61 and CD-45-negative properties. Further studies are indicated to validate this protocol in canine blood products.

A major challenge for MP quantification and phenotyping is the lack of standardization for flow cytometry, specifically in veterinary medicine due to sparse publications to date. Substantial research has been published evaluating the methodologies and limitations of MP isolation, enumeration, and characterization.[52-58] Standard flow cytometry is the current gold standard for MP quantification. However, standard flow cytometers only reliably detect particles greater than 500 nanometers. A large portion of MPs, specifically those with higher procoagulant potency, fall below this threshold and are often underestimated due to poor resolution of standard flow cytometers. For this reason, we used a high-resolution flow cytometer (BD LSRFortessa) in combination with fluorescent sub-micron particle size reference beads to validate our technique for optimal MP detection and quantification. The LSRFortessa was able to detect nanoparticles as small as 0.22 micrometers based on reference beads. Detection of an event smaller than 0.22 micrometers may have been unreliable and excluded from the MP gate. Therefore, a percentage of the MPs present in our samples could have been excluded from quantification by the gates used to exclude autofluorescent debris. We know that a significant proportion of MPs are annexin-V-negative and may have been missed with this antibody-mediated identification method.[59] McEntire *et al*[56] suggested that lactadherin may be a more sensitive label for detection and enumeration of MPs. Aung *et al*[10] contradicted these findings and suggested that there was no difference in MP quantification between labeling with annexin-V and lactadherin. Therefore, further studies are indicated to investigate the gold standard for MP quantification and labelling to improve detection sensitivity.

There were several limitations of this study. Sample size was small, and there was no power calculation prior to conduction of the experiment. Therefore, the results could have been underpowered to detect differences between the pre- and post-LRF absolute MP concentrations.

In addition, there is substantial controversy among centrifuge protocols for MP analysis. Sample preparation and centrifugation conditions have a significant effect on MP concentrations.[13] We elected to use a centrifugation protocol that was previously published in veterinary medicine for equine blood. The difference in species may or may not have skewed our results. If the centrifuge speed is too high, the MPs are pelleted and therefore not able to be quantified from the supernatant (PPP). As a result, the MP concentration could have been underestimated. Lastly, there is no canine compatible glycophorin-A to facilitate validated quantification of EDMPs in our study. We make the assumption based on reported CD surface proteins that our annexin-V-positive, CD-61 and CD-45-negative population of events are EDMPs.

In conclusion, our results indicate that EDMPs are the most prevalent population of MPs in canine stored pRBCs. Our findings do not support effective MP reduction with LRF. This was the first report of MP phenotyping in canine stored pRBCs using a modified multilabel flow cytometry protocol. The results of this study help us further investigate protocols for MP quantification in veterinary medicine to advance our knowledge of the role of MPs in transfusion medicine and patient outcome. Stored transfusions may have an increased risk for transfusion reactions, sepsis, or thrombosis by administering excessive amounts of MPs in pRBCs to patients with elevated circulating MP levels. For products that have undergone prestorage leukoreduction, MPs can still play a major role in transfusion reactions. Based on published knowledge thus far, we have not found the best way to eliminate adverse effects of stored transfusions. Therefore, the safest transfusion is no transfusion, and restrictive transfusion protocols should be implemented for our veterinary patients.

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Figure 1. *Flow diagram of cell salvage processing and filtration apparatus.* (A) Heparinized saline; (B) Inoculated blood; (C) Cardiotomy chamber; (D) Cell salvage machine; (E) Waste collection; (F) Post-wash RBCs; (G) Leukocyte reduction filter 1; (H) Post-filtration1 RBCs; (I) Leukocyte reduction filter 2; (J) Post-filtration2 RBCs. Samples for culture and bacterial enumeration were collected at points B, F, H, and J.

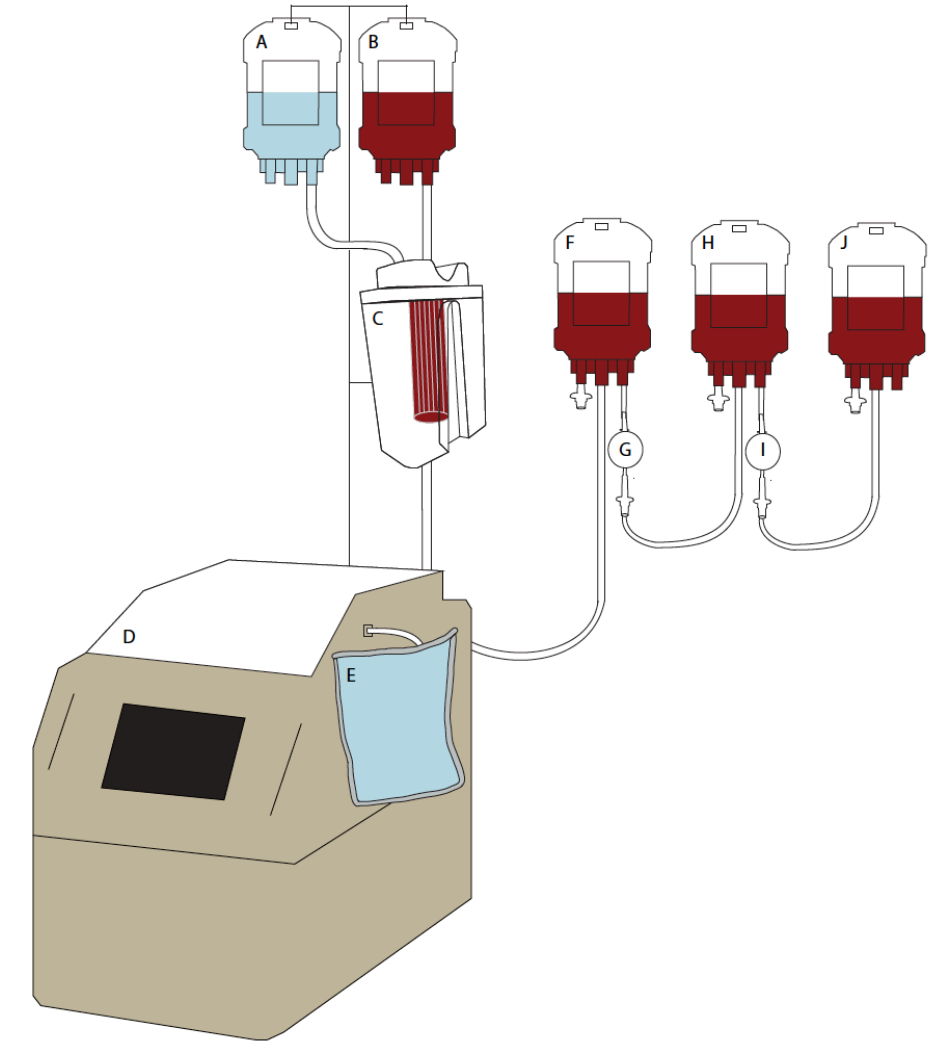

Figure 2. *Dot plots illustrating flow cytometric analysis of platelet-poor plasma (PPP) collected from expired canine packed RBCs pre- and post-filtration for sample unit 4.* **A-C**: Dot plots representing pre-LRF analysis for unit 4; **D-F**: Dot plots representing post-LRF analysis for unit 4. **(A)** Pre-LRF FSC (forward scatter) versus compensated FITC dot plot with FITC negative microparticle gate in lower left corner, and FITC positive microparticle gate in lower right corner. The y-axis describes forward scatter (FSC-A) or particle size and the x-axis describes annexin-V-FITC staining (comp-FITC-A). **(B)** Pre-LRF FITC negative- derived compensated BV 421 versus APC dot plot. The y-axis describes CD45-BV 421 staining (comp-BV 421-A) and the x-axis describes CD61-APC staining (comp-APC-A); Lower left quadrant events are FITC-/BV421-/APC-. Lower right quadrant events are FITC-/BV421-/APC+. Upper left quadrant events are FITC-/BV421+/APC-. Upper right quadrant events are FITC-/BV421+/APC+. **(C)** Pre-LRF FITC positive- derived compensated BV 421 versus APC dot plot. The y-axis describes CD45-BV 421 staining (comp-BV 421-A) and the x-axis describes CD61-APC staining (comp-APC-A); Lower left quadrant events are FITC+/BV421-/APC-. Lower right quadrant events are FITC+/BV421-/APC+. Upper left quadrant events are FITC+/BV421+/APC-. Upper right quadrant events are FITC+/BV421+/APC+. **(D)** Post-LRF FSC (forward scatter) versus compensated FITC dot plot with FITC negative microparticle gate in lower left corner, and FITC positive microparticle gate in lower right corner. The y-axis describes forward scatter (FSC-A) or particle size and the x-axis describes annexin-V-FITC staining (comp-FITC-A). **(E)** Post-LRF FITC negative- derived compensated BV 421 versus APC dot plot. The y-axis describes CD45-BV 421 staining (comp-BV 421-A) and the x-axis describes CD61-APC staining (comp-APC-A); Lower left quadrant events are FITC-/BV421-/APC-. Lower right quadrant events are FITC-/BV421-/APC+. Upper left quadrant events are FITC-/BV421+/APC-. Upper right quadrant events are FITC-/BV421+/APC+. **(F)** Post-LRF FITC positive- derived compensated BV 421 versus APC dot plot. The y-axis describes CD45-BV 421 staining (comp-BV 421-A) and the x-axis describes CD61-APC staining (comp-APC-A); Lower left quadrant events are FITC+/BV421-/APC-. Lower right quadrant events are FITC+/BV421-/APC+. Upper left quadrant events are FITC+/BV421+/APC-. Upper right quadrant events are FITC+/BV421+/APC+. Analysis was performed on high flow rate, concluding with 2500 counting beads (sizing bead gate in upper right quadrant labeled beads). FITC-A-positive microparticles were counted as events based on size (<1 µm, defined by beads in sizing bead gate) and positive fluorescence (square gate in upper left quadrant labeled MPs). Event signals with FITC-A < 10^1 were defined FITC-negative (debris) based on control sample.

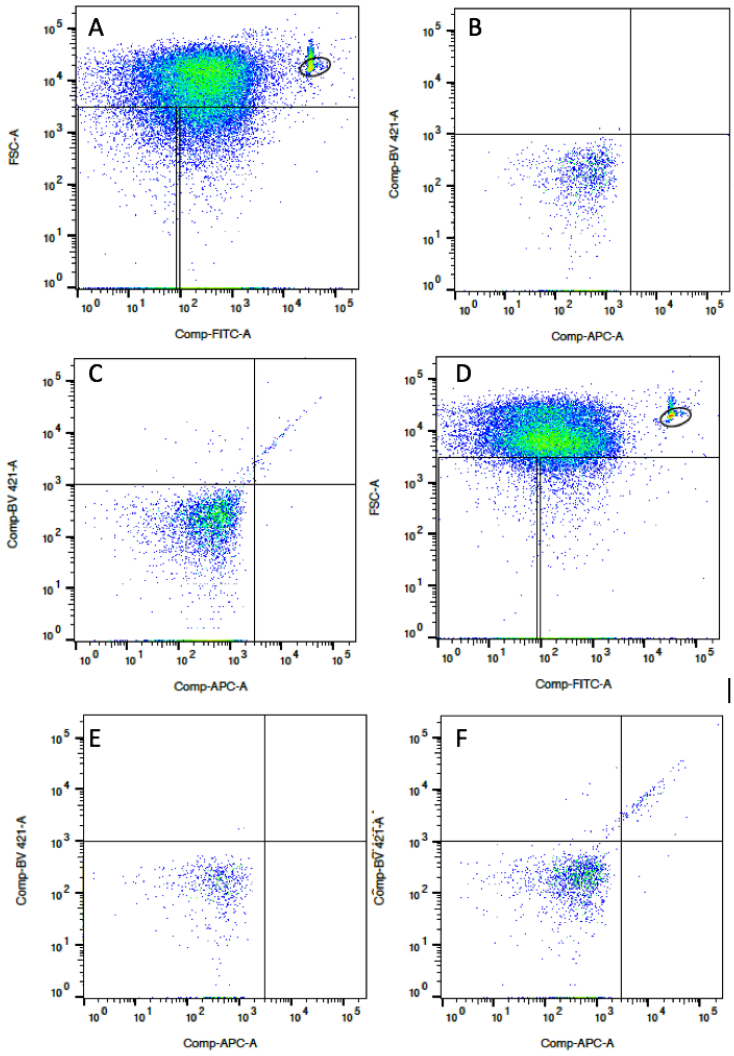

Table 1. Mean wash duration of cell salvage washing for treatment days 1, 2, and 3.

Treatment Day	Mean Wash Duration (minutes ± SD)
Day 1	17.9 ± 2.8
Day 2	18.2 ± 4.5
Day 3	17.9 ± 1.6

Table 2. Annexin-V-positive pre- and post-LRF microparticle (MP) concentrations per µL in expired canine packed RBC platelet-poor plasma (PPP) by MP phenotype. (LRF = leukoreduction filtration; MP = microparticle; EDMP = erythrocyte-derived microparticle; LDMP = leukocyte-derived microparticle; PDMP = platelet-derived microparticle)

MP Phenotype	Pre-LRF [MP] (MPs/µL)	Post-LRF [MP] (MPs/µL)
EDMP	3,208.71 ± 7,489.54	1,762.75 ± 3,210.40
LDMP	273.10 ± 182.47	279.06 ± 176.55
PDMP	27.81 ± 25.92	9.43 ± 10.60

Table 3. Mean bacteria concentrations for pre-wash, post-wash, post-first filtration, and post-second filtration blood samples.

Bacteria	Pre-wash Concentration* (CFU/mL) ± SD	Post-wash Concentration† (CFU/mL) ± SD	Post-LRF1 Concentration‡ (CFU/mL) ± SD	Post-LRF2 Concentration§ (CFU/mL) ± SD	Total reduction, %
E. coli	1128 ± 634.52	178 ± 278.95	0.03 ± 0.1	0 ± 0	99.9
S. pseudintermedius	773.33 ± 210.09	64 ± 67.49	0 ± 0	0 ± 0	100.0
P. aeruginosa	1100.67 ± 529.51	64.67 ± 110.76	0 ± 0	0 ± 0	100.0

* $P < 0.0001$ for comparison of initial pre-wash concentrations with correlation to bacterial reduction.

† $P < 0.0001$ for comparison of pre-wash and post-wash concentrations.

‡ $P < 0.0001$ for comparison of pre-wash and post-LRF1 concentrations.

§ $P < 0.0001$ for comparison of pre-wash and post-LRF2 concentrations.

cfu = colony forming units, LRF = leukoreduction filtration, SD = standard deviation

4. CONCLUSIONS

Alternatives to ABT have changed transfusion medicine for the better and allowed reduction of ABT use in our patients. The results of this study have advanced our knowledge of transfusion medicine as it applies to veterinary patients, specifically with the use of CSW and LRF for intraoperative autotransfusion and application of LRF to stored blood products.

CSW and LRF proved to be effective in removing bacteria from whole dog blood. The leukoreduction filter is an easy, inexpensive addition to cell salvage washing that, in combination with CSW, has the ability to remove bacteria from blood. This approach could be applied to intraoperative autotransfusion of blood in veterinary patients, even for clean procedures. Future clinical trials in client-owned patients are warranted to determine whether this autotransfusion process improves patient outcome. The outcome should be compared with those of current standard protocols (allogeneic blood transfusions) in dogs with intracavity hemorrhage with bacterial contamination.

Microparticles in store ABT are coming to the forefront of transfusion medicine to investigate the role in transfusion-associated complications and role in immunomodulation. Our results indicate that EDMPs are the most prevalent population of MPs in canine stored pRBCs. Our findings do not support effective MP reduction with LRF. This was the first report of MP phenotyping in canine stored pRBCs using a modified multilabel flow cytometry protocol. The results of this study help us further investigate protocols for MP quantification in veterinary medicine to advance our knowledge of the role of MPs in transfusion medicine and patient outcome. Stored transfusions may have an increased risk for transfusion reactions, sepsis, or thrombosis by administering excessive amounts of MPs in pRBCs to patients with elevated

circulating MP levels. For products that have undergone prestorage leukoreduction, MPs can still play a major role in transfusion reactions.

Based on published knowledge thus far, we have not found the best way to eliminate adverse effects of stored transfusions. Therefore, the safest transfusion is no transfusion, and restrictive transfusion protocols should be implemented for our veterinary patients.